Tracing evolution: Molecular phylogeny of Acochlidia (Heterobranchia, Gastropoda)

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To my parents Eva and Michael

CONTENTS

ABSTRACT

Sediment covered ocean floors constitute one of the largest and at the same time least explored habitats on Earth, still hiding a largely unknown species diversity. This marine mesopsammic habitat also harbors some of the remaining enigmas within heterobranch gastropods, which are puzzling due to their unclear phylogenetic position, their aberrant morphologies and the lack of knowledge regarding their biology and diversity. The limited data available for these neglected taxa rather obscured than improved phylogenetic assessments on Heterobranchia and has left significant gaps in the evolutionary history of this hyperdiverse clade of slugs and snails.

The spatially restricted interstitial environment constrains the morphology of its inhabitants, favoring flexible and vermiform body shapes, i.e., the reduction of shells in mesopsammic gastropods. Acochlidia are the most prominent clade of meiofaunal slugs with regard to local densities and species diversity. Traditionally classified as their own order among opisthobranch sea slugs, acochlidian slugs display a long history of controversial placements, which leave their phylogenetic relationships among euthyneuran Heterobranchia unresolved. Despite of being a small clade (with only 27 species validated prior to this thesis), Acochlidia are globally distributed and comprise a remarkable morphological and ecological diversity. Acochlidian slugs inhabited the marine mesopsammon and successfully transitioned between marine, limnic and potentially (semi-) terrestrial environments, where these slugs exhibit an epibenthic lifestyle. In the flexibility of habitats, Acochlidia reflect the ecological diversity of euthyneuran heterobranchs at a small scale, allowing for studies of morphological adaptations to the mesopsammon and an evaluation of morphological requirements that enabled these slugs to overcome the physical barriers related to transitions out of the marine habitat.

In order to establish a phylogeny of Acochlidia as backbone to understand their complex evolution and to clarify their origin among Heterobranchia, I aimed to optimize taxon sampling (i.e., perform an ‘all-species’ approach) by re-collecting all described species at their type localities for molecular purposes. My efforts to optimize the character sampling comprise a multi-locus molecular approach which combines nuclear and mitochondrial markers, quality tests at the levels of primary sequences and alignments, and a critical evaluation of different phylogenetic approaches. Additionally, I contributed to an accurate and detailed morphological dataset via advanced 3D-microanatomy from histological semithin-sections in conjunction with immunocytochemical and ultrastructural investigation (via scanning and transmission electron microscopy) of different organ systems.

The presented multi-locus molecular phylogeny on the origin of Acochlidia placed acochlidian slugs and other traditional opisthobranch or 'lower heterobranch' taxa in pulmonate relationships, clearly rendering the long-standing taxa of Opisthobranchia and Pulmonata non-monophyletic. In a substantial reorganization of euthyneuran systematics, a new classification of Heterobranchia was established naming several higher taxa such as Euopisthobranchia (including most of the traditional opisthobranch lineages, but excluding Actenoidea and Nudipleura) and Panpulmonata (uniting paraphyletic Pulmonata with 'opisthobranch' Sacoglossa and Acochlidia and former 'lower heterobranch' Glacidorboidea and Pyramidelloidea). So far, further multi-locus studies on different samplings of heterobranch taxa reveal very similar topologies, and initial phylogenomic approaches on gastropod systematics also support what is emerging as the 'new phylogenetic hypothesis of Heterobranchia'. Therein, my study on Acochlidia has demonstrated how the inclusion of small, previously neglected taxa can significantly contribute to solving deep phylogenetic relationship puzzles.

Based on the established molecular phylogeny, heterobranch slugs colonized the mesopsammic habitat several times independently. The aberrant external morphology of Acochlidia supposedly evolved from an abnormally developing panpulmonate ancestor via progenesis. The role of progenesis in the evolution of meiofaunal slugs is reviewed herein. Shared morphological features of mesopsammic slugs (e.g., accessory ganglia, calcareous spicules and adhesive glands) are confirmed as convergent adaptations to the spatially restricted environment; their functional aspects, however, still require further comparative studies.

In the course of this thesis, my colleagues and I successfully re-collected approximately 85% of the described acochlidian species and added nearly another 50% of yet undescribed species diversity. The generated comprehensive molecular phylogeny of Acochlidia is highly congruent with morphological approaches and therefore provides a robust framework to trace their evolution in time and space. Based on ancestral area chronograms and ancestral character state reconstructions, Acochlidia originated in the mid Mesozoic Jurassic from a marine, mesopsammic ancestor, who was adapted to tropical waters. The two major acochlidian subclades emerging in the Jurassic exhibit remarkably different evolutionary histories: The Microhedylacea are highly adapted to the marine mesopsammon and present a stunning example of morphological and ecological stasis. Their specialization likely contributed to survival through major geological extinction events and to the present-day global distribution with high local densities of specimens. But it also resembles an evolutionary dead-end road to morphological and ecological diversity, pointing to an irreversibility of excessive regressive

evolution. In contrast, basal meiofaunal Hedylopsacea retained or regained some morphological complexity and exhibit a remarkable flexibility in habitats. Their transitions out of the mesopsammon into limnic and (semi-) terrestrial habitats are connected to a secondary increase of body size and the reestablishment of a benthic lifestyle. The molecular phylogeny in conjunction with detailed microanatomical redescriptions allow for reconstruction of the evolution of e.g., reproductive and excretory features and an evaluation of their impact for the evolution of Acochlidia.

My thesis demonstrates the quality and accuracy of 3D-microanatomy for species descriptions in meiofauna. It also reveals the limitations of even high-end morphological approaches to delineate species of mesopsammic slugs reliably. Morphological species delimitation is frequently hampered by a combination of regressive evolution and simplified organ systems (providing few diagnostic characters) with high degrees of intraspecific variation potentially related to progenetic development in the evolution of meiofaunal taxa. Molecular species delineation on the other hand is complicated by the rarity of meiofaunal slugs, which are often discovered as singletons only. Therefore, previously established workflows on large-scale species delineation of hyperdiverse taxa need to be modified in order to deal with the problems symptomatic (yet certainly not exclusive) to many meiofaunal taxa. Herein, I present a workflow for molecularly founded, but nevertheless integrative, species delineation for elusive taxa, which aims to make best use of all (scattered) sources of data available. It presents a time-consuming form of 'deep-taxonomy', designed to complement 'turbo-taxonomic' approaches in cases where the evolutionary history of taxa urges to do so, e.g., to successfully tackle the taxonomic deficit in many meiofaunal taxa.

Workflows of species delineation must not terminate in the discovery of independently evolving lineages without proceeding to the final step of species description. In denying the discovered entities formal recognition, this form of molecular driven taxonomy betrays its own justification by enhancing instead of reducing impediments in taxonomy. My thesis discusses the use of molecular characters for taxonomy and practically demonstrates that, and how, molecular diagnostic characters can be included into the traditional taxonomic framework, either as additional source of data or even as backbone for descriptions of cryptic species. Ideally, this final step should be implemented in automated species delineation procedures in the near future to bridge the gap between species discovery and description – reuniting taxonomy.

By combining molecular sequence data with modern morphological analyses, I traced the evolution of Acochlidia through geological times and followed their pathways in and out of the

mesopsammon. My thesis contributes to transforming an enigmatic, neglected taxon to a well-studied clade, providing the evolutionary background for further in-depth revision of its sister taxa and novel lineages likely to be discovered. Moreover, it offers a methodological example of how to address the diversity of elusive taxa to avoid this part of global biodiversity slipping through automated approaches of modern taxonomy, thereby missing its potential information for our understanding of the evolution of life on Earth.

INTRODUCTION

Background - Our understanding of the evolution of organisms and the diversity of life on Earth, depends on phylogenetic reconstructions of the origin of taxa and their natural relationships. In the past two decades, molecular approaches have revolutionized systematics and radically altered our traditional perspective of the evolution of the animal kingdom (see e.g., results proposed by Halanych et al. 1995, Aguinaldo et al. 1997, Giribet 2002, Dunn et al. 2008, Giribet 2008). This upheaval concerns major relationships among metazoan phyla – e.g., offering evidence of a relationship of lophophorates with Mollusca and Annelida (Lophotrochozoa) (Halanych et al. 1995) – and paves a path through the systematic hierarchy, challenging classic concepts of molluscan evolution (Giribet et al. 2006, Wilson et al. 2010b, Kocot et al. 2011, Smith et al. 2011), gastropod relationships (Aktipis et al. 2008), the systematics of Euthyneura (Vonnemann et al. 2005, Klussmann-Kolb et al. 2008) and nearly all orders, families or genera in which molecular approaches have been conducted so far (e.g., Wade et al. 2001, Wollscheid-Lengeling et al. 2001, Klussmann-Kolb & Dinapoli 2006, Malaquias et al. 2008, Göbbeler & Klussmann-Kolb 2010, Dinapoli et al. 2011, Weigand et al. 2013).

Among gastropod molluscs, the morphology-based concept of Heterobranchia (Haszprunar 1985, 1988) was supported by subsequent cladistic analyses (Ponder & Lindberg 1997) and also withstood a first multi-marker molecular approach (Dinapoli & Klussmann-Kolb 2010). The Heterobranchia comprise the informal 'Lower Heterobranchia', which form a basal, paraphyletic grade of different taxa formerly classified as Allogastropoda (Haszprunar 1988, Ponder & Lindberg 1997) and the Euthyneura (Haszprunar 1985, 1988, Ponder & Lindberg 1997, Dinapoli & Klussmann-Kolb 2010). Milne Edwards (1848) divided the Euthyneura into Opisthobranchia and Pulmonata, a traditional concept of the two euthyneuran subtaxa that has been largely adopted ever since, particularly because it conveniently bears a rough resemblance to the ecological division between sea slugs on the one hand and limnic and terrestrial slugs and snails on the other. Based on morphological characters, however, neither Opisthobranchia nor Pulmonata are well supported monophyla (Haszprunar 1985, Dayrat & Tillier 2002). Due to the high degree of homoplasy (Dayrat & Tillier 2002), cladistic analyses of morphological data of Euthyneura have failed to resolve the relationships between the major opisthobranch and pulmonate taxa. Molecular phylogenetic approaches based on various mitochondrial or nuclear markers either also left the monophyly of Opisthobranchia and Pulmonata unsupported or even clearly rejected it by clustering opisthobranch clades among pulmonates (Grande et al. 2004a, Vonnemann et al. 2005, Klussmann-Kolb et al. 2008). Next to the unclear monophyly of the

traditional higher taxa, the relationships among major opisthobranch and pulmonate taxa remained unresolved, while contradicting topologies have been recovered by different morphological and molecular analyses based on various markers (e.g., Dayrat et al. 2001, Dayrat & Tillier 2002, Grande et al. 2004a, b, Vonnemann et al. 2005, Wägele & Klussmann-Kolb 2005, Klussmann-Kolb et al. 2008, Wägele et al. 2008). Euthyneura are one of the few higher taxa of invertebrates having members which have successfully switched habitats between marine, limnic and terrestrial systems several times independently in their evolutionary history (Barker 2001, Klussmann-Kolb et al. 2008, Mordan & Wade 2008). They thus present a promising field of research on the morphological and biological adaptations or precursors allowing these slugs and snails to overcome the physical barriers represented by habitat transitions that few other invertebrates were able to cross. However, comparative morphological and behavioral studies, as well as efforts to locate habitat shifts within a biogeographic and ecological background were impeded by a lack a robust phylogenetic hypothesis on Euthyneura.

Prior to this thesis, a well-supported euthyneuran phylogeny has been hampered by a lack or underrepresentation of small, aberrant groups, which have tended to 'jump' around in phylogenetic analyses and weaken support for the sister group relationships at the deeper nodes. Especially problematic were the small, little known meiofaunal clades such as Acochlidia, Platyhedylidae, Philinoglossidae or Rhodopemorpha. They are all characterized by members having minute vermiform bodies, frequently lacking coloration, eyes and appendages (Swedmark 1964, 1968a, Arnaud et al. 1986) and have often been recovered in sister group relationships based on morphological evaluation (Salvini-Plawen & Steiner 1996, Wägele & Klussmann-Kolb 2005). Schrödl and Neusser (2010), however, demonstrate in their cladistic approach how Acochlidia cluster with every other meiofaunal slug included in the analyses. They conclude that external features and shared anatomical characteristics (e.g., presence of calcareous spicules and accessory ganglia) present convergent adaptations to the interstitial habitat, which likely mask the true phylogenetic signal (Schrödl & Neusser 2010). Consequently, morphological approaches may be insufficient to trace the evolutionary pathways of meiofaunal slugs into the mesopsammon.

The Acochlidia (also referred to as Acochlidiacea) are the most prominent group of meiofaunal slugs concerning species diversity and local densities of specimens (Poizat 1984, Arnaud et al. 1986, Wawra 1987). In traditional classifications, they form an independent order of the 'Opisthobranchia' (Bouchet et al. 2005). At the beginning of this thesis, the Acochlidia comprised 27 valid species (Wawra 1987). This little known and poorly described group was

considered ‘fascinating’ among malacologists (Dayrat & Tillier 2002), as they combine worldwide distribution with remarkable ecological and morphological diversity in a small number of lineages. The majority of Acochlidia is minute in size (1-4 mm) and inhabits interstitial spaces in marine sands, though some lineages have successfully colonized freshwater habitats and re-attained ‘gigantic’ body sizes of up to 3cm (Wawra 1987, Schrödl & Neusser 2010). Additionally, recent discoveries of Aitengidae have revealed an evolutionary pathway within Acochlidia which led to an amphibious lifestyle in the marginal zones of terrestrial systems (Swennen & Buatip 2009, Neusser et al. 2011a). Therefore, Acochlidia reflect the ecological diversity of Euthyneura at a small scale, representing manageable species diversity and allowing for a detailed, comparative approach to address major habitat shifts and the forces that drive them.

Taxon sampling - The enthusiasm about the availability of new molecular character sets, which ideally are independent from direct ecological pressure and therefore not influenced by convergent adaptations, has also been accompanied by skepticism on how trustworthy these new phylogenies are. Doubts were further enhanced by contradicting hypotheses based on different genetic markers or resulting from changes in taxon sampling. It has been demonstrated over the past few years that the accuracy of molecular phylogenetics largely depends on the amount and quality of chosen molecular markers and on adequate taxon sampling (e.g., Bininda-Emonds & Stamatakis 2006, Nabhan & Sarkar 2012). The latter should ideally provide a representation of the diversity of the taxon and include basal rather than highly derived representatives in order to avoid false clusters influenced e.g., by long-branch attraction. Consequently, efforts have to focus on optimizing taxon and character sampling, and new phylogenetic hypotheses have to be critically evaluated in light of present morphological data and the likelihood of implemented evolutionary scenarios.

At the beginning of this thesis, the Acochlidia were comprised of 27 valid species, though some new, undescribed lineages had already been discovered in the field (M. Schrödl, T. Neusser, pers. comm.) indicating a higher actual than described diversity. The nevertheless manageable acochlidian diversity allowed for an ‘all-species-approach’ to address their evolution. To optimize the ingroup taxon sampling, I collected Acochlidia worldwide with the workgroup led by PI Michael Schrödl and a series of international collaborators (please see Additional file 1 for names and institutions that provided material and a list of all sampling localities), focusing on type localities of valid species (see Additional file 2) but also covering unexplored regions ‘in between’. Figure 1 shows the type localities of the valid species of Acochlidia (based on

Wawra 1987, Schrödl & Neusser 2010) and sampling localities of the present study (also marking unsuccessful re-collection attempts and type localities of the species described within this thesis). Most sampled acochlids show a highly scattered and irregular distribution, which is typical for many marine meiofaunal organisms (e.g., Andrade et al. 2011). They can thus easily be missed even when sampling intensely in a single area. Negative sampling records in meiofauna, therefore, by no means demonstrate the absence of a certain taxon from this region. Nevertheless, the information was included in Figure 1, as it is unlikely that these areas represent one of the major centers of acochlidian abundance and diversity. Imprecise information on type localities in combination with poorly detailed descriptions and putatively closely related, yet still undiscovered lineages in the same area of distribution partially hinder reliable assignment of collected specimens to species such as '*Parhedyle' gerlachi* (Marcus & Marcus, 1959) from the Maldives or *Pseudunela eirene* Wawra, 1988 from the Andaman Sea. My own re-collecting attempts at the type localities of *Tantulum elegans* Rankin, 1979 (St. Vincent, February 2009) and *Palliohedyle weberi* (Bergh, 1895) (Flores, Indonesia, September 2008) were unsuccessful. In addition, *Asperspina riseri* (Morse, 1976) could not be found any longer at its type locality in 2011 (T. Morse, pers. comm.). Material of *Asperspina loricata* (Swedmark, 1968) and *Asperspina murmanica* (Kudinskaya & Minichev, 1978) could be obtained successfully by international cooperators, but unfortunately, DNA extraction and/or amplification of the material failed. All acochlidian material analyzed in the present study in listed in Additional file 3. Voucher material collected in my thesis is deposited in and accessible through the Mollusca Section of the Bavarian State Collection of Zoology (ZSM, Munich). Aliquots of all extracted DNA are stored long term and available through the DNAbank network (at the ZSM, http://www.dnabank-network.org/; see Additional file 3 for museums and accession numbers). All published sequences are deposited in the GenBank sequence database provided by the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/genbank/, see Additional file 3, for accession numbers). Major phylogenic trees are deposited in TreeBase (see Chapter 1, 10 and 13, for accession numbers).

Origin of Acochlidia - Chapter 1 of my thesis provides a multi-marker molecular phylogeny to clarify the origin of Acochlidia and other enigmatic meiofaunal lineages. To do so, we build upon the existing broad euthyneuran sampling of Klussmann-Kolb et al. (2008) based on four genetic markers (nuclear 18S rRNA and 28S rRNA and mitochondrial 16S rRNA and cytochrome oxidase subunit I (COI)), which included representatives from all major

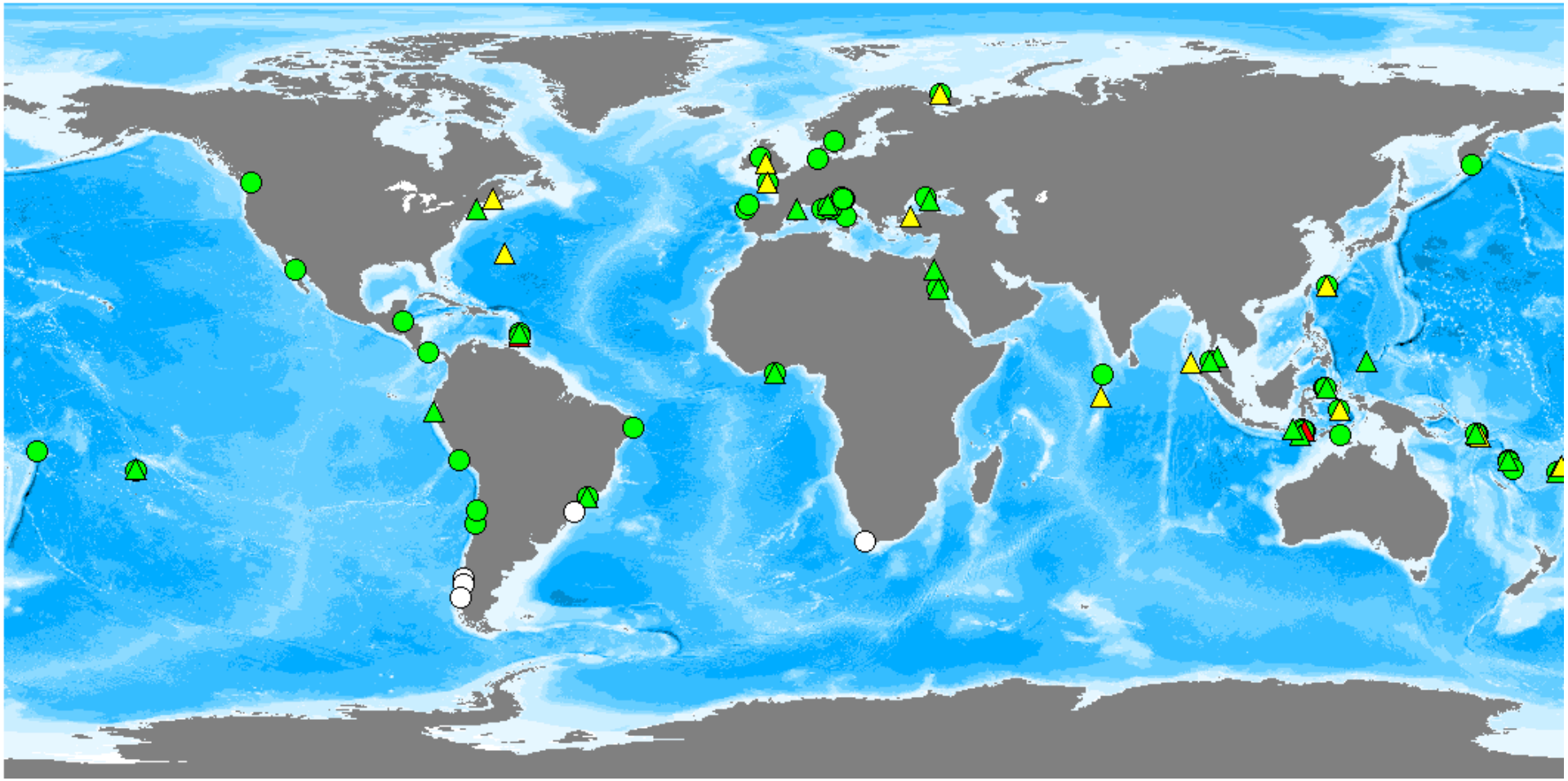


Figure 1: Sampling and type localities of Acochlidia. Triangles indicate type localities, dots sampling efforts in the course of this study (for collectors, coordinates and details on the collecting sites, see Additional material 1). green represents successful (re-)collecting events, yellow marks type localities which were not sampled during this thesis and red those which were sampled extensively but without success. Unsuccessful sampling in formerly unexplored areas is marked by white dots.

euthyneuran lineages. We supplemented and refined the sampling by including more meiofaunal lineages as well as all the sister groups of Acochlidia previously suggested in the literature (e.g., diaphanid *Toledonia* or philinoglossid *Philinoglossa praelongata* (Sommerfeldt & Schrödl 2005, Wägele & Klussmann-Kolb 2005)). The consequences of our analyses for the systematics of Heterobranchia are further reviewed in **Chapter 2**. In the absence of any fossil record of meiofaunal slugs, I established a molecular clock of Euthyneura to estimate the times of origin and diversification for the major clades and locate their evolution in a historical framework. Most critical for molecular clock estimates is the choice of calibration points (i.e., dated fossils or geographic events) (Renner 2005, Wilke et al. 2009) and the underlying phylogenetic hypothesis. An opposing scenario to our phylogeny of Euthyneura combined with considerably deviating molecular clock estimates is commented on in **Chapter 3**. For recent developments in heterobranch systematics based on phylogenomic approaches, see Discussion (Euthyneuran relationships inferred from other markers).

Diversity and evolution of Acochlidia – Traditionally, the taxonomy of gastropods relies on external morphological characters; even in 2006, approximately 80% of new gastropod species descriptions were based solely on shell characters (Bouchet & Strong 2010). This practice can be problematic given the partially high intraspecific variability of shell characters in different ecomorphs (see e.g., Hauswald et al. 2008). In absence of a shell, the taxonomy of meiofaunal slugs is still mainly based on external morphology, presence and type of calcareous spicules and radula characteristics (Kowalevsky 1901b, Arnaud et al. 1986, Wawra 1987). The anatomical data provided in most original descriptions shows little detail (Kowalevsky 1901b, Marcus 1954, Salvini-Plawen 1973) and/or frequently requires reinvestigation due to inadequate methodology in the past. Therefore, few encodable characters with sufficient coverage across acochlidian taxa are available for phylogenetic analyses (Schrödl & Neusser 2010). In my thesis, I contributed to the morphological knowledge on Acochlidia by providing detailed microanatomical studies using advanced computer-based 3D-visualization. Comparative morphology of major organ systems and ultrastructural investigations of e.g., sperm morphology is used to mine for additional characters suitable for phylogenetic reconstructions. In general, we aimed for detailed redescription of at least one representative of each acochlidian genus to generate an extensive and accurate morphological dataset, allowing us to reliably interpret the evolution of organ systems across Acochlidia.

The detailed exploration of the central nervous system is of special interest, as it provides the data necessary to establish homology assumptions across euthyneuran taxa and evaluate

putative synapomorphies for higher clades previously proposed (e.g., presence of a procerebrum and cerebral gland in pulmonates (Van Mol 1967) or Pentaganglionata hypothesis (Haszprunar 1985)). A histological account on putative additional characters in the central nervous system and sensory organs across different species of Acochlidia is provided in **Chapter 4**. In addition to computer-based 3D-reconstruction from histological semithin sections, I applied immunocytochemistry (staining of FMRFamide and Tyrosine Hydroxylase) in conjunction with confocal laser scanning microscopy to redescribe the nervous system of the minute Mediterranean *Parhedyle cryptophthalma* (Microhedylidae s.l.), a little known acochlid inhabiting the highly energetic wave zone (see **Chapter 5**).

Among Acochlidia different modes of sperm transfer are reported. Copulation – as was suspected to occur e.g. in *Tantulum elegans* (Rankin 1979, Neusser & Schrödl 2007) – is the likely basal state in Acochlidia. Several hedylopsacean lineages have evolved armed copulatory organs, likely used for hypodermic injection (Haase & Wawra 1996, Schrödl & Neusser 2010) and most microhedylacean lineages are aphallic and transfer sperm via spermatophores randomly attached to the mates (Swedmark 1959, Morse 1994). In **Chapters 6** and **7**, we investigate in microanatomical and ultrastructural detail extreme cases of protandry in sequential hermaphrodites, involving a complete sex change and the unique occurrence of secondary gonochorism; we also discuss their evolution based on the current phylogeny and as adaptations to different environments.

Next to the generation of reliable morphological data, the main objective of my thesis was to establish a molecular phylogeny of Acochlidia based on multiple markers to trace major events in acochlidian evolution such as habitat shifts, and to reconstruct character evolution. Acochlidia reflect the ecological diversity of Euthyneura at small scale, presenting several habitat transitions in and out of the mesopsammon, and beyond the fully marine system into brackish waters in Pseudunelidae (Neusser & Schrödl 2009), limnic habitats in Acochlidiidae (Wawra 1974, 1979, 1987, Haynes & Kenchington 1991, Brenzinger et al. 2011a) and (semi-) terrestrial systems in Aitengidae (Swennen & Buatip 2009, Neusser et al. 2011a). We used an integrative approach combining muli-marker molecular phylogenies with 3D-microanatomy to evaluate adaptations and potential morphological precursors for habitat shift in relation to the marine sister taxa: to freshwater habitats (**Chapter 8** on *Strubellia*) and (semi-)terrestrial habitats (**Chapter 9** on Aitengidae). Summarizing the efforts of the workgroup over the past years, including the results of the present PhD thesis, and drawing heavily on the thesis written by Dr. Timea Neusser, we transformed a poorly known enigmatic taxon to one of the gastropod clades with the most detailed morphological knowledge available. This knowledge is evaluated

in light of an established comprehensive molecular phylogeny of Acochlidia. This includes approximately 85% of the described species diversity, as well as another 50% of yet undescribed, novel forms in order to address the driving forces in their evolution from miniaturization to 'secondary gigantism' and key morphological features enabling habitat shifts in and out of the mesopsammon (see **Chapter 10**). To further trace the evolutionary history of Acochlidia in time and space, I established ancestral area chronograms, estimated shifts in diversification rates and inferred ancestral states of major ecological traits. This provides a rough timeframe for the diversification and radiation of acochlidian taxa and allows for a discussion on vicariance events and the paleo-environmental background in which both morphological key features of certain lineage have evolved and major habitat shifts have occurred.

The marine mesopsammon is one of the largest, yet least explored habitats on Earth, and the taxonomic deficit is correspondingly high. Sampling for meiofaunal slugs in marine sands worldwide revealed an enormous amount of hidden diversity. This is partly due to novel lineages directly recognizable as such via external characteristics in the field, and partly to cryptic lineages pointed to by long internal branches in generated molecular phylogenies – creating an urgent need for efficient and reliable species delineation in meiofaunal slugs.

Species delineation and integrative taxonomy - Fundamental for taxonomy and species delineation is the underlying species concept. This is not merely a philosophical question, but is also reflected in practice by the deviating number of recognized species discovered in delimitation approaches (Isaac et al. 2004). The importance of 'species' for all fields of biology has encouraged numerous attempts to formulate a species concept that aims to capture adequately the diversity of living forms (for a summary of the different concepts see e.g., Mayden 1999, Wägele 2001, de Queiroz 2005a, 2007, Hausdorf 2011). But despite all these efforts, a consensus has yet to be reached. The search for a unified species concept across all fields of biology may be in fact idealistic, considering the diversity of living forms and the variety of patterns which drive their evolution. Traditionally, species have been addressed as morphospecies, i.e. entities of individuals which present the same morphological characters, distinguishing them from other groups of organisms (Simpson 1961). However, due to conflicting data from uniform morphologies and biological traits (e.g., low reproductive output) the morphological species concept is inapplicable to my dataset of meiofaunal slugs. It clearly fails to capture cryptic speciation, which is suspected to occur frequently in taxa with low dispersal abilities (see e.g., Wilson et al. 2009).

The biological species concept defines species as groups of individuals which actually or potentially interbreed, produce fertile offspring and are thus reproductively isolated (Mayr 1942). So far, no practical test for interbreeding has been conducted successfully in meiofaunal slugs under laboratory conditions. However, even a successful interbreeding experiment would not reliably prove the absence of significant barriers to interbreeding under natural conditions (e.g. via behavioral means or competitive disadvantages). In allopatric populations, a special, distinct morphology of reproductive organs may allow for indirect inferences of reproductive isolation. Anatomical data on the reproductive system of meiofaunal slugs is frequently uninformative, however, when it comes to testing for reproductive isolation, since sperm transfer via hypodermic injection or spermatophores lack evident key and lock mechanisms – at least at the histological level. Genetic isolation via haplotypes and genetic distances, as absolute or relative values, is often used to infer the existence of distinct species (see e.g., Hebert & Gregory 2005, Hajibabaei et al. 2007). But, strictly, the isolation reflected in separate haplotype networks can only count as reproductive isolation in line with the biological species concept if the lineages occur sympatrically, with specimens having the chance to interbreed naturally. Further requirements are, e.g. an ideal sampling of populations, ideal genetic markers (i.e., not suffering from incomplete lineage sorting) and ideal population genetic analyses. Applying a biological species concept to allopatric populations using molecular markers that are not directly involved in the development of intrinsic reproductive barriers (speciation genes (e.g., Krug 2011)) is as problematic as e.g., using morphological (dis)similarity of features not directly involved in reproductive success.

Under the phylogenetic species concept, a 'species' may be defined as a group of individuals descending from a common ancestor and possessing at least one derived trait that differentiates the group from its ancestor (Eldredge & Cracraft 1980). To maintain the monophyly of species, speciation under the phylogenetic species concept must lead to two descendant species which replace the ancestral one. Phylogenetic species form monophyla and may be diagnosable by a unique combination of character states (see e.g., Nixon & Wheeler 1990). This concept is applicable even to cryptic meiofaunal slugs; however, it has its own shortcomings, such as non-concordance between gene and species trees resulting in poly- or paraphyletic species (see e.g., Funk & Omland 2003) and the objective identification of species monophyla.

Currently most popular among scientists is a lineage-based species concept that extracts the common element of the previously competing species concepts (Wiens 2007). This 'unified species concept' defines 'species' as independently evolving (segments of) metapopulation lineages (i.e., ancestor-descendant series), using the formerly secondary species criteria of

different species concepts as operational criteria, i.e. lines of evidence supporting the species status (de Queiroz 1998, 1999, 2007). Hausdorf (2011) has criticized the circularity of this approach, due to the absence of an independent, self-contained definition of 'population', which only shifts the problem of defining 'species' to defining 'population'. Usually, a 'population' is defined as a group of conspecific individuals which commonly inhabit a certain geographic area (Hausdorf 2011). Inferring metapopulations, i.e. populations in allopatry, would introduce an assumption on biological species through the backdoor (Hausdorf 2011). I agree, though also see advantages in de Queiroz' (2007) proposal of combining the evidence of criteria used under different species concepts (such as monophyly, intrinsic reproductive isolation and diagnosibility). Biological species criteria such as direct observations are hardly applicable to elusive meiofaunal slugs; while discontinuous distributions of morphological characters or non-overlapping genetic clusters in syntopic populations represent evidence of intrinsic reproductive barriers, the value of such differences in potentially allopatric populations can only be estimated. In the absence of practicable alternatives, I thus apply in the present thesis the unified, lineage-based species concept, following de Queiroz (1999, 2007), integrating all available lines of evidence, e.g. from geography, morphology, biology, and genetics.

Defining species as independent evolutionary lineages clearly shifts the problems formerly connected to species concepts to species delineation. This raises the question of which criteria are considered relevant and informative for species delimitation, as well as how to integrate different lines of evidence in a taxonomic framework: With some taxonomic training and the use of light-microscopy, the identification of meiofaunal slugs at the family and genus level can be conducted in the field, relying on external morphology and some internal features such as calcareous spicules and radula characteristics (see Additional file 4: Key for the identification of meiofaunal slugs in the field). Across taxa of meiofaunal slugs (i.e. Acochlidia, Rhodopemorpha and Philinoglossidae), however, species level identification based on these traditional characters is in most cases impossible, and requires advanced microanatomical or molecular approaches. Numerous recent studies – including my own contributions during my work on the present thesis – have successfully demonstrated the power of advanced computer based 3D-microanatomy for species (re-) descriptions in meiofaunal slugs and highlighted the accuracy, reliability and vast amount of information generated by this method (e.g., Neusser et al. 2006, Neusser & Schrödl 2007, Jörger et al. 2008, Neusser et al. 2009a, Neusser et al. 2009b, Jörger et al. 2010a, Brenzinger et al. 2011c, Brenzinger et al. 2013b). Nevertheless, these studies have also revealed the limitations of 3D-microanatomy when it comes to species delineation. Because these approaches are so time-consuming, the advantages in terms of

minute detail go at the cost of estimating intraspecific vs. interspecific variability of characters. Moreover, some lineages of meiofaunal slugs show reduced complexity in all major organ systems, which provide hardly any anatomical differences. Today, integrative taxonomy is generally considered best practice, applying, however, different workflows of integration, deviating in the mode of integration from different sources (e.g., morphology, molecular sequences, behavior or geography) to delineate and describe species (see Discussion on Workflows on integrative species delineation) (Dayrat 2005, Padial & De La Riva 2010, Padial et al. 2010). Applied to Acochlidia, the combination of morphological and molecular data in integrative approaches via cumulation (*sensu* Padial et al. 2010) has provided the key to delineated species successfully (see **Chapter 11** on *Pseudunela*).

In integrative approaches, especially via congruence (*sensu* Padial et al. 2010), the major taxonomic challenge consists in identifying the usefulness of characters for species delineation and distinguishing between trivial and informative data before the process of integration (Valdecasas et al. 2008). Worldwide Acochlidia sampling efforts have revealed a striking morphological uniformity especially within Microhedylacea. In the absence of phylogenetic signals, parts of the clade cannot be resolved even on the genus or family level in cladistics analyses of morphological characters (Schrödl & Neusser 2010). Furthermore, assignment of new collected material to existing species based on morphological data is dubious. Applying traditional taxonomic characters to the globally collected lineages, distribution areas of the valid species would need to be expanded considerably, resulting in several amphi- or circum-oceanic species. Even advanced histological microanatomy fails to provide distinguishing characters, e.g., between Mediterranean and Black Sea *Pontohedyle milaschewitchii* and Western Atlantic *P. brasilensis*, which resulted in synonymisation (Jörger et al. 2007). These wide distribution areas create what is known as the 'meiofauna paradox' (Giere 2009) given the assumed low dispersal abilities of meiofaunal slugs and raises concern about overlooked cryptic diversity. Barcoding and molecular species delineation have been advocated as a fast and efficient means to uncover cryptic lineages and address the 'taxonomic impediment' in times of biodiversity crisis (e.g., Blaxter 2004, Hebert & Gregory 2005, Hajibabaei et al. 2007). DNA barcoding in the strict sense is a means of species identification and relies on a comprehensive sequence database (DeSalle et al. 2005, DeSalle 2006). With the vast majority of the habitats of marine meiofauna still unexplored, being able to find corresponding sequences in public databases that match new collected material cannot be expected for decades to come. Effective methods of molecular species delineation are needed to identify novel lineages and reliably delimit them from existing material. Population genetics and the establishment of haplotype networks are the most straightforward method for addressing the genetic diversity in a sample

and thus to distinguish between intra- and interspecific variability of a putative flock of species (Avise et al. 1987). This has been applied to comparably densely sampled Mediterranean *Microhedyle* populations (see **Chapter 12**), but is unfeasible for other world-wide distributed acochlidian genera characterized by a high degree of singletons from far distant localities.

The rarity of sampled specimens is one of the major limitations of molecular species delineation approaches in our dataset and of many if not most other datasets on marine invertebrates (Bouchet et al. 2002, Albano et al. 2011, Lim et al. 2012). We used the world wide sampled specimens of *Pontohedyle* (Microhedylidae s.l., Acochlidia) as a touchstone for testing and critically evaluating the performance of different approaches of molecular species delineation (see **Chapter 13**) on a difficult real-world dataset with a high degree of singletons and in the absence of a comparable 'gold standard' such as morphology in putatively cryptic species. The study aims to establish a workflow of molecular but nevertheless integrative species delineation in elusive and putative undersampled groups of marine invertebrates.

Once cryptic lineages have been discovered as independently evolving lineages (no matter if termed as OTUs, MOTUs or candidate species), hypotheses on the species status of these discovered entities exist in the literature, which require the final step of formal species description and proper voucher documentation. If this final step of 'DNA taxonomy' is left out, impediments in taxonomy will be enhanced rather than reduced by molecular species delineation approaches. Various previous studies have supplemented or solely based species descriptions on diagnostic characters from molecular sequence data, but in different unstandardized forms (summarized in Goldstein & DeSalle 2011). In my thesis (see **Chapter 14**) I exemplarily describe several cryptic species of meiofaunal slugs based on diagnostic nucleotides, exploring and discussing the practical challenges of molecular taxonomy, such as the nature of a diagnostic molecular character, the critical evaluation of homology assumptions in alignments and the necessary requirements for molecular-based species descriptions to ensure traceability and sustainability in future research. The presented work on molecular taxonomy aims to encourage discussion among taxonomists in order to establish best practice on integrating molecular data into the Linnaean system and traditional taxonomy.

Aims of the thesis - To summarize, my PhD thesis pursues three major aims: 1) To establish a molecular phylogeny and molecular clock of euthyneuran slugs by supplementing existing molecular datasets with the goal of clarifying the origin of Acochlidia and identifying potential sister groups, therein providing a robust hypothesis to trace evolutionary pathway(s) of marine slugs into the interstitial habitat. 2) To optimize both taxon sampling of Acochlidia, based on

worldwide sampling efforts at type localities and beyond, and character sampling (quantitatively and qualitatively) by means of sophisticated 3D-microanatomy, ultrastructural data and mainly molecular markers. This allows the establishment of a multi-marker molecular phylogeny of Acochlidia, which is then comparatively discussed with morphology-based approaches and used as a backbone for reconstructing the evolutionary history of Acochlidia. The evolution of characters and habitat transitions to freshwater and semi-terrestrial systems is discussed in light of the present phylogeny. 3) Given both the power and the limitations of morphology-based approaches with regard to species delineation in meiofaunal slugs, my final aim is to explore the utility of molecular species delineation in elusive taxa and to establish an integrative, molecular founded workflow for these groups with putative undersampled diversity.

RESULTS

Publications included in this thesis

First author publications:

Jörger KM, Heß M, Neusser TP, Schrödl M (2009) Sex in the beach: spermatophores, dermal insemination and 3D sperm ultrastructure of the aphallic mesopsammic *Pontohedyle milaschewitchii* (Acochlidia, Opisthobranchia, Gastropoda). *Marine Biology* 156: 1159-1170.

Jörger KM, Stöger I, Kano Y, Fukuda H, Knebelsberger T, Schrödl M (2010a) On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia. *BMC Evolutionary Biology* 10: 323

Jörger KM, Kristof A, Klussmann-Kolb A, Schrödl M (2010b) Redescription of the meiofaunal gastropod *Parhedyle cryptophthalma*, with focus on nervous system and sensory organs (Acochlidia, Panpulmonata). *Spixiana* 33: 161-170.

Jörger KM, Wilson NG, Norenburg JL, Schrödl M (2012) Barcoding against a paradox? Combined molecular species delineation reveals multiple cryptic lineages in elusive meiofaunal sea slugs. *BMC Evolutionary Biology* 12: 245.

Jörger KM, Schrödl M (2013) How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology* 10: 59.

Jörger KM, Brenzinger B, Neusser TP, Martynov AV, Wilson NG, Schrödl M (in review) Panpulmonate habitat transitions: tracing the evolution of Acochlidia (Heterobranchia, Gastropoda). *Molecular Phylogenetics and Evolution.*

Senior author publications:

Eder B, Schrödl M, **Jörger KM** (2011) Systematics and redescription of the european meiofaunal slug *Microhedyle glandulifera* (Kowalevsky, 1901) (Heterobranchia: Acochlidia): evidence from molecules and morphology. *Journal of Molluscan Studies* 77: 388-400.

Co-author publications:

Brenzinger B, Neusser TP, **Jörger KM**, Schrödl M (2011) Integrating 3D microanatomy and molecules: natural history of the Pacific freshwater slug *Strubellia* Odhner, 1937 (Heterobranchia, Acochlidia) with description of a new species. *Journal of Molluscan Studies* 77: 351-374.

Kohnert P, Neusser TP, **Jörger KM**, Schrödl M (2011) Time for sex change! 3D-reconstruction of the copulatory system of the 'aphallic' *Hedylopsis ballantinei* (Gastropoda, Acochlidia). *Thalassas* 27: 113-119.

Neusser TP, **Jörger KM**, Schrödl M (2007) Exploring cerebral features in Acochlidia (Gastropoda: Opisthobranchia). *Bonner Zoologische Beiträge* 55: 301-310.

Neusser TP, Fukuda H, **Jörger KM**, Kano Y, Schrödl M (2011) Sacoglossa or Acochlidia? 3D-reconstruction, molecular phylogeny and evolution of Aitengidae (Gastropoda: Heterobranchia). *Journal of Molluscan Studies* 77: 332-350.

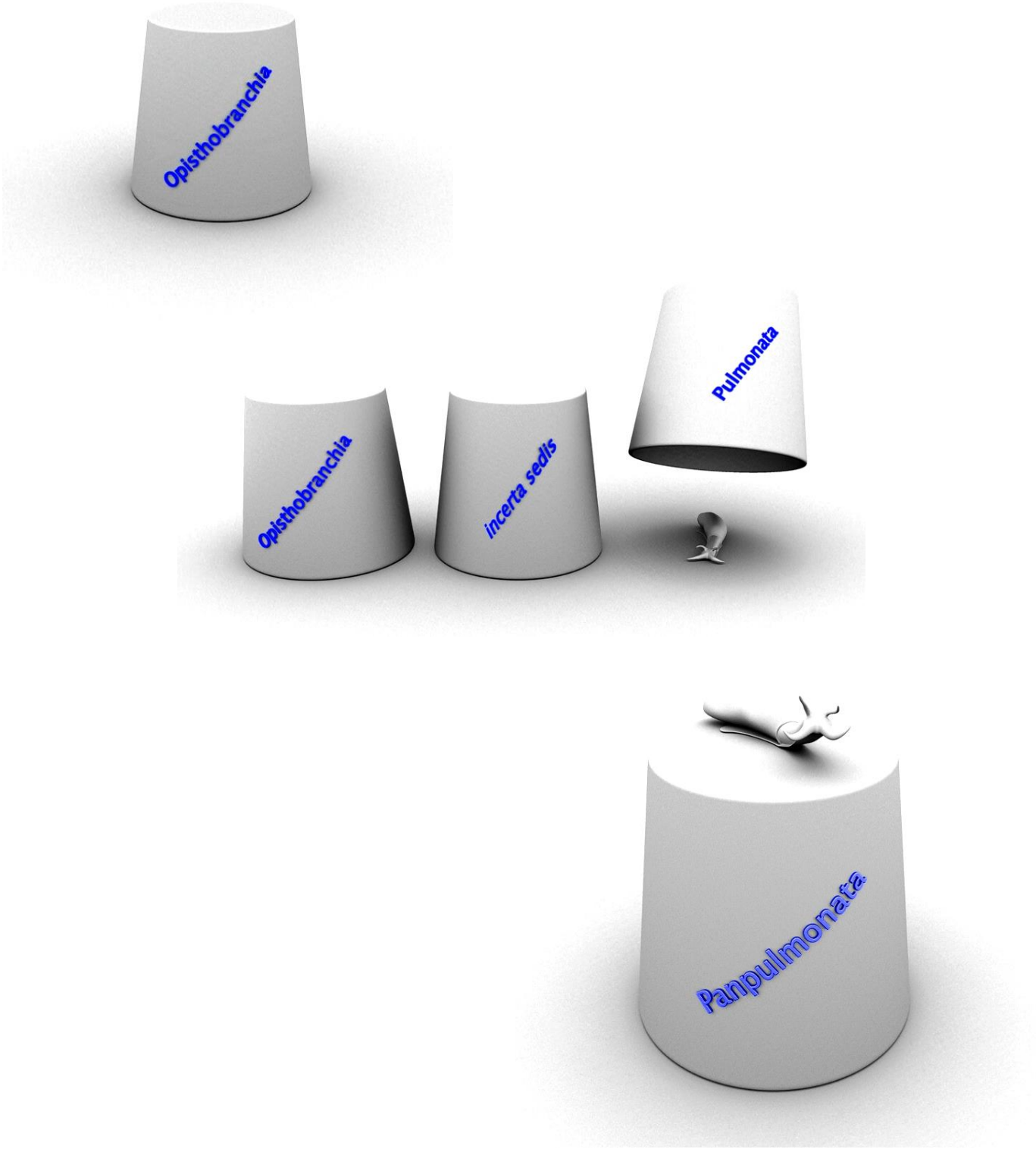
Neusser TP, **Jörger KM**, Schrödl M (2011) Cryptic species in tropic sands - Interactive 3D anatomy, molecular phylogeny and evolution of meiofaunal Pseudunelidae (Gastropoda, Acochlidia). *PLoS ONE* 6: e23313.

Schrödl M, **Jörger KM**, Klussmann-Kolb A, Wilson NG (2011) Bye bye 'Opisthobranchia'! A review on the contribution of mesopsammic sea slugs to euthyneuran systematics. *Thalassas* 27: 101-112.

Schrödl M, **Jörger KM**, Wilson NG (2011) A reply to Medina et al. (2011): Crawling through time: Transition of snails to slugs dating back to the Paleozoic based on mitochondrial phylogenomics. *Marine Genomics* 4: 301-303.

The publications in the following chapters are not arranged chronologically, but are grouped in accordance with the major aims of the thesis.

I. ORIGIN OF ACOCHLIDIA



Chapter 1. On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia

Jörger KM, Stöger I, Kano Y, Fukuda H, Knebelsberger T, Schrödl M (2010) On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia. *BMC Evolutionary Biology* 10: 323.

A pdf of the article is available at:

http://www.biomedcentral.com/1471-2148/10/323

Jörger et al. BMC Evolutionary Biology 2010, 10:323
http://www.biomedcentral.com/1471-2148/10/323

BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia

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Abstract

Background: A robust phylogenetic hypothesis of euthyneuran gastropods, as a basis to reconstructing their evolutionary history, is still hindered by several groups of aberrant, more or less worm-like slugs with unclear phylogenetic relationships. As a traditional "order" in the Opisthobranchia, the Acochlidia have a long history of controversial placements, among others influenced by convergent adaptation to the mainly meiofaunal habitats. The present study includes six out of seven acochlidian families in a comprehensive euthyneuran taxon sampling with special focus on minute, aberrant slugs. Since there is no fossil record of tiny, shell-less gastropods, a molecular clock was used to estimate divergence times within Euthyneura.

Results: Our multi-locus molecular study confirms Acochlidia in a pulmonate relationship, as sister to Eupulmonata. Previous hypotheses of opisthobranch relations, or of a common origin with other meiofaunal Euthyneura, are clearly rejected. The enigmatic amphibious and insectivorous Aitengidae *incerta sedis* clusters within Acochlidia, as sister to meiofaunal and brackish Pseudunelidae and limnic Acochlidiidae. Euthyneura, Opisthobranchia and Pulmonata as traditionally defined are non-monophyletic. A relaxed molecular clock approach indicates a late Palaeozoic diversification of Euthyneura and a Mesozoic origin of the major euthyneuran diversity, including Acochlidia.

Conclusions: The present study shows that the inclusion of small, enigmatic groups is necessary to solve deep-level phylogenetic relationships, and underlines that "pulmonate" and "opisthobranch" phylogeny, respectively, cannot be solved independently from each other. Our phylogenetic hypothesis requires reinvestigation of the traditional classification of Euthyneura: morphological synapomorphies of the traditionally defined Pulmonata and Opisthobranchia are evaluated in light of the presented phylogeny, and a redefinition of major groups is proposed. It is demonstrated that the invasion of the meiofaunal habitat has occurred several times independently in various euthyneuran taxa, leading to convergent adaptations previously misinterpreted as synapomorphies. The inclusion of Acochlidia extends the structural and biological diversity in pulmonates, presenting a remarkable flexibility concerning habitat choice.

Background

Since the introduction of the Heterobranchia concept by Haszprunar [1,2], considerable advances have been achieved, solving the phylogeny of certain heterobranch groups (i.e. "families" or "orders") on morphological (e.g. Mikkelsen [3] on Cephalaspidea; Jensen [4] on Sacoglossa; Wägele and Willan [5] on Nudibranchia, Klussmann-Kolb [6] on Aplysiidae) and molecular levels (e.g. Wollscheid-Lengeling et al. [7] on Nudibranchia; Wade et al. [8] on Stylommatophora; Klussmann-Kolb and Dinapoli [9] on Pteropoda). Members of the Euthyneura - the major heterobranch clade - have conquered marine, limnic and terrestrial habitats from the deep sea to the high mountains. As a result they form one of the most successful and diverse groups within Gastropoda, and even within Mollusca as regards species numbers and ecological diversity. Quite some effort has been dedicated to revealing relationships in the taxon, and to

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supporting or rejecting the respective monophyly of traditional higher groupings such as Pulmonata and Opisthobranchia. Nevertheless, the phylogeny of the Euthyneura has remained partially unresolved and heavily discussed [see e.g. [10-17]]. While morphological analyses face the problem of convergent developments that might mask the true phylogenetic signal, and depend on the coding procedure for morphological characters [18], single-marker molecular analyses are challenged in choosing a suitable marker, and multi-locus molecular studies stand and fall with the available taxon sampling.

One major problem in molecular studies is that highly aberrant or derived taxa of uncertain taxonomic relationships "jump around" in phylogenetic analyses and weaken the phylogenetic signal for higher taxa. Members of such groups are often hard to obtain (especially for molecular purposes); thus, the groups are frequently either excluded from phylogenetic analyses or only included with a low number of representatives, resulting in poor overall taxon sampling. One attempt to support future phylogenetic approaches on a higher taxonomic level (i.e. Heterobranchia or Gastropoda) is to provide data on small enigmatic groups and their phylogenetic relationships step by step.

The Acochlidia, a traditional "order" of the Opisthobranchia since their establishment by Odhner [[19]; as Acochlidiacea], form one of the unsolved mysteries within Euthyneura [18]. Being a small group with only 28 valid species worldwide, these slugs are morphologically and biologically highly aberrant and diverse, comprising a series of unusual characters (e.g. secondary gonochorism, lack of copulatory organs, asymmetric radulae) [see e.g. [20-23]]. Most acochlidians live interstitially in marine sands, while some have conquered limnic systems (uniquely within opisthobranch gastropods). Their monophyly is widely accepted [20,22,24,25] especially since a proposed sister group relationship of the acochlidian family Ganitidae with Sacoglossa (based on the dagger-shaped radula teeth, see [26]) could be rejected based on a comprehensive parsimony analysis of morphological characters [22]. During the last years a series of studies have redescribed key acochlidian taxa in great detail, including 3D reconstructions [27-32], and added considerably to the morphological and biological knowledge of this previously little understood group. A first comprehensive cladistic analysis of their phylogeny is now established [22], but the identity of their sister group remains uncertain. Most recent morphological analyses suggested a common origin with either the equally enigmatic Rhodopemorpha [10], the diaphanid cephalaspidean *Toledonia* [25], or with runcinid or philinoid cephalaspideans [22,33]. However, morphology-based analyses by Schrödl and Neusser [22], demonstrated that Acochlidia usually group with other mesopsammic taxa, if any were included (i.e. with the sacoglossan *Platyhedyle*, the rhodopemorph *Rhodope* or the cephalaspideans *Philinoglossa* or *Philine exigua*). Thus, it is likely that convergent adaptations to the interstitial habitat mask the truly phylogenetic signals. Molecular markers independent from direct ecological pressures suggested an unresolved basal opisthobranch origin for Acochlidia ([34] based on nuclear 18S rRNA and 28S rRNA). A first combined multi-gene dataset led to the surprising result of Acochlidia clustering in a pulmonate relationship, united in a clade with Pyramidelloidea, Amphiboloidea and Eupulmonata [17]. However, only three derived acochlids [see [22]] were included, with partially missing data. Therefore this unexpected result requires re-examination based on complete multi-locus data and a more focused taxon sampling, including all previously suggested potential sister groups of Acochlidia. Most recently, another curiosity with potential affinities to Acochlidia has been described: the amphibious and insectivorous sea slug *Aiteng ater* from mangrove mud in Thailand [35]. Due to its unusual combination of morphological characters (prepharyngeal nerve ring, presence of ascus, uniseriate radula) it was placed in a new family, Aitengidae, with unclear phylogenetic relationships and affinities to Sacoglossa, Acochlidia and Cephalaspidea. A similar but still undescribed species was found in Japan, which was available for the present study. Morphologically it clearly belongs to the Aitengidae, but shows differences to *A. ater* at genus or species level (own unpublished data). Its affinity to *A. ater* is confirmed by comparison of the mitochondrial 16S rRNA-sequences (K. Händeler, pers. comm.).

The present study aims to clarify the origins and phylogenetic relationships of Acochlidia and potentially related enigmatic taxa such as Aitengidae, based on a combined molecular dataset from nuclear and mitochondrial markers. For the first time, representatives of six out of seven acochlidian families [22] are analysed in the context of a broad taxon sampling that includes other meiofaunal slugs (*Philinoglossa praelongata*, *Philine exigua*, *Smeagol phillipensis*) and most euthyneuran sub-groups. Furthermore, the potentially related *Gascoignella nukuli* (as a representative of Platyhedylidae) and an undescribed species of Aitengidae are included in the present study. Since there is no fossil record of Acochlidia or any other mesopsammic Euthyneura, we apply a molecular clock approach to estimate divergence times for these groups. On the basis of our phylogenetic hypothesis we discuss evolutionary trends and potential consequences for euthyneuran classification in general.

Results

Neighbournet analysis

The neighbournet graph created by SplitsTree 4 (see Additional File 1) visualises a generally high conflict in

the data (shown by a netlike structure with edges of similar length), and high substitution rates displayed by long terminal branches in many taxa. There is no clade-supporting pattern for the monophyly of Opisthobranchia or of Pulmonata on the basis of our dataset. Of the major traditional heterobranch taxa only Acteonoidea and Nudipleura show a clear split support (visualised by long parallel edges); some split support is present for Pyramidelloidea, Cephalaspidea s.s., Anaspidea, Umbraculoidea, pteropod Gymnosomata and Thecosomata, Amphiboloidea and Siphonarioidea. No pattern supporting any of the other opisthobranch or pulmonate groups can be found, mainly due to affinities of individual species to neighbouring groups. No split pattern indicates a relationship between the different meiofaunal heterobranchs such as Acochlidia, *Smeagol phillipensis* and Philinoidea (*Philinoglossa praelongata* and *Philine exigua*) (see Additional File 1).

The monophyly of the Acochlidia receives no split support. A very weak signal supports a grouping of Acochlidia together with some pulmonate taxa, but there is no indication for affinities to other opisthobranch taxa. The acochlidian subgroups Hedylopsacea and Microhedylacea receive no split support, due to some common support for *Hedylopsis* (Hedylopsacea) and *Asperspina* (Microhedylacea). The enigmatic Aitengidae sp. receives split support grouped with acochlidian Pseudunelidae and Acochlidiidae, and shows no affinity to Sacoglossa or Cephalaspidea.

Phylogenetic analysis

Examination of differences in incongruence length between the four genetic markers - 18S rRNA, 28S rRNA, 16S rRNA and cytochrome *c* oxidase subunit I (COI) - using the ILD-test implemented in PAUP* [36] revealed that the phylogenetic signal is improved in the combined data set (p-value of 0.01). Thus a concatenated dataset was used for phylogenetic analyses. The likelihood values of the different partitions of the dataset were compared via the Akaike Information Criterion (AIC) and the separation into 5 partitions (one each for 18S, 28S and 16S; COI separated in the two partitions 1st and 2nd position and 3rd position) improved the likelihood significantly (see Additional File 2). The dataset aligned with MAFFT, masked with Gblocks and analysed in 5 partitions led to the best likelihood value, thus it is presented herein as the most probable phylogenetic hypothesis based on our data (see Figure 1). For comparison of the different analytical approaches and the resulting differences in tree topology and related support values, see Table 1.

The Euthyneura form a monophyletic group without significant bootstrap support (BS) in ML-analyses, or posterior probability (PP) in Bayesian analyses. They do not include the Acteonoidea (sister to "lower heterobranch" Rissoelloidea) in most of our analyses, but include the Pyramidelloidea and Glacidorboidea as sister group to Amphiboloidea. Within the Euthyneura the Opisthobranchia clearly result as non-monophyletic. At the basis of the Euthyneura the Nudipleura split off, with high internal support. The clade of the remaining euthyneuran taxa receives good support (85 BS/1.0 PP). First, an opisthobranch clade (no significant BS/1.0) is composed of Umbraculoidea, Runcinacea, Cephalaspidea s.s., Anaspidea and Pteropoda, with Umbraculoidea as the most basal branch. The runcinid *Runcina africana* forms the sister group to the Anaspidea and the well backed (82/1.0) Pteropoda (Gymnosomata and Thecosomata), and the above combined are sister to the remaining Cephalaspidea s.s., with high support for monophyly of Cephalaspidea s.s. (100/1.0). Internally the Cephalaspidea s.s. are poorly resolved, and their internal topology differs between the RAxML and Bayesian analyses (see Table 1). The mesopsammic *Philine exigua* and *Philinoglossa praelongata* do not form a clade: *P. praelongata* clusters with *Scaphander lignarius*, whereas no clear sister group relationship could be identified for *P. exigua*.

The Pulmonata as traditionally defined result as non-monophyletic due to the inclusion of the opisthobranch groups Sacoglossa and Acochlidia and of the "lower" heterobranch Pyramidelloidea and Glacidorboidea. The pulmonate clade is significantly supported (75/1.0), but internally characterised by an unstable topology, with no or low support concerning the sister group relationships between the major groups. Siphonarioidea and Sacoglossa form a clade (lacking significant support) sister to the remaining taxa (see Figure 1). In the analyses of the ALISCORE dataset Siphonarioidea form the most basal group, followed by a split-off of the Sacoglossa (see Table 1). The monophyletic Sacoglossa (98/1.0) combine clades with shelled and shell-less representatives, with *Gascoignella nukuli* (Platyhedylidae) as the most basal offshoot of the latter. Siphonarioidea + Sacoglossa are recovered as sister group to a clade composed of (Glacidorboidea + (Amphiboloidea + Pyramidelloidea)) + (Hygrophila + (Eupulmonata + Acochlidia)). Apart from Acochlidia, the monophyly of all higher taxa is well supported: Amphiboloidea (100/1.0), Pyramidelloidea (99/1.0), Hygrophila (86/1.0) and Eupulmonata (93/1.0). However, relations between these taxa are poorly resolved, not supported, and vary within the different analyses (see Table 1). In all our analyses Amphiboloidea cluster with Glacidorboidea and Pyramidelloidea. Thus Thalassophila (= Siphonarioidea and Amphiboloidea) and Basommatophora (= Thalassophila and Hygrophila) are left as polyphyletic. The Eupulmonata (Stylommatophora, Systellommatophora, Ellobioidea, Trimusculoidea and Otinoidea) are recovered sister to

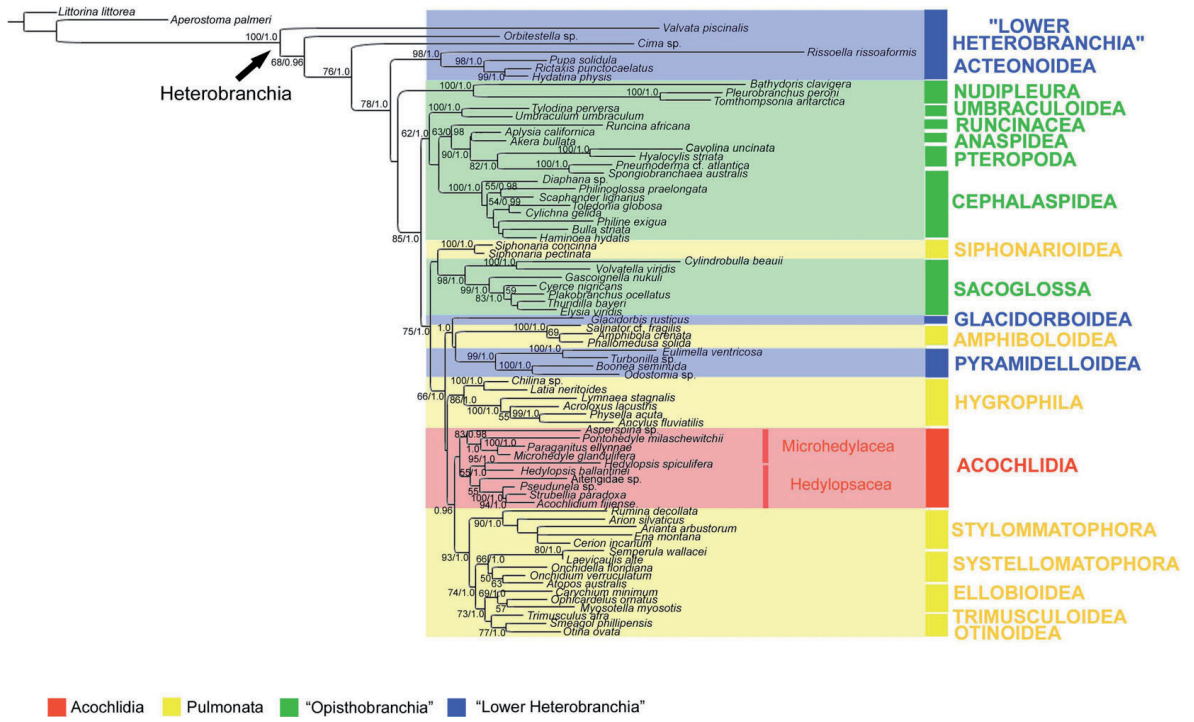

Figure 1 Origin of Acochlidia within Heterobranchia. RAxML analysis based on the combined dataset (18S, 28S, 16S, COI), masked with Gblocks. Bootstrap support and posterior probabilities given (only BS ≥ 50 and PP ≥ 0.95 are considered relevant and thus shown).

Table 1 Summary of the different analyses conducted

Sequence alignment/ masking/ phylogenetic analysis	Model of sequence evolution	Length of alignment	Monophyly of Acochlidia and sister group relationship	Changes within the tree topology compared to Figure 1
MAFFT + Gblocks + RAxML	GTRCAT GTR + GAMMA	3641 bp	see Figure 1	see Figure 1
MAFFT + ALISCORE + RAxML	GTRCAT GTR + GAMMA	3926 bp	Acochlidia monophyletic (no BS support) Aitengidae basal within Hedylopsacea; Acochlidia sister to (Hygrophila + (Glacidorboidea + (Amphiboloidea + Pyramidelloidea))) (no BS support)	Anaspidea non-monophyletic; different internal topology of Cephalaspidea s.s. (Philine exigua basal to remaining taxa); Siphonarioidea and Sacoglossa form no clade, but Siphonarioidea + (Sacoglossa + remaining pulmonate taxa)
MAFFT + Gblocks + MrBayes	GTR + G + I	3641 bp	Acochlidia monophyletic (no significant PP); sister group to Eupulmonata (0.96 PP)	basal tritomy within Euthyneura: (Acteonoidea + Rissoelloidea)/Nudipleura/remaining Euthyneura; different internal topology of Cephalaspidea s.s. (Philine exigua basal to remaining taxa), ((Glacidorboidea + Amphiboloidea) + Pyramidelloidea)
MAFFT + ALISCORE + MrBayes	GTR + G + I	3926 bp	Acochlidia monophyletic (no significant PP) Aitengidae basal within Hedylopsacea; Acochlidia sister to (Hygrophila + (Glacidorboidea + Amphiboloidea + Pyramidelloidea)) (no significant PP)	Anaspidea non-monophyletic; different internal topology of Cephalaspidea s.s. (Philine exigua basal to remaining taxa); Siphonarioidea and Sacoglossa form no clade, but Siphonarioidea + (Sacoglossa + remaining pulmonate taxa); Nudipleura form a basal clade with (Acteonoidea + Rissoelloidea)

The table lists the different methods of masking the alignment, phylogenetic approaches and models of sequence evolution used for the different analyses, as well as the resulting differences in tree topology (bootstrap support = BS; posterior probability = PP).

Acochlidia. Within Eupulmonata Stylommatophora (90/1.0) form the basal group; Systellommatophora (no significant BS/1.0) is sister to a clade Ellobioidea + (Trimusculoidea + Otinoidea), the latter comprising *Smeagol phillippensis* and *Otina ovata*.

Acochlidia are recovered as monophyletic but with no significant support. The internal phylogeny of the Acochlidia is composed of the two monophyletic traditional suborders Hedylopsacea (with Hedylopsidae, Pseudunelidae and Acochlidiidae) and Microhedylacea (with Asperspinidae and Microhedylidae including Ganitidae), and is congruent with the morphology-based phylogeny of Acochlidia proposed by Schrödl and Neusser [22]. Additionally the enigmatic Aitengidae sp. clusters within the Hedylopsacea as sister group to Pseudunelidae and Acochlidiidae (see Figure 1) or basal within Hedylopsacea.

In analyses of Gblock datasets Acochlidia are sister to Eupulmonata (see Figure 1), in ALISCORE based analyses they cluster sister to Hygrophila + (Glacidorboidea + Amphiboloidea + Pyramidelloidea) (see Table 1). To assess the level of confidence of the "best" tree (i.e. pulmonate relationship of Acochlidia), we calculated the p-values of an alternative topology (Acochlidia cluster within Opisthobranchia) in combination with the "best" tree topology. Based on the resulting p-values of the AU test the alternative hypothesis is highly significantly rejected (AU value = 0).

Molecular clock

The phylogenetic hypothesis obtained with the software BEAST (see Figure 2) based on the concatenated four-marker Gblocks dataset largely confirms the topology obtained from RAxML and MrBayes (see Figure 1). Based on the three fossil calibration points the Euthyneura originated already in the Palaeozoic, probably in the Carboniferous or Permian. The diversification of Euthyneura with the rise of many extant taxa started approximately in the late Palaeozoic (Permian) and major divergence events occurred in the Mesozoic. On the basis of our analysis the pulmonate clade (also including Sacoglossa, Acochlidia, Pyramidelloidea and Glacidorboidea) first appeared in the late Palaeozoic to early Mesozoic, approximately at the Permian/Triassic transition. The split between Eupulmonata and Acochlidia took place in the Mesozoic, between the Triassic and Jurassic periods. The diversification of Acochlidia is estimated to have happened in the Jurassic with the split between Hedylopsacea and Microhedylacea. Aitengidae split off from Pseudunelidae and Acochlidiidae in the Cretaceous. The transition to limnic habitats within Acochlidiidae appears as a comparatively recent event dating to the Palaeogene.

According to our data, major opisthobranch groups originated also in the Mesozoic (e.g. Cephalaspidea s.s. estimated to the Jurassic, Sacoglossa approximately Triassic/early Jurassic period, Pteropoda to the Cretaceous).

For comparison and to evaluate the impact of removing ambiguous parts of the alignment on molecular clock analyses, we repeated the analysis with the raw (i.e. uncut) alignment of our data (again using the concatenated four-marker dataset in five partitions). Even though the topology varied slightly from the one in the previous analysis, the estimated divergence times stayed surprisingly constant, supporting the rough estimate given above.

Discussion

Implications for the phylogeny of Heterobranchia

Our results on the origin of Acochlidia - in congruence with previous molecular studies on Euthyneura based on the same molecular markers [14,17] - necessitate the reconsideration of current classification concepts. Redefinitions below aim to observe continuity in traditional nomenclature and cause the unavoidable minimum of changes in terminology.

Euthyneura

The monophyly of Euthyneura (traditionally uniting Opisthobranchia and Pulmonata) has been widely accepted and well supported [13,18,37], even though their eponymous apomorphy - the euthyneury - has been revealed as convergent development [1,2]. Euthyneuran monophyly was recently questioned due to inclusion of "lower Heterobranchia" Pyramidelloidea unresolved within Pulmonata [13,15,16] or sister to Amphiboloidea [14,17]. Some other morphological studies place Pyramidelloidea as sister to Euthyneura [10,33]. Dinapoli and Klussmann-Kolb [14] argued to include them within Euthyneura, which has also been supported by morphological analysis [13]. Latest molecular data on Pyramidelloidea support an euthyneuran origin and indicate a relationship with Glacidorboidea and Amphiboloidea [38]. Our data again recovers Pyramidelloidea as sister to Amphiboloidea within pulmonates (see Figure 1), but with no significant support. In addition to nucleotide sequences [[14,15,17], present study], data from mitochondrial gene arrangements [16], a "morpho-molecular" synapomorphy (20 bp deletion in 16S rRNA helix of Pyramidelloidea and Euthyneura, see [11]) as well as morphology (presence of a euthyneurous nervous system with giant nerve cells) all support the inclusion of Pyramidelloidea within Euthyneura. When first describing Glacidorboidea, Ponder [39] placed them within Pulmonata and discussed a relationship to Amphiboloidea. However, Haszprunar [2] moved them to "lower Heterobranchia". The first molecular data on Glacidorboidea confirmed a pulmonate relationship [14]. This is again supported by our data.

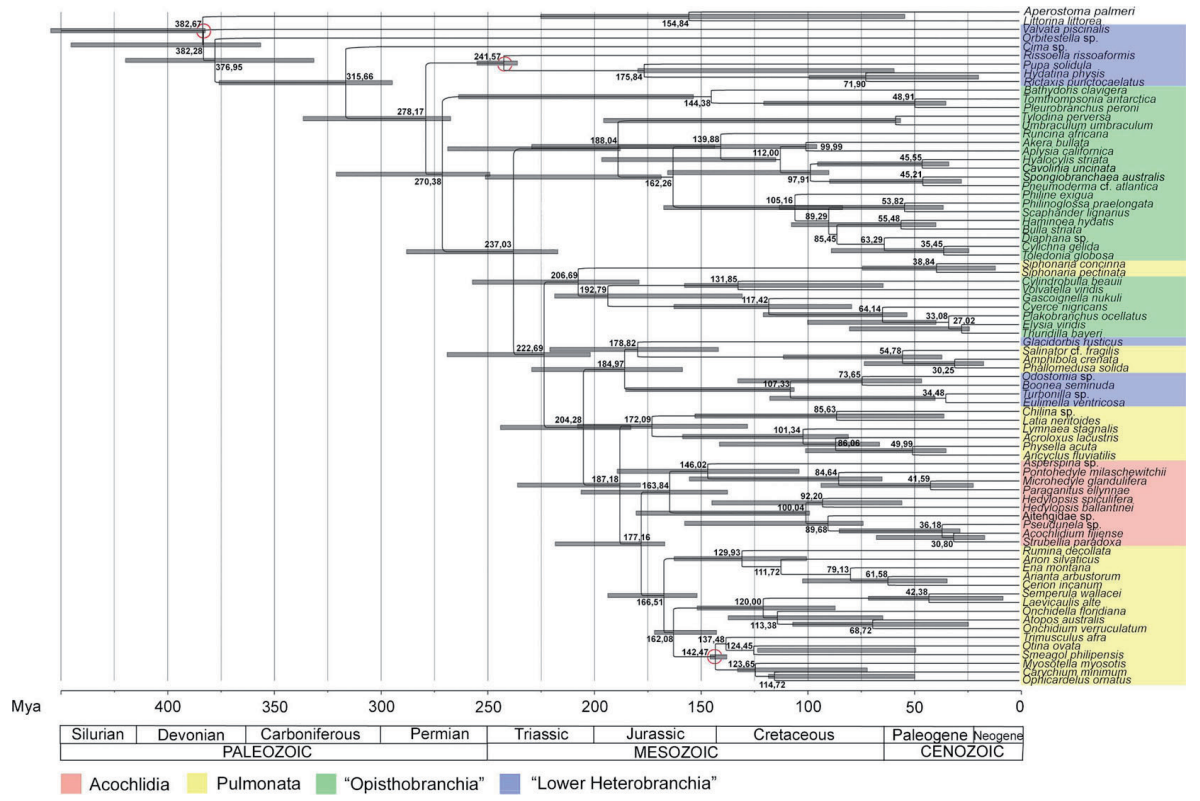

Figure 2 Chronogram of Heterobranchia. Showing estimated divergence times obtained from BEAST v1.5.3 under the uncorrelated lognormal relaxed clock model. Numbers at nodes refer to node ages in this presented tree (maximum-clade-credibility-tree); bars express 95% highest posterior density (HPD) (only presented for nodes with a PP > 0.5). Red circles indicate calibration points. Geological timescale is based on the International Stratigraphic Chart by the International Commission on Stratigraphy (2009).

"Opisthobranchia"

While the monophyly of several opisthobranch subgroups (e.g. Pteropoda, Cephalaspidea s.s., Nudipleura) receives good support, the monophyly of the Opisthobranchia in a traditional sense is rejected in all recent studies, regardless of whether the latter are molecular or morphological [e.g. [14,17,40]]. This is confirmed by our multi-locus molecular approach (see Figure 1) and supported by the results of the AU test. Thus, "Opisthobranchia" as traditionally defined should be considered as non-monophyletic.

As in previous studies we can clearly distinguish at least two clades (i.e. basal Nudipleura and Umbraculoidea + Runcinacea + Anaspidea + Pteropoda + Cephalaspidea s.s.) within "Opisthobranchia" that lead towards the pulmonate level of organisation.

Only one of our analyses indicates the Acteonoidea sister to Nudipleura (see Table 1). This clade that had resulted repeatedly in molecular studies with still limited "lower heterobranch" taxon sampling, either in a derived position [34,41] or as a basal offshoot within Euthyneura [15,17]. A recent molecular phylogeny on Acteonoidea suggest a common origin with lower heterobranch Rissoelloidea and a sister group relationship to Nudipleura [42]. While the basal position of Acteonoidea was commonly accepted [33,40], some authors doubted the basal position of Nudipleura, which was originally considered as a highly derived taxon, and suspect rate heterogeneity and deviant base composition as causing this unnatural grouping [17,34]. Based on potential synapomorphies in the reproductive system (presence of a ciliary stripe within the ampulla, androdiaulic or triaulic pallial gonoduct), Ghiselin [43] already suggested a relationship between Acteonoidea and Nudipleura. However, Acteonoidea form a well-supported "lower heterobranch" clade with Rissoelloidea, (see Figure 1; Table 1), confirming results by Aktipis et al. [44] and Dinapoli and Klussmann-Kolb [14]. The latter authors also recovered Nudipleura as the first offshoot of Euthyneura, which is confirmed by our study. Salvini-Plawen and Steiner [10] grouped Umbraculoidea with Nudipleura, but none of the recent molecular or morphological studies support such a relationship [17,33,34].

A common clade including Umbraculoidea, Anaspidea, Cephalaspidea s.s. and Pteropoda was already well supported in previous molecular analyses [9,14,17], and monophyly of a clade Anaspidea + Pteropoda received strong support in one previous study [12]. The present results confirm Cephalaspidea s.s., including Diaphanidae, but excluding Runcinidae as suggested in a previous analysis [45]. In our study *Runcina africana* groups with Anaspidea and Pteropoda, as in the Bayesian analysis of the concatenated 18S rRNA, 28S rRNA and COI dataset of the more comprehensive cephalaspidean phylogeny by Malaquias et al. [45]. The latter authors thus proposed to reinstate Runcinacea as a taxonomic category equivalent to Cephalaspidea s.s.. However, different analyses of the same authors led to different placements of Runcinacea, e.g. as sister to the remaining Cephalaspidea s.s.; hence the group's origin was left unresolved. Surprisingly our study indicates independent origins for the mesopsammic *Philine exigua* (Philinidae) and *Philinoglossa praelongata*(Philinoglossidae). The internal topology of Cephalaspidea s.s. is weakly supported in our study, but a more complete cephalaspidean sampling also rendered Philinoidea paraphyletic (based on 18S and 28S) [45].

Based on our results and in congruence with the topology in previous studies [14,17], we suggest to unite Umbraculoidea, Anaspidea, Runcinacea, Pteropoda and Cephalaspidea s.s. in the new clade Euopisthobranchia (see Figure 3), presenting a monophyletic remainder of the "Opisthobranchia" as traditionally defined. Previous studies [9,18] discussed the gizzard (i.e. a muscular oesophageal crop lined with cuticula) with gizzard plates as homologous apomorphic structures supporting a clade composed of Cephalaspidea s.s., Pteropoda and Anaspidea. A gizzard with gizzard plates probably originated in herbivorous taxa in which it worked like a grinding mill, thus might be secondarily reduced in carnivorous groups within Cephalaspidea s.s. and Gymnosomata [9]. Klussmann-Kolb and Dinapoli [9] considered the gizzard in Umbraculoidea as non-homologous with the one in the previous groups, on account of the absence of gizzard plates or spines. This contradicted Salvini-Plawen and Steiner [10], who had proposed the gizzard to be a synapomorphy of the larger clade of Paratectibranchia (Pteropoda, Cephalaspidea and Anaspidea) and Eleutherobranchia, secondarily lost in Nudipleura but still present in Umbraculoidea. As coded in Wägele and Klussmann-Kolb [33], our phylogenetic hypothesis supports homology of the gizzard in Umbraculoidea with the gizzard with gizzard plates and spines in the other euopisthobranchian taxa. Thus, the structure is proposed as a synapomorphy of Euopisthobranchia.

"Pulmonata"

The monophyly of Pulmonata as traditionally defined has been well supported in morphological analyses (see e.g. [10,13]) and molecular studies [8,46]. However, doubts have arisen recently due to molecular studies which recovered additional taxa (e.g. Pyramidelloidea, Sacoglossa or Acochlidia) within "Pulmonata" [14,17], or to novel studies based on mitochondrial gene arrangements [16] which rendered "Pulmonata" polyphyletic. Based on our phylogenetic hypothesis (Figure 1) "Pulmonata" as traditionally defined is non-monophyletic due to the inclusion of Pyramidelloidea, Glacidorboidea, Sacoglossa and Acochlidia. On the premise of monophyletic Euthyneura, with basal Nudipleura and monophyletic Euopisthobranchia (see discussion above), the remaining euthyneuran taxa necessarily form a clade, in our study supported with maximum posterior probability (1.0) and significant bootstrap support (75%) (see Figure 1). Even though the topology within this pulmonate clade is unstable and not well resolved (see Table1), for practical reasons and due to the assumptions of monophyletic Euthyneura and Euopisthobranchia we suggest the new taxon Panpulmonata to unite Siphonarioidea, Sacoglossa, Glacidorboidea, Pyramidelloidea, Amphiboloidea, Hygrophila, Acochlidia and Eupulmonata (see Figure 3). The scientific meaning of the name "Pulmonata" and the corresponding major feature of those animals being "air-breathers" surely are not applicable to the novel panpulmonate groups Acochlidia, Sacoglossa and Pyramidelloidea, but also not for traditional pulmonate taxa such as Siphonarioidea or Hygrophila, most members of which lack permanently air-filled lungs. The term Panpulmonata is chosen for continuity in terminology. While certain pulmonate groups are well supported morphologically and molecularly (i.e. Eupulmonata and Hygrophila), unambiguous synapomorphies for Panpulmonata are hard to find (see discussion below).

Siphonarioidea and Sacoglossa form a clade sister to the remaining Panpulmonata (see Figure 3). While Haller [47] classified Siphonarioidea as opisthobranchs (e.g. on account of the presence of a gill), nowadays they are usually considered as "primitive" pulmonates, either grouped at the basis of the remaining Pulmonata [37,46] or united with Amphiboloidea as basommatophoran Thalassophila [48]. Molecular studies rendered Basommatophora and Thalassophila paraphyletic and indicated a close relationship of Siphonarioidea to Sacoglossa, either both within Opisthobranchia [16], at their basis [15], or basal to the remaining Pulmonata [[14,17], present study] as sister groups or separate clades. However, all studies show weak support at these nodes, and the positions of siphonariids and sacoglossans as well as

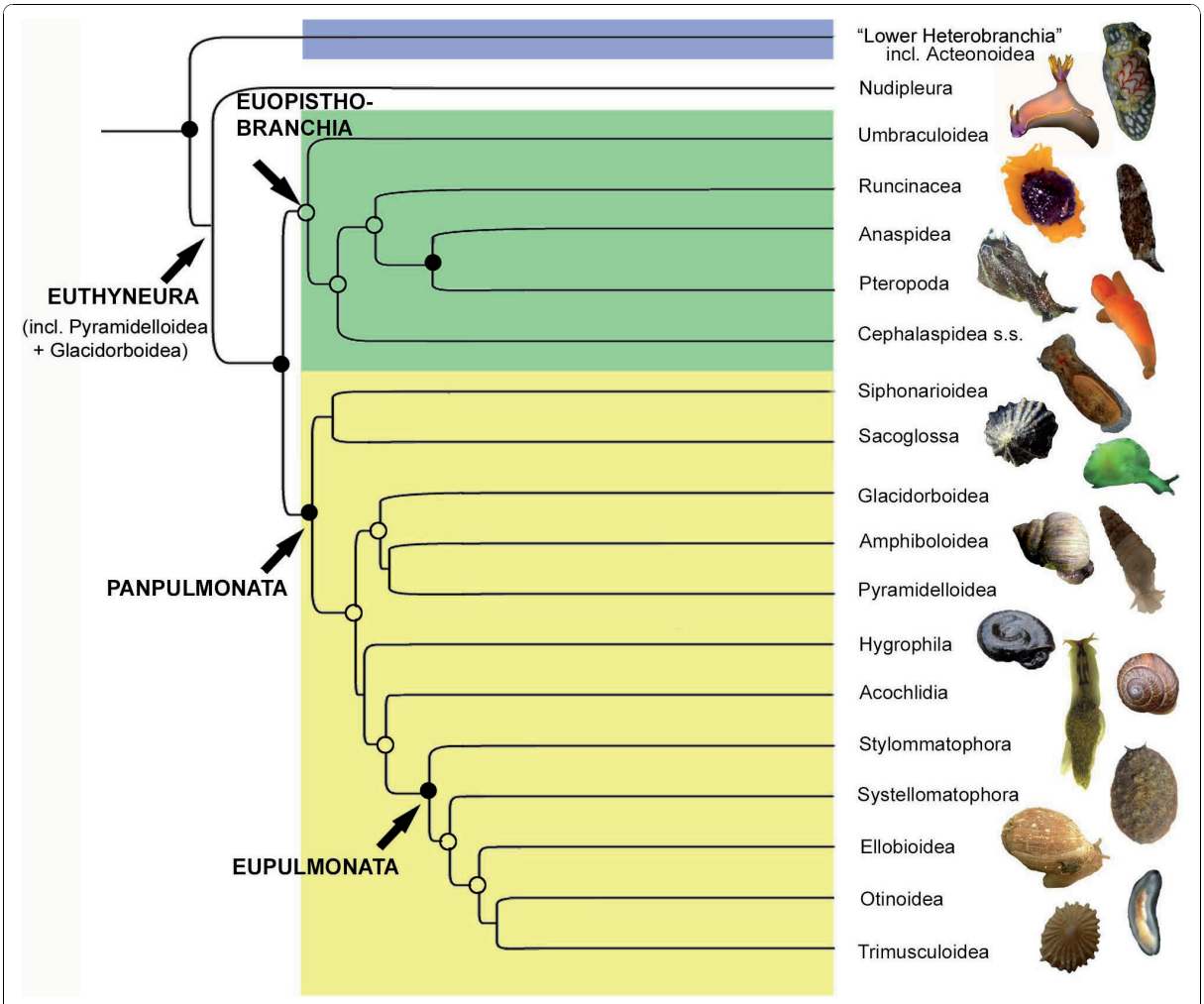

Figure 3 Proposed reclassification of Euthyneura, discussed groups shown. Nodes with significant support (i.e. > 75% bootstrap support (BS) and > 0.95 posterior probability (PP)) marked by dots; nodes with > 0.95 PP but low BS marked by circles. (Note: "Lower Heterobranchia" does not form a clade in the present study (see Figure 1), the branches are collapsed in the present Figure for illustration purposes.)

their relationship still need confirmation by other character sets and improved taxon sampling.

In the present study the monophyly of Sacoglossa is well supported and also the split into shelled Oxynoacea and Plakobranchacea is well backed (see Figure 1). Both suborders are also well supported morphologically [4]. Platyhedylidae stand basally within the latter, as sister to Limapontioidea plus the remaining Plakobranchoidea. Jensen [4] placed Platyhedylidae at the basis of Plakobranchoidea but already pointed out their unclear relationships.

Hygrophila, Amphiboloidea and Eupulmonata are all well supported monophyletic groups in the present study, but their sister group relationships are not well resolved and receive little to no support.

Origin of Acochlidia

All groups previously discussed as having an affinity or closer relationship to Acochlidia were included in the present study to reveal their phylogenetic relationships. Only the enigmatic Rhodopemorpha are lacking, but a recent molecular phylogeny based on nuclear and mitochondrial markers shows no affinities between Acochlidia and Rhodopemorpha [49], and the morphological characters common to both groups can be explained as convergent developments (see discussion below and [22]). A phylogenetic relationship of Acochlidia with the diaphanid *Toledonia*, which was suggested based on similar radula characteristics [25], is rejected by the present molecular data and also resulting from morphological analyses [22]. Morphological studies indicated a

common origin for small Runcinacea and Cephalaspidea (i.e. mesopsammic *Philinoglossa* and *Philine exigua*) with Acochlidia [22,33]. However, Schrödl and Neusser [22] showed the liability of the topology to inclusion of other interstitial taxa such as *Rhodope* and *Platyhedyle*, which always resulted as direct sister groups to Acochlidia in various analyses. The authors thus concluded that the convergent adaptations to the interstitial habitat (e.g. worm-shaped body, development of spicules, loss of pigmentation) mask the true phylogenetic signal. This interpretation is supported by our SplitsTree analysis (see Additional File 1) and the present molecular results (see Figure 1), which clearly signal independent evolutionary origins for all the different mesopsammic Heterobranchia included here.

Previous molecular analyses placed the Acochlidia basally in an unresolved opisthobranch level [34] or surprisingly clustered them in an unresolved pulmonate relationship [17]. While any opisthobranch affinities are rejected based on split support (see Additional File 1), based on the AU test and based on phylogenetic analysis, the pulmonate relationship of Acochlidia is confirmed in this study (see Figure 1), which presents a much better acochlidian taxon sampling and highly likely topology within Acochlidia (see discussion below). Even though support for their direct sister group relationships are low and the topology varies between the different analyses, all analyses performed in the present study placed Acochlidia within pulmonates (see Table 1). This grouping based on molecular markers requires a re-evaluation of morphological characters and earlier, potentially biased homology assumptions, and a search for potential synapomorphies uniting Acochlidia with pulmonates. Three anatomical characters are generally accepted as true synapomorphies of the "Pulmonata" as traditionally defined: the pallial cavity opening by means of a pneumostome, presence of a procerebrum (with cerebral gland and double cerebro-connectives) and the existence of medio-dorsal (cerebral) bodies [13,50].

1) Pallial cavity opening by means of a pneumostome

Although denied by some earlier authors, the pulmonary cavity of "Pulmonata" is today generally considered as homologous to the pallial cavity of non-pulmonate gastropods [51]. Whereas the loss of a gill and the presence of a "lung" certainly is a matter of multiple convergence paralleled in several prosobranch clades, the acquisition of a pneumostome (i.e. a small respiratory opening) is considered as synapomorphic for "Pulmonata" [13,18,48]. Dayrat and Tillier [[13], see also references therein] pointed out that the pneumostome of Siphonarioidea is not contractile, and their phylogenetic hypothesis [13] favoured homology with the pneumostome of the remaining Pulmonata. On the other hand, at least some siphonariids are reported to open and close their pneumostome [e.g. [52]]. A morphocline from a wide open pallial cavity to a narrow, nearly closed one (i.e. presence of pneumostome) is present in both "Opisthobranchia" and "Pulmonata"; thus the presence of a pneumostome in general cannot be considered as a pulmonate synapomorphy [53]. Barker [53] also questioned the synapomorphic contractile pneumostome, which might have evolved independently in different pulmonate taxa, e.g. in Eupulmonata and some Siphonarioidea. The presence of a small opening seems to be variable, indeed, and might depend on the habitat. For example, the truly subtidal marine *Williamia* (Siphonarioidea) have a wide opening [54], while intertidal *Siphonaria* have a small one (i.e. a contractile or non-contractile pneumostome). The opening is wide also in subtidal shell-bearing Sacoglossa [3], whereas the pallial cavity is usually reduced in shell-less Sacoglossa. Pyramidelloidea also have a wide opening. In general within "Pulmonata" the "lung" undergoes a series of reductions; e.g., the tiny *Smeagol climoi* only has a small pallial cavity without respiratory function [51], as do larger Onchidiidae. A small, reduced pallial cavity can still be found in the quite basal acochlidian *Hedylopsis ballantinei* [55] (as *Hedylopsis* sp.), while all remaining Acochlidia studied so far entirely lack such a cavity [22,30]. All hedylopsacean nervous systems described in detail contain an osphradial ganglion [25,29,31,32], which can be interpreted as a remainder of an osphradium that was reduced in the course of the reduction of the pallial cavity. A group of derived, benthic and limnic acochlidians have developed a sensory, osphradium-like organ [56] like the one reported for the basal ellobiid *Ovatella* [57].

2) Presence of a procerebrum

The procerebrum of "Pulmonata" is defined as an accessory lobe linked to the cerebral ganglion via two connectives, associated to the optic, tentacular and peritentacular nerves [58]. Its homology with the opisthobranch rhinophoral ganglion has long been discussed [2,47,59]. The configuration of the cerebral nerves and associated ganglia is complex in Acochlidia. The labiotentacular nerve arises ventrally from the cerebral ganglion; the rhinophoral ganglion usually gives rise to the rhinophoral nerve (with Hancock's nerve branching off), and the optic ganglion to the optic nerve ([31,32,56] and own unpublished data). However, in *Pseudunela cornuta* the optic nerve splits off from the rhinophoral nerve, and no nerves arise from the optic ganglion [29]. A similar arrangement occurs in *Hedylopsis spiculifera* and *H. ballantinei*, except that the optic ganglion is lacking [25,60]. In the microhedylaceans *Pontohedyle* and *Microhedyle* the rhinophoral nerve emerges directly from the cerebral ganglion, and eyes nestle directly on it ([27], own unpublished data); thus the additional ganglion might refer to either the

rhinophoral or the optic ganglion. Tillier et al. [46] discussed a potential homology between the optic ganglion in "Opisthobranchia" and the pulmonate procerebrum. In Acochlidia double cerebral connectives could be identified for the rhinophoral ganglion in *Tantulum elegans* [60], the optic (but not the rhinophoral) in *Strubellia paradoxa* [56], and for the unclear optic/rhinophoral ganglion in *Pontohedyle milaschewitchii* and *Microhedyle glandulifera* ([27] as rhinophoral ganglion, own unpublished data). The variable development of cerebral features in Acochlidia makes homologisation difficult at this time. Rhinophoral and optic ganglia are closely related to and might develop from the cerebral ganglion, and they share common features with the pulmonate procerebrum. Based on our phylogenetic hypothesis, the plesiomorphic state for Panpulmonata might be separate rhinophoral and optic ganglia that have been fused various times independently. However, the presence of so-called "globineurons" - neurons with densely packed, small, round nuclei - in Eupulmonata [58,61] appears to be a synapomorphy for this clade.

Additionally, the presence of a cerebral gland - a small, tube-like structure involved in the formation of the procerebrum - is considered as characteristic for the pulmonate nervous system [58,61]. This ectodermal structure may form a tube-like process from the procerebrum towards the lateral head region, or it may be reduced to a small epithelial cavity attached or enclosed within the procerebrum [58,61]. No structure similar to the cerebral gland has been described for Acochlidia, but due to the small size of the cerebral gland and the previously unknown pulmonate affinities of Acochlidia it might have been overlooked in morphological studies; hence, ultrastructural reinvestigations of acochlidian nervous systems are needed in the future. The cerebral gland is lacking also in other pulmonate taxa, e.g. Amphiboloidea [58], which either raises doubts about their pulmonate affinities [46] or suggests that the structure might have been lost secondarily. Moreover, Tardy [62,63] described a similar invagination involved in the formation of the rhinophoral ganglion in different nudibranchs. In light of the present phylogenetic hypothesis, with Nudipleura as the most basal euthyneuran offshoot, this might indicate that the formation of the rhinophoral ganglion (and the homologous procerebrum) involving an ectodermal invagination is plesiomorphic within Euthyneura, and that there are remnants (or paedomorphotic reinstatements) of this structure in adults of (some) pulmonate taxa.

3) Presence of medio-dorsal (= cerebral) bodies

(Medio-)dorsal bodies (also termed cerebral bodies) are endocrine organs situated dorsally of the cerebral ganglia in "Pulmonata" [13], but considerable variation exists within the main pulmonate groups as regards the structure and innervation of the dorsal bodies [58,61,64]. Similar structures closely attached to the cerebral ganglia have been found in several Acochlidia: First described as "dorsal bodies" [25], they were later renamed "lateral bodies" by Neusser et al. [60], due to their more lateral position to the central nervous system and the unclear homology to pulmonate dorsal bodies. Since dorsal bodies in Pulmonata play a role in female reproduction [64], they might be fully developed in female adults only, thus might have been overlooked in some studies of gonochoristic acochlidian species or of hermaphrodites with "sex change". Further ultrastructural data on acochlidian "lateral bodies" and their potentially neurosecretory function are needed to evaluate homology with pulmonate structures. Moreover, pulmonate dorsal bodies might be homologous to the juxtaganglionar organs of some opisthobranchs [60], and thus might represent a plesiomorphic character of Panpulmonata and a potential synapomorphy of Euthyneura.

In addition, the presence of an unpaired dorsal jaw, which probably originated through the fusion of the paired lateral jaws [65], has been discussed as a potential synapomorphy of "Pulmonata" [18,48]. The presence of a pair of dorso-lateral jaws is a plesiomorphic character state for Euthyneura [13,65], but that condition has been reduced various times independently in "Opisthobranchia" and "Pulmonata" [18]. A dorsal, unpaired jaw might have evolved at the basis of Panpulmonata, and then have been secondarily reduced various times independently (e.g. in Onchidiidae, *Amphibola*) [18]. In Acochlidia, jaw-like structures are reported only for the derived microhedylacean family Ganitidae (as paired jaws), and as unclear "cuticular elements" for *Microhedyle glandulifera* (see [22] for citations). According to the derived position of Ganitidae in morphological [22] and molecular analyses (present study), these structures may represent either secondary developments (potentially related to the specialised dagger-shaped radula) or paedomorphic structures; however, studies of Acochlidia larvae are still overdue.

The only potential synapomorphy of "Opisthobranchia" is the presence of a rhinophoral nerve with a thickened basis (i.e. rhinophoral ganglion) and of associated sensory structures such as Hancock's organ [66]. Based on our phylogenetic hypothesis the presence of a rhinophoral nerve has to be considered as a plesiomorphic character within Euthyneura, and thus for Panpulmonata. The rhinophoral ganglion, and potentially the optic ganglion, is considered as homologous with the pulmonate procerebrum. Rhinophoral nerve and Hancock's organ have been reduced various times independently, probably correlated with the reduction of the rhinophores and/or habitat changes.

In summary, we are currently unable to find clear morphological synapomorphies which support a placement of Acochlidia within pulmonate taxa, as sister to Eupulmonata. In the light of our phylogenetic hypothesis, conventional pulmonate synapomorphies appear to be plesiomorphies or convergences within pulmonate taxa. On the other hand, no morphological characters currently contradict that molecular phylogenetic hypothesis, nor do they favour any alternative relationships, since morphological characters common to the mesopsammic heterobranchs are shown to be convergent developments, and the potential synapomorphy of Acochlidia with "Opisthobranchia" has to be considered as plesiomorphic.

The aberrant morphology of Acochlidia in relation to its proposed sister groups remains problematic. In his ontological studies on the nudibranch *Aeolidiella alderi*, Tardy [62] reported an abnormal development in some larvae that leads to a visceral hump separated from the head-foot complex in juvenile stages, thereby closely resembling external morphology in Acochlidia (see fig. 20 in [62]). According to Tardy [62] these abnormal developmental forms are also known from pulmonate Stylommatophora. Progenesis is discussed as a principle in the evolution of meiofaunal taxa [67], and acochlidian morphology might have evolved by retention of the juvenile characters of an aberrant developmental form of an early pulmonate.

Monophyly and phylogeny of Acochlidia

The monophyly of Acochlidia is well supported morphologically [20,22,24] and also backed by previous molecular studies [17,34]. Our study, which includes all valid acochlidian families except for the monotypic Tantulidae, also recovers Acochlidia as monophyletic but with low posterior probability and bootstrap support. The low bootstrap values for Acochlidia and some internal acochlidian taxa (e.g. Hedylopsacea) might be caused by their relatively early (Mesozoic) divergence times (see Figure 2): recent acochlidian taxa probably constitute but a remnant of much larger diversity in evolutionary history.

The acochlidian internal topology confirms the morphological analysis of Schrödl and Neusser [22], showing the same family relationships, but with better resolution within Microhedylacea: the genus *Pontohedyle* splits off at the basis of the Microhedylidae s.l. (including Ganitidae) with the closely related genera *Microhedyle* and *Paraganitus*. The hedylopsacean family Acochlidiidae includes the genera *Strubellia* and *Acochlidium* as proposed by Arnaud et al. [68] and Schrödl and Neusser [22]. Puzzling is the position of the enigmatic Aitengidae within Acochlidia, either as sister to Pseudunelidae and limnic Acochlidiidae (see Figure 1) or basal within Hedylopsacea (see Table 1). Aitengidae shows some of the general, but not unique, features of Acochlidia, such as the lack of a shell, reduction of mantle cavity, the praepharyngeal (circumpharyngeal) nerve ring, and the radula with a descending and ascending limb. This taxon also shares some features with limnic Acochlidiidae: the radula with a strong rhachidian tooth specialised in egg feeding, as also reported for *Strubellia* sp. [56]; the large, internal lateral eyes closely associated with the cerebral ganglia; and the presence of a foot groove and a branched digestive gland like reported for the genera *Acochlidium* and *Palliohedyle* [69,70]. On the other hand, Aitengidae lacks several acochlidian characteristics: the division of the body into head-foot complex and visceral hump; presence of 1-2 head appendages (with characteristic innervation of the rhinophores); and the ability to retract the head-foot complex into the visceral hump. However, in the absence of a separated visceral hump *A. ater* is able to retract its head under the notum. The presence of spicules is confirmed for Aitengidae sp., and the "parasites" described for *A. ater* might represent spicules instead (T. Neusser, pers. comm.). Re-examination of the doubtful "ascus" in *A. ater* is necessary; examination of Aitengidae sp. showed no true (i.e. sacoglossan-like) ascus containing old teeth, just a radula slightly bent at the end (own unpublished data). The presence of an ascus is currently accepted as a unique synapomorphy of Sacoglossa [4], and any sacoglossan relationship is clearly rejected by SplitsTree analysis (see Additional file 1) and phylogenetic analyses in the present study.

At the present stage of knowledge, molecular data suggests an inclusion of Aitengidae within Acochlidia, as sister to Pseudunelidae and Acochlidiidae. Detailed description by semithin serial sectioning and 3D reconstruction of the Aitengidae sp. used in the present study, together with focused redescription of *A. ater*, are needed as a basis to evaluating phylogenetic relationships of Acochlidia and Aitengidae in the future. This should be supported by a comprehensive molecular phylogeny of Acochlidia, including the two known species of Aitengidae.

Evolutionary traits in Euthyneura

Invasion of the interstitial habitat

Our study supports earlier assumptions that invasion of the interstitial habitat has occurred various times independently within the Euthyneura [22,68,71], probably by benthic, sand-dwelling or temporarily (i.e. juvenile) mesopsammic ancestors of the nudibranch genera *Embletonia* and *Pseudovermis*, the cephalaspidean *Philinoglossa* and *Philine exigua*, the sacoglossan *Platyhedyle*, some members of the Rhodopemorpha *incertae sedis* (*Helminthope* and some *Rhodope*), and the Acochlidia

[22,68]. The pulmonate genus *Smeagol* is found in gravel or pebble beaches on the undersides of stones; due to the relatively large body size in some species (e.g. up to 14 mm in *S. manneringi*[72]), it cannot be generally assigned to the meiofauna.

Major convergent adaptations to this spatially limited and unstable habitat are the worm-shaped body, loss of shell, and reduction of head appendages and pigmentation [21]. The development of subepidermal, calcareous spicules in Acochlidia, Rhodopemorpha and potentially *Platyhedyle* can also be considered as an adaptation to the interstitial habitat, probably serving to stabilise certain body parts during movements through the interstices [27], even though the occurrence of spicules is not limited to the mesopsammon. As far as is known, Acochlidia represent the most successful group of Heterobranchia in the mesopsammon concerning species diversity and abundance [27]. Key features for their success probably are an initial heterochronic miniaturisation and two different evolutionary trends towards a rapid, imprecise sperm transfer [23]. Additionally, adaptation to (temporarily) brackish waters with the development of a complex excretory system in Hedylopsacea [22,29] allows colonisation of shallow sands with freshwater impact (by groundwater or rain), overcoming limitations to deeper, truly marine sands.

Colonisation of freshwater and terrestrial habitats

It is undisputed and again confirmed by the present study that the "Pulmonata" have a marine origin [see e. g. [17,18]]. The hygrophilian radiation in the freshwater system is the most successful within "Pulmonata" [17], in terms of diversity and abundance, but not a unique event in pulmonate evolutionary history. Dinapoli and Klussmann-Kolb [14] already showed that the invasion of freshwater within pulmonate taxa took place at least twice, in Hygrophila and in the enigmatic *Glacidorbis*. According to our study, the colonisation of freshwater in Panpulmonata has occurred at least one more time in Acochlidia. Schrödl and Neusser [22] showed that within Acochlidia the freshwater colonisation already occurred twice independently, with a radiation of the Indo-Pacific Acochlidiidae and the single Caribbean *Tantulum elegans* (Tantulidae, not included in the present study). Thus, the development of a complex kidney within Hedylopsacea [29] as an adaptation to (temporarily) brackish water can be considered as a precursor to the invasion of limnic systems in Acochlidia. Acochlidian invasion of freshwater originated probably from a mesopsammic ancestor with temporary freshwater tolerance [32], or via a semi-terrestrial habitat as reported for Aitengidae [35]. Our study thus highlights the high diversity and flexibility of pulmonate habitats ranging from marine to temporarily brackish, permanently brackish, limnic and terrestrial environments. The still enigmatic *Aiteng ater* (Aitengidae) lives "amphibiously" and tolerates marine to brackish waters, but there are no observations of these animals truly leaving the water [35]. The species' mangrove habitat is comparable to that of representatives of, e.g., the pulmonate Onchidiidae, and is classified as marginal zones from which the transition to terrestrial habitat probably originated [17]. Similar to the limnic habitat, terrestrial environments have been colonised various times independently [53]. The present study indicates a least four independent pathways to the terrestrial habitat: in Amphiboloidea, Stylommatophora, Systellommatophora and Ellobioidea.

Molecular clock and estimation of divergence times in Acochlidia

The use of molecular clocks to estimate divergence times is controversially debated, due to conflicting results from different studies and disparities with paleontological or archaeological data [73-76]. Criticism focuses on the major problems such as faulty calibration, impact of rate heterogeneity among lineages, and "time dependency of molecular rates" [73,75-77]. Some of the problems could be solved by the relaxed clock approach [78], and despite all pitfalls and criticism, molecular clock approaches have helped considerably to reveal the evolutionary history of life, especially when it comes to divergence times of groups with poor or no fossil record [75,76,79]. Thus, we consider it a valuable methodology to roughly estimate divergence times for tiny, sluggish gastropods for which there is no fossil record. Molecular clock dating stands and falls with the accuracy with which genetic distances can be estimated [80]; thus we consider the removal of ambiguous (i.e. potentially non-homologous) sites from the alignment as problematic. It seems common use to run the molecular clock analyses with reduced datasets (e.g. [14,81-83]), but the crucial question, how this will affect the molecular dating, has remained unaddressed. The exclusion of highly saturated positions - e.g., in some cases the 3rd codon position of the COI sequence (see e.g. [84]) - can be justified by the biasing effect of saturation on the molecular clock. It can be argued that ambiguous parts of the alignment are often highly variable and might suffer from saturation, but on the other hand the exclusion of a series of non-saturated sites might result in underestimated divergence times. However, our Beast analysis of the raw, uncut dataset provided estimations of divergence times very similar to those from the Gblocks dataset (not shown). Nevertheless, we recommend to critically compare data from masked and raw alignments for molecular clock analyses, and to stay mindful of the potentially underestimating effect on divergence times.

The only molecular clock data on Heterobranchia [14] available prior to the present study suffers from

unreliable calibration, which is considered as the most sensible and critical part of divergence time estimations [76]. There is no objective way to assign fossils to a certain point of a stem line in a recent phylogeny, thus the age of the fossil has to be taken as the minimum age of the split between the extant taxon it is assigned to and its sister group [80]. In [14] the fossil ages were assigned to the diversification of Heterobranchia, Acteonoidea and Omalogyridae, respectively, rather than to the splits from the corresponding sister groups, which led, e.g., to the surprising Pre- to early Cambrian split between Vetigastropoda and Apogastropoda. Our molecular clock was calibrated to the split between Caenogastropoda and Heterobranchia; thus molecular dating of this node is biased (i.e. depends directly on calibration features). However, fossil data shows two clearly different lineages by the mid-Devonian, thus indicating a pre- or early Devonian split of Apogastropoda [85,86]. According to our study euthyneuran gastropods already emerged in the Palaeozoic Permian, diverting from the "Lower Heterobranchia", but all major radiations of Euthyneura occurred in the early Mesozoic. According to paleontological data the oldest opisthobranchs appeared in the Triassic (about 220 Mya), the earliest pulmonates in the Jurassic (about 190 Mya) [85,86].

Based on their phylogenetic hypothesis from morphological data and the fossil record of cephalaspidean outgroups, Schrödl and Neusser [22] suspected a Jurassic time frame for the origin of Acochlidia. Their inferred sister group relationships are different from the present study, but the early divergence time is supported by our molecular clock approach, which places the origin of Acochlidia in the late Triassic to early Jurassic and their major diversification in the Jurassic. In the present study the Eupulmonata as sister group to Acochlidia show similar origin and diversification times, and so do the Hygrophila. Tillier et al. [46] inferred divergence times from branch lengths in a molecular distance tree (based on partial 28S sequences), indicating a similar Jurassic time frame for Eupulmonata and slightly younger for Hygrophila. This corresponds with fossil data, which reports a first occurrence in the late Jurassic (approx. 150 Mya) [46]. Based on fossils, diversification times of eupulmonate groups such as Stylommatophora can be dated to the late Cretaceous, when most extant families appear [87].

According to our data most acochlidian families appeared in the Jurassic or Cretaceous, only Ganitidae, Pseudunelidae and Acochlidiidae have a Palaeogene origin. These old splits on the family and even genus levels (see *Hedylopsis*, Figure 2, diverging in the Cretaceous) might indicate either that the extant diversity of Acochlidia is only a small remnant of high diversity in former times, or that known acochlidian diversity is just the tip of the iceberg still waiting to be discovered.

Based on fossil data the major diversification of "opisthobranch" taxa in a traditional sense took place comparatively recently, at the beginning of the Cenozoic (around 60 Mya), with the first records of Sacoglossa, Anaspidea and Thecosomata [86]. However, due to more or less reduced shells the fossilization probability is low. Our study suggests that most extant "opisthobranch" taxa, e.g. Sacoglossa, Cephalaspidea s.s., Pteropoda, Umbraculoidea and Anaspidea, have a Mesozoic origin. Ambiguous is the basal euthyneuran position of the Nudipleura and the resulting estimates of an old age (late Palaeozoic) and diversification (middle Mesozoic). This contradicts previous molecular clock analyses on Nudipleura, which indicated a Triassic origin and Jurassic diversification [82]. These discrepancies clearly result from major differences in tree topology (basal vs. derived position). Moreover, while our study includes only three nudipleuran representatives (poor ingroup taxon sampling), Göbbeler's and Klussmann-Kolb's [82] analysis lacks comprehensive heterobranch outgroup sampling. Future studies are needed to resolve the origin of Nudipleura within the Heterobranchia.

Conclusions

Our multi-locus molecular study including six out of seven acochlidian families and the recently established Aitengidae confirms a pulmonate relationship of Acochlidia, which was traditionally placed within Opisthobranchia. The enigmatic Aitengidae cluster within Acochlidia. Previously assumed morphological synapomorphies of Pulmonata (pallial cavity with pneumostome, procerebrum with cerebral gland, and presence of medio-dorsal bodies) appear as either homoplastic or plesiomorphic in light of the present phylogenetic hypothesis, as does the potential opisthobranch synapomorphy (presence of rhinophoral nerve). At present, morphological characters neither justify a placement of Acochlidia within Pulmonata, nor do they favour any opisthobranch relationships that would contradict the molecular hypothesis. The aberrant acochlidian morphology might have resulted from ancestral progenesis and paedomorphic retention of the morphology of an abnormally developed juvenile.

The present study once more underlines the respective non-monophyly of Euthyneura, Opisthobranchia and Pulmonata as defined traditionally. We demonstrate the necessity for inclusion of small, enigmatic groups to solve deep-level phylogenetic relationships, and highlight that the "pulmonate" and "opisthobranch" phylogenies cannot be solved independently from each other. Clarification of remaining enigmas such as Rhodopemorpha, and of well supported taxa with unclear relationships such as Pyramidelloidea or Sacoglossa, is needed for future advances. The reclassification suggested herein

defines 1) Euthyneura as including Pyramidelloidea and Glacidorboidea; 2) Euopisthobranchia as including Umbraculoidea, Cephalaspidea s.s., Runcinacea, Anaspidea and Pteropoda, but excluding Acteonoidea and Nudipleura, as well as Sacoglossa and Acochlidia; and 3) Panpulmonata as composed of Siphonarioidea, Sacoglossa, Hygrophila, Amphiboloidea, Pyramidelloidea, Glacidorboidea, Eupulmonata and Acochlidia. The present results based on standard molecular markers require confirmation from other character sets (e.g. rare genomic changes, mitochondrial gene arrangements, additional molecular markers) and careful (re-)examination of morphological characters and homology assumptions in the light of the new phylogenetic hypothesis. Our molecular clock analysis estimates a Mesozoic origin for all major panpulmonate taxa. The poorly supported topology within Panpulmonata might be promoted by the old age of this group, which potentially stands for a series of radiation and extinction events in history, resulting in poor taxon representation in present times.

The present study shows that the mesopsammon was colonised various times independently within Euthyneura, resulting in a series of convergent adaptations to the interstitial habitat. The inclusion of Acochlidia within pulmonate taxa extends the structural and biological diversity of the pulmonate clade, which exhibits remarkable flexibility in habitat choice, with various transitions from marine to limnic and terrestrial habitats.

Methods

Taxon sampling

A total of 78 gastropod taxa were investigated in the present study. As new material, nine acochlidian taxa and five additional enigmatic and hard-to-obtain euthyneuran taxa with potential acochlidian relationships were included (see Table 2). Specimens were collected by hand or extracted from sand samples following the method described by Schrödl [88], usually anaesthetised with $MgCl_2$, and fixed in 96% ethanol. Reference specimens and DNA vouchers of sequences generated in this study are deposited at the Bavarian State Collection for Zoology (ZSM); sampling localities, reference material and DNA Bank accession numbers (http://www.dnabank-network.org) of our own data are listed in Table 2. Other sequences were retrieved from GenBank (for accession numbers see Table 3). Outgroups were chosen to include all major euthyneuran and several further heterobranch taxa. Special focus was given to mesopsammic representatives and groups previously discussed as potentially related to Acochlidia. Of these potential relatives only Rhodopemorpha are missing in our study, but a Rhodopemorpha-Acochlidia relationship can be clearly rejected based on molecular markers [49].

DNA extraction, PCR and sequencing

Genomic DNA was extracted from tissue samples of the foot or from entire specimens using the DNeasy Blood and Tissue Kit (Qiagen, Hilden Germany). Four markers were amplified: nuclear 18S rRNA (approx. 1800 bp), 28S rRNA (approx. 1020 bp), mitochondrial 16S rRNA (approx. 300-400 bp), and cytochrome *c* oxidase subunit I (COI - approx. 650 bp). For PCR protocols and primers used, see additional file 3. Successfully amplified PCR products were purified using ExoSapIT (USB, Affymetrix, Inc.). Cycle sequencing and the sequencing reaction were performed by the sequencing service of the Department of Biology Genomic Service Unit (GSU) of the Ludwig-Maximilians-University Munich, using Big Dye 3.1 kit and an ABI 3730 capillary sequencer. All fragments were sequenced in both directions using the PCR primers. All sequences have been deposited at GenBank (see Table 3 for accession numbers). The Gblock alignment and the resulting tree were deposited in TreeBASE (http://www.treebase.org, accession number 10801).

Sequence editing and alignment

All sequences generated in this study were checked for contaminations with BLAST searches [89] implemented in the GenBank database on the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Reconciliation of forward and reverse reads was carried out in BioEdit 7.0.5. [90]. MAFFT v6 [91] was used to generate sequence alignments for each gene region, using the default settings (automatically chosen models for 18S, 28S, COI: FFT-NS-i; for 16S: L-INS-i). The alignment of the protein coding COI gene was corrected manually according to the amino acids. The individual MAFFT alignments were parsed 1) using Gblocks [92,93] with the default settings for less stringent selection, 2) with ALISCORE v1.0 [94] using the default parameters, or c) left unmasked.

Phylogenetic analysis

For an *a priori* analysis of variation in the phylogenetic signal a split-decomposition analysis was performed using SplitsTree v4.6 [95].

The best-fit model of nucleotide substitution for each gene was selected using Modeltest 3.7 [96] via the Akaike Information Criterion (AIC). The incongruence length difference (ILD) test [97] was carried out in Paup 4.0b10 [36]. This test was conducted with heuristic searches and 100 replicates to evaluate incongruence between single markers.

Table 2 Information on the material generated for the present study

Taxon	Family	Locality	Museums Nr.	DNA Bank voucher Nr.
Acochlidia				
Hedylopsis spiculifera	Hedylopsidae	Istria Croatia/Corse France, Mediterranean Sea	ZSM Mol 20080951/ZSM Mol 20080955	AB35081816 AB35081817
Hedylopsis ballantinei	Hedylopsidae	Sinai, Egypt, Red Sea	ZSM Mol 20090244	AB34858170
Pseudunela sp.	Pseudunelidae	Mounparap Island, Vanuatu, Pacific	ZSM Mol 20080393	AB35081809
Strubellia paradoxa	Acochlidiidae	Ambon, Indonesia, Indo-Pacific	Berlin Moll 193944	AB34858174
Acochlidium fijiense	Acochlidiidae	Vitilevu, Fiji, Pacific	ZSM Mol 20080063	AB34404244
Asperspina sp.	Asperspinidae	Kamtschatka, Russia, North Pacific	ZSM Mol 20090171	AB35081833
Microhedyle glandulifera	Microhedylidae	Istria, Croatia, Mediterranean Sea	ZSM Mol 20081019	AB35081799
Pontohedyle milaschewitchii	Microhedylidae	Istria. Croatia, Mediterranean Sea	ZSM Mol 20080054/ZSM Mol 20080925	AB34404241
Paraganitus ellynnae	Ganitidae	Guadalcanal, Solomons, Pacific	ZSM Mol 20080170	AB34404203
Sacoglossa				
Gascoignella nukuli	Platyhedylidae	Pak Phanang Bay, Thailand, Gulf of Thailand	ZSM Mol 20090182	AB344011928
Volvatella viridis	Volvatellidae	Bonotsu, Kagoshima, Japan, Pacific	-	-
Aitengidae sp.	Aitengidae	Hisamatsu, Miyako Island, Okinawa, Japan, Pacific	-	-
Cephalaspidea				
Philine exigua	Philinidae	Guadalcanal, Solomons, Pacific	ZSM Mol 20080752	AB34401927
Philinoglossa praelongata	Philinoglossidae	Istria, Croatia, Mediterranean Sea	ZSM Mol 20080917	AB34500041

The table lists the species names, collecting localities, reference numbers of museum vouchers (ZSM = Bavarian State Collection for Zoology; Berlin = Museum of Natural History, Berlin) and DNA vouchers deposited in the DNA Bank of the ZSM.

Maximum likelihood analyses were performed using RAxML 7.0.3 [98] adapting the program parameters to the alignment as described in the manual ("hard & slow way" - with 10 parsimony starting trees and 6 different rate categories). Additionally 200 multiple inferences were executed on the original alignment and 1000 bootstrap replicates were generated. Analyses were run under the GTR Gamma model as recommended in the manual [98] and the caenogastropod taxa *Littorina littorea* and *Aperostoma palmeri* were defined as outgroups. The alignment was analysed in different partition sets: one partition, two partitions (18S + 28S + 16S combined; COI separate), three partitions (18S + 28S + 16S combined; COI with codons partitioned to 1^{st} + 2^{nd} separate from 3^{rd}), four partitions (separated by gene regions), and five partitions (18S, 28S, 16S, COI 1^{st} + 2^{nd}, COI 3^{rd}). To test whether partitioning significantly improves the likelihood values of the dataset, we compared the likelihood values of all partitions via the Akaike Information Criterion.

Bayesian phylograms were generated from the Gblocks and ALISCORE alignments with MrBayes 3.1.2 [99]. The general time-reversible model was used for both datasets, with invariant site frequency and gamma-shape parameter estimated from the data (GTR + I + G). The 'shape', 'proportion of invariant sites', 'state frequency' and 'substitution rate' parameters were estimated for each gene separately. Each codon position in the amino-acid coding COI was also allowed to have different parameters; hence the alignments had six partitions of parameters. Two parallel runs were made for 5×10^6 generations (with a sample frequency of 1000), using a default value of four Markov chains. Quality and ESS values (effective sampling size) of each run were checked in Tracer 1.5.3. The first 2000 trees for each run were discarded to ensure that the four chains reached stationarity. The consensus tree and posterior probabilities were computed from the remaining 6000 trees (3000 trees × 2 runs).

To evaluate support for our tree topology an alternative topology (grouping Acochlidia with Opisthobranchia) was tested in comparison to the "best" tree topology by using the Approximately Unbiased Test [100]. The hypothetic topology was computed with RAxML [98] using the -g option for the constraint ML tree. The p-values of the sitewise log likelihoods combined with the "best" topology were estimated using Treefinder [101].

Molecular clock

Approximate divergence times were calculated using the relaxed molecular clock approach [78] implemented in the software BEAST 1.5.3 [102]. For molecular clock analysis the concatenated Gblock-dataset was analysed in five partitions as for the phylogenetic analyses.

Calibration points were chosen for groups with stable and well supported nodes in the phylogenetic

Table 3 GenBank accession numbers of the sequences used in the present study

Taxon	Family	Species	18S	28S	16S	COI
Caenogastropoda	Cyclophoridae	*Aperostoma palmeri*	DQ093435	DQ279983	DQ093479	DQ093523
	Littorinidae	*Littorina littorea*	X91970	AJ488672	DQ093481	AY345020
"Lower" Heterobranchia	Orbitestellidae	*Orbitestella* sp.	EF489352	EF489377	EF489333	EF489397
	Valvatidae	*Valvata piscinalis*	FJ917223/FJ917222	FJ917224	FJ917248	FJ917267
	Cimidae	*Cima* sp.	FJ917206.1	FJ917228.1	FJ917260.1	FJ917279.1
	Rissoellidae	*Rissoella rissoaformis*	FJ917214.1	FJ917226.1	FJ917252.1	FJ917271.1
	Pyramidellidae	*Turbonilla* sp.	EF489351	EF489376	EF489332	EF489396
	Pyramidellidae	*Boonea seminuda*	AY145367	AY145395	AF355163	-
	Pyramidellidae	*Eulimella ventricosa*	FJ917213.1	FJ917235.1	FJ917255.1	FJ917274.1
	Pyramidellidae	*Odostomia* sp.	AY427526.1	AY427491.1	FJ917256.1	FJ917275.1
	Glacidorbidae	*Glacidorbis rusticus*	FJ917211.1	FJ917227.1	FJ917264.1	FJ917284.1
Acteonoidea	Acteonidae	*Pupa solidula*	AY427516	AY427481	EF489319	DQ238006
	Aplustridae	*Hydatina physis*	AY427515	AY427480	EF489320	GQ845174.1
	Acteonidae	*Rictaxis punctocaelatus*	EF489346	EF489370	EF489318	EF489393
Nudipleura	Bathydorididae	*Bathydoris clavigera*	AY165754	AY427444	AF249222	AF249808
	Pleurobranchidae	*Tomthompsonia antarctica*	AY427492	AY427452	EF489330	DQ237992
	Pleurobranchidae	*Pleurobranchus peroni*	AY427494	AY427455	EF489331	DQ237993
Umbraculoidea	Umbraculidae	*Umbraculum umbraculum*	AY165753	AY427457	EF489322	DQ256200
	Tylodinidae	*Tylodina perversa*	AY427496	AY427458	-	AF249809
Anaspidea	Akeridae	*Akera bullata*	AY427502	AY427466	AF156127	AF156143
	Aplysiidae	*Aplysia californica*	AY039804	AY026366	AF192295	AF077759
Pteropoda	Pneumodermatidae	*Pneumoderma cf. atlantica*	DQ237970	DQ237989	-	DQ238003
	Pneumodermatidae	*Spongiobranchaea australis*	DQ237969	DQ237988	-	DQ238002
	Cavoliniidae	*Hyalocylis striata*	DQ237966	DQ237985	-	-
	Cavoliniidae	*Cavolinia uncinata*	DQ237964	DQ237983	-	DQ237997
Runcinacea	Runcinidae	*Runcina africana*	DQ923473	DQ927240	-	DQ974680
Cephalaspidea s.s.	Bullidae	*Bulla striata*	DQ923472.1	DQ986694.1	DQ986632.1	DQ986567.1
	Phillinoglossidae	*Philinoglossa praelongata*	AY427510	AY427475	HQ168411*	-
	Scaphandridae	*Scaphander lignarius*	EF489348	EF489372	EF489324	-
	Haminoeidae	*Haminoea hydatis*	AY427504	AY427468	EF489323	DQ238004
	Philinidae	*Philine exigua*	HQ168425*	HQ168438*	HQ168412*	HQ168450*
	Diaphanidae	*Diaphana* sp.	-	EF489373	EF489325	EF489394
	Diaphanidae	*Toledonia globosa*	EF489350	EF489375	EF489327	EF489395
	Cylichnidae	*Cylichna gelida*	EF489349	EF489374	EF489326	-
Sacoglossa	Volvatellidae	*Volvatella viridis*	HQ168426*	HQ168439*	HQ168413*	HQ168451*
	Cylindrobullidae	*Cylindrobulla beauii*	EF489347	EF489371	EF489321	-
	Platyhedylidae	*Gascoignella nukuli*	HQ168427*	HQ168440*	HQ168414*	HQ168452*
	Caliphyllidae	*Cyerce nigricans*	AY427500	AY427463	EU140843	DQ237995
	Plakobranchidae	*Plakobranchus ocellatus*	AY427497	AY427459	DQ480204	DQ237996
	Elysiidae	*Thuridilla bayeri*	AF249220	AY427461	DQ480206	DQ471271
	Elysiidae	*Elysia viridis*	AY427499	AY427462	AY223398	DQ237994
Sacoglossa (?)	Aitengidae	*Aitengidae* sp.	HQ168428*	HQ168441*	HQ168415*	HQ168453*
Acochlidia	Hedylopsidae	*Hedylopsis ballantinei*	HQ168429*	HQ168442*	HQ168416*	HQ168454*
	Hedylopsidae	*Hedylopsis spiculifera*	HQ168430*	HQ168443*	HQ168417*	HQ168455*
	Pseudunelidae	*Pseudunela* sp.	HQ168431*	HQ168444*	HQ168418*	HQ168456*
	Acochlidiidae	*Strubellia paradoxa*	HQ168432*	HQ168445*	HQ168419*	HQ168457*
	Acochlidiidae	*Acochlidium fijiense*	HQ168433*	HQ168446*	HQ168420*	HQ168458*
	Asperspinidae	*Asperspina* sp.	HQ168434*	HQ168447*	HQ168421*	-
	Microhedylidae	*Pontohedyle milaschewitchii*	HQ168435*	AY427484	HQ168422*	HQ168459*
	Ganitidae	*Paraganitus ellynnae*	HQ168436*	HQ168448*	HQ168423*	HQ168460*
	Microhedylidae	*Microhedyle glandulifera*	HQ168437*	HQ168449*	HQ168424*	HQ168461*

Table 3 GenBank accession numbers of the sequences used in the present study *(Continued)*

Siphonarioidea	Siphonaridae	*Siphonaria pectinata*	U86321	DQ279993	AY377627	AF120638
	Siphonaridae	*Siphonaria concinna*	EF489334	EF489353	EF489300	EF489378
Amphiboloidea	Amphibolidae	*Amphibola crenata*	EF489337	EF489356	EF489304	-
	Amphibolidae	*Phallomedusa solida*	DQ093440	DQ279991	DQ093484	DQ093528
	Amphibolidae	*Salinator cf. fragilis*	-	EF489355	EF489303	EF489381
Hygrophila	Latiidae	*Latia neritoides*	EF489339	EF489359	EF489307	EF489384
	Chilinidae	*Chilina* sp.	EF489338	EF489357	EF489305	EF489382
	Acroloxidae	*Acroloxus lacustris*	AY282592	EF489364	EF489311	AY282581
	Lymnaeidae	*Lymnaea stagnalis*	EF489345	EF489367	EF489314	EF489390
	Physidae	*Physella acuta*	AY282600	EF489368	AY651241	AY282589
	Planorbidae	*Ancylus fluviatilis*	AY282593	EF489365	EF489312	AY282582
Stylommatophora	Arionidae	*Arion silvaticus*	AY145365	AY145392	AY947380	AY987918
	Helicidae	*Arianta arbustorum*	AY546383	AY014136	AY546343	AY546263
	Enidae	*Ena montana*	AY546396	-	AY546356	AY546276
	Cerionidae	*Cerion incanum*	-	AY014060.1	-	-
	Subulinidae	*Rumina decollata*	-	13794085:464-1292	AY345050	AY345050
Systellommatophora	Onchidiidae	*Onchidium verruculatum (§)*	AY427522	AY427487	EF489316	EF489391
	Onchidiidae	*Onchidella floridiana*	AY427521	AY427486	EF489317	EF489392
	Veronicellidae	*Laevicaulis alte*	X94270.1	AY014151.1		
	Veronicellidae	*Semperula wallacei*	-	DQ897671.1	DQ897675.1	DQ897673.1
	Rathouisiidae	*Atopos australis*	-	AY014152.1	-	-
Trimusculoidea	Trimusculidae	*Trimusculus afra*	EF489343	-	EF489309	EF489388
Otinoidea	Otinidae	*Otina ovata*	EF489344	EF489363	EF489310	EF489389
	Smeagolidae	*Smeagol phillipensis*	FJ917210	FJ917229	FJ917263	FJ917283
Ellobioidea	Carychiidae	*Carychium minimum*	EF489341	EF489361	EF489308	EF489386
	Ellobiidae	*Ophicardelus ornatus*	DQ093442	DQ279994	DQ093486	DQ093486
	Ellobiidae	*Myosotella myosotis*	EF489340	EF489360	AY345053	EF489385

Sequences generated within this study are marked with *; (§) in GenBank as "*O. verrucosum*", which is not a valid name, thus treated as *O. verruculatum*. (" - " indicates missing sequences).

hypothesis and decently documented fossil record with clear identification to recent taxa. Minimum constraints for three nodes were chosen based on the fossil record: 1) split between Caenogastropoda and Heterobranchia based on the oldest known fossil of the Heterobranchia (*Palaeocarboninia janke*) recorded from the Middle Devonian (390 Ma) [85]; 2) the split between Acteonoidea and its sister group based on acteonoid fossils with a minimum age of 240 Ma ([103], A Nützel pers. comm.) and 3) the split of Ellobioidea and their sister group based on ellobiid fossils with a minimum age of 140 Ma ([86], A Nützel pers. comm.). We calibrated using a hard minimum bound (i.e. the divergence data cannot be younger than the oldest known fossil); the probability that the divergence event occurred above the minimum date declines according to a gamma distribution, such that 95% of the posterior density falls within the range [x - x + 10%] [see [104]]. Calibration nodes were not fixed as monophyletic.

The analyses were run with the relaxed uncorrelated lognormal clock model under the Yule process using the GTR+G+I substitution model (chosen from Modeltest 3.7 [96] via the Akaike Information Criterion) for all markers. The MCMC was run ten times independently, generating 10^6 generations each, and sampled every 1000 steps. The single runs were combined with LogCombiner 1.5.3, with the first 10^5 samples each discharged as burn-ins. The runs were checked for quality and sufficient ESS (effective sample size) in Tracer 1.5.3. All trees were combined to produce a consensus tree using TreeAnnotator 1.5.3, with the first 1000 trees of each dataset discharged as burn-in.

To evaluate the potential effect on molecular dating of removing ambiguous sites from the alignment, the BEAST runs were repeated with the raw alignments (i.e. mainly uncut; only longer ends of some sequences removed due to the use of different primers) alignments, generating 10×10^6 generations and following the method described above.

Additional material

Additional file 1: Neighbournet graph on the origin of Acochlidia. Generated with Splits Tree v4.6 from the concatenated, four marker dataset masked with Gblocks, visualising highly conflicting signal at the basis of the Acochlidia. Representatives of meiofaunal taxa highlighted in boldface, showing the absence of a common phylogenetic signal.

Additional file 2: Likelihood values of different partitions

Additional file 3: PCR protocols and primers used [105-107].

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Authors' contributions
KMJ, MS, YK and HF sampled the material. KMJ, IS, TK and YK generated the molecular data. KMJ and YK conducted the phylogenetic and network analysis. KMJ performed the molecular clock approach. KMJ wrote the initial version of the manuscript; all authors contributed to the discussion of the results and the preparation of the final manuscript. MS planned and supervised the study. All authors have read and approved the final manuscript.

Competing interests
The authors declare that they do not have competing interests.

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Chapter 2. Bye bye "Opisthobranchia"! A review on the contribution of mesopsammic sea slugs to euthyneuran systematics

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BYE BYE "OPISTHOBRANCHIA"! A REVIEW ON THE CONTRIBUTION OF MESOPSAMMIC SEA SLUGS TO EUTHYNEURAN SYSTEMATICS

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Key words: Mollusca, Heterobranchia, morphology, molecular phylogeny, classification, evolution.

ABSTRACT

During the last decades, textbook concepts of "Opisthobranchia" have been challenged by morphology-based and, more recently, molecular studies. It is no longer clear if any precise distinctions can be made between major opisthobranch and pulmonate clades. Worm-shaped, mesopsammic taxa such as Acochlidia, Platyhedylidae, Philinoglossidae and Rhodopemorpha were especially problematic in any morphology-based system. Previous molecular phylogenetic studies contained a very limited sampling of minute and elusive meiofaunal slugs. Our recent multi-locus approaches of mitochondrial COI and 16S rRNA genes and nuclear 18S and 28S rRNA genes ("standard markers") thus included representatives of most mesopsammic "opisthobranchs" within a comprehensive euthyneuran taxon set.

The present study combines our published and unpublished topologies, and indicates that monophyletic Rhodopemorpha cluster outside of Euthyneura among shelled basal heterobranchs, acteonids are the sister to rissoellids, and Nudipleura are the basal offshoot of Euthyneura. Furthermore, Pyramidellidae, Sacoglossa and Acochlidia cluster within paraphyletic Pulmonata, as sister to remaining "opisthobranchs". Worm-like mesopsammic heterobranch taxa have clear independent origins and thus their similarities are the result of convergent evolution. Classificatory and evolutionary implications from our tree hypothesis are quite dramatic, as shown by some examples, and need to be explored in more detail in future studies.

We do not claim that these concatenated "standard marker" gene trees reflect the true phylogeny of all groups; exploring additional, suitable markers is required. We do claim, however, that improved taxon sampling and improved data quality (such as sequences, alignments) were beneficial towards

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revealing relationships of higher euthyneuran taxa, and that phylogenetic hypotheses based on this data set are converging. The traditional taxon concept of Opisthobranchia is clearly artificial and thus obsolete. Novel phylogenetic hypotheses, as disturbing they may be at first glance, give us the opportunity and perhaps the obligation to refine our approaches and rethink older paradigms. Most importantly, we see no more way to explore morphology, systematics and evolution of "opisthobranchs" separately from "lower heterobranchs" and "pulmonates".

INTRODUCTION

Milne Edwards (1848) split the gastropods into Prosobranchia, Pulmonata and Opisthobranchia. The latter two taxa are usually combined as Euthyneura. Both researchers and amateurs easily associate opisthobranchs as marine slugs or snails, with a more or less reduced or internalized shell, having an almost bilaterally symmetrical body and either a head shield or head tentacles, whereas pulmonates appear almost exclusively related to limnic and terrestrial habitats. Unconventional taxa such as interstitial worm-like forms, limnic opisthobranchs and marine pulmonates occur, but are obviously too exceptional to challenge the practical value of the traditional Opisthobranchia-Pulmonata concept. The often beautifully coloured and bizarrely shaped approx. 6000 opisthobranch species thus are treated as belonging to a clade in virtually all older field guides and zoological textbooks (e.g. Westheide & Rieger, 2007), current molluscan classifications (e.g. Bouchet & Rocroi, 2005), and reviews (e.g. Schmekel & Portmann, 1982, Schmekel, 1985, Rudman & Willan, 1998), including the most recent one by Wägele *et al.* (2008) that was published within a compendium on molluscan phylogeny and evolution (Ponder & Lindberg, 2008). Recent comprehensive field guides on Caribbean and Indo-Pacific opisthobranchs, however, left monophyly open (Valdés *et al*., 2006, Gosliner *et al*., 2008).

There has always been a certain disagreement with regards to which major subtaxa should be included into Opisthobranchia (Gosliner, 1981). Commonly accepted "core groups" are Cephalaspidea, Anaspidea, Thecosomata, Gymnosomata, Sacoglossa, Acochlidia, Tylodinoidea (=Umbraculida) and Nudipleura, the latter consisting of side-gilled Pleurobranchomorpha and Nudibranchia, which are the sea slugs in a strict sense. Some taxa with more or less well-developed helicoidal shells such as Acteonoidea (see Mikkelsen, 1996 vs. 2002) and Pyramidelloidea (e.g. Fretter & Graham, 1949) and the limpet-like Siphonarioidea have also occasionally been discussed as part of Opisthobranchia (see review by Wägele *et al*., 2008). While the worm-like Rhodopemorpha were either seen as turbellarians or transitional forms between worms and gastropods in early approaches, most modern authors treated them as euthyneurans or integral part of opisthobranchs (e.g. Haszprunar & Heß, 2005).

Establishing the Heterobranchia concept, Haszprunar (1985, 1988) reconstructed an apomorphy-based phylogeny implying a progressive evolution from simple "allogastropod" (="lower heterobranch") taxa such as Valvatoidea, Architectonicoidea and Pyramidelloidea towards Pentaganglionata (=Euthyneura). Haszprunar's phylogeny showed Acteonoidea (Architectibranchia) as the sister to monophyletic Pulmonata (including pentaganglionate Rhodopemorpha), which was itself the sister to remaining opisthobranchs (including vermiform Smeagolidae), rendering "Opisthobranchia" paraphyletic. Haszprunar thus was the first to phylogenetically infer and discuss the artificial nature of Opisthobranchia rather than comparing similarities and modifying the inclusiveness of the concept. Using cladistic analyses on a morphological dataset, Salvini-Plawén & Steiner (1996) recovered monophyletic Euthyneura, and Pulmonata plus Thecosomata as sister to remaining Opisthobranchia including Rhodopemorpha (as Rhodopida) as sister to equally shell-less and small-sized Acochlidia and Gymnosomata. Dayrat & Tillier (2002) found Pyramidelloidea within euthyneuran taxa and summarized an unresolved euthyneuran topology with

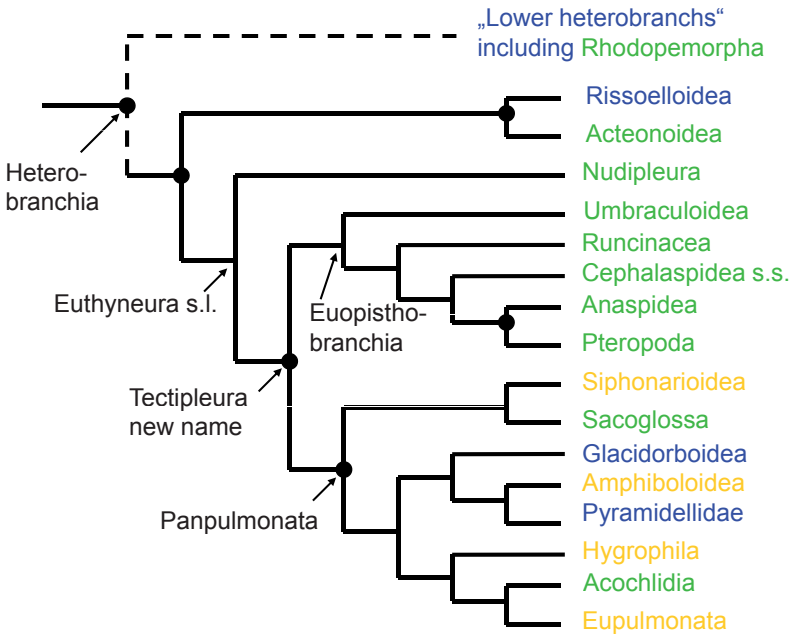

Figure 1:
"Opisthobranch" phylogeny as inferred from "standard genes" analyses, combining results by Jörger et al. (2010) and Wilson et al. (2010); robustly supported nodes (bootstrap support >75 and posterior probability >0.95) indicated by black dots. Taxa formerly regarded as opisthobranchs in green, pulmonate taxa in yellow, "lower heterobranch" taxa in blue. Note that the assemblage of "Lower heterobranchs including Rhodopemorpha" is paraphyletic but collapsed for illustrative purposes.

monophyletic Pulmonata arising as one of many clades from an opisthobranch grade of organization. An even more comprehensive morphology-based parsimony analysis by Wägele & Klussmann-Kolb (2005) showed Pteropoda (Gymnosomata plus Thecosomata) as sister to Pulmonata plus remaining Opisthobranchia, but this is contradicted by a more focused molecular study (Klussmann-Kolb & Dinapoli, 2006). In the study by Wägele & Klussmann-Kolb (2005) the remaining Opisthobranchia included a clade of exclusively interstitial (and/or small sized) cephalaspidean subtaxa, Rhodopemorpha and Acochlidia as sister to Sacoclossa, rendering Cephalaspidea polyphyletic. In the light of the latest morphology-based cladistic analysis focussing on Acochlidia (Schrödl & Neusser, 2010), such results are in doubt. While resolving inner relationships of Acochlidia quite nicely, other mesopsammic euthyneurans included, regardless their supposed affiliation, had a tendency to cluster with Acochlidia; Schrödl & Neusser (2010) explained that by parallel concerted reductions of body-size and organs, but also by convergent evolution of vermiform bodies having a set of special organs as adaptations to a special habitat. Summarizing, 1) the Heterobranchia concept has always conflicted with a monophyletic Opisthobranchia, 2) no morphology-based analyses have recovered a monophyletic Opisthobranchia, 3) morphology-based analyses are mislead by problems of interpreting morphological similarities and a generally high degree of parallelism (Gosliner, 1981, 1991); in particular, convergences displayed by small-sized slugs that occur in many subgroups may outnumber characters showing true phylogenetic signal, and thus lead to unreliable or completely wrong topologies.

Molecular markers, in contrast, offer an extremely large number of characters (via nucleotide sequences)

and many genes such as rRNA genes may not be directly influenced by habitat-specific ecological selective pressures. Early molecular approaches on opisthobranch phylogeny counted with single genes (partial 16S rDNA, Tholleson, 1999a,b, Wägele *et al*., 2003; 18S rDNA, Wollscheid & Wägele 1999; partial 28S rDNA, Dayrat *et al*., 2001), for relatively small sets of taxa. Whenever pulmonates were included in such analyses, opisthobranchs were not recovered as monophyletic unless the taxon definitions were extraordinarily modified. The same happened to the mitochondrial genome-based data sets of Grande *et al*. (2004a,b, 2008) and Medina *et al*. (2011). Vonnemann *et al*. (2005) were the first to combine the more conservatively evolving nuclear 18S and 28S rRNA gene fragments sequenced from a larger and more representative euthyneuran taxon set (including 3 different acochlidian species), recovering monophyletic Opisthobranchia as sister to potentially paraphyletic Pulmonata, but only in Maximum Parsimony analysis of the combined data set. Successively extending the taxon sampling to further pulmonate subgroups and especially to lower heterobranchs, using a combined set of mitochondrial CO1, 16S rRNA gene fragments, and nuclear 18S rRNA (complete) plus 28S rRNA genes (D1-3), and applying Maximum Likelihood algorithms became the standard for further analyses. None of the studies increasing in sophistication (e.g. Klussmann-Kolb *et al*., 2008, Dinapoli & Klussmann-Kolb, 2010) recovered a monophyletic Opisthobranchia, usually due to acochlidian, but also sacoglossan and pyramidelloidean taxa clustering among pulmonates.

Since we failed to trace the origin of Acochlidia in morphology-based frameworks (Schrödl & Neusser, 2010), we carefully designed molecular studies including representatives of all the hard-to-find groups with interstitial slugs and all but one acochlidian families, plus all taxa that were mentioned to be potentially related to some of them (Jörger *et al*., 2010, Wilson *et al*., 2010). Special attention was paid to alignments and to the potential effect of ambiguous alignment portions, which were masked and more or less rigorously removed by the programs Aliscore and Gblocks (see Jörger *et al*., 2010 for details). The topology showing best likelihood resulting from Jörger *et al*. (2010) rejected all traditional hypotheses on the origin of Acochlidia, but indicate a pulmonate relationship of Acochlidia. In particular, tree hypotheses were considered as robust and reliable enough to propose a reclassification of Euthyneura, abandoning the taxon name and concept of Opisthobranchia.

The present paper combines results of Jörger *et al*. (2010), Dinapoli & Klussmann-Kolb (2010), Dinapoli *et al*. (2011) and some preliminary data on the origin of Rhodopemorpha (see Wilson *et al*., 2010), and reviews and discusses the status of Opisthobranchia in the light of improving data sets and analytical methods. Finally, it gives some examples how new phylogenetic hypotheses affect old paradigms on opisthobranch evolution, and recommends facing the consequences of changing concepts.

Challenging the Opisthobranchia concept

Combining the results on the origin of Acochlidia by Jörger *et al*. (2010) with a preliminary analysis on the origin of Rhodopemorpha by Wilson *et al*. (2010) by hand shows a consensus topology (Fig. 1) that radically differs from traditional heterobranch classifications. Monophyletic Rhodopemorpha cluster among basal, shelled lower heterobranchs with high support; thus, based on molecular data, Rhodopemorpha are preliminary not related to any of the euthyneuran taxa or even to dorid nudibranchs as was suspected based on morphological data before. The Opisthobranchia are polyphyletic: Acteonoidea plus Rissoelloidea is the sister to Euthyneura, with Nudipleura as first euthyneuran offshoot. Pulmonates in a traditional sense are paraphyletic, including the "opisthobranch" clades Sacoglossa and Acochlidia, and the potential lower heterobranchs *Glacidorbis* and Pyramidellidae, and thus were called Panpulmonata by Jörger *et al*. (2010). The remaining opisthobranchs form a clade called Euopisthobranchia by Jörger *et*

al. (2010). Several, but not all of these nodes (Fig 1; see Jörger *et al*., 2010) are robustly supported and the topologies slightly vary according to different analyses and parameters. Nevertheless, Opisthobranchia are not recovered monophyletic under any circumstances. In particular, worm-like sluggish opisthobranch taxa undoubtedly have independent origins and thus structural, functional and biological similarities evolved convergently due to selective pressure in an extreme habitat (Jörger *et al*., 2010, Schrödl & Neusser, 2010, Wilson *et al*., 2010). Bootstrap support and posterior probability values are high for most of the morphologically well-defined opisthobranch and pulmonate subclades usually treated as superfamilies or (sub)orders (collapsed to terminal taxa in Fig 1). Excluding the historically enigmatic Rhodopemorpha and Acteonoidea conceptually still results in paraphyletic Opisthobranchia at best, with Nudipleura as sister to all other euthyneurans, and both Sacoglossa and Acochlidia clustering among pulmonate taxa. Constraining the analyses of Jörger *et al*. (2010) towards monophyletic Opisthobranchia was highly significantly rejected based on their data. Excluding Acochlidia or Sacoglossa or both from an Opisthobranchia concept still does not render them monophyletic. Standard molecular markers clearly reject the monophyly of Opisthobranchia under any historic or reasonable taxon definition, and the topology (Fig. 1) differs from any morphology-based classifications, apomorphy-based reconstructions and, in particular, cladistic analyses that, thus, all were misled.

New trees, new truths?

By showing the non-monophyly of Euthyneura, Opisthobranchia and Pulmonata in a traditional sense, our standard marker based tree hypothesis (Fig. 1) is consistent to most previous molecular analyses available, regardless of using single genes, combinations of nuclear and mitochondrial genes or mitochondrial genomic data. More problematic than showing the deficiency of traditional classifications, however, is to present a convincing alternative: data sets, methods used and resulting topologies may greatly differ depending on the data used and there is no way of a direct numerical evaluation of how reliably these trees reflect evolutionary history.

However, there is some evidence that the design and performance of molecular studies on heterobranchs evolved over time, and thus there is hope that some of the latest topologies are superior to previous ones. Early single gene analyses (e.g. Thollesson, 1999a,b) were limited by still poor taxon and character sampling, simplistic alignment tools and parsimony as a single optimization criterion. Studies using mitochondrial genes (Grande *et al*. 2004a,b) or mitochondrial genomes (Grande *et al*., 2002, 2008, Medina *et al*., 2011) also were based on inadequate and unrepresentative heterobranch taxon sampling, the signal to noise ratio of markers remains untested, and topologies still differ. Supplementing the landmark studies on combined 18S and 28S rRNA genes by Vonnemann *et al*. (2005) by further taxa and using the whole set of what we now call "standard" genes of Klussmann-Kolb *et al*. (2008) and Dinapoli & Klussmann-Kolb (2010), our current approaches (Jörger *et al*., 2010, Wilson *et al*., 2010) use a multi-locus set of a truly representative taxon sampling i.e. several lineages of lower heterobranchs, all previously recognized or suspected euthyneuran clades, and all the enigmatic interstitial target taxa in question are included, plus assumed relatives of Rhodopemorpha such as dorid nudibranchs and several runcinids. In addition, the few European acochlidian taxa used in previous analyses (e.g. Vonnemann *et al*. 2005, Dinapoli & Klussmann-Kolb, 2010) were shown to be highly derived ones; especially *Hedylopsis spiculifera*, but also the microhedylacean species *Pontohedyle milaschewitschii* and *Microhedyle glandulifera* showed long branches due to aberrantly evolved loci in comparison to other, more slowly evolving acochlidian species from other parts of the worlds oceans (Jörger *et al*., 2010). Selecting a sufficient number of basal and slow-evolving taxa from old groups is clearly beneficial for minimizing branch

lengths and the effects of signal erosion (e.g. Wägele & Mayer, 2007). On the data quality side, state of the art procedures have been applied to minimize errors and noise, i.e. sequences were checked by BLAST searches and hypervariable regions of the alignments removed by masking programs, and only the most recent studies (e.g. Dinapoli & Klussmann-Kolb, 2010, Holznagel *et al*., 2010, Jörger *et al*., 2010, Dayrat *et al*., 2011, Dinapoli *et al*., 2011,) used both ML and Bayesian analyses, which is beneficial to reveal and control for effects of different evolutionary rates among lineages (e.g. Paps *et al*, 2009). While Holznagel *et al*. (2010) limited their study on partial 28S of an incomplete panpulmonate sampling, i.e. lacking Sacoglossa and Acochlidia, the more representative and comprehensive standard gene studies by Dinapoli & Klussmann-Kolb (2010) and Jörger *et al*. (2010) seem to converge towards a topology that is largely congruent to Fig. 1. We thus assume that this topology will be fairly robust to taxon addition. In particular, adding several more species of Pyramidellidae to the standard gene set, Dinapoli *et al*. (2011) already confirmed the Pyramidellidae as part of a common clade with *Glacidorbis* and Amphiboloidea. Göbbeler & Klussmann-Kolb (2010) showed that the node of Rissoelloidea and Acteonoidea is robust to adding representatives of all acteonoidean families.

Despite all these efforts to optimize taxon sampling, data quality, and alignment procedures, neighbornet analyses by Dinapoli & Klussmann-Kolb (2010) and Jörger *et al*. (2010) show a still high level of conflict in the data, with split support for some groups only. Since none of the well-supported nodes in the tree is contradicted by the split analyses, we do not interpret this as general evidence against our tree but as a warning that the power of our standard marker set for resolving heterobranch evolution has its limitations. The topology shown herein (Fig. 1) needs to be tested and refined by a truly independent set of molecular markers showing a high signal to noise ratio and minimizing the risk of alignment artefacts, i.e. conservative, protein coding nuclear genes.

Violating morphology?

Our phylogenetic consensus hypothesis (Fig. 1) is based on a large and representative taxon sampling, and on alignments of several thousands of nucleotides; its major weakness is due to just 4 - and always the same - "standard genes" involved. However, most of the traditionally accepted heterobranch taxa on order or family level such as Nudipleura, Acochlidia, Sacoglossa, Eupulmonata and Ellobioidea were recovered as robustly supported lineages. These molecular results are congruent with morphology-based ideas, and thus are likely to represent the evolutionary history. This also implies that both morphology-based inference and standard genes are informative at least at these levels. What remains problematic are the interrelationships between such major clades that have just poorly supported and sometimes incongruent trees based on standard markers. There is no doubt that much of the conflict with previous morphology-based hypotheses (e.g. Wägele & Klussmann-Kolb, 2005 as the most comprehensive one) is due to misconceptions that based on misinterpretations of homology and on extreme levels of homoplasy in the latter, as already suspected by Gosliner (1981) and Gosliner & Ghiselin (1984). The best examples refer to mesopsammic, convergently evolved worm-like taxa (Fig. 1) all showing a similar set of reductions and innovations (e.g. Jörger *et al*., 2010, Schrödl & Neusser, 2010), that are obviously adaptive to their special habitat. Moreover, at present, we are not able to present any conspicuous apomorphies for the recently established clades, except for Euopisthobranchia having evolved an oesophageal/gizzard cuticle (Jörger *et al*., 2010). Morphology thus has to be re-examined carefully and *a priori* homology assumptions might have to be changed according to *a posteriori* relationships unravelled. On the other hand, even some of the most intriguing relationships proposed by recent molecular analyses (Dinapoli & Klussmann-Kolb, 2010; 2011; Jörger *et al*., 2010) may fit within a morphological framework. *Glacidorbis* clusters within pulmonates,

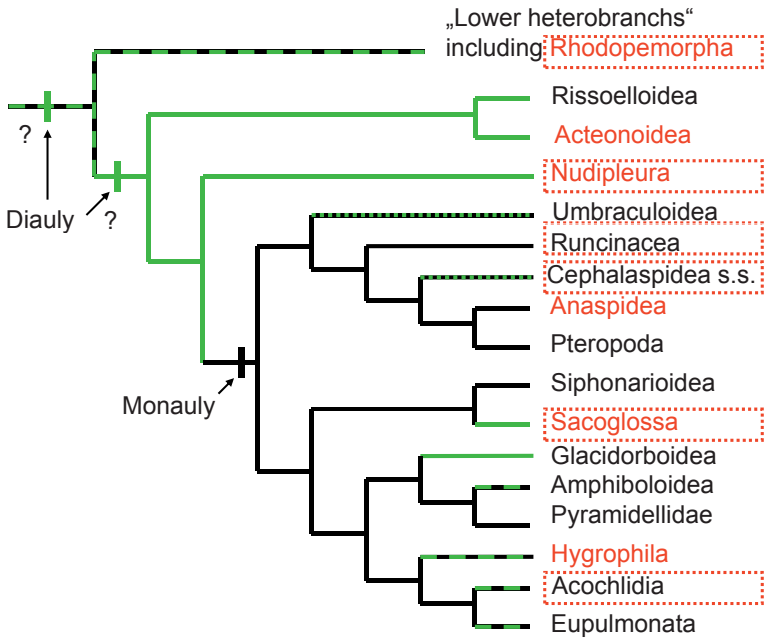

Figure 2:
Evolution of "opisthobranchs". Taxa with interstitial members are framed in red; mesopsammic habitat is basal in Acochlidia and possibly Rhodopemorpha only; meiofaunal subclades, all more or less vermiform and showing an array of further adaptations, thus evolved many times independently among Heterobranchia. Taxa with at least one secure pentaganglionate stage known in at least a single species (see Dayrat & Tillie,r 2000) are marked red; the only regularly pentaganglionate higher taxa may be Rhodopemorpha (juveniles only of Rhodope, adults of Helminthope) and Acteonoidea (adult). Stem lineages of taxa showing monaulic reproductive systems are colored black, those having androdiaulic (including triaulic) conditions are green; clades with mixed states are broken black/green, and clades with just exceptional and/or non-basal androdiaulic taxa are dotted black/green. Note that it is parsimonious to assume that monauly evolved in the common, tectipleuran ancestor of Euopisthobranchia and Panpulmonata. If so, true androdiauly (gonoducts split into oviduct and vas deferens proximal to the female gland mass) re-evolved in the stem lineages of Sacoglossa, Glacidorboidea and within several other panpulmonate subclades. Also note that a variety of structurally differing monaulic and diaulic conditions occur and that different authors use different terms; e.g. the special androdiaulic condition occurring in some acochlidians is called monaulic by Valdés et al. (2010). In the light of novel phylogenetic hypotheses, the characters and evolution of heterobranchs need to be re-examined in much greater depth.

i.e. as sister of Amphiboloidea, as suggested by Ponder (1986), rather than being related with lower heterobranchs as proposed by Haszprunar (1985, 1988). The Pyramidellidae *sensu stricto*, (i.e. all those Pyramidelloidea having a buccal stylet rather than a complex jaw apparatus as in Murchisonellidae) is an integral part of Euthyneura even when comparing mitochondrial genomes (Grande *et al*., 2008). This placement is supported by central nervous features such as the possession of giant nerve cells and a rhinophoral ganglion (see Huber, 1993). Siphonarian intertidal (or even fully marine) limpets were suggested to be opisthobranchs (Haller, 1892) or most basal pulmonates based on their morphology (Hyman, 1967), which fits with their position as early panpulmonate descendants of an opisthobranch grade. As discussed by Jörger *et al*. (2010), morphological features usually suggested to be synapomorphic for pulmonates are either plesiomorphic, poorly explored, or of limited significance. Even more straightforward, accepting the proposed homology of the pulmonate procerebrum and opisthobranch rhinophoral ganglia (Haszprunar, 1988) that has received increasing evidence from results of several microanatomical studies (e.g. Huber, 1993, Neusser *et al*. 2007), there is not a single putative synapomorphy left for Opisthobranchia (Jörger *et al*., 2010).

Summing up, it is the absence of contradiction, rather than unambiguous support, which makes the novel euthyneuran phylogenetic hypothesis presented by Jörger *et al*. (2010) and herein alluring. Still, the monophyly of Pulmonata and Opisthobranchia are clearly rejected by current knowledge (Fig. 1) and this fact cannot be longer ignored.

Consequences

Accepting the core topology presented here (Fig. 1), or just parts of it, has dramatic consequences for opisthobranch (and pulmonate) research.

First, neither "Opisthobranchia" nor "Pulmonata" can be retained as monophyletic taxa and thus have to be abandoned from our thinking and the literature. A reclassification has been proposed by Jörger *et al*. (2010) recently, modifying old names according to new concepts, i.e. Nudipleura as sister to a clade composed of Euopisthobranchia plus Panpulmonata; the latter, well-supported clade (Fig. 1) is named Tectipleura herein. In particular, polyphyletic "Opisthobranchia" do not even form a grade that can be characterized by any conspicuous set of plesiomorphies. Traditional "Opisthobranchia" thus are nothing else than an artificial assemblage of usually marine slugs or snails with limpet-like, bivalved or bubble shells showing tendencies of reduction or internalization, having a more or less detorted and externally bilateral symmetrical body with usually at least one pair of head tentacles or a head shield, including many exceptions. Rather than having a phylogenetic or evolutionary or even merely descriptive value, the "Opisthobranchia" concept is of historical and –to many of us– emotional value "only".

Second, hypotheses on structures, functions or any other features, homology, character polarity, and evolution of opisthobranchs have to be reassessed in the light of new phylogenetic evidence. Some of the rampant parallelism assigned to Opisthobranchia is actually attributable to a taxon misconception, while even higher levels of homoplasy are indicated e.g. by the independent origins of meiofaunal groups showing an array of independently derived features (e.g. Jörger *et al*., 2010, Schrödl & Neusser, 2010). Intriguingly, basal Rhodopemorpha are one of the few taxa supposedly showing a pentaganglionate condition (in juveniles and/or adults), but, according to Figs. 1 and 2, are not part of the Pentaganglionata (=Euthyneura) *sensu* Haszprunar, a concept that has been criticized before (Dayrat & Tillier, 2000). Additionally, rhodopemorphs are euthyneurous slugs that are not part of Euthyneura (Fig. 2). The simple, monaulic condition of the reproductive system was taken for granted to be plesiomorphic for Opisthobranchia (e.g. Ghiselin, 1966, Gosliner, 1981, Valdés *et al*., 2010). Structurally more complex diaulic conditions with separate male and female gonoducts were thought to have evolved from such a "primitive" level of organization, either as a single event or in multiple convergence (Valdés *et al*., 2010), with the condition in pulmonates unclear (Wägele *et al*., 2008). Widening the taxonomic focus and mapping monaulic and diaulic conditions on our novel topology (Fig. 2) may question these paradigms at least. It appears that (andro)diauly evolved at least once already in the heterobranch stemline and was plesiomorphically retained in Nudipleura. Opisthobranch monauly thus evolved at least once from a diaulic condition, possibly already in the common ancestor of Euopisthobranchia and Panpulmonata; monauly may be a synapomorphy of Tectipleura. While basal clades of Euopisthobranchia are monaulic, a few androdiaulic taxa exist ("triaulic" *Anidolyta*, certain *Ringicula* spp; Valdés *et al*., 2010), indicating secondary androdiauly. Also, some secondary, more or less incomplete structural and functional subdivisions of gonoducts may occur in certain subtaxa, e.g. leading to a sometimes called "oodiaulic" system in Anaspidea (Gosliner, 1994) or some cephalaspidean genera (Rudman & Willan, 1998, Valdés *et al*., 2010). The situation within panpulmonates is very complex showing a mosaic of (primary or secondary) monaulic and diaulic conditions in many major subgroups (Fig. 2),

implying much homoplasy involved. Androdiauly in panpulmonates is structurally heterogeneous, e.g. the vas deferens may split off the hermaphroditic duct in a proximal ("true androdiauly") or in more distal position ("special androdiauly", e.g. of some Acochlidia), and may run freely in the body cavity or in association to the body wall (as a "sunken" or "closed" sperm groove) (e.g., see Hubendick, 1978; Golding *et al*., 2008; Schrödl & Neusser, 2010). Complex evolutionary scenarios proposed by Visser (1977, 1988) trusted on a direct descent of pulmonates from prosobranch ancestors that is, however, rejected by all modern phylogenetic results. The actual variation, homology and evolution of heterobranch genital systems clearly merit detailed comparative and integrative exploration. Even more fundamentally changing our view, rather than being a "crown group" the opisthobranchs including the diverse Nudipleura and Euopisthobranchia now may be considered as just moderately species rich and successful early offshoots of the panpulmonate stem line, leading to much higher ecological and species diversity therein (Fig. 1).

Third, and of practical importance, in future studies on traditional opisthobranch (or pulmonate) taxa it is no longer tenable to just define and use "Opisthobranchia" (or "Pulmonata") as an ingroup, as a taxon concept, or just as a point of reference, without proving its monophyly by using an adequate heterobranch taxon sampling. In simple words, there is no more way to study opisthobranchs without considering lower heterobranchs and pulmonates, and vice versa. Instead, the traditionally isolated research communities on basal heterobranch, opisthobranch or pulmonate taxa have to recognize that barriers are perceived rather than of a systematic nature; the earlier we combine our knowledge and efforts the better it is for furthering our branch of science.

Fourth: Yes, we now advocate for renaming the International Opisthobranch workshops as Heterobranch workshops, to bring people together!

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Chapter 3. A reply to Medina et al. (2011): Crawling through time: Transition of snails to slugs dating back to the Paleozoic based on mitochondrial phylogenomics

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Letter to the Editor

A reply to Medina et al. (2011): Crawling through time: Transition of snails to slugs dating back to the Paleozoic based on mitochondrial phylogenomics

The most recent approach on opisthobranch phylogeny by Medina et al. (2011) merits both credit and criticism. On one hand, it contributes new and valuable data; no less than 13 complete euthyneuran gastropod mitochondrial genomes were sequenced, and concatenated amino acids were analyzed in a total set of 25 gastropods. The phylogenetic tree resulting from maximum likelihood analyses is fully resolved, and the support for non-critical nodes was very high. However, there are a number of significant problems that arise in this paper, that need to be discussed.

Medina et al. (2011) assert that their paper substantiates mitogenomic approaches as a reliable approach to solving difficult phylogenetic problems or nodes, and positively reviews similar earlier research (Grande et al., 2002, 2004a,b, 2008). Yet paradoxically, the cited studies showed high node support for alternative contradictory topologies. These unconventional relationships, e.g. Patellogastropoda as a maximally supported sister of Euthyneura (Grande et al., 2008), could easily be explained by limited taxon sampling. Earlier studies emphasized the potential power of apomorphies derived from mitochondrial gene rearrangements for euthyneuran systematics (e.g. Grande et al., 2002). Yet Medina et al. (2011) apparently found no major apomorphic gene rearrangements to support their topology; instead, they refer to an 'ancestral opisthobranch arrangement' without any explicit reconstruction given.

Fundamentally, the taxon sampling used by Medina et al. (2011) is still limited, highly selective, and based on the predefined concept of reciprocal monophyly of the traditional euthyneuran groups 'Opisthobranchia' and 'Pulmonata'. To represent the ingroup, the authors include six traditional opisthobranch orders, however, problematic major groups such as Tylodinoidea, Runcinoidea, Thecosomata, Gymnosomata, Acochlidia and Rhodopemorpha are lacking, and are crucial to testing monophyly of 'Opisthobranchia'. *A priori* definitions of 'Opisthobranchia' and 'Pulmonata' as monophyletic sister taxa contradict most, if not all, recent papers addressing heterobranch relationships. This includes those based on morphology (Wägele and Klussmann-Kolb, 2005) or molecular markers (Grande et al., 2008; Dinapoli and Klussmann-Kolb, 2010; Göbbeler and Klussmann-Kolb, 2010; Jörger et al., 2010; Dinapoli et al., 2011; all papers available prior to final submission of Medina et al., 2011), and also a recent molecular phylogeny by one of the authors (Dayrat et al., 2011). Apart from acteonoids resulting as opisthobranchs, and pyramidellids resulting as pulmonates, no lower heterobranchs (sensu Allogastropoda) were included, and further outgroups chosen are distant and unjustified. Arguably construed as *a posteriori* sampling selection, Medina et al. (2011) also discard several available pulmonate mitogenomes with no justification other than claiming they show 'particularly long branches'. Our own preliminary amino acid alignments on all available gastropod genomes, however, do not show any specific irregularities within these taxa (own observations). It is a pity that Medina et al. (2011) do not discuss the changes in tree topology that these 'long branch taxa' might have caused, especially since monophyly of 'Pulmonata' and its potential sister group relationship to 'Opisthobranchia' is affected when these taxa are added (see Grande et al., 2008).

The outcome of this taxon selection regime is a euthyneuran tree topology that, taken alone and unrooted, shows some similarity to results from comprehensive multi-locus studies (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Dayrat et al., 2011). It still differs regarding the relative positions of e.g. Acteonoidea and Sacoglossa, and, in particular, the position of the euthyneuran root. While the sampling of 'lower heterobranch' outgroups has been dramatically improved in recent multi-locus studies, the analysis by Medina et al. (2011) still uses very distant caenogastropod outgroups. Unsurprisingly, and undiscussed by Medina et al. (2011), phylogenetic distance is reflected by the long caenogastropod and euthyneuran stem lines, and long pulmonate branches. Compared with well-rooted topologies from recent multi-locus studies (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010), long branches and misrooting are likely responsible for Medina et al.'s (2011) euthyneuran bifurcation into 'monophyletic' Opisthobranchia and Pulmonata. These taxa were, however, made monophyletic only by redefinition.

A key problem to the interpretation of the topology presented by Medina et al. (2011) remains with the appearance of the pulmonate *Siphonaria* among opisthobranch clades. Although re-defining morphological features of *Siphonaria* to a supposedly opisthobranch relationship, the authors did not discuss any contradictory evidences, e.g. topologies of recent multi-locus studies (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Dayrat et al., 2011), records of closable pneumostomes from some siphonariids (e.g. Marshall and McQuaid, 1992), or several other traditional putative synapomorphies for pulmonates that are also present in *Siphonaria*, such as cerebral glands, mediodorsal bodies, and a procerebrum with double connectives (e.g. Van Mol, 1967; Saleuddin et al., 1997). Ignoring older ideas that pulmonary and mantle cavities of pulmonates and opisthobranchs are homologous (Ruthensteiner, 1997) and a recent reclassification of Euthyneura with *Siphonaria* as a basal (pan)pulmonate (Jörger et al., 2010), the authors missed hypotheses that explain both the (plesiomorphic) similarity of *Siphonaria* with (eu)opisthobranchs and the presence of (possibly also plesiomorphic) 'pulmonate features'. Instead, 'Opisthobranchia' sensu Medina et al. (2011) is maintained as monophyletic only by briefly reinterpreting the morphology of *Siphonaria* to fit into their concept of 'Opisthobranchia'. Furthermore, in conflict with most modern literature (e.g. Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010; Valdés et al., 2010), Acteonoidea are treated as opisthobranchs by Medina et al. (2011) without any discussion. The same applies for the formerly 'lower heterobranch' pyramidellid *Pyramidella* nesting within 'Pulmonata'; a typical result in recent molecular studies (Klussmann-Kolb et al., 2008; Dinapoli and

1874-7787/$ – see front matter
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Klussmann-Kolb, 2010; Jörger et al., 2010; Dayrat et al., 2011; Dinapoli et al., 2011), but this fact remains unaddressed by the authors. Most tellingly, 'Opisthobranchia' and 'Pulmonata' sensu Medina et al. (2011) are the only clades that do not garner *any* bootstrap support. We suggest that they may instead represent artificial assemblages of heterobranch taxa, as indicated by recent direct sequencing approaches on much more representative taxon sets (Dinapoli and Klussmann-Kolb, 2010; Göbbeler and Klussmann-Kolb, 2010; Jörger et al., 2010; Dinapoli et al., 2011).

Instead of acknowledging limitations regarding study design and outcomes, Medina et al. (2011) choose to name major clades, which is usually seen as a proxy for confidence in such hypotheses. The clade Acteopleura (Acteonoidea plus Nudipleura) had resulted from earlier direct sequencing analyses (Vonnemann et al., 2005), but was shown to be an artifact of limited taxon sampling in subsequent studies using a broader set of lower heterobranchs (*Rissoella*, not Nudipleura, is sister to Acteonoidea; Göbbeler and Klussmann-Kolb, 2010; Jörger et al., 2010). Their clade Siphoglossa (Sacoglossa plus *Siphonaria*) also resulted previously from analyses with more representative taxon sets (Jörger et al., 2010), but has never achieved any bootstrap support in past or current maximum likelihood analyses, and must be considered dubious. Finally, the clade Placoesophaga (Cephalaspidea plus Anaspidea), as defined by the esophageal cuticle, is synonymous to Euopisthobranchia sensu Jörger et al. (2010).

Medina et al. (2011) were the first to run molecular clock analyses for gastropods using whole mitochondrial genomes and recover a much earlier origin of euthyneuran, pulmonate and opisthobranch gastropods than suspected from fossils and earlier molecular clock approaches on multi-locus datasets (Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010). Rather than suggesting their limited data set and resulting topology is responsible for unrealistic (as compared to current knowledge on fossil record) time estimates, this discrepancy was explained by the generally poor heterobranch fossil record and earlier miscalibrations. However, Medina et al. (2011) apparently miscalibrated their own analysis using first appearances of potential stemline fossils as minimal node ages, i.e. divergence of extant members of these clades (see Jörger et al., 2010 for details). The same misunderstanding occurred when criticizing Dinapoli and Klussmann-Kolb (2010) for apparently much too recent ages of 'Siphonariidae' (in fact two *Siphonaria* species, 22 Mya) and 'Cephalaspidea' (just two taxa, 49 Mya), while the oldest fossils date back to 161 and 208 Mya, respectively; the dated stemlines, however, fit quite well with such fossil ages.

Although the timing of divergence is a major point of Medina et al. (2011), their BEAST analysis is not adequately described, calibration priors were counter intuitively set as normal distributions (minimum clade ages are unlikely to be represented by a normal distribution), and apparently only a single run was attempted before being abandoned. For example, on page 53 the authors claim 'In the Bayesian analysis all nodes are supported by 100% posterior probability values', which is simply not true according to their Fig. 1. Also, since no Bayesian analysis apart from a BEAST run is mentioned in the Material and methods section, where do the Bayesian analysis and posterior probabilities mentioned in Fig. 1 come from? If they derive from an interrupted BEAST run not reaching convergence, these support values must be disregarded. Using R8s instead, Medina et al. (2011) dated the Euthyneura node, i.e. the divergence of extant euthyneuran taxa, at 523 Mya, in the early Cambrian period. Stemline euthyneurans (or stem heterobranchs, since Acteonoidea are included) then must be much older — perhaps more than a billion years! How old would stemline apogastropods, gastropods or molluscs have to be then? Authors or any other experts involved in the review process should have doubted such results, could have explored the reasons for this, and could have required giving confidence intervals in an improved approach. The resulting reinterpretation of fossils and discussion of evolutionary scenarios in Medina et al. (2011) depends on unreliable topologies and highly unlikely time scales.

Concluding, both 'Opisthobranchia' and 'Pulmonata' were biased towards monophyly by definition and by selection. The problematic study design by Medina et al. (2011) clearly weakens the significance of topological results used for proposing a reclassification of 'Opisthobranchia', a taxon that is contradicted by almost all other recent studies (see review by Schrödl et al., 2011). Neither molecular timing, nor evolutionary conclusions by Medina et al. (2011) are convincing, and alternative interpretations must be considered. While generating an impressive amount of novel data, 'Crawling through time' has highlighted how slow progress can be in understanding sea slug evolution.

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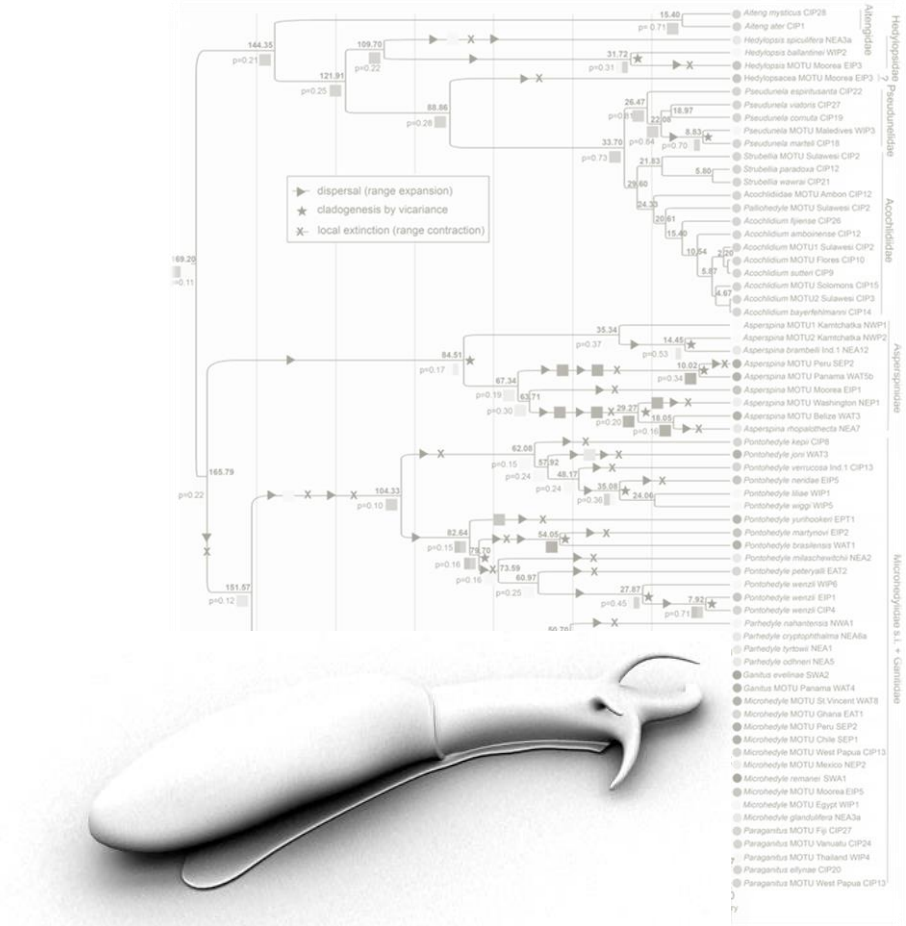
29 April 2011

II. PHYLOGENY AND EVOLUTION OF ACOCHLIDIA



Character mining and advanced 3D-microanatomy

Chapter 4. Exploring cerebral features in Acochlidia (Gastropoda: Opisthobranchia)

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Exploring Cerebral Features in Acochlidia (Gastropoda: Opisthobranchia)*

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Abstract. Histological semithin sections of the marine acochlidian species *Hedylopsis spiculifera* (Kowalevsky, 1901), *H. ballantinei* Sommerfeldt & Schrödl, 2005, *Microhedyle remanei* (Marcus, 1953) and *Asperspina murmanica* (Kudinskaya & Minichev, 1978) and of the limnic *Tantulum elegans* Rankin, 1979 were (re)examined for different cerebral features: 1) the number of cerebro-rhinophoral connectives, 2) the presence of Hancock's organs, 3) the relative position and size of the eyes, the length and diameter of the optic nerve, and the presence of an optic ganglion, and 4) cellular aggregates attached to the cerebral ganglia. We describe novel structures such as double cerebro-rhinophoral connectives in *T. elegans*, and "lateral bodies" in *H. spiculifera*, *H. ballantinei* and *A. murmanica*. Cerebral features are discussed as a promising additional set of characters for phylogenetic analysis. However, (ultra)structural comparisons of acochlidians with basal opisthobranchs and pulmonates are overdue.

Keywords. Cerebral nerves, "lateral bodies", dorsal bodies, Hancock's organ, optic ganglion.

1. INTRODUCTION

Acochlidian opistobranch gastropods show high morphological and biological diversity. However, the number of useful characters for phylogenetic analyses is still limited by the paucity of comparative data available. The central nervous system (cns) of several euthyneurous taxa was described (e.g. Haszprunar & Huber 1990; Huber 1993; Mikkelsen 2002), comprising data about cerebral nerves and sensory organs. The value of these data in phylogenetic studies is evident (Dayrat & Tillier 2002; Mikkelsen 1996). In contrast, several of the species (re)descriptions in Acochlidia do not include any information on the cns (e.g. Haynes & Kenchington 1991; Hughes 1991; Kirsteuer 1973; Marcus & Marcus 1955, 1959; Salvini-Plawen 1973; Wawra 1979, 1980, 1988). Other authors limited their descriptions of the cns to the main ganglia on the (pre)pharyngeal nerve ring and the visceral nerve cord (e.g. Bergh 1895; Bücking 1933; Challis 1968, 1970; Doe 1974; Hertling 1930; Kowalevsky 1901; Kudinskaya & Minichev 1978; Küthe 1935; Marcus 1953; Marcus & Marcus 1954; Morse 1976; Swedmark 1968; Wawra 1989; Westheide & Wawra 1974). Unfortunately, the identification of the small and hardly separated ganglia on the visceral nerve cord is problematic. Even detailed histological descriptions, such as that of *Tantulum elegans* by Rankin (1979), can be considerably misleading and thus cannot be trusted (see Neusser & Schrödl 2007). Furthermore, very few studies give data about cerebral nerves and sensory organs reflecting the complexity of the acochlidian cns. Huber (1993) gave a detailed overview of the cns in marine heterobranchs and determined the number of cerebral nerves in Acochlidia to only two (the labiotentacular nerve and the proximally joint oral and rhinophoral nerve) plus the static nerve. Sommerfeldt & Schrödl (2005) confirmed these three nerves plus optic nerves for *Hedylopsis spiculifera* and *H. ballantinei*. The authors emphasized the presence of large rhinophoral ganglia, from which the joint oral and rhinophoral nerve arise, and that was overlooked in *H. spiculifera* by Huber (1993). The terminology and the homology of the different cerebral nerves in Acochlidia are still uncertain.

Data about sensory organs are sparse, often consisting only in the affirmation of presence or absence of easily identified structures, such as eyes (e.g. Challis 1970; Marcus 1953; Marcus & Marcus 1955; Westheide & Wawra1974). Hancock's organs, the primary chemosensory organs in architectibranchs and cephalaspideans (Mikkelsen 1996, 2002), were thought to be absent in Acochlidia (e.g. Neusser et al. 2006; Sommerfeldt & Schrödl 2005; Wawra 1987). However, Hancock's organs like structures were reported from *Microhedyle glan-*

Table 1 . Comparison of cerebral features in different acochlidian species. +: present, –: absent, ?: not detected.

feature \ species	*Hedylopsis spiculifera*	*Hedylopsis ballantinei*	*Asperspina murmanica*	*Tantulum elegans*	*Microhedyle remanei*
Double cerebro-rhinophoral connective	?	?	?	+	?
Hancock´s organ	?	?	?	+	?
Eyes	+ pigmented	+ pigmented	–	+ reduced unpigmented	–
Eyes externally visible	dorsal and lateral well visible	dorsal and lateral hardly visible	–	not visible	–
Eyes position	posterior to the rhinophores (in some distance)	slightly posterior to the rhinophores (at their base)	–	slightly anterolateral to the cerebral ganglion	–
Eye size in diameter	25 µm	30 µm	–	20 µm	–
Optic nerve	long, undulated	long, undulated	–	short, not undulated	–
Optic nerve diameter	6–7 µm	6–7 µm	–	3 µm	–
Optic ganglion (diameter)	–	–	–	+ (18 µm)	–
Lateral bodies	+	+	+	–	–
Cells above cerebral commissure	?	?	+	?	?

dulifera (Kowalevsky, 1901) and *Pontohedyle milaschewitchii* (Kowalevsky, 1901) by Edlinger (1980a, b), and recently confirmed for *P. milaschewitchii* (Jörger et al. in press). Additionally, our re-examination of *Tantulum elegans* revealed the presence of a small Hancock's organ in this species too (Neusser & Schrödl 2007).

Among representatives of four traditional acochlidian families (Hedylopsidae, Asperspinidae, Tantulidae and Microhedylidae), the present study (re)investigates a number of special cerebral nervous features using histological sections. As far as information is available, these characters are compared with other acochlidian species and are evaluated as a possible set of characters for future phylogenetic analysis.

2. MATERIAL

Serial semi-thin sections of five different acochlidian species were available for re-examination by light microscopy: one series (section thickness: 1.5 µm) of *Hedylopsis spiculifera*, Zoologische Staatssammlung München, ZSM N° 20070391 (Secche della Meloria, Livorno, Italy, September 2005) and one paratype series (section thickness: 2 µm) of *Hedylopsis suecica* Odhner, 1937, Swedish Museum of Natural History, SMNH N° 27211; *H. suecica* was considered as a synonym of *H. spiculifera* by Wawra (1989) and confirmed by Sommerfeldt & Schrödl (2005). Five paratype series (section thickness: 2 µm) of *Hedylopsis ballantinei*, ZSM N° 20004766/1, 20004767, 20004768, 20004769 and N° 26X (Dahab, Gulf of Aqaba, northern Red Sea, October 1999). Six series (section thickness: 1.5 µm) of *Microhedyle remanei*, ZSM N° 20070079, 20070080, 20070081, 20070082, 20070083 and 20070084 (southwest of Castle Roads, Bermuda Islands, July 1999). Four series (section thickness: 1.5 µm) of *Asperspina murmanica*, ZSM N° 20062163, 20062164, 20062165 and 20062167 (Yarnyshnaya Bay, Barents Sea, Russia, August 2005). Four original paratype series (section thickness: 3 µm) and two recently prepared paratype series (section thickness: 1.5 µm) of *Tantulum elegans*, Royal Ontario Museum, Canada, ROM N° 8E1 and 2F0 (Golden Grove, St. Vincent, West Indies, July 1972). All sections, except the original paratype series of *T. elegans*, were stained with methylene blue-azure II according to Richardson et al. (1960).

3. CEREBRAL FEATURES EXAMINED

3.1. Rhinophoral ganglia and cerebro-rhinophoral connectives

A comparative overview of all examined features in the different species is given in Table 1.

All species re-examined herein, except *Microhedyle remanei*, have a pair of true rhinophoral ganglia, i.e. large ganglia separated into a nuclei-free medulla and a cortex composed of cell bodies. The rhinophoral ganglia of *M. remanei* are not subdivided into cortex and medulla; instead the nuclei are distributed homogeneously all over the ganglion (see NEUSSER et al. 2006, fig. 3d). Serial sections of *Hedylopsis spiculifera*, *H. ballantinei* and *M. remanei* show only a single nerve (approx. 5–10 µm in diameter) that connects the cerebral ganglion to the rhinophoral one. In one specimen of *Tantulum elegans* examined, we found two nerves connecting the cerebral ganglion with the rhinophoral ganglion (Fig. 1). Both nerves are thin (approx. 7 µm in diameter) and lie close together (distance between them approx. 3µm). Nevertheless, the transition between the cerebral ganglion and the rhinophoral ganglion is well identifiable due to the presence of dark stained fibres (Fig. 1A, D).

3.2. Sensory organs

3.2.1. Hancock's organ and nerve

Paired, small and ciliated invaginations posterior to the head appendages and innervated by cerebral nerves are present in *Tantulum elegans* (see NEUSSER & SCHRÖDL 2007, fig. 4b). Neither such organs of similar shape could be detected in *Hedylopsis spiculifera*, *H. ballantinei* and *Microhedyle remanei*, or cerebral nerves innervating the region where Hancock's organs are present in other acochlidian species.

3.2.2. Eyes, optic nerves and optic ganglia

Asperspina murmanica and *Microhedyle remanei* are eyeless and lack any optic nerve or optic ganglion. Both *Hedylopsis* species have pigmented lens eyes (Fig. 3A, B) that, however, differ in size and relative position. The eyes of *H. spiculifera* are clearly visible externally (Fig. 2A, B) from dorsal and lateral and reach up to 25 µm in diameter (Fig. 3A). They are located on the rather lateral side of the head (Fig. 2B), and are in some distance posterior to the rhinophores (Fig. 2A, B) and anterior of the cerebral ganglia. In contrast, the eyes of *H. ballantinei* are hardly detectable by external view (Fig. 2C) even though they are slightly larger (approx. 30 µm in diameter) (Fig. 3B). Furthermore, they are situated closer together and are just posterior to the rhinophores (Fig. 2C). The optic nerves show approx. 6-7 µm in diameter in both species (Fig. 3A, B). They arise from the rhinophoral ganglia and are highly undulated. An optic ganglion is absent in *H. spiculifera* as well as in *H. ballantinei*. In contrast, *Tantulum elegans* develops a very short and thin optic nerve (approx. 3 µm in diameter) leading to a reduced unpigmented eye of approx. 20 µm in diameter (Figs. 1, 3C). The optic nerve arises from a small optic ganglion (approx. 18 µm in diameter) that is subdivided into the outer cortex and the inner medulla (Fig. 3D). It is attached laterally to the cerebral ganglion, both of which are surrounded by a thin layer of connective tissue (Fig. 3D). No nerves can be detected by light microscope examination connecting the cerebral with the optic ganglion.

3.3. Aggregates attached to the cerebral ganglia

3.3.1. "Lateral bodies"

A "lateral body" as defined herein consists of a more or less hemispherical cluster of cells that is lying laterally on the surface of each cerebral ganglion. Under a light microscope, the cells of the "lateral bodies" cannot be distinguished from the neuron bodies situated in the cortex of the cerebral ganglion. Each "lateral body" is surrounded by a separate, relatively thin sheath of connective tissue and together with the cerebral ganglion by a second common and thick one. "Lateral bodies" are present in *Hedylopsis spiculifera* (Fig. 4A), *H. ballantinei* (Fig. 4B) and *Asperspina murmanica* (Fig. 4C). The "lateral body" lacks any subdivision. The nuclei are more or less uniformly distributed over the entire "lateral body". There are no nerves visible under the light microscope connecting the cerebral ganglion with the "lateral body", and there are no nerves arising from the latter. None of the specimens examined of *Microhedyle remanei* and *Tantulum elegans* had "lateral bodies".

3.3.2. Cells near the cerebral commissure

Additionally, we could find several cells of uncertain origin and function dispersed in the connective tissue above the cerebral commissure in *Asperspina murmanica* (Fig. 4D). In contrast to the "lateral bodies", these cells are not tightly attached to each other, and are not enclosed by an individual sheath of connective tissue. No data about the presence or absence of these cells can be given for *Hedylopsis spiculifera*, *H. ballantinei* and *Tantulum elegans*, due to very compressed tissue layers.

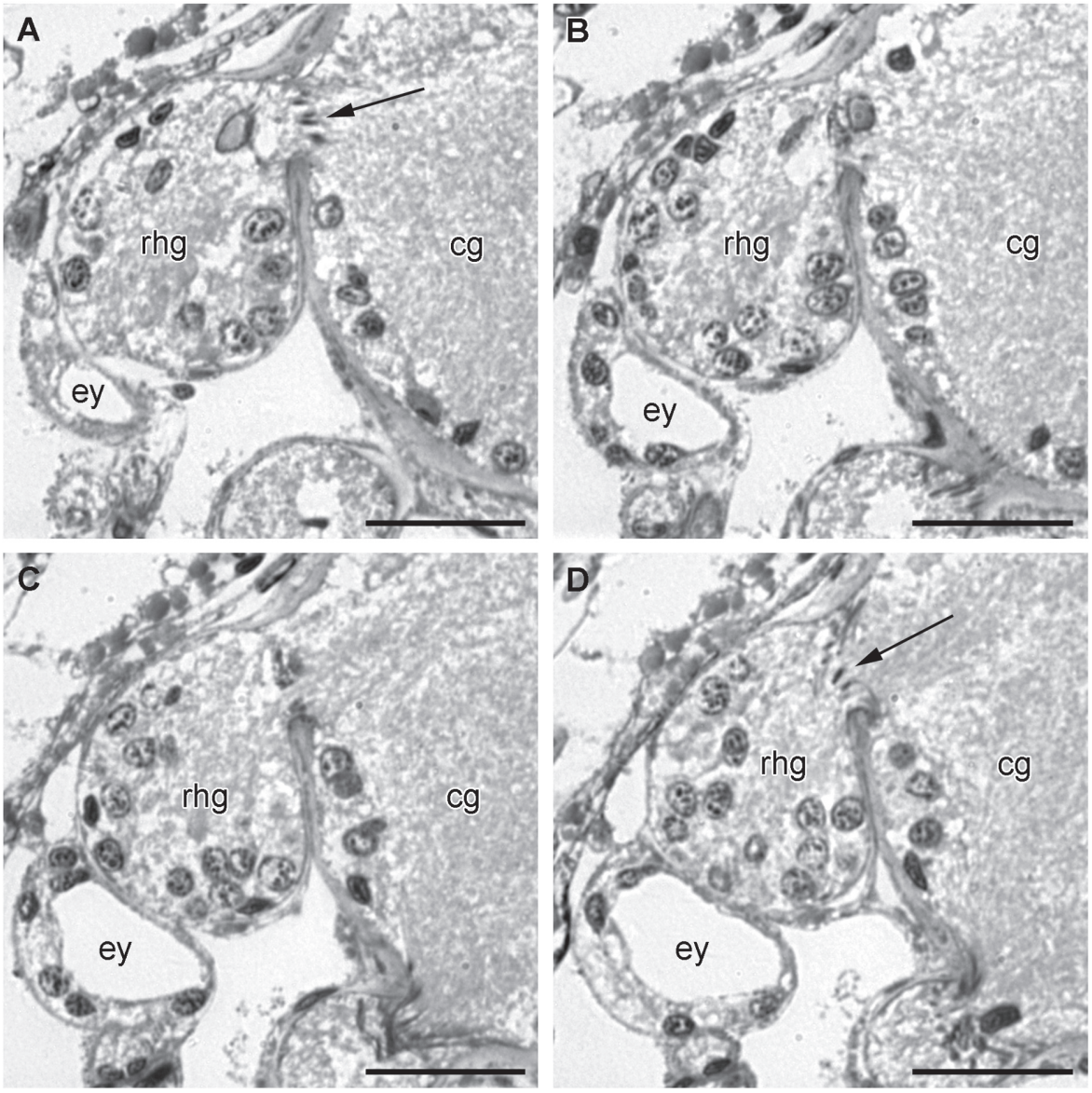

Fig. 1. Double cerebro-rhinophoral connective in *Tantulum elegans*. Four consecutive cross sections of series ROM N° 8E1, 3.slide, 6. ribbon, section N° 17–20. A: section N° 17, first cerebro-rhinophoral connective. B and C: section N° 18 and 19, respectively, without connective. D: section N° 20, second cerebro-rhinophoral connective. cg cerebral ganglion; ey eye; rhg rhinophoral ganglion; arrow, indicates fibres of the cerebro-rhinophoral connective. Scale bars A–D: 15 µm.

4. DISCUSSION

4.1. Rhinophoral ganglia and number of cerebro-rhinophoral connectives

The presence of rhinophoral ganglia were reported for *Hedylopsis spiculifera* and *Tantulum elegans* (see Rankin 1979; Wawra 1989), but both descriptions lack histological data of the rhinophoral ganglia. Recently, rhinophoral ganglia were described in detail for *Hedylopsis ballantinei* (see Sommerfeldt & Schrödl 2005), *Microhedyle remanei* (see Neusser et al. 2006), *T. elegans* (see Neusser & Schrödl 2007) and *Pontohedyle milaschewitchii* (see Jörger et al. in press). Due to their position anterodorsally of the cerebral ganglia and their similar innervation the homology of the rhinophoral ganglia can be assumed for all acochlidian species studied herein. In contrast to *Hedylopsis* species, *Asperspina murmanica* and *T. elegans*, rhinophoral ganglia of *P. milaschewitchii* and *M. remanei* are not separated into medulla and cortex. The presence

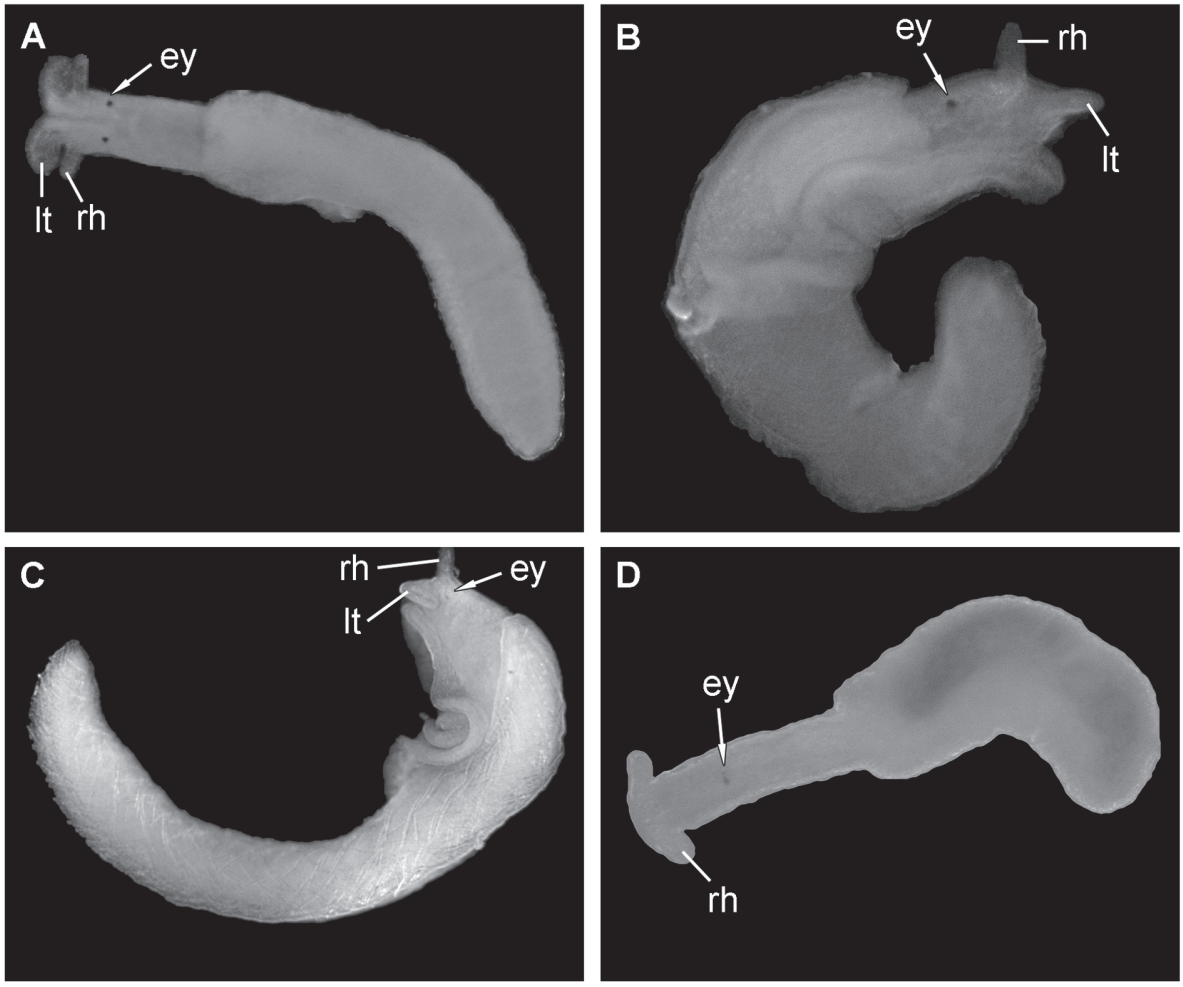

Fig. 2. Position of eyes in different acochlidian species, external view. A: *Hedylopsis spiculifera*, dorsal view, length 3.5 mm. B: *Hedylopsis spiculifera*, lateral view, length 3.5 mm. C: *Hedylopsis ballantinei*, lateral view, length 5 mm. D: *Pontohedyle milaschewitchii*, dorsal view, length 2.5 mm. ey eye; lt labial tentacle; rh rhinophore.

of rhinophoral ganglia within *P. milaschewitchii* that is lacking any rhinophores might be explained by a modified, e.g. neurosecretory function. *Microhedyle remanei*, however, possesses rhinophores and cell bodies evenly distributed within the rhinophoral ganglia.

Of all the specimens here studied, the double connection between the cerebral ganglia and rhinophoral ganglia could only be detected in one specimen of *Tantulum elegans*, and is only clearly visible on the right side of the nervous system. Unfortunately, the identification of these thin nerves depends critically upon preservation and staining conditions as well as on the cutting plane. Tiny nerves can thus be overlooked and easily misinterpreted, or be invisible even on semi-thin serial sections. While "detected" usually means "present", "not detected" does not necessarily mean "absent". The cerebro-rhinophoral connective has been identified by the presence of dark stained fibres. Haszprunar (1985, figs. 19, 20) described similar fibres occurring at the transition between two different ganglia in *Discotectonica discus* Philippi, 1844. A double cerebro-rhinophoral connective has also been found in *Pontohedyle milaschewitchii* (see Jörger et al. in press); both nerves are even thinner than those in *T. elegans*. There is no reliable data on further acochlidians.

Haszprunar & Huber (1990) described a double cerebro-rhinophoral connective for the enigmatic opisthobranchs *Rhodope veranii* Kölliker, 1847 and *Rhodope transtrosa* Salvini-Plawen, 1989, as well as a double connective attaching the cerebral ganglion with the procerebrum in the pulmonate *Smeagol manneringi* Climo, 1980. In fact, the double cerebro-rhinophoral connective of the acochlidian cns resembles the general pulmonate condi-

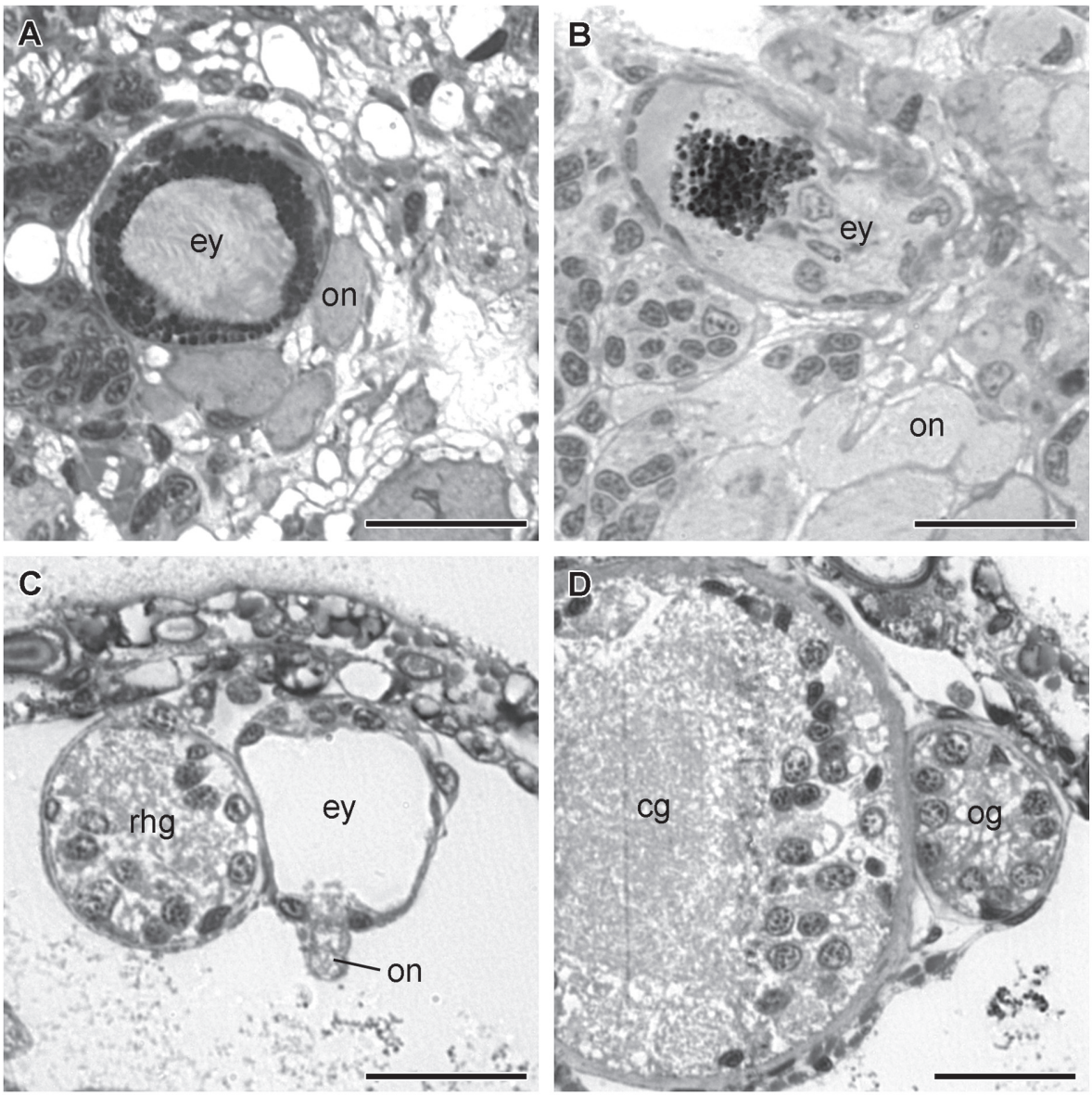

Fig. 3. Eyes and optic ganglion (cross sections). A: Pigmented eye in *Hedylopsis spiculifera* ZSM N° 20070391. B: Pigmented eye in *Hedylopsis ballantinei* ZSM N° 20004766/1. C: Unpigmented eye in *Tantulum elegans* ROM N° 8E1. D: Optic ganglion attached to the cerebral ganglion in *Tantulum elegans* ROM N° 8E1. cg cerebral ganglion; ey eye; og optic ganglion; on optic nerve; rhg rhinophoral ganglion. Scale bars A–D: 15 µm.

tion (Van Mol 1967). Therefore, the potential homology of acochlidian rhinophoral ganglia to the procerebrum of pulmonates should be investigated in detail.

4.2. Sensory organs

4.2.1. Hancock's organ

We were not able to detect any Hancock's organ like structures in the species examined herein except for *Tantulum elegans* which shows a pair of epidermal folds on the side of the head (Neusser & Schrödl 2007). Such folds were reported for *Pontohedyle milaschewitchii* and *Microhedyle glandulifera* and regarded as Hancock's organs by Edlinger (1980a, b), i.e. as true homologues of the primary chemosensory organs in architectibranchs and cephalaspids (see Mikkelsen 1996). According to their similar position, cerebral innervation, (although more tiny) structure, and probable sensory function, a general homology can be suspected. Some doubts persist, such as the

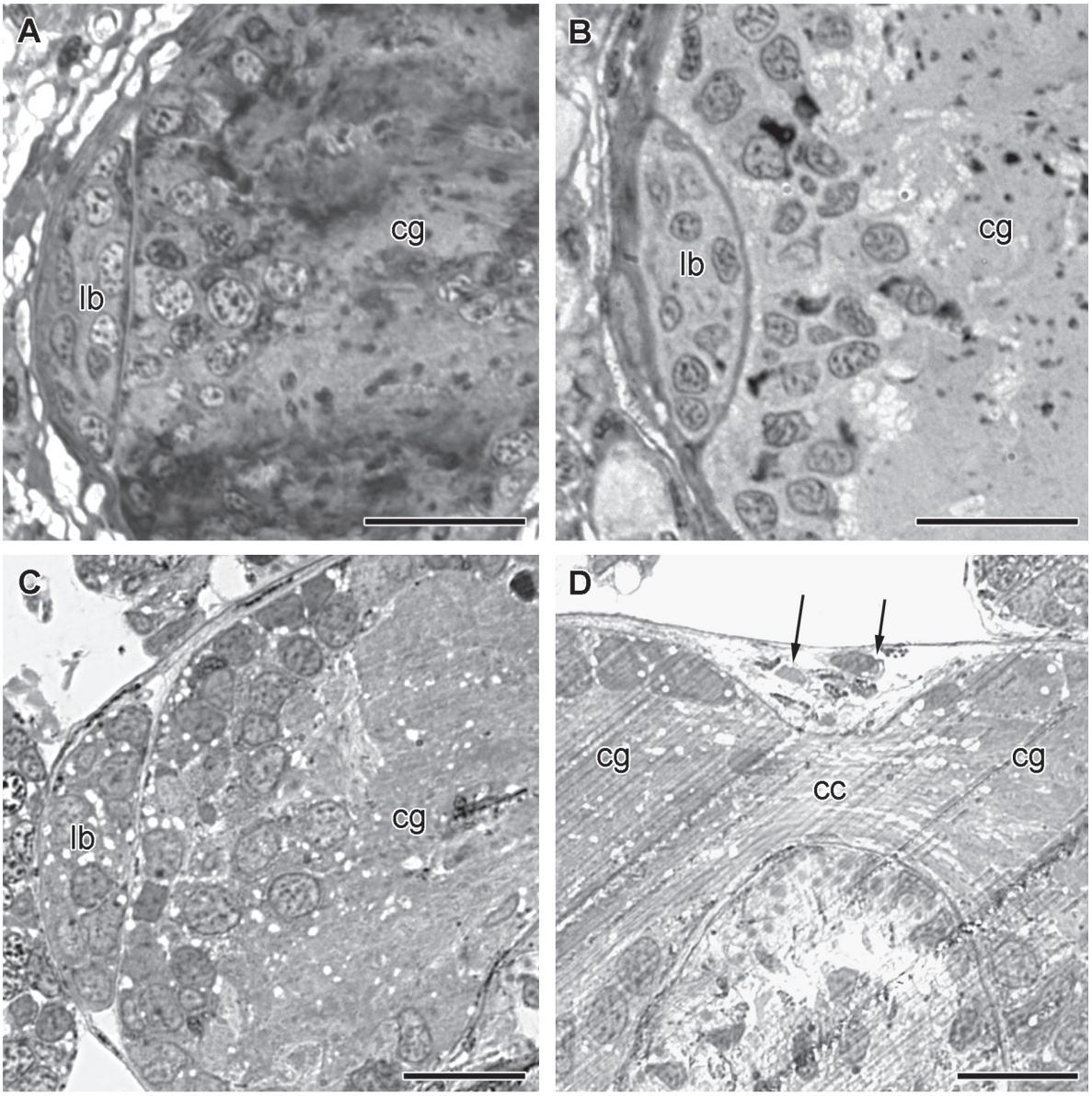

Fig. 4. Aggregates attached to the cerebral ganglia (cross sections). A: "Lateral body" in *Hedylopsis spiculifera* ZSM N° 20070391. B: "Lateral body" in *Hedylopsis ballantinei* ZSM N° 20004766/1. C: "Lateral body" in *Asperspina murmanica* ZSM N° 20062163. D: Cells above cerebral commissure in *Asperspina murmanica* ZSM N° 20062163. cc cerebral commissure; cg cerebral ganglion; lb "lateral body"; arrow, cells near cerebral commissure. Scale bars A–D: 15 µm.

yet unclear homology of euthyneuran cerebral nerves, the unknown origin of the Acochlidia and reports of acochlidian "Hancock's organs" from only a few and supposedly derived microhedylid species, i.e. *P. milaschewitchii* and *M. glandulifera,* and the enigmatic *T. elegans*.

4.2.2. Eyes, optic nerves and optic ganglia

In the past, the description of acochlidian eyes often was limited to the affirmation of presence or absence of these sensory organs. Eyes are absent in all *Asperspina* species, *Microhedyle remanei*, *Ganitus evelinae* Marcus, 1953, *Paraganitus ellynnae* Challis, 1968 and *Pontohedyle verrucosa* Challis, 1970 (see Challis 1968, 1970; Kudinskaya & Minichev 1978; Marcus 1953; Morse 1976; Salvini-Plawen 1973; Swedmark 1968). Our results show that the position, size and development of eyes in Acochlidia examined herein differ considerably.

The eyes of *Hedylopsis spiculifera* are clearly visible externally from a dorsal and lateral view. In the freshwater acochlidian species *Strubellia paradoxa* (Strubell, 1892) and *Acochlidium fijiense* Haynes & Kenchington, 1991 the eyes are clearly observable only in lateral view (unpubl. data of MS). In contrast, the eyes of the marine *Microhedyle glandulifera* (see Kowalevsky 1901; Marcus & Marcus 1955; Odhner 1952), *Hedylopsis ballantinei* (Fig. 2C) and *Pontohedyle milaschewitchii* (Fig. 2D) are externally not that clearly visible through the head tissue. Westheide & Wawra (1974) observed that eyes of *Parhedyle cryptophthalma* (Westheide & Wawra, 1974) were not visible externally in living specimens, and only as two small pigmented spots in preserved specimens. Eyes in *Pseudunela cornuta* (Challis, 1970) are poorly developed and not visible externally (Challis 1970, as *Hedylopsis cornuta*).

The eyes of *Hedylopsis spiculifera* and *H. ballantinei* are both located dorsolaterally in the body cavity; while the eyes of *H. ballantinei* are situated at the base of the rhinophores, in *H. spiculifera* they are somewhat more posteriorly. A similar dorsolateral eye position at or close to the base of the rhinophores is already known from the limnic acochlidian species *Acochlidium amboinense* Strubell, 1892, *Palliohedyle weberi* (Bergh, 1895) and *Strubellia paradoxa* (see Bergh 1895; Bücking 1933; Küthe 1935). In contrast, the eyes of *Pontohedyle milaschewitchii* are located more posteriorly and closer together (Fig. 2D). Westheide & Wawra (1974) described a similar eye position in the marine acochlidian *Parhedyle cryptophthalma*.

The optic nerve is short in *Strubellia paradoxa* (see Küthe 1935). The well-developed eyes of *Acochlidium amboinense*, *Palliohedyle weberi* and *S. paradoxa* were described as attached anterodorsally to anterolaterally on the cerebral ganglia (Bergh 1895; Bücking 1933; Küthe 1935), thus the optic nerves are probably short as well. The eyes of *Pontohedyle milaschewitchii* are directly attached to the cerebral ganglia (Jörger et al. in press), as are the eyes of *Parhedyle cryptophthalma*, *Microhedyle nahantensis* (Doe, 1974), *M. glandulifera* and *M. odhneri* (Marcus, 1955) (see Doe 1974; Marcus & Marcus 1955; Westheide & Wawra 1974). The optic nerve is moderately long but thin in *Tantulum elegans*, while long and thick in both *Hedylopsis* species. The long optic nerves observed herein may be phylogenetically informative in Acochlidia.

All eyes described for Acochlidia are pigmented, except those of *Tantulum elegans* (present study) and of *Microhedyle nahantensis* (see Doe 1974). The "poorly developed" eyes of *Pseudunela cornuta* described by Challis (1970) should be reinvestigated.

The eye size differs within the species: whereas eyes of *Hedylopsis spiculifera* and *H. ballantinei* measure approx. 25 and 30 µm, respectively, eyes in *Pontohedyle milaschewitchii* reach approx. 20 µm (Jörger et al. in press). The largest eye size known from an acochlidian species is 0.52 mm and was reported for the limnic *Palliohedyle weberi* (see Bergh 1895).

The optic ganglion in *Tantulum elegans* was first described by Neusser & Schrödl (2007) and is regarded to be a true ganglion with subdivision into cortex and medulla (see Neusser et al. 2006). More specifically, it is enclosed in a thin layer of connective tissue together with and attached to the cerebral ganglion. This feature should not be confused with the "lateral bodies" described in the present study, since the latter are lying inside the thick layer of connective tissue from the cerebral ganglion (see below). So far there are only two reports of ganglia being surrounded by a common layer of connective tissue with the cerebral ganglia: the rhinophoral ganglia of *T. elegans* (see Neusser & Schrödl 2007), and the rhinophoral ganglia of *Pontohedyle milaschewitchii* (Jörger et al. in press).

The presence of an optic ganglion only in *T. elegans* is surprising, since eyes are unpigmented in this species, while for species possessing more well-developed eyes (e.g. both *Hedylopsis* species and *Pontohedyle milaschewitchii*) this character is lacking. Either there are some unknown sensory abilities involved in at least one ontogenetic stage, or both eyes and optic ganglia are evolutionary remnants of organs in the process of being reduced. The optic ganglia of *Tantulum* do no more fuse with the rhinophoral ganglia, as may be the case in both *Hedylopsis* species with large rhinophoral ganglia bearing optic nerves. We urgently need ontogenetic evidence for the development of acochlidian central nervous structures.

The presence of optic ganglia, the origin and length of optic nerves, eye position in terms of situation and proximity to the cerebral ganglion, as well as eye size and structure should be reinvestigated in all acochlidian species, since these may be easily accessible and phylogenetically informative characters (see Mikkelsen 1996).

4.3. Aggregates attached to the cerebral ganglia

4.3.1. "Lateral bodies"

Sommerfeldt & Schrödl (2005) described "dorsal bodies" attached to the cerebral ganglion in the acochlidian *Hedylopsis ballantinei*. We herein confirm the presence of such organs for both *Hedylopsis* species and *A. murmanica*. Their position is, however, more lateral than dorsal. We thus propose to use the term "lateral bodies" for

such acochlidian structures until more detailed and comparative data are available to assess their homology to pulmonate dorsal bodies.

The "lateral bodies" of the re-examined acochlidian species are characterized by a group of neuronal cells that are enclosed within the thick connective tissue layer surrounding the cerebral ganglion. The dorsal bodies of basommatophoran pulmonates consist of a pair of similar neuronal cell clusters that are, however, enclosed in a thin sheath of connective tissue, and are situated dorsally on the cerebral ganglia. Basommatophoran dorsal bodies can lie close together and appear as one group in *Helisoma* Swainson, 1840 and *Planorbarius* Duméril, 1806, or they can be distinguished as two separate tissue masses, as in *Ancylus* Mueller, 1774, *Lymnaea* Lamarck, 1801 and *Siphonaria* Sowerby, 1823 (Saleuddin 1999; Saleuddin et al. 1997; Takeda & Ohtake 1994).

Sommerfeldt & Schrödl (2005) described the "lateral bodies" of *Hedylopsis spiculifera* and *H. ballantinei* being subdivided into an outer cortex and an inner medulla. According to Saleuddin (1999), most of the dorsal bodies of basommatophoran pulmonates develop a cortex with nuclei and an inner medulla with cell processes that lie very close to the cerebral ganglia. In "lateral bodies" of *H. spiculifera*, *H. ballantinei* and *Asperspina murmanica*, no such clear subdivision into cortex and medulla was found; instead all nuclei are distributed more or less uniformly. Similarly, the basommatophoran pulmonate *Siphonaria pectinata* Linnaeus, 1758 is described to possess dorsal bodies without clear separation into cortex and medulla (Saleuddin et al. 1997).

The function of the "lateral bodies" in *Hedylopsis spiculifera*, *H. ballantinei* and *Asperspina murmanica* is unclear. Due to the absence of visible nerves arising from these aggregations, the "lateral bodies" are possibly not sensory but secretory organs. The role of dorsal bodies in pulmonates as an endocrine organ involved in female reproduction is quite well known (Saleuddin 1999). Furthermore a putative endocrine gland, called the juxtaganglionar organ, has been described in several opisthobranch species (e.g. Switzer-Dunlap 1987). However, the homology of these structures is still unclear. Future studies by means of transmission electron microscopy and (immuno)histochemical studies are needed to understand homologies and functions. Disregarding our deficient knowledge, within acochlidians the presence of "lateral bodies" in members of Hedylopsidae, Asperspinidae and Tantulidae versus their absence in two members of Microhedylidae (*Pontohedyle milaschewitchii, Microhedyle remanei)* may represent characters with a phylogenetic signal.

4.3.2. Cells near the cerebral commissure

For the first time in an acochlidian species we describe several cells that are loosely dispersed within the connective tissue above the cerebral commissure in *Asperspina murmanica*. Due to its position such a cell aggregation resembles the dorsal bodies of stylommatophoran pulmonates (e.g. *Theba pisana* Mueller, 1774, *Helix aspersa* Mueller, 1774 and *Achatina fulica* Ferussac, 1821) which were described as diffusely scattered cells within the connective tissue sheath of the cerebral ganglion and located near the cerebral commissure (Saleuddin 1999; Saleuddin et al. 1997; Takeda & Ohtake 1994). The presence, structure, origin and function of these cells in acochlidians cannot be revealed by light microscopy alone but requires ultrastructural studies.

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Chapter 5. Redescription of the meiofaunal gastropod *Parhedyle cryptophthalma*, with focus on nervous system and sensory organs (Acochlidia, Panpulmonata)

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Redescription of the meiofaunal gastropod *Parhedyle cryptophthalma*, with focus on nervous system and sensory organs

(Acochlidia, Panpulmonata)

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Jörger, K. M., Kristof, A., Klussmann-Kolb, A. & Schrödl, M. 2010. Redescription of the meiofaunal gastropod *Parhedyle cryptophthalma*, with focus on nervous system and sensory organs (Acochlidia, Panpulmonata). Spixiana 33 (2): 161-170.

Parhedyle cryptophthalma (Westheide & Wawra, 1974) is a poorly known meiofaunal slug from the Mediterranean, inhabiting intertidal sands under direct wave impact. The present study is the first redescription of the acochlid *P. cryptophthalma*, confirming original results on general morphology, integumental spicules, and aberrant radula morphology by light and scanning electron microscopy. Our focus was on the central nervous system and sensory organs, using 3D reconstruction based on serial semi-thin sections and immunocytochemistry (staining of FMRFamide and Tyrosine Hydroxylase) in conjunction with confocal laser scanning microscopy. There is a mass of, yet undifferentiated, accessory ganglia anterior to the cerebral ganglia, typical for microhedylacean acochlidians. Apart from the common setting of ganglia (paired rhinophoral, cerebral, pedal, pleural, and buccal ganglia and three distinct ganglia on the visceral nerve cord), we found a putative osphradial ganglion for the first time in the microhedylacean clade. No osphradium, no Hancock's organ and, in contrast to the original description, no pigmented eyes could be detected. Bundles of sensory cilia were found laterally on the head-foot complex and scattered cilia are present on the head appendages. FMRFamidergic immunoreactivity was detected in all cerebral ganglia but not in the accessory ganglia. Tyrosine Hydroxylase (TH) expression and aldehyde-induced fluorescence was observed in cerebral ganglia such as pedal ganglia, in labiotenctacular, rhinophoral, and pedal nerves and in single neurons in the anterior region of the foot sole. Central nervous and sensory features may greatly vary among acochlidians and other heterobranch taxa, and comprehensive comparative approaches are necessary to reveal their presence, function, homology, and evolution.

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Introduction

Acochlidia are the most diverse and abundant group of meiofaunal heterobranch gastropods. Inhabiting shallow subtidal sands worldwide some species may occur intertidally in protected places such as reef lagoons. In contrast, *Parhedyle cryptophthalma* (Westheide & Wawra, 1974) is known to occur on Mediterranean beaches with direct wave impact. Despite recent advances in acochlidian morphology and anatomy (Neusser et al. 2006, Neusser & Schrödl 2007, Jörger et al. 2008, Neusser et al. 2009a,b, Brenzinger et al. in press), very little is still known about the biology of these mainly marine mesopsammic slugs and the adaptation of the sensory and locomotory system to their interstitial habitat. Being one of the smallest acochlids with an adult size of only 1.6 mm the original description applying traditional light microscopy lacks considerable detail, especially regarding fine nervous structures. Moreover, a trend towards reduction within organ systems ("regressive evolution"), e.g. with aphallic males and reduced reproductive systems, is observed in the acochlidian Microhedylacea clade (Swedmark 1959, 1968, Neusser et al. 2009b, Schrödl & Neusser 2010). The small amount of distinguishing characters paired with a lack of morphological knowledge on some genera – in first place the genus *Parhedyle* – thus still results in partially unresolved relationships within Microhedylacea (for phylogeny, see Schrödl & Neusser 2010).

The genus *Parhedyle* Thiele, 1931 was characterized as a member of gonochoristic Microhedylidae having two pairs of highly mobile head tentacles and irregularly shaped spicule plates in the integument (Wawra 1987). The type species *Parhedyle tyrtowii* (Kowalevsky, 1901) (as *Hedyle*) from the Black Sea was described to have clearly visible pigmented eyes and a symmetric radula with formula 2.1.2 by Kowalevsky (1901), while the Mediterranean *P. cryptophthalma* (as *Microhedyle*) has cryptic eyes and, uniquely among microhedylids, an asymmetric radula with a formula of 1.1.2 was found by light microscopical examination (Westheide & Wawra 1974). No details on central nervous or sensory features are known.

Traditionally, Acochlidia formed an order of the "Opisthobranchia" (see e.g. Dayrat & Tillier 2003, Wägele & Klussmann-Kolb 2005), but recent multilocus molecular data revealed pulmonate relationships (Klussmann-Kolb et al. 2008, Jörger et al. 2010). These recently proposed relationships question traditional homology assumptions within Heterobranchia and require re-evaluation of problematic character sets such as the nervous system. Investigations characterizing serotonergic, FMRFamidergic, and/or cathecholaminergic parts of the nervous system have shown to provide phylogenetically useful characters across different taxa (Dickinson & Croll 2003, Croll & Dickinson 2004, Wanninger 2009, Heuer et al. 2010, Kristof & Klussmann-Kolb 2010, Worsaae & Rouse 2010). Moreover, spatial distribution and immunoreactivity of neurites are indicating their function (see Faller et al. 2008, and references therein). Among Acochlidia Hochberg (2007) provided the first detailed information on the chemistry of the nervous system in a species of microhedylacean *Asperspina*. He revealed the presence of an extensive serotoneric network in the central nervous system, with similarities to model heterobranchs such as *Aplysia*, in the presence of serotoneric pericarya in cerebral and pedal ganglia and absence from pleural ganglia (Hochberg 2007). Due to its minute body size *Parhedyle cryptophthalma* offers the possibility of immunocytochemical studies on whole-mount specimens for visualization by confocal laser scanning microscopy (cLSM).

Here, we redescribe the nervous system and associated sensory structures of *P. cryptophthalma* by 3D reconstruction from histological semi-thin serial sections. To gain insights in the function of certain nervous structures, this data is supplemented by the characterization of FMRFamidergic and chatecholaminergic parts of the nervous system by means of immocytochemistry in conjunction with cLSM. The results are compared to other acochlidian nervous systems and previous homology assumptions are discussed.

Material and methods

Sampling

Specimens of *Parhedyle cryptophthalma* were recollected at Arco Felice, near Naples, Italy, a locality that was reported in the original description by Westheide & Wawra (1974). Approximately 70 individuals were extracted from sand samples collected in the high intertidal (wave zone) following the method described by Schrödl (2006). All specimens were anaesthetized with $MgCl_2$ prior to fixation to prevent retraction. Specimens were identified using light-microscopy and 10 individuals were fixed in 75 % ethanol for radulae preparation. For histology and scanning electron microscopy (SEM) specimens were fixed in 4 % glutardialdehyde.

Histology and 3D reconstruction

For serial semi-thin sectioning 20 glutardialdehyde-fixed specimens were embedded in Spurr's low viscosity epoxy resin (Spurr 1969). Specimens were rinsed three times in 0.2 M sodium cacodylate buffer (0.1 M NaCl and 0.35 M sucrose, pH 7.2), followed by post-fixation in buffered 1 % OsO_4 for 1.5 h in the dark. Sub-

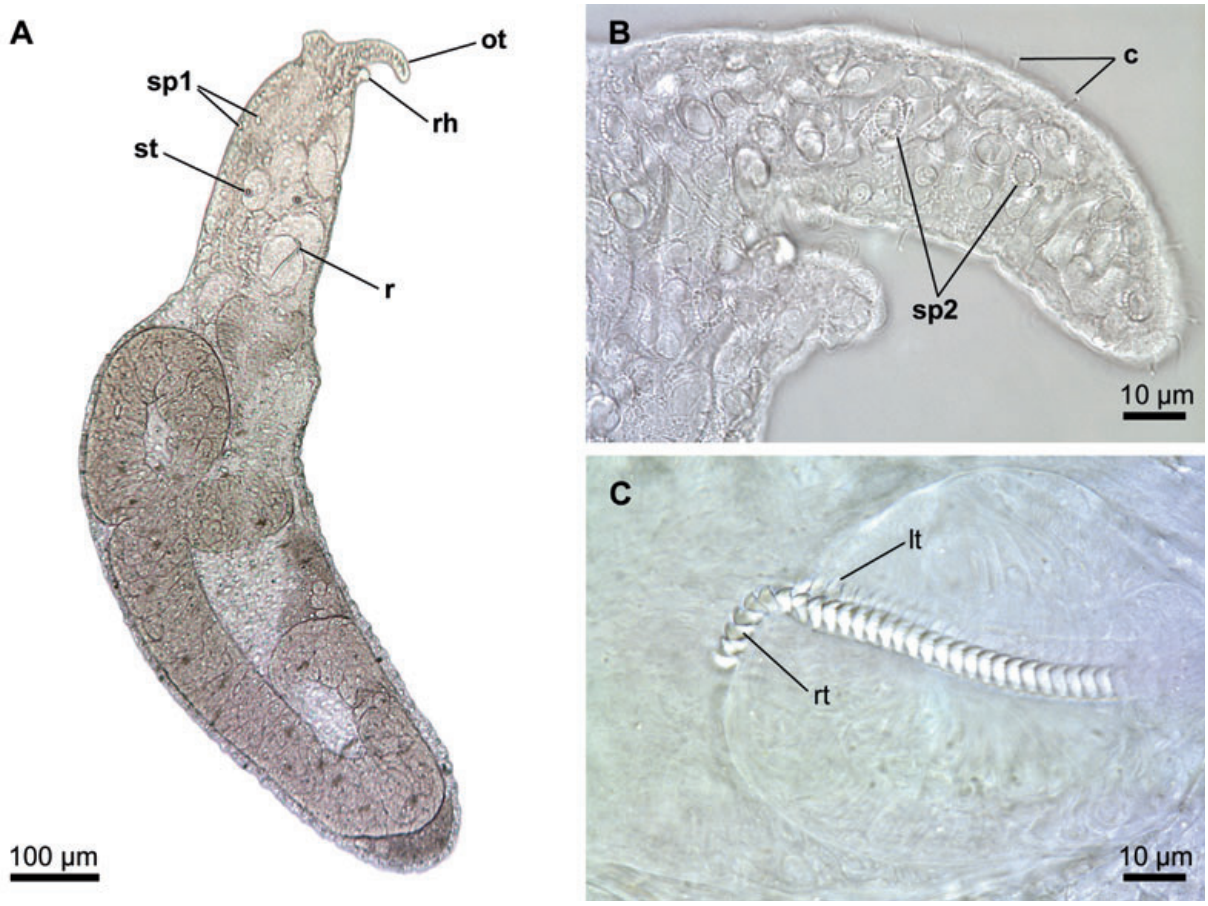

Fig. 1. Light microscopy. **A.** External morphology of a living specimen. **B.** Oral tentacle and rhinophore, with scattered sensory cilia and circular bead-chain spicules (type 2). **C.** Typical J-shaped radula with ascending and descending limb. Abbreviations: **c**, cilia; **lt**, lateral tooth; **ot**, oral tentacle; **r**, radula; **rh**, rhinophore; **rt**, rhachidian tooth; **sp1**, plate-like spicules (type 1); **sp2**, circular bead-chain spicules; **st**, statocyst.

sequently, specimens were decalcified in 1 % ascorbic acid overnight and dehydrated in a graded acetone series. Four series of serial semi-thin sections (1.0-1.5 µm thickness) were prepared using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) and contact cement on the lower cutting edge to form ribbons (Ruthensteiner 2008). Sections finally were stained with methyleneazure II (Richardson et al. 1960) and are deposited at the Bavarian State Collection of Zoology (ZSM Mol 20071113, 20071923, 20071925, 20071931). Digital photographs were taken of every section with a CCD microscope camera (Spot Insight, Diagnostic Instruments, Sterling Heights, USA) mounted on a DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). A computer-based 3D-reconstruction of the nervous system of *Parhedyle cryptophthalma* was conducted with the software AMIRA 5.2 (Visage Imaging GmbH, Germany), following in principle the procedure explained by Neusser et al. (2006) and Ruthensteiner (2008).

Scanning electron microscopy (SEM)

10 entire specimens of *Parhedyle cryptophthalma* were stepwise dehydrated in graded acetone series and critical-point-dried in 100 % acetone in a Baltec CPD 030. After mounted on SEM stubs with self adhesive carbon stickers, the dried specimens were sputtered with gold in a Polaron Sputter Coater for 120 sec. SEM examination of the sensory organs of *P. cryptophthalma* were carried out using a LEO 1430VP SEM at 10-15 kV. For the preparation of radulae 10 specimens were macerated over night in 10 % KOH, remaining tissue was removed mechanically under a stereomicroscope. Radulae were rinsed in Aqua bidest., mounted on SEM stubs, sputtered, and examined as described above.

Immunolabelling and confocal laser scanning microscopy

Specimens were prepared basically following the protocol described by Kristof & Klussmann-Kolb (2010): 15 specimens of *P. cryptophthalma* were anaesthetized with 7 % $MgCl_2$ and fixed in 4 % paraformaldehyde (PFA) in

0.1 M phosphate-buffered saline (PBS, pH 7.3) for 5 hours at 4 °C. Subsequently, the specimens were rinsed three times (2 × 5 min, 1 × 60 min) in PBS (= PBS rinse). After decalcification (0.5 M EDTA for three hours), followed by another PBS rinse, specimens were permeabilized for 24 hours (4 % Triton X-100 in PBS) and incubated with the rabbit anti-FMRFamide (Immunostar, Hudson, Wisconsin, USA, diluted in PBS to 1:500 final working concentration) in blocking solution (1 % goat serum in PBT (= PBS with 1 % Triton X-100)) for 72 hours at 4 °C. After primary labelling, specimens were rinsed three times for 15 min in PBS and incubated for 24 hours in 1:50 dilution of goat anti-rabbit antibodies labelled either with fluorescein isothiocynate (FITC) or rhodamine (TRITC) (Jackson ImmunoResearch Laboratories, West Groove, PA, USA) at 4 °C. This was again followed by several rinses in PBS, before the specimens were mounted on glass slides in a 3:1 mixture of glycerol to TRIS-buffer (0.5 M) with 2 % propyl gallate added to prevent fading (Longin et al. 1993).

For Tyrosine Hydroxilase (TH) labelling (marks catecholaminergic cells) 15 specimens were anaesthetized (see above) and fixed in 99 % Methanol (+1 % acetic acid) for 30 min at −20 °C and subsequently transferred for 10 min each in a decreasing methanol series (70 %, 50 %, 30 %), followed by several PBS rinses. Specimens were decalcified, permeabilized (see above), and incubated with a monoclonal TH antibody (Immunostar, Inc. Hudson, WI, USA) (1:500 dilution in PBS) in blocking solution (1 % goat serum in PBT (= PBS with 1 % Triton X-100)) for 72 hours at 4 °C. This was followed by several rinses in PBS and incubation in secondary antibody (sheep anti-mouse) conjugated to TRITC for 24 hours at 4 °C. After several rinses in PBS the specimens were mounted on glass slides as described above.

Autofluorescence were excluded by negative controls, in which specimens were processed without incubation in primary antibody. Positive controls involved parallel processing of *Aeolidiella stephanieae* Valdés, 2005 and *Haminoea japonica* (Pilsbry, 1895) larvae with known staining patterns (Kristof & Klussmann-Kolb 2010, and C. Schulze (Zoological Museum Hamburg) pers. comm.).

The immunolabelling was viewed with a Leica TCS SP5 confocal laser-scanning microscope, generating optical sections at 0.1-0.5 µm intervals. All sections were digitally merged to maximum projections and edited with general imaging software.

Results

Taxonomic identification of *Parhedyle cryptophthalma* conducted in the field was based on the locality and special type of habitat (described in the original literature, see Westheide & Warwra 1974), and general morphology (Fig. 1A-C). Light microscopy on living specimens revealed irregular, plate-like calcareous spicules and circular bead-chain spicules (see Fig. 1A,B) as described by Westheide & Wawra (1974). Preliminary identification was confirmed by SEM examination of the unusual radulae. The radula is asymmetric, with formula 1-1-2; the first right lateral is narrow and the second lateral is broad (Fig. 2A), what is unique among known acochlidians. Identification as *P. cryptophthalma* is further supported by clear genetic distinction to the only other described congener *Parhedyle tyrtowii* (Kowalevsky, 1901) that was collected at the type locality in the Black Sea (unpublished data).

All specimens available for the present study were sub-adult juveniles with the genital system not yet (fully) developed.

Central nervous system (CNS)

The CNS of (sub-adult) *Parhedyle cryptophthalma* confirms the general setting within Acochlidia and ganglia are identified following previous species (re-)descriptions (see e. g. Neusser et al. 2006). The prepharyngeal CNS consists of the paired cerebral, rhinophoral, pedal, pleural and buccal ganglia and three distinct ganglia on the visceral nerve cord (respectively the left parietal ganglion, the fused visceral/subintestinal ganglion and the fused right parietal/supraintestinal ganglion), see Fig. 3. An additional unpaired, putative osphradial ganglion (Fig. 3A,B) was found attached to the right parietal/supraintestinal ganglion (Fig. 3B). Anterior to the cerebral and rhinophoral ganglia there is an undifferentiated mass of supposedly nervous tissue, interpreted as accessory ganglia (Fig. 3D). Cerebral and rhinophoral ganglia are located pre-pharyngeally, pedal and pleural ganglia in the anterior region of the pharynx, and the ganglia of the visceral nerve cord near the posterior end of the pharynx; the buccal ganglia lie post-pharyngeal. All ganglia are situated very close together. Cerebral as well as pedal ganglia are connected via short, strong commissures; short cerebro-pedal, cerebro-pleural, and pleuro-pedal connectives could be detected. The rhinophoral ganglion was identified as such due to its position anterolateral of the cerebral ganglion and a thin connective with the cerebral ganglion, even though no nerve could be detected emerging from this small ganglion and the putative rhinophoral nerve emerges dorsally from the cerebral ganglion (see below).

Three cerebral nerves could be detected emerging from the cerebral ganglion (for identification see previous redescriptions e.g. Jörger et al. 2008): 1) slightly ventrally the labiotentacular nerve, 2) more dorsally the dorsal nerve, herein interpreted as the rhinophoral nerve, and 3) the thin static nerve.

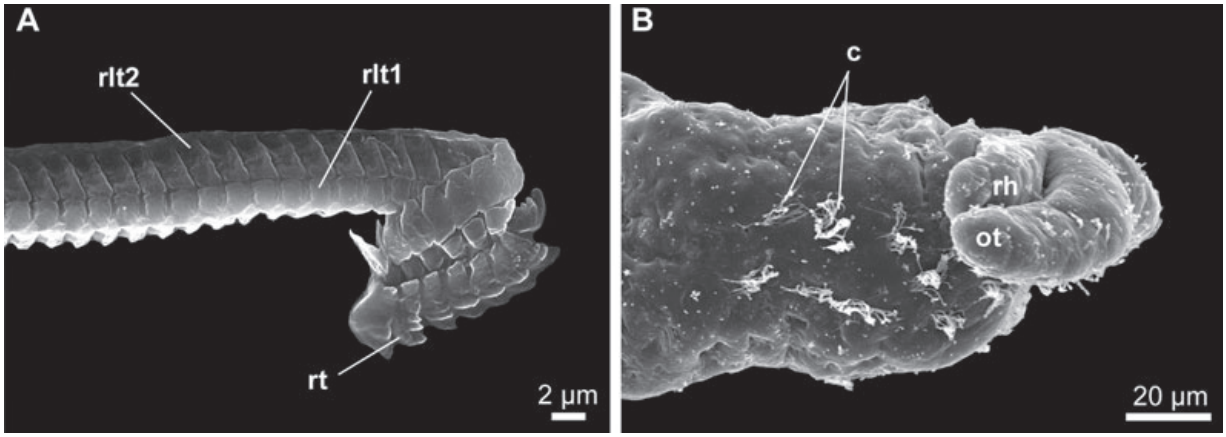

Fig. 2. Scanning electron micrographs. **A.** Right lateral view of radula with rhachidian tooth (with one central and three lateral cusps) and two lateral teeth on the right side. **B.** Lateral view of the head-foot complex showing scattered bundles of cilia. Abbreviations: **c**, cilia; **ot**, oral tentacle; **rh**, rhinophore; **rlt1**, right lateral tooth 1; **rlt2**, right lateral tooth 2; **rt**, rhachidian tooth.

Sensory structures

Parhedyle cryptophthalma possesses two pairs of head appendages (Figs 1A, 2B). Ventrally located are the curved, roundish and only slightly tapered oral tentacles. Rhinophores are slightly shorter than the oral tentacles, and are pointed upwards in living, crawling specimens. Using light microscopy, scattered cilia, supposedly with sensory function are found on oral tentacles and rhinophores (Fig. 1B). SEM examination of the head-foot complex reveals additional bundles of cilia in the anterior region of the head-foot complex (Fig. 2B).

Surprisingly, the eponymous "cryptic eyes" could not be detected in our material from Italy. No pigment anterior to the cerebral ganglia was found on histological sections or by light microscopy of living specimens. However, minute unpigmented globules are identified nestling anterior to the cerebral ganglia. Those might be interpreted as remainder or ontogenetic precursor of eyes (see discussion).

Each pedal ganglion bears a comparably large statocyst (Fig. 3A,E; containing one statolith each), which is attached dorsally near the posterior end of the ganglion (also clearly visible using light microscopy, see Fig. 1A).

No Hancock's organ (i. e. paired ciliated grooves posterior to the head appendages, commonly innervated by part of the rhinophoral nerve) could be detected with SEM examination of the head-foot or on the histological sections.

Immunolabelling and cLSM

Successful labelling against FMRFamides was only achieved in completely retracted specimens, where allocation of labelled nuclei within the concentrated CNS was difficult. FMRFamide immunoreactivity could be detected in all ganglia, but no reactivity was observed in the accessory ganglia anterior to the cerebral ganglia (see Fig. 4A).

TH-like immunoreactivity could be detected in the anterior region of the cerebral ganglion and the labiotentacular and rhinophoral nerves (Fig. 4B). Additionally, TH-immunoreactivity was observed in the centre of the pedal ganglia, within a bifurcating pedal nerve leading ventrally to the anterior part of the foot and in a series of single neurons bordering the anterior region of the foot near the mouth opening (Fig. 4C).

Discussion

Taxonomy

The genus *Parhedyle* is among the most ill-defined acochlidian taxa, comprising two (or three) species that are only known by their original descriptions (Wawra 1987, Schrödl & Neusser 2010). While not resolved as a monophylum in the cladistic analysis by Schrödl & Neusser (2010), *Parhedyle tyrtowii* and *Parhedyle cryptophthalma* are quite similar. Both are agile slender microhedylids with two pairs of slender, highly mobile tentacles. Irregular calcareous spicule plates and pearl-chain like aggregations were known from *P. tyrtowii* and *P. cryptophthalma* (see Kowalevsky 1901, Westheide & Wawra 1974). The re-examination of *P. cryptophthalma* by SEM confirms the presence of an asymmetric radula with a formula of 1.1.2. *P. cryptophthalma* thus is the only member of the gonochoristic Microhedylidae sensu lato with an asymmetric radula. Interestingly, the first right lateral is a much narrower plate than the

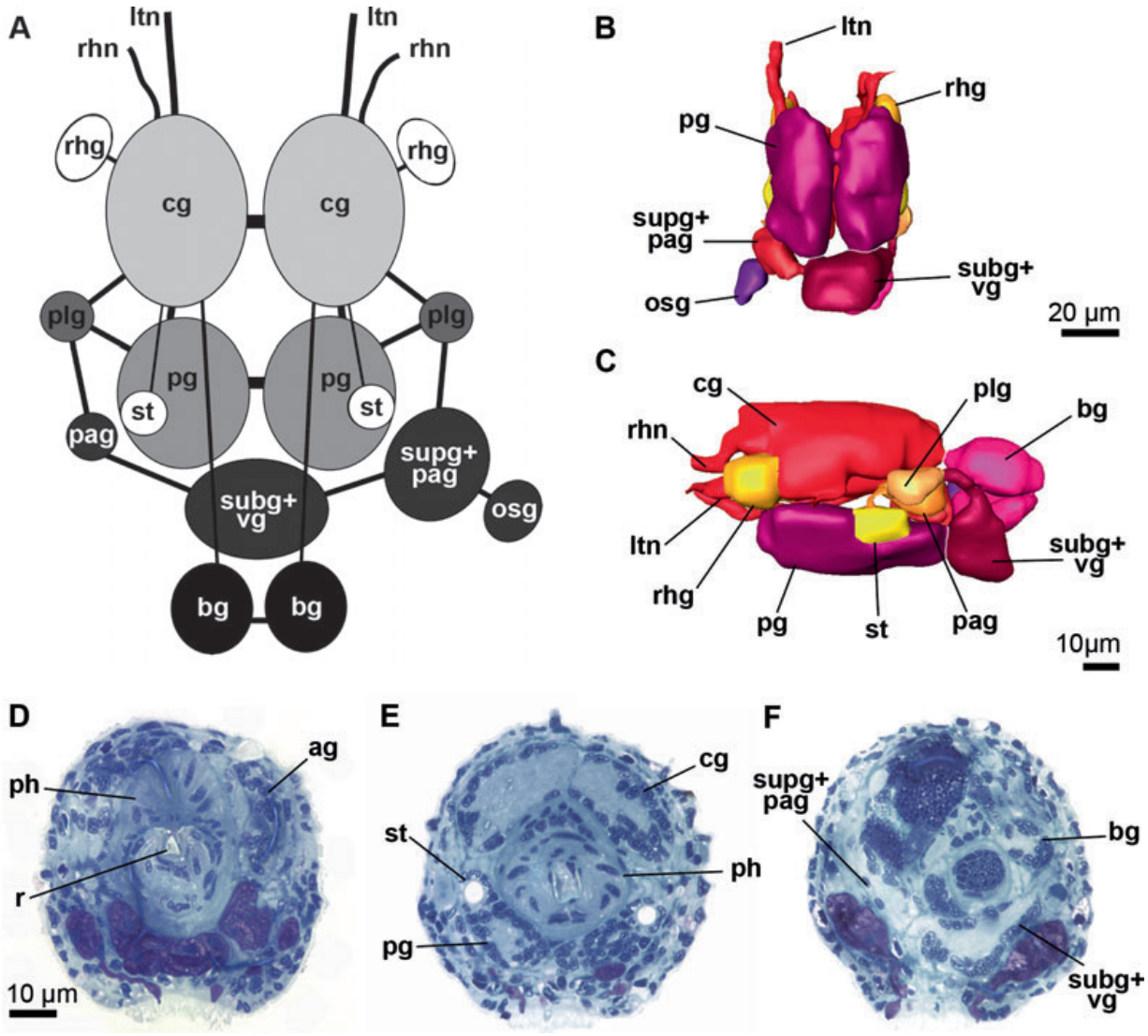

Fig. 3. Central nervous system (CNS). **A.** Schematic overview, dorsal view. **B-C.** 3D reconstruction of the CNS based on histological semi-thin sections; ventral view (**B**), lateral left view (**C**). **D-F.** Histological semi-thin sections (1 µm); undifferentiated mass of accessory ganglia anterior to cerebral ganglia (**D**), cerebral and pedal ganglia with statocysts (**E**), ganglia of visceral nerve cord (**F**). Abbreviations: **ag**, accessory ganglia; **bg**, buccal ganglion; **cg**, cerebral ganglion; **ltn**, labiotentacular nerve; **osg**, osphradial ganglion; **pag**, parietal ganglion; **pg**, pedal ganglion; **ph**, pharynx; **plg**, pleural ganglion; **r**, radula; **rhn**, rhinophoral nerve; **rhg**, rhinophoral ganglion; **st**, statocyst; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **vg**, visceral ganglion.

second, rectangular one. This is in contrast to all hedylopsacean and *Asperspina* species with an asymmetric radula; here, the first right lateral is a broad rectangular plate and the second tooth a narrow plate or spine-like (Schrödl & Neusser 2010). According to Kowalevsky (1901: figs 38, 40), *P. tyrtowii* has a narrow first lateral and a broad second one as well; the originally described symmetric condition in the latter species needs to be re-examined. While the presence versus absence of well-visible, pigmented eyes clearly distinguish the species, morphological and genetic evidences may finally confirm *P. tyrtowii* and *P. cryptophthalma* as belonging to a monophyletic genus *Parhedyle*. The just rudimentarily known *Parhedyle gerlachi* (Marcus & Marcus, 1959) from the Maldives (Indian Ocean) was tentatively related to *Parhedyle* (see Wawra 1987). However, flat oral tentacles and a radula with single lateral teeth (formula 1.1.1) do not support a closer relationship to *Parhedyle*. *P. gerlachi* needs re-examination before conclusions on its relationships can be made.

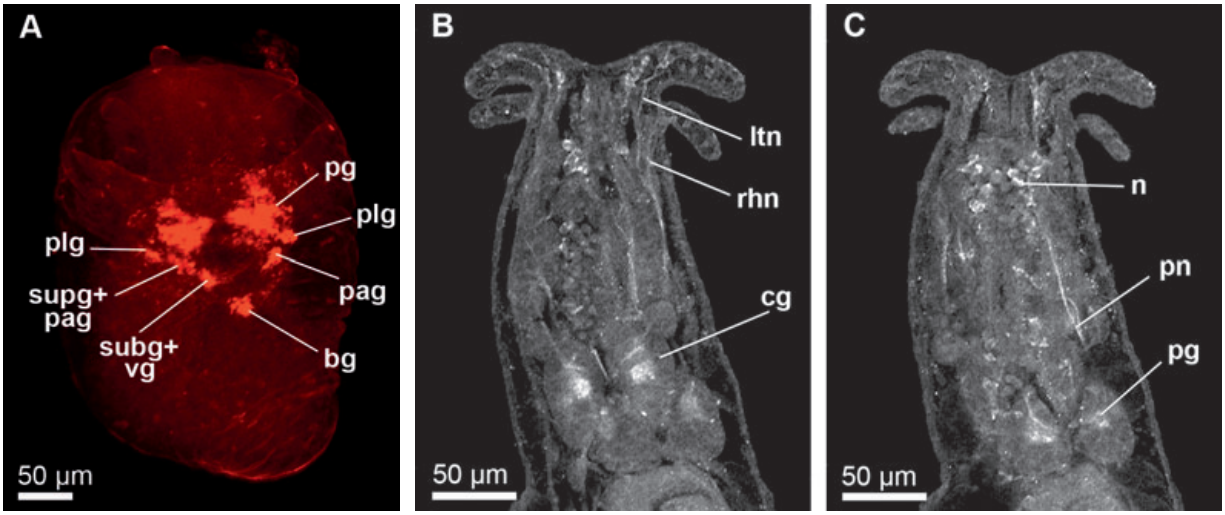

Fig. 4. Expression of neurotransmitters. **A.** FMRF-amidergic expression in ganglia, labelled in a completely retracted specimen (ventral view). **B-C.** TH-immunoreactivity, in cerebral ganglia, labiotentacular and rhinophoral nerves (**B**), in pedal ganglia, pedal nerves and scattered neurons at the foot sole (**C**). Abbreviations: **bg**, buccal ganglion; **cg**, cerebral ganglion; **ltn**, labiotentacular nerve; **n**, neurons in anterior part of the foot; **pag**, parietal ganglion; **pg**, pedal ganglion; **plg**, pleural ganglion; **pn**, pedal nerve; **rhn**, rhinophoral nerve; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **vg**, visceral ganglion.

Central nervous system

The condition of the prepharyngeal nervous system observed in *Parhedyle cryptophthalma* confirms the general setting of ganglia in microhedylid Acochlidia (Neusser et al. 2006, Jörger et al. 2008, Neusser et al. 2009b). Additionally, we found an unpaired ganglion attached to the right parietal/supraintestinal ganglion for the first time in Microhedylacea. Such unpaired ganglia are present in all hedylopsacean Acochlidia described in detail (Wawra 1989, Sommerfeldt & Schrödl 2005, Neusser & Schrödl 2007, 2009, Neusser et al. 2009a, Brenzinger et al. in press) and were regarded as osphradial ganglia even in the absence of detected osphradia. For the first time in Acochlidia, B. Brenzinger (pers. comm.) detected an osphradium in a comparably large, limnic hedylopsacean *Strubellia* species from the Solomon Islands. While the osphradium is well-visible as a bright spot on dark-pigmented living specimens, its histological detection is difficult (B. Brenzinger pers. comm.). Thus, this small unpaired ciliated groove, similar to the Hancock's organ, might have been overlooked in previous histological studies on tiny meiofaunal acochlids. The non-detection of an osphradium in the preliminary immunocytochemical approach herein does not necessarily mean its absence. The osphradial ganglion in hedylopsaceans and *P. cryptophthalma* may be a mere relic in species without (well-) developed osphradium. However, new functions – potentially related to copulation – are indicated by a thick penial sheath nerve leaving this ganglion in *Tantulum elegans* (see Neusser & Schrödl 2007).

Accessory ganglia

Accessory ganglia are aggregations of nervous tissue, closely associated to the cerebral ganglia and differentiated from "true" ganglia by the lack of division into cortex and medulla (Neusser et al. 2006). They are characteristic for Microhedylacea but were also found occasionally in some hedylopsacean acochlids (Wawra 1987, Schrödl & Neusser 2010). The present study on subadult *Parhedyle cryptophthalma* suggests that accessory ganglia develop later in ontogeny, after the development of the "true" ganglia. This supports earlier observations on juvenile *Pontohedyle milaschewitchii* (Kowalevsky 1901) with developing accessory ganglia (see Jörger et al. 2008). The function of accessory ganglia is still a matter of speculation, e. g. as adaptation to minute body sizes (Haszprunar & Huber 1990) or involved in reorganization of the reproductive system in sequential hermaphrodites (Neusser & Schrödl 2007). In contrast to "true" ganglia, no FMRFamide immunoreactivity could be detected in the accessory ganglia investigated herein. This might, however, be a result of the not fully developed stage of the accessory ganglia, which might have no expression of neurotransmitters, yet. Comparable studies on adult specimens are needed for further conclusions. TH-expression is observed in the cerebral ganglia and cerebral nerves inner-

vating the head appendages (labiotentacular and rhinophoral nerve, respectively). The presence in nerves leading to the sensory head appendages and the accumulation of TH neurites near the mouth opening suggests an involvement in the transmission of sensory stimuli, confirming general consideration that catecholamines are involved in contact chemoreception and mechanoreception (Croll et al. 2003, Faller et al. 2008). Considering the interstitial lifestyle of acochlidians the accessory ganglia might be necessary to be able processing all the sensory input from the surrounding cathecholaminergic neurites. In a Caribbean *Asperspina* species Hochberg (2007) found serotonergic expression in cerebral and pedal ganglia and in one ventral "accessory ganglion" connected to the pedal ganglion (with similar expression pattern as the pedal ganglion). As pointed out by the author this is rather surprising, since accessory ganglia are normally associated to cerebral ganglia in all Acochlidia studied in detail. Most of Hochberg's specimens were mounted left-side down, so observations were concentrated on the right lateral view, lacking information on the left body part. Assuming that the laser scanner could have penetrated more than half of the whole body width, or that a slight dislocation of left side ganglia occurred in the fixed specimens, there is a certain possibility that the "accessory ganglion" of *Asperspina* sp. refers to the left pedal ganglion. Reinvestigation on the potential serotonergic expression in accessory ganglia is needed.

Sensory structures

Eyes. Surprisingly, we were unable to detect the eponymous cryptic eyes in our *Parhedyle cryptophthalma*. Westheide & Wawra (1974) reported the eyes to be invisible in squeezed material, alive or fixed. Only within brightened, fixed material the describing authors were able to detect two "tiny spots of pigment" anterior to the cerebral ganglia. We were unable to detect any pigment anterior to the cerebral ganglia in the sectioned series and also on observed living material. Since our study specimens were sub-adult juveniles, the observed unpigmented nervous globules might develop into eyes within fully developed adults. However, available data from other microhedylid species such as juvenile *Pontohedyle milaschewitchii* and *Microhedyle glandulifera* (Kowalevsky 1901) suggest that fully developed, pigmented eyes are already present in an early juvenile stage (unpublished data). The unpigmented structures found in *P. cryptophthalma* might thus rather be interpreted a remainder of eyes developed in an earlier ontogenetic stage. In general, the presence and amount of pigmentation within the eyes of Acochlidia seems to be variable between and even within one population, e.g. observed in *Pseudunela* sp. from Indonesia (T. Neusser pers. comm.).

Hancock's organ. Hancock's organs (i.e. paired ciliated grooves behind the oral tentacles with supposedly chemosensory function) have been reported from acochlidians in three different families: in the small interstitial *Tantulum elegans* Rankin, 1979 (Tantulidae) (Neusser & Schrödl 2007), *Pontohedyle milaschewitchii* and *Microhedyle glandulifera* (both Microhedylidae) (Edlinger 1980a,b, Jörger et al. 2008) and the large benthic *Strubellia paradoxa* (Strubell, 1892) (Acochlidiidae) (Brenzinger et al. in press). However they were not detected in several other acochlidians examined in detail (Neusser & Schrödl 2009, Neusser et al. 2009a,b), among them other representatives of the family Microhedylidae (Neusser et al. 2007). We were also unable to detect Hancock's organs in *Parhedyle cryptophthalma*. Small ciliated grooves can easily be overlooked or misinterpreted as epidermal folds on retracted specimens or badly preserved material, thus Hancock's organs might have been overlooked in previous studies or even within the present one. The presence might also be related to a certain function corresponding e.g. to differences in habitat. However, this seems unlikely at the present stage of knowledge with Hancock's organs present/absent from marine interstitial species and present in (some?) limnic ones. Earlier authors suggest a potential homology of Hancock's organs within "Opisthobranchia"; i.e. of the epidermal grooves in Acochlidia with Hancock's organs of Acteonoidea and within Cephalaspidea sensu stricto, based on the similar position and innervations by a split of the rhinophoral nerve (Edlinger 1980a,b, Neusser et al. 2007). The recently revealed panpulmonate relationship of Acochlidia (Jörger et al. 2010) at first glance may contradict such an assumption due to phylogenetic distance, implying several independent losses in intermediate panpulmonate lineages. However, assuming homology of opisthobranch rhinophoral ganglia and pulmonate procerebrum (Jörger et al. 2010), at least parts of the ancestral, lower heterobranch actenoidean-like Hancock's organs could be homologous to the more or less elaborated epidermal folds and rhinophores, i.e. all sensory structures and tentacles innervated by the rhinophoral nerve, throughout the Euthyneura.

There is increasing knowledge on acochlidian microanatomy, greatly benefiting from a wide range of light microscopical, ultrastructural, histological and immunocytochemical techniques that are applied to a variety of formerly poorly or unknown taxa. Con-

firming aberrant features such as the special setting and shape of radula teeth in *Parhedyle cryptophthalma* is as important for future phylogenetic and evolutionary analyses as the revealing of new structures, such as the osphradial ganglion. Regarding central nervous and sensory organs, our understanding is still fragmentary. Latest anatomical redescriptions of acochlidian nervous systems underline the variability of nervous structures, especially the setting of cerebral nerves and the presence of sensory structures even within closely related species (see Sommerfeldt & Schrödl 2005, Neusser et al. 2006, Neusser & Schrödl 2007, Neusser et al. 2007, Jörger et al. 2008, Neusser & Schrödl 2009, Neusser et al. 2009a,b, Brenzinger et al. in press, present study). The presence/absence of rhinophoral or optic ganglia, the number and location of cerebral nerves, the innervation pattern of sensory structures and head appendages are highly variable and not entirely congruent with phylogenetic hypotheses no matter if based on morphological characters (Schrödl & Neusser 2010) or on molecular markers (Jörger et al. 2010). Thus, homology assumptions and generalization of reductions or inventions of cerebral features within Acochlidia, and even more across different heterobranch taxa, is difficult at the present stage of knowledge. A comprehensive comparative approach using a combination of detailed histological reexamination combined with immunocytochemical labelling against different neurotransmitters is needed to gain further insights into this quite variable character set within Heterobranchia.

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Chapter 6. Sex in the beach: spermatophores, dermal insemination and 3D sperm ultrastructure of the aphallic mesopsammic *Pontohedyle milaschewitchii* (Acochlidia, Opisthobranchia, Gastropoda)

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ORIGINAL PAPER

Sex in the beach: spermatophores, dermal insemination and 3D sperm ultrastructure of the aphallic mesopsammic *Pontohedyle milaschewitchii* (Acochlidia, Opisthobranchia, Gastropoda)

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Abstract Sperm transfer via spermatophores is common among organisms living in mesopsammic environments, and is generally considered to be an evolutionary adaptation to reproductive constraints in this habitat. However, conclusions about adaptations and trends in insemination across all interstitial taxa cannot be certain as differences in mode of insemination via spermatophores do exist, details of insemination are lacking for many species, and evolutionary relationships in many cases are poorly known. Opisthobranch gastropods typically transfer sperm via reciprocal copulation, but many mesopsammic Acochlidia are aphallic and transfer sperm via spermatophores, supposedly combined with dermal fertilisation. The present study investigates structural and functional aspects of sperm transfer in the Mediterranean microhedylacean acochlid *Pontohedyle milaschewitchii*. We show that spermatophore attachment is imprecise. We describe the histology and ultrastructure of the two-layered spermatophore and discuss possible functions. Using DAPI staining of the (sperm-) nuclei, we document true dermal insemination in situ under the fluorescence microscope. Ultrastructural investigation and computer-based 3D reconstruction from TEM sections visualise the entire spermatozoon including the exceptionally elongate, screw-like keeled sperm nucleus. An acrosomal complex was not detected. From their special structure and behaviour we conclude that sperm penetrate epithelia, tissues and cells mechanically by drilling rather than lysis. Among opisthobranchs, dermal insemination is limited to mesopsammic acochlidian species. In this spatially limited environment, a rapid though imprecise and potentially harmful dermal insemination is discussed as a key evolutionary innovation that could have enabled the species diversification of microhedylacean acochlidians.

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Introduction

The interstitial habitat is characterised by extreme ecological conditions, requiring various morphological adaptations of its inhabitants (Swedmark 1968a). The small dimensions of the lacunary system restricts the interstitial fauna to elongate microforms (seldom exceeding 3 mm in size), and wave action in the intertidal or shallow subtidal zone creates a dynamic, mechanically labile habitat (Swedmark 1959, 1964). Minute body size generally results in a low number of gametes, which demands economisation and high effectiveness in reproduction in the mesopsammon (Swedmark 1959, 1968a; Ax 1969; Clark 1991). Thus, mechanisms of direct sperm transfer, i.e. copulation, hypodermic injection and epidermal application via spermatophores are dominant in securing impregnation (Ax 1969).

Epidermal application via spermatophores is reported from many different interstitial invertebrate groups, such as annelids, nematodes, copepods, kinorhynchs, gastrotrichs

and opisthobranchs (see e.g. Teuchert 1968; Ax 1969; Rice 1978; Brown 1983; Clark 1991; Schrödl and Neusser, in press). Reproduction via spermatophores has thus been regarded as a characteristic adaptation to the interstitial habitat (Swedmark 1959, 1968a; Ax 1969). Three potential ways of insemination can be differentiated: (1) spermatophores placed on the female gonopore, e.g. in the kinorhynch *Kinorhynchus phyllotropis* (see Brown 1983); (2) spermatophores placed somewhere on the body wall and sperm migration to the genital pore, (3) spermatophores placed somewhere on the body surface and sperm intruding into the wall. The latter type is called dermal insemination, it occurs, e.g. in the polychaete *Hesionides arenaria* (see Westheide and Ax 1965).

Within opisthobranch gastropods sperm transfer via spermatophores is rare (Mann 1984); the usual mode of reproduction is reciprocal copulation (Schmekel 1985). Direct observations of spermatophores exist for the cephalaspideans *Haminoea hydatis* and *Cylichna arachis* (see Perrier and Fischer 1914 as *Haminoea*) and *Runcina ferruginea* (see Kress 1985), as well as for the nudibranchs *Aeolidiella glauca* (see Haase and Karlsson 2000; Karlsson and Haase 2002), *Tenellia fuscata* (see Chambers 1934 as *Embletonia*) and *Polycera quadrilineata* (see von Ihering 1886). In all these taxa, spermatophores are placed at the genital pore or sperm migrate towards it externally (see Table 1). Within the Acochlidia, a small traditional opisthobranch "order," most of the minute, mesopsammic species also possess spermatophores and are assumed to transfer sperm by dermal insemination (Swedmark 1968a, b; Westheide and Wawra 1974; Morse 1976, 1994; Neusser et al. 2007; Schrödl and Neusser, in press). Opposed to the usually hermaphroditic opisthobranchs, some acochlids are gonochoristic, including the study species *Pontohedyle milaschewitchii* (Kowalevsky, 1901) (Jörger et al. 2008).

Dermal insemination via spermatophores in Acochlidia raises many functional questions: (1) How does sperm penetrate the epidermis of the recipient? (2) How does sperm move through the body cavity and tissue of the recipient? (3) Is the dermally intruding sperm the fertilising sperm, and, if so, (4) how and where does fertilisation take place? (5) Are there functional morphological adaptations, e.g. in the sperm ultrastructure, for such a mode of sperm transfer? And (6) how did dermal insemination and related structures evolve? Most of these questions have never been adequately addressed. The only detailed ultrastructural data on acochlidian sperm available refer to *Microhedyle remanei*, an aphallic, spermatophore producing species (see Neusser et al. 2007). In having a helically coiled nucleus, a complex mitochondrial derivative enclosing the axoneme, coarse fibres and one glycogen helix, sperm of *M. remanei* conform to the model of a typical, reciprocally copulating opisthobranch (Healy 1982, 1993; Healy and Willan 1984). However, an elsewhere obligatory acrosomal complex has not been detected, and the long nucleus of *M. remanei* shows conspicuous spiral keels (Neusser et al. 2007). A recent comprehensive phylogenetic analysis (Schrödl and Neusser, in press) gives robust support for reconstructing the evolution of major acochlidian subgroups around potential key innovations such as certain reproductive features and modes.

The special method of acochlidian sperm transfer via spermatophores is herein investigated in a common Mediterranean species, *P. milaschewitchii*. The present study provides for the first time detailed histological and ultrastructural data of an acochlidian spermatophore. DAPI staining and fluorescence microscopy allows direct observations of dermal insemination following the attachment of the spermatophore. The first 3D-reconstruction from ultrathin serial sections of a gastropod spermatozoon helps to visualise the complex sperm ultrastructure of *P. milaschewitchii*, and enables conclusions on functional and evolutionary aspects of acochlidian reproduction.

Table 1 Spermatophore types in opisthobranch gastropods

Transfer of spermatophore	Who?	Requirements for reproductive system/spermatophore	Literature
Type I: spermatophores introduced into female genital pore (copulation)	*Polycera quadrilineata, Tenellia fuscata, Haminoea, Runcina*[a]	Male copulatory apparatus to place spermatophore in genital opening	von Ihering, 1886; Perrier and Fischer, 1914; Chambers, 1934; Kress, 1985
Type II: spermatophores attached to body wall → sperm migrates to genital opening	*Aeolidiella glauca* (Nudibranchia)	"Anchoring device" that fixes spermatophore to mates body	Haase and Karlsson 2000; Karlsson and Haase 2002
Type III: spermatophores attached to body wall → sperm penetrates tissue	Microhedylacea, *Hedylopsis ballantinei* (?) (Acochlidia)	Aphallic; "anchoring device" that fixes spermatophore to mates body; lytic process that dissolves the epidermis, spermatozoa able to penetrate tissue	For literature see Schrödl and Neusser (in press)

[a] However, Ghiselin (1963) reported that the penis does not penetrate deeply in *Runcina* and Kress (1985) observed spermatophores also attached to the body surface, which she interpreted as a result of crowding effects, the fate of these spermatozoa is unknown

Materials and methods

Sand samples were collected by snorkelling at various collecting sites in Istria, Croatia (Mediterranean Sea) at a depth range between 5 and 9 m in June 2005, July 2007 and 2008. Specimens of *P. milaschewitchii* were extracted from the samples following the method described by Schrödl (2006). Up to 50 individuals were haltered for up to 2 weeks in glass Petri dishes (diameter 10–12 cm) with sand granules and checked daily for the occurrence of spermatophores. In July 2008, freshly extracted specimens contained spermatophores. Spermatophores were investigated by light microscopy and prepared for semi- and ultrathin sectioning.

Specimens with attached spermatophores were slowly anaesthetised using 7% $MgCl_2$ solution to prevent retraction. They were fixed for structural analysis in 4% glutaraldehyde buffered in 0.2 M sodium cacodylate (0.1 M NaCl and 0.35 M sucrose, pH 7.2), rinsed in the same buffer, followed by post-fixation in 1% OsO_4 buffered in 0.2 M cacodylate buffer (0.3 M NaCl, pH 7.2) for 1.5 h in darkness. After being rinsed in 0.2 M cacodylate buffer (0.3 M NaCl, pH 7.2), decalcification was effected using ascorbic acid. Stepwise dehydration was undertaken by graded acetone series. Specimens were then embedded in Spurr's low viscosity epoxy resin (Spurr 1969). Semithin sections (1 µm) of two mature females were cut to approach the region of interest using glass knives with a RMC MT 6000-XL (RMC Inc.) ultramicrotome. For orientation within the block and to gauge the approach to the target, semithin sections were stained according to Richardson et al. (1960) and checked under the light microscope. Ultrathin sections were prepared using glass knives or a diamond knife (MC 3270, Diatome 35°) at 80 nm (pale gold reflection) in the same ultramicrotome. The ultrathin sections were picked up using copper slot-grids (Agar Scientific G2500C), covered with a thin layer of formvar. For better contrast the selected ultrathin sections were stained with uranyl acetate and lead citrate after Reynolds (1963). They were analysed, using a transmission electron microscope EM 900 (Zeiss). Sperm morphology was partially reconstructed 3-dimensionally from serial ultrathin sections using AMIRA® software (TGS Template Graphics Software, Inc., USA). A voucher specimen (ZSM Mol 20080920), the semi- and ultrathin sections (ZSM Mol 20060519, 20060520) and the original TEM-negatives are deposited in the "Zoologische Staatssammlung München" (ZSM), Mollusca Section.

For observation of insemination following the attachment of the spermatophore, three living females of *P. milaschewitchii* with attached spermatophores were stained in a 1% DAPI-solution, for about 4–12 h in complete darkness. The stained sperm nuclei were observed under the fluorescence microscope (Leica DM RBE; 20×/0.5, 63×/1.32 oil; DAPI filterset) for about 30 min in each animal.

Results

Spermatophores

In total >20 spermatophores were found to be attached to specimens of *P. milaschewitchii*; the development or the transfer of the spermatophore to the recipient was not observed directly. The spermatophores were placed on different positions on the visceral hump (Fig. 1a), as well as on the head–foot complex. Spermatophores were not exclusively attached to females, but were also encountered once on a male and a juvenile, and an additional spermatophore was found attached to a sand granule. No differences in the placement of the spermatophores was noted between freshly extracted specimens and specimens kept under laboratory conditions.

The spermatophores in *P. milaschewitchii* are straight, elongate capsules with a rounded apical tip (Fig. 1b) and vary in length between 150 and 600 µm. In cross-sections they are oval and measure about 20 µm × 45 µm in diameter (Fig. 1c). The spermatophores are tightly packed with a dense mass of sperm; the spermatozoa are randomly orientated in all directions (Fig. 2a). Methylene blue-stained semithin sections show the mass of spermatozoa surrounded by a relatively thick basophilic dark blue inner layer and an outer layer composed of a non-stained inner region and an outer thin basophilic dark blue-stained border (Fig. 1c). TEM-examination reveals that the inner layer is composed of electron-dense, tightly arranged globules which form an irregular thick layer (varying between 0.3 and 0.75 µm in width; Fig. 2b). The globules reach a diameter of up to 80 nm. The outer layer is composed of a loose fibrous inner part, which bears large unstained spaces and an outer border formed of electron-dense minute globules (Fig. 2b). The width of the outer layer is also irregular, varying between 0.6 and 1.3 µm. Between the randomly orientated sperm various granules, globules and vacuoles with different electron density are found. Under the light microscope a "central filament" could be observed within the sperm mass, extending nearly the entire length of the spermatophore (Fig. 1b); it could, however, not be located on semithin or ultrathin sections, and might thus just be a central rotation axis for intruding sperm. No special anchoring features could be detected at the attachment site. Near the point of attachment the spermatophore is surrounded by loose transparent material (light microscopic observation, see Fig. 1b). Semithin sections show the membranes of a spermatophore open towards the epidermis of the recipient. At the point of attachment the epidermal cells of the recipient are lysed and spermatozoa can be observed within the tissue of the female (Fig. 1d).

The spermatophore empties gradually (Fig. 1e shows a partly emptied spematophore). Semithin sections reveal that not all spermatozoa successfully intrude through the

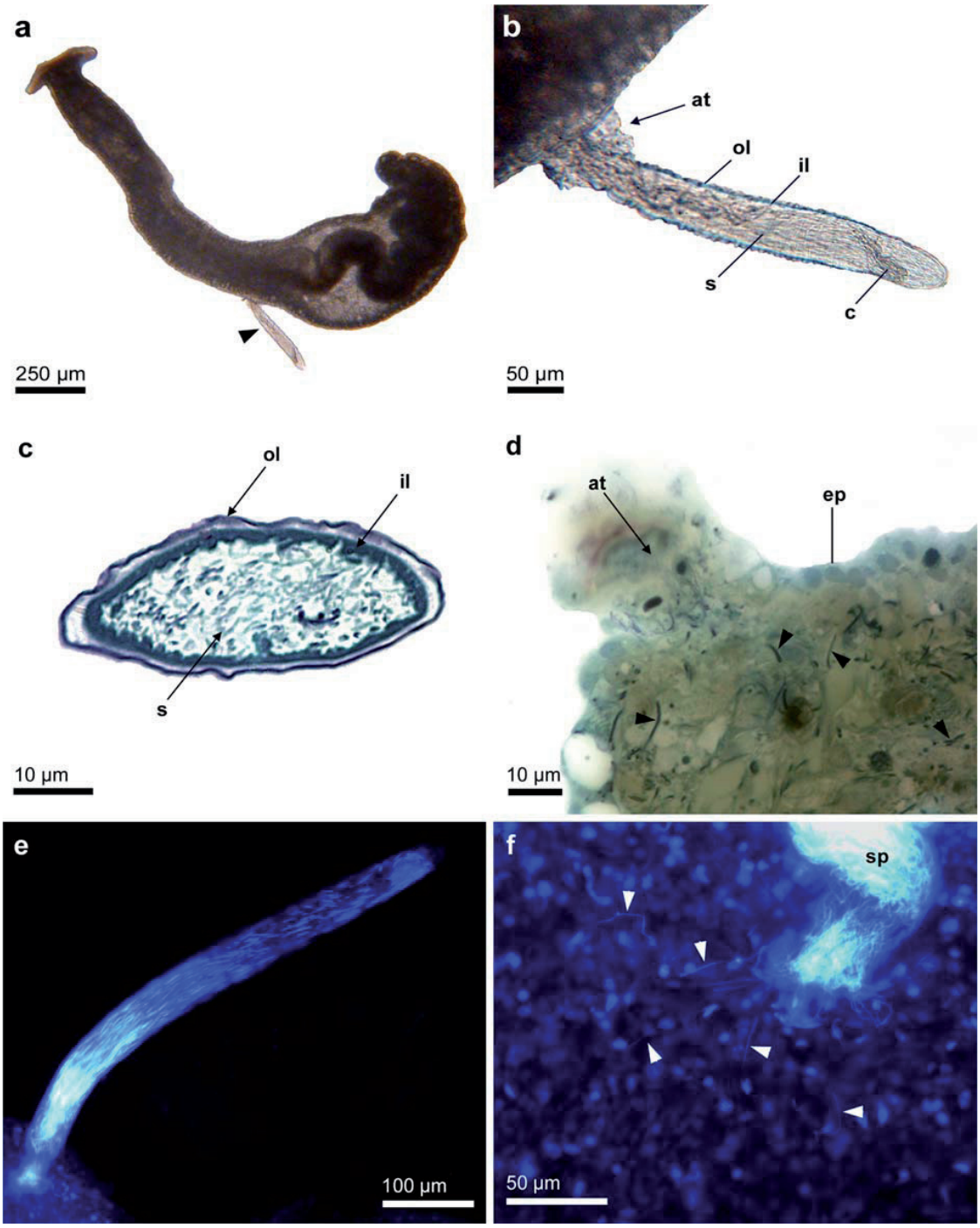

Fig. 1 Spermatophore of *Pontohedyle milaschewitchii* (light microscopy). **a** Female *P. milaschewitchii* with a spermatophore (*arrowhead*) attached to the left-anterior region of the visceral hump. **b** Close up of spermatophore filled with spermatozoa. **c** Semithin cross-section of spermatophore. **d** Attachment site of the spermatophore showing the lysed epidermal cells of the recipient (*arrowheads* showing intruded spermatozoa). **e** Fluorescence micrograph of the spermatophore attached to the body wall stained with DAPI (sperm nuclei *highlighted*). **f** Close up of attachment site of spermatophore and intruding spermatozoa (*arrowheads*; DAPI fluorescence). *at* Attachment site, *c* "central filament", *ep* epidermis, *il* inner layer, *ol* outer layer, *s* sperm, *sp* spermatophore

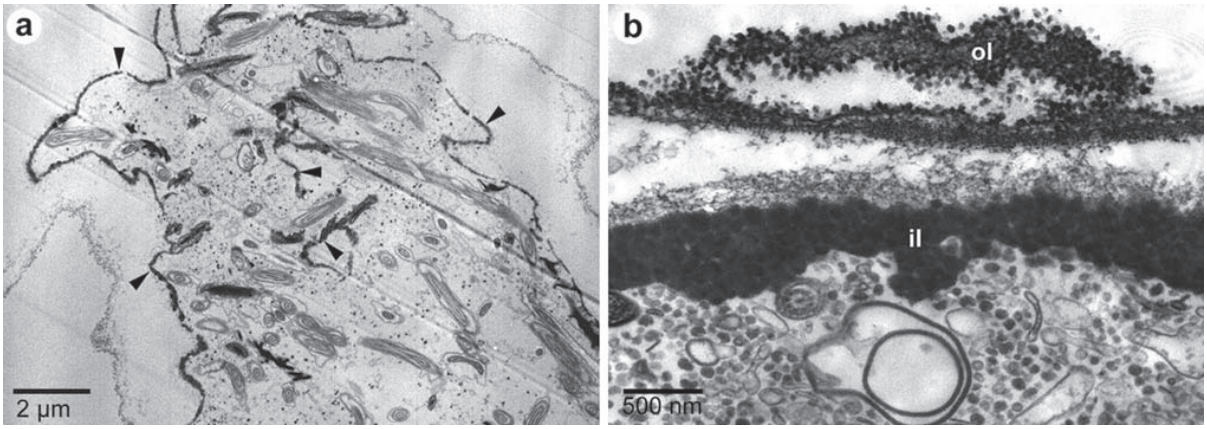

Fig. 2 TEM micrographs of a spermatophore of *P. milaschewitchii*. **a** Overview of the distal region of a spermatophore containing randomly orientated spermatozoa in a matrix with granulae and vesicles (*arrowheads* represent inner layer of spermatophore). **b** Close up of the layered wall of the spermatophore. *il* Inner layer, *ol* outer layer

epidermis of the recipient, but that some spermatozoa move along the outer surface of the epidermis. Under the fluorescence microscope the DAPI-stained elongate nuclei of the spermatozoa could be observed intruding into the body of the female and spreading out in all directions (Fig. 1f). Allosperm was found in the cavity of the visceral hump, as well as head–foot complex, e.g. single spermatozoa next to the eyes and cerebral ganglia of the recipient. The continuous discharge of the spermatozoa could be observed for about 0.5 h. From this observation and the fact that the spermatophore was already attached for at least 12 h (duration of DAPI staining) it can be concluded that the entire discharge takes several hours. Even though the spermatozoa are orientated in all directions within the spermatophore, while discharging they seem to orientate in the direction of the attachment site and the sperm mass displays a spiral arrangement while emptying.

Sperm ultrastructure

As described above the spermatozoa were irregularly orientated within the examined spermatophores. Therefore more or less randomly orientated cutting-profiles had to be examined. The terminology used in the following is based on Thomson (1973) and Healy and Willan (1991). The spermatozoa of *P. milaschewitchii* are comprised of a head, a mid-piece and a tail (i.e. annulus and glycogen piece), all continuously sheathed by the plasma membrane (Fig. 3a). The overall length of the spermatozoon is approximately 55–60 µm (light microscopic observation).

Acrosomal complex and nucleus

An acrosomal complex could not be detected, even though various spermatozoan apical tips were studied. The nucleus reaches a length of approximately 20–25 µm and can be subdivided into three morphologically distinct regions: the apical, the mid and the basal nuclear region (Figs. 3a, 4a–f). All three regions are helically coiled and the content is highly electron-dense; the apical and the mid region additionally bear helical keels. In the apical region of the nucleus the "screw-thread" of a single keel spirals with about 0.4 µm per convolution (Figs. 3b, 4a). The keel in this region is relatively thin and sometimes the tips of the keels are pointed distally. In the mid region of the nucleus the "screw-thread" of the three keels is narrower than in the apical region and the three keels are compact (Figs. 3c, 4b, f). The basal nuclear region differs from the other parts of the nucleus by the absence of keels and an heterogenous electron-dense appearance (Figs. 3a, 4g). The inner electron-dense region is surrounded by a fibrous, less electron-dense outer ring. In cross-sections the basal nuclear region is circular to oval (Fig. 3a, d). The nuclear diameter decreases from the basal to the apical nuclear region.

Neck region and mid-piece

A bell-shaped centriolar derivative fills a relatively shallow invagination at the base of the nucleus (Figs. 3d, 4g, h). A sub-nuclear ring is present at the base of the nucleus (see arrowheads in Fig. 4h). The central axoneme emerges from the centriolar derivative and extends throughout the mid-piece into the glycogen piece. The axoneme shows the typical formation of microtubules: nine doublets surrounding a central pair. In the sperm mid-piece the nine doublets seem to be slightly thickened (coarse fibres?) in comparison to the doublets of the axoneme in the tail region. Intra-axonemal granules occur throughout the whole length of the axoneme; in longitudinal sections these granular deposits

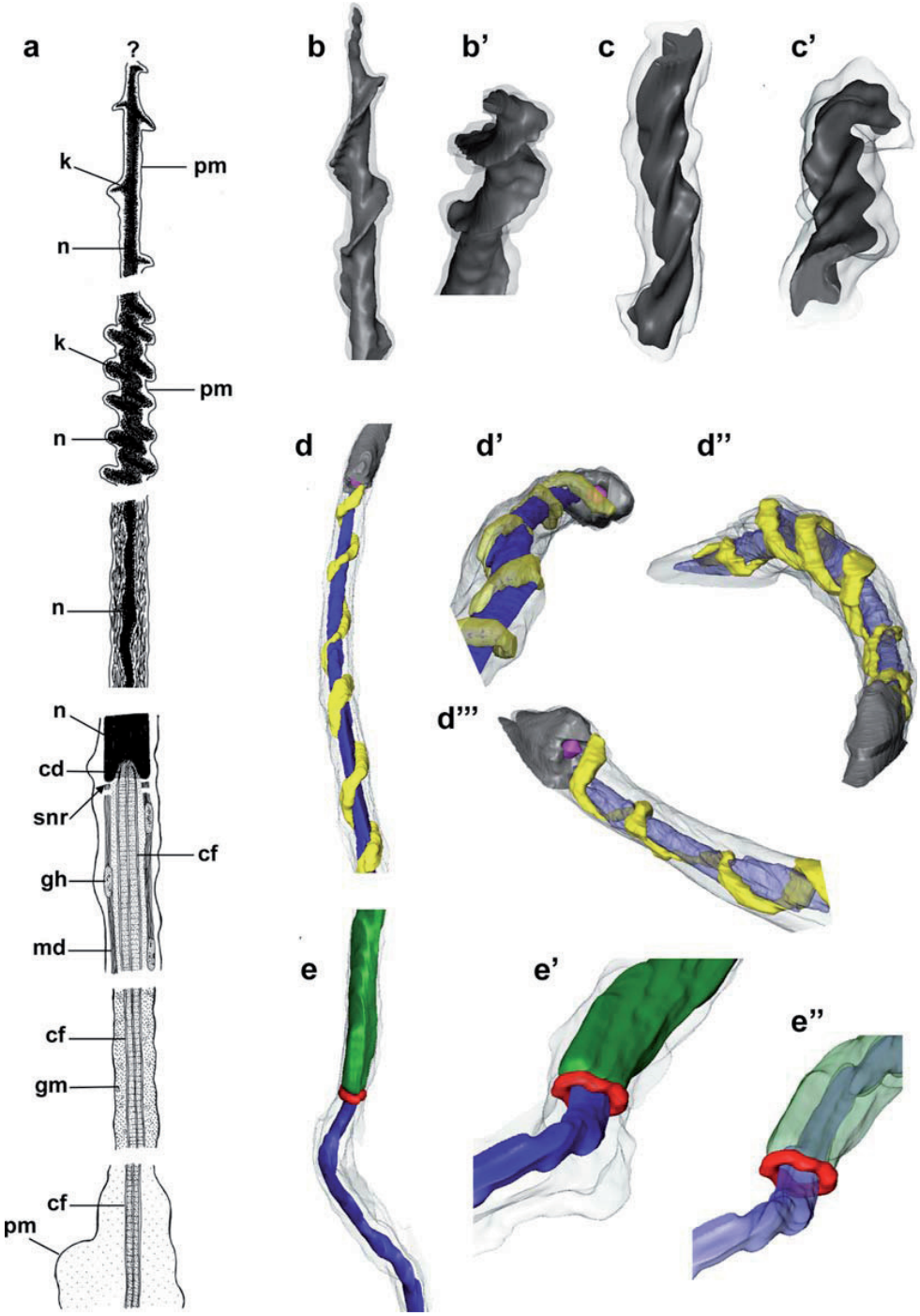

Fig. 3 Schematic overview and 3D-reconstructions of a sperm cell of *P. milaschewitchii*. **a** Schematic overview of the different structural elements. **b**–**e** 3D-reconstruction from ultrathin section series in different perspectives and transparencies. **b**, **b′** Corkscrew-shaped, one-keeled tip of the sperm nucleus with surrounding plasma membrane. **c**, **c′** Middle region of the sperm nucleus (three keels) with surrounding plasma membrane. **d**–**d‴** Middle region of the sperm cell at transition to the nucleus. **e**–**e″** Transition from mid-piece to sperm tail. *cd* Centriolar derivative, *cf* central flagellum, *gh* glycogen helix, *gm* glycogen material, *k* nuclear keel, *md* mitochondrial derivative, *n* nucleus, *pm* plasma membrane, *snr* sub-nuclear ring

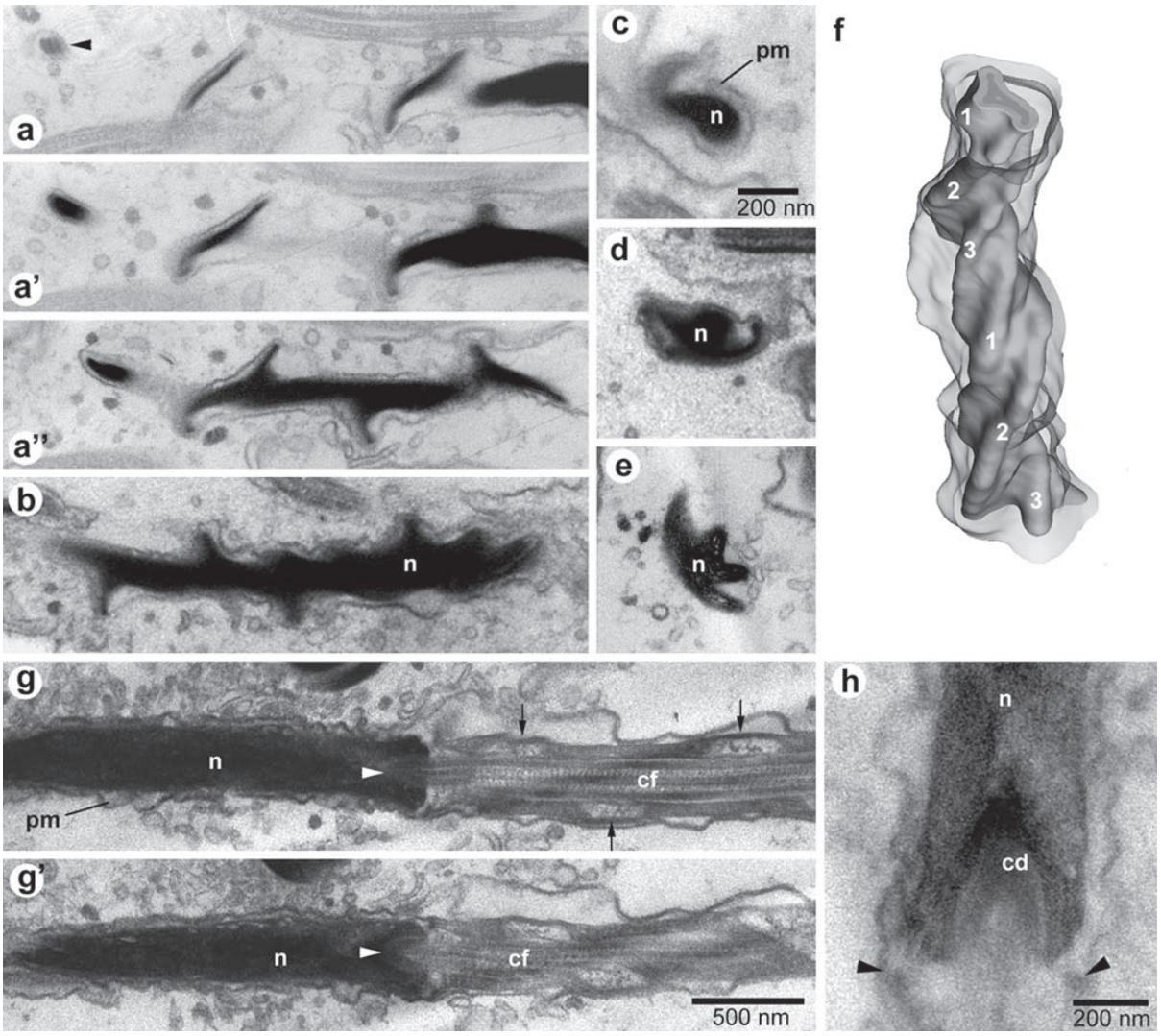

Fig. 4 TEM micrographs of sperm nucleus and mid-piece in *P. milaschewitchii*. **a**–**a**″ Longitudinal section series (*z*-spacing 80 nm) at the very tip (*arrowhead*) of a sperm nucleus. Note corkscrew-like convolution of the terminal nuclear keel (compare with Fig. 3b). **b** Longitudinal section in the distal half of the nucleus with three intertwisted rounded keels (compare with Fig. 3c). **c**–**e** Cross-sections through the sperm nucleus showing different aspects of the nuclear keels, **c** near the tip, **d**, **e** at different locations in the distal half. **f** 3D-reconstruction of the sperm nucleus in the distal half with three intertwisted keels (1–3; surrounding plasma membrane displayed transparently). **g**, **g**′ Neighbouring longitudinal sections through a single sperm cell at the transition of the nucleus to the mid-piece. Note the centriolar derivative (*arrowhead*) and the helically coiled glycogen helix (*arrows*) within the mitochondrial derivative (compare with Fig. 3d). **h** Longitudinal section of transition of nucleus to tail (*arrowheads* sub-nuclear ring). *cd* Centriolar derivative, *cf* central flagellum, *n* nucleus, *pm* plasma membrane (where not indicated: magnification as in **g**′)

appear arranged in thin bars orientated in a 90° angle to the microtubules (Figs. 3a, 4g). In the mid-piece the axoneme is surrounded by a ring of lamellar organised matrix components; paracrystalline mitochondrial derivatives could not be detected. One single glycogen helix runs a spiral course around the mid-piece, rising about 0.75 µm per convolution. The glycogen helix is about 0.25–0.30 µm wide and contains granular deposits (Figs. 4g, 5a). It is well developed in the post-nuclear region but diminishes in the later course of the mid-piece (Fig. 5d). In cross-sections the mid-piece is round and has a diameter of about 0.40 µm (Fig. 5b, c).

Glycogen piece and annulus

The transition point of the mid-piece to the glycogen piece is marked by the presence of an annulus, i.e. a simple, electron-dense ring (Figs. 3e, 5e, g, h). Here the tube

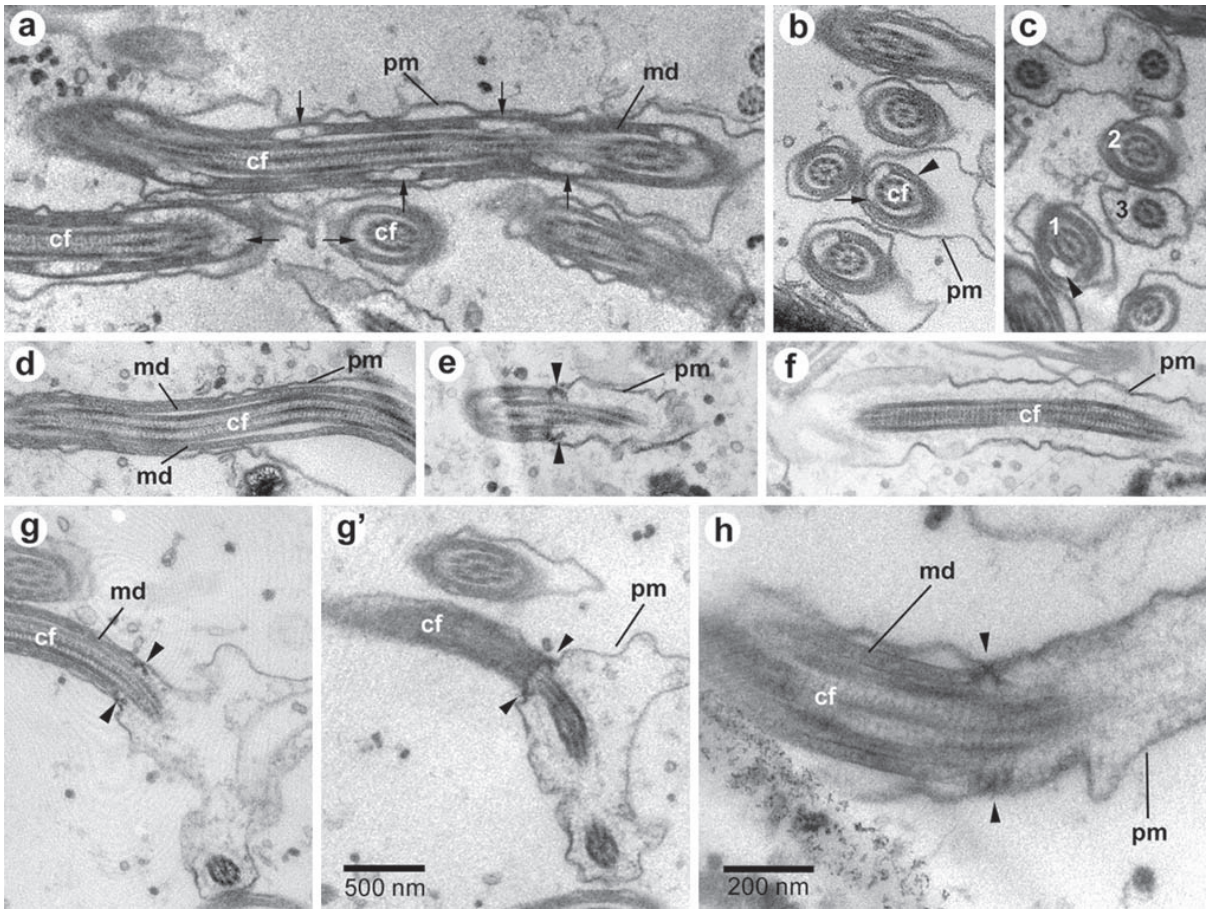

Fig. 5 TEM micrographs of sperm mid-piece and tail in *P. milaschewitchii*. **a** Oblique section through the frontal halves of sperm mid-pieces with glycogen helices (*arrows*) within the mitochondrial derivatives. **b**, **c** Cross-sections of sperm tails and mid-pieces at different positions along the cell. *1* represents mid-piece with glycogen helix (*arrowhead*), *2* represents mid-piece without helix, *3* represents tail without mitochondrial derivative. **d**–**f** Longitudinal sections, **d** back half of a sperm mid-piece: mitochondrial derivative without glycogen helix. **e** Transition from mid-piece to tail. Note annulus (*arrowheads*). **f** Sperm tail (behind annulus) without mitochondrial derivative. **g**, **g′** Two neighbouring planes ($\Delta z = 80$ nm) of a sperm cell at the transition from mid-piece to tail (*arrowheads* represent annulus). **h** Transition from mid-piece to tail showing annulus (*arrowheads*). *cf* Central flagellum, *md* mitochondrial derivative, *pm* plasma membrane (where not indicated: magnification as in *g′*)

of mitochondrial matrix disappears and the axoneme continues the glycogen piece surrounded by some loose granular material (probably glycogen according to Thompson 1973), and the plasma membrane (Fig. 5e, f). The surrounding plasma membrane becomes partly degenerated and widened towards the distal end of many spermatozoa (Fig. 5g). In the distal tail region the axoneme sometimes turns and twists within the lose membrane. It remains unclear whether this is an artefact or the normal appearance of the spermatozoan plasma membrane. The axoneme in this region has a diameter of about 0.2 µm. In the distal tail region the granules disappear; the axoneme persists and forms the posterior tip of the spermatozoon.

Discussion and conclusions

Spermatophores

Pontohedyle milaschewitchii produces spermatophores that consist of the sperm mass surrounded by two capsular layers, i.e. an inner globular and an outer fibrous one. A similar assembly of two layers was reported by Kress (1985) for the cephalaspidean opisthobranch *R. ferruginea* (Runcinidae), but the layers differ greatly in dimensions from those in *P. milaschewitchii*: the globular inner layer of *R. ferruginea* is comprised of large, comparably loosely arranged globules with a lamellar structure and a diameter of 10 µm (about 100× the size of those of *P. milaschewitchii*). In

contrast, the outer layer of *R. ferruginea* is comparably thin (0.3–0.7 µm; 0.6–1.3 µm in *P. milaschewitchii*) and is composed of an inner more fibrillar and an outer more flocculent structure. Kress (1985) suggested a sticky property for the outermost layer functioning in attachment of the spermatophore. She also tested the spermatophore components with different enzymes, revealing a predominant lipid character of the globules in the inner layer. The function of the lipid globules in *R. ferruginea* and probably in *P. milaschewitchii* remains unclear; a protective (water-proof) and/or lytic function involving dissolution of the epidermis is probable for *P. milaschewitchii*.

The exact place of spermatophore production in *P. milaschewitchii* is not known. Probably sperm is covered by fluids/sheaths in the prostatic region of the vas deferens (Ghiselin 1966). All the described acochlidian spermatophores are elongate, tube- or spindle-shaped, tightly packed with sperm and are comparably long in relation to the body size, ranging from 80 to 900 µm (Swedmark 1968a, b; Westheide and Wawra 1974; Morse 1994). Sizes of acochlidian spermatophores appear to be highly variable intraspecifically: the spermatophores of *P. milaschewitchii* varied from 150 to 600 µm, while Swedmark (1968a) described them as "very small". The size of spermatophores in Acochlidia might thus depend on factors such as nutrition and the frequency of spermatophore placement, and may not be a reliable taxon specific character.

Transfer of spermatophores

Uniquely within spermatophore-possessing acochlidians with genital openings on the right side of the body, the vas deferens in *P. milaschewitchii* opens above the mouth. Jörger et al. (2008) suspected that this frontal opening at the sensory head could be advantageous for placing spermatophores more precisely onto the mate. However, data shows that spermatophores are still attached in a rather imprecise way, not only to females, but occasionally also to males, juveniles, and, in some cases, even to the substratum. *P. milaschewitchii* thus seems to be generally able to (chemically?) detect conspecifics in the mesopsammic environment, but not to differentiate efficiently between appropriate and inappropriate mates.

In *P. milaschewitchii*, spermatophores were found attached over the entire body surface. Attachment was in general more frequent on the visceral hump, which also accounts for the largest available body area. Poizat (1986) observed 40–45 spermatophores in *P. milaschewitchii* and *M. glandulifera* randomly distributed over the body surface, but with a higher percentage attached to the dorsal, posterior region of the visceral hump; Swedmark (1968b) reports a similar situation for *Asperspina brambelli*. None of these studies detected a higher percentage of spermatophores placed at or near to the female genital opening; we thus conclude that acochlidian spermatophores are more or less randomly anchored to mates. The higher placement-rates in the dorsal–posterior region of the visceral hump might be explained by an advantage in approaching (or chasing?) the mate. Additionally it might be advantageous for the intruding sperm due to proximity to the gonad.

Dermal insemination

How do sperm penetrate the epidermis of the recipient? We observed a lysis of epidermal cells at the attachment site of the spermatophore in *P. milaschewitchii*. This partly confirms earlier observations of Morse (1994) and Swedmark (1968a) on other microhedylacean acochlidians. Swedmark (1968a) assumed that an autolysis of epidermal cells occurs under the influence of allosperm. It remains, however, unclear whether lysis is induced by sperm or by parts of the spermatophore.

Our staining experiments with DAPI showed that most sperm successfully penetrates the body wall at the point of spermatophore attachment and then moves into the body of the recipient spreading out in all directions through the body fluid and tissue. Marcus (1953) also found that sperm of spermatophores on female microhedylacean *Ganitus evelinae* penetrates the skin directly. This special mode of dermal insemination, showing active spermatozoan migration through a dissolved (or at least partly dissolved) integument, is likely the same for all other aphallic microhedylacean acochlidian species. This is in contrast to other spermatophore-transferring opisthobranchs, where spermatophores are either placed directly into or near to the genital opening (see Table 1), or where spermatophores are attached to the body and the sperm migrate externally towards the genital pore as in the nudibranch *Aeolidiella glauca* (see Haase and Karlsson 2000; Karlsson and Haase 2002). Occasionally, spermatozoa of *A. glauca* bury their heads into the integument; however they do not penetrate deeply into the tissue (Karlsson and Haase 2002). At present, members of the Acochlidia are the only opisthobranchs with true dermal insemination (see Table 1).

Dermal fertilisation

Since there is no allosperm storing organ or obvious fertilisation chamber in *P. milaschewitchii* (Jörger et al. 2008), fertilisation probably occurs directly in the gonad. This would require actively migrating allosperm to (1) locate the oocytes, and (2) not only penetrate the (lysed?) body wall and body cavity of the mate, but also the epithelia of the gonad and oocytes. Our observations of sperm spreading through the entire body cavity of mature female *P. milaschewitchii* indicate that spermatozoan taxis, if present, is

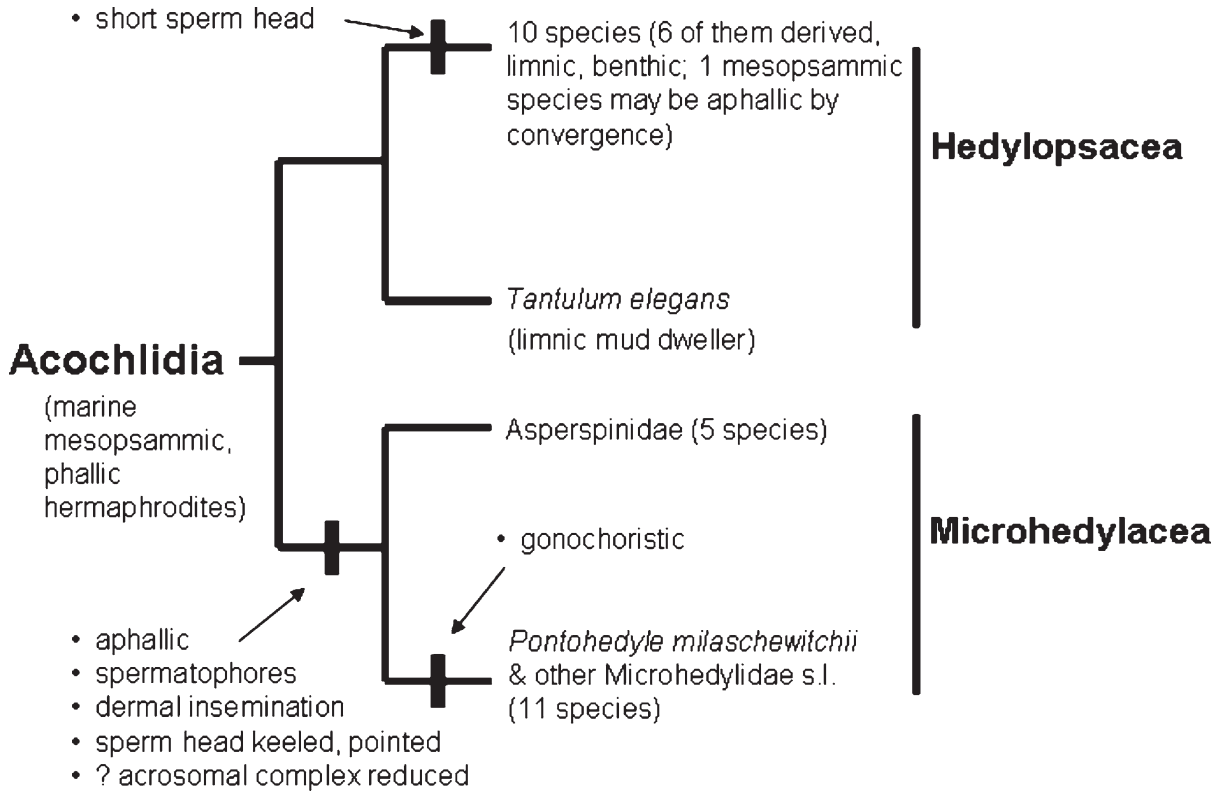

Fig. 6 Evolution of sperm structure, spermatophores and dermal insemination in the Acochlidia. Topology and apomorphies modified after Schrödl and Neusser (in press). The evolution of sperm transfer via spermatophores, dermal insemination and screw-like keeled sperm heads are regarded as key innovations leading to greater species diversification of Microhedylacea in the marine interstitial

not very efficient. Instead, given the large quantities of sperm in the body cavity, single spermatozoans probably encounter and penetrate the gonad by chance; potential chemotaxis might be limited to finding the relatively large oocytes of *P. milaschewitchii* within the gonad.

Curiously, allosperm of acochlidians with dermal insemination appear to be able to penetrate and thus perforate any cells, tissues and organs. This is indicated by histological data of Marcus (1953) who found "many" allosperm not only in the haemocoel but also within the digestive gland, connective tissue and nerve fibres of female *M. remanei*. There is neither certain information on how long allosperm may survive in the body of a recipient, nor any estimation on the damage which an excess of allosperm might cause to an individual.

Sperm ultrastructure: special adaptations to dermal insemination?

The spermatozoa of *P. milaschewitchii* correspond to the general characteristics of opisthobranch sperm (Thompson 1973; Healy 1982, 1993; Healy and Willan 1984; Fahey and Healy 2003). Remarkable features in *P. milaschewitchii* are the long and strongly keeled nucleus and the potential lack (or at least extremely small size) of the acrosome. With a length of 20–25 µm the strongly keeled nucleus of *P. milaschewitchii* ranges among the longest reported sperm nuclei within the opisthobranchs (Franzén 1955; Thompson 1973). The spermatophore-bearing *M. remanei* also presents a fairly long and keeled nucleus with a minimal length of 11 µm (Neusser et al. 2007). Based on light microscopical data, nuclei are long and keeled in other, generally aphallic microhedylacean species as well (Schrödl and Neusser, in press; Fig. 6). In contrast, *Hedylopsis spiculifera* and other hedylopsacean acochlidians that usually copulate or use hypodermic injection have short sperm heads (Sommerfeldt and Schrödl 2005; Schrödl and Neusser, in press). Such differences in sperm morphology may be attributed to the differing biology of fertilisation (Franzén 1955). Nuclear elongation in bivalves and gastropods has been correlated with larger, yolky eggs (Franzén 1983; Wilson and Healy 2002). In fact, many microhedylacean species produce comparably large yolky eggs (see e.g. Swedmark 1968b; Westheide and Wawra 1974). Thompson (1973) concluded that keels on spermatozoa convert uni-planar flagellation into helical progression, particularly in a viscous medium, which strongly

suggests that prominent keels at the nucleus may enhance sperm movement (Wilson and Healy 2002). While long and keeled sperm nuclei also occur in other opisthobranchs with reciprocal copulation (see e.g. Kubo and Ishikawa 1981; Healy 1982, 1993), the corkscrew shaped, pointed sperm nucleus of *P. milaschewitchii* and other microhedylaceans might be an evolutionary adaptation allowing efficient movement through the body cavity of females.

All opisthobranchs previously studied in sufficient detail possess an acrosomal complex (of varying size and shape), with the exception of microhedylacean acochlids such as *M. remanei* (see Neusser et al. 2007) and *P. milaschewitchii* (present study). Careful redescription of previously acrosome-lacking molluscs often revealed tiny acrosomal vesicles (see Kubo and Ishikawa 1981 for aplysiid opisthobranchs; Buckland-Nicks et al. 1988 for chitons). We were unable to detect an ultrastructurally differentiated acrosome at the tip of the sperm nucleus and we thus conclude that it is either truly absent, or a very small acrosomal vesicle (i.e. <80 nm, missed by the cutting plane). In comparison to well-developed acrosomal complexes (i.e. acrosomal vesicle and pedestal) in other opisthobranch groups (see e.g. Healy and Willan 1984, 1991 on some Notaspidea and Nudibranchia), the acrosome in microhedylacean acochlids is reduced. As mentioned by Healy (1993) on Rissoellidae and Omalogyridae, there might be a correlation between the elongation of the nucleus and the reduction of the acrosome. A potential reduction in importance of the acrosome in microhedylacean acochlids might also be correlated to the drilling mechanism of the "corkscrew"-shaped nucleus.

Future studies on sperm ultrastructure of closely related acochlids and especially on spermatid development in Acochlidia in general are needed to settle the issue of presence or absence of acrosomes and potential correlations to the drilling sperm movement presented in this study.

Dermal insemination—a success story in the interstitial?

Spermatophores are generally considered as characteristic of interstitial organisms (Ax 1969) and as an adaptation to the mesopsammic habitat, evolved convergently within different groups of invertebrates (Clark 1991). But what makes sperm transfer via dermal application of spermatophores so advantageous? Life in the lacunary system of the interstitial is influenced by limited space availability and instability of the habitat due to movement of sand by waves and currents (Swedmark 1964; Ax 1969). For mesopsammic acochlidians such as *P. milaschewitchii* it might already be mechanically difficult to locate and approach a potential mate, but it is even harder to synchronise sexual activities and engage in (reciprocal) copulation which is the typical mode for benthic opisthobranchs (Schrödl and Neusser, in press). Of 27 valid acochlidian species only a few taxa such as the mud-dwelling *Tantulum elegans* and the limnic *Strubellia* may still copulate (Neusser and Schrödl 2007; Schrödl and Neusser, in press). *Hedylopsis spiculifera*, another basal mesopsammic species, uses hypodermic injection of sperm via a hollow penial spine (see Sommerfeldt and Schrödl 2005), a fast but imprecise and to a certain degree violent way of sperm transfer. The vast majority of the 20 known mesopsammic acochlidian species, however, i.e. all 16 described microhedylaceans, lost the copulatory organ and are very likely to transfer sperm via spermatophores and dermal insemination as shown for *P. milaschewitchii* (see Fig. 6). Disadvantages to dermal sperm transfer include sperm loss by misplacement of spermatophores, disorientation of sperm within the recipient, and damage to mates through lysing of integument and perforating inner organs. However, these disadvantages are evolutionarily outweighed by the benefits of transferring sperm to any available body portions of a potential mate while "passing by."

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Chapter 7. Time for sex change! 3D-reconstruction of the copulatory system of the ‘aphallic’ *Hedylopsis ballantinei* (Gastropoda, Acochlidia)

Kohnert P, Neusser TP, **Jörger KM**, Schrödl M (2011) Time for sex change! 3D-reconstruction of the copulatory system of the 'aphallic' *Hedylopsis ballantinei* (Gastropoda, Acochlidia). *Thalassas* 27(2): 113-119.

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TIME FOR SEX CHANGE! 3D-RECONSTRUCTION OF THE COPULATORY SYSTEM OF THE 'APHALLIC' *Hedylopsis ballantinei* (GASTROPODA, ACOCHLIDIA)

KOHNERT P, NEUSSER TP, JÖRGER KM & SCHRÖDL M

Key words: Mollusca, Panpulmonata, morphology, hypodermal injection, penial stylet, protandry, sequential hermaphroditism.

ABSTRACT

Within hedylopsacean acochlidians an evolutionary trait from a simple unarmed copulatory system towards complex hypodermal injection systems was recognized. This culminates in a large, trap-like spiny rapto-penis of several limnic Acochlidiidae having a sperm injection stylet plus an additional injection system with an accessory gland. The only exception was the mesopsammic hedylopsacean species *Hedylopsis ballantinei* Sommerfeldt & Schrödl, 2005, since it was assumed to be aphallic. Specimens with mature autosperm and oogonia in the hermaphroditic gonad showed no trace of any male copulatory organs. Sperm transfer via spermatophores was thus suggested, as known to occur in the generally aphallic microhedylaceans. The present study re-examines several series of semithin sections used for the original description. Additionally, one specimen of *H. ballantinei* was newly collected near the type locality in the Red Sea. It is externally identical with but smaller than the original specimens. The specimen was embedded into Spurr's resin and serially cut into semithin histological sections. Reproductive systems were compared in detail and that of a specimen in the male phase was 3-dimensionally reconstructed using AMIRA software. The copulatory organs comprise the posterior-leading vas deferens passing into a voluminous tubular prostate, a presumable paraprostate and a bipartite penis with a large apical, hollow penial stylet and with a cuticular, solid thorn on top of the basal swelling. As already known for *H. spiculifera* (Kowalevsky, 1901), its European sister species, *H. ballantinei* thus is a sequential hermaphrodite with sex change. The male phase precedes the female one, in which male copulatory organs completely disappear. Sperm transfer is likely by hypodermal injection. *Hedylopsis ballantinei* in the male phase has an external sperm groove, while specimens in the female phase possess a ciliary field; the latter may have a function related to building or placing the egg mass. *Hedylopsis ballantinei* now fits well with evolutionary traits observed within other hedylopsacean acochlidians known in detail.

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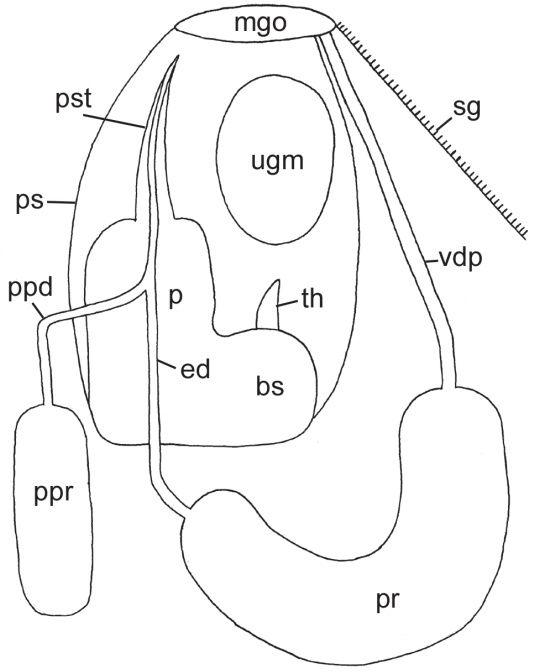

Figure 1:
Schematic overview of the male cephalic copulatory organs with associated glands of Hedylopsis ballantinei. Abbreviations: bs, basal swelling; ed, ejaculatory duct; mgo, male gonopore; p, penis; ppd, paraprostatic duct; ppr, paraprostate; pr, prostate; ps, penial sheath; pst, hollow penial stylet; sg, external sperm groove; th, solid thorn; ugm, unidentified glandular mass; vdp, posterior-leading vas deferens. Not to scale.

INTRODUCTION

Most recently, opisthobranch gastropods were shown to be an artificial assemblage, with the traditional order Acochlidia clustering within a (pan)pulmonate relationship (Jörger *et al*., 2010; Schrödl *et al*., this volume). Both molecular and morphology-based phylogenetic analyses (Jörger *et al*., 2010; Schrödl & Neusser, 2010) indicate a basal acochlidian split into generally regressive, meiofaunal Microhedylacea (Neusser *et al*., 2009) and morphologically and ecologically more variable Hedylopsacea, including marine, brackish water and limnic species of variable body sizes (e.g. Neusser & Schrödl, 2007, 2009; Brenzinger *et al*., 2011). Within hedylopsacean acochlidians an evolutionary trait from a simple, unarmed copulatory system towards complex hypodermal injection systems was recognized (Schrödl & Neusser, 2010). This culminates in the large, trap-like spiny rapto-penis of several limnic Acochlidiidae, having a sperm injection stylet plus an additional injection system with an accessory gland (Haase & Wawra, 1996). The only exception in this evolutionary scenario of evolving a more and more complex and probably violent copulatory apparatus was the mesopsammic hedylopsacean species *Hedylopsis ballantinei* Sommerfeldt & Schrödl, 2005, since it was assumed to be aphallic. The few specimens available had mature autosperm and oogonia in the hermaphroditic gonad, but showed no trace of any copulatory organs (Sommerfeldt & Schrödl, 2005). Sperm transfer via spermatophores was thus suggested, as known to occur in the generally aphallic microhedylaceans.

The present study examines old and new material of different-sized *H. ballantinei* from serial histological sections for the presence of reproductive organs. Male copulatory organs were identified, labeled and 3-dimensionally reconstructed using AMIRA software, and compared to other hedylopsacean copulatory systems.

MATERIAL AND METHODS

One specimen of *Hedylopsis ballantinei* was newly collected approx. 600 m north of the type locality (Inmo Reef) in Mashraba (28°29`42`` N, 34°31`04`` E), Dahab, Egypt in August 2009. A sample of coarse coral sand was obtained by snorkeling from 6 m depth by night. The specimen was extracted from the sand sample according to the method described by Schrödl (2006). The specimen was relaxed with isotonic $MgCl_2$-solution and was preserved in 4 % glutardialdehyde buffered in 0.2 M sodium cacodylate (0.1 M NaCl and 0.35 M sucrose, pH 7.2). Following a post-fixation in buffered 1 % OsO_4 for 1.5 h in the dark, the specimen was decalcified in 1 % ascorbic acid overnight and dehydrated in an acetone series (30, 50, 70, 90, 100 %). For semithin sectioning the specimen was embedded in Spurr's low viscosity resin (Spurr, 1969) and a series of ribboned serial semithin sections of 1.5 µm thickness was prepared using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) and contact cement on the lower cutting edge to form ribbons (Ruthensteiner, 2008). Finally, the sections were stained with methylene-azure II (Richardson *et al.,* 1960) and were deposited at the Mollusca Section of the Bavarian State Collection of Zoology (ZSM), Germany (ZSM Mol 20100856). Additionally, we (re-) examined five series of serial semithin sections (2 µm) of *Hedylopsis ballantinei* which were available at the ZSM by light microscopy: ZSM Mol 20100855, ZSM Mol 20004766/1, ZSM Mol 20004767, ZSM Mol 20004768 and ZSM Mol 20004769. The series N° 20100855 revealed *H. ballantinei* to possess mature male copulatory organs. Digital photographs of every slice of the latter series were taken with a CCD microscope camera (Spot Insight, Diagnostic Instruments, Sterling Heights, USA) mounted on a DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). The image resolution was reduced to 50 % and images were contrast enhanced, unsharp masked and converted to 8bit greyscale format with standard image editing software. A detailed computer-based 3D-reconstruction of the body surface and the male reproductive system was performed using the software AMIRA 5.2.2 (Visage Imaging GmbH, Germany) as outlined by Ruthensteiner (2008).

RESULTS

The re-examination of the semithin section series used for the original description of *Hedylopsis ballantinei* (Sommerfeldt & Schrödl, 2005) and for the examination of the excretory system (Fahrner & Haszprunar, 2002, as *Hedylopsis* sp.), did not provide new data on the male reproductive system. The newly collected specimen was in the female phase with mature female reproductive organs, but lacking any male copulatory organs. In contrast, the examination of a series of semi- and ultrathin sections (ZSM Mol 20100855) showed a male specimen of *H. ballantinei* with mature complex copulatory organs. The 3D reconstruction by Amira and the following description of the male genital system of *H. ballantinei* is based on series N° 20100855.

Hedylopsis ballantinei is a sequential, protandric hermaphrodite with an external sperm groove (Figs. 1; 2A,B) in the male phase and a ciliary field in the female phase. The external sperm groove connects the posterior reproductive system from the female gonopore (Fig. 2D) to the male gonopore (Fig. 1) and the cephalic male copulatory organs (Figs. 1; 2A-C). The latter include a large bipartite penis with an apical hollow stylet, a very voluminous prostate, a potential paraprostate and an accessory gland (Figs. 1; 2C) with unknown function and homology.

The posterior-leading vas deferens (Figs. 1; 2A,B) leads from the male genital opening (Fig. 1) which is situated at the base of the right rhinophore, to the tubular, glandular prostate (Figs. 1; 2A,B,F). The ejaculatory duct (Fig. 1) emerges from the latter and enters the muscular penis (Figs. 1; 2A-C). A second glandular mass, the sac-like paraprostate

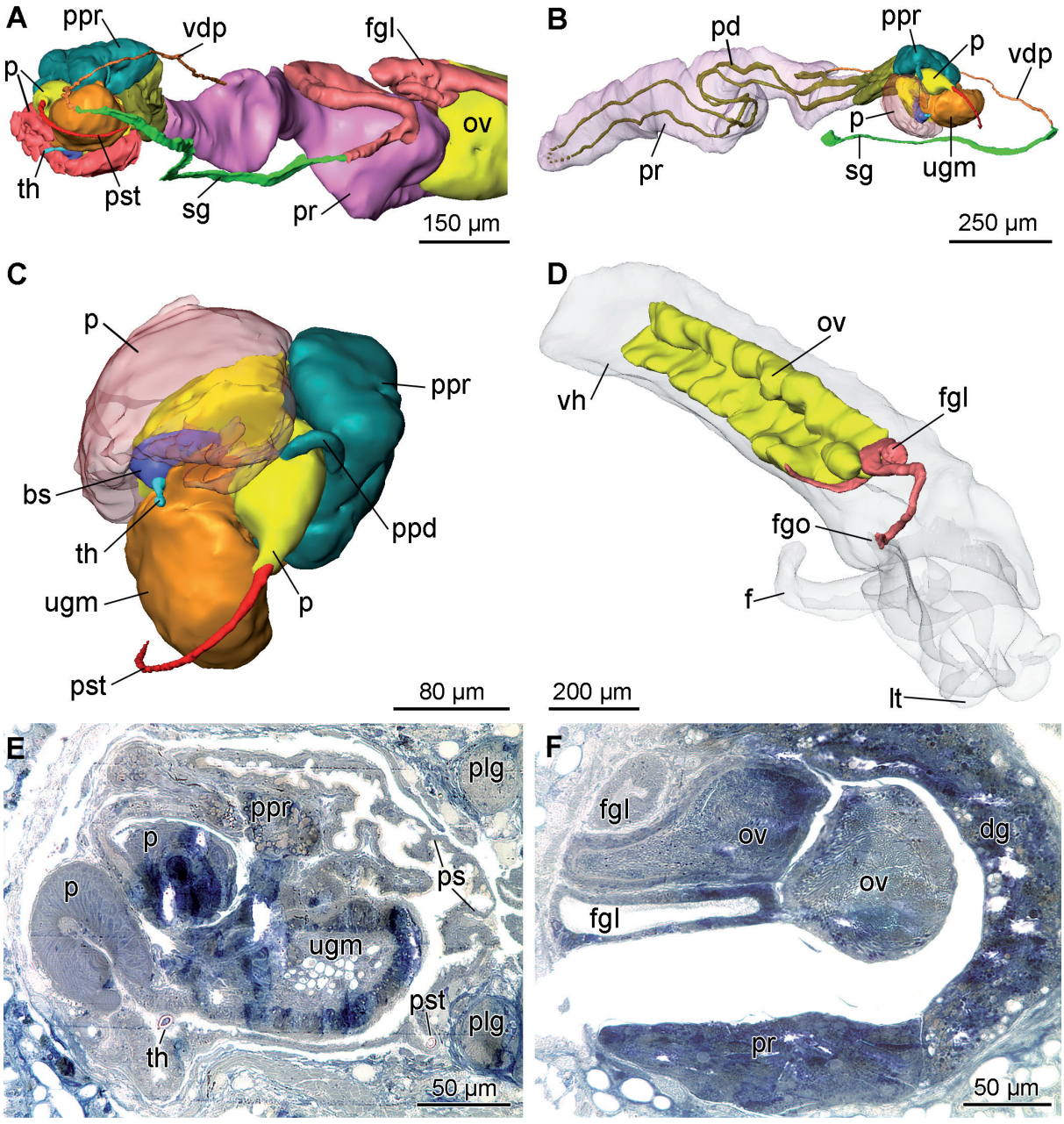

Figure 2:
3D-reconstruction and histological semithin sections of the male reproductive system of Hedylopsis ballantinei. A, Hermaphroditic reproductive system (ventral view); B, Male cephalic copulatory organs (right view); C, Penis and basal swelling with glands and armature (anterior view); D, Body with ovotestis and female glands (right anterolateral view); E, Penis, penial stylet and basal thorn; F, Ovotestis, prostate and female glands. Abbreviations: bs, basal swelling; dg, digestive gland; f, foot; fgl, female glands; fgo, female gonopore; lt, labial tentacle; ov, ovotestis; p, penis; pd, prostatic duct; plg, pleural ganglion; ppd, paraprostatic duct; ppr, paraprostate; pr, prostate; ps, penial sheath; pst, hollow penial stylet; sg, external sperm groove; th, solid thorn; ugm, unidentified glandular mass; vdp, posterior-leading vas deferens; vh, visceral hump.

(Figs. 1; 2A-C,E), is much smaller than the prostate and connected to the penis via the paraprostatic duct (Figs. 1; 2C). The latter enters the penis in the upper part and joins the ejaculatory duct. Together they discharge at the top of the penial papilla into a curved, hollow penial stylet (Figs. 1; 2A,C,E) of approx. 160 µm length. A muscular basal swelling with a solid thorn of approx. 40 µm (Figs. 1; 2A,C,E) is attached to the base of the penis. Near the muscular penis an additional, unidentified glandular mass (Figs. 1; 2B,C,E) with yet unknown function was detected. The bipartite penis and the unidentified glandular mass are surrounded by the thin-walled penial sheath (Figs. 1; 2E).

DISCUSSION

Among hedylopsacean acochlidians, *H. ballantinei* was exotic in lacking any detectable cephalic male reproductive organs. The presence of mature autosperm and egg cells in the hermaphroditic gonad of aphallic specimens led Sommerfeldt & Schrödl (2005) to assume that *H. ballantinei* is an aphallic hermaphrodite species rather than a sequential hermaphrodite as *Hedylopsis spiculifera*. However, our results show a specimen of *H. ballantinei* having complex male reproductive organs, while others do not possess any. We thus conclude that *H. ballantinei* is a sequential hermaphrodite with a male, phallic phase preceding a female, aphallic phase, just as it was described for *H. spiculifera* by Wawra (1989). The function, if any, of testis remainders in aphallic, early (?) female stages is unknown. All hedylopsacean species known to date thus have copulatory organs, in contrast to microhedylaceans that are all aphallic during their entire ontogeny (e.g. Neusser *et al*., 2009). The external sperm groove of *Hedylopsis* in the male phase is likely to transform into the ciliary field that was observed in the female phase of specimens of *H. ballantinei* by Sommerfeldt & Schrödl (2005); a function related to handling the egg mass can be inferred.

Sequential hermaphroditism with complete reduction of copulatory organs occur in some, but not all hedylopsacean clades, i.e. in the genus *Hedylopsis*, *Strubellia*, and possibly in *Tantulum* (Wawra, 1989; Neusser & Schrödl, 2007; Brenzinger *et al*., 2011). In contrast, *Pseudunela*, *Acochlidium* and *Palliohedyle* may be protandric but then simultaneous hermaphrodites during most of their ontogeny (Bücking, 1933; Haynes & Kenchington, 1991; Wawra, 1980; Neusser & Schrödl, 2009; Neusser *et al*., 2009). Mapping this feature on an acochlidian consensus tree (Neusser *et al*., 2009) reveals an ambiguous scenario. Possibly, hedylopsaceans are sequential hermaphrodites either ancestrally or evolved ontogenetic resorption of copulatory systems after the offshoot of *Tantulum* from the stemline, with re-evolution of simultaneous hermaphroditism in *Pseudunela* and the common ancestor of *Acochlidium* and *Palliohedyle*.

The anterior male copulatory system of *H. ballantinei* is quite complex, resembling that of its congener *H. spiculifera* in having an external sperm groove leading to a cephalic posterior-leading vas deferens with a well-developed prostate and a muscular penial papilla tipped with a hollow stylet. The dimensions of the penial stylets cannot be compared due to lacking data on the stylet length of *H. spiculifera*. Obviously, sperm is transferred to the mate via injection rather than via spermatophores as assumed originally for *H. ballantinei* (see Sommerfeldt & Schrödl, 2005). In absence of any allosperm receptacles (Sommerfeldt & Schrödl, 2005), hypodermal injection is likely. Imprecise sperm transfer into the body cavity was observed from *H. spiculifera* by Wawra (1989) who detected a penial stylet in the visceral sac of a mature female specimen. In both species the penis is bipartite having a basal swelling with a solid, cuticular thorn. The copulatory organs of *H. ballantinei* differ from those of *H. spiculifera* by the presence of a rather well-developed gland, a putative paraprostate, which connects through a duct to the ejaculatory duct within the penis.

Table 1:
Comparison of the male genital system within Hedylopsis. (? = no data available).

	Hedylopsis spiculifera (Kowalevsky, 1901)	*Hedylopsis ballantinei* Sommerfeldt & Schrödl, 2005	
Data source	Wawra (1989)	Sommerfeldt & Schrödl (2005)	present study
Type of hermaphroditism	sequential	simultaneous	sequential, protandric
Complex, cephalic male copulatory organs	penis with hollow stylet and basal thorn, prostate, penial gland of unknown function and homology	absent	large bipartite penis with apical hollow penial stylet (approx. 160 µm) and basal thorn (approx. 40 µm), voluminous prostate, potential paraprostate, plus accessory gland of unknown function and homology
Sperm transfer via	hypodermic injection	spermatophore	hypodermic injection
Function of ciliary field	?	for handling spermatophore	probably involved in egg mass deposition

Specimens of *H. spiculifera* have a small "penial gland" in a corresponding location that, however, opens separately at the base of the penial stylet. A comparison of the male reproductive features within *Hedylopsis* is given in Table 1.

Potentially homologous, more elaborate paraprostatic systems present in higher hedylopsaceans (Neusser & Schrödl, 2009; Neusser *et al*., 2009; Brenzinger *et al*., 2011) are separated from the ejaculatory duct and exit via own stylets on the tip of the basal swelling that is developed into a larger, so-called basal finger (according to Haase & Wawra, 1996). The copulatory system found in *H. ballantinei* thus represents a formerly unknown, intermediate condition in hedylopsaceans and is in line with the idea of progressively evolving more and more elaborate copulatory organs with various glands and injection systems (Neusser *et al*., 2009; Schrödl & Neusser, 2010).

CONCLUSIONS

1. *Hedylopsis ballantinei* is a sequential protandric hermaphrodite with sex change.
2. *H. ballantinei* has a large and complex cephalic copulatory organ with an apical hollow stylet, a solid thorn and two accessory gland systems, all of which completely disappear in the early female phase. Some male parts of the gonad, however, may still persist after the loss of the copulatory organs.
3. The presence of an apical penial stylet and a basal thorn resembles that of *Hedylopsis spiculifera;* but the arrangement of glands is unique.
4. As a phallic species transferring sperm via hypodermic impregnation and lacking any allosperm receptacles, *H. ballantinei* now much better resembles its Mediterranean/ eastern Atlantic sister species *H. spiculifera*, and fits well with evolutionary traits observed within hedylopsacean acochlidians.

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Molecular phylogeny and evolution of Acochlidia

Chapter 8. Integrating 3D microanatomy and molecules: natural history of the Pacific freshwater slug *Strubellia* Odhner, 1937 (Heterobranchia, Acochlidia) with description of a new species

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INTEGRATING 3D MICROANATOMY AND MOLECULES: NATURAL HISTORY OF THE PACIFIC FRESHWATER SLUG *STRUBELLIA* ODHNER, 1937 (HETEROBRANCHIA: ACOCHLIDIA), WITH DESCRIPTION OF A NEW SPECIES

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ABSTRACT

Forming a small group of mainly marine meiofaunal slugs, the Acochlidia have recently been separated from the traditional opisthobranch gastropods and placed within a mixed clade of pulmonates, Sacoglossa and Pyramidelloidea on the basis of molecular data. In the light of this new phylogenetic framework, we examined several populations of a comparatively giant *Strubellia* (Acochlidiidae *s. l.*) found in rivers of the Solomon Islands and Vanuatu, combining microanatomical and molecular methods (interactive three-dimensional models are given in the online version). Novel features include an extended set of nerves, a 'cephalic gland' of unknown function and an osphradium, all detected here for the first time in Acochlidia. The protandric genital system is characterized by three receptacles in the male phase, a possibly secondary open seminal groove and a complete reduction of the elaborate cephalic copulatory apparatus during ontogeny. Combined evidence from copulatory features and DNA sequences indicate a specific separation between the type species *S. paradoxa* (Strubell, 1892) from Ambon and the eastern Melanesian *Strubellia wawrai* n. sp. Live observations show the species to feed on the highly mineralized egg capsules of limnic Neritidae using a special piercing radula. Limnic Pacific acochlidians are suggested to be amphidromic, as are their prey organisms. A unique type of adhesive larva, observed in an *Acochlidium* species, indicates a possible dispersive stage in Acochlidiidae. Molecular phylogeny confirms the morphology-based placement of *Strubellia* as sister taxon to other Acochlidiidae.

INTRODUCTION

The Acochlidia consist of about 30 described species of heterobranch slugs that are characterized by a rather uniform external morphology, showing a freely projecting and uncurled visceral sac (giving the order its name) and one or two pairs of head appendages. For long time considered as one of the classic orders of the 'Opisthobranchia', morphological studies have repeatedly failed to place the taxon conclusively (e.g. Dayrat & Tillier, 2002; Wägele & Klussmann-Kolb, 2005) and molecular studies of Heterobranchia have cast further doubt on this classification (Klussmann-Kolb *et al.*, 2008). The most recent molecular studies with a direct focus on the group have consistently retrieved Acochlidia in a new monophylum comprising the Sacoglossa, Pyramidelloidea and the 'pulmonate' groups (all together called Panpulmonata), with acochlidians (including the recently described Aitengidae; Swennen & Buatip, 2009; Neusser *et al.*, 2011a) as sister group to Eupulmonata (Jörger *et al.*, 2010a). However, morphological synapomorphies of the panpulmonate group have not yet been identified.

Most acochlidian species are tiny inhabitants of worldwide marine interstitial sand habitats (Arnaud, Poizat & Salvini-Plawen, 1986). Internal phylogenetic relationships derived from morphology indicate a basal split into the completely meiofaunal Microhedylacea and partially meiofaunal Hedylopsacea, a relationship that has been confirmed by recent molecular approaches (Wawra, 1987; Jörger *et al.*, 2010a; Schrödl & Neusser, 2010). The hedylopsaceans also contain—uniquely among shell-less Gastropoda—two independent lineages that have colonized freshwater streams of tropical volcanic islands: the minute Caribbean *Tantulum elegans*

Table 1. Collection localities of *Strubellia wawrai* n. sp. on Guadalcanal, Solomon Islands (1–4) and Espiritu Santo, Vanuatu (5–8).

Number	Locality	Coordinates
1	Mataniko River, near Tavaruhu (3 km upstream)	S 9°27.377′, E 159°57.447′
2	Mataniko River, near Tavaruhu (3.5 km upstream)	S 9°27.517′, E 159°57.490′
3	Kohove River, Tanasawa bridge (at sea level)	S 9°25.333′, E 159°54.164′
4	Lungga River, near Mbetikama (6 km upstream)	S 9°26.916′, E 160°02.448′
5	Wounaouss River, Tapuntari Cascades (800 m upstream)	S 15°34.320′, E 167°00.159′
6	Puelapa River (Rowa River, 200 m upstream)	S 15°34.664′, E 167°01.902′
7	Wenoui River (350 m upstream)	S 15°34.826′, E 167°02.879′
8	Adson River (5 km upstream)	S 15°33.397′, E 166°58.112′

Rankin, 1979 (from St Vincent; see Neusser & Schrödl, 2007) and the radiation of comparatively giant Indo-Pacific Acochlidiidae (*sensu* Arnaud *et al.*, 1986). The latter family comprises the genera *Acochlidium* and *Strubellia*, the first acochlidians discovered by the Austrian naturalist A. Strubell (1892); the type species for both genera were described from a stream on the island of Ambon (Amboina) in the Molucca archipelago of Indonesia (Bücking, 1933; Küthe, 1935). Together with the enigmatic *Palliohedyle* Rankin, 1979, several acochlidiid species have been described from island streams of Indonesia, Palau, the Solomon Islands and Fiji (Bergh, 1895; Bayer & Fehlmann, 1960; Wawra, 1979, 1980; Haynes & Kenchington, 1991; own unpublished data).

Since the discovery of *Strubellia paradoxa* (Strubell, 1892) on Ambon (Küthe, 1935; original material redescribed by Brenzinger *et al.*, 2011), populations of *Strubellia* have been discovered some 3,500 km away on Guadalcanal, Solomon Islands (Starmühlner, 1976). This geographically separate population was described as the "rediscovery of *Strubellia paradoxa*" by Wawra (1974, 1988). Further examinations of island stream malacofauna showed the genus to occur even further south on Efate and Espiritu Santo Islands, both Vanuatu (Haynes, 2000; present study). In all locations, *Strubellia* is known to share its habitat with numerous limnic Neritidae and can be found hiding under calcareous rocks in brackish water from close to the river's mouth to as far as 5 km upstream. A fifth population is presently known only from a single juvenile collected on Sulawesi, Indonesia (present study).

The Indo-Pacific limnic species are generally large-bodied (crawling individuals are up to at least 4 cm long, compared to the millimetre-scale marine mesopsammic acochlidians); they should thus be ideal candidates in the search for shared morphological characters uniting Acochlidia and their panpulmonate relatives. They are also relatively easy to keep in an aquarium; observations on their biology are nevertheless scarce and mostly limited to descriptions of habitat. Life history is unknown except for the observation that *Acochlidium* veligers do not survive in fresh water (Haynes & Kenchington, 1991; own observations). Assuming an amphidromous lifestyle as in many other invertebrates found in similar habitats (see McDowall, 2007; Kano, 2009), the questions how metamorphosed individuals manage to return and maintain reproductive populations, or how they have colonized widely separated islands, remain unanswered.

We observed and examined numerous specimens from Guadalcanal and Vanuatu, using three-dimensional (3D) microanatomical reconstruction from serial semithin sections and scanning electron microscopy (SEM). Molecular data from *Strubellia* specimens from all five known localities and from closely related hedylopsacean taxa were compared in order to reveal their origin and relationships. Based on morphological and molecular evidence, the eastern Melanesian *Strubellia* is described as a new species and the evolution of the genus is discussed in the light of these new data.

MATERIAL AND METHODS

Collection and cultivation

About 90 specimens of *Strubellia wawrai* n. sp. were collected on northwestern Guadalcanal, Solomon Islands, in October 2007; further specimens from Espiritu Santo Island, Vanuatu, were collected during the Santo Expedition in September 2006 (see Table 1 for collection localities). All specimens were collected by hand in shallow water of freshwater streams flowing into the sea. The slugs were most commonly found aggregating in small groups on the underside of loose limestone rocks at the river's edge, up to 5 km upstream. In most places the rocks showed covering of algae; freshwater neritids were abundant in most places.

Living specimens were observed in petri dishes. Four specimens from Kohove River, Guadalcanal, were kept alive for several months in a small and shallow glass aquarium with a few flat rocks. Water was regularly replenished with tap water that had been allowed to stand for several days beforehand; the aquarium was ventilated by an aerating pump. Specimens were fed different types of fish feed, egg masses of *Physa* snails and egg capsules of freshwater neritids (*Neritina* cf. *natalensis*). The neritids were acquired from a zoo store and kept in a separate aquarium with added pieces of wood; chips of wood with freshly laid egg capsules were placed with the *Strubellia* specimens. Photographs of feeding specimens were made through a stereo microscope using a handheld digital camera.

For further studies, specimens were anaesthetized using menthol crystals sprinkled onto the water surface, fixed in 1.5% glutardialdehyde buffered with 0.2 M sodium cacodylate (pH 7.2) and stored in 75% ethanol for histological study or 96% ethanol for molecular analysis.

Serial sectioning and 3D reconstruction

Glutardialdehyde-fixed specimens were postfixed in 0.01 M cacodylate buffer/0.35 M sucrose (pH 7.2) and 1% osmium tetroxide. After decalcifying in 1% ascorbic acid, specimens were dehydrated in a graded acetone series and infiltrated overnight with Spurr's low-viscosity epoxy resin (Spurr, 1969) diluted with one part 100% acetone. Infiltrated specimens were placed on embedding grids, covered with pure epoxy resin and left to polymerize for 24 h at 60°C.

Serial sections of 1.5 μm were cut with Ralph glass knives (first half of series ZSM Mol-20071895) or a Histo Jumbo diamond knife (Diatome, Biel, Switzerland—all other series) with a Microm HM 360-rotation microtome (Zeiss, Germany) (Table 2). Serial sections were collected on cleaned microscopy slides, stained with methylene blue/azure II (Richardson, Jarett & Finke, 1960) and sealed with araldite. Slides were then mapped from 600-dpi greyscale scans; single sections were photographed through a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany) with mounted Spot CCD camera (Spot Insight, Diagnostic Instruments, Sterling Heights,

Table 2. Material used for morphological and phylogenetic analyses.

Species	Locality	Museum number of voucher and use of specimens			
Strubellia wawrai n. sp.	Solomons, loc. 1	ZSM Mol-*20071895* (used for 3D); *20071881*, *20071883*, *20071886*, 20071887, 20071890 (further serial sections)			
	Solomons, loc. 2	ZSM Mol-20071796 (entire lot used for SEM)			
	Solomons, loc. 3	ZSM Mol-*20071894* (used for 3D); 20071877, 20071880, *20071892* (further serial sections)			
	Vanuatu, loc. 5	ZSM Mol-20071105 (used for 3D)			
	Vanuatu, loc. 6	ZSM Mol-20071106 (used for 3D)			
		Museum number of voucher	DNA voucher DNA Bank	GenBank accession number	
				16S rRNA	COI
	Solomons, loc. 3	ZSM Mol-20080014	AB34404271	JF819728*	JF819756*
	Solomons, loc. 3	ZSM Mol-20080015	AB34404208	JF819729*	JF819757*
	Solomons, loc. 3	ZSM Mol-20080016	AB34404250	JF819730*	JF819758*
	Solomons, loc. 1	ZSM Mol-20080017	AB34404264	JF819731*	JF819759*
	Solomons, loc. 1	ZSM Mol-20080018	AB34404255	JF819732*	JF819760*
	Solomons, loc. 1	ZSM Mol-20080019	AB34404256	JF819733*	JF819761*
	Solomons, loc. 4	ZSM Mol-20071810	AB34404212	JF819734*	JF819762*
	Vanuatu, loc. 7	ZSM Mol-20071117	AB34404234	JF819735*	JF819763*
	Vanuatu, loc. 7	ZSM Mol-20080150	AB34404205	JF819736*	JF819764*
	Vanuatu, loc. 5	ZSM Mol-20080072	AB34404207	JF819737*	—
	Vanuatu, loc. 5	ZSM Mol-20080148	AB34404251	JF819738*	—
Strubellia paradoxa	Kemeri, Ambon, Indonesia	Berlin Moll 193943	AB35081823	JF819739*	—
	Watatiri, Ambon, Indonesia	Berlin Moll 193944	AB34858174	HQ168419	HQ168457
Strubellia sp.	Tambala River, Manado, Sulawesi, Indonesia	ZSM-Mol 20100339	AB35081762	JF819740*	JF819765*
Palliohedyle sp.	Tambala River, Manado, Sulawesi, Indonesia	ZSM-Mol 20100356	AB35081794	JF828040	JF828032
Acochlidium fijiense	Lami River, Viti Levu, Fiji	ZSM-Mol 20080063	AB34404244	HQ168420	HQ168458
Pseudunela espiritusanta	SE Espiritu Santo, Vanuatu	ZSM-Mol 20080117	AB34404289	JF819750	JF819775
Pseudunela marteli	Oyster Island, Vanuatu	ZSM-Mol 20080393	AB35081809	HQ168418	HQ168456
Hedylopsis ballantinei	'INMO' Reef, Dahab, Egypt, Red Sea	ZSM-Mol 20090244	AB34858170	HQ168416	HQ168454

The table lists the species names, collecting localities (number refers to Table 1), reference numbers of museum vouchers (ZSM, Bavarian State Collection of Zoology; Berlin, Museum of Natural History, Berlin), DNA vouchers deposited in the DNA Bank of the ZSM and GenBank accession numbers. Numbers in italics indicate designated paratypes; asterisks mark the sequences generated for the present study.

MI, USA). Series of photographs were downsized to *c.* 400 megabytes by conversion to 8-bit greyscale and a resolution of 800 × 600 pixels and then imported to AMIRA 4.1 software (TGS Europe, Mercury Computer Systems, Mérignac, France) for 3D reconstruction. Labeling of organ systems was done manually, with interpolation and surface-smoothing features applied to create 3D surfaces, in general following the method described by Ruthensteiner (2008). Reconstructions of four specimens are used herein: one 'male' from Vanuatu (every eighth section was photographed for the model, resulting in a virtual stack of 871 photos; Figs 4A; 9C–E), one 'female' from the Solomon Islands (693 photos, every 4th; Figs 4E; 9A, B, F) and two further specimens for the CNS (Solomon Islands: 439 photos, every section photographed, Fig. 4B, D, F; Vanuatu: 479 photos, every 2nd; Fig. 4C). All sections are deposited in the Mollusca Department, Bavarian State Collection of Zoology, Munich, Germany (see Table 2 for museum numbers).

Interactive 3D model

The interactive 3D models in the online PDF version were prepared according to Ruthensteiner & Heß (2008), although using the 3D tools of Deep Exploration v. 5.5 (Right Hemisphere EMEA, Germany) and Adobe Acrobat v. 9.0 Professional Extended (Adobe Systems GmbH, Germany) to create interactive models of the original Amira surface files. Separate surface files of each organ were exported into the former program, then grouped into a complex model and rendered. An interactive figure was then created by importing these rendered models as backdrops of Figure 4; different views of the organ systems were prefabricated to allow the reader rapidly to get a general idea of the models. Click on the interactive Figure 4A–D for models of the general anatomy and on Figure 4E, F for a more detailed model of the CNS.

Scanning electron microscopy

Several specimens were dissected and spicules, radulae and copulatory stylets were removed and cleared from tissue in diluted KOH or Proteinase K (20 µl in 180 µl ATL Tissue lysis buffer; Qiagen, Hilden, Germany; after Holznagel, 1998). The undissolved sheath of radulae was removed using tungsten minutien needles before flattening the radula. After rinsing with distilled water, samples were mounted on aluminum stubs with sticky carbon tabs, sputter coated with gold (120 s at 2.4 kV) and examined in a LEO 1430 VP scanning electron microscope (15 kV; 2×10^{-5} mbar).

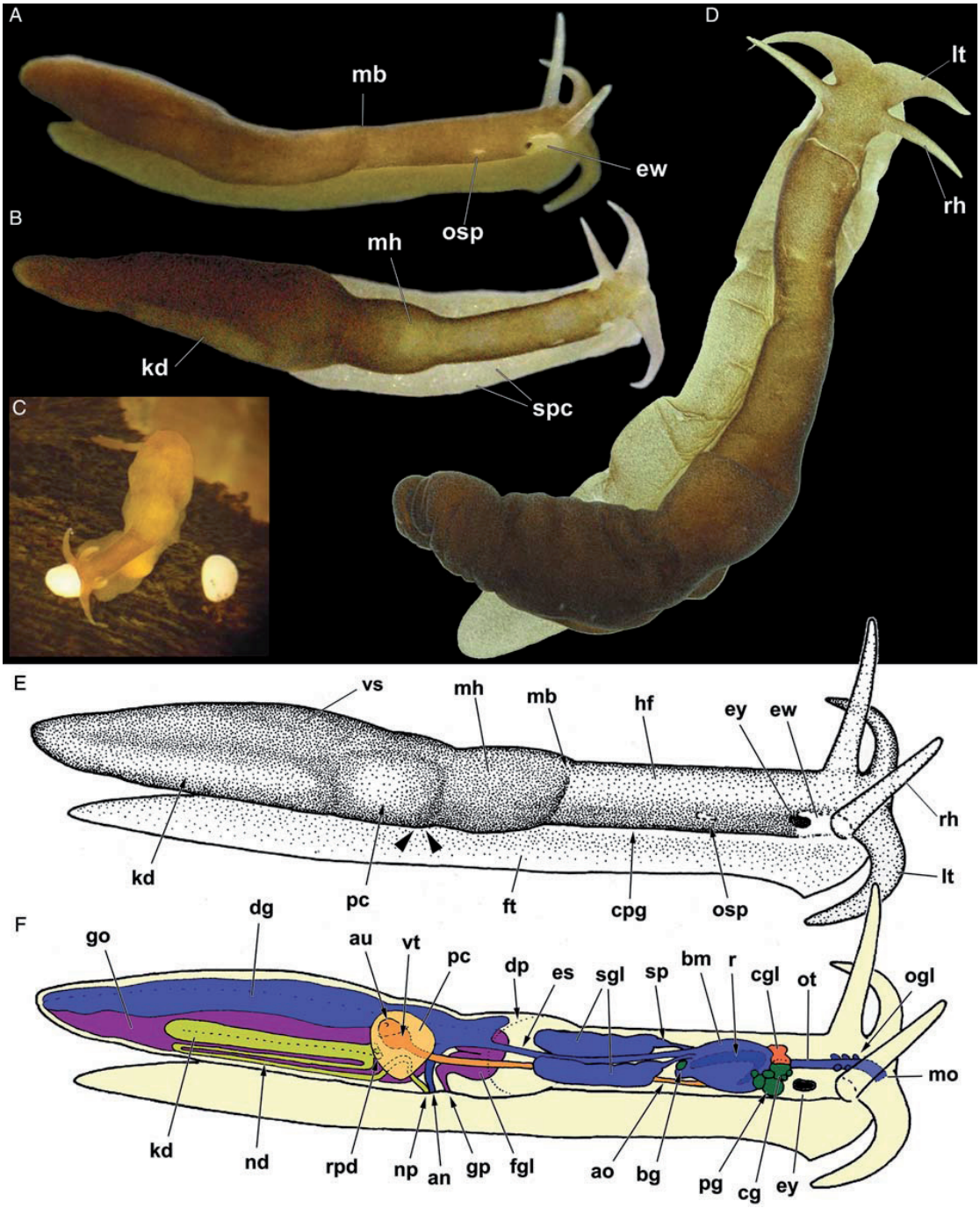

Figure 1. Live specimens and general schematic overview of the anatomy of *Strubellia wawrai* n. sp. **A–D.** External morphology of living specimens from Kohove River, Guadalcanal, Solomon Islands (**A–C**) and Tapuntari Cascades, Wounaouss River, Espiritu Santo, Vanuatu (**D**). **A.** Young specimen, *c.* 8 mm, right view. **B.** 20 mm specimen, dorsal view. **C.** Juvenile feeding on egg capsule of *Neritina* cf. *natalensis* attached to wood (experimental setting). **D.** Adult, at least 30 mm, dorsal view. **E.** Overview of external morphology, based on young specimen A, right view. **F.** Composite of internal anatomy, female phase. Abbreviations: an, anus; ao, aorta; au, auricle; bg, buccal ganglion; bm, buccal mass; cg, cerebral ganglion; cgl, "cephalic gland"; cpg, cephalopedal groove; dg, digestive gland; dp, diaphragm separating body cavities of head–foot complex and visceral sac; es, esophagus; ey, eye; ew, translucent patch over eye ('eye-window'); fgl, female gland mass; ft, foot; go, gonad; gp, genital pore; hf, head–foot complex; kd, kidney; lt, labial tentacle; mb, anterior border of mantle; mh, mantle 'hood'; mo, mouth opening; nd, nephroduct; np, nephropore; ogl, oral glands; osp, osphradium; ot, oral tube; pc, pericardium; pg, pedal ganglion; r, radula; rh, rhinophore; rpd, renopericardioduct funnel; sgl, salivary glands; sp, salivary pump; spc, spicule; vs, visceral sac; vt, ventricle. Arrowheads: position of nephropore/anus (left) and genital pore (right).

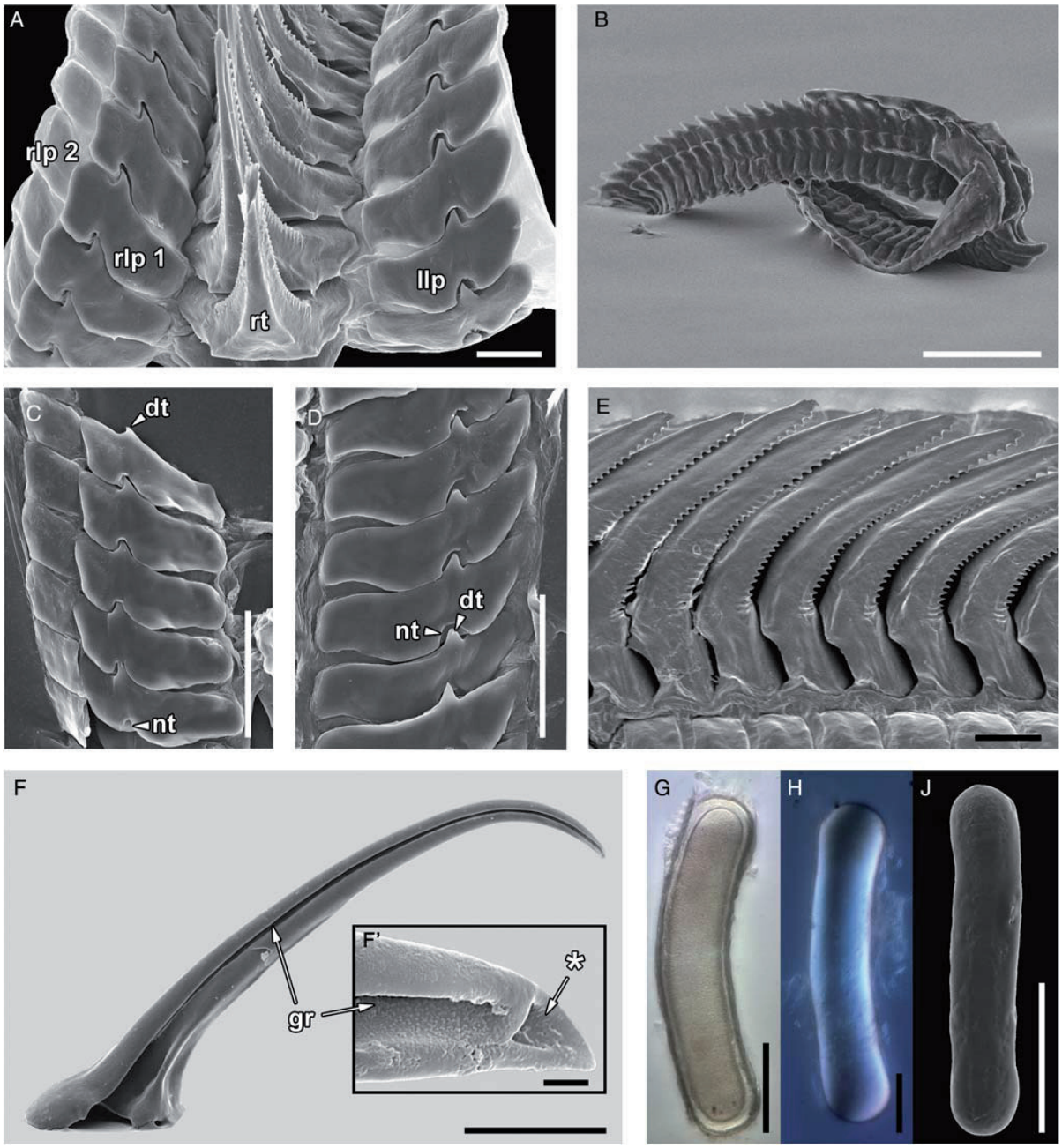

Figure 2. Microscopic views of radula (SEM), stylet of basal finger (SEM) and spicules surrounding the buccal mass (SEM, light microscopy) of *Strubellia wawrai* n. sp. **A, F, F′.** Vanuatu specimen; **others**: Solomon Islands. **A.** Functional part of radula. **B.** Complete hook-shaped radula. **C.** Right lateral teeth. **D.** Left lateral teeth. **E.** Rhachidian teeth, left view. **F.** Stylet of basal finger. **F′.** Detail of stylet tip. **G.** Spicule, phase contrast. **H.** Spicule, lateral illumination. **J.** Spicule, SEM. Abbreviations: dt, denticle; gr, groove; llp, left lateral plate; nt, notch; rlp 1 and 2, first and second right lateral plates; rt, rhachidian tooth; *, opening of hollow stylet. Scale bars: **A, C–E** = 20 μm; **B** = 100 μm; **F** = 150 μm; **F′** = 3 μm; **G, H, J** = 50 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

Molecular analysis

Genomic DNA was extracted from tissue samples of the foot or entire specimens using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. Two mitochondrial markers, partial 16S rRNA (400 bp) and cytochrome *c* oxidase subunit I (COI; 650 bp), respectively, were amplified using PCR (for PCR protocols and primers, see Table 3). PCR products were purified using ExoSapIT (USB, Affymetrix, Inc.); cycle sequencing and the sequencing reaction were performed by the sequencing service of the Department of Biology Genomic Service Unit (GSU) of the Ludwig-

Table 3. PCR protocols and primers used for the sequences generated within this study.

Gene	Primer	Sequence 5′–3′	Reference	PCR program
16S	16S-H	CGC CTG TTT ATC AAA AAC AT	Simon *et al.* (1994)	98°C 30 s (98°C 5 s, 48–55°C 5 s, 72°C 25 s) × 35–40,
	16S-R	CCG GTC TGA ACT CAG ATC ACG T	Simon *et al.* (1994)	72°C 60 s (Phire polymerase, New England Biolabs)
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer *et al.* (1994)	94°C 3 min (94°C 60 s, 45–48°C 60 s, 72°C 90 s) × 35–
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al.* (1994)	40, 72°C 3 min (Taq polymerase, Sigma)
	COI long r	TAA AGA AAG AAC ATA ATG AAA ATG	Stothard & Rollinson (1997)	

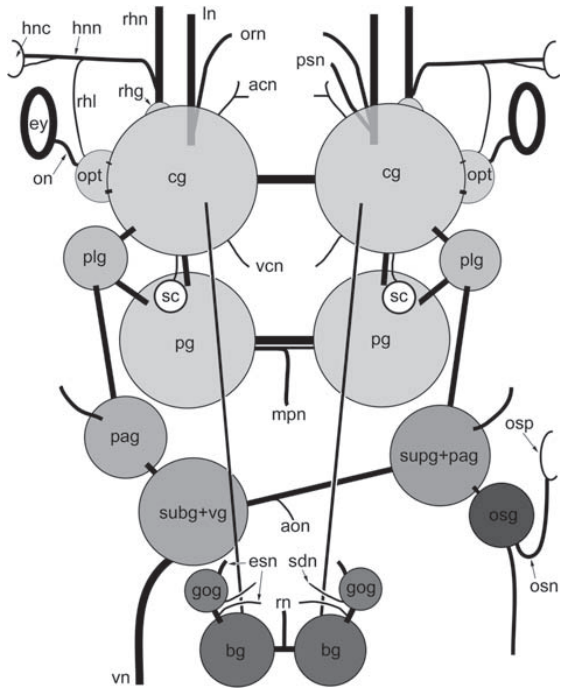

Figure 3. Schematic overview of the CNS (pedal nerves omitted for clarity) of *Strubellia wawrai* n. sp., dorsal view. Abbreviations: acn, anterior cerebral nerve; aon, aortic nerve; bg, buccal ganglion; cg, cerebral ganglion; esn, esophageal nerves; ey, eye; gog, gastroesophageal ganglion; hnc, Hancock's organ; hnn, Hancock's organ nerve; ln, labial tentacle nerve; mpn, median pedal nerve; on, optic nerve; opt, optical ganglion; orn, oral nerve; osg, osphradial ganglion; osn, osphradial nerve; osp, osphradium; pag, parietal ganglion; pg, pedal ganglion; plg, pleural ganglion; psn, penial sheath nerve; rhg, rhinophoral ganglion; rhl, rhinoporal looping nerve; rhn, rhinophoral nerve; rn, radular nerve; sc, statocyst; sdn, salivary duct nerve; subg, subintestinal ganglion; supg, supraintestinal ganglion; vcn, ventral cerebral nerve; vg, visceral ganglion; vn, visceral nerve. Not to scale.

Maximilians-University Munich, using Big Dye 3.1 kit and an ABI 3730 capillary sequencer. All fragments were sequenced on forward and reverse strand. DNA vouchers are stored at the DNAbank of the Bavarian State Collection of Zoology; sequences are deposited at GenBank (see Table 2 for accession numbers). Sequences were edited using Sequencer (Gene Codes Corporation). We applied a Blast search (Altschul *et al.*, 1990) on each sequence to check for potential contamination (http://blast.ncbi.nlm.nih.gov/Blast.cgi). MUSCLE v. 3.8.31 (Edgar, 2004) was used to create the alignments of each marker, subsequently the COI alignment was checked manually according to the translation into amino acids. Maximum-likelihood analyses of the concatenated dataset (in two partitions) were performed using RAxML v. 7.0.3 (Stamatakis, 2006) under the GTR + G model (selected for the concatenated dataset under the Akaike information criterion with jModeltest; Posada, 2008) and 1,000 bootstrap replicates were generated. Outgroups were chosen according to previous morphological and molecular hypotheses on acochlidian phylogeny (Jörger *et al.*, 2010a; Schrödl & Neusser, 2010) and retrieved from GenBank (Table 2). *Hedylopsis ballantinei* Sommerfeldt & Schrödl, 2005 was defined as outgroup.

For both markers, intra- and inter-specific variation was evaluated using Species Identifier, available from TaxonDNA (http://taxondna.sourceforge.net; Meier *et al.*, 2006) and used to cluster sequences based on pairwise distances (testing thresholds from 1 to 10%). Additionally, we calculated haplotype networks for both markers using TCS 1.21 (Clement, Posada & Crandall, 2000); the COI alignment was shortened, until all sequences had the same length; default settings (95% probability of parsimony) were used.

SYSTEMATIC DESCRIPTION

Heterobranchia *sensu* Haszprunar, 1985a
Panpulmonata Jörger *et al.*, 2010a
Acochlidia sensu Wawra, 1987
Hedylopsacea *sensu* Wawra, 1987
ACOCHLIDIIDAE *sensu* Arnaud *et al.*, 1986

Strubellia Odhner, 1937

Strubellia wawrai n. sp.

Strubellia paradoxa—Wawra, 1974: 8–10. Starmühlner, 1976: 473–656. Wawra, 1988: 163–172 (not *Acochlidium paradoxum* Strubell, 1892 = *Strubellia paradoxa*).
Strubellia sp. Haynes, 2000: 101–111.

Type material: Holotype: ZSM Mol-20100718; complete specimen stored in 75% ethanol; 7 mm preserved body length; collected in Mataniko River, Guadalcanal, Solomon Islands (locality 1, Table 1), 8/9 October 2007 by K. Jörger & Y. Kano. Paratypes: nine complete specimens stored in 75% ethanol (lot: ZSM Mol-20071797), same lot as the holotype; six serially sectioned specimens mounted on microscope slides [Mataniko River ZSM Mol-20071881, 20071883 (partial series), 20071886, 20071895; Kohove River: 20071892 (partial series), 20071894]; all paratypes collected 8/9 October 2007, together with holotype (Table 2).

Etymology: Named in honour of Erhard Wawra (1945–1994) for his pioneering work on the biology and systematics of Acochlidia and particularly the *Strubellia* of the Solomon Islands.

Interactive model: In addition to the 3D images (Figs 4, 9), see also the interactive 3D models of *Strubellia wawrai* n. sp. that can be accessed by clicking onto Figure 4A–D (general anatomy) and E, F (CNS) in the online PDF version of this article.

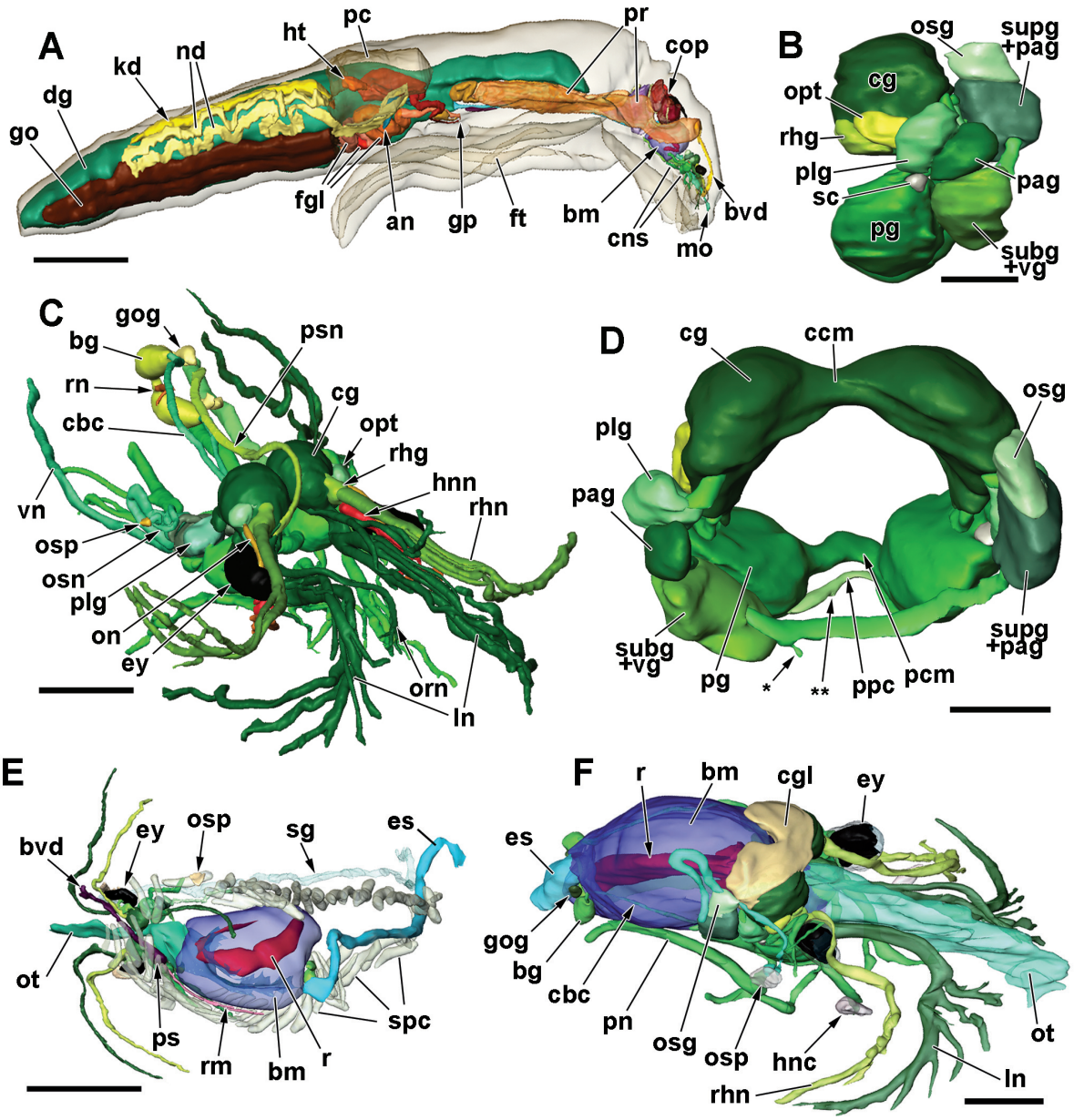

Figure 4. Three-dimensional reconstruction of general anatomy and CNS of *Strubellia wawrai* n. sp. from Vanuatu (**A, C**) and Solomon Islands (**B, D–F**). **A.** General anatomy, right view. **B.** Main ganglia, left view. **C.** CNS, anterior right view. **D.** Main ganglia, posterodorsal view. **E.** CNS with spicule grid and rudimentary penial sheath, dorsal view. **F.** CNS and buccal mass, anterior right view. Abbreviations: an, anus; bg, buccal ganglion; bm, buccal mass; bvd, posterior-leading vas deferens; cbc, cerebrobuccal connective; ccm, cerebral commissure; cg, cerebral ganglion; cgl, 'cephalic gland'; cns, central nervous system; cop, copulatory apparatus; dg, digestive gland; es, esophagus; ey, eye; fgl, nidamental glands; ft, foot; go, gonad; gog, gastroesophageal ganglion; gp, genital pore; hnc, Hancock's organ; hnn, Hancock's nerve; ht, heart; kd, kidney; ln, labial tentacle nerve; mo, mouth opening; nd, nephroduct; on, optic nerve; opt, optical ganglion; orn, oral nerve; osg, osphradial ganglion; osn, osphradial nerve; osp, osphradium; ot, oral tube; pag, parietal ganglion; pc, pericardium; pcm, pedal commissure; pg, pedal ganglion; plg, pleural ganglion; pn, pedal nerve; ppc, parapedal commissure; pr, prostate; ps, penial sheath; psn, penial sheath nerve; r, radula; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; rm, retractor muscle of penial sheath; rn, radular nerve; sc, statocyst; sg, sperm groove; spc, spicules; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve; asterisks: branching points of nerves. Scale bars: **A** = 2 mm; **B, D** = 100 μm; **C, F** = 200 μm; **E** = 500 μm. The interactive 3D models of *S. wawrai* n. sp. can be accessed by clicking onto **A**–**D** (general anatomy) and **E, F** (CNS) in the online PDF version of this article. Rotate model by dragging with left mouse button pressed; shift model: same action + ctrl (or dragging with left and right mouse buttons pressed); zoom: use mouse wheel. Select or deselect (or change transparency of) components in the model tree, switch between prefab views or change surface visualization (e.g. lighting, render mode, crop etc.). Interactive manipulation requires Adobe Reader 7 or higher.

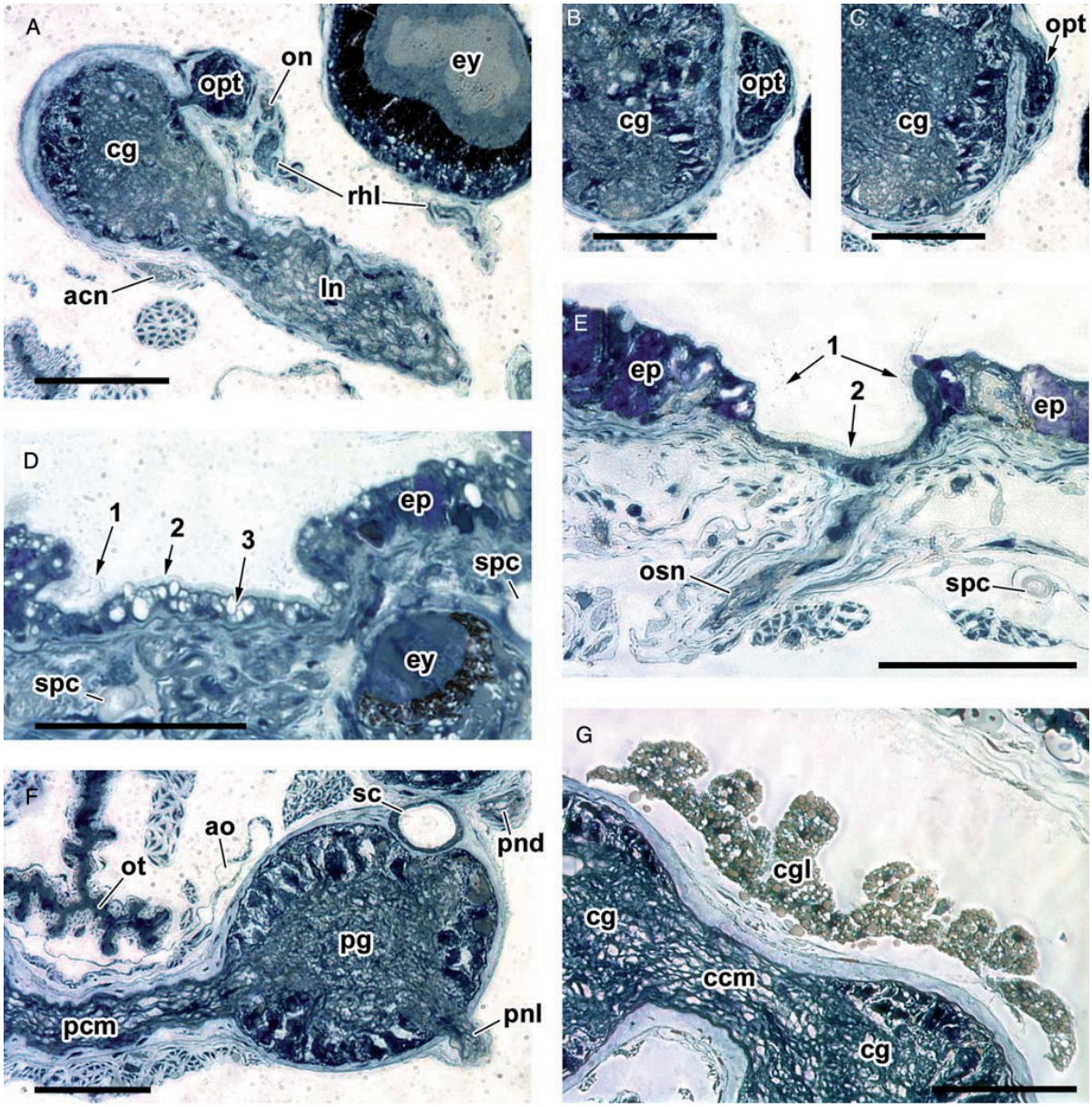

Figure 5. Semithin sections of the CNS and sensory organs (Solomon Islands specimens) of *Strubellia wawrai* n. sp. **A–C.** Cerebral ganglion and double cerebro-optic connectives. **D.** Hancock's organ. **E.** Osphradium. **F.** Pedal ganglion and statocyst. **G.** Cephalic gland dorsally to cerebral ganglia. Abbreviations: acn, anterior cerebral nerve; ao, aorta; ccm, cerebral commissure; cg, cerebral ganglion; cgl, cephalic gland; ep, epidermis; ey, eye; ln, labial tentacle nerve; on, optic nerve; opt, optic ganglion; osn, osphradial nerve; ot, otal tube; pcm, pedal commissure; pg, pedal ganglion; pnd, dorsal pedal nerve; pnl, lateral pedal nerve; rhl, rhinophoral looping nerve; sc, statocyst; spc, spicules; 1, multiciliated cells; 2, microvillous border; 3, vacuolate cells. All scale bars = 50 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

External morphology: External appearance is of a typical hedylopsacean acochlidian: elongate head–foot complex with two pairs of pointed head appendages; foot separated from body by longitudinal cephalopedal groove; uncoiled, shell-less visceral sac projecting freely behind foot, especially in fully grown specimens (Fig. 1). Epidermis appearing velvety smooth under stereo microscope; visceral sac slightly grainier. Body coloration orange to rusty brown in living specimens; foot, head appendages and translucent patch above the eye (Fig. 1A, E: ew) brighter, pale yellow; large specimens appear darker. Eyes visible externally as black dots, digestive gland as orange tube. Spicules in foot and head appendages visible as refracting bodies. Osphradium a keyhole-shaped brighter spot on right side of head–foot (Fig. 1A). Alcohol-fixed material light yellow-brown.

Crawling specimens usually between 6 and 12 mm, up to 20 mm (Solomon Islands specimens; Fig. 1B) or 35 mm long (Vanuatu; Fig. 1D). In younger specimens, visceral sac straight

and slightly shorter than foot with foot tip visible in dorsal view; larger specimens with visceral sac longer and appearing somewhat ragged and bent, with tip often pointing to right side. Pericardial space and beating of heart sometimes visible ('heart-bulb') at anterior right of visceral sac. Spacious haemocoel cavity into which head–foot can be retracted located between 'heart-bulb' and anterior mantle border (mantle 'hood' just anterior to position of diaphragm separating head–foot from visceral sac; Fig. 1). When disturbed, animals retract head–foot into this cavity and contract, visceral sac then curved, foot folded and tucked into concave side of visceral sac, head appendages project partially from underneath mantle 'hood'.

Front end of foot semicircular, edges slightly flaring; posterior end with pointed tip; foot sole wider than dorsal head–foot. Head appendages of about equal length; each appendage showing rod-like spicules sorted longitudinally. Labial tentacles slightly flattened in cross-section, held parallel to ground in crawling specimens, medially forming upper lip. Rhinophores round in cross-section, held erect.

General histology: Musculature consisting of blue staining fibres either spanning body cavity independently, or associated closely with organs. Body wall musculature a mesh of outer circular and inner longitudinal fibres. All parts of digestive system surrounded by longitudinal muscle fibres; circular fibres apparent only around salivary ducts. Transversal muscular diaphragm (Fig. 1F: dp) is punctured by aorta, oesophagus and visceral nerve, and is located at base of visceral sac, separating body cavities of head–foot and visceral sac (see mantle 'hood' above).

Connective tissue fills most spaces in foot (dense aggregates of cells), and flanks of head–foot and anterior visceral sac (less dense aggregates). Aggregates separated from central body cavity by thin longitudinal sheath of connective tissue; aggregates consisting of rather large, irregularly shaped cells staining homogeneously light blue, filled with darker grains and few yellow-stained vesicles.

Calcareous spicules embedded in most of connective tissue. In serial sections of decalcified animals only spicule cavity remaining, apparently enclosing spicule in living animals; chamber usually containing remnants of dissolved spicules visible as smaller, translucent body consisting of concentric layers of undissolved matter. Spicules themselves cylindrical, straight or slightly bent with slightly thickened, rounded tips, giving a dumb-bell-like shape. Spicules glassy transparent but strongly refracting (Fig. 2H) under light microscope. Spicule surface smooth (Fig. 2J), interior slightly yellowish to brown in phase-contrast due to organic material (Fig. 2G). Concentric lamination evident in broken spicules viewed with SEM. Spicules size differing greatly: very small and short spicules around oral opening and oesophagus; long and thin ones arranged longitudinally inside cephalic appendages, forming continuous row from labial tentacles into upper lip. Highest number of spicules (80–120 μm long) embedded in dense connective tissue of foot. Largest spicules (up to 300 μm) sorted in at least two parallel strips dorsolaterally of central nervous system (CNS) and buccal mass, forming a grid of interdigitating pieces ('cephalic spicule grid'; Fig. 4E).

Large anterior pedal gland located in anterior body cavity, ventrally to pharynx and CNS; distal part consists of paired lobes of thick glandular epithelium surrounding central lumen; cells filled with very small granules staining dark or light blue. Lobes of this gland merge anteriorly, connecting to short and wide epidermal duct leading into strongly ciliated, V-shaped longitudinal groove on dorsal side of anterior foot margin, ventrally to mouth opening. Further clusters of round foot glands located in entire foot ventrally to connective tissue, between dorsoventral muscle fibres; glands most numerous in anterior foot. Glandular cells containing many small dark blue grains, some yellow vesicles; cells open onto foot sole through very thin ducts.

Digestive system: Digestive system closely resembling that of other acochlidians: oral tube elongate, followed by bulbous pharynx containing hook-shaped radula, followed by paired salivary glands and oesophagus; direct connection into large digestive gland filling large part of visceral sac; intestine short with anal opening on right anterior side of visceral sac (Fig. 1E). No histologically detectable differentiated stomach. Ciliation of digestive tract detectable only in two places: at short strip in proximal part of oesophagus (where it projects from pharynx) and inside intestine.

Mouth opening a vertical slit located underneath upper lip; the following rather long oral tube surrounded by lateral clusters of oral glands opening into oral tube through thin ducts; oral gland cells staining dark blue (peripheral) or pale pink (closer to oral tube). Strong pair of pharynx protractors running from posterior end of oral tube to rhinophores; another pair running posteroventrally. Posterior end of oral tube is lined with thin cuticle. Pharynx egg-shaped, complex mass of muscle surrounding pharyngeal cavity; muscle surrounds posterior tip of radula (Fig. 4E, F). Pharynx protrusible anteriorly in slightly sucker-like fashion, surrounded by circular margin of epidermal tissue. Haemocoel lacunae present within pharynx, between fibres of pharyngeal muscles, supporting radula laterally and ventrally. Pharyngeal cavity lined with thin epithelium covered by equally thick, clear blue-staining cuticle (up to 15 μm thick); cavity with three longitudinal furrows, appearing as three-pointed star in cross-section (vertical furrow extending dorsally of radula). Radula originates in posterior tip of pharynx; ribbon originally still folded, embedded between large cells. Folded, upper branch runs anteriorly, emerging into pharyngeal cavity and spreading open. Radula then curves down, open part with old and worn teeth leading posteriorly again for about half length of upper branch (Fig. 2B). Radula asymmetric: single left lateral plate, prominent rhachidian tooth, two right lateral plates per row. Radular formula 40–60 × 1.1.2 (number of tooth rows in small Solomon Islands to large Vanuatu specimens, respectively). Rhachidian teeth with rectangular base and very slender, blade-like and pointed median cusp, its margins serrated (*c.* 30 or more small denticles per side) (Fig. 2A, E). Under light microscope, youngest rhachidian teeth appearing more translucent and with slimmer base than following teeth; median cusps of oldest rhachidian teeth generally worn down to stumps. First lateral plates of both sides flat and rectangular; each plate equipped with strong denticle on border to next younger plate, this border with notch into which denticle of other plate fits (Fig. 2A). Small and diamond-shaped second lateral plate on right side of radula; inner border straight, right first lateral plate appearing equally cut-off (Fig. 2A, C). Left lateral plates slightly wider than right ones (65 *vs* 50 μm in same row), outer border more rounded (Fig. 2A, D).

Salivary glands paired, connecting to posterior end of pharynx via thin salivary ducts. Each gland with two longitudinal lobes (resembling figure-of-eight in cross-section) formed by columnar cells densely filled with dark blue-stained granules. Central collecting duct strongly ciliated, showing bulbous salivary pump distal to glandular tissue (Fig. 1F: sp); spindle-shaped pumps and following salivary ducts surrounded by circular muscle fibres (contrasting with all other muscular linings of digestive system); salivary ducts opening anteriorly into lateral folds of pharyngeal cavity.

Oesophagus a simple tube projecting from posterodorsal side of pharynx; distal oesophagus widens gradually before

connecting to lumen of digestive gland. Digestive gland a long sac usually filling most of visceral sac (in mature specimens gonad more voluminous). Outer surface of gland with irregular transverse folds; inner surface highly enlarged by glandular epithelium with high columnar cells forming bundles projecting into lumen. Epithelial cells filled with numerous small blue-stained vesicles; large, spherical, yellow-stained vacuoles in an apical position make up large part of glandular mass (Fig. 7A, C). Intestine rather short and thick, emerging from digestive gland dextrolaterally to distal oesophagus. Inner surface of intestine folded longitudinally, strongly ciliated. Intestine gradually thinning towards anal opening; opening hard to detect in most specimens but very close to renal pore, both openings sometimes forming an invaginated and ciliated common cavity (possibly an artifact due to fixation).

Central nervous system—cerebral nerve ring: CNS euthyneurous, slightly epiathroid (i.e. pleural ganglia closer to cerebral than to pedal ganglia), following general acochlidian bauplan (Fig. 3). Prepharyngeal nerve ring consisting of paired cerebral, pedal and pleural ganglia; three ganglia on visceral nerve cord plus osphradial ganglion; paired buccal ganglia posterior to pharynx. Further elements: paired optic and rhinophoral ganglia (on anteroventral sides of cerebral ganglia), paired gastro-oesophageal ganglia dorsally on each buccal ganglion. Serial sections reveal numerous nerves (Figs 3, 4).

Cerebral ganglia largest ganglia, largely spherical; cerebral commissure strong (Figs 4D, 5G). Cerebropleural connective slightly shorter than cerebropedal one; static nerve very thin, emerging close to base of cerebropleural connective and running parallel to it to paired statocysts. Statocysts embedded in top of each pedal ganglion. Cerebrobuccal connectives thin, very long, running posteriorly within pharyngeal musculature laterally to dorsal branch of radula (Fig. 4F).

Labiotentacular nerves very thick (diameter *c.* 50 μm), emerging medioventrally from each cerebral ganglion; nerve splits early into thinner oral branch (running to upper lip) and thick part (to tip of labial tentacles, with thinner branches repeatedly running to anterior side of tentacles; Fig. 4C, F). Right labial nerve of some specimens with further branch extending posterodorsally, innervating penial sheath (Fig. 4C: psn).

Rhinophoral ganglion located at anteroventral part of cerebral ganglion between labiotentacular nerve and optic ganglion (Fig. 4B). Rhinophoral ganglion elongate and pear-shaped; thicker portion containing few peripheral cell bodies and connecting to cerebral ganglion by short connective, thinner part running smoothly into rhinophoral nerve. Rhinophoral nerve splitting into three branches close to its origin: thickest part continues into rhinophores (without much further branching); second, thinner part innervates Hancock's organs posterior to rhinophoral bases; third (thinnest) branch looping backwards and apparently connecting to anteroventral side of optic ganglion (Fig. 3: rhl).

Optic ganglion hemispherical, attached to cerebral ganglion laterally but separated by independent layer of connective tissue (Fig. 5B). Double, very short cerebro-optic connectives, posterior one stronger (Fig. 5A, C); third connective detected in single specimen. Optic nerve thin, rather long, joining to posteroventral portion of eye; thin and looping second nerve connecting to Hancock's organ's branch of rhinophoral nerve (see above).

Two further cerebral nerves detectable: (1) thin nerve leaving cerebral ganglion medially (Figs 3, 5A: acn), running anteroventrally along paired cephalic blood vessels before splitting into branches running towards rhinophores and to the mouth opening; (2) thin nerve emerging from posteroventral side of cerebral ganglion (Fig. 3: vcn), running into muscular lining of cephalic blood vessels.

Mass of loosely aggregated and apparently glandular cells in body cavity above cerebral ganglia and cerebral commissure ('cephalic gland'); containing numerous vacuoles staining light yellow. Gland mass without detectable connection to ganglia except for some thin fibers (connective tissue?); symmetric lobes extending slightly down sides of cerebral ganglia (Figs 4F, 5G).

Pedal ganglia spherical, only slightly smaller than cerebral ganglia; joined by thick pedal commissure (Fig. 5F) and thinner, longer parapedal commissure; very thin nerve splitting off parapedal commissure just left of midline (Fig. 4D), running to median part of foot sole and anterior pedal gland.

Six further pairs of pedal nerves detected, all running to body flanks: anteroventral, ventrolateral, posteroventral and posterodorsal nerves rather thick and running along body sides in posterior direction (except for first one); additional thin antero- and posterodorsal nerves running to sides, the former one apparently joining to anteroventral pedal nerve close to eye.

Central nervous system—visceral loop and buccal ganglia: Visceral cord with three medium-sized to large ganglia, connecting beneath anterior part of pharynx (Fig. 4B, D; nomenclature after Haszprunar, 1985a; Sommerfeldt & Schrödl, 2005): (1) left parietal ganglion (small, thin nerve running to left body side); (2) fused subintestinal/visceral ganglion (large, left of midline; giant nerve cells and very thick visceral nerve running posteriorly); (3) fused supraintestinal/right parietal ganglion (medium sized, thin nerve running to right body side). Latter ganglion with osphradial ganglion (small, cap-shaped) on posterodorsal side (Fig. 5D), both ganglia enclosed by common sheath of connective tissue. Osphradial ganglion with two nerves, one looping upwards first before running posteriorly; second: osphradial nerve innervating osphradium on anterior right body side (Fig. 4F). Ganglia on visceral nerve cord joined by short to very short connectives, only ganglia (2) and (3) with long connective passing obliquely between pharynx and aorta; thin nerve emerging from left third of long connective running downward into musculature of aorta (Fig. 4D: asterisk).

Visceral nerve strongest nerve posterior to CNS (diameter *c.* 25 μm) and running posteriorly into visceral sac, slightly left of midline (Fig. 4C: vn); nerve identifiable by surrounding longitudinal muscle fibres throughout entire length; nerve passes through diaphragm close to aorta and oesophagus.

Buccal ganglia paired, medium-sized, situated on posterodorsal side of pharynx at emerging point of oesophagus. Buccal commissure short, running ventrally to oesophagus; thin, apparent radular nerve emerging from middle of commissure, leading forward into muscular mass of pharynx (Fig. 4C, F).

Gastro-oesophageal ganglia (small, bean-shaped) on top of each buccal ganglion, connected by short vertical connective; thin oesophageal nerve from upper part of connective leading medially into muscular sheath of oesophagus; another thin nerve running from base of each gastro-oesophageal ganglion into sheath surrounding salivary ducts (Fig. 3: esn, sdn).

Sensory organs: Eyes located dorsolaterally to slightly anteriorly to cerebral ganglia, underneath translucent patch of epidermis visible in living animals (Fig. 1A, B); eyes bean-shaped, *c.* 130 μm long, facing anterolaterally (Fig. 4C, F), surrounded by thin layer of connective tissue; innervation by thin optic nerves. Prismatic (sensory?) cells with distinct nuclei form cup-shaped outer layer of eye, followed by layer of grainy black pigment; grey-blue staining irregular band (possibly sensory microvilli) between pigment layer and otherwise acellular and

light blue-staining lens (Fig. 5A). Lens covered distally by cornea consisting of single layer of flat cells.

Statocysts paired, hollow spheres (diameter 25 μm) with flat, slightly ciliated cells forming outer wall (Fig. 5F); remnants of layered single statolith inside fluid-filled cavity visible in some sections. Statocysts embedded in dorsal part of each pedal ganglion (Fig. 4B); static nerve originating in cerebral ganglia.

Hancock's organs posterior to base of each rhinophore, located inside zone of brighter epidermis over eyes; exact dimensions of organs detectable only in serial sections, there appearing as shallow patches of thin epidermis, resembling osphradium in histology (dense microvillous border, several multiciliated cells), differing in presence of rounded, apparently glandular cells with clear lumen (Fig. 5D); innervation by lateral branches of rhinophoral nerves.

Osphradium a small pit on right body side, visible in living animals as keyhole-shaped spot paler than surrounding epidermis (Fig. 1A); in serial sections a pit about 40 μm deep and 60 μm long, lined with very thin epidermis showing strong microvillous border (Figs 4F, 5E); several cells with bundles of cilia *c*. 25 μm long found inside pit but mainly close to rim; osphradial nerve emerging from osphradial ganglion, splitting up distally.

Multiciliated cells similar to putative sensory cells in Hancock's organs and osphradium found interspersed within normal epidermal cells on labial tentacles and rhinophores.

Circulatory and excretory systems: Pericardial complex located in anterior right of visceral sac, with externally visible 'heart bulb' indicated by beating of heart in living animals (Fig. 1B). Pericardial complex formed by spacious pericardium enveloping two-chambered heart; elongate kidney and looping nephroduct extending posteriorly along right side of visceral sac (Figs 4A, 6). Renal pore situated on anteroventral right, close to anal opening. Aorta extending into head–foot, passing between pharynx and pedal commissure, distally dividing into paired vessels (Figs 5F, 7); vessels terminating laterally of oral tube. In large Vanuatu specimens, second branch of aorta detectable, running posteriorly into visceral sac.

Pericardium formed by very thin wall breached in three places: (1) dorsally at venous connection between haemocoel and atrial lumen; (2) anteroventrally, where aorta extends from ventricle into body; (3) posterolaterally to heart where ciliated renopericardioduct drains off into kidney (Fig. 6). Pericardial lumen free of cells, except for few vacuolated cells at anteroventral wall which appears to wrap around distal part of nephroduct.

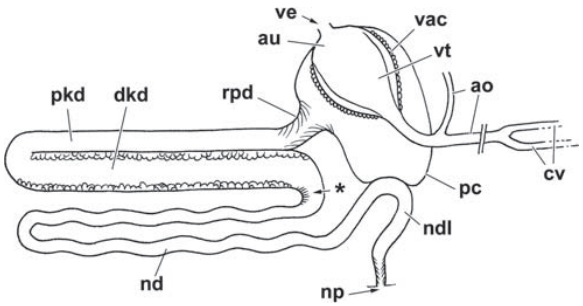

Figure 6. Schematic overview of the circulatory and excretory systems of *Strubellia wawrai* n. sp., right view. Abbreviations: ao, aorta; au, auricle; cv, paired cephalic vessels; dkd, distal kidney lumen; nd, nephroduct; ndl, nephroduct loop; np, nephropore; pc, pericardium; pkd, proximal kidney lumen; rpd, renopericardioduct; vac, vacuolated epicardium on ventricular wall; ve, venous opening; vt, ventricle; *, ciliated intersection between kidney and nephroduct. Not to scale.

Heart consisting of thin-walled auricle and muscular, ovoid ventricle. Haemocoel on right side of visceral sac connected to auricle by small hole (diameter 10 μm); opening visible only in single series where auricle clearly distinguishable from ventricle (Fig. 7A); auricle collapsed in most other cases. Ventricle continuous with auricle in its wall, ovoid form appearing more constant; ventricular wall much thicker, formed by mesh of striated muscle fibres staining blue-grey, some fibres appearing to cross ventricular lumen, forming muscular bridges (Fig. 7B).

Inside of ventricular wall covered with irregular cells, some staining darker blue or with yellow-stained vacuole; conspicuous large cells embedded in former layer and interspersed freely in the ventricular lumen: cells elongate and ovoid, showing a central body stained light grey, with concentric layers somewhat resembling a spicule.

Outer wall of ventricle covered with irregularly bordered, conspicuous lining at least as thick as muscular layer of wall. Epicardial lining consisting of vacuolate cells staining light blue to grey, with flat nuclei sorted apically staining slightly darker (Fig. 7E).

Tip of ventricle continuing into thick aorta, wall consisting of longitudinal muscle fibres, internal surface smooth. Aorta leaving pericardium on medioventral side, running anteriorly and passing through diaphragm close to oesophagus and visceral nerve, splitting into paired vessels formed only by strips of muscle fibres and membranous wall ventrally to cerebral nerve ring; cephalic vessels spacious, running parallel to oral tube (Figs 5F, 6), terminating close to mouth.

Excretory system consisting of short but well-developed renopericardioduct, elongate kidney and long and looping nephroduct. Renopericardioduct longitudinally folded, connecting to pericardium via funnel-shaped opening containing conspicuous ciliary flame; cuboidal lining with bundles of strong cilia projecting into pericardium and renopericardial duct (Fig. 7C, E), leading into kidney.

Kidney elongate, extending along two thirds of visceral sac; longitudinal interior wall separating lumen into hairpin-like loop connected only at kidney's posterior end (Figs 6, 9A, B): proximal part of lumen (running front to back) lying more ventrally, lined with regular epithelium with dense microvillous border (Fig. 7C, F); distal part of kidney lumen (running back to front) lying dorsally, more voluminous and lined with epithelium with shorter microvillous border, conspicuous unstained vacuoles giving wall spongy appearance (Fig. 7D) and accounting for much of kidney's volume. Connection to nephroduct through constriction of only about 3 μm diameter (in direct proximity to the renopericardioduct funnel), followed by short patch of dense ciliation (Fig. 7C: triple asterisk). Undulating nephroduct running posterior to tip of kidney and looping forward again; nephroduct interconnected by single muscle fibres in at least one place; lined with smooth epithelium staining light blue, with interspersed yellow-stained vesicles and a slight microvillous border (Fig. 7G). Distal loop of nephroduct differing slightly in histology (epithelium staining darker, showing fewer yellow vesicles but possibly colorless, irregular vacuoles), arching upward before running downward again towards nephropore (Fig. 9A, B); appearing to be closely associated with fold of pericardium.

Nephropore formed by ciliated and invaginated part of epidermis, situated next to anal opening or inside invaginated cloaca (artifact?), on dextroventral anterior visceral sac.

Genital system: Presence of allosperm receptacles in males, and females with rudimentary 'male' features indicate protandric hermaphroditism (as in *S. paradoxa* from Ambon). Examined specimens from Solomon Islands only juveniles and two functional 'females' (one with vestigial bursa copulatrix and penial sheath;

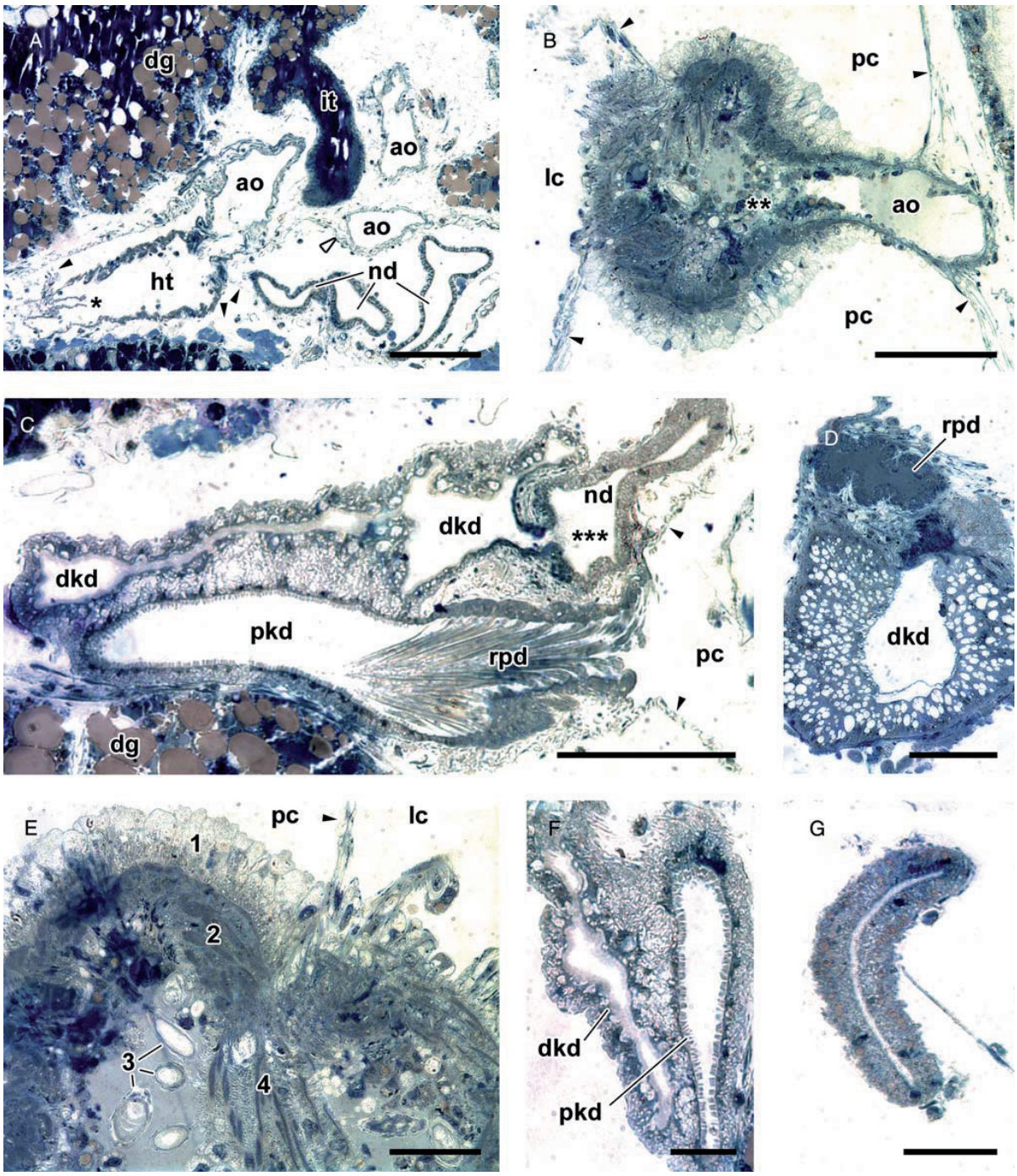

Figure 7. Semithin sections of the circulatory and excretory systems of *Strubellia wawrai* n. sp. (Solomon Islands specimens). **A.** Heart, longitudinal section. **B.** Pericardium and heart, cross-section. **C.** Anterior portion of excretory system, longitudinal section. **D.** Anterior portion of excretory system, cross-section. **E.** Wall of ventricle, cross-section. **F.** Proximal and distal kidney lumina, cross-section. **G.** Nephroduct, suspended by muscle fiber, cross-section. Abbreviations: ao, aorta; dg, digestive gland; dkd, distal kidney lumen; ht, heart; it, intestine; lc, hemocoel lacunae dorsally to pericardium; nd, nephroduct; pc, lumen of pericardium; pkd, proximal kidney lumen; rpd, renopericardioduct; black arrowheads: wall of pericardium; white arrowhead: peritoneal membrane; *, venous opening of heart to hemocoel lacunae; **, loose cells inside heart; ***, ciliated intersection between kidney and nephroduct; 1, vacuolate epicardium; 2, muscular wall of ventricle; 3, cells containing spicule-like body; 4, muscle fibers spanning ventricle. Scale bars: **A–C** = 100 μm; **D, E** = 50 μm; **F, G** = 25 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

Figs 4E, 9F); Vanuatu specimens containing one juvenile and one female (gonad filled with oocytes, nidamental glands developed) but with apparently functional cephalic copulatory apparatus and two allosperm receptacles (Figs 4A, 9C, D, E).

Posterior genital system consisting of acinar gonad, proximal receptaculum seminis filled with sorted spermatozoa and glandular gonoduct leading to genital opening on anterior right of visceral sac. Ampulla thin-walled, wide; detected only in single specimen. Gonad consisting of numerous almost spherical acini, filling much of visceral sac in functionally female specimens. Each acinus formed by thin epithelial wall, filled with large spherical oocytes containing high numbers of vesicles staining brilliantly blue, with colorless vesicles filling gaps in between; acini connected to gonoduct by thin ducts (Fig. 8A). Collecting gonoduct surrounded by muscle fibres but collapsed in both specimens; strong ciliation apparent; following last acinus a very short piece of gonoduct from which receptaculum seminis (thick-walled and blind-ending sac) emerges laterally. Receptacle lined with simple blue-staining epithelium forming an undulated inner wall; numerous spermatozoa are embedded with their heads into wall. Heads of spermatozoa visible only at high magnifications as stronger refracting bodies; head short, not screw-shaped, diameter about 1 μm; flagella forming pink-stained, dense and streaked mass inside receptacle (Fig. 10B: arrowheads and asterisk).

Following receptaculum seminis another short piece of gonoduct, leading into female gland mass. Glandular mass tubular throughout, forming several stout loops in anterior visceral sac; strongly stained, columnar glandular cells surround lumen only from one side (Fig. 10A); lumen a longitudinal fold projecting in between glandular cells. Glandular cells up to almost 100 μm high, filled with granular secretions. Three differently staining zones along glandular gonoduct: (1) proximal part staining dark blue; (2) distal part blue with strong pinkish tone; (3) part in between appearing blue with slightly greenish hue (Fig. 9D, F). Distal part of glandular epithelium becomes thinner with diameter of strongly ciliated gonoduct lumen appearing to increase before opening to outside through genital pore.

Single female Solomon Island specimen with vestigial bursa copulatrix consisting of very thin duct (10 μm diameter; emerging from gonoduct close to genital opening) and almost spherical terminal bulb close to upper intestine (Fig. 9F); bulb stained very dark blue inside. Same individual with distal gonoduct containing several oval bodies with pink-stained and grainy vesicle and fully developed ciliated sperm groove running from genital opening to base of right rhinophore. Thin tube entering body and running posteriorly from anterior end of sperm groove: posterior-leading vas deferens passing cerebral commissure dorsally and terminating in elongate blind sac (an empty and reduced penial sheath); reduced, thread-like penial retractor muscle extending posteriorly from sac, ending freely in body cavity (Fig. 4E).

Cephalic male copulatory organs: One Vanuatu specimen with elaborate male and female features: external sperm groove between female genital opening and base of right rhinophore, connecting to fully developed male copulatory organs surrounded by penial sheath at left of pharynx. Copulatory organs consisting of muscular basal finger, considerably smaller penis and their associated paraprostatic and prostatic glandular systems, respectively (Figs 4A, 8B).

Posterior-leading vas deferens connected to voluminous, tubular prostate gland; prostate continuing into long and curled ejaculatory duct, entering muscular penis at its base; ejaculatory duct opening to exterior through penial papilla at tip of penis. Solid spine of *c*. 150 μm width situated next to penial papilla (Fig. 9E). Blind ending glandular paraprostate a longer and thinner tube than prostate, strongly coiled (Fig. 9C: ppr). Paraprostatic duct emerging from paraprostate and connecting to muscular basal finger, entering basal finger approximately in middle of curved muscle; duct opening apically via curved hollow stylet of about 750 μm length. Stylet with cuticular groove running along its side (Figs 2F, 10D–H). Penis and basal finger muscles interconnected at their base; both structures surrounded by thin-walled penial sheath meeting posterior-leading vas deferens before opening to exterior at base of right rhinophore.

Behaviour and feeding: Living specimens collected by hand under rocks in shallow water at sides of streams. Aggregations of up to 25 individuals found under single calcareous rocks, hidden in grooves and pits of undersurfaces. Exposure to light causes animals to move; specimens kept in a Petri dish moved around without pause until hiding place was presented. On smooth surfaces, movement was fast, about 7 mm/s, with head moving from left to right, labial tentacles held parallel to ground. Movement appeared to be caused by ciliary motion (visible in animals crawling upside down at water surface: fine particles on water surface were quickly drawn away from front margin of foot) and supported by clear mucus as observable in specimens suspended by thread of mucus from water surface.

Three small individuals (probably juveniles) were cultivated in a small aquarium for about 5 months. When supplied with calcareous egg capsules of freshwater neritids *Strubellia* individuals were observed to aggregate on the egg capsules after a few minutes. Other types of food (fish feed, algae tabs, gelatinous egg masses of *Physa* sp.) did not lead to any reaction. Individuals remained on egg capsule with anterior border of foot and mouth pressed onto capsule's surface, head appearing slightly contracted (head appendages bent backwards, eyes not visible; Fig. 1C). Slow peristaltic dilatations of entire visceral sac observed during this apparent feeding posture, accompanied by slow but strong pumping motions of heart. Each feeding period up to 15 min; between two and three egg capsules fed on per individual. Some egg capsules fed on by more than one individual, others were ignored. Continuous supply of neritid eggs over longer period of time proved difficult; specimens shrank during time in aquarium.

Molecular phylogeny: The RAxMC-tree based on 16S rRNA and COI genes recovers the monophyletic genus *Strubellia* (bootstrap support, BS = 100) as sister taxon to the genera *Acochlidium* and *Palliohedyle* (Fig. 11), all three genera forming the large-bodied and limnic family Acochlidiidae (*sensu* Arnaud *et al*., 1986). Sampling of 13 *Strubellia* individuals reveals three clades: a basal and yet undescribed branch from Sulawesi (known only from single individual) as sister taxon to a clade formed of *S. paradoxa* from Ambon (BS = 100) and a clade consisting of all sampled individuals from Solomon Islands and Vanuatu (BS = 96). Specimens from Vanuatu are nested within populations from the Solomons.

Statistical parsimony analyses generate two independent haplotype networks (not shown) for partial 16S rRNA: *S. paradoxa* and a network uniting *S. wawrai* n. sp. populations from Solomons and Vanuatu (no 16S rRNA sequence was available for *Strubellia* from Sulawesi). Four independent networks were generated based on partial COI (reduced to 571 bp, to analyse sequences of same length): *S. paradoxa*, *Strubellia* sp. (Sulawesi), *S. wawrai* n. sp. from Solomons, and from Vanuatu.

Intraspecific variation is generally very low: in 16S rRNA (438 bp) 0.0% in *S. paradoxa* ($n = 2$), 0.68–0.91% in *S. wawrai* n. sp. from Solomons ($n = 7$) and 0.45–0.68% in *S. wawrai* n. sp. from Vanuatu ($n = 4$). Uniting both populations of *S. wawrai* n. sp. ($n = 11$), intraspecific variation ranges from 0.45 to 1.14% in 16S rRNA. Lowest interspecific variation in 16S rRNA between *S. paradoxa* and *S. wawrai* n. sp. is 4.1%; a higher selected

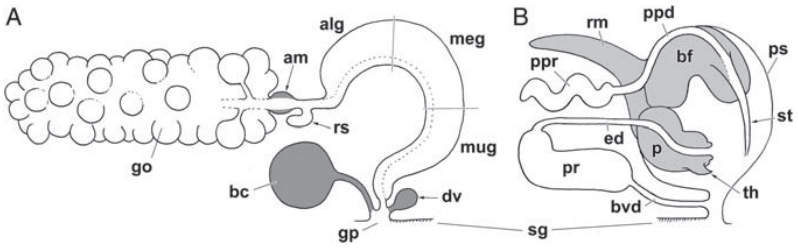

Figure 8. Schematic overview of the genital system and copulatory apparatus of *Strubellia wawrai* n. sp. **A.** Genital system, dark grey areas indicate organs that become reduced in the female phase. **B.** Copulatory apparatus. Abbreviations: alg, albumen gland; am, ampulla; bc, bursa copulatrix; bf, basal finger; bvd, posterior-leading vas deferens; dv, diverticle; ed, ejaculatory duct; go, gonad; gp, genital pore; meg, membrane gland; mug, mucus gland; p, penis; ppd, paraprostatic duct; ppr, paraprostate; pr, prostate; ps, penial sheath; rm, retractor muscle; rs, receptaculum seminis; sg, sperm groove; st, stylet of basal finger; th, spine. Not to scale.

threshold clusters both species together. In COI (571 bp) intraspecific variation ranges between 1.57 and 1.92% for *S. wawrai* n. sp. from the Solomons ($n = 7$) and 0.7% for *S. wawrai* n. sp. from Vanuatu ($n = 2$); uniting both populations ($n = 9$) the variation ranges between 2.1 and 2.8%. Interspecific variation is comparably high ranging between 12.25 and 13.31% in *S. paradoxa* and *S. wawrai* n. sp. and between 14.36 and 15.06% in *Strubellia* sp. from Sulawesi and *S. wawrai* n. sp.

DISCUSSION

Comparative morphology of the cerebral nerve ring

The CNS of *Strubellia wawrai* n. sp. has been described briefly from dissected material by Wawra (1974, as *S. paradoxa*). The general organization of ganglia resembles that of *S. paradoxa* and other hedylopsacean acochlidian species, e.g. *Pseudunela espiritusanta* (Neusser & Schrödl, 2009; Brenzinger *et al.*, 2011). Examination of serially sectioned specimens revealed several additional features, such as the previously undetected parapedal commissure and several thin cerebral nerves that complement the set of nerves beyond what is generally detectable in small mesopsammic acochlidians. Among the usually present nerves are three comparatively large anterior cerebral nerves also shown in Wawra's drawing (1974: fig. 7); we regard the nerves numbered 1.1–1.3 therein to be the labial tentacle nerve, the Hancock's and the rhinophoral nerve, respectively. *Strubellia* shows two small ganglia attached to the cerebral ganglia, as do all other hedylopsaceans: The "procerebral lobe" described by Wawra (but not depicted) can be suspected at the base of the rhinophoral and Hancock's nerve and likely refers to the rhinophoral ganglion herein. The optic ganglion appears to have been overlooked by Wawra; his "optic" nerve is shown to arise directly from the cerebral ganglion and thus might alternatively be the oral nerve which extends close to the labial tentacle nerve.

The homology of opisthobranch rhinophoral or optic ganglia and the pulmonate procerebrum (with double connectives to the cerebral ganglion) has been suggested previously (e.g. Haszprunar, 1988; Haszprunar & Huber, 1990; Huber, 1993) and a general homology of the sensory innervation among Euthyneura appears more and more likely (Jörger *et al.*, 2010a, b). Comparison of these ganglia among Acochlidia might however hint at a common anlage of both the optic and rhinophoral ganglion: the presence of a looping nerve interconnecting both (present in *S. wawrai* n. sp. and *Tantulum elegans*; Neusser & Schrödl, 2007), the variable origin of the optic nerve (usually from the optic ganglion, in *P. cornuta* it splits off from the rhinophoral nerve; Neusser, Heß & Schrödl, 2009a) and finally the presence of double connectives in one ganglion or the other. A double cerebro-rhinophoral connective is present in *Tantulum*, the microhedylacean *Pontohedyle milaschewitchii* (Kowalevsky, 1901) and *Microhedyle glandulifera* (Kowalevsky, 1901) (Jörger *et al.*, 2008; Neusser & Schrödl, 2009; own unpublished data); *S. wawrai* n. sp. is the only known species with a double cerebro-optic connective.

Differences from the CNS of *S. paradoxa* involve the apparent lack of the small cerebral nerves, the Hancock's nerve and Hancock's organs, but are likely to be artefacts (see Brenzinger *et al.*, 2011). The only evident difference between the CNS of *S. wawrai* n. sp. from the Solomon Islands and from Vanuatu is the 'penial' nerve in the examined specimen from Vanuatu, which might be present only in mature male specimens and could therefore not be detected in the female specimens from the Solomon Islands.

Visceral loop, osphradial ganglion and arrangement of buccal ganglia

Wawra (1974) described the typical acochlidian visceral nerve cord with three separate ganglia; we identify the ganglia herein as a left parietal, a fused subintestinal/visceral and a fused right parietal/supraintestinal ganglion, respectively. Nerves splitting from the connective joining the latter two ganglia and from the parietal ganglia have not been reported for any other acochlidian so far.

The additional ganglion attached to the fused right parietal/supraintestinal ganglion was mentioned by Wawra (1974); it is known for all hedylopsacean species examined in detail and also for the microhedylacean *Parhedyle cryptophthalma* (Westheide & Wawra, 1974; Jörger *et al.*, 2010b; Schrödl & Neusser, 2010). Judging from its position on the right side of the visceral loop, the ganglion was hypothesized to be homologous with the osphradial ganglion of other euthyneurans (Wawra, 1989; Huber, 1993; Sommerfeldt & Schrödl, 2005); this interpretation can be confirmed with the detection of an osphradium in *S. wawrai* n. sp. The presence of two nerves in *S. wawrai* n. sp. and a bifurcating nerve in *Pseudunela espiritusanta* suggests more than one function of the osphradial ganglion. In *Tantulum elegans*, the single nerve leaving the osphradial ganglion was mentioned to terminate close to the copulatory apparatus and hence assumed to be a "genital" or "penial" nerve (Neusser & Schrödl, 2007); innervation of the copulatory apparatus in *S. wawrai* n. sp., however, appears to be mainly by the nerve of cerebral origin mentioned above.

Buccal ganglia posterior to the pharynx are present in all Acochlidia, and associated gastro-oesophageal ganglia are known from several hedylopsacean species but not (yet) *Hedylopsis ballantinei* (Sommerfeldt & Schrödl, 2005; Wawra, 1988, 1989; Schrödl & Neusser, 2010) and also the microhedylaceans *Asperspina murmanica* (Kudinskaya & Minichev, 1978) and *Microhedyle glandulifera* (Neusser, Martynov & Schrödl, 2009b; Eder, Schrödl & Jörger, 2011). In *S. wawrai* n. sp., this arrangement of ganglia innervates the salivary ducts, musculature of the oesophagus and the radula as can be shown from

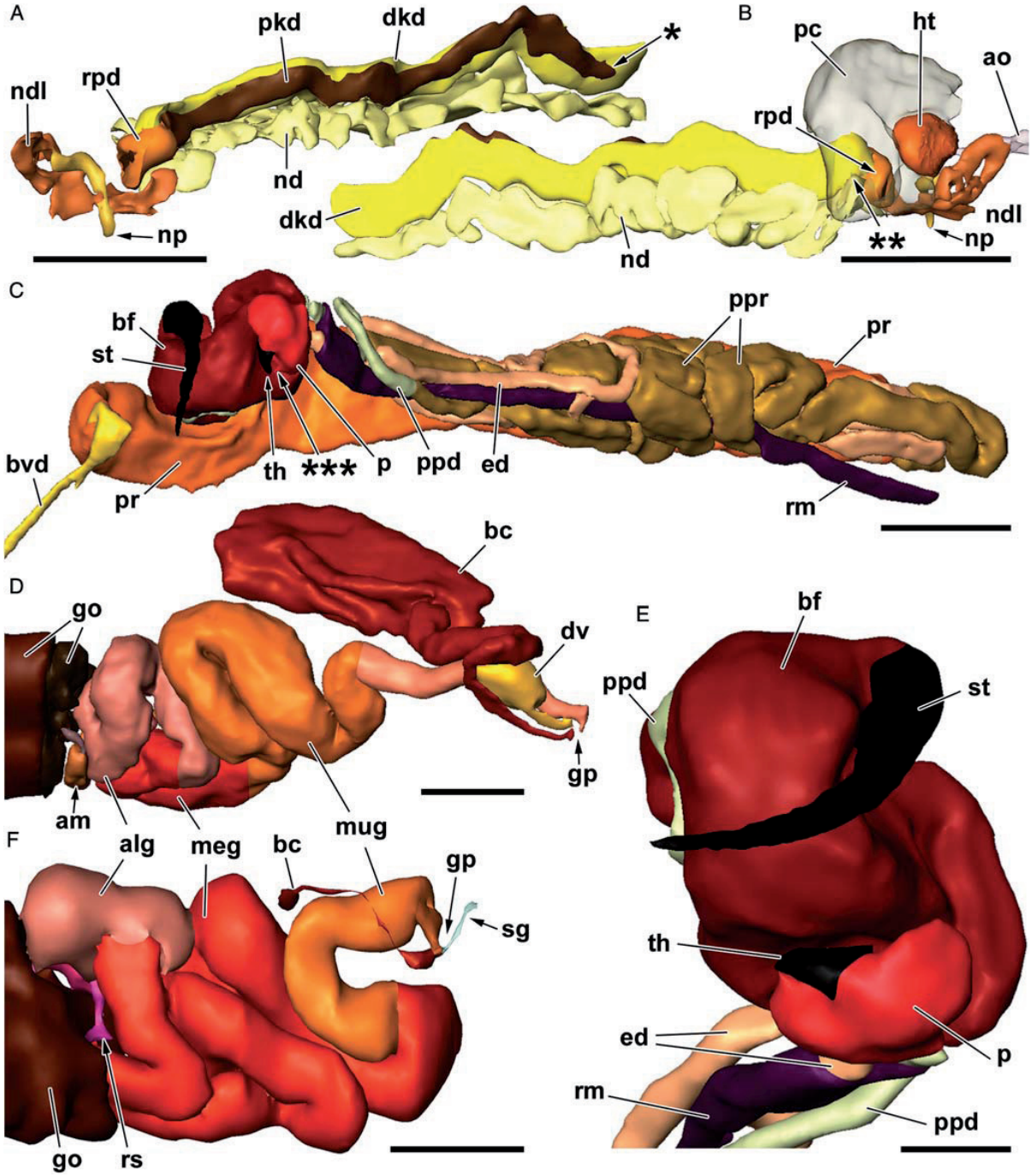

Figure 9. Three-dimensional reconstructions of the excretory, circulatory and reproductive systems of *Strubellia wawrai* n. sp. from Solomon Islands (**A, B, F**) and Vanuatu (**C–E**). **A.** Excretory system, left view. **B.** Excretory and circulatory systems, right view. **C.** Anterior male copulatory organs and (para-)prostatic glandular systems, left view. **D.** Nidamental glands and bursa copulatrix, right view. **E.** Penis and basal finger, left view. **F.** Nidamental glands and rudimental bursa copulatrix, ventral view. Abbreviations: am, ampulla; alg, albumen gland; ao, aorta; bc, bursa copulatrix; bf, basal finger; bvd, posterior-leading vas deferens; dkd, distal kidney lumen; dv, diverticle; ed, ejaculatory duct; go, gonad; gp, genital pore; ht, heart; meg, membrane gland; mug, mucus gland; nd, nephroduct, ndl, nephroduct loop; np, nephropore; p, penis; pc, pericardium; pkd, proximal kidney lumen; pr, prostate; ppd, paraprostatic duct; ppr, paraprostate; rm, retractor muscle; rpd, renopericardioduct; rs, receptaculum seminis; sg, sperm groove; st, stylet; th, spine; *, connection between proximal and distal kidney lumina; **, connection between distal kidney lumen and nephroduct; ***, position of ejaculatory duct opening. Scale bars: **A, B** = 500 μm; **C** = 600 μm; **D, F** = 400 μm; **E** = 200 μm.

three pairs of nerves plus the unpaired radular nerve, again most of which have not been detected in other acochlidians.

The detection of a high number of previously unknown cerebral features, all possibly bearing useful phylogenetic information, again highlights the usefulness of serial sectioning and accompanying 3D reconstruction.

Osphradium

The observation of a pit-shaped osphradium is the first mention of this sensory organ in Acochlidia. In living *S. wawrai* n. sp. from Guadalcanal, the osphradium is clearly visible as a paler spot on the right body side. A similar spot is also visible in living *Acochlidium* sp. from the same locality, in this case rather inconspicuously on the anterior border of an otherwise darkly pigmented bar (own unpublished data). Interestingly, two previous accounts on the aforementioned genera mention body openings in the position of the osphradium: *S. paradoxa* was erroneously displayed to have the anus in the position of the osphradium (Rankin, 1979: 72) and the original account of *A. amboinense* Strubell, 1892 described the copulatory apparatus to open in this place (Bücking, 1933: fig. 2), contradicting observations from other sources or species (e.g. Küthe, 1935; Haase & Wawra, 1996; Brenzinger *et al.*, 2011).

The position of the osphradium—far anterior to what can be considered the mantle border (see Fig. 1A)—appears strange, since the chemosensory organ is usually part of the mantle cavity organs including the gill, anus, genital opening and nephropore (e.g. Thompson, 1976). Apparently the osphradium has moved to a more anterior position after the loss of the mantle cavity in acochlidians. While it appears possible that the osphradium as a discrete organ is expressed only in the large-bodied species, it is also likely to have simply been overlooked so far in the minute interstitial species. These taxa should be critically (re-)investigated regarding the presence of a possible osphradium by searching for the osphradial nerve and its targeted epithelium as part of the epidermis.

Judging from light-microscopical observations, the osphradium of *S. wawrai* n. sp. resembles the organ of the cephalaspidean *Philine* (a pit-like structure with a flat bottom; Edlinger, 1980) and can accordingly be divided into two zones: a microvillous inner zone and a ciliated border forming the rim (Fig. 5E), similar to the condition described for the cephalaspidean *Scaphander lignarius* (Linnaeus, 1758) by Haszprunar (1985b). Since ultrastructural research on the osphradial sensory epithelia has been used to test phylogenetic hypotheses, examination of the organ in *Strubellia* might reveal features shared with other Panpulmonata that have retained the osphradium.

Hancock's organs

Hancock's organs are cerebrally innervated chemosensory organs situated on the sides of the head; they are present in most shelled opisthobranch gastropods (see Göbbeler & Klussmann-Kolb, 2007). Previously assumed to be missing in Acochlidia (see e.g. Wawra, 1987; Sommerfeldt & Schrödl, 2005), the organs were detected in four mesopsammic species including one *Pseudunela* species (Edlinger, 1980; see Neusser, Jörger & Schrödl, 2007; Neusser *et al.*, 2011b; own unpublished data). As in the latter species, the Hancock's organs of *S. wawrai* n. sp. are ciliated epidermal depressions located posterior to the labial tentacles; each organ is innervated by a lateral branch of the rhinophoral nerve. The organs can only be reliably detected in specimens where the head is not strongly retracted into the visceral sac and are thus likely to be overlooked, as was probably the case in *S. paradoxa*.

Oophagy and radular characters

An asymmetric radula with a formula of $n \times 1.1.2$ is present in most hedylopsaceans and has been regarded as a possible synapomorphy of all Acochlidia (Schrödl & Neusser, 2010). Wawra (1974) described the radula of Solomon Island *S. wawrai* n. sp. (as *S. paradoxa*) with a formula of $n \times 2.1.2$, but later corrected this to $n \times 1.1.2$ (Wawra, 1979); the latter can be confirmed by our study. *Strubellia paradoxa* was also originally described with a formula of $n \times 2.1.2$ (Küthe, 1935). Reexamination of *S. paradoxa* showed that on the left side there is just a single tooth (Brenzinger *et al.*, 2011). The genus shares with *Acochlidium* (and *Aiteng ater* Swennen & Buatip, 2009) the finely serrated rhachidian teeth (e.g. Haynes & Kenchington, 1991; Swennen & Buatip, 2009; Neusser *et al.*, 2011a), however the very elongate rhachidians appear to be a synapomorphy for *Strubellia*. There are no clear differences in tooth morphology separating *S. paradoxa* and the Solomon Islands/Vanuatu populations. Counts of radular rows do not show consistent differences among populations and the only connection appears to be with size or ontogenetic stage: very large individuals of *S. wawrai* n. sp. from Vanuatu had *c.* 55–60 rows of teeth, medium-sized specimens from the Solomon Islands showed between 48–51 rows (Wawra, 1974, 1979) and 40–46 rows (this study).

The observation of cultured *S. wawrai* n. sp. feeding on egg capsules of *Neritina* cf. *natalensis* is the first direct observation of feeding in Acochlidia. Only *Acochlidium amboinense* has been mentioned to have "animal remains in the stomach" (Bergh, 1895), while the meiofaunal *Pontohedyle milaschewitchii* was suggested to be an unspecialized detritus grazer due to its preference of substrates with microbial mats (Hadl *et al.*, 1969; Edlinger, 1980; see Schrödl & Neusser, 2010).

Clusters of neritid egg capsules were seen on rocks at most sampling localities in the Solomon Islands and are an energy-rich potential food source. However, these capsules are strongly reinforced by conchiolin and diatoms or sand grains derived from the food (Andrews, 1935), a fact that appears to deter predation effectively. Only recently have other neritids been shown to feed facultatively on egg capules of other species (Kano & Fukumori, 2010). *Strubellia wawrai* n. sp. appears to be equipped with a radula specialized for piercing the hard-shelled capsules: the rhachidian teeth are more elongate than in any other acochlidian genus and show considerable wear in the older part of all examined radulae. The finely serrated rhachidians are most likely used to create a slit in the egg capsules through which the contents of the capsules are sucked out, as is suggested by the peristaltic movement of the visceral sac during feeding and the duration of each feeding interval. The sucker-like aspect of the lips surrounding the protruding pharynx is probably related to this mode of feeding. An oophagous habit can also be assumed for *S. paradoxa*, which shows no major differences in microhabitat or radular morphology (Brenzinger *et al.*, 2011). The closely related *Acochlidium* species all share the same habitat (as far as can be deduced from the literature) and exhibit highly similar radular morphology (the rhachidian teeth are wider and less dagger-shaped). One might suggest a similar mode of feeding in this genus, perhaps involving niche differentiation with regard to the durability of egg capsules that are fed on; not all egg capsules are equally reinforced and most harden further after their deposition on the rock (Kano & Fukumori, 2010). During the feeding experiment, a specimen of *Acochlidium* from Guadalcanal was attracted to the presented egg capsules but did not feed (own observations).

Spicules

Subepidermal spicules are found in a number of shell-less heterobranchs and are there considered to be an adaptation to life

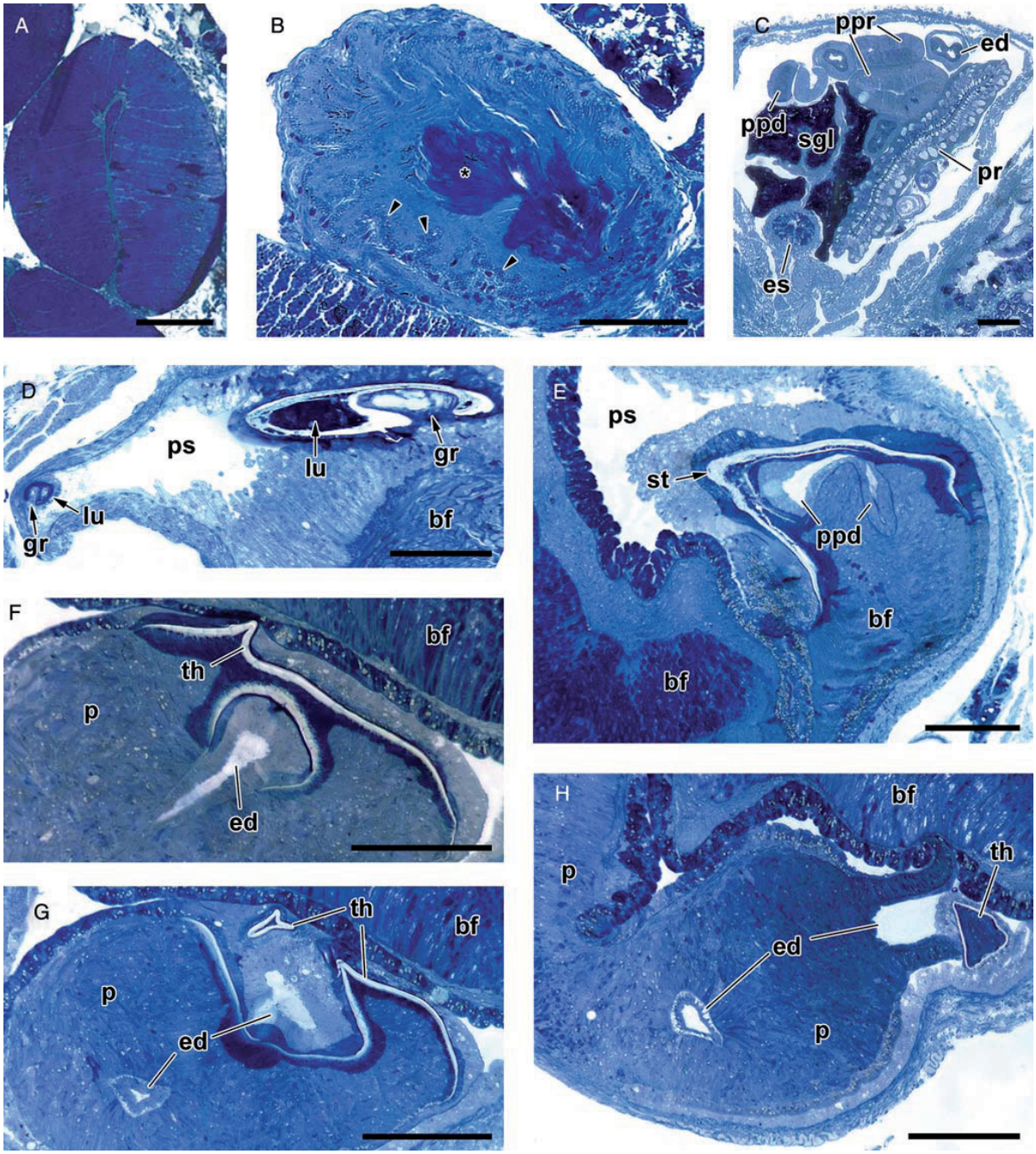

Figure 10. Semithin sections of the genital system of *Strubellia wawrai* n. sp. from Solomon Islands (**A, B**; posterior genital system in female phase) and Vanuatu (**C–H**; parts of copulatory apparatus). **A.** Membrane gland showing acentral lumen. **B.** Receptaculum seminis filled with spermatozoa, heads along the wall. **C.** (Para-)prostatic glandular system. **D.** Hollow stylet of basal finger (tip on the left, close to the base on the right). **E.** Basal finger at base of stylet. **F, G.** Penis with ejaculatory duct and thorn embedded in epithelium. **H.** Trumpet-shaped penial papilla and tip of thorn. Abbreviations: bf, basal finger; ed, ejaculatory duct; es, esophagus; gr, groove of basal finger stylet; lu, lumen of basal finger stylet; p, penis; ppd, paraprostatic duct; ppr, paraprostate; pr, prostate; ps, lumen of penial sheath; sgl, salivary gland; st, stylet of basal finger; th, spine of penis; arrowheads: sperm heads; asterisk: mass of flagella. Scale bars: **A, B** = 50 μm; **C–H** = 100 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

in the marine interstitial environment (see Rieger & Sterrer, 1975 for a review), functioning as either protective or stabilizing skeletal elements. In some doridoidean nudibranchs, defensive calcareous spicules have also been suggested to be calcium reservoirs (Cattaneo-Vietti *et al.*, 1995). Spicules are present in most acochlidians (Jörger *et al.*, 2008; Schrödl & Neusser, 2010); members of the mesopsammic *Asperspina* and *Hedylopsis* have evolved a secondary spicule 'shell' that surrounds the

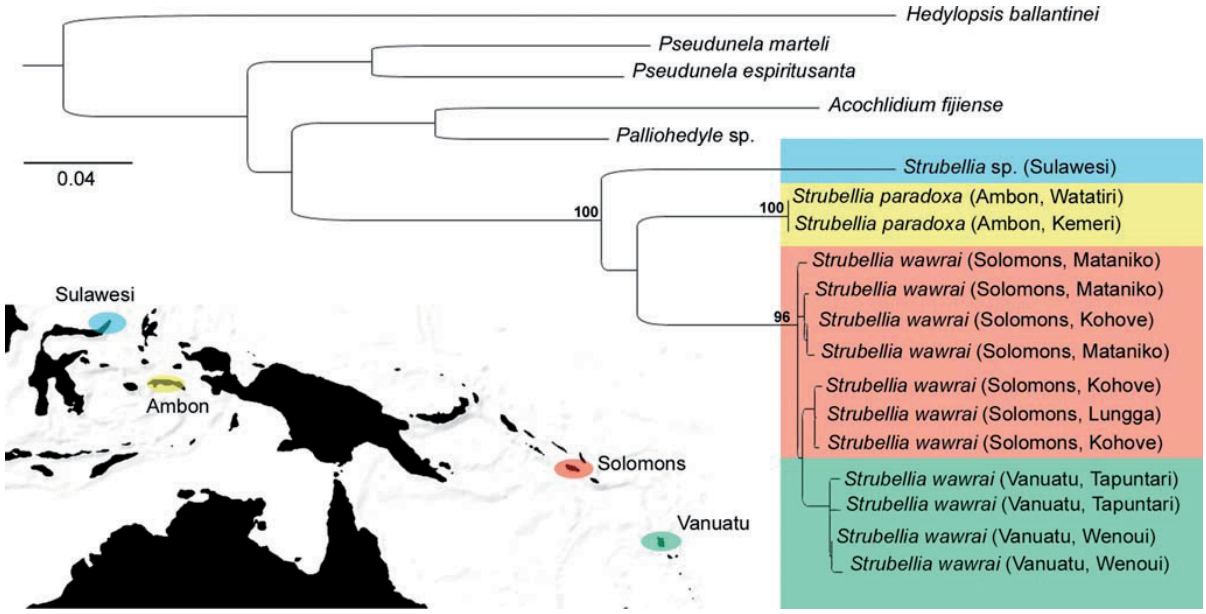

Figure 11. RAxML tree of the genus *Strubellia*, based on a concatenated dataset of mitochondrial COI and 16S rRNA (1113 bp) and colour coded distribution map of the different *Strubellia* species. Bootstrap values given above nodes.

visceral sac (e.g. Swedmark, 1968; Schrödl & Neusser, 2010). Wherever present, acochlidian spicules are calcareous, more or less elongate or forming concrements of irregularly formed grainy material.

In form, relative size and distribution, *Strubellia* spicules resemble those of *Pseudunela* or *Acochlidium* (see Bayer & Fehlmann, 1960; Neusser & Schrödl, 2009) but, judging from their location within the body, they do not function as protective elements (the lowest density of spicules is found in the dorsal surface of the visceral sac, the part of the body which remains most prominent in contracted animals). Rod-shaped spicules with blunt ends are found most numerously in the foot, in the head appendages, at the base of the visceral sac and in parallel strips dorsolaterally of the pharynx ('cephalic spicule grid'). A skeletal function appears likely for the former three examples, in a position where the spicules might well function, in bulk, as stabilizing agents. A protective function (for the CNS) seems reasonable only for the cephalic spicules, as has already been suggested for *S. paradoxa* by Küthe (1935). We hypothesize an additional function of this spicular arrangement, namely acting as a supporting structure during feeding: the spicules might interlock and thus stabilize the pharyngeal region, while the head is pressed hard onto the neritid egg capsules in order to puncture their walls with the radula. Similar aggregations of spicules close to the pharynx have been reported in other acochlidian genera: in the microhedylacean *Asperspina* and *Pontohedyle* (Jörger *et al.*, 2008) and as a "post-pharyngeal spicule collar" in the hedylopsacean *Tantulum elegans* (Neusser & Schrödl, 2007; Schrödl & Neusser, 2010); in *Acochlidium bayerfehlmanni* Wawra, 1980 (Bayer & Fehlmann, 1960; as *A. amboinense*) spicules are stated to form "a ring around the esophagus" similar to the situation found in *Strubellia*.

Cephalic gland

The loose aggregation of cells covering the cerebral ganglia was present in all individuals examined in this study, but has not been reported for any acochlidian species, including *S. paradoxa*. Neusser *et al.* (2007, 2009b) mention both "cells above the cerebral commissure" and "lateral bodies" attached to the cerebral ganglia in the interstitial acochlidians *Asperspina murmanica* and *Hedylopsis ballantinei*; these cells were, however, embedded within the connective sheath of the cerebral commissure. Supposedly endocrine "dorsal bodies"—surrounded by a connective sheath and associated with the cerebral ganglia—are common among pulmonates, where there is considerable diversity regarding structure and innervation (e.g. Boer, Slot & van Andel, 1968); they have been shown to be more active during female reproduction (Saleuddin, Ashton & Khan, 1997). In *S. wawrai* n. sp. there appears to be no connective sheath and there are no histologically detectable differences between juveniles and mature specimens.

In histology (loose tissue with yellow-stained vesicles visible in serial sections) and position the structure also resembles the 'blood' gland found in some anthobranch nudibranchs, e.g. the doridoidean *Corambe lucea* Marcus, 1959 (Schrödl & Wägele, 2001) and the dexiarchian *Doridoxa* (Schrödl, Wägele & Willan, 2001). However, the presence of apparently osmiophilic, yellow-staining vesicles indicates fatty substances, as are present in the digestive gland, possibly implying a function as an additional fat-storing structure. Ultrastructural research on cell anatomy and affiliation to the CNS is needed for conclusive identification of this organ, which might represent an apomorphy for either *Strubellia* or Acochlidiidae.

Heart and kidney

Only few shell-less heterobranchs venture into habitats that are regularly influenced by freshwater, e.g. some nudibranchs and sacoglossans (Barnes, 1994). The excretory system of the sacoglossan *Alderia modesta* (Lovén, 1844), found on partially brackish intertidal mudflats (Evans, 1951), has been examined in detail but lacks a heart and shows no apparent elaboration of its sac-like kidney (Fahrner & Haszprunar, 2001). Members of the recently described Aitengidae (also Acochlidia) live

amphibiously among mangroves or coastal rocks, and show an elaborate system of branched dorsal vessels (resembling the condition found in many plakobranchioid sacoglossans) which might originate from a histologically similar and sac-like kidney (Swennen & Buatip, 2009; Neusser *et al*., 2011a). Neither condition appears very similar to that found in *Strubellia*.

The circulatory and excretory systems of *S. wawrai* n. sp. show several apparent morphological adaptations to permanent life in fresh water, namely specialized cell types in the heart, elongated lumina of the kidney and nephroduct, and possibly the loop in the distal nephroduct. A strongly vacuolated epicardium and discrete thick-walled cells inside the lumen of the heart have been described only from *S. paradoxa* and the brackish-water *Pseudunela espiritusanta* (Neusser & Schrödl, 2009; Brenzinger *et al*., 2011). These cells possibly involve a novel site of ultrafiltration (on the ventricle) and aggregations of rhogocytes, however in both cases ultrastructural investigation is needed to identify those cell types. Muscular bridges spanning the lumen of the heart, presumably an aspect of an enhanced circulation, have been mentioned for *Acochlidium amboinense* (Bücking, 1933) and *S. paradoxa*.

In *Strubellia* there appears to be a functional division of otherwise elongated excretory lumina, judging from the separation of at least three histologically different zones (proximal and distal kidney lumina and nephroduct). The presence of the conspicuous upward loop of the distal nephroduct, which is closely associated with the pericardial wall, hints at the presence of a fourth zone involved in the modification of the primary urine. Again, ultrastructural studies are needed to test these observations derived from light microscopy.

Elongation of excretory lumina has been shown to be a feature of hedylopsaceans and is conspicuously present in the coastal mesopsammic *Pseudunela cornuta* (Challis, 1970) and *P. espiritusanta* (Neusser & Schrödl, 2009; Neusser *et al*., 2009a, Neusser, Jörger & Schrödl, 2011b) and the more basal but limnic *Tantulum elegans* (Neusser & Schrödl, 2007). All of these species display an elongate kidney with divided lumina and U-shaped nephroduct with distal loop. Members of the marine mesopsammic genus *Hedylopsis* also show the elongate, complex kidney, but have a short nephroduct (Fahrner & Haszprunar, 2002; Sommerfeldt & Schrödl, 2005). This means that the elaborate excretory system found in *Strubellia* is already more or less present in marine or brackish-water *Pseudunela* species (Neusser *et al*., 2011b) and thus further adaptations to life in freshwater are likely to have happened on an ultrastructural level.

There is only scarce information on the circulatory and excretory systems of *Acochlidium* species, although it appears to be more sophisticated. Bücking (1933) mentioned a branching vessel on the dorsal side of the visceral sac (superficially similar to that found in sacoglossans or Aitengidae) and the presence of multiple renopericardial funnels. It should be critically compared with the condition found in *Strubellia* to trace the evolution of characters in these organ systems that are crucially important in the colonization of limnic habitats.

Genital ontogeny

As was confirmed for *S. paradoxa* by Brenzinger *et al*. (2011), *S. wawrai* n. sp. appears to be a sequential, protandric hermaphrodite, as is otherwise known only for *Tantulum elegans* and *Hedylopsis* species among Acochlidia (Wawra, 1989; Neusser & Schrödl, 2007; Kohnert *et al*., 2011). The change of sex during ontogeny can be deduced (1) from the presence of two allosperm receptacles in otherwise male specimens and (2) the presence of intermediate stages (females with bursa copulatrix, seminal groove and copulatory apparatus still present but in various stages of reduction) (Wawra, 1988; present study). Sperm transfer appears to be via copulation and mainly in the male phase, after which the sex changes to a female state (gonad producing oocytes; female gland mass developed) while the strictly male genital features become reduced. This change is likely to be rapid since intermediate stages have rarely been found in previous studies of *Strubellia* species (Küthe, 1935; Wawra, 1988).

Genital system (posterior part)

The genital system of *S. wawrai* n. sp. was largely described by Wawra (1974, 1988; as *S. paradoxa*), assuming first gonochorism and then sequential hermaphroditism. We can confirm the description of the posterior genital system with a full set of sperm storing organs, i.e. the ampulla for autosperm and two allosperm receptacles (receptaculum seminis, bursa copulatrix), which is a condition known from the marine *Pseudunela cornuta* and the brackish-water *P. espiritusanta*, among Acochlidia. However, in both the latter species the receptaculum seminis is situated more proximally to the gonad than the sac-like ampulla (Neusser & Schrödl, 2009; Neusser *et al*., 2009a); this is in contrast to *S. paradoxa* and *S. wawrai* n. sp. where the receptaculum seminis is distal to the tubular ampulla. Except for its functional change during ontogeny, the gonad of *Strubellia* varies from the aforementioned genus by the separation into distinct follicles and the high number of eggs, both features shared with *Acochlidium fijiense* (Haynes & Kenchington, 1991; Haase & Wawra, 1996), probably reflecting a higher reproductive potential per individual. The female gland mass, developed from the very long gonoduct in 'males', is tubular all along and shows three histologically separable parts. This organ system is highly variable among *Pseudunela* and other acochlidians (but see Neusser *et al*., 2011b), where usually at least some of the glands are sac-like extensions and sometimes there appear to be only two different glands; the situation in *Acochlidium* species is unclear (see Schrödl & Neusser, 2010; Brenzinger *et al*., 2011).

The bursa copulatrix, reduced in the female phase, is similar to that of the marine hedylopsaceans in its morphology (bulbous, with thinner stalk) and its location next to the genital opening. *Acochlidium* on the other hand has been described to lack any allosperm receptacles due to its supposedly hypodermal mode of insemination (Haase & Wawra, 1996). The genital diverticulum next to the genital opening is a feature known also from *S. paradoxa* (Brenzinger *et al*., 2011); its variability in size (largest in one specimen from Vanuatu) and reduction in females hint at a function in copulation.

Strubellia shares the supposedly 'primitive' open seminal groove connecting to the genital opening distal to the bursa with *Hedylopsis spiculifera* Kowalevsky, 1901 (see Wawra, 1989). Other hedylopsaceans have been described to have a closed vas deferens that splits off the distal gonoduct proximal to the bursa and runs below the epidermis of the right body side (e.g. Neusser *et al*., 2009a; see Schrödl & Neusser, 2010). We suggest that the open seminal groove is not a plesiomorphic character *per se*, but is likely connected with ontogenetic sex change; as a transient feature, the duct remains only as a groove and is not sunk below the epidermis.

Cephalic copulatory apparatus

We disagree with Wawra's (1974) description of the cephalic copulatory apparatus which was based on dissected material missing the penis and associated glands; as in the description of *S. paradoxa* by Küthe (1935), the basal finger was erroneously interpreted as the penis. The copulatory organs of *S. wawrai* n. sp. consist of two distinct muscles with connected (para-)

prostatic glandular systems as in *S. paradoxa*, resembling the *Pseudunela* species known in detail (Neusser & Schrödl 2009; Neusser *et al.*, 2009a, 2011b). *Strubellia*, however, lacks the hollow penial stylet and instead features a solid spine near the penial opening, precluding sperm transfer by hypodermal injection which is believed to occur in *Pseudunela*, *Acochlidium* and a number of heterobranchs that possess one or several hollow penial stylets as an extension of the distalmost vas deferens (see Gascoigne, 1974; Haase & Wawra, 1996; Neusser *et al.*, 2009a).

The long and hollow stylet of the basal finger, however, appears to be used for (hypodermal) injection of the paraprostatic secretion; only in *Strubellia* does the stylet have the longitudinal groove. Both muscle and chitinous elements are more pronounced in *Strubellia* than in other genera, which imply a relatively higher importance of the paraprostatic system in this genus. Stylet morphology (and perhaps that of the penial spine) may also present a possibility to distinguish at least male specimens from the two *Strubellia* species by SEM: the basal finger stylet of *S. wawrai* n. sp. appears to be more elongate than that of *S. paradoxa* and shows a bent or slightly hooked tip (Table 4). This distinction is however only based on few male specimens and disregards the possibility of the stylet being flexible, as is mentioned for the chitinous penial stylets of some sacoglossan species (Gascoigne, 1974).

The paraprostatic duct has been mentioned to split at the base of the stylet in *S. paradoxa* and *S. wawrai* n. sp. from Guadalcanal (Küthe, 1935; Wawra, 1974; Brenzinger *et al.*, 2011), whereas it is undivided in the specimen from Vanuatu. This feature is of unclear function and may again be related to the individual stage of ontogeny, but is hard to detect and deserves reexamination.

Species-level relationships

Molecular data indicate that there are three separate lineages in the genus *Strubellia*, the first offshoot known only from the single juvenile specimen from Sulawesi examined herein. More material is needed to establish this population as a new species.

The eastern Melanesian specimens of *S. wawrai* n. sp. form a clade that is sister group to *S. paradoxa* from Ambon, Indonesia. Both clades receive strong bootstrap support and sequence divergence in COI (*c.* 12–13%) is relatively high. Both Species Identifier and parsimony network analyses indicate specific differences between *S. paradoxa* and *S. wawrai* n. sp. Given the 3,500-km distance between Ambon and the Solomon islands, this divergence is not surprising. Separation of *S. wawrai* n. sp. by only morphological means is not straightforward, since most organ systems previously used to separate acochlidian species are highly similar. However, there are some differences in parts of the copulatory apparatus, including length and curvature of the basal finger stylet (elongate and apically curved vs. rather stout and short in *S. paradoxa*; Brenzinger *et al.*, 2011) and form of the penial spine that might be useful features discernible by SEM. In both cases these differences refer to few mature individuals only, so ranges of intraspecific or ontogenetic variations remain poorly known. Variations in radular row counts, as already mentioned, are likely to be attributable to the size of the individuals examined. The presence of a second lateral plate in *S. paradoxa* has to be formally confirmed (Brenzinger *et al.*, 2011).

Summing up, potential differences in relevant parts of the copulatory organs, together with genetic evidence, leave little doubt that the populations from Ambon and Melanesia represent distinct species.

On a population level, the observed size disparity between mature specimens of *S. wawrai* n. sp. from the Solomon Island and Vanuatu is an obvious morphological difference, especially since female individuals from Vanuatu with remaining male genitalia were larger than already fully female specimens from Guadalcanal (Table 4). This observed delay in ontogeny is hard to explain given knowledge of the genetic similarity between the populations, but is perhaps attributable to ecological factors. Observed differences in the size of the genital diverticulum and the distal division of the paraprostatic duct (present/absent) are also likely to be variable during ontogeny. Analysis of molecular divergence shows that the Guadalcanal and Espiritu Santo populations of *S. wawrai* n. sp. are very similar, with the clade comprising the latter population nested inside the former, indicating that the split is too recent to be obvious from COI divergence. We therefore regard the two populations as a single species that might be close to separating into two species, with geographic separation preventing regular gene flow.

Habitats and dispersal

The localities discovered in this study fit well with the described habitat regarding physical and chemical properties, i.e. limestone slabs at the edge of shallow streams carrying mineral-rich and slightly alkaline water. *Strubellia* species co-occur with neritid gastropods (Starmühlner, 1976; Haynes, 2000). This is significant, since we observed *S. wawrai* n. sp. feeding on neritid eggs, resolving a longstanding mystery. In addition we know that different species and populations occur in limnic systems of more or less distant islands and archipelagos surrounded by sea.

Table 4. Comparison of morphological data of *Strubellia wawrai* n. sp. and *S. paradoxa*.

	S. wawrai n. sp.			*S. paradoxa* (Strubell, 1892)	
Reference	Wawra (1974, 1979, 1988)	Present study	Present study	Küthe (1935)	Brenzinger *et al.* (2011)
Collecting site	Guadalcanal, Solomon Is	Guadalcanal, Solomon Is	Espiritu Santo, Vanuatu	Ambon, Indonesia	Ambon, Indonesia
Max. recorded body size	~2.5 cm	~2.0 cm	~3.5 cm	~2 cm	~1 cm
Radula formula	48–51 × 1.1.2	43–46 × 1.1.2	59 × 1.1.2	48–56 × 2.1.2	38 × 1.1.2
1st lateral tooth denticle	Present	Present	Present	Absent	Present
Length of basal finger stylet	1 mm	?	0.75–1 mm	0.5 mm	0.6 mm
Stylet form	Elongate, tip hooked	?	Elongate, tip bent	Rather stout	Rather stout
Distal paraprostatic duct	Divided (Wawra, 1974: fig. 4)	?	Undivided	Divided	Divided
Genital diverticle	Small	?	Large	?	Small
Penial thorn	?, curved	?	Concave, curved	Flat (?), curved	Flat, curved

So, how do limnic slugs, generally hiding away under rocks during the day, disperse to or maintain gene flow between different localities, as is implied by the molecular analysis? Other stream gastropods with similar lifestyles, such as the numerous neritid species occurring in the rivers of Indo-West Pacific islands, reach distant islands by means of planktonic larvae (Haynes, 1988; Myers, Meyer & Resh, 2000) and regularly recolonize them; juveniles of at least one species even migrate by sometimes 'hitchhiking' upstream on the shell of larger individuals (Kano, 2009). Assuming a similarly amphidromic life with larvae hatching in freshwater and returning to it after a period of time and metamorphosis in the sea (see McDowall, 2007) would explain the observed distribution in *Strubellia*—but there are yet no observations of eggs or larvae of *Strubellia*. However, *Acochlidium fijiense* is known to produce gelatinous egg masses from which veligers hatch that are apparently not able to survive in fresh water (Haynes & Kenchington, 1991). In seawater, these veligers quickly metamorphosize into 'adhesive'-type larvae which remain alive for at least 2 months and glue themselves e.g. to the wall of the Petri dish they are kept in (own observations on *Acochlidium* sp.). This shows that limnic *Acochlidium*, and possibly already the common ancestor with *Strubellia*, have evolved a specialized larval type that might be able to disperse between islands of archipelagos leading to the colonization of rivers, involving a neritid-like amphidromic lifestyle. On one hand, these adhesive larvae, if quickly glued to a substratum outside the river, could avoid being drifted away too far into the ocean. Following juvenile neritids on their necessary movement upstream (possibly while glued to a shell during metamorphosis) and then feeding on their eggs would be a novel and efficient kind of symbiosis. On the other hand, it seems possible that this type of larva is able to use more mobile and far-ranging organisms as vectors between islands (planktonic organisms, fish, birds, boats). While acochlidiid larvae can survive in the laboratory for months without any movement or food uptake, metamorphosized juveniles would have to feed. Such juveniles would still be in the size range of most marine acochlidians (1–2 mm) and are not likely to prey on adult food, i.e. strongly mineralized neritid egg capsules. A juvenile stage feeding on microbial mats, mucus, algae or detritus is thus hypothesized. Field observations and laboratory experiments are needed to confirm the hypothesized life-history traits of *Strubellia*.

Larvae sticking to floating or swimming objects may therefore be the 'missing' dispersive stages explaining interisland dispersal, such as from the Solomon Islands to Vanuatu in the case of *S. wawrai* n. sp., or the colonization of Palau or Fiji in the case of *Acochlidium bayerfehlmanni* and *A. fijiense* (Bayer & Fehlmann, 1960; Haynes & Kenchington, 1991). Since limnic Acochlidiidae are estimated to have originated in the Palaeogene (Jörger *et al*., 2010a), this long period would present a timeframe to have enabled dispersal via island-hopping, facilitated by lower sea levels and shorter distances between islands in Indonesia during much of the period. Dispersal to the west might have been limited by deeper-water currents being deflected at the border of the Southeast Asian continental shelf, as is indicated by Wallace's-line distributional patterns of marine organisms with pelagic larval stages (Barber *et al*., 2000). The lack of records of acochlidiids west of the Wallace line hints at a similar limitation. On the other hand, it appears likely that numerous populations of acochlidiids are yet to be discovered and also that many have become extinct.

Phylogeny of Strubellia *and evolution of characters*

The molecular phylogeny of the acochlidiids shows *Strubellia* to have originated in Indonesia. The genus is sister group to the morphologically more derived *Acochlidium* and *Palliohedyle*, these in turn being sister group to the marine interstitial Pseudunelidae. This configuration is congruent with the previously proposed phylogenies of Acochlidia, based on morphology (Schrödl & Neusser, 2010) or molecular markers (Jörger *et al*., 2010a).

According to the new results, the apomorphies for Acochlidiidae are the limnic habitat, benthic and probably amphidromic lifestyle, accompanied by large body size and distinct epidermal pigmentation, and the finely serrated rhachidian teeth. The visible distinction of the anterior mantle border and heart 'bulb', complex kidneys and the bipartite copulatory organs with spines and associated glands are already present in the mesopsammic *Pseudunela* species (Neusser & Schrödl, 2009; Neusser *et al*., 2009a, 2011b).

Presence of an osphradium and oophagy might represent further apomorphies; however, we suggest that the presence of epidermal sensory cells is likely at least in the hedylopsacean species with an osphradial ganglion. Furthermore, we suggest that a piercing-and-sucking mode of feeding is typical for Acochlidia, since all share the muscular pharynx, a slender radula that appears ill-equipped for grazing, and sometimes arrays of spicules surrounding the pharynx. For the meiofaunal species, instead of grazing, sucking liquid contents from soft, encapsulated food such as large-bodied protists or eggs of sand-dwelling organisms might explain the coloration of some species' digestive glands (e.g. brown or green in *Pontohedyle milaschewitchii*; Jörger *et al*., 2008), the lack of both abraded teeth and mineral residues in the digestive system. The sacoglossan-like monostich radula of the microhedylacean Ganitidae (Challis, 1968) would thus be specialized for a specific type of food, but not a unique mode of feeding within the group. Given the similarity of the pharynx and radula (slender ribbon, triangular median tooth with serrated margins, flat or reduced laterals) in Sacoglossa (especially the basal *Cylindrobulla*; Mikkelsen, 1998), Aitengidae (Swennen & Buatip, 2009; Neusser *et al*., 2011a), Amphibolidae (Golding, Ponder & Byrne, 2007) and Glacidorbidae (Ponder, 1986; Ponder & Avern, 2000), the suggested mode of feeding by piercing and sucking might represent a basal panpulmonate feature. Somewhat similar to *Strubellia*, both Sacoglossa and *Aiteng ater* are known to feed by puncturing internally soft food (siphonal algae and insect pupae, respectively) and sucking out the contents (Jensen, 1980, 1981; Swennen & Buatip, 2009); some Sacoglossa are also known to feed on the more or less gelatinous egg masses of opisthobranch gastropods (see Jensen, 1980; Coelho, Malaquias & Calado, 2006). However, some Euopisthobranchia *sensu* Jörger *et al*. (2010a) show similar, narrow radulae with serrated rhachidian and flat lateral teeth, e.g. species of the cephalaspidean *Toledonia* (Marcus, 1976; Golding, 2010), and also several nudibranchs (such as the oophagous aeolidioidean *Favorinus*; Schmekel & Portmann, 1982), making it difficult to detect phylogenetic patterns. An example is the proposed relationship of *Toledonia* and Acochlidia on the basis of radular morphology (Gosliner, 1994), which according to more recent hypotheses clearly represents a case of convergent evolution (Jensen, 1996; Sommerfeldt & Schrödl, 2005; Jörger *et al*., 2010a; Schrödl *et al*., 2011). Furthermore, a slender piercing radula is also present in *Omalogyra atomus* (Philippi, 1841) ('lower Heterobranchia'; Bäumler *et al*., 2008).

Synapomorphies of *Strubellia* appear to be the reddish-brown pigmentation, very slender rhachidian teeth, three receptacles in the male phase, the genital diverticulum, the enhancement of the basal finger with the stylet having a lateral groove, and the possession of a single flat hook on the penis instead of a hollow penial stylet.

The organization of the posterior genital system of *Strubellia* essentially conforms to the 'classic' idea of plesiomorphic monauly that was suggested to be the condition found in the hermaphroditic "opisthobranch common ancestor" (Ghiselin, 1966; Gosliner & Ghiselin, 1984), however the condition of *Strubellia* is fundamentally different. All hedylopsaceans are (special) androdiaulic hermaphrodites (Schrödl & Neusser, 2010; Schrödl *et al*., 2011) except for *Strubellia* (and *Hedylopsis* species; see Wawra, 1989; Sommerfeldt & Schrödl, 2005; Kohnert *et al*., 2011). The derived phylogenetic position of *Strubellia* (Jörger *et al*., 2010a; Schrödl & Neusser, 2010) suggests either a reversal to a monaulic system (with sperm and oocyte pathways not separated anatomically but in time, with a secondarily open seminal groove) or multiple developments of diauly among Acochlidia. The presence of allosperm receptacles already in the male phase might have led to the evolution of defined breeding seasons in *Strubellia*, hinted at by the strong skew among sexes revealed from sampling in all known localities: specimens were either predominantly juvenile, or only either male or female (Küthe, 1935; Wawra, 1988; present study). This might also be related to the observation that *Strubellia* generally aggregates in groups: If *Strubellia* has defined breeding seasons (possibly the rainy seasons accompanied by changes in riverine water levels) then aggregations of numerous specimens might mate after which the specimens change sex synchronously, spawn and then either die or fully reduce their genital organs, as was suggested for *A. fijiense* (Haynes & Kenchington, 1991). This appears at least possible, since complete reduction of the large copulatory apparatus during ontogeny can be deduced from the observations presented here, and a strong reduction of body size likely connected with a reduction of organs has been observed after periods of starvation in the specimens maintained in aquaria for this study.

Strubellia differs externally from *Acochlidium* and *Palliohedyle* by its more slender body, elongate visceral sac (versus leaf-shaped and flattened) and uniform reddish coloration (*vs* greenish-brown and black pigmentation), making it externally more similar to the aforementioned *Pseudunela* species (e.g. Haynes & Kenchington, 1991; own observations). According to the literature, internal differences from the better-known *Acochlidium* species include shape of the rhachidian teeth (very elongate in *Strubellia vs* triangular), morphology of the penis (relatively small with single apical thorn in *Strubellia vs* large and multi-spined; e.g. Wawra, 1979, 1980; Haase & Wawra, 1996) and basal finger (larger than the penis and with long stylet in *Strubellia*), the mode of genital ontogeny (protandric hermaphroditism in *Strubellia vs* hermaphroditism; Haynes & Kenchington, 1991) and the elaboration of visceral organs (multiple renopericardial funnels, digestive gland lobes, praeampullary gonoducts and branched, dorsally situated vessels connected to the heart in *Acochlidium*; Bücking, 1933; Haase & Wawra, 1996). Since the only comprehensive anatomical description of an *Acochlidium* species is very old (Bücking, 1933) and the only detailed study of the genital system includes characters that are still unclear (e.g. a connection between the ampulla and the digestive gland; Haase & Wawra, 1996), revision of the aforementioned anatomical features is urgently needed to trace the evolution of these unique limnic slugs.

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Chapter 9. Sacoglossa or Acochlidia? 3D-reconstruction, molecular phylogeny and evolution of Aitengidae (Gastropoda: Heterobranchia)

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SACOGLOSSA OR ACOCHLIDIA? 3D RECONSTRUCTION, MOLECULAR PHYLOGENY AND EVOLUTION OF AITENGIDAE (GASTROPODA: HETEROBRANCHIA)

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ABSTRACT

The amphibious 'bug-eating slug' *Aiteng ater* Swennen & Buatip, 2009 shows a worm-like, compact body shape lacking any cephalic tentacles or body processes. Anatomically it has been described as showing an unusual mix of sacoglossan and acochlidian characters, thus the systematic affinities are uncertain. The species is redescribed here with an integrative microanatomical and molecular approach. All major organ systems were three-dimensionally reconstructed from serial histological sections using AMIRA software. *Aiteng ater* has a prepharyngeal nerve ring with separate cerebral and pleural ganglia rather than cerebro-pleural ganglia, and no sacoglossan-like ascus is detectable histologically. The radula is triseriate rather than uniseriate, showing one lateral tooth on each side of the rhachidian tooth. A well-developed two-chambered heart is present. The vas deferens in *A. ater* splits off distal to the female glands. The intestine is short and opens into a small mantle cavity. Long cavities in the connective tissue are remains of dissolved calcareous spicules. Only a few characters thus remain to support a closer relationship of *A. ater* to Sacoglossa, i.e. the *Gascoignella*-like body shape lacking cephalic tentacles, the presence of an elysiid-like system of dorsal vessels, and an albumen gland consisting of follicles. Additionally we describe in microanatomical detail an equally small and vermiform new aitengid species from Japan. *Aiteng mysticus* n. sp. differs from *A. ater* in habitat, body size and colour, central nervous system and presence of a kidney. Both aitengid species resemble acochlidians in the retractibility of the head, by possessing calcareous spicules, a prepharyngeal nerve ring with separated cerebral and pleural ganglia, a triseriate radula with an ascending and descending limb, but without sacoglossan-like ascus, and a special diaulic reproductive system. The prominent rhachidian tooth of Aitengidae, which is used to pierce insects and pupae in *A. ater*, and the large, laterally situated eyes closely resemble the anatomy of members of the limnic Acochlidiidae. The acochlidian nature of *Aiteng* is strongly indicated by our molecular analysis, in which it forms a basal hedylopsacean offshoot or the sister clade to limnic Acochlidiidae and brackish or marine Pseudunelidae within Hedylopsacea. Such a topology would, however, imply that Aitengidae have lost the most characteristic acochlidian apomorphy, the subdivision of the body into a headfoot complex and a free, elongated visceral hump. Also, the absence of cephalic tentacles gives the Aitengidae an appearance that is very different to other, strictly aquatic Acochlidia. Differences of the external morphology and the internal anatomy are discussed in the light of a habitat shift of Aitengidae within the Acochlidia.

INTRODUCTION

The Acochlidia and Sacoglossa were traditionally regarded as taxa of the 'Opisthobranchia' in morphological (e.g. Jensen, 1996; Dayrat & Tillier, 2002; Wägele & Klussmann-Kolb, 2005; Schrödl & Neusser, 2010) as well as molecular (e.g. Grande *et al.*, 2004; Vonnemann *et al.*, 2005; Händeler *et al.*, 2009) studies. Recent molecular studies (e.g. Klussmann-Kolb

et al., 2008; Dinapoli & Klussmann-Kolb, 2010; Jörger *et al*., 2010) have changed our understanding of the phylogeny of Heterobranchia considerably. With a comprehensive euthyneuran taxon set, an analysis of mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rRNA genes and nuclear 18S and 28S rRNA genes has revealed the traditional 'Opisthobranchia' as polyphyletic (see Schrödl *et al*., 2011). Both Sacoglossa and Acochlidia have been shown to be part of an early (pan)pulmonate radiation (Jörger *et al*., 2010). The internal acochlidian topology revealed by molecular markers is congruent with that obtained by our morphology-based cladistic analysis (Schrödl & Neusser, 2010). However, a still undescribed putative member of the recently established Aitengidae Swennen & Buatip, 2009, named 'himitsu namekuji' (English: secret slug) when the specimens were found in Japan, clustered among hedylopsacean acochlids in the molecular analyses (Jörger *et al*., 2010).

The family Aitengidae was established as a monotypic sacoglossan family with a possible affinity to Acochlidia (Swennen & Buatip, 2009). Its sole species, the mysterious 'bug-eating slug' *Aiteng ater* Swennen & Buatip, 2009 was included into the 'top ten list of bizarre new species 2010' by the International Institute for Species Exploration at Arizona State University (http://species.asu.edu/Top10). *Aiteng ater* lives amphibiously in a mangrove forest in Thailand. The body length is 8–12 mm and the body shape is worm-like, lacking any cephalic tentacles or body processes. Anatomically it was described as showing an unusual mix of acochlidian and sacoglossan features, such as the prepharyngeal nerve ring characteristic for the Acochlidia, but the uniseriate radula, an ascus, a ramified digestive gland, a system of dorsal vessels and the albumen gland consisting of follicles—features which are all characteristic for Sacoglossa. The head and back of the slug bear strange 'white cigar-shaped bodies', which were interpreted as parasites by Swennen & Buatip (2009). *Aiteng ater* was preliminarily placed within Sacoglossa, but the authors expressed their doubts and the systematic affinities remained uncertain. The present study aims to re-examine *A. ater* with a microanatomical approach using computer-based three-dimensional (3D) reconstructions, as used e.g. for Acochlidia (Neusser *et al*., 2006; Neusser & Schrödl, 2007, 2009; Jörger *et al*., 2008, 2009; Neusser, Heß & Schrödl, 2009a; Neusser, Martynov & Schrödl, 2009b; Brenzinger *et al*., 2010; Neusser, Jörger & Schrödl, 2011) and to compare it to the 'secret slug' from Japan, which is also reconstructed in the present study in the same way. Combining evidence from detailed micromorphological descriptions and molecular analyses of both aitengid species we aim to clarify the systematic relationships and evolutionary history of the Aitengidae.

MATERIAL AND METHODS

Material

One paratype of *Aiteng ater* was obtained from the Zoological Museum, University of Amsterdam (ZMA) for semithin sectioning. One specimen of *A. ater* was collected at the type locality by Dr Swennen (Prince of Songkla University, Thailand) in October 2009 and was provided for the examination of the radula. Several specimens of *Aiteng mysticus* n. sp. were collected by H.F. and Y.K. on different islands of Okinawa Prefecture, Ryukyu Islands, Japan, in April 1992, March 1993, May 2008 and June 2009. The latter specimens were relaxed in 7.5% $MgCl_2$, fixed in 10% formalin and preserved in 75% ethanol for semithin sectioning and scanning electron microscopy (SEM) or fixed in 99% ethanol for molecular studies. Details of collecting sites are given in Table 1 and a summary of all material used in the morphological study in Table 2.

Table 1. Collecting date and localities of *Aiteng mysticus* n. sp. in Okinawa Prefecture, Ryukyu Islands, Japan.

Locality no.	Locality	GPS data	Date/collected by
1	Shimozaki, Nikadori, Hirara, Miyako Island	24°49′49″N, 125°16′42″E	04.1992 and 05.2008/HF, YT
2	Matsubara, Hirara, Miyako Island	24°47′01″N, 125°16′05″E	05.2008/HF, YT
3	Nakamoto, Kuroshima Island	24°13′42″N, 123°59′58″E	03.1996/YK
4	NW of Yonaguni Airport, easternmost corner of Higashi-bokujô, Yonaguni Island	24°28′04″N, 122°58′15″E	06.2009/HF, YT

HF, Hiroshi Fukuda; YK, Yasunori Kano; YT, Yuki Tatara.

Table 2. Material examined for morphological study.

Species	Locality (no., see Table 1)	Type of investigation and storage	Museum no.
Aiteng mysticus n. sp.	1	Specimen in 75% ethanol (H)	ZSM Mol 20110185
		Section series (P)	ZSM Mol 20110186
		Radula on SEM stub (P)	ZSM Mol 20110187
		Specimen in 99% ethanol (P)	NSMT Mo 77319
Aiteng mysticus n. sp.	2	Section series (P)	ZSM Mol 20110188
		Specimen in 99% ethanol (P)	OKCAB M21473
Aiteng mysticus n. sp.	4	Specimen in 5% formalin and radula on SEM stub (P)	OKCAB M21474
Aiteng ater	Pak Phanang Bay, Gulf of Thailand	Section series (P)	ZMA 409068
		Radula on SEM stub	ZSM Mol 20110189

Abbreviations: H, holotype; NSMT, National Museum of Nature and Science, Tokyo, Japan; OKCAB, Laboratory of Conservation of Aquatic Biodiversity, Faculty of Agriculture, Okayama University, Japan; P, paratype; ZMA, Zoological Museum, University of Amsterdam, The Netherlands; ZSM, Bavarian State Collection of Zoology, Germany.

Embedding and sectioning

Specimens were decalcified in Bouin's solution overnight and dehydrated in an acetone series (70, 90, 100%). For semithin sectioning two specimens of *A. mysticus* were embedded in Spurr's low-viscosity resin (Spurr, 1969) and the paratype of *A. ater* was embedded in Epon (Luft, 1961). Three series of ribboned serial semithin sections of 2 μm thickness were prepared using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) with contact cement on the lower cutting edge to form ribbons (Ruthensteiner, 2008). Sections were stained with methylene-azure II (Richardson, Jarett & Finke, 1960). The sections of *A. mysticus* were deposited at the Bavarian State Collection of Zoology, Germany (ZSM), Mollusca Section (ZSM Mol 20110186 and 20110188); the sections of *A. ater* were deposited at ZMA (ZMA 409068).

3D reconstruction

Digital photographs of every second section were taken with a CCD microscope camera (Spot Insight, Diagnostic Instruments, Sterling Heights, MI, USA) mounted on a DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). Images were converted to 8-bit greyscale format, contrast enhanced and unsharp masked with standard image-editing software. A computer-based 3D reconstruction of all major organ systems was conducted with the software AMIRA 5.2 (Amira Visaging GmbH, Germany) following the procedure of Ruthensteiner (2008). The 3D reconstruction of *A. ater* was based on the paratype series and that of *A. mysticus* on the series ZSM Mol 20110188.

Scanning electron microscopy

One specimen of *A. mysticus* from Miyako Island, Japan, preserved in 75% EtOH, one specimen of the same species from Yonaguni Island, Japan, preserved in 5% formalin and one specimen of *A. ater* from Thailand were used for SEM examination of radulae. Specimens were macerated in 10% KOH overnight. Remaining tissue was removed with fine dissection pins. Radulae were mounted on specimen stubs and sputter-coated with gold for 135 s (SEM-Coating-System, Polaron) and examined with a LEO 1430 VP (Leo Elektronenmikroskopie GmbH, Oberkochen, Germany) at 15 kV.

Molecular studies

One alcohol-preserved specimen of *A. ater* from the type locality was available for molecular study. DNA was extracted by K. Händeler (University of Bonn, Germany) using the Qiagen Blood and Tissue Kit according to manufacturer's recommendations. Four genetic markers were sequenced following the protocols and using the same primers as described by Händeler *et al.* (2009) for partial mitochondrial COI and 16S rRNA genes, and following Jörger *et al.* (2010) for nuclear 18S rRNA and partial 28S rRNA genes. Sequences were edited using Geneious Pro™ 5.1 (Biomatters Ltd). To supplement sequence data available from public databases we additionally sequenced the sacoglossan *Platyhedyle denudata* and the acochlidian *Parhedyle cryptophthalma*, *Ganitus evelinae* and *Palliohedyle* sp. as described above (see Table 3 for collection details and Table 4 for GenBank accession numbers).

The sampled Aitengidae were analysed in a dataset containing 35 heterobranch taxa with a focus on Acochlidia and Sacoglossa (Table 4). We aimed to cover known acochlidian and sacoglossan diversity by including at least one representative of each genus for Acochlidia (only lacking monotypic *Tantulum elegans*) and one sacoglossan representative per family following the classification of Jensen (1996). Other outgroups were chosen to cover a variety of euopisthobranch and panpulmonate taxa (see Jörger *et al.*, 2010). The alignments for each marker were generated using Muscle (Edgar, 2004). To remove ambiguous regions the alignments of 18S, 28S and 16S rRNA were masked with Gblocks (Castresana, 2000; Talavera & Castresana, 2007) using the options for a less stringent selection; the COI alignment was checked manually according to translation into amino acids. We performed maximum-likelihood analyses using RAxML v.7.0.3 (Stamatakis, 2006) according to the programmer's instructions ('hard and slow way') of the concatenated datasets combining 18S + 28S, 18 + 28S + COI, 18S + 28S + COI + 16S and 28S + COI + 16S with the GTR + Γ + I model, chosen via the Akaike Information Criterion implemented in jModeltest (Posada, 2008) and with one partition for each marker. The acteonoid *Rictaxis punctocaelatus* was defined as outgroup.

SYSTEMATIC DESCRIPTIONS

AITENGIDAE Swennen & Buatip, 2009
Aiteng Swennen & Buatip, 2009

Type species: *Aiteng ater* Swennen & Buatip, 2009, by original designation.

Aiteng ater Swennen & Buatip, 2009
(Figs 1–4, 5A, 6)

Aiteng ater Swennen & Buatip, 2009: 495–500, figs 1B–M, 2A–H.

Table 3. Collection data of the species for which molecular data were generated.

Species	ZSM no.	Locality	GPS data	Date/collected by
Aiteng ater	—	Pak Phanang Bay, Thailand, Gulf of Thailand	8°29′18″N, 100°10′55″E	09.2007/CS
Aiteng mysticus n. sp.*	—	Matsubara, Miyako, Okinawa, Japan	24°47′01″N, 125°16′05″E	05.2008/HF,YT
Aiteng mysticus n. sp.§	—	Shimozaki, Nikadori, Miyako, Okinawa, Japan	24°49′49″N, 125°16′42″E	05.2008/HF,YT
Palliohedyle sp.	Mol 20100356	Tambala River near Manado, Sulawesi, Indonesia	1°24′11″N, 124°41′08″E	11.2009/KJ
Pontohedyle milaschewitchii	Mol 20080054	Cap Kamenjak, Istria, Croatia, Mediterranean Sea	44°46′03″N, 13°54′58″E	09.2005/KJ
Parhedyle cryptophthalma	Mol 20100584	Bacoli, Naples, Italy, Mediterranean Sea	40°47′19″N, 14°03′54″E	09.2009/MS
Ganitus evelinae	Mol 20100328	Sina da Pedra, Ilhabela, Brazil, Atlantic Ocean	23°46′43″S, 45°21′33″W	03.2010/MS
Platyhedyle denudata	Mol 20091351	Secche della Meloria, Livorno, Italy, Mediterranean Sea	43°33′01″N, 10°13′08″E	09.2009/MS

CS, Cornelis Swennen; HF, Hiroshi Fukuda; KJ, Katharina Jörger; MS, Michael Schröd; YT, Yuki Tatara; ZSM, Bavarian State Collection of Zoology, Germany.
*as Aitengidae sp. in Jörger *et al.* (2010). §COI sequence only.

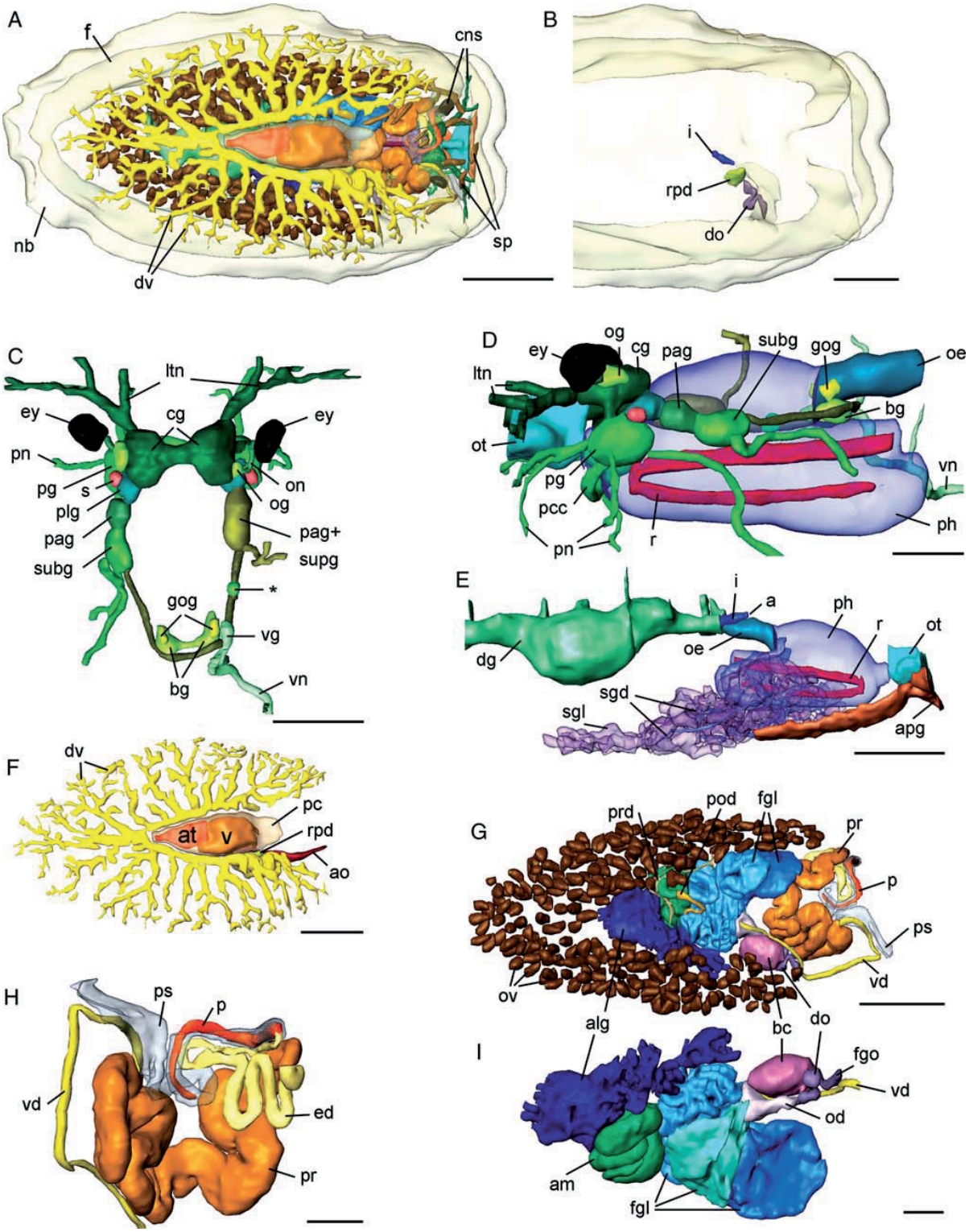
Table 4. Taxon sampling and GenBank accession numbers for the gene sequences used in the present study.

Taxon	Family	Species	18S	28S	16S	COI
PANPULMONATA						
Incerta sedis	Aitengidae	*Aiteng ater*	JF828036*	JF828037*	JF828038*	JF828031*
		Aiteng mysticus n. sp.§	HQ168428	HQ168441	HQ168415	HQ168453
Acochlidia	Hedylopsidae	*Hedylopsis ballantinei*	HQ168429	HQ168442	HQ168416	HQ168454
	Pseudunelidae	*Pseudunela* sp.†	HQ168431	HQ168444	HQ168418	HQ168456
	Acochlidiidae	*Strubellia paradoxa*	HQ168432	HQ168445	HQ168419	HQ168457
	Acochlidiidae	*Acochlidium fijiense*	HQ168433	HQ168446	HQ168420	HQ168458
	Acochlidiidae	*Palliohedyle* sp.	—	JF828039*	JF828040*	JF828032*
	Asperspinidae	*Asperspina* sp.	HQ168434	HQ168447	HQ168421	—
	Microhedylidae	*Pontohedyle milaschewitchii*	HQ168435	JF828043*	HQ168422	HQ168459
	Microhedylidae	*Parhedyle cryptophthalma*	—	JF828041*	JF828042*	JF828033*
	Microhedylidae	*Microhedyle glandulifera*	HQ168437	HQ168449	HQ168424	HQ168461
	Ganitidae	*Paraganitus ellynnae*	HQ168436	HQ168448	HQ168423	HQ168460
	Ganitidae	*Ganitus evelinae*	—	JF828044*	JF828045*	JF828034*
Sacoglossa	Volvatellidae	*Volvatella viridis*	HQ168426	HQ168439	HQ168413	HQ168451
	Cylindrobullidae	*Cylindrobulla beauii*	EF489347	EF489371	EF489321	—
	Juliidae	*Julia exquisita*	—	GQ996653	EU140895	GQ996661
	Oxynoidae	*Oxynoe antillarum*	FJ917441	FJ917466	FJ917425	FJ917483
	Platyhedylidae	*Gascoignella nukuli*	HQ168427	HQ168440	HQ168414	HQ168452
	Platyhedylidae	*Platyhedyle denudata*	—	JF828046*	—	JF828035*
	Caliphyllidae	*Cyerce nigricans*	AY427500	AY427463	EU140843	DQ237995
	Plakobranchidae	*Plakobranchus ocellatus*	AY427497	AY427459	DQ480204	DQ237996
	Elysiidae	*Elysia viridis*	AY427499	AY427462	AY223398	DQ237994
	Limapontiidae	*Limapontia nigra*	AJ224920	AY427465	—	—
	Boselliidae	*Bosellia mimetica*	AY427498	AY427460	EU140873	GQ996657
	Hermaeidae	*Hermaea cruciata*	—	GU191025	GU191042	GU191058
Siphonarioidea	Siphonaridae	*Siphonaria concinna*	EF489334	EF489353	EF489300	EF489378
Amphiboloidea	Amphibolidae	*Phallomedusa solida*	DQ093440	DQ279991	DQ093484	DQ093528
Hygrophila	Lymnaeidae	*Lymnaea stagnalis*	EF489345	EF489367	EF489314	EF489390
Stylommatophora	Arionidae	*Arion silvaticus*	AY145365	AY145392	AY947380	AY987918
Systellommatophora	Onchidiidae	*Onchidella floridana*	AY427521	AY427486	EF489317	EF489392
Glacidorboidea	Glacidorbidae	*Glacidorbis rusticus*	FJ917211.1	FJ917227.1	FJ917264.1	FJ917284.1
EUOPISTHOBRANCHIA						
Umbraculoidea	Tylodinidae	*Tylodina perversa*	AY427496	AY427458	—	AF249809
Anaspidea	Akeridae	*Akera bullata*	AY427502	AY427466	AF156127	AF156143
Cephalaspidea s.s.	Diaphanidae	*Toledonia globosa*	EF489350	EF489375	EF489327	EF489395
'LOWER HETEROBRANCHIA'						
Acteonoidea	Acteonidae	*Rictaxis punctocaelatus*	EF489346	EF489370	EF489318	EF489393

*Sequences generated in the present study. §Aitengidae sp. in Jörger *et al.* (2010), described as new in the present study. †*P. marteli* Neusser *et al.* (2011).

Central nervous system (CNS) (Fig. 1A, C, D): CNS euthyneurous with paired cerebral (cg), optic (og), pedal (pg), pleural (plg), buccal (bg) and gastro-oesophageal ganglia (gog) and four distinct ganglia on visceral nerve cord (Figs 1C, 2B, 3). All ganglia prepharyngeal, except buccal and gastro-oesophageal ganglia (Fig. 1D). Cerebral, pedal and pleural ganglia linked by short connectives forming prepharyngeal nerve ring (Figs 1D, 2B, 3). Cerebral ganglia (Figs 1C, 2B, 3) linked by short commissure. Labiotentacular nerve (ltn) (Figs 1C, D, 2A, 3) emerges anteriorly from cerebral ganglion. Optic

Figure 1. 3D reconstruction of *Aiteng ater*. **A.** General microanatomy, dorsal view. **B.** Mantle cavity, dorsal view. **C.** Central nervous system, dorsal view. **D.** CNS and anterior part of digestive system, left view. **E.** Digestive system (only main branch of digestive gland reconstructed), right view. **F.** Circulatory and excretory systems, dorsal view. **G.** Reproductive system, dorsal view. **H.** Anterior copulatory organs, ventral view. **I.** Female reproductive system including sperm storing receptacles, right view. Abbreviations: a, anus; alg, albumen gland; am, ampulla; ao, aorta; apg, anterior pedal gland; at, atrium; bc, bursa copulatrix; bg, buccal ganglion; cg, cerebral ganglion; cns, central nervous system; dg, digestive gland; do, distal oviduct; dv, dorsal vessel; ed, ejaculatory duct; ey, eye; f, foot; fgl, female gland; fgo, female gonopore; gog, gastro-oesophageal ganglion; i, intestine; ltn, labial tentacle nerve; nb, notum border; od, oviduct; oe, oesophagus; og, optic ganglion; on, optic nerve; ot, oral tube; ov, ovotestis; p, penis; pag, parietal ganglion; pc, pericardium; pcc, pedal commissure; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; pn, pedal nerve; pod, postampullary gonoduct; pr, prostate; prd, preampullary gonoduct; ps, penial sheath; r, radula; rpd, renopericardioduct; s, statocyst; sgd, salivary gland duct; sgl, salivary gland; sp, spicule cavity; subg, subintestinal ganglion; supg, supraintestinal ganglion; v, ventricle; vd, vas deferens; vg, visceral ganglion; vn, visceral nerve; *, aggregation of nerve cells. Scale bars: **A** = 700 μm; **B, E** = 500 μm; **C** = 300 μm; **D, H, I** = 200 μm; **F, G** = 600 μm.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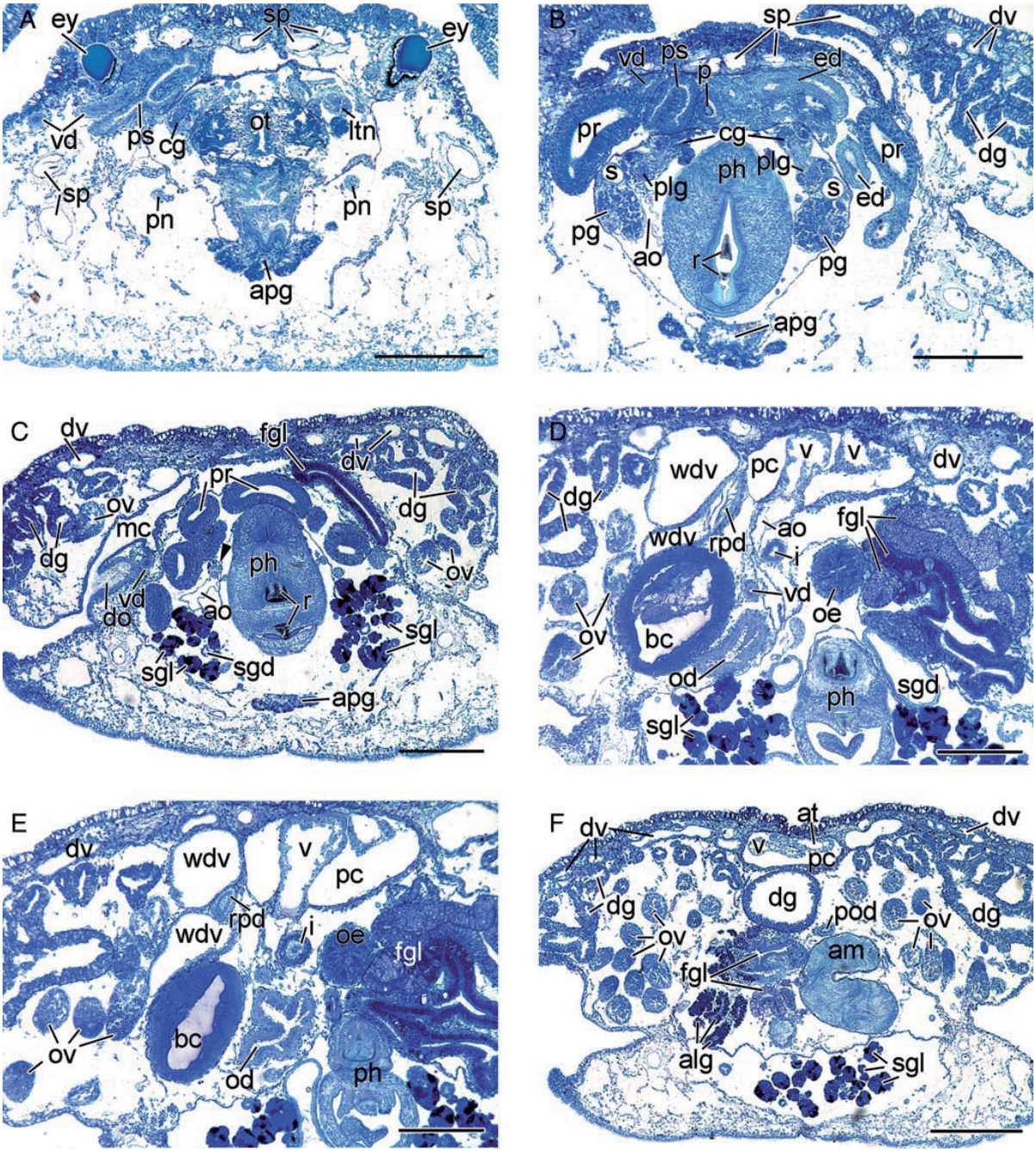

Figure 2. Histological cross-sections of *Aiteng ater*. **A.** Eyes, vas deferens and penial sheath. **B.** Ganglia, prostate. **C.** Mantle cavity. **D.** Dorsal vessels, renopericardioduct. **E.** Bursa copulatrix, ovotestis. **F.** Ampulla. Abbreviations: alg, albumen gland; am, ampulla; ao, aorta; apg, anterior pedal gland; at, atrium; bc, bursa copulatrix; cg, cerebral ganglion; dg, digestive gland; do, distal oviduct; dv, dorsal vessel; ed, ejaculatory duct; ey, eye; fgl, female gland; i, intestine; ltn, labial tentacle nerve; mc, mantle cavity; od, oviduct; oe, oesophagus; ot, oral tube; ov, ovotestis; p, penis; pc, pericardium; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; pn, pedal nerve; pod, postampullary gonoduct; pr, prostate; ps, penial sheath; r, radula; rpd, renopericardioduct; s, statocyst; sgd, salivary gland duct; sgl, salivary gland; sp, spicule cavity; v, ventricle; vd, vas deferens; wdv, wide lumen of dorsal vessel; arrowhead, aggregation of nerve cells on visceral nerve cord. Scale bars: **A, B** = 250 μm; **C** = 300 μm; **D, E** = 200 μm; **F** = 400 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

ganglion (Figs 1C, 3) attached laterally to each cerebral ganglion. Optic nerve (on) (Figs 1C, 3) emerges from optic ganglion innervating pigmented eye (ey) of 150 μm (Figs 1C, D, 2A, 3). Precerebral accessory ganglia absent. Pedal commissure (Fig. 1D) longer than cerebral commissure. Statocyst (Figs 1C, D, 2B, 3) attached dorsally to each pedal ganglion

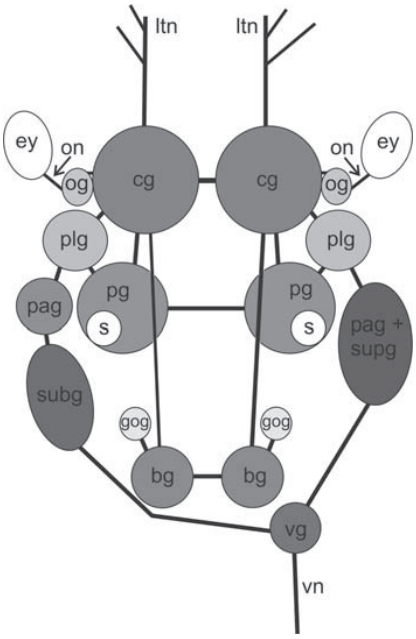

Figure 3. Schematic overview of the central nervous system of *Aiteng ater* (dorsal view). Abbreviations: bg, buccal ganglion; cg, cerebral ganglion; ey, eye; gog, gastro-oesophageal ganglion; ltn, labial tentacle nerve; og, optic ganglion; on, optic nerve; pag, parietal ganglion; pg, pedal ganglion; plg, pleural ganglion; s, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve. Not to scale.

(Figs 1D, 2B, 3). Pleural ganglion (Figs 1C, 3) connected to visceral nerve cord by very short connective. Four separate ganglia on visceral nerve cord (Figs 1C, 3): left parietal ganglion (pag), subintestinal ganglion (subg), small visceral ganglion (vg) and fused supraintestinal/right parietal ganglion (pag + supg). Aggregation of few cells on visceral nerve cord (Figs 1C, 2C) between visceral ganglion and fused supraintestinal/right parietal ganglion. No osphradial ganglion and no histologically differentiated osphradium detected. Paired buccal ganglia (Figs 1C, D, 3) posterior to pharynx, short buccal commissure ventrally to oesophagus. Small gastro-oesophageal ganglion (Figs 1C, D, 3) dorsally to each buccal ganglion.

Digestive system: Anterior pedal gland (apg) (Figs 1E, 2A–C) discharging ventrally of mouth opening to exterior. Oral tube (ot) (Figs 1E, 2A) short. Radula (r) U-shaped (Figs 1D, E, 2B, C), 1–1.2 mm long, embedded within muscular pharynx (ph) (Fig. 1D, E, 2B–E). Ascending and descending limbs almost equally long (Fig. 1D), each terminating in muscular bulb. Radula formula 57 × 1.1.1, 33 rows of teeth on upper ramus, 24 rows of teeth on lower one. Each row consists of rhachidian tooth and one lateral tooth on each side. Lower ramus without any lateral teeth in oldest part, only *c*. 7 of youngest teeth of lower ramus with lateral teeth (Fig. 4A). Triangular rhachidian tooth (Fig. 4A–C) with one large, projecting central cusp (cc). Central cusp with up to 20 lateral denticles (ld) on each side (Fig. 4B, C). Distance between lateral denticles increasing towards tip of central cusp. Right lateral tooth (ltr) (Fig. 4B, D) plate-like with one pointed, well-developed denticle (d) (Fig. 4B, D) and 10–15 smaller denticles (sd) on anterior margin (Fig. 4D). Prominent notch (n) on posterior margin in which denticle of anterior lateral tooth fits. Posterior margin with emargination on inner side of tooth. Left lateral tooth (ltl) (Fig. 4A, E) plate-like with two well-developed, pointed denticles on anterior margin, two prominent notches (n) on posterior one. Jaws absent. Oesophagus (oe) (Figs 1D, E, 2D, E) short, ciliated. One pair of large, folliculate salivary glands (sgl) (Figs 1E, 2C–F) connected via salivary gland ducts (sgd) (Figs 1E, 2C, D) at transition between pharynx and oesophagus. No distinct stomach detected. Digestive gland (dg) (Figs 1E, 2B–F) ramified, consisting of long main branch extending posteriorly and several smaller lateral branches only partly reconstructed. Intestine (i) (Figs 1E, 2D, E) densely ciliated, short. Anus (a) (Fig. 1E) opens on right side of body posterior to female gonopore into narrow and deep cavity (Fig. 1B).

Circulatory and excretory systems: Circulatory and excretory systems dorsal to digestive system. Circulatory system with wide, thin-walled pericardium (pc) surrounding large two-chambered heart (Figs 1F, 2D–F, 5A) with anterior ventricle and posterior atrium (Figs 1F, 2D–F, 5A). Aorta (Figs 1F, 2D, 5A) extending to head from anterior of ventricle. Renopericardioduct (rpd) (Figs 2D, E, 5A) well developed, densely ciliated, next to mantle cavity (Figs 1B, 2C); it connects to extensive system of ramified dorsal vessels (Figs 1A, F, 5A). The latter with very thin epithelium with minute vacuoles (Fig. 2C–F) inside cells extending to notum border. Part of dorsal vessels connected to renopericardioduct wider (wdv) than other branches of dorsal vessels (Figs 2D, E, 5A). However, histologically both parts look identical; distinct kidney with characteristic large, highly vacuolated cells absent. Nephroduct and nephropore not detected.

Reproductive system: Reproductive system ventral to digestive system, hermaphroditic and showing a special androdiaulic condition (Fig. 6). Ovotestis (ov) with follicles (Figs 1G, 2D–F, 6) located in semicircle over whole visceral sac. Tiny ducts emerge from follicles, joining in preampullary gonoduct (prd) (Fig. 6). Large tubular ampulla (am) (Figs 1I, 2F, 6) with autosperm in disorder. Sperm heads short. Receptaculum seminis absent or not developed in examined specimen. Four nidamental glands (Figs 1G, I, 2D–F, 6) from proximal to distal: ramified albumen gland (alg) discharges into postampullary gonoduct (Figs 1I, 2F, 6), followed by three glands with different histological and staining properties. Distal part of nidamental glands extends to right side of body where hermaphroditic duct bifurcates into internal vas deferens (vd) and short oviduct (od) (Figs 1I, 2D, 6). Bursa copulatrix (bc) large (Figs 1G, I, 2D,E, 6), splits off oviduct, without pronounced bursal stalk. Distal oviduct (do) opens through female gonopore (fgo) (Figs 1I, 2C, 6) at right side of body into narrow and deep cavity (Fig. 1B, 2C). Female gonopore considerably anterior to anus. Internal vas deferens (Figs 1G, H, 2A, 6) extends subepidermally up to head connecting to long, tubular prostate gland (pr) (Figs 1G, H, 2B, C, 6). Muscular ejaculatory duct (ed) (Figs 1H, 2B, 6) arises from prostate, discharges at top of penis (p) (Figs 1H, 2B, 6). Penis slender, without any stylet or spine, partially surrounded by thin-walled penial sheath (ps) (Figs 1H, 2A, B, 6).

Remarks: Our microanatomical results substantially revise the original description of *A. ater*, with discrepancies related to all organ systems (summary in Table 5). The original description of the CNS of *A. ater* is limited to mentioning four prepharyngeal ganglia, two of them being the fused cerebro-pleural ganglia. Instead, our reconstruction clearly shows the cerebral and pleural ganglia being separated rather than fused. We supplement the original description with the presence of the paired optic, buccal and gastro-oesophageal ganglia and four

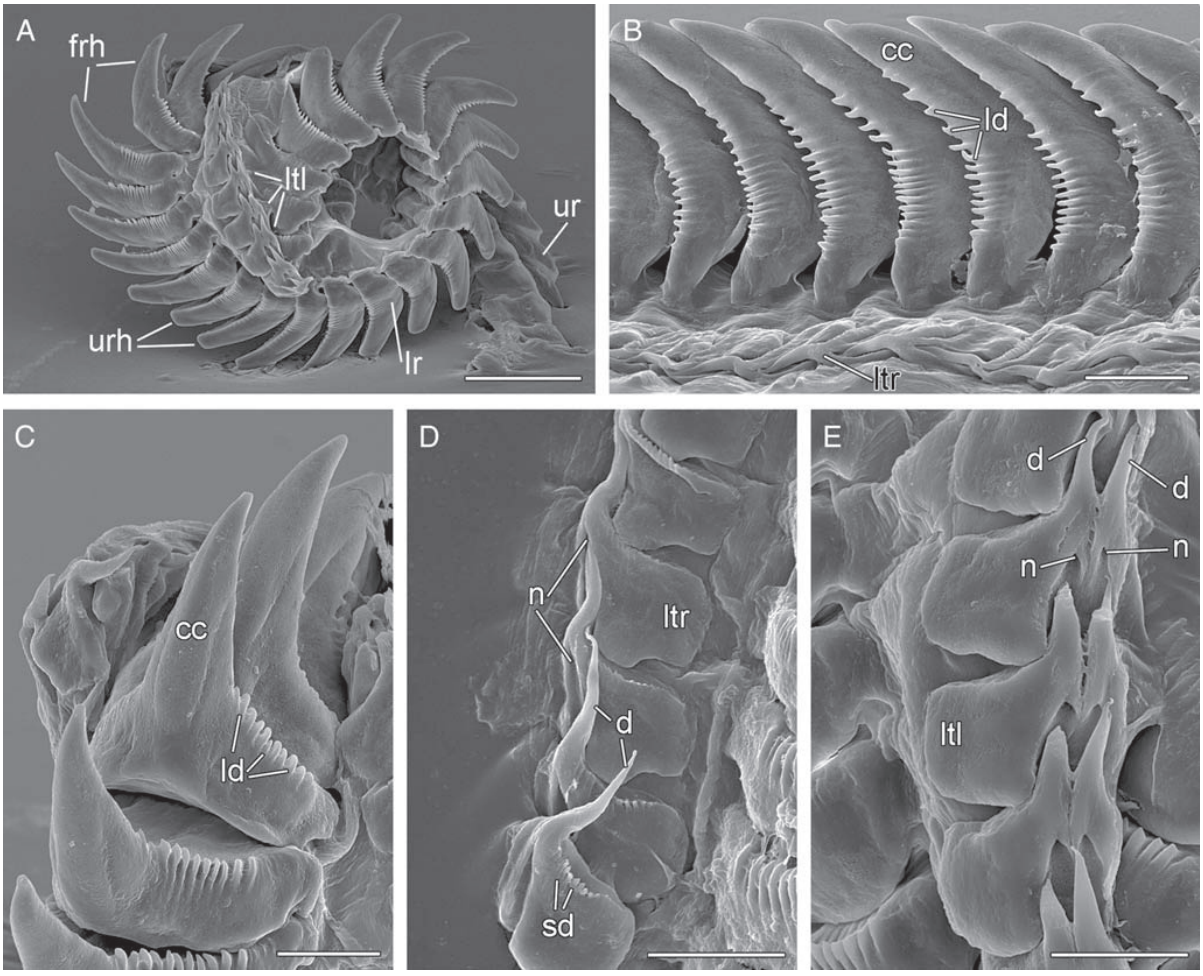

Figure 4. SEM micrographs of the radula of *Aiteng ater*. **A.** Radula, left view. **B.** Rhachidian teeth, right view. **C.** Rhachidian teeth, anterior view. **D.** Right lateral teeth. **E.** Left lateral teeth. Abbreviations: cc, central cusp; d, denticle; frh, functional rhachidian tooth; ld, lateral denticle; lr, lower ramus; ltl, left lateral tooth; ltr, right lateral tooth; n, notch; sd, small denticle; ur, upper ramus; urh, used rhachidian tooth. Scale bars: **A** = 60 μm; **B**–**E** = 20 μm.

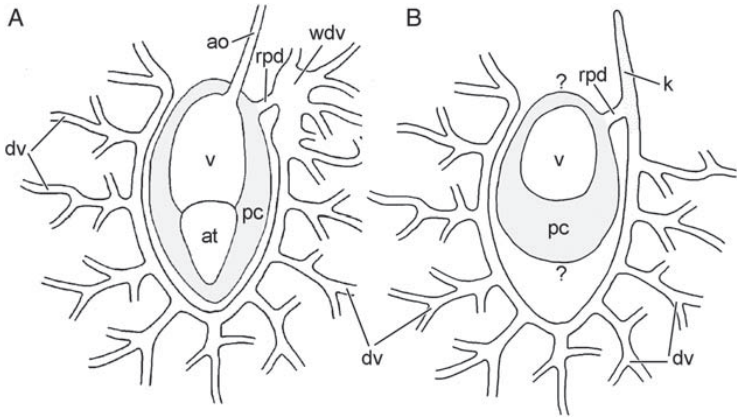

Figure 5. Schematic overview of the circulatory and excretory systems (dorsal view). **A.** *Aiteng ater*. **B.** *Aiteng mysticus* n. sp. Abbreviations: ao, aorta; at, atrium; dv, dorsal vessel; k, kidney; pc, pericardium; rpd, renopericardioduct; v, ventricle; wdv, wide lumen of dorsal vessel; ?, no data available. Not to scale.

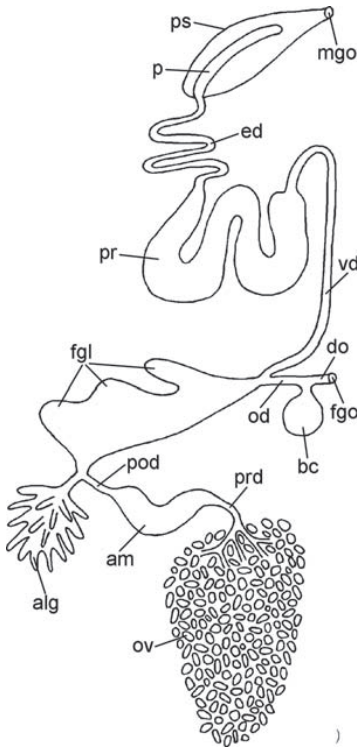

Figure 6. Schematic overview of the reproductive system of *Aiteng ater* (dorsal view). Abbreviations: alg, albumen gland; am, ampulla; bc, bursa copulatrix; do, distal oviduct; ed, ejaculatory duct; fgl, female gland; fgo, female gonopore; mgo, male gonopore; od, oviduct; ov, ovotestis; p, penis; pod, postampullary gonoduct; pr, prostate; prd, preampullary gonoduct; ps, penial sheath; vd, vas deferens. Not to scale.

ganglia on the visceral nerve cord. Additionally, there is an aggregation of several cells on the visceral nerve cord between the visceral ganglion and the fused right parietal-supraintestinal ganglion, which is not considered as a true ganglion herein. Our data about the digestive system match generally with the original description; however, a histologically distinct stomach could not be detected. This is consistent with other acochlidian species originally described with a stomach, e.g. *Asperspina murmanica* (Kudinskaya & Minichev, 1978) or *Pontohedyle milaschewitchii* (Kowalevsky, 1901), that were shown to possess a distal cavity of the digestive gland rather than a distinct stomach (Jörger *et al.*, 2008; Neusser *et al.*, 2009b). The intestine in *Aiteng ater* is short rather than long and opens into a deep and narrow cavity that was not mentioned by Swennen & Buatip (2009); probably, this cavity was misinterpreted as the intestine opening to the exterior. This narrow but deep cavity, receiving the anal and female genital openings and, likely, the (nondetected) opening of the closely associated excretory system, is herein interpreted as a putative mantle cavity. In the absence of other typical mantle cavity organs such as gills or osphradia, and without ontogenetic evidence, such an interpretation is speculative. However, the marine hedylopsacean *Hedylopsis ballantinei* was described as possessing a similarly small mantle cavity in which the anus, nephropore and gonopore open and that has a special cell type not observed on the normal body integument (Fahrner & Haszprunar, 2002; Sommerfeldt & Schrödl, 2005). In contrast, the originally reported presence of a large longitudinally separated mantle cavity in *Asperspina murmanica* could be rejected in our re-examination; here the body orifices open directly to the exterior (Neusser *et al.*, 2009b). Though situated in a similar position, the mantle cavity in *A. ater* is a deep cavity with a small opening rather than a transversal ciliated groove as in elysiid sacoglossans (Jensen, 1992); whether or not the latter also represents a reduced and modified mantle cavity should be clarified by comparing the microanatomy of shelled and shell-less sacoglossans in histological detail.

Table 5. Comparison of *Aiteng ater* with *A. mysticus* n. sp.

	Aiteng ater Swennen & Buatip, 2009	*Aiteng ater* Swennen & Buatip, 2009	*Aiteng mysticus* n. sp.
Data source	Swennen & Buatip (2009)	Present study	Present study
Habitat	Mangrove forest	See orig. description	On or underside of rocks
Body size (mm)	8–12 (alive)	3.5 (preserved)	4–6 (alive)
Body colour	Grey-black	See orig. description	Brownish, pale
CNS	Prepharyngeal	Prepharyngeal	Prepharyngeal
Fused cerebro-pleural ganglia	Present	Absent	Absent
No. of ganglia on visceral nerve cord	?	4	2 or 3
Oesophagus	Short	Short	Long
Radula	Uniseriate	Triseriate	Triseriate
Radula length (μm)	<900	1,200	900
Radula formula	59–67 × 0.1.0	57 × 1.1.1	70 × 1.1.1
Rhachidian tooth	cc projecting, 6–10 ld	cc projecting, 20 ld	cc large, 7–9 ld
No. of denticles on right lateral tooth	?	1 large, 10–15 small	1 large, 4–6 small
No. of denticles on left lateral tooth	?	2 large, no small	1 large, 12–13 small
Ascus	Present	Absent	Absent
Intestine	Long	Short	Short
Heart	?	Two-chambered	One-chambered
Kidney	?	Indistinct from dorsal vessels	Present
Vas deferens splits off	Postampullary duct	Female glands	Female glands
Small mantle cavity	Absent	Present	Present
Endoparasites	Present	Absent	Absent
Spicules	Absent	Present	Present

Abbreviations: cc, central cusp; ld, lateral denticle; ?, no data available.

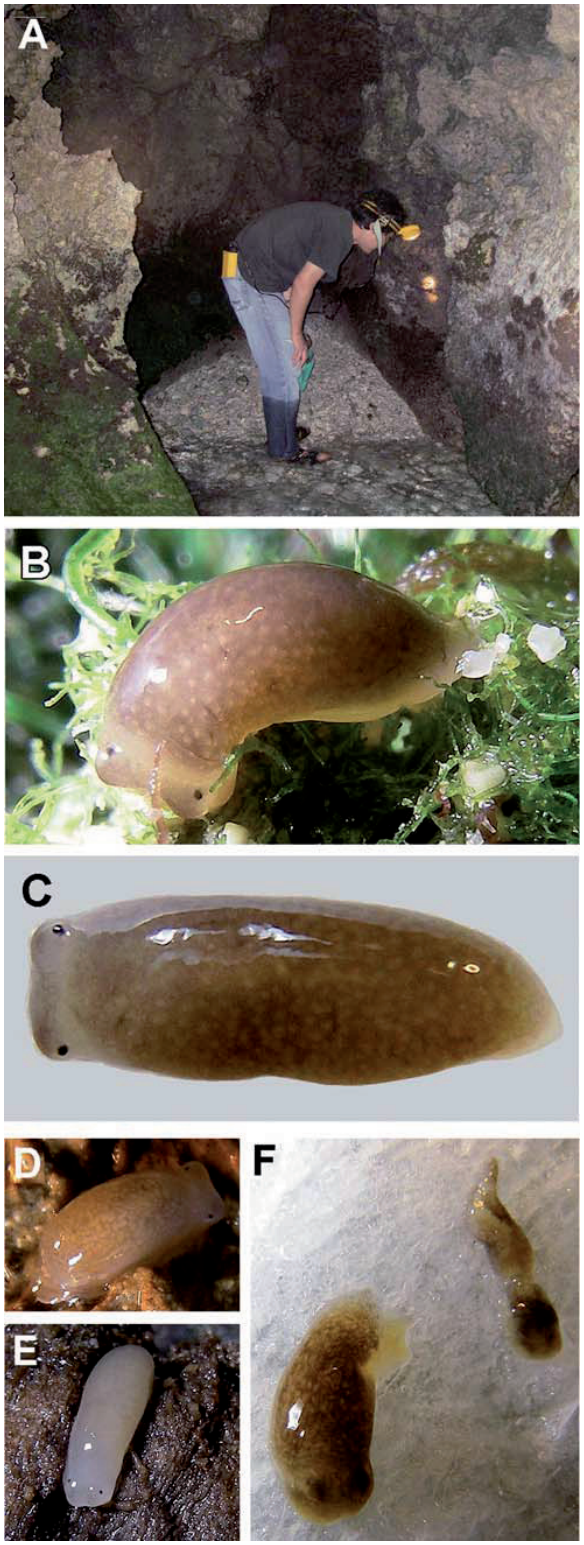

Figure 7. Habitat and external morphology of *Aiteng mysticus* n. sp. **A.** Coastal cavern on Miyako Island, Okinawa, Japan. **B–D, F.** Living specimens of *c.* 5 mm on Miyako Island. **B.** On algae. **C.** Brownish coloration. **D.** Pale coloration. **E.** Pale coloration (Yonaguni Island). **F.** Autotomy.

The radula in *A. ater* was reported as being uniseriate with only one rhachidian tooth per row, but our histological sections suggested the presence of one lateral tooth on each side. The examination by SEM clearly confirms the presence of a triseriate radula with a rhachidian tooth and one lateral tooth on each side (the latter of which is lacking in the oldest rows of the descending limb). In contrast to the original description we could not detect any sacoglossan-like ascus and there are no broken teeth at the posterior end of the descending limb in the pharynx. However, both radular limbs terminate in a separate muscular bulb.

Besides mentioning heart beats there are no more data about the circulatory system in the original description. Our reconstruction shows *A. ater* with a well-developed two-chambered heart, an aorta emerging from the ventricle, and the renopericardioduct connecting to a widened lumen of the dorsal vessel system. Our results for the reproductive system match well with the original data with one difference: whereas in the original description the postampullary hermaphroditic duct splits into vas deferens and oviduct, in our study the vas deferens splits off distal to the female glands, i.e. spermatocytes have to pass the female glands before entering the internal vas deferens and being transported to the male copulatory organs.

Swennen & Buatip (2009) reported "white, cigar-shaped bodies of different sizes" distributed "under the skin and loose on other organs in some specimens" of *A. ater* and supposed these were endoparasites. We cannot confirm this finding; instead our histological sections indicate the presence of subepidermal spicules (Figs 1A, 2A, B), which are distributed over the whole body, but concentrate in the head. We suppose these spicules have been misinterpreted in the original description as the endoparasites, as the latter dissolved later in the laboratory in an acidic solution (C.K. Swennen, personal communication).

***Aiteng mysticus* new species**

(Figs 5B, 7B–F, 8–10)

Type material: Holotype: in 75% ethanol, *c.* 3 mm (ZSM Mol 20110185). Type locality Shimozaki, Nikadori, Hirara, Miyako Island, Okinawa, Japan, 24°49′49″N, 125°16′42″E.

Paratypes: two section series (ZSM Mol 20110186, ZSM Mol 20110188), one radula on SEM stub (ZSM Mol 20110187), two specimens in 99% ethanol (NSMT Mo 77319, OKCAB M21473) and one in 5% formalin with radula on SEM stub (OKCAB M21474). For localities see Table 1.

Etymology: After the Japanese common name 'himitsu namekuji' (English: secret slug), given to the specimens when they were found.

Material examined: See Table 2.

Distribution: Known from Miyako Island, Kuroshima Island and Yonaguni Island (Okinawa Prefecture, Ryukyu Islands, Japan).

Habitat: The specimens were found in two different habitats. In Nikadori, Miyako Island, the animals were found on the surface of notches and lateral walls of small caves formed by erosion caused by strong waves (Fig. 7A), on shores of white limestone facing the open sea. In the intertidal zone were many small crevices which were usually moist with seawater and covered with two algae, *Caulacanthus ustulatus* (Gigartinales: Caulacanthaceae) and *Cladophora herpestica* (Cladophorales: Cladophoraceae). The specimens were

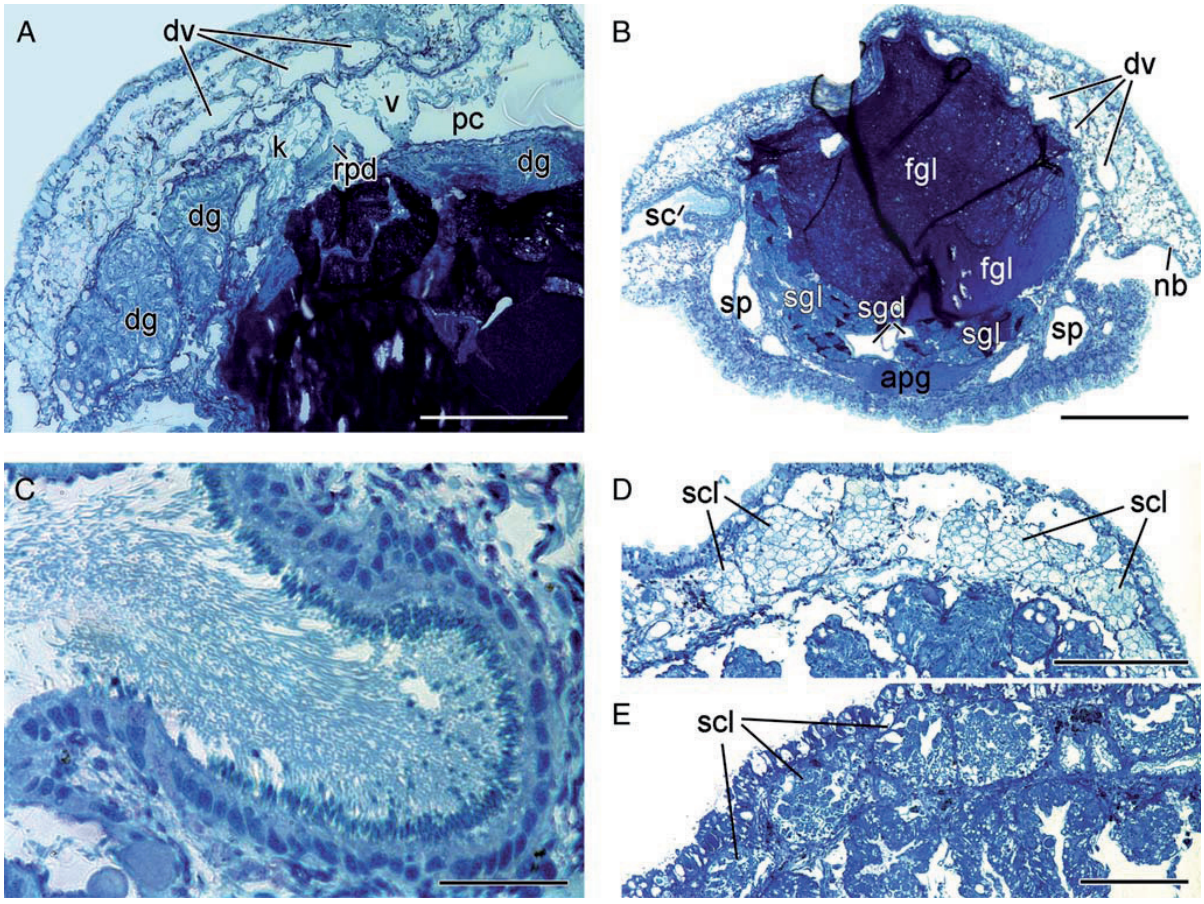

Figure 8. A–**D.** Histological cross-sections of *Aiteng mysticus* n. sp. **A.** Kidney, pericardium. **B.** Female glands, spermatocytes under notum border. **C.** Spermatocytes. **D.** Supporting cells. **E.** Supporting cells in *Aiteng ater*. Abbreviations: apg, anterior pedal gland; dg, digestive gland; dv, dorsal vessel; fgl, female gland; k, kidney; nb, notum border; pc, pericardium; rpd, renopericardioduct; sc, spermatocytes; scl, supporting cells; sgd, salivary gland duct; sgl, salivary gland; sp, spicule cavity; v, ventricle. Scale bars: **A** = 150 μm; **B** = 200 μm; **C** = 20 μm; **D, E** = 100 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

observed crawling just above the high tidal line at night from 11 p.m. to 5 a.m., together with *Paludinella* sp. and *Angustassiminea* sp. (both Assimineidae), *Pedipes jouani*, '*Allochroa*' aff. *affinis* and *A. layardi* (all Ellobiidae). While the ellobiids occurred in high numbers, *Ai. mysticus* was rare and it was hard to find more than two individuals in the same locality in one night. As reported for most of the ellobiid species found in the same habitat (Fukuda, 1996), *A. mysticus* is truly nocturnal and rapidly disappears after sunrise. In the same habitat the large chiton *Acanthopleura spinosa* (Chitonidae) was often found alive at midnight. Sasaki, Hamaguchi & Nishihama (2006) reported the distribution and habitat of *Ac. spinosa* in Miyako Island, and *Ai. mysticus* was also collected from one of their localities. The habitat of *Ai. mysticus* in Kuroshima Island was similar to Nikadori, but *Ac. spinosa* was not found. In Yonaguni Island, *Ai. mysticus* was found in a narrow space among rocks at the innermost part of a spacious cave (about 10 m in width and length) similar to the Nikadori habitat. The inside of the cave was always dark and humid. The accompanying molluscan species were the same as those of Nikadori, with the addition of *Ditropisena* sp. (Assimineidae) and the ellobiid *Microtralia* sp.

Aiteng mysticus was also found in Matsubara, Miyako Island, however the habitats differ considerably. This site was a brackish area neighbouring a small mangrove swamp on a narrow (about 10 m) river estuary at the innermost part of a small bay. Many rocks of various sizes lay on flat, sandy-mud bottom in the intertidal. *Aiteng mysticus* was found alive beneath large rocks (30–50 cm diameter) deeply buried in mud in the upper intertidal zone, during daytime. The underside of these rocks was usually wet. *Angustassiminea* sp. and several other ellobiid species (e.g. *Blauneria quadrasi*, *Laemodonta monilifera*, *L.* aff. *minuta*, *L. octanflacta*, *L. typica*, *Melampus fasciatus*, *Me. granifer*, *Me. parvulus*, *Me. sculptus*, *Melampus* sp., *Microtralia* sp. and *Pedipes jouani*; see Fukuda, 1996) were also found.

The two habitats mentioned above were rather different from each other, but *Angustassiminea* sp., *Pedipes jouani* and *Microtralia* sp. were observed in both. Among them, *P. jouani* was considered to be restricted to notches or caves in the rocks. Judged from the presence of *P. jouani* and *Aiteng mysticus*, the two habitats may share some environmental conditions that are suitable for these two species. Two specimens of *Ai. mysticus* from the two habitats were found to share exactly the same COI sequence (see below), supporting their conspecific status.

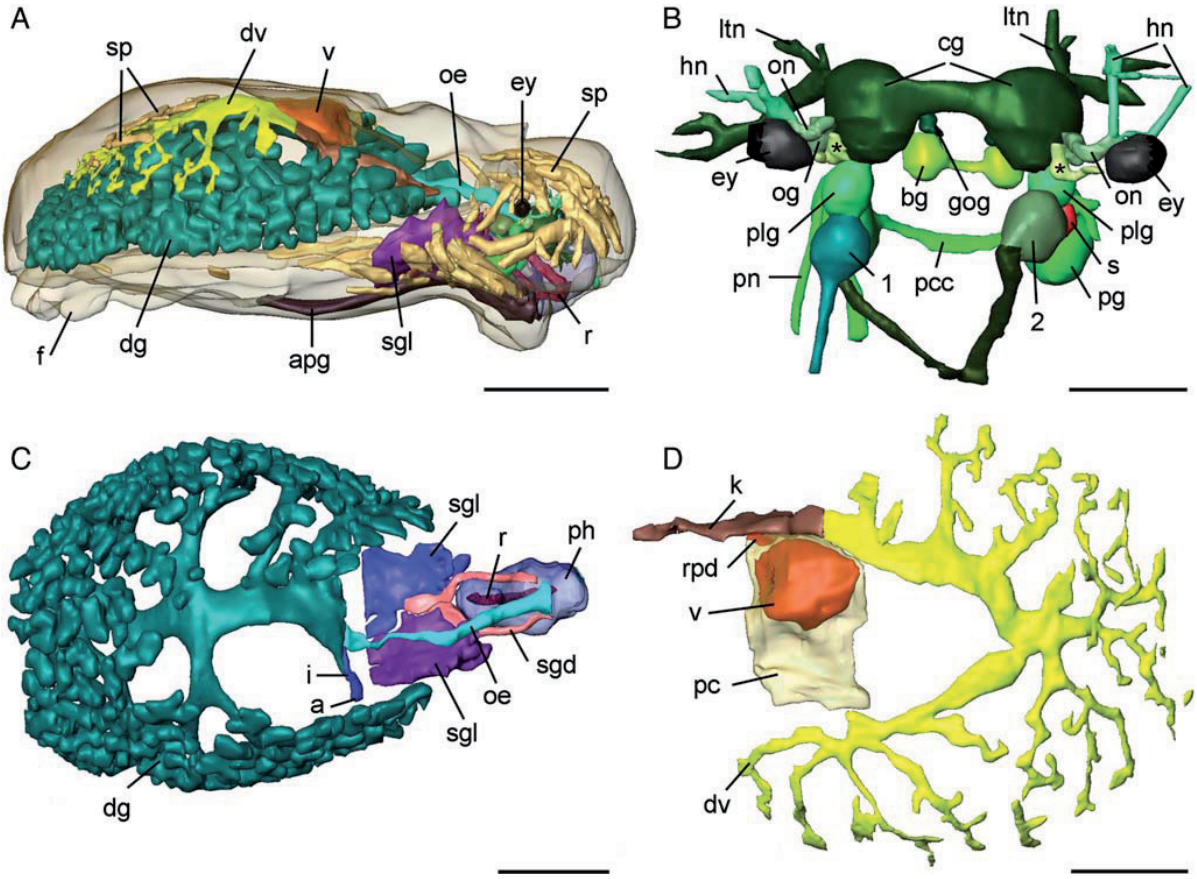

Figure 9. 3D reconstruction of *Aiteng mysticus* n. sp. **A.** General microanatomy, right view. **B.** Central nervous system, dorsal view. **C.** Digestive system, dorsal view. **D.** Circulatory and excretory systems, dorsal view. Abbreviations: a, anus; apg, anterior pedal gland; bg, buccal ganglion; cg, cerebral ganglion; dg, digestive gland; dv, dorsal vessel; ey, eye; f, foot; gog, gastro-oesophageal ganglion; hn, Hancock's nerve; i, intestine; k, kidney; ltn, labial tentacle nerve; oe, oesophagus; og, optic ganglion; on, optic nerve; pc, pericardium; pcc, pedal commissure; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; pn, pedal nerve; r, radula; rpd, renopericardioduct; s, statocyst; sgd, salivary gland duct; sgl, salivary gland; sp, spicule cavity; v, ventricle; 1,2, ganglia on the visceral nerve cord; *, ganglion attached to the cerebral ganglion. Scale bars: **A, C** = 400 μm; **B** = 150 μm; **D** = 300 μm.

External morphology of living specimens: Slug-like, lacking cephalic tentacles or other body processes (Fig. 7B, C). Length *c*. 5 mm. Dorsal surface glossy from copious mucus. Dorsal mantle pale to purplish brown. Brown coloration (Fig. 7B–D) variable in intensity, some individuals (e.g. from Yonaguni Island; Fig. 7E) paler than others. Large, vacuolated supporting cells visible as many distinct white granules through translucent skin of dorsal mantle (Figs 7, 8D). Head with pair of short, round bulges with distinct black eyes at postero-lateral corners. Head colour almost same as on dorsal mantle. Dorsal foot around head with thin pigment of same colour as dorsal mantle. Shallow transverse groove across anterior part of foot (uncertain whether or not this is an artefact by contraction). Sole flat, elongate oval, pale beige, without pigmentation. It consists of propodium and rest of foot: propodium occupies anterior 1/6 of whole foot; weak constriction on both sides at posterior end of propodium. Indistinct longitudinal groove on centre from portion just posterior to propodium to posterior end of foot. Foot simple, round. Lateral sides of foot pale beige without pigments.

Possible autotomy observed in one individual from Nikadori (Fig. 7F). While kept alive in small container, posterior edge of mantle and foot suddenly separated from rest of animal. This happened automatically without disturbance, but might have been a reaction to change of environmental condition from field to laboratory. The individual was still alive and crawled after this.

Central nervous system: CNS of *Aiteng mysticus* euthyneurous, prepharyngeal (Fig. 9B); arrangement of ganglia mainly as in *A. ater* (Fig. 3). Paired cerebral ganglia (cg) connected by short cerebral commissure. Labiotentacular nerve (ltn) (Fig. 9B) emerges from cerebral ganglion anteriorly. Optic ganglion (Fig. 9B) attached laterally to each cerebral ganglion; connective not detected. Optic nerve (on) arises from optic ganglion innervating pigmented eye (ey) of 100 μm (Fig. 9A, B). Hancock's nerve (Fig. 9B) splits off optic nerve innervating Hancock's organ. Small ganglion (Fig. 9B) attached to cerebral ganglion posterior to optic ganglion with unknown function. Precerebral accessory ganglia absent. Paired pedal ganglia (pg) ventral to cerebral ganglia; pedal commissure (Fig. 9B) considerably longer than in *A. ater*. Statocyst small, attached to each pedal ganglion. Pleural ganglion (plg) smaller than cerebral and pedal ganglia, posterior to both;

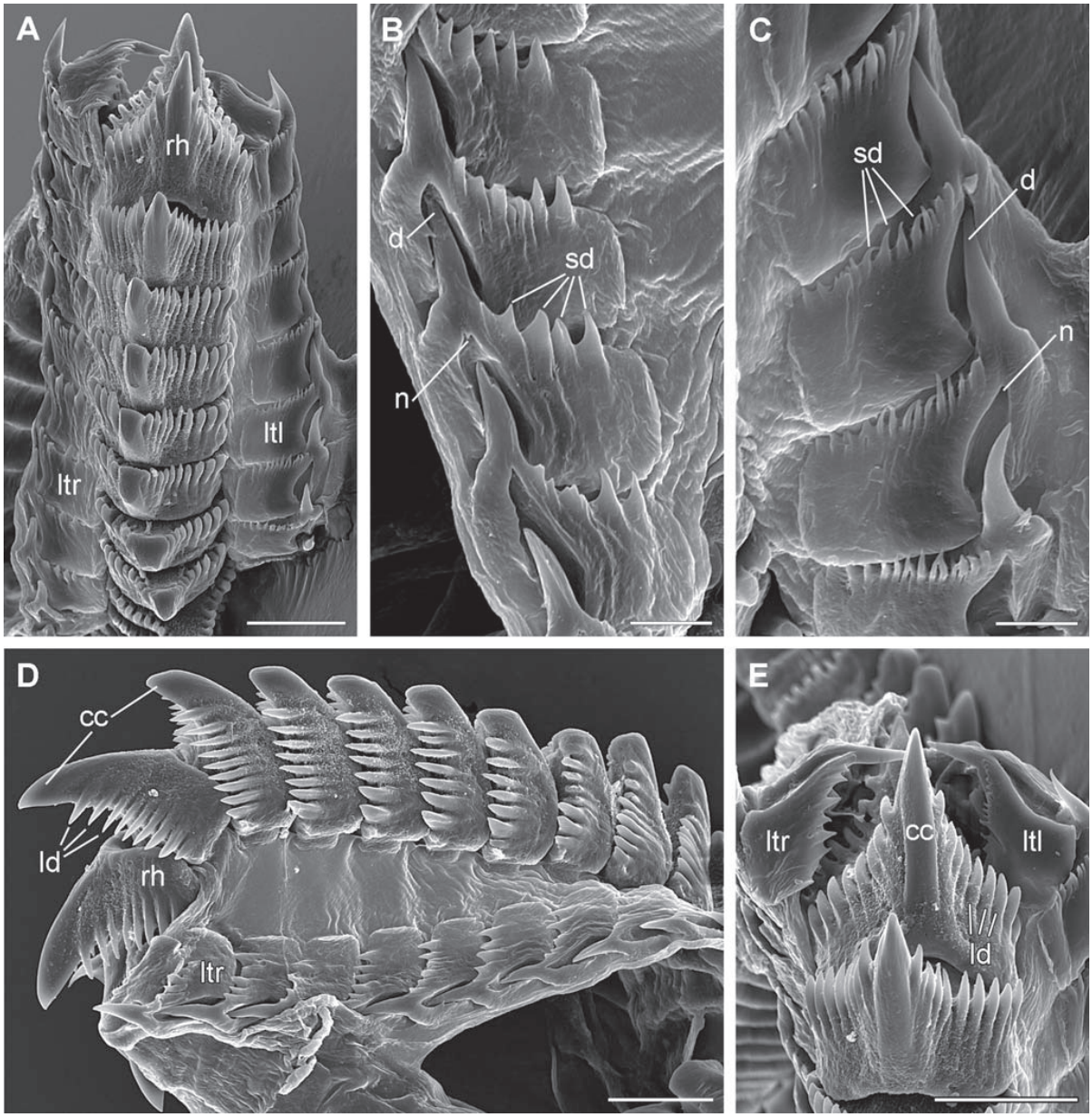

Figure 10. SEM micrographs of the radula of *Aiteng mysticus* n. sp. **A.** Rows of radular teeth (anterior view). **B.** Right lateral teeth. **C.** Left lateral teeth. **D.** Rhachidian teeth, right view; **E.** Rhachidian teeth, anterior view. Abbreviations: cc, central cusp; d, denticle; ld, lateral denticle; ltl, left lateral tooth; ltr, right lateral tooth; n, notch; rh, rhachidian tooth; sd, small denticle. Scale bars: **A, D, E** = 20 μm; **B, C** = 6 μm.

pleural ganglion (Fig. 9B) clearly separated from cerebral ganglion. Visceral nerve cord with only two large ganglia (Fig. 9B), both at ends of visceral nerve cord next to pleural ganglia. In one specimen three ganglia on visceral nerve cord. No osphradial ganglion, no histologically differentiated osphradium detected. Buccal ganglion (bg) just posterior to pharynx; however, in 3D reconstruction shifted more anteriorly because buccal apparatus was somewhat withdrawn in this specimen. Small gastro-oesophageal ganglion (gog) dorsal to each buccal ganglion.

Digestive system: Digestive system closely resembles that of *A. ater*. Anterior pedal gland (apg) (Figs 8B, 9A) discharges ventrally of mouth to exterior. Oral tube (ot) very short. Radula (r) U-shaped (Fig. 9A, C), 900 μm long, within muscular pharynx (ph) (Fig. 9C). Ascending and descending limbs almost equally long, each terminating in muscular bulb. Radula formula 70 × 1.1.1, 26 rows of teeth on upper ramus, 44 rows on lower one. Each radular row with triangular rhachidian tooth and one lateral tooth on each side (Fig. 10A). Lower ramus without any lateral teeth in oldest

part, only *c*. 16 of youngest teeth of lower ramus bear lateral teeth. Rhachidian tooth (Fig. 10D, E) with one large central cusp (cc) with 7–9 thinner, pointed lateral denticles (ld) on each side (Fig. 10D, E). All lateral denticles of almost same size. Right lateral tooth (ltr) (Fig. 10B, D) elongated plate-like with one prominent, pointed denticle (d) on anterior margin and well-developed notch (n) on posterior one, in which denticle of anterior lateral tooth fits. Additionally, 4–6 small denticles (sd) (Fig. 10B) on inner side of right lateral tooth. Left lateral tooth (ltl) (Fig. 1C) with same shape as right one with one large denticle and well-developed notch, but anterior margin with 12 or 13 small denticles (Fig. 1C) which look smaller and thinner than on right side. Jaws absent. Oesophagus (oe) (Fig. 9C) long, ciliated. Paired salivary glands (sgl) large (Figs 8B, 9A, C) with numerous small follicles reconstructed only in part. Follicles connected by small ductules before uniting in broad salivary gland ducts (sgd) (Figs 8B, 9C) that discharge at posterior of pharynx. Digestive gland (dg) (Figs 8A, 9A, C) ramified, extending to posterior end of visceral sac, as in *A. ater*. Intestine (i) (Fig. 9C) densely ciliated, short. Anus opens on right side of body posterior to female gonopore into small mantle cavity.

Circulatory and excretory systems: Circulatory and excretory systems dorsal to digestive system (Fig. 9A). Circulatory system with one-chambered heart surrounded by thin-walled pericardium (Figs 5B, 8A, 9A, D). Aorta and atrium not detected. Renopericardioduct (rpd) (Figs 5B, 8A, 9D) well developed, densely ciliated, connected to kidney (Figs 5B, 9D) with highly vacuolated cells (Fig. 8A). Kidney is one anterior branch of ramified dorsal vessel system (Fig. 5B); can be distinguished only histologically; whereas dorsal vessels have very thin epithelium (Fig. 8A) with minute vacuoles inside cells, kidney is characterized by highly vacuolated tissue with large vacuoles. Nephroduct and nephropore not detected.

Reproductive system: Reproductive system of *A. mysticus* not reconstructed in 3D due to very compressed tissue; general anatomy as in *A. ater* (Fig. 6). Reproductive system hermaphroditic, special androdiaulic, ventral to digestive system. Ovotestis (ov) with follicles united by small ductules discharging into preampullary gonoduct. Ampulla large, tubular. Sperm heads short. Receptaculum seminis absent or not developed in examined specimen. Albumen gland with follicles, discharges into postampullary gonoduct. Other nidamental glands very compressed in examined specimens, cannot be distinguished clearly from each other. Hermaphroditic duct bifurcates into internal vas deferens and short oviduct. Bursa copulatrix large, splits off oviduct. Bursal stalk connects to distal oviduct which opens through female gonopore into small mantle cavity at right side of body. Internal vas deferens subepidermally on right side of body wall up to head, connects to glandular prostate; prostate tubular, coiled. Ejaculatory duct muscular, arises anteriorly from prostate, connects to slender penis lacking any armature. Penis surrounded by thin-walled penial sheath. Male gonopore opens to exterior on right side of body near eye. In one examined specimen spermatocytes (Fig. 8B, C) under notum on right body side. Spermatocytes all directed with their heads to body wall filling notum rim from head up to female gonopore.

Remarks: Autotomy is known from several nudibranch species which detach their cerata, e.g. in *Janolus* (Schrödl, 1996), and parts of their mantle (e.g. *Discodoris* sp.; Fukuda, 1994: pl. 40, fig. 793) or even their whole mantle as in *Berthella martensi* (see Rudman, 1998). However, autotomy of the foot as in *A. mysticus* is only known from a few gastropods, such as the vetigastropod *Stomatella varia* (see Taki, 1930) or the sacoglossans *Oxynoe panamensis* and *Lobiger serradifalci* (see Lewin, 1970).

Noteworthy is the triseriate radula of *A. mysticus* (and *A. ater*) in which the lateral teeth are not present over the whole length of the descending limb and only the youngest rows of the lower ramus and the whole upper ramus bear lateral teeth. The oldest, i.e. no more functional rows of the lower ramus consist only of the rhachidian tooth. This phenomenon is unknown to us and is not observed in any sacoglossan or acochlidian species. The triseriate radula of the Acochlidia bears lateral teeth in all tooth rows, although the lower limb is usually considerably shorter than the upper limb (Schrödl & Neusser, 2010). If we imagine the oldest teeth rows (without lateral teeth) eliminated in the aitengid species, the radula could be perfectly an acochlidian one. On the other hand, nonshelled sacoglossan species have smaller, preradular teeth in front of the normal teeth rows (Jensen, 1996). However, the presence of such preradular teeth in Aitengidae is not likely as the teeth on the lower limb have the same appearance as the younger teeth, only the central cusps are used and more worn.

Our observation of the spermatocytes situated in the notum rim with their heads directed to the body wall in *A. mysticus* is peculiar. This specimen had mature female glands and a filled ampulla could not be detected, thus autosperm might have been just released. If these spermatocytes were autosperm, the question arises why they are situated under the notum rim; perhaps autosperm were released accidentally when the animal was disturbed, but in this case we would expect the spermatocytes unorientated rather than directing their heads to the wall. Thus, it is probable that these spermatocytes are allosperm. As there is a penis in *A. mysticus*, sperm are perhaps transferred by the copulatory organ and attached to the body and not near or directly inside the genital pore by copulation. Similarly, in the nudibranch *Aeolidiella glauca* a spermatophore is attached to the mate's body and sperm migrate externally towards the gonopore (Haase & Karlsson, 2000; Karlsson & Haase, 2002).

Molecular phylogeny: Two specimens of *Aiteng mysticus* from different habitats on Miyako Island (Table 3) were found to share the same COI sequence, supporting their conspecificity. Independent of the combination of molecular markers *A. ater* and *A. mysticus* always cluster together in a highly supported Aitengidae clade (see Fig. 11 for ML tree based on the 28S + COI + 16S dataset; trees from other gene combinations not shown). In all analyses Aitengidae cluster outside of the well-supported monophyletic Sacoglossa and within acochlidian Hedylopsacea. Their position within Hedylopsacea, however, varies according to the different genes combined for analysis: in 18S + 28S and 18S + 28S + COI trees Aitengidae form the sister group to a clade uniting marine and brackish Pseudunelidae with limnic Acochlidiidae (trees not shown). When 16S is included in the dataset Aitengidae form the sister group to all remaining Hedylopsacea (Hedylopsidae, Pseudunelidae and Acochlidiidae). Monophyly of Acochlidia (uniting Microhedylacea and Hedylopsacea) is poorly supported and in some analyses not recovered at all due to pulmonate taxa separating both clades (e.g. *Glacidorbis* or *Hygrophila*). This may be a result of the taxon set that was selected to cover acochlidian and sacoglossan families, rather than to comprehensively represent all other major euthyneuran groups, as done by Jörger *et al*. (2010). Acochlidian relationships recovered in the present study are congruent with a previous morphology-based hypothesis (Schrödl & Neusser, 2010), only the paraphyly of Ganitidae is surprising. The Sacoglossa form a well-supported clade in all analyses, with a division into shell-bearing Oxynoacea (including *Cylindrobulla*) and shell-less Plakobranchacea, with Platyhedylidae as most basal offshoot. Internal sacoglossan

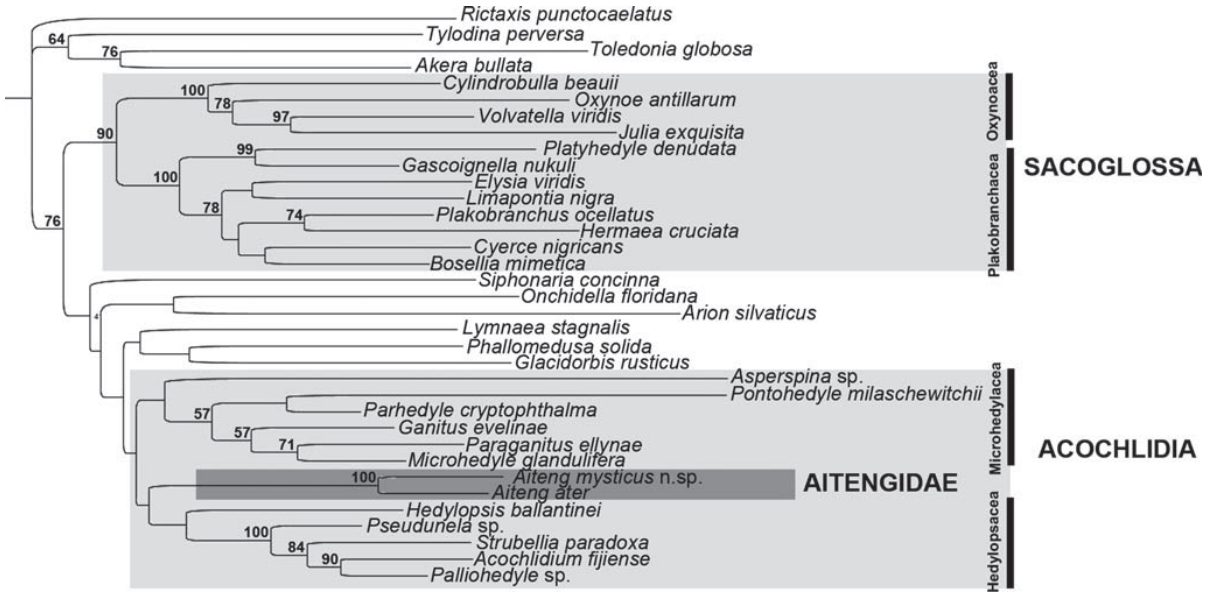

Figure 11. Maximum-likelihood tree generated with RAxML based on the concatenated 28S + COI + 16S dataset, clustering monophyletic Aitengidae basal within Hedylopsacea (bootstrap values >50% given above nodes) *Pseudunela* sp. = *P. marteli* Neusser *et al.*, 2011.

Table 6. Comparison of characteristic sacoglossan and acochlidian features with those of Aitengidae.

	Sacoglossa	Acochlidia	Aitengidae
Retractibility of the head	−	+	+
Calcareous spicules	−	+	+
CNS	Postpharyngeal	Prepharyngeal	Prepharyngeal
Cerebral and pleural ganglia separated	−	+	+
Radula	Uniseriate	Triseriate	Triseriate
Ascending and descending limb	+/−	+	+
Ascus	+	−	−
Branched digestive gland	+/−	+/−	+
Cephalic tentacles	−	+	−
Dorsal vessel system	+/−	−(+)	+
Albumen gland follicled	+	−	+

+, present; −, absent.

relationships slightly differ between the different analyses and resolved clades within Plakobranchacea are not entirely congruent with previous morphological analyses (Jensen, 1996).

DISCUSSION

Aitengid taxonomy

Our specimens from Japan can be clearly distinguished from *Aiteng ater* from Thailand by the habitat, the external morphology, the internal anatomy and perhaps by their feeding habits. *Aiteng ater* inhabits a dense mangrove forest high in the intertidal, which is not covered by the sea during high tides (Swennen & Buatip, 2009), but the specimens are always associated with small pools of water in the mud. In contrast, *Aiteng mysticus* n. sp. from Japan is found on rocky shores in the upper intertidal in tiny crevices of small sea caves that are usually wet by sea water; or, it is found in a brackish area neighbouring a mangrove swamp on the underside of large, wet rocks deeply embedded in mud in the upper intertidal zone. Although these various habitats are quite different, they all provide a wet and shaded environment without direct exposure to sunlight. Furthermore, both species show a higher activity during the night.

The external morphology of *A. ater* is quite different from that of *A. mysticus*: the body size of *A. ater* is 8–12 mm (Swennen & Buatip, 2009) whereas mature specimens of *A. mysticus* are smaller with a body length of 4–6 mm. The living coloration of *A. ater* is grey-black (Swennen & Buatip, 2009), but brownish or pale in *A. mysticus*.

The internal anatomy is different in nearly all organ systems. At the present stage of knowledge we do not consider the absence/presence of the tiny Hancock's nerve or the small additional ganglion attached to the cerebral ganglion as suitable for species identification, as these tiny structures can be easily overlooked. However, the number of ganglia on the visceral nerve cord differs more clearly between the species: two or three in *A. mysticus*, but (at least) four in *A. ater*. The digestive system is very similar in both aitengid species, but with great differences in radular structure: whereas the rhachidian tooth in *A. ater* has one large, projecting central cusp with up to 20 lateral denticles on each side, in *A. mysticus* there is one large central cusp with 7–9 thinner, pointed lateral denticles on each side. Furthermore, the lateral denticles are smaller in the *A. ater* and the distance between them increases towards the tip of the central cusp, whereas in *A. mysticus* they are larger and evenly spaced. The right lateral teeth in both species bear one pointed, well-developed denticle; in *A. ater* there are 10–15 very small denticles on the anterior margin, whereas *A. mysticus* has only 4–6 small denticles, which are considerably stronger than those of the species from Thailand. Additionally, there is an emargination on the posterior margin of the inner side of

the right lateral teeth in *A. ater*, which is absent in the Japanese species. There are great differences in the left lateral teeth: whereas there are two well-developed, pointed denticles without small denticles on the anterior margin in *A. ater*, there is only one large denticle but accompanied by 12 or 13 small denticles in *A. mysticus*.

The circulatory and excretory systems show major differences between the two species. Whereas a well-developed two-chambered heart is present in *A. ater*, we could only detect a one-chambered heart in *A. mysticus*; however, the epithelium of the pericardium and the atrium is very thin and both organs may collapse artificially. Thus, we do not consider the absence of an atrium as species-specific yet. The thin epithelium of the dorsal vessel system with small vacuoles looks histologically similar in both species. However, in *A. ater* the renopericardioduct connects to a widened lumen of the dorsal vessels, while in *A. mysticus* it is connected to a kidney. The latter is an anterior branch of the dorsal vessel system, but looks histologically very different and shows the characteristic tissue of the kidney with large vacuoles. Concerning the reproductive system we could not detect major differences between the two aitengid species.

The morphological and anatomical differences found in our study are paralleled by the molecular results, which show that our Japanese specimens belong to the family Aitengidae, but are distinct from *A. ater*. In all analyses *A. ater* and *A. mysticus* formed a highly supported clade (bootstrap 100%). Genetic similarities between the two *Aiteng* species are 89% in 16S rRNA and 85% in COI sequences.

Sacoglossa or Acochlidia?

Aiteng ater was described with an unusual mix of sacoglossan and acochlidian characters and the authors doubtfully suggested a sacoglossan relationship. A comparison of sacoglossan and acochlidian features is given in Table 6. Our results show that only a few characters remain that indicate a closer relationship to Sacoglossa: (1) the absence of any cephalic tentacles similar to e.g. the semi-terrestrial *Gascoignella aprica* (Jensen, 1985) or *Platyhedyle denudata* (Rückert, Altnöder & Schrödl, 2008); (2) the presence of an elysiid-like system of dorsal vessels, as in *Elysia* (Marcus, 1982; Jensen, 1996); (3) the albumen gland consisting of follicles as e.g. in the limapontioid *Hermaea* (Jensen, 1996). There are two ambiguous characters that are characteristic of at least some sacoglossan and acochlidian species: (1) the radula with an ascending and a descending limb present in all acochlidian species known in detail (Neusser *et al*., 2006, 2009a, b; Neusser & Schrödl, 2007, 2009; Jörger *et al*., 2008; Brenzinger *et al*., 2010) and e.g. in the sacoglossan *Ascobulla* (Jensen, 1996); (2) the branched digestive gland which has been reported from the limnic *Acochlidium fijiense*, *A. amboinense* and *Palliohedyle weberi* (Bergh, 1895; Bücking, 1933; Haynes & Kenchington, 1991) and which is present e.g. in the sacoglossan *Limapontia* and *Hermaea* (Jensen, 1996).

Finally, aitengids resemble acochlidians by (1) retractibility of the head; (2) presence of calcareous spicules; (3) prepharyngeal nervous system; (4) separated cerebral and pleural ganglia; (5) triseriate radula; (6) absence of a sacoglossan-like ascus; and (7) the "special androdiaulic reproductive system" (Schrödl, *et al*., 2011) as present in *Tantulum elegans*, *Pseudunela cornuta* and *P. espiritusanta* (Neusser & Schrödl, 2007, 2009; Neusser *et al*., 2009a). Furthermore, the large, laterally situated eyes of Aitengidae closely resemble the anatomy in members of the large, limnic acochlidian family Acochlidiidae (e.g. in *Strubellia paradoxa*) (Brenzinger *et al*., 2010); as well as the prominent rhachidian tooth of members of Aitengidae, which is used to pierce insects and pupae in *A. ater* and for piercing neritid egg capsules in *Strubellia* (Brenzinger *et al*., 2011). The case for the originally suspected sacoglossan relationship of *Aiteng* is clearly weakened and, based on our morphological results, the affinity to Acochlidia, in particular to limnic Acochlidiidae, is more evident. Morphological features alone, however, might not be sufficient to reveal correctly the systematic relationships of aberrant species inhabiting special habitats (see e.g. Schrödl & Neusser, 2010). Thus, supporting molecular evidence is needed.

In a recent multilocus molecular analysis, *A. mysticus* (as Aitengidae sp.) also clusters within hedylopsacean Acochlidia (Jörger *et al*., 2010); however, only a single aitengid species and single representatives of acochlidian families were included. Here we present a focused taxon sampling for Acochlidia and Sacoglossa and new sequence data for *A. ater*. Acochlidian rather than sacoglossan relationships for Aitengidae are again supported. Their position within Hedylopsacea, however, cannot be ascertained at the present stage of knowledge, differing depending on the molecular markers included: they are sister to a clade of marine/brackish Pseudunelidae and limnic Acochlidiidae in analysis of 18S + 28S (with or without COI); but sister to all remaining Hedylopsacea when 16S is included (see Fig. 11). A hedylopsacean origin of Aitengidae reflects morphological similarities discussed above. Any inner acochlidian origin would, however, imply that Aitengidae have lost the most characteristic acochlidian apomorphy (Sommerfeldt & Schrödl, 2005; Schrödl & Neusser, 2010), which is the subdivision of the body into a headfoot complex and a free, elongated visceral hump. Furthermore, the absence of cephalic tentacles gives the Aitengidae a compact external appearance that is very different from other marine or limnic Acochlidia.

Habitat shift

The question is whether or not these external differences between Aitengidae and other Acochlidia, and perhaps also some peculiar anatomical features, might be evolutionarily related to the habitat shift from an ancestrally aquatic to an amphibious lifestyle.

The cephalic head appendages and the free, elongated visceral sac of 'normal' aquatic acochlidian species are supported in shape while under water, but in air, e.g. during collecting, they collapse to an amorphous mass. Obviously, elongate head appendages on land should be hydrostatic and/or provided with muscles as in terrestrial stylommatophoran pulmonates, or must be reduced. Following the putative acochlidian relationship of Aitengidae, this implies that in *Aiteng* the ancestral rhinophores (as e.g. in the marine acochlidians *Pontohedyle milaschewitchii* and *Ganitus evelinae*; Marcus, 1953; Jörger *et al*., 2008) were lost, and labial tentacles became short lobes that fused to a velum. The compact body shape of aitengids with a short stout head might be also interpreted as an adaptation to an amphibious lifestyle, with the visceral hump connected to the foot on all its length guaranteeing better stability and minimizing the body surface.

Calcareous spicules in the connective tissue are already present in aquatic acochlidians, and in aitengids spicules are present but do not build an elaborate skeleton. However, the notum of aitengids shows a unique layer of large, vacuolated supporting cells. This layer almost certainly contributes to a more stable and robust body shape in Aitengidae. Probably the notal layer also provides some mechanical protection as well as protection from desiccation. By analogy, the sea slug *Corambe* shows a thickened protective notum that, however, hinders the diffusion of oxygen through the notal tissue and thus likely induced the multiplication of hyponotal gills (Martynov *et al*., 2011; Martynov & Schrödl, 2011). Despite the presence of the special notal supporting cell layer in *Aiteng*, the diffusion of oxygen is probably sufficient when animals are

exposed to air. If submerged for a long period, the compact gill-less animals may have a problem. Under any conditions, cells of the body wall need to be supplied with oxygen and other substances, and waste removed. We speculate that these and perhaps other functions might be carried out by the dorsal vessel system lying directly below the supporting cell layer, extending in fine ramifications to the notum border. Thus, the presence of the thin-walled dorsal vessel system of the Aitengidae, which is a modified portion of the kidney, is assumed to enhance respiratory, secretory and excretory processes in a secondarily amphibious lineage and, as such, might also be explained by the habitat shift.

Similar dorsal vessels exist in elysiid and some other non-shelled sacoglossans. Jensen (1992) assumed an excretory or osmoregulatory function, but also discussed a possible homology with the gills of the shelled sacoglossan species; so far neither cellular structures of sacoglossan dorsal vessels, nor the connections to the circulatory or excretory system, nor homologies with e.g. atrial, pericardial or renal tissue have been sufficiently explored. Accepting the phylogenetic distance between aitengids and elysiids, these vessel systems evolved convergently. Dorsal vessels have been discussed earlier as a 'negative gill' in sacoglossan species having functional kleptoplasts, i.e. species in which an excess of the oxygen produced must be transported away from the tissue (Jensen, 1996, and references therein). However, Aitengidae do not incorporate and maintain active plastids as do some sacoglossan species (Wägele *et al*., 2011) and therefore such a function is not imaginable in *Aiteng*.

The dark body coloration of aitengid species might be a protection from UV radiation to which these species could be exposed, in contrast to other acochlidian species which live hidden in sand or under stones. This coincides with the mostly nocturnal activity of Aitengidae preventing an excessive exposure to sunlight.

Regarding acochlidians, Bücking (1933) reported vessels emerging from the heart bulb and extending over the whole dorsal surface of the visceral sac in the limnic *Acochlidium amboinense* and suggested a respiratory function. Wawra (1979) observed vessel-like structures in *Palliohedyle sutteri*. However, both observations were based on preserved specimens only. Other limnic Acochlidiidae, such as *A. fijiense* and *A. bayerfehlmanni* were described to lack any vessels (Wawra, 1980; Haynes & Kenchington, 1991). Preliminary re-examinations of *A. amboinense* and *A. bayerfehlmanni* show both species to possess a dorsal vessel system that is, however, less ramified than in aitengids (own unpublished data). Thus a histological survey on all known Acochlidiidae is necessary to confirm the presence or absence of dorsal vessels and to clarify the homology and the function of such vessels in the large limnic Acochlidiidae. Only if they are part of the excretory rather than circulatory system, could acochlidiid and aitengid dorsal vessels be synapomorphic and thus support a sistergroup relationship, as suggested by further potential morphological apomorphies and some molecular analyses discussed above.

Finally, the habitat shift might induce a change in the feeding habits. While the prominent rhachidian tooth in *Strubellia* is used to feed on neritid egg capsules (Brenzinger *et al*., 2011), other molluscan eggs might not be available in the new habitat outside the water, but instead insects and pupae as in the case of *Aiteng ater*. The food source of *Aiteng mysticus* was not observed in the field. This species can be found frequently on intertidal algae, but shows no sign of feeding on algae. Furthermore, its pale coloration argues against any food containing plastids. Although the rhachidian tooth of *A. mysticus* is not as prominent as in *A. ater*, a grazing feeding habit is not likely. We assume that the food resource of *A. mysticus* is present on the algae and might consist of animal eggs or pupae similar to its congener from Thailand.

Conclusion

Aitengidae are small but highly specialized amphibious slugs, now known from two species from the Indian and Pacific Oceans. Traditional morphological means such as dissections and light microscopy gave a glimpse of the acochlidian relationship of *Aiteng ater*. Applying 3D-reconstruction methods to soft parts and SEM radula examinations substantially supplement and refine the original description of *A. ater* and reveal several putative apomorphies indicating the acochlidian nature of Aitengidae. Molecular data additionally support Aitengidae clustering within Acochlidia as a more or less basal offshoot of Hedylopsacea, implying a switch from aquatic to amphibious lifestyle. Considerable external dissimilarities and even aberrant anatomical structures such as the layer of vacuolated notal cells and the kidney that is modified into a highly ramified system of dorsal vessels can be explained as aitengid autapomorphies that evolved (or further elaborated) during that habitat shift. Surveying tropical slug diversity in different, not only aquatic, habitats may reveal further and perhaps even more specialized and aberrant creatures. Integrating biological observations such as 'bug-eating' with (micro)morphological and genetic data allows us to reconstruct an evolutionary scenario that turns a 'mysterious slug' into an instructive and amazing example of animal evolution.

AUTHORS' CONTRIBUTIONS

H.F. and Y.K. collected the material of *Aiteng mysticus*. H.F. wrote the sections on habitat and external morphology of *A. mysticus*. T.P.N. and H.F. examined the radula by SEM. K.M.J. and Y.K. carried out the molecular studies. T.P.N. carried out the morphological analyses and drafted the first manuscript version that was discussed and improved jointly. M.S. planned and supervised the study.

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Chapter 10. Panpulmonate habitat transitions: tracing the evolution of Acochlidia (Heterobranchia, Gastropoda)

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Panpulmonate habitat transitions: tracing the evolution of Acochlidia (Heterobranchia, Gastropoda)

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Abstract

Habitat transitions from marine to terrestrial and limnic systems have played a key role in the evolution and diversification of euthyneuran slugs and snails. Well-supported euthyneuran phylogenies with detailed morphological data provide the opportunity to study the historical, biological and ecological background in which these habitat shifts took place. Acochlidian slugs are 'basal pulmonates' with uncertain relationships to other major panpulmonate clades. They present a unique evolutionary history with representatives in the marine mesopsammon, but also benthic lineages in brackish water, limnic habitats and (semi-)terrestrial environments. We present the first comprehensive molecular phylogeny on Acochlidia, based on a global sampling that covers nearly 85 % of the described species diversity, and additionally, nearly doubles known diversity by undescribed taxa. Our phylogenetic hypotheses are highly congruent with previous morphological analyses, confirm all included recognized families and genera and thus provide a robust framework to trace the evolutionary history of Acochlidia. We further establish an ancestral chronogram for Acochlidia, document changes in diversification rates in their evolution via the birth-death-shift-model and reconstruct the ancestral states for major ecological traits. Based on our data, Acochlidia originated from a marine, mesopsammic ancestor adapted to tropical waters, in the mid Mesozoic Jurassic. We found major tectonic events influenced the biogeography of two major subclades with remarkably different evolutionary histories. The Microhedylacea are morphologically highly-adapted to the marine mesopsammon. They show a circum-tropical distribution with several independent shifts to temperate and temperate cold-habitats, but remained in stunning morphological and ecological stasis since the late Mesozoic. Their evolutionary specialization, including a remarkable and potentially irreversible meiofaunal syndrome, guaranteed long-term survival and locally high species densities also presented a dead-end road to morphological and ecological diversity. In contrast, the Hedylopsacea are characterized by morphological flexibility connected series of independent habitat shifts out of the marine mesopsammon, conquering (semi-)terrestrial and limnic habitats while reestablishing a benthic lifestyle and secondary 'gigantism' out of interstitial ancestors with a moderate adaptations to the mesopsammic world. The major radiations and habitat shifts in hedylopsacean families occur in the central Indo-West Pacific in the Paleogene. In the Western Atlantic only one enigmatic representative is known probably presenting a relict of a former pan-Tethys distribution of the clade. This study on acochlidian phylogeny and biogeography adds another facet of the yet complex panpulmonate evolution and shows the various parallel pathways in which these snails and

slugs invaded non-marine habitats. Given the complex evolutionary history of Acochlidia, which represent only a small group of Panpulmonata, this study highlights the need to generate comprehensively-sampled species-level phylogenies before addressing the larger picture.

Keywords: meiofauna, Euthyneura, sea slugs, progenesis, morphological stasis, habitat shifts

Introduction

Habitat transition from sea to land or freshwater and vice versa are considered infrequent across all metazoan clades, except for tetrapod vertebrates (Vermeij and Dudley, 2000). The general rarity of habitat shifts in the evolution of animal kingdom is likely due to the adaptive costs that a new physical environment demands, for example respiration, osmoregulation, reproduction and defensive features, and the competitive disadvantage of the new invaders against well-adapted incumbents (Vermeij and Dudley, 2000; Vermeij and Wesselingh, 2002). The evolution of euthyneuran gastropods, however, defies such generalizations: euthyneuran snails and slugs inhabit all aquatic and terrestrial habitats and pulmonate taxa especially have shown numerous independent shifts between habitats (Barker, 2001; Dayrat et al., 2011; Klussmann-Kolb et al., 2008; Mordan and Wade, 2008). The invasion of new habitats (especially terrestrial ones) played a key role in the diversification of euthyneuran gastropods leading to highest species richness and ecological diversity among this class of Mollusca (Mordan and Wade, 2008). Studying the historical context and the ecological and biological precursors, which facilitated habitat shifts in euthyneuran gastropods, should facilitate comparative evaluation in the future. Why can the barriers preventing habitat transition be overcome more easily by some groups of invertebrates than by others?

To study the evolution of features such as habitat transitions, a robust phylogenetic hypothesis on the sister group relationships among major clades and between taxa inhabiting different environments is required. Unfortunately, until recently, the phylogeny of Euthyneura could not be resolved satisfactorily (Brenzinger et al., 2013; Wägele et al., 2013) via cladistic analyses of morphological markers, probably due to a high degree of homoplasy (Dayrat and Tillier, 2002). Molecular phylogenetics has contradicted the

traditional division of Euthyneura in ‘Opisthobranchia’ and ‘Pulmonata’ (Grande et al., 2004; Klussmann-Kolb et al., 2008) and retrieved some former opisthobranch and ‘lower heterobranch’ clades in pulmonate relationships (Jörger et al., 2010b; Klussmann-Kolb et al., 2008; Schrödl et al., 2011a). For continuity in terminology, these newly allied groups are recognized with traditional pulmonates using the taxon name Panpulmonata (Jörger et al., 2010b). The new classification of Euthyneura is consistently retrieved based on molecular ‘standard markers’ (nuclear 18S and 28S rRNA and mitochondrial 16S rRNA and cytochrome oxidase subunit I) (e.g., Dayrat et al., 2011; Dinapoli and Klussmann-Kolb, 2010; Dinapoli et al., 2011; Jörger et al., 2010b; Klussmann-Kolb et al., 2008), but none of these molecular studies provides well-supported sister group relationships among the major panpulmonate taxa. The new classification was not supported by combining data from mitochondrial genomes analyzed for ‘Opisthobranchia’ (Medina et al., 2011) and ‘Pulmonata’ (White et al., 2011). However, mitogenomics does not seem suitable to resolve basal euthyneuran relationships (Bernt et al., 2013; Stöger and Schrödl, 2013). The first phylogeneomic approaches do not contradict panpulmonate relationships (Kocot et al., 2011; Kocot et al., 2013; Smith et al., 2011), but more comprehensive taxon sampling is required to resolve the panpulmonate tree reliably. Although considerable advances have been achieved with regards to well-supported monophyly of many major euthyneuran clades, ambiguities in the phylogenetic relationships among the major panpulmonate taxa still hinder overall evolutionary approaches, such as the transitions between aquatic and terrestrial habitats (Dayrat et al., 2011). Thus, at the current stage of knowledge, the evolution of panpulmonates can only be addressed via focusing on well-supported, undisputed clades, adding step-by-step to the complex picture.

One of the suitable panpulmonate clades to study habitat transitions in is Acochlidia. This major slug lineage – with regards to species diversity and local densities of specimens per m² – inhabits the interstitial spaces of the marine intertidal and shallow subtidal sands. Despite their global distribution and complex evolutionary history, known species diversity is still manageable with only 43 acochlidian species described at present. Based on the current phylogenetic hypothesis on Acochlidia (Jörger et al., 2010b; Schrödl and Neusser, 2010), their evolution comprises several habitat shifts 1) into the mesopsammon in the putative marine, benthic ancestor and 2) out of the mesopsammon - uniquely for interstitial gastropods - reestablishing a benthic lifestyle in a brackish environment in Pseudunelidae (Neusser and Schrödl, 2009), limnic habitats in Acochlidiidae (see e.g., Brenzinger et al., 2011b; Bücking, 1933; Haynes and Kenchington, 1991; Wawra, 1974, 1979) and

Tantulidae (Neusser and Schrödl, 2007; Rankin, 1979) and (semi-)terrestrial habitats in Aitengidae (Neusser et al., 2011a; Swennen and Buatip, 2009). In the past years, detailed microanatomical redescriptions from representatives of all seven acochlidian families and most of the 13 genera were conducted (Brenzinger et al., 2011a; Brenzinger et al., 2011b; Eder et al., 2011; Jörger et al., 2010a; Jörger et al., 2008; Jörger et al., 2007; Kohnert et al., 2011; Neusser et al., 2011a; Neusser et al., 2006; Neusser et al., 2009a; Neusser et al., 2011b; Neusser et al., 2009b; Neusser and Schrödl, 2007, 2009). In combination with a cladistic approach towards the phylogeny of Acochlidia based on a comprehensive matrix of morphological and biological characters (Schrödl and Neusser, 2010), these studies provide a reliable morphological dataset to study morphological adaptations preceding or resulting from habitat shifts and compare different evolutionary strategies across Acochlidia. Integrative approaches also demonstrated the limits of morphology, however, revealing a high degree of pseudocryptic or fully cryptic speciation in mesopsammic lineages (Jörger et al., 2012; Neusser et al., 2011b) and suspected misleading signal in morpho-anatomical phylogenetic analyses due to convergent adaptations (Schrödl and Neusser, 2010).

In the present study, we aim to establish a molecular phylogeny of Acochlidia with a comprehensive panpulmonate outgroup sampling and including all valid acochlidian species. Over the past years and with the support of a series of international collaborators (please see Acknowledgements for details) we successfully recollected at type localities worldwide (see Fig. 1) and are currently able to cover approx. 85 % of the described acochlidian diversity and adding another 50 % previously unknown lineages. In addition to generating this phylogeny, we also aimed to combine carefully calibrated molecular clock analyses and various model-based ancestral area reconstruction analyses to retrieve ancestral area chronograms as basis for hypotheses on vicariance events and dispersal in Acochlidia. Moreover, we wanted to reconstruct the history of major ecological traits (habitat, lifestyle and climate) by inferring ancestral states. Based on phylogenetic trees the rate of evolution that created present-day diversity can be evaluated. Typically, rates of diversification changes (speciation minus extinction) are detected via slope changes in lineage-through-time plots (LTT). We also wanted to implement a recent powerful likelihood approach that can detect shifts in the rate of diversification and avoid stochastic errors e.g. when only a small number of specimens is available (Stadler, 2011a). Moreover, it can account for incomplete taxon sampling (Stadler, 2011a), a fact which needs to be

considered in acochlidian evolution with the vast majority of the world's mesopsammic habitat still unsampled (Jörger et al., 2012; Neusser et al., 2011b).

With this combination of up-to-date approaches to reconstruct the evolutionary history of Acochlidia, we aim to provide knowledge on the biogeographic background of habitat shifts in Acochlidia and reevaluate morphological, biological and behavioral traits which might have triggered or resulted from these transitions. The role of progenesis in the evolution of this meiofaunal taxon is discussed, as is the impact of secondary 'gigantism' to cope with the physical requirements in freshwater and terrestrial habitats. The conclusions on the evolutionary history of Acochlidia aim to add a piece to the complex puzzle of the evolution and radiation of hyperdiverse panpulmonates and provide insights on requirements and consequences of habitat shifts in marine invertebrates.

Material and methods

Sampling and fixation

Sampling effort for Acochlidia was conducted in intertidal and subtidal sands worldwide, whenever possible covering type localities of nominal species (see Fig. 1 and Additional material 1 for collecting sites). In total, 36 described and 30 undescribed lineages (identified as molecular operational taxonomic units –MOTUs) are included here. Limnic specimens were collected by hand from the undersides of stones in rivers and streams. All material was observed alive and whenever possible photographed in the field. Limnic specimens were anesthetized prior to fixation using menthol crystals. Meiofaunal specimens were extracted from sand sampled using a $MgCl_2$-seawater solution and careful decantation technique (Schrödl, 2006) and again anesthetized with $MgCl_2$ prior to fixation to prevent retraction of the head-foot complex. Material was fixed in 75 % ethanol (for preparation of hard structures, such as radulae, spicules and copulatory stylets), 96-99 % ethanol (molecular analyses) and 2.5-4 % glutardialdehyde buffered in cacodylate (for histology and ultrastructure).

Unfortunately, our analyses lack one family of Acochlidia: monotypic Tantulidae. *Tantulum elegans* Rankin 1979 was discovered by Rankin (1979) in the muddy interstices of a freshwater mountain spring of the Caribbean Island of St. Vincent. The original type material is unsuitably fixed for sequencing attempts and was not obtained from the Royal Ontario Museum. New recollection attempts at the type locality (by KMJ) in February 2009

failed. The described locality could be localized precisely based on the available literature (Harrison and Rankin, 1976; Rankin, 1979), but has changed considerably probably due to agriculture and the (only) spring where *Tantulum* occurred had disappeared at least during that time of year.

DNA extraction, amplification and sequencing

DNA was extracted from entire specimens (in minute meiofaunal taxa) or from foot tissue (limnic taxa) using the DNA Qiagen Blood and Tissue Kit or the Macherey-Nagel NucleoSpin Tissue Kit. We followed the manufacture's extraction protocol, with overnight tissue lysis. We amplified four genetic standard markers via polymerase chain reaction (PCR): mitochondrial cytochrome c oxidase subunit I (COI) and 16S rRNA, and nuclear 18S rRNA and 28S rRNA using the same protocols and primers as listed in Jörger et al. (2010b). Successful PCR products were cleaned up using ExoSap IT (Affymetrix Inc.) (COI, 16S rRNA) or Macherey-Nagel NucleoSpin Gel and PCR Clean Up (28S rRNA). Cycle sequencing and sequencing reactions were performed by the Sequencing Service of the LMU using the PCR primers, Big Dye 3.1 and an ABI capillary sequencer. Voucher specimens are deposited at the Bavarian State Collection of Zoology (ZSM, Munich), DNA aliquots are stored and publicly available through the DNAbank network (http://www.dnabank-network.org) and all sequences are deposited to GenBank at NCBI (http://www.ncbi.nlm.nih.gov/genbank/) (see Table 1 for accession numbers).

Phylogenetic analyses

Sequences were edited with BioEdit (Hall, 1999) and Geneious 5.2 (Drummond et al., 2010). All sequences generated in this study were checked for putative contamination using BLAST searches (Altschul et al., 1990) to compare our sequences with published sequences in GenBank (http://blast.ncbi.nlm.nih.gov). Outgroup selection followed the latest phylogenetic hypothesis on the origin of Acochlidia (Jörger et al., 2010b) and aimed to cover all major lineages of Panpulmonata and also includes more distantly related Euopisthobranchia and a 'lower heterobranch'. Outgroup sequences were retrieved from GenBank (see Table 2 for accession numbers). Alignments for each marker were generated using MAFFT (E-ins-I- option) (Katoh et al., 2005). Ambiguous positions in the alignment were removed using Aliscore (Misof and Misof, 2009). Comparative masking with Gblocks (Talavera and Castresana, 2007) (options for a less stringent selection) resulted in the

removal of 300 more nucleotide positions but the topology of the final ML-tree was not affected. The COI alignment was checked manually and by translation into amino acids for potential shift in the reading frame and for stop codons. Sequences were concatenated using Sequence Matrix (Meier et al., 2006), and BioEdit (Hall, 1999) for the translated COI alignment.

Models were selected using jModeltest (Posada, 2008) from 5 substitution schemes and 40 models for each individual marker and the concatenated dataset. This resulted in the GTR+gamma+I-model for all four markers. We conducted maximum-likelihood analyses of the concatenated for-marker dataset, analyzed in four partitions corresponding to each marker. In our RAxMLv7.2.8 analyses we followed 'the hard and slow way' in RAxMLv7.0.4 Manual and generated five parsimony starting trees, applied 10 different rate categories, 200 random starting trees and non-parametric bootstrapping on 1000 trees. *Orbitestella vera* (Powell 1940) (Orbitestellidae, 'lower Heterobranchia') was defined as the outgroup.

Molecular dating

Molecular clock analyses – To estimate divergence times in Acochlidia we conducted relaxed molecular clock analyses using BEAST v1.6.1 (Drummond and Rambaut, 2007) on our concatenated four marker dataset. All analyses were run with the relaxed uncorrelated lognormal model in four partitions corresponding to each marker and under the Yule process using the GTR+Gamma+I substitution model for each marker. We selected calibration points, which we considered most reliable (i.e. taxa with a decent fossil record and comparably reliable assignation to present day taxa) and most conservative (i.e. potential older representatives with controversial assignment were ignored). A gamma-shaped distribution prior was selected setting a hard prior on the minimum age of the respective node, highest probability was assigned to the ages selected from literature and a 10 % range was chosen for the distribution curve. Fossil timing follows Tracey et al. (1993) and Bandel (1994) using five calibration points: the minimum age of the Anaspidea was set to 190 mya, Ellobiidae to 145 mya, Siphonariidae to 150 mya and Lymnaeidae to 140 mya. Moreover, we calibrated the closure of the Isthmus of Panama to 2.1 mya, accommodating the possibility of subsequent submersion after the closure about 3.4 mya (Cox and Moore, 2010). Analysis with all 5 calibration points was run for 100 000 000 generations. Results were analyzed in Tracer v1.5, and all values reached good effective sampling sizes. To produce a consensus tree, all trees were combined in TreeAnnotator v1.6.1, with the first 10

000 trees discharged as burn-in. To evaluate the impact of each calibration point on the time estimations, we performed sensitivity analyses and ran five additional BEAST analyses (30 000 000 generations each) omitting one calibration point in each of the analyses.

Rate of evolution – we applied two different methods to detect changes in the rate of evolution of Acochlidia. As input tree we used the ultrametric tree from our molecular clock analyses in BEAST and removed outgroups and multiple individuals of one species prior to analyses in R. We generated lineage-through-time plots (LTT) in R using the ape package (Paradis et al., 2004). Additionally, we conducted maximum-likelihood analyses applying the birth-death-shift process (Stadler, 2011a) to infer diversification rate changes in TreePar (Stadler, 2011b). As described by Stadler (2011a) the likelihoods of the model allowing *m* rate shifts were compared to the model allowing *m+1* rate shifts via the likelihood ratio test, applying a 0.99 criterion.

Ancestral area reconstruction

We used two different algorithms of ancestral area reconstruction: Statistical Dispersal-Vicariance analysis (S-DIVA) in RASP 2.1b (Yu et al., 2012) and dispersal-extinction-cladogenesis model (DEC) implemented in Lagrange (Ree et al., 2005; Ree and Smith, 2008). We coded the areas based on the biogeographic realms of the Marine Ecosystem of the World (MEOW) system designed for the world's coastal and shelf areas (Spalding et al., 2007). In general, meiofaunal Acochlidia lack a planktonic larval stage, thus dispersal abilities are considered low (Jörger et al., 2012; Neusser et al., 2011b; Swedmark, 1968). Additional to the MEOW realms, we further subdivided Eastern/Western regions of the Atlantic and Pacific, and separating the Pacific and Atlantic Coast of temperate South America, which resulted in 12 coded areas for the included material: Western Indo-West Pacific (WIP), central Indo-West Pacific (CIP), Eastern Indo-West Pacific (EIP), tropical Eastern Pacific (EPT), Southeast Pacific (SEP), Northeast Pacific (NEP), Northwest Pacific (NWP), tropical Western Atlantic (WAT), Northwest Atlantic (NWA), Southwest Atlantic (SWA), tropical Eastern Atlantic (EAT), Northeast Atlantic (NEA) (see Figure 1). No subdivisions into provinces of the MEOW were adopted, as they contradict distribution ranges based on molecular analyses and haplotype networks in comparably wide spread microhedylacean Acochlidia (Eder et al., 2011; Jörger et al., 2012). Ranges of ancestral areas should resemble those of recent representatives (Clark et al., 2008, Hausdorf, 1998), we thus allowed for a maximum of two areas at each ancestral node. For the input tree in both analyses, we used our chronogram generated with BEAST using all calibration points

discussed above, with outgroups removed. Multiple individuals from one species distributed in the same area were removed for ancestral area reconstruction. In Lagrange analyses, we included five dispersal matrices to accommodate differences in dispersal probabilities during the changing geological history of Earth: 1) from mid Jurassic (175 mya) to early Cretaceous (120 mya) prior to the breakup of Gondwana; 2) Early Cretaceous (120 mya) to late Cretaceous (90 mya) as the circum Tethyan Seaway allowed worldwide exchange of tropical coastal faunas, and the South Atlantic formed; 3) late Cretaceous (90 mya) to Miocene (20 mya) characterized by the ongoing Tethyan Seaway and completed formation of the Atlantic and Beringia prohibiting exchange between Northwest Pacific and North Atlantic fauna; 4) Miocene (20 mya) to Pliocene (3.5 mya): closure of Tethyan Seaway isolates Indo-West Pacific from Atlantic/ Pacific fauna, Bering Strait opens; 5) Pliocene (3.5 mya) to present: closure of the Panamanian Isthmus separates Western Atlantic from Eastern Pacific. Dispersal probabilities range from 0.1 between disconnected, separated areas and 1.0 between directly adjacent coastal areas. Dispersal between areas separated by one intermediate realm was set to 0.25, as were adjacent areas with supposed dispersal barriers for Acochlidia (e.g. trans-oceanic ranges or present day East Pacific barrier). For comparison, we ran a more constrained analyses restricting dispersal to (directly) adjacent regions.

Transitions in ecological traits

We reconstructed ancestral states of ecological traits (climate: tropical, temperate, temperate-cold; habitat: marine, amphibious-marine, brackish, limnic; life style, interstitial, benthic) using MESQUITE 2.75 (Maddison and Maddison, 2011) on our fully resolved maximum-likelihood topology. For comparison, we also repeated analyses using the slightly different topology generated in Bayesian analyses conducted with BEAST. To assess ancestral states, we performed maximum-parsimony and maximum-likelihood analyses, in the latter applying the Mk1 model (assuming all changes between states are equally probable). The selection of a more complex model was not supported using the likelihood ratio test from results in Bayes Traits (Pagel and Meade, 2006).

Results

Phylogenetic analyses

We retrieved our hypothesis on the phylogeny of Acochlidia from a maximum-likelihood analysis conducted with RAxML 7.2.8 on the concatenated four marker dataset (nuclear

18S rRNA, 28rRNA and mitochondrial 16S rRNA and COI) analyzed in four partitions corresponding to each marker. An overview of the topology with the sister group relationships of Acochlidia and the relationships among acochlidian families, is provided in Fig. 2. We included 57 outgroup taxa resembling a broad range of panpulmonate taxa to resolve the origin of Acochlidia within Panpulmonata. Unfortunately, as in previous analyses (Jörger et al., 2010b; Klussmann-Kolb et al., 2008) none of the sister group relationships of major panpulmonate subgroups is statistically supported (i.e. receives bootstrap values (BS) $\geq$ 75). But in all analyses conducted herein Acochlidia are monophyletic and are sister to a clade comprised of Hygrophila+(Pyramidelloidea+(Glacidorboidea+Amphiboloidea)). We included 36 out of 43 valid acochlidian species and 30 individuals belonging to roughly 20 or more putatively undescribed species, identified here as molecular operational taxonomic units (MOTUs) (see Fig. 3). The included taxa represent all recognized acochlidian families and genera, apart from the monotypic Tantulidae, with *Tantulum elegans* Rankin, 1979 being unavailable for molecular analyses. All acochlidian (super-)families inferred from cladistic analyses of morphological characters (Schrödl and Neusser, 2010) resulted monophyletic. In concordance with morphological analyses, Acochlidia split into Hedylopsacea (comprised of Hedylopsidae+(Aitengidae+(Hedylopsacea *incertae sedis*+(Pseudunelidae +Acochlidiidae)))) and Microhedylacea (comprised of Asperspinidae+Microhedylidae s.l. (i.e. including paraphyletic Ganitidae)). Neither the sistergroup relationship of Hedylopsacea and Microhedylacea, nor the deep splits within these clades, however, are supported by BS$\geq$ 75. Most recognized acochlidian families receive high statistical support (BS 75-100), but not Pseudunelidae and Acochlidiidae (see Fig. 3). One undescribed species of Hedylopsacea, which also unites a unique combination of morphological characters (KMJ unpublished data), resulted as the sister clade of Pseudunelidae+Acochlidiidae and thus formed a still unnamed 'family-level' clade (Hedylopsacea MOTU Moorea in Fig. 3). The topology of our analyses was concordant between the different phylogenetic approaches (maximum-likelihood, Figs. 2,3; Bayesian inference, Fig. 4) with the exception of amphibious Aitengidae, either being sister to all remaining hedylopsacean taxa (Fig. 4) or sister to Pseudunelidae+(Hedylopsacea sp.+Acochlidiidae) (Figs. 2,3). In our analyses all recognized acochlidian genera were monophyletic, apart from *Microhedyle*, which is paraphyletic due to the inclusion of *Paraganitus*, a result concordant with morphological analyses, but in the latter it is paraphyletic due to inclusion of Ganitidae (*Paraganitus*+*Ganitus*) (Schrödl and Neusser,

2010). With the exception of the genus *Pseudunela*, all monophyletic acochlidian genera are statistically highly supported (see Fig. 3). Next to the undetermined clade 'Hedylopsacea sp.', another undescribed species 'Acochlidiidae sp.', forms a separate 'genus-level' clade sister to *Palliohedyle+Acochlidium*. All remaining MOTUs cluster within recognized genera.

Molecular dating and rate of evolution

We performed sensitivity analyses by six independent molecular clock analyses to test the effect of different calibration points to our dataset (see material and methods for details). Differences for our time estimates on acochlidian evolution only vary slightly among the different analyses and no general pattern could be observed that one calibration point rejuvenates or artificially ages the recovered time estimates (see Additional material 2 for resulting node ages and ranges of 95 % highest posterior densities (HPD)). The values reported below and presented in Fig. 4 refer to our main molecular clock analyses using all calibration points listed in the material and method section. For the entire chronogram with uncollapsed outgroups see Additional file 3. According to our data, most major panpulmonate clades originated in the Mesozoic Jurassic (i.e. Siphonarioidea, Sacoglossa, Stylommatophora, (Systellommatophora+Ellobioidea), Hygrophila, (Glacidorboidea+ Amphiboloidea), Pyramidelloidea and Acochlidia), the split between Systellommatophora/Ellobioidea and the split between Glacidorboidea/Amphiboloidea are dated to the early Cretaceous (see Additional file 3). The origin of Acochlidia is dated to the mid Jurassic 176.6 mya (HPD: 207.9–171.0 mya). The diversification into the two major acochlidian clades – Hedylopsacea and Microhedylacea – occurred shortly after but still in Jurassic times 169.2 mya (HPD: 200.9–159.9 mya). The diversification of the Hedylopsacea into the recent families started in the late Jurassic to early Cretaceous with the origin of Aitengidae estimated to 144.4 mya (175.4–97.0 mya), the origin of Hedylopsidae to 121.8 mya (HPD: 160.1–83.5 mya) and the split between Hedylopsacea *incertae sedis* and (Pseudunelidae+Acochlidiidae) estimated to 88.9 mya (HPD: 113.0–48.5mya). Pseudunelidae and Acochlidiidae originate in the Paleogene 33.7 mya (HPD: 58.3–32.0 mya). Genera of Acochlidiidae originate during late Paleogene/early Neogene: the origin of *Strubellia* is estimated to 29.6 mya (HPD: 50.6–27.1 mya), the origin of undescribed Acochlidiidae sp. to 24.3 mya (HPD: 40.5–19.9 mya), and the origin of *Palliohedyle* and *Acochlidium* to 20.6 mya (HPD: 34.4–16.2 mya). The valid microhedylacean families

Asperspinidae and Microhedylidae are estimated to have a Mesozoic origin at 165.8 mya (HPD: 193.3–150.4 mya), with the radiation of *Asperspina* and *Pontohedyle* starting in the Cretaceous 84.5 mya/104.3 mya (HPD: 113.7–71.3 mya/133.5–83.5 mya). The origin of *Parhedyle* is estimated to the late Cretaceous/early Paleogene (56.3 mya; HPD: 87.5–44.6 mya). The origin of *Ganitus* and *Paraganitus* is estimated to the late Paleogene 43.2 mya resp. 26.6 mya (HPD: no estimate for *Ganitus* resp. 41.7–20.2 mya for *Paraganitus*).

To detect changes in the rate of diversification of Acochlidia we reconstructed a lineage-through-time (LTT) plot. Ignoring the time period prior to 100 mya (because the log number of lineages is too low), the LTT showed a continuous slope with no major changes (see Fig. 5A). One minor period of stasis was present at 40–35 mya followed by a slight increase in diversification, abating again slightly at 10 mya till present. Additionally, we used the likelihood approach with the birth-death-shift process described by Stadler (2011a) on our phylogeny to determine rate changes in the diversification of Acochlidia through time. In all tested scenarios, diversification rates were low with minimum 0.02 and maximum 0.12 per million years. Testing sampling intensities between 90-25 % the likelihood ratio test supported no shifts in rate changes (see Fig. 5B). Diversification rates remained constant between 0.021–0.025 per million years. With sampling intensities lowered to 10 (and 5 %), the model supported one rate shift in the evolution of Acochlidia at approximately 37 mya with the rates of diversification increasing from 0.026 (0.030) to 0.061 (0.075). An alternative scenario testing 1 % sampling intensity also supported one rate shift in evolution of Acochlidia but resulted in a considerable decrease (from 0.118 to 0.024) at approximately 123 mya (see Fig. 5B).

Ancestral area reconstruction of Acochlidia

Independent of the chosen approach (DEC model in Lagrange or S-DIVA), or the different models of dispersal allowed between the defined ocean ranges, no ancestral areas with robust support values could be recovered for the basal nodes in Acochlidia. In all approaches, their evolutionary history involves numerous dispersal (range expansion), vicariance and local extinction (range contraction) events. In Lagrange, the less constricted model (favoring dispersal via neighboring areas but not entirely prohibiting dispersal between unconnected areas, see Material and Methods) received better likelihood values and is presented in Fig. 4. Since the S-DIVA analysis was conducted without restrictions on area distributions, several ancestral areas include ‘impossible’ distribution ranges (across unconnected areas). Thus, S-DIVA results are only reported when not contradicted by

geography. The paleotopology of the continents changes considerably throughout the evolutionary history of Acochlidia and especially modern Ocean ranges are difficult to assign to Mesozoic ranges. Recent Acochlidia are restricted to costal and shelf areas, we thus aim to approximately allocate recent continental margins to the historic topologies at the ages of the reconstructed nodes (see also Discussion). The North-West Pacific (NWP) and the central Indo-West Pacific (CIP) both receive comparable support values in DEC analyses (relative probability p_r=0.11 each, referring to the North-Eastern part of the Tethys Ocean and the Western margin of the Panthalassic/ Pacific Ocean) as ancestral area for the diversification of Acochlidia (S-DIVA additionally includes the Eastern Indo-Pacific (EIP) and Western Atlantic Ocean (WAT) as potential ancestral areas). The radiation of Hedylopsacea originated in the waters nowadays belonging to the CIP (Eastern Tethys Ocean - p_r=0.21 S-DIVA: 0.5 for CIP, 0.5 for CIP+EIP). One major dispersal event occurred at the base of Hedylopsidae via the Western Indo-Pacific (WIP, i.e. Western part of the Tethys) into the North Atlantic (NEA) and also to EIP, in the Cretaceous all well-connected via the circum-equatorial Tethyan Seaway. Based on DEC, two vicariance events (in *Hedylopsis* and *Pseudunela*) occurred isolating sister species in WIP and CIP.

The biogeographic history of Microhedylacea is highly complex and characterised by numerous dispersal, local extinction and vicariance events (see Fig. 4). The primary radiation of Microhedylacea most likely (p_r=0.22) occurred in the waters nowadays belonging to the NWP (Western part of the Panthalassas/ Pacific Ocean, S-DIVA: 0.25 for CIP or WAT) with an eastward dispersal and range expansion to the North-East Pacific in the stemline of Asperspinidae. A vicariance event split the two asperspinid clades with the NWP-clade dispersing westward around the Asian continent into the North Atlantic. The NEP asperspinid clade (p_r=0.37, S-DIVA: 1.0 NEP) dispersing south-eastward via the East Pacific (EPT) into the Western Atlantic (WAT) and back again in *Asperspina* MOTU Washington (NEP) and from WAT eastward to NEA. The basal radiation of Microhedylidae s.l. most likely occurred in NEA (in late Jurassic resembled by the forming Atlantic Ocean, p=0.12). Along the stemline of *Pontohedyle* two major dispersal events occurred eastward via WIP into CIP (i.e. through the western part of the Tethys to the eastern part). One of the two major *Pontohedyle* clades disperses via the WIP and from this western part of the Tethys Ocean westward via the North East Atlantic into the Western Atlantic and also eastward into the Eastern Indo-West Pacific (see Fig. 4). The other shows a similar complex picture with several dispersals events among the waters of the Tethyan Seaway in both directions westward and eastward. Our data for example indicates three

independent dispersal events in *Pontohedyle* into EIP: twice eastward via CIP and once westward from the East Pacific. The genus *Parhedyle* (restricted to the North Atlantic) most likely (p_r=0.30) originated in NEA and dispersed into NWA (ancestral area reconstructed with S-DIVA: 1.0 NEA+NWA). A vicariance event split the ancestral population of (*Ganitus*+'*Microhedyle*') and ('*Microhedyle'*+*Paraganitus*), which was distributed across the Atlantic (p_r=0.50) into an originally Western Atlantic clade including *Ganitus* (p_r=0.50, S-DIVA: 1.0 WAT) (with one dispersal event back to the tropical East Atlantic and one into the Pacific) and one clade which originated in NEA+WIP (p_r=0.22) and dispersed westward via WAT to SWA and into the North-East Pacific and three times independently to the East (e.g. along the stemline of *Paraganitus,* with WIP+CIP as ancestral area of its radiation p_r=0.50, S-DIVA: 1.0 CIP).

Ancestral state reconstruction of ecological traits

We performed ancestral state reconstructions of three major ecological traits (climate, habitat and life style) using maximum-likelihood (values derived from maximum-parsimony are reported when differing). We also coded the traits in our outgroup taxa, but results might not be representative for these clades due to limited taxon sampling (see discussion). Based on our analyses the Acochlidia derive from a marine (likelihood (lh) 0.96) and benthic (lh 0.99) common ancestor with Hygrophila+(Pyramidelloidea+(Glacidorboidea+Amphiboloidea)) and originated in temperate waters (lh 0.98) (see Fig. 6). Along the acochlidian stemline, the ancestral acochlid inhabited tropical waters (lh 0.97) and invaded the interstitial habitat (lh 0.83; most parsimonious state: benthic or interstitial). Rerunning the ancestral state reconstruction without outgroup taxa, the ancestor of Acochlidia was clearly (lh 0.99) marine, interstitial, and inhabiting tropical waters. This also affects likelihoods for the hedylopsacean radiation originating from a marine, mesopsammic and tropical ancestor (lh 0.99).

The origin of the hedylopsacean radiation clearly occurred in tropical waters (lh 0.99), with *Hedylopsis spiculifera* currently being the only representative found in temperate waters. The radiation of the hedylopsacean clade shows a remarkable flexibility in habitat choice: the hedylopsacean ancestor likely inhabited the marine (lh0.99) mesopsammon (lh 0.84, most parsimoniously benthic or interstitial) and shifts to a benthic life style occurred in parallel three times: 1) along the stemline of Aitengidae with a shift to amphibious-marine habitat, 2) within Pseudunelidae with a shift to brackish habitat, and 3) at the base of

Acochlidiidae with the invasion of the limnic system. Alternatively (but with weaker statistical support lh 0.21), the ancestor of Aitengidae+(Hedylopsacea sp.+(Pseudunelidae+Acochlidiidae)) resumed a benthic lifestyle with two independent invasions back into the mesopsammon in Hedylopsacea sp. and Pseudunelidae. Within Pseudunelidae+Achochlidiidae hypotheses on the ancestral lifestyle are unclear: either the common ancestor was 1) benthic (lh 0.48) and within Pseudunelidae, the slugs are secondarily mesopsammic or 2) mesopsammic (lh 0.52) and the transition to a benthic lifestyle occurred twice independently (within Acochlidiidae and for *Pseudunela espiritusanta*). Testing the alternative topology suggested by Bayesian inference (see Fig. 4) with Aitengidae forming a basal offshoot to the remaining Hedylopsacean clades only slightly weakens the likelihood for an interstitial hedylopsacean ancestor (lh 0.78), but does not affect the results on ancestral character states described above and shown in Fig. 6. The entire diversification of Microhedylacea occurred in the marine mesopsammon, shifts are restricted to climatic zones, originating in tropical waters and inhabiting temperate zones at least five times independently (Fig. 6). *Asperspina* is the only acochlidian clade with members occurring in temperate-cold waters, but there is only a low probability that the asperspinid ancestor already inhabited temperate (lh 0.22) or temperate-cold waters (lh 0.20). Adaptation to cold waters probably (lh 0.88) occurred along the (*A. brambelli+Asperspina* MOTU Kamtschatka) stemline and between temperate and tropic waters another three times independently (Fig. 3). Additionally, the radiation of the genus *Parhedyle* occurred in temperate waters (lh 0.89) and it is currently the only genus with no representatives known from tropical waters. The ancestor of *Pontohedyle* inhabited tropical waters. *Pontohedyle milaschewitchii* is the only lineage in the tropical *Pontohedyle* clade, which secondarily adapted to temperate waters. Within paraphyletic *Microhedyle* an adaptation to temperate waters occurred twice independently in the Eastern Pacific and the Northern Atlantic.

Discussion

Acochlidian origin and phylogeny

The exact position of Acochlidia within panpulmonate euthyneurans remains unresolved in this study. Schrödl and Neusser (2010) demonstrated that parsimony analyses of morpho-anatomical characters easily lead to a grouping of Acochlidia with other minute, mesopsammic taxa as an artifact of multiple convergent adaptations. This explained previous phylogenetic hypotheses that are incompatible with recent molecular results, such

as the idea of a common origin with Rhodopemorpha (Salvini-Plawen and Steiner, 1996) or equally minute Runcinacea and Cephalaspidea (Wägele and Klussmann-Kolb, 2005). Molecular analyses have considerably rearranged the classical view on euthyneuran phylogeny, commonly rejecting monophyly of 'Opisthobranchia' and 'Pulmonata' and placing the traditional opisthobranch order Acochlidia in (pan)pulmonate relationships (Jörger et al., 2010b; Klussmann-Kolb et al., 2008). 'Opisthobranchia' are also in conflict with morphological analyses (Dayrat and Tillier, 2002; Haszprunar, 1985; Wägele et al., 2013; Wägele et al., 2008). Revisiting morphological characters of Euthyneura (*sensu* Jörger et al. (2010b)) in the light of the new phylogenetic hypothesis, the presence of rhinophores innervated by N3 (*nervus rhinophoralis*) might be an apomorphy for Euthyneura (Brenzinger et al., 2013; Wägele et al., 2013) and a gizzard (i.e. muscular oesophagial crop lined with cuticula) apomorphic for Euopisthobranchia (Jörger et al., 2010b). Currently, the double rooted rhinophoral nerve or the double rooted procerebrum presents the only putative morphological apomorphy for the highly diverse panpulmonate clade including Acochlidia (Brenzinger et al., 2013). Molecular studies on euthyneuran phylogeny provided high bootstrap support for most higher panpulmonate taxa (Dayrat et al., 2011; Dinapoli and Klussmann-Kolb, 2010; Jörger et al., 2010b; Klussmann-Kolb et al., 2008), but fail to recover decent support values on the relationships among major clades within Panpulmonata. Acochlidia are suggested to be sister to (Pyramidelloidea+Amphiboloidea)+Eupulmonata (Klussmann-Kolb et al., 2008) or Eupulmonata (Jörger et al., 2010b). In comparison to these previous studies the outgroup sampling herein was optimized and targeted to include representatives of all major lineages among the putative panpulmonate relatives. Despite this denser taxon sampling we again fail to reconstruct sister group relationships of Acochlidia reliably, i.e. with significant support. In the present analyses Acochlidia are sister to a clade comprised of Hygrophila+(Pyramidelloidea+(Amphiboloidea+Glacidorboidea)), all together sister to Eupulmonata (see Fig. 2). Our analyses indicate an explosive radiation of panpulmonate diversity (expressed by very short branches at the base of the higher taxa, see Figs. 3, 4). The chosen 'standard marker' set (i.e. partial mitochondrial COI and 16S rRNA and nuclear 18S rRNA and 28S rRNA), offers the broadest taxon sampling currently available for any molecular markers in Mollusca (Stöger et al., in press), but unfortunately at the present stage seems to be incapable of solving the deep panpulmonate relationships reliably. This is probably handicapped by the old Mesozoic origin of Panpulmonata in combination with a very rapid diversification into the higher taxa in the Mesozoic Jurassic (see Fig. 4 and

Additional material 3). The application of new molecular markers with potential phylogenetic signal for deep euthyneuran splits, such as phylogenomic datasets (e.g., Kocot et al., 2013), but at the same time allowing for a similarly broad and representative taxon sampling as gathered using the 'standard markers', are overdue.

In contrast to ambiguous sister group relationships, the monophyly of Acochlidia is currently undisputed based on morphology (Schrödl and Neusser, 2010) and molecular markers (present study; Jörger et al., 2010b). Even though the two major acochlidian clades – Hedylopsacea and Microhedylacea – differ remarkably in their evolutionary history (see discussion below), they share a unique combination of characters (e.g. characteristic separation into head-foot complex and visceral hump, presence of calcareous spicules and a pre-pharyngeal nerve ring with separated cerebral and pleural ganglia and a rhinophoral nerve innervating the rhinophore) (Schrödl and Neusser, 2010). Many of these features can be interpreted as either plesiomorphic in the new heterobranch tree or apomorphic through progenetic reductions, i.e. result of the 'meiofaunal syndrome' (Brenzinger et al., 2013). The recently discovered Aitengidae (Swennen and Buatip, 2009), which were placed into hedylopsacean Acochlidia based on microanatomy and molecular data, conflict with the classical acochlidian bauplan by lacking the division into fully retractable head-foot complex and visceral hump (Neusser et al., 2011a). Our present phylogenetic hypothesis implies that Aitengidae have lost this most striking acochlidian apomorphy secondarily, as several of the features have been reduced secondarily in other genera as well (e.g. rhinophores in microhedylacean *Pontohedyle* and *Ganitus*). The monophyly of Acochlidia was again recovered in our study independent of the type of analyses (maximum-likelihood see Fig. 3 or Bayesian interference see Fig. 4). Even though the monophyly was not well supported – probably suffering from the same effects as discussed above for Panpulmonata in general – there is currently no doubt that Acochlidia are monophyletic.

The first detailed classification of Acochlidia conducted by Rankin (1979), which led to the establishment of 19 genera in 13 families and four suborders for only 25 nominal species, was heavily criticized by all subsequent authors (Arnaud et al., 1986; Schrödl and Neusser, 2010; Wawra, 1987). The alternative classification erected by Wawra (1987) rendered the system of Rankin (1979) obsolete and was largely confirmed by first cladistic analyses of Acochlidia relying on over a hundred biological and morphological characters (Schrödl and Neusser, 2010). Our molecular phylogenetic hypothesis (see Fig. 3) is largely compatible with the one based on morphological markers (Schrödl and Neusser, 2010, see fig.3). Therefore, most of the potential synapomorphies hypothesized for higher acochlidian clades

and major evolutionary scenarios are confirmed, such as the progressive elaboration of copulatory organs within hedylopsaceans (Neusser et al., 2009a). Also the apomorphic, successive reduction of reproductive organs among microhedylaceans is supported, with loss of copulatory organs in the common ancestor of Asperspinidae plus Microhedylidae (s.l.), and evolution of secondary gonochorism in the latter (e.g., Schrödl and Neusser, 2010; Sommerfeldt and Schrödl, 2005). Unfortunately, our analyses lack monotypic Tantulidae. Thorough 3D-microanatomy from the type material showed mixed and partially unique characters states for *Tantulum* (e.g. complex, unarmed anterior copulatory complex), which justifies the erection of a monotypic family by Rankin (1979), based on its unique ecology and morphological features (Neusser and Schrödl, 2007). Cladistic analyses (Schrödl and Neusser, 2010) based on morphological and biological characters resolved *Tantulum* as basal offshoot of Hedylopsacea, i.e. sister to (Hedylopsidae+(Pseudunelidae+Acochlidiidae)). Due to additional inclusion of Aitengidae and the undescribed Hedylopsacea sp. into Hedylopsacea, *Tantulum* cannot be plotted unambiguously onto our molecular tree any longer. Tantulidae retains several supposedly plesiomorphic features (e.g. an unarmed copulatory organ) and likely diverges quite early among Hedylopsacea; it probably originates in the late Mesozoic (i.e. Cretaceous) (see basal Hedylopsacean diversification in Fig. 4).

All of the included acochlidian families recognized by Schrödl and Neusser (2010) were monophyletic, largely with high bootstrap supports (see Fig. 3). The only disagreement to morphological approaches is paraphyletic Ganitidae (*Ganitus*+*Paraganitus*). Ganitidae which receives high support in morphological analyses, is nested here within paraphyletic *Microhedyle,* and was thus included in a broader definition of Microhedylidae *s.l.* (Schrödl and Neusser, 2010). Within Microhedylacea, only few distinguishing characters are present, leaving most of the clade unresolved in morphological analyses (see Schrödl and Neusser, 2010). The monophyly of Ganitidae is supported mainly by the common characteristics of the dagger-shaped radula and the related pharyngeal characteristics (i.e. paired cuticular mandibles) (Schrödl and Neusser, 2010). The paired cuticular mandibles are discussed to serve as functional 'odontophore' and counterparts for the muscles moving the radula (Challis, 1968; Marcus, 1953). Gosliner (1994) originally suspected a relationship of Ganitidae with Sacoglossa, which possess a strikingly similar dagger-shaped radula, used for piercing algae. All currently available morphological and molecular data on Ganitidae clearly reject a sacoglossan relationship of this family and places it rather in a derived microhedylacean relationship (present study; Jörger et al., 2010b; Neusser et al., 2011a;

Schrödl and Neusser, 2010). Thus, radula similarities seem to be based on functional convergence rather than common ancestry, supported by considerable differences in pharyngeal morphology (i.e. presence of mandibles, longitudinal pharynx musculature and absence of an ascus in Ganitidae) (B. Eder, pers. comm.; Challis, 1968; Marcus, 1953). Based on our molecular data, the dagger-shaped radulae and remarkably similar pharyngeal structures (B. Eder, pers. comm.) of *Ganitus* and *Paraganitus* also refer to convergent modifications potentially based on a shared feeding strategy in the mesopsammon.

In contrast to morphological analyses, our study successfully resolved the Microhedylidae s.l. confirming monophyletic *Pontohedyle*, *Parhedyle*, *Ganitus* and *Paraganitus*, but rendering *Microhedyle* paraphyletic. The paraphyly of *Microhedyle* is concordant with morphological analyses, but due to the high degree of homoplasy and few morphological apomorphies supporting the genera, we are currently unable to diagnose the different clades within *Microhedyle* based on morphological characters and thus leave '*Microhedyle*' for a future taxonomic revision of Acochlidia. As discussed previously, *Microhedyle nahantensis* and *M. odhneri* need to be transferred to *Parhedyle* based on molecular data and the presence of a special type of asymmetric radulae (formula 1.1.2, with inner right lateral tooth smaller than outer one) (Eder et al., 2011, own unpublished data), diagnostic for the genus *Parhedyle* (Wawra, 1987).

Additionally, our study detected 30 additional acochlidian lineages, which could not be assigned directly to valid species, and were determined as molecular operational taxonomic units (MOTUs) herein. All MOTUs were independent lineages in phylogenetic analyses, but only some were supported by unique morphological characters recognizable in the field and via light microscopy (e.g. Hedylopsacea sp. or Acochlidiidae sp., own unpublished data). Recent studies have shown that acochlidian diversity can best be tackled by using an integrative taxonomic approach making use of all available character sets, e.g. microanatomy, DNA sequence data and biogeography (Jörger et al., 2012; Neusser et al., 2011b). The presented MOTUs, thus, still require comparative microanatomical approaches with their sister groups, a molecular species delineation approach capable of dealing with low numbers of specimens, as described by Jörger et al. (2012), and molecular diagnoses (see Jörger and Schrödl, in press) in addition to morphological ones, if any exist. This is beyond the scope of the present paper.

Timeframe and biogeography of acochlidian evolution

The Mesozoic origin - Previous molecular clock analyses on euthyneuran gastropods indicated a rapid radiation into the modern higher taxa in the early to mid Mesozoic (Jörger et al., 2010b; Klussmann-Kolb et al., 2008; Stöger et al., in press). Our present molecular clock estimates – relying on the same set of genetic markers – supported these previous diversification estimates. The variation in fossil calibration points and the stability throughout our sensitivity analyses herein (see Additional material 2) shows that the estimations do not rely on a single calibration point but correspond well to a broad set of euthyneuran fossils. Earlier terrestrial gastropods fossils from the Paleozoic interpreted as pulmonates (Solem, 1985; Solem and Yochelson, 1979) are contradicted by our time trees (Additional material 3). Pulmonate affinities of such fossils also would imply long gaps in the fossil record. Our results thus support earlier criticism (Dayrat et al., 2011; Mordan and Wade, 2008) that suggests the few detectable characters of such potential Palaeozoic pulmonate shells might as well refer to prosobranch lineages. Considerably earlier molecular clock estimates dating the radiation of euthyneurans to the Cambrian and the appearance of (pan)pulmonate lineages to Ordovician and Silurian (Medina et al., 2011) based on mitogenomic data was likely driven by erroneous coding procedures and biased sampling (Schrödl et al., 2011b), or that mitogenomic datasets available to date are simply not suitable to resolve basal euthyneuran topologies correctly (Stöger and Schrödl, 2013).

The rapid mid Mesozoic radiation of euthyneuran and especially panpulmonate taxa (and thus the origin of Acochlidia) might be an outcome of the breakup of the supercontinent Pangaea and Panthalassas Ocean (approx. 180 mya) and resultant isolation of marine biotas, which in general boosted marine diversity (Cox and Moore, 2010; Lomolino et al., 2010). Based on this hypothesis (which relies on molecular clock data), the origination of the major panpulmonate clades including Acochlidia might be characterized as part of the successful recovery fauna of the Triassic/Jurassic turnover.

For gastropod taxa with a Mesozoic origin and poor fossil record, precise areas of origin are difficult to determine and ancestral area reconstructions based on molecular phylogenies could often only support assumptions of origins in the Tethyan realm, without further specification (Frey and Vermeij, 2008; Malaquias and Reid, 2009; Ozawa et al., 2009; Reid et al., 2010). As shown in previous studies on Mesozoic radiations for other taxa, Acochlidia originated when shelf areas of oceans varied dramatically from the current profiles and it is thus difficult to assign recent distribution ranges to historic areas. Based on

current knowledge, Acochlidia are limited to rather shallow shelf areas; we thus aimed to allocate recent continental margins to the historic topologies at the ages of the reconstructed nodes and discuss the biogeography accordingly. While there is strong support that the ancestral acochlid inhabited the marine mesopsammon in tropical waters (see Fig. 6), none of our ancestral area reconstructions (DEC-model and S-DIVA) provided reliable support values for the ancestral area of Acochlidia (see Fig. 4). The most likely ancestral areas for Acochlidia based on the DEC model is the North-Eastern part of the Tethys Ocean and the Western margin of the Panthalassic/Pacific Ocean, but only with marginal higher relative probabilities than other regions of the Tethys or the forming Atlantic Ocean. Although our data clearly indicated a circum-tropical distribution of Acochlidia throughout the Tethys, Pacific and Atlantic Ocean (see Discussion below), the precise region of origin could not be solved. The evolution of Microhedylacea is characterized by numerous dispersal, vicariance and local extinction events (see Fig. 4). The indicated local extinction events might either truly relate to extinction events in the corresponding areas or refer to still unsampled microhedylacean lineages in the different areas of the world's oceans. The complex picture on microhedylacean biogeography with sister clades in non-connected areas of distribution, and somewhat long internal branches, demonstrates the high degree of missing diversity with the current ancestral chronogram only able to present current knowledge. In the present study we included almost 85 % of the described acochlidian species-level diversity and added approximately another 50 % by putatively new species (left as undetermined MOTUs for future research). Nevertheless, our sampling map (see Fig. 1) shows 'white spots' of large biogeographic areas, which are still relatively unexplored for meiofaunal molluscs. In particular, the Western Indo-Pacific (former Western Tethys Region) which was revealed as a major area of transition and dispersal for many microhedylacean lineages (see Fig. 4) and discussed as center of origin and diversification in the Oligocene and early Miocene for marine molluscan biodiversity (Harzhauser et al., 2007) is virtually unsampled, as is the vast majority of the Eastern Atlantic. Based on their patchy occurrence and with large parts of tropical sands still unsampled, we are probably only scraping at the surface of the expected recent acochlidian diversity, which still remains to be discovered. Evidently, this might have major influence on biogeographical hypotheses, but our robust phylogenetic hypothesis – concordant between previous morphological (Schrödl and Neusser, 2010) and molecular analyses (Jörger et al., 2010b) – nevertheless, allows for the reconstruction of ancestral area chronograms and the reflection how historic geographic events triggered acochlidian diversification:

Distributing circumtropical and beyond - A warm-tropical circum-equatorial seaway (Tethyan Seaway) formed during the early Cretaceous (Cox and Moore, 2010; Lomolino et al., 2010), and throughout the Cretaceous and Paleogene it served as gateway for marine biotas between the tropical world's oceans (Frey and Vermeij, 2008). This connectivity is well reflected in our dataset on Acochlidia by numerous dispersal events across the Tethys Ocean, the forming Atlantic and East Pacific Ocean (see Fig. 4). The Tethyan Seaway is described as a strong westward directed current, providing better dispersal conditions westward than eastward (Cox and Moore, 2010). This is not supported, however, by our data, which shows frequent changes in the direction of dispersal events even within a clade (see e.g. *Pontohedyle* and *Asperspina* in Fig. 4). The current abated once the Tethyan Seaway narrowed by the convergence of Africa with Eurasia in Oligocene and Miocene, enabling an eastward dispersal from the East Pacific to the Western Atlantic (Lomolino et al., 2010). The independent dispersal events in Asperspinidae eastwards via the tropical East Pacific into the Caribbean Sea predate the narrowing and closure of the Tethyan Seaway, estimated to the early Paleogene, but the dispersal into the Atlantic might indeed have occurred after the weakening of the circum-tropical current. Mesopsammic Acochlidia are considered as poor dispersers due to the lack of a planktonic larval stage (veliger larvae remain in the interstices of sand grains (Swedmark, 1968)). Thus, currents at local scales, the presence of continuous coastlines and the uplift of ocean floors creating shallow shelf seas or terranes, providing stepping stones for dispersal probably had larger influence on their historic biogeography than major oceanic current systems in the past.

In the Mesozoic, terranes in the central Pacific are thought to have served as stepping stones which allowed dispersal into the Eastern Indo-Pacific (Grigg and Hey, 1992). However, our data presented five independent dispersal events in Hedylopsacea and Microhedylacea from the CIP to EIP (the island of Moorea) not limited to the Mesozoic, but instead ranging between Upper Cretaceous and Late Miocene (88–7 mya). If we are not severely underestimating acochlidian dispersal abilities, this indicates that remote Eastern Indo-West Pacific islands were continuously accessible either via island stepping stones or seamounts.

The East Pacific Barrier (EPB, approx. 5000km of deep water separating the Indo-West Pacific fauna from the tropical East Pacific fauna) is today generally considered as one of the most effective barriers to the dispersal of marine shallow-water fauna and data from fossil coral suggests that it was largely in place throughout the Cenozoic (Grigg and Hey, 1992). Based on our global phylogeny the ancestors of *Pontohedyle* (Microhedylidae *s. l.*) crossed the EPB once in an eastward dispersal event (approx. 82 mya) and once westward

(approx. 54 mya). The ancestor of *Asperspina* MOTU Moorea also dispersed in westward direction from the Northeast Pacific (approx. 63 mya) (see Fig. 4). Reports on gene exchange or closely related species spanning the EPB (e.g., in gastropod species with long larval stages (Reid et al., 2010), echinoderms (Lessios et al., 1998) and tropical fish (Lessios and Robertson, 2006)) indicate that this potent barrier is not 'impassable'. The span of the EPB by a presumably poor disperser like *Pontohedyle* or *Asperspina*, however, is still puzzling. All acochlidian dispersal events dated to Upper Cretaceous till late Paleocene/early Eocene, and fossil data on coral suggest that the EPB was less effective during the Cretaceous (Grigg and Hey, 1992). The long stemlines of the discussed species, however, might also indicate that the evolutionary history of these clades is not well covered by our dataset and might lack e.g. Western Atlantic sister clades which could reverse the dispersal picture.

Asperspinidae were reconstructed with an ancestral range spanning the North Pacific and presented the only clade with a putative trans-arctic dispersal in the late Paleogene into NEA, which is in concordance with the usual dispersal direction of most other Mollusca in the Trans-Artic Interchange between the two northern Oceans (Vermeij, 1991). Asperspinidae also show the highest flexibility to ocean temperatures and include the only currently known polar acochlid *Asperspina murmanica* (see Kudinskaya and Minichev, 1978; Neusser et al., 2009b), which unfortunately could not be included in the present analyses. The tropical origin of Asperspinidae received high statistical support in our analyses and the cold water adaptation likely evolved at the base of the NWP/NEA clade in the late Eocene (see Figs. 3, 4). This correlates to a shift of the warm-temperate zones southward in the circumpolar region following a series of temperature declines after the mid Eocene and the establishment of a cold-temperate regime (Briggs, 2003).

Major vicariance events shaping the evolutionary history of Acochlidia - Our molecular clock estimates, the pan-Tethyan, Central American and Pacific distribution of acochlidian taxa and the supposed poor dispersal abilities of Acochlidia, all suggest that the biogeography of the clade is shaped by major vicariance events during the Mesozoic and Cenozoic: the closure of the Tethyan Seaway (Tethyan Terminal Event - TTE) in the Miocene (approx. 18 mya), collision of Australia and New Guinea with Eustralasia forming the modern Wallacea province (25 mya) and the Pliocene uplift of the Isthmus of Panama (3.4 resp. 2.1 mya) (Cox and Moore, 2010; Lomolino et al., 2010).

The Terminal Tethyan Event in the Miocene (TTE, i.e. the vicariance event preventing exchange between the Atlantic and Indo-Pacific regions) (Cox and Moore, 2010; Lomolino et al., 2010) is reflected in our data in the split between Mediterranean and North East Atlantic *Microhedyle glandulifera* and its sister clade - the (central) Indo-West Pacific radiation of *Paraganitus*. The split was estimated slightly prior to the TTE by our molecular clock analyses (approximately 40–18 mya, see Fig. 4 and Additional material 3). Data from other gastropod species pairs, which presumably originated by Tethyan vicariance, however, also predate the final closure of the seaway in the Miocene (e.g., Malaquias and Reid, 2009; Reid et al., 2010), probably related to the proceeding isolation of the Tethyan realm prior to the final closure. Assuming a Tethyan divergence via vicariance between *M. glandulifera* and *Paraganitus*, one would expect an eastward dispersal pattern in the historic biogeography of *Paraganitus*, which rather presents a mixed picture with westward tendencies (see Fig. 4).

Nowadays, the central Indo-West Pacific forms a hotspot in marine biodiversity, but based on fossil records of Mollusca the faunal diversity was rather poor until the late Paleogene (Frey and Vermeij, 2008). During Oligocene times the Western Tethys region potentially served as center of origin and diversification of molluscan taxa, which shifted southeast after the TTE (Harzhauser et al., 2007). Our data showed that 1) hedylopsacean Aitengidae, Pseudunelidae and Acochlidiidae evolved *in situ* in the central Indo-Pacific Ocean and 2) no *in situ* splits within the central Indo-West Pacific occurred prior to the mid Paleogene. The estimated timeframe of the origin of the families predates, however, the closure of Tethyan Seaway but no data on relict species in other parts of the world suggest an origin elsewhere. The late Oligocene and Miocene is known for being a time in which the diversity of marine shallow-water fauna increased, likely influenced by the availability of new shallow-water habitats formed by the collision of Australia and New Guinea with the southeastern edge of Eurasia (Williams and Duda, 2008). Williams and Duda (2008) showed increased rate of cladogenesis in different unrelated gastropod genera in the Indo-West Pacific in the beginning of late Oligocene/early Miocene. This is in concert with the increased diversification especially in hedylopsacean Indo-West Pacific clades starting in the late Oligocene, which likely holds major responsibility for the detected overall increase in diversification rates of Acochlidia in the Oligocene by the birth-death-shift model (see Fig. 5B).

Moreover, our data showed two recent specification events by vicariance in the Indo-West Pacific: in hedylopsacean *Pseudunela marteli* (CIP) and *Pseudunela* MOTU Maledives

(WIP), and microhedylacean *Paraganitus* from CIP and *Paraganitus* MOTU Thailand (WIP). Strong currents in recent central Indo-West Pacific waters should facilitate the dispersal of larvae and be responsible for the invisibility of the Wallace's Line (sharp transition between terrestrial faunas of eastern and western Indonesia) in marine taxa (Barber et al., 2000). But Barber et al. (2000) showed that Pleistocene ocean basins are still reflected in the genetic structure of shrimp populations, which might resemble relicts of Indian and Pacific populations separated by the emergence of the Sunda and Sahul continental shelves, suggesting the presence of a marine Wallace's line perpendicular to the terrestrial Wallace's line. Our data by far predates the Pleistocene Indonesian continental shelf formations, but might represent relicts reflecting the better connectivity between Indian and Pacific populations prior to tectonic events in the Wallacea region in the early Neogene. An integrative species delineation approach, however, still needs to confirm the species status of the identified MOTUs; a putative conspecificity between the different populations would imply genetic exchange between the recent CIP and WIP populations, thus contradicting assumptions on a marine Wallace's line, while a subdivision of MOTUs would further support it.

Following the TTE the North Eastern Atlantic, viz. Mediterranean suffered from an impoverishment in faunal diversity, due to the Miocene cooling, Messinian salinity crisis and glacial events in late Pliocene and Pleistocene (Harzhauser et al., 2007). Although our data recovered NEA frequently as ancestral area of microhedylacean clades throughout the Mesozoic and Paleogene, only comparably few lineages persisted or still radiated in NEA after the TTE. The only entirely North Atlantic clade *Parhedyle* originated in the NEA prior to the closure if the Tethyan Seaway and dispersed westward into the NWA, following the typical unidirectional expansion of molluscan species across (or around) the Atlantic (Vermeij, 2005). Our molecular clock estimate on the radiation of NEA *Parhedyle* (approx. 6.4 mya) slightly predated the Messinian salinity crisis, but in absence of records of these *Parhedyle* species outside the Mediterranean, it is likely that the radiation occurred in the Mediterranean during reinvasion after the crisis.

Based on our data, Western Atlantic species of Acochlidia presented relictual Tethys origins, inhabiting the area prior to the TTE (in *Pontohedyle*) or showed origins in the New World (WAT+EPT) prior to the closure of the Isthmus of Panama (in *Asperspina*) (see Fig. 4). *Ganitus* and western Atlantic *Microhedyle* radiated in the Western Atlantic. Two 'soft barriers' for dispersal in marine taxa in the Atlantic (Briggs and Bowen, 2013) are reflected in our data, the vicariance event splitting eastern and western Atlantic *Microhedyle* and

Asperspina species (open water expanse of the mid-Atlantic) and the vicariance event splitting the two *Ganitus* MOTUs, which according to our time tree slightly predated the freshwater discharge of the Amazon river (11 mya, see Briggs and Bowen, 2013). In a previous study on microhedylid *Pontohedyle* population this soft barrier between Caribbean and Brazilian provinces, however, still allows for gene exchange (Jörger et al., 2012). Vicariance events potentially related to the formation of the Isthmus of Panama (approx. 3 mya, see Cox and Moore, 2010) are reflected twice within our dataset: *Asperspina* MOTU Peru (SEP) and *Asperspina* MOTU Panama (WAT) and *Microhedyle remanei* (SWA, but also reported from WAT) and *Microhedyle* MOTU Mexico (NEP), even though this implies a series of still unsampled (or extinct) intermediate populations.

Evolutionary hotspots - To summarize the discussed biogeography with regard to evolutionary hotspots of Acochlidia, our reconstructions of ancestral ecological traits like climate (Fig. 6) clearly suggest that the tropical regions provided the species pools of Acochlidia, from which temperate regions were populated several times independently. In concert with data on other gastropod taxa (see e.g., Williams, 2007; Williams and Duda, 2008), Acochlidia show a latitudinal gradient in taxonomic diversity and diversification concentrated in the tropics (see Figs. 1, 6). Generalizing, the Indo-West Pacific – especially its central region – is remarkably diverse in comparison to marine geographic regions of the Eastern Pacific or Atlantic, with the Western Atlantic showing an intermediate diversity of marine taxa and the Eastern Atlantic and Pacific harboring the lowest diversity (Briggs, 2007). The vast majority of worldwide mesopsammic fauna is still unexplored, but our data already offers some indication on the centers of acochlidian evolution. The tropical Western Atlantic is equally well-sampled as the central Indo-Pacific (concerning number of stations), however, only one enigmatic hedylopsacean species (*Tantulum elegans*) has been reported, while the central Indo-West Pacific harbors the vast majority of hedylopsacean lineages and acts as cradle for the recent Cenozoic diversification. Hypothetically, including *Tantulum* into our ancestral area chronogram, late Mesozoic Hedylopsacea probably had a circum-tethyal distribution. One can speculate that *Tantulum* presents a relict in an unstable geological area, which was majorly affected by the mass extinction events like the Cretaceous-Paleogene (approx. 66 mya) or Eocene-Oligocene event (approx. 33 mya). Fossil data suggests that up to 90 % of the molluscan fauna was eliminated in Gulf of Mexico during each event (Hansen et al., 2004); similar ranges might be expected for the Caribbean Sea, but none of the events can be traced on our diversification rate estimates (see Fig. 5). Since no marine hedylopsacean has yet been discovered in the Western

Atlantic, no rough dating or speculations on the evolutionary background of the shift from marine to freshwater in Tantulidae can be done. *Tantulum* remains truly enigmatic as the only minute, interstitial or mud-dwelling slug in freshwater (Neusser and Schrödl, 2007; Rankin, 1979). While the sluggish bauplan in freshwater is unique to Acochlidia in general, all other known acochlidian limnic slugs reestablished a benthic lifestyle.

Habitat shifts

Into the mesopsammon – The interstitial or mesopsammic fauna is one of the most diverse on Earth and comprises minute representatives from most major lineages of Metazoa, including several exclusively meiofaunal clades (Rundell and Leander, 2010; Worsaae et al., 2012). The physical parameters of the interstitial habitat of marine sands are challenging for the inhabitants, but likely persisted throughout the history of Eukaryota, and a meiofaunal lifestyle might have evolved even prior to the Cambrian with numerous independent colonization events since then (Rundell and Leander, 2010). A previous study based on molecular analyses discussed at least five independent interstitial invasions within heterobranch slugs and dated colonization events range between the Mesozoic and Cenozoic (Jörger et al., 2010b). Based on our data herein the invasion of the mesopsammon already occurred along the stemline of Acochlidia and could thus be estimated to Lower to Middle Jurassic (see Fig. 4, Additional material 3).

The restricted space in the interstitial habitat requires great morphological and biological adaptations for its minutely-sized inhabitants. Even though the exact origin of Acochlidia within panpulmonates remain unresolved, no evidence exists at present that any of the sister groups are derived from meiofaunal ancestors, thus it is likely that the miniaturization of Acochlidia occurred along with the transition into the mesopsammon in the acochlidian ancestor. Many meiofaunal taxa show paedomorphic traits (i.e. morphological characters present in juveniles or larvae of closely related species) discussed as result of progenesis (accelerated sexual maturation in relation to somatic development) or neoteny (retardation of somatic development in relation to sexual maturity) (Brenzinger et al., 2013; Rundell and Leander, 2010; Westheide, 1987; Worsaae et al., 2008; Worsaae et al., 2012). Little is still known of the influence of progenesis in the evolution of meiofaunal slugs, mainly due to a lack of ontogenetic data and uncertain phylogenetic affinities. Within the basal heterobranch Rhodopemorpha, strong evidence for a progenetic origin is reported, based on paedomorphic traits in adults including features like the pentaganglionate stage of the

visceral loop and a protonephridial organization of the kidney (Brenzinger et al., 2013). Jörger et al. (2010b) suggests a progenetic origin of the acochlidian ancestor by relating the typical acochlidian morphology (shell-less yet free visceral hump) to the phenotype of an abnormally developed larva reported in the nudibranch *Aeolidiella alderi* and some stylommatophoran Eupulmonata (Tardy, 1970), leading to a very similar external morphology.

Life in the interstitial spaces of sand grains evidently constrains morphology and leads to a series of convergent adaptations in the different taxa (e.g., vermiform body shape), termed the 'meiofaunal syndrome' (Brenzinger et al., 2013). Our study confirms assumptions based on morphological data that the ancestral acochlid had already invaded the interstitial habitat, which was related to major morphological adaptations (e.g. minute, worm-shaped body, loss of shell, detorsion resulting in symmetric body condition) (Schrödl and Neusser, 2010). The ancestral acochlid probably possessed a simple sac-like kidney that is typical for marine Euthyneura (Neusser et al., 2011b), and had a hermaphroditic, phallic, monaulic reproductive system, transferring sperm via copulation (Schrödl and Neusser, 2010). Miniaturized body plans often combine simplified organ systems with morphological novelties and inventions (Hanken and Wake, 1993; Westheide, 1987). The microhedylacean clade lacks novel morphological inventions entirely, and rather present a line of 'regressive evolution' (i.e. as simplification and reduction of organ systems (Swedmark, 1964, 1968)). All microhedylacean Acochlidia studied in sufficient detail (Eder et al., 2011; Jörger et al., 2008; Jörger et al., 2007; Neusser et al., 2006; Neusser et al., 2009b) have a simple sac-like kidney, as described as ancestral for Acochlidia (Neusser et al., 2011b). So far no habitat transitions are known for Microhedylacea. Most members of the clade are limited to truly marine and subtidal sands, but minor tolerance to changes in salinity concentration seem to be present in e.g. in the microhedylacean species reported from the Black Sea *P. milaschewitchii* and *Parhedyle tyrtowii* (Kowalevsky, 1901). Few microhedylacean species (e.g. *Asperspina murmanica, A. riseri and Pontohedyle verrucosa*) occur in the intertidal, which is characterized by temporary changes in salinity either due to rain or desiccation. No specific adaptations of the excretory or circulatory system, however, are reported in these slugs (Challis, 1970; Kowalevsky, 1901; Morse, 1976; Neusser et al., 2009b) and thus do not seem to be mandatory to deal with the temporary osmotic stress in the intertidal.

In regards to the reproductive system, some hedylopsacean Acochlidia are protandric hermaphrodites and the ontogenetic loss of male genital organs was interpreted as the precursor for the evolution of secondary gonochorism at the base of Microhedylidae s.l.

(Schrödl and Neusser, 2010), which might be related to a second progenetic event in acochlidian evolution. Microhedylacea are characterized by the loss of copulatory organs and the usage of spermatophores for sperm transfer (Jörger et al., 2009; Schrödl and Neusser, 2010). The male genital opening is usually located dextrolaterally or shifted anteriorly and spermatophores are randomly attached to the mates followed by dermal insemination (Jörger et al., 2009; Morse, 1994; Swedmark, 1968). Spermatophores are a common development across different meiofaunal taxa and considered as adaptation to the spatially restricted and unstable interstitial habitat, favoring imprecise but fast sperm transfer (Jörger et al., 2009). What serves as advantageous mode of reproduction in the mesopsammic world might present an evolutionary disadvantage, however, in transition to freshwater or semi-terrestrial habitats where directly injected sperm via stylets or regular copulation is more common. From the origin of Microhedylidae s.l in the mid-Jurassic (approx. 166 mya), this family remained in almost complete morphological stasis: minor changes occurred only in form, shape and number of spicules, the secondary reduction of the rhinophores (twice independently in *Pontohedyle* and *Ganitus*) and modifications of the radula (i.e. small second right lateral tooth in *Parhedyle* and twice independently the evolution of a dagger-shaped radula with corresponding modifications of the pharynx in *Ganitus* and *Paraganitus*). Other than that, due to the reduced stage of all organ systems, even advanced microanatomy failed to recover distinguishing features even at the genus level (for details on the anatomy see e.g. Eder et al., 2011; Jörger et al., 2010a; Jörger et al., 2008; Jörger et al., 2007; Neusser et al., 2006). Moreover, intraspecific or even intraindividual (right and left side) variation, especially within the nervous system, often exceeds interspecific morphological variation in these highly cryptic lineages. This high intraspecific variability is discussed as consequence from miniaturization and paedomorphosis, frequently involving late forming structures, which are then individually truncated in development (Hanken and Wake, 1993). The most striking example of morphological stasis in Microhedylacea is a recently discovered world-wide radiation in *Pontohedyle* slugs which present clearly independently evolving genetic lineages but are entirely cryptic based on traditional taxonomic characters (i.e. external morphology, spicules and radula features). Even microanatomy does not reveal reliable diagnostic characters (Jörger et al., 2012).

In direct contrast to the morphological reduction and simplification in Microhedylacea, the evolution of Hedylopsacea is driven by novel inventions and increasing organ complexity. The development of a complex kidney at the base of Hedylopsacea probably plays a key

role for the ecological diversification of the clade, serving as precursor to habitat shifts which enables to handle osmotic stress (Brenzinger et al., 2011b; Neusser et al., 2009a; Neusser et al., 2011b; Neusser and Schrödl, 2009).

Out of the mesopsammon – The evolutionary pathway away from the meiofaunal lifestyle is even less studied and understood than the way in. Meiofaunal animals may have played a major role in the earliest diversification of bilaterians (Rundell and Leander, 2010; Worsaae et al., 2012), but few reports document the reversion of miniaturized forms re-establishing a benthic life-style and evolving a 'secondary gigantism' in body size (see e.g., Westheide, 1982; Worsaae and Kristensen, 2005). In an example known for annelids, morphological features which were reduced as previous adaptation to the interstitial life (e.g. setae) were not reestablished with increasing body size (Westheide, 1982). Our ancestral state reconstruction of Acochlidia supports a mesopsammic hedylopsacean ancestor and three independent ways out of the interstitial habitat and to larger body sizes: in Aitengidae, *Pseudunela espiritusanta* and Acochlidiidae (see Fig. 6). Alternatively, but less supported in the analyses, the benthic lifestyle could have been reestablished only once (or twice) in an ancestral lineage within Hedylopsacea, then again Pseudunelidae (and Hedylopsacea sp.) would have secondarily recolonized the interstitial habitat. Currently, all transitions out of the mesopsammon are always into habitats characterized by non-marine salinities (brackish, limnic, amphibious) (see Fig. 6). In absence of any macrofaunal marine Hedylopsacea, the 'secondary gigantism' seems a consequence of the habitat transition into brackish water, freshwater and terrestrial systems rather than the necessary precursor of the habitat switch e.g., to be able to cope with osmoregulatory requirements. The hypothesis, that habitat shifts primarily depend on the ability of efficient osmoregulation independent of the overall body size is further supported by the presence of the limnic, yet minute and interstitial *Tantulum elegans*. Some recently discovered deep-sea benthic slugs, however, show putative hedylopsacean relationships (TPN unpublished data) and might shed new light on hedylopsacean habitat shifts out of the mesopsammon in the future.

Shifting from marine to (semi-)terrestrial and limnic habitats – Evolutionary transitions between aquatic and terrestrial habitats as well as between marine and freshwater systems are comparatively rare in the animal kingdom (Vermeij and Dudley, 2000). Therefore considerable barriers probably exist among the habitats related to osmoregulation,

desiccation and novel predator-prey interactions (Vermeij and Dudley, 2000; Vermeij and Wesselingh, 2002). The key to the diversification of particularly panpulmonate Euthyneura was the invasion of freshwater and terrestrial habitats, and the drivers of those habitat transitions are of major interest (Barker, 2001; Klussmann-Kolb et al., 2008). Earlier assumptions that pulmonate taxa secondarily invaded the marine habitat via terrestrial pathways (Solem, 1985) are nowadays replaced by a consensus on the marine origin of (Pan)Pulmonata (Dayrat et al., 2011; Klussmann-Kolb et al., 2008). This is confirmed by our ancestral state reconstruction, in which most deep panpulmonate nodes were highly supported as marine (lh 0.96 -1.0, reconstruction not shown). Truly terrestrial panpulmonates are found within Stylommatophora, Systellommatophora and Ellobioidea and several independent evolutionary pathways led to the life on land in pulmonate slugs and snails (Barker, 2001; Dayrat et al., 2011), probably via marine marginal zones and amphibious-marine transition stages (Klussmann-Kolb et al., 2008). Klussmann-Kolb et al. (2008) suggested that the colonization of freshwater in Euthyneura was a unique evolutionary event (in Hygrophila) directly from marine habitat via an aquatic pathway. Our ancestral area reconstruction confirmed the proposed aquatic pathway into freshwater in Hygrophila and also for Glacidorboidea, 'amphibious-limnic' lineages in Ellobioidea and limnic Acochlidia. This also indicates that conclusions on a unique transition event into freshwater, however, were premature and do not reflect the highly complex picture of habitat transitions in Panpulmonata.

Because of incomplete taxon sampling, conclusions from ancestral area reconstructions across these phylogenies have to be carefully evaluated. Although it is ideal to all major lineages of each outgroup clade, ideally with a basal rather than derived internal position, the selected taxa may not necessarily reflect the ecological variability and basal state of habitat within their clade, e.g., the non-inclusion of the putatively basal marine *Williamia* within Siphonarioidea resulted in the amphibious semi-terrestrial ancestral state for the diversification of the clade (results not shown). Due to the uncertainty of panpulmonate sister group relationships and the incomplete ecological representation of our panpulmonate outgroups (e.g., Siphonarioidea, Hygrophila and Ellobioidea) we refrain from reporting ancestral characters states across Panpulmonata in detail, including dating ancestral areas for the involved transitions. Nevertheless, our present and previous molecular clock analyses (Jörger et al., 2010b; Klussmann-Kolb et al., 2008) indicate that some of the major habitat transitions (e.g., land invasion by the stylommatophoran ancestor and shift to freshwater by the ancestor of Hygrophila) date back to the Mesozoic. In contrast, our study

on Acochlidia revealed comparably recent habitat transitions within Hedylopsacea: According to our analyses, freshwater was invaded only once in the ancestor of Acochlidiidae (see Fig. 6). Another, independent transition to freshwater occurred in the Western Atlantic Tantulidae (Schrödl and Neusser, 2010), a taxon unfortunately unavailable for molecular approaches (see Discussion above). The semi-terrestrial habitat was invaded once in Aitengidae and permanently brackish water once at the base of Pseudunelidae by *Pseudunela espiritusanta* (see Fig. 6). All habitat shifts occurred in the central Indo-West Pacific. The habitat shifts to limnic and brackish water were dated herein to the late Paleogene. Unfortunately, the stemline of Aitengidae spans nearly 130 my hindering assumptions on the timeframe in which the transition to a (semi-) terrestrial lifestyle occurred, but it is likely that they fall in the same timeframe in which the forming Wallacea region offered ideal conditions for shifts in habitat. During the Paleogene the Central Indo-West Pacific region was under major geological reformation and lots of new shallow shelf areas and islands appeared (Cox and Moore, 2010; Lomolino et al., 2010). This 1) provided comparatively pristine habitats with low levels of competition and predation for newcomers, which is discussed as beneficial for habitat shifts (Vermeij and Dudley, 2000) and 2) boosted marine diversity in general, thus raising the competition and predation pressure in the old habitat. Based on our phylogenetic hypotheses the (semi-)terrestrial habitat in Aitengidae was invaded by a marine ancestor and can be considered part of the marginal zone between marine and terrestrial habitat. With regard to the colonization of limnic habitats however, paticular hypotheses of brackish habitats serving as stepping stones into freshwater ones are not supported by our phylogeny and the reconstruction of ancestral character states, including those on temporarily brackish habitats (e.g., intertidal mesopsammic *Pseudunela cornuta* influenced by rain) and permanently brackish waters (e.g., *P. espiritusanta*) (Neusser et al., 2009a; Neusser and Schrödl, 2009). But more intermediate taxa inhabiting zones with decreasing salinity are necessary to reconstruct the evolutionary scenario of the invasion of limnic systems in Acochlidia.

Morphological and behavioral adaptations

Habitat shifts require adaptation to the new physical environment; to deal with osmotic stress, risk of desiccation and adapt life strategies concerning reproduction, predator avoidance and available food sources (Mordan and Wade, 2008). In the evolution of Hedylopsacea, the complexity of the excretory and circulatory systems increased in taxa

that conquered new habitats, compared with their fully marine, mesopsammic sister groups. Next to 'secondary gigantism', which improves the volume/surface ratio for osmoregulation, the excretory system of limnic and brackish Acochlidiidae and Pseudunelidae potentially increases effectiveness by evolving a long, looped nephroduct (which is short in fully marine sister species of Pseudunelidae) (Neusser et al., 2009a; Neusser et al., 2011b; Neusser and Schrödl, 2009). 'Enhanced' nephroducts are known for limnic panpulmonates (Smith and Stanisic, 1998), where it is long and looping as in some hedylopsacean Acochlidia (e.g. Hygrophila, *Acroloxus* and *Ancylus* (Delhaye and Bouillon, 1972)) or bladder-like (*Glacidorbis* (Ponder, 1986)). The nephroduct is also elaborate in terrestrial Systell- and Stylommatophora, but not modified in the remaining coastal or amphibious panpulmonate taxa (e.g. Siphonariidae (Delhaye and Bouillon, 1972) or sacoglossan *Gascoignella* (Kohnert et al., 2013)). Additionally, specialized heart cells in limnic *Strubellia*, which probably enhance circulation, likely present another novel adaptive feature to life in freshwater (Brenzinger et al., 2011b). The dorsal vessel system (modified part of the kidney) in Aitengidae, was proposed as an adaptation to a semi-terrestrial lifestyle, potentially used for oxygen supply in a gill-less animal (Neusser et al., 2011a).

Analogous to the increasing complexity in excretory and circulatory systems, several 'novel' or reinvented reproductive features also evolved in Hedylopsacea. The Acochlidiidae developed a progressively large and complex copulatory apparatus, with additional glands and cuticular injection systems, culminating in the 'giant' phallus equipped with several rows of cuticular spines termed 'rapto-penis' (Schrödl and Neusser, 2010). In addition, sperm storage organs such as a seminal receptacle and a bursa copulatrix (gametolytic gland) are absent in basal acochlidians, but reappear in derived hedylopsaceans (Schrödl and Neusser, 2010). On one hand, this increasing diversity and complexity of male and female reproductive organs, paired with an obviously traumatic type of mating (Lange et al., 2013), suggests an arms race between individuals and perhaps even sexes in these hermaphrodites. Large reproductive organs are coupled with secondarily large body sizes and a benthic lifestyle. On the other hand, this re-establishment of reproductive features present in euthyneuran outgroups but putatively reduced in basal mesopsammic acochlidian lineages, shows that the ontogenetic source of these structures might persist and can be reactivated in larger individuals again. This implies, however, that the function of theses developmental pathways is maintained by other selective constrains, otherwise a reactivation after such long timescales is considered impossible (Marshall et al., 1994).

The mode of reproduction is further modified in limnic Acochliididae, which produce a large number of eggs in contrast to the low reproductive output in the marine sister taxa. Benthic, limnic acochlidiids are likely amphidromic, i.e. have a marine planktonic larval stage and recolonize freshwater as juveniles (Brenzinger et al., 2011b). The freshwater fauna of tropical oceanic islands in the central Indo-West Pacific is dominated by amphidromous species (Crandall et al., 2010). This life strategy evolved several times independently among invertebrates, likely because it facilitates (re-)invasion of island habitats and unstable stream environments (Crandall et al., 2010; McDowall, 2007). This long-distance marine planktonic dispersive stage promotes a population structure similar to those in marine species with planktonic larvae, and is characterized by little genetic structure among the populations of different island archipelagos (Crandall et al., 2010; Kano and Kase, 2004). The dispersal abilities of the unique 'adhesive larvae' reported for Acochlidiidae are unknown and it remains speculative whether other animals are used as dispersal vectors or whether the glue is used on the substrate to avoid being swamped to the open sea (Brenzinger et al., 2011b). In the future population genetic analyses might give insights about the connectivity of populations on different islands and thus allow conclusions on the fate of the larvae after being washed into the sea. It is likely that these changes in habitat and mode of reproduction result in entirely different population structures, with rather well-connected populations and widespread species in limnic taxa versus a high degree of endemism and small distributions in marine, mesopsammic Acochlidia.

The pathway into the mesopsammon is thought to be advantageous in avoiding predator pressure (Palmer, 1988). In general, slugs should be more vulnerable to predation than snails, as the gastropod shell serves as protection against predators. However, sea slug lineages which lack a protective shell have independently evolved a series of defensive mechanisms such a cleptocnides, acid glands or secondary metabolites (for summary see Wägele and Klussmann-Kolb, 2005). In Acochlidia, no special defensive features are known apart from their unique ability to retract their head-foot complex into their visceral hump (Schrödl and Neusser, 2010), showing similar behavior to snails withdrawing into their shell. The acochlidian visceral hump can be equipped with a dense arrangement of calcareous spicules in Asperspinidae and Hedylopsidae forming a secondary 'spicule shell' (Schrödl and Neusser, 2010; Sommerfeldt and Schrödl, 2005). The ability to retract the anterior is present in all mesopsammic Acochlidia, and may be an effective defense against (small-sized) meiofaunal predators by increasing the body diameter to avoid being

swallowed whole, and additionally protects essential body parts against bites. Secondarily benthic Acochlidia can only slightly or partially retract, which may result from the changes in overall morphology (e.g., the flattened leaf-like visceral sac in *Acochlidium* and *Palliohedyle*). But probably the strategy is not particularly efficient against large-sized benthic predators such as crabs or fish and might have lost its evolutionary significance in benthic environments. Interestingly, no marine benthic acochlidians are described at present, thus acochlidian evolution lacks evidence for the most direct habitat shift (from the marine mesopsammon back to a benthic marine lifestyle, see discussion above). The transitions to a benthic lifestyle in Acochlidia occur where habitats present comparatively low predator pressure, such as freshwater systems, or by dwelling in brackish water like *Pseudunela espiritusanta* (Neusser and Schrödl, 2009), and in the latter, is supported by behavioral predator avoidance such as hiding beneath stones during daytime. The semi-terrestrial Aitengidae is an exception, and lives in the marine intertidal habitat which is very stressful due to highly fluctuating temperatures and salinities, the risk of desiccation and the presence of many predators such as crabs, other arthorpods or sea birds. Behavioral stress avoidance, like hiding into the damp crevices of intertidal rocks during daytime in *Aiteng mysticus* (Neusser et al., 2011a), might have been the key to successfully colonize this habitat. Whether or not Aitengidae possess additional defensive features, for example via chemical substances, still needs to be explored in future research.

Given the late Paleogene timeframe and the ancestral area (Wallacea) for the major habitat shifts in Acochlidia, the driving forces for the transitions still remain unclear. Food sources of Acochlidia are largely unknown, but it is likely that they are highly specialized feeders, e.g., preying on the eggs of co-occurring species. It can be speculated that the availability of new food sources with less competition might play a role, e.g. *Aiteng ater*, which specializes on feeding on insect pupae (Swennen and Buatip, 2009). Moreover, potential co-evolution between limnic Acochlidia like *Strubellia* and the neritid gastropods whose eggs they feed on (Brenzinger et al., 2011b), should be investigated in future research combining gut content analyses, molecular clock analyses and the habitat shifts in both groups.

Conclusions

The Acochlidia provide an astonishing example of two major evolutionary clades differing enormously in habitat transition flexibility. The hedylopsacean evolution presents a mosaic of habitat transitions between aquatic and (semi-) terrestrial habitats shifting from the

mesopsammon to epibenthic lifestyle and vice versa, which corresponds to a series of novel morphological developments and increasing complexity e.g., in excretory and reproductive features within some hedylopsacean clades. Consequently, Hedylopsacea comprise high morphological plasticity and ecological diversity with their major diversity hotspot in the central Indo-West Pacific, and morphological divergent lineages are still expected in further research. Conversely, their sister clade Microhedylacea remained in almost entire morphological and ecological stasis since the late Mesozoic. Miniaturization, organ simplifications and specialization led to a highly successful clade, which is worldwide distributed with several transitions to temperate and even temperate-cold waters, occurs in locally high species densities and has apparently successfully survived or recolonized areas after major extinction events. But this evolutionary success of Microhedylacea by taking the 'regressive' and specialized pathway into the mesopsammon apparently also forms a dead-end road concerning morphological or ecological diversification. The currently known Microhedylacean diversity is largely cryptic, which is also to be expected for their still undiscovered lineages. Adding about 30 new MOTUs, the present study again confirms the existence of hidden marine diversity, and highlights the more general need for integrative species delimitation and the potential for molecular description of cryptic species.

The comparatively small and well-studied clade of Acochlidia demonstrates the high degree of habitat flexibility in panpulmonates, evoking potential complexity in the evolutionary history of other less-known panpulmonate clades. The present study shows how habitat transitions can be placed in space, time and biological context, once a robust, integrative species-level phylogeny is established, and despite limitations such as still undiscovered diversity. Even though panpulmonate relationships cannot be satisfactorily resolved at present, converging molecular clock data from sensitivity analyses with different fossil calibration points already indicate that different shifts in habitat in Panpulmonata occurred in different Mesozoic and Cenozoic timeframes and therefore various geological and ecological backgrounds. The ancestral marine habitat of the basal panpulmonate Acochlidia and other deep panpulmonate nodes is the originator of the panpulmonate evolution in the mid Mesozoic, but habitat shifts need to be addressed individually across each clade.

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Tables

Table 1: List of museums numbers of voucher material, DNA voucher numbers (all ZSM) and GenBank numbers of the material of Acochlidia analyzed in the present study. * marks sequences generated within this study, all remaining sequences were retrieved from GenBank.

Institutions: AM – Australian Museum, Sydney; SI – Smithsonian Institute, Washington, D.C.; ZSM – Bavarian State Collection of Zoology, Munich. DNA and sequences generated by KMJ if not stated otherwise: KH – Katharina Händeler – University of Bonn; YK – Yasunori Kano, University of Tokyo; NW – Nerida Wilson, Scripps Institution of Oceanography, La Jolla.

Taxon	**Locality**	**Museums number**	**GenBank accession numbers of generated sequences**				**DNA-bank number**
HEDYLOPSACEA							
Hedylopsidae			**18S rRNA**	**28S rRNA**	**16S rRNA**	**COI**	
Hedylopsis spiculifera	NEA-3a	ZSM Mol 20080951	HQ168430	HQ168443	no data	HQ168455	AB35081816
	NEA-4	ZSM Mol 20080955	no data	no data	HQ168417	KF709352	AB35081817
	NEA-8	ZSM Mol 20080389	no data	KF709319	KF709245	KF709351	AB35081764
	NEA-11	ZSM Mol 20081016	KF709275	no data	KF709246	KF709353	AB34404246

Hedylopsis ballantinei	WIP-2	ZSM Mol 20090244	HQ168429	HQ168442	HQ168416	HQ168454	AB34858170
Hedylopsis MOTU Moorea	EIP-3	AMC.4760 56.001	KF709276	no data	KF709247	KF709354	AB34402008
Hedylopsacea indet.							
Hedylopsacea MOTU Moorea	EIP-4	AMC.4760 59.001	KF709277	KF709320	no data	KF709355	AB34402051
Pseudunelidae							
Pseudunela cornuta	CIP-19	ZSM Mol 20071809	JF819754	KF709321	JF819748	JF819774	AB34404215
Pseudunela viatoris	CIP-27	ZSM Mol 20080020	JF819751	no data	JF819741	JF819766	AB34404247
	CIP-5	ZSM Mol 20070953	no data	KF709322	JF819745	JF819770	AB34404276
Pseudunela marteli	CIP-18	ZSM Mol 20080022	JF819753	no data	JF819746	JF819771	AB34404252
	CIP-23	ZSM Mol 20080393	HQ168431	HQ168444	HQ168418	HQ168456	AB35081809
	CIP-13	ZSM Mol 20100381	KF709278	KF709323	KF709248	KF709356	AB34402060
Pseudunela MOTU Maledives	WIP-3	ZSM Mol 20110029	KF709279	KF709324	KF709249	KF709357	AB34402077
Pseudunela espiritusanta	CIP-22	ZSM Mol 20080117	JF819755	KF709325	JF819749	JF819775	AB34404289
Acochlidiidae							
Strubellia paradoxa	CIP-12	ZMB Moll. 193.944	HQ168432	HQ168445	HQ168419	HQ168457	AB34858174
Strubellia wawrai	CIP-16	ZSM Mol 20080016	KF709280	no data	JF819730	JF819758	AB34404250
	CIP-17	ZSM Mol 20071810	no data	KF709326	JF819734	JF819762	AB34404212
	CIP-21	ZSM Mol 20080150	KF709281	KF709327	JF819736	JF819764	AB34404205
Strubellia MOTU Sulawesi	CIP-2	ZSM Mol 20100339	KF709282	KF709328	JF819740	JF819765	AB35081762
Acochlidiidae MOTU Ambon	CIP-12	ZMB Moll. 193.966	KF709283	KF709329	KF709250	KF709358	AB35081841
Palliohedyle MOTU Sulawesi	CIP-2	ZSM Mol 20100356	KF709284	JF828039	JF828040	JF828032	AB35081794
Acochlidium fijense	CIP-26	ZSM Mol 20080063	HQ168433	HQ168446	HQ168420	HQ168458	AB34404244
Acochlidium amboinense	CIP-12	ZMB Moll. 193.942a	KF709285	KF709330	KF709251	KF709359	AB35081759
Acochlidium bayerfehlmanni	CIP-14	ZSM Mol 20080384	KF709286	no data	KF709252	KF709360	AB35081800
Acochlidium sutteri	CIP-9	ZSM Mol 20080911	KF709287	KF709331	KF709253	KF709361	AB35081785
Acochlidium MOTU Flores	CIP-10	ZSM Mol 20080897	KF709288	KF709332	KF709254	KF709362	AB35081777
Acochlidium MOTU Sulawesi	CIP-2	ZSM Mol 20100341	KF709289	KF709333	KF709255	KF709363	AB34500502
	CIP-3	ZSM Mol 20100359	no data	no data	KF709256	KF709364	AB34402017

Acochlidium MOTU Solomons	CIP-15	ZSM Mol 20080159	KF709290	KF709334	KF709257	KF709365	AB34404232
Aitengidae							
Aiteng ater	CIP-1	extracted by KH	JF828036	JF828037	JF828038	JF828031	AB34858187
Aiteng mysticus	CIP-28	generated by YK	HQ168428	HQ168441	HQ168415	HQ168453	no DNA aliquot at ZSM
MICROHEDYLACEA							
Asperspinidae							
Asperspina brambelli	NEA-12	ZSM Mol 20100576	no data	JQ410991	JQ410990	JQ410924	AB34402042
	NEA-12	ZSM Mol 20100573	KF709291	no data	KF709258	KF709366	AB34402038
Asperspina rhopalotecta	NEA-7	ZSM Mol 20080409	KF709292	KF709335	KF709259	KF709367	AB35081814
Asperspina MOTU Peru	SEP-2	ZSM Mol 20080560	KF709293	no data	KF709260	KF709368	AB35081775
Asperspina MOTU1 Kamtchatka	NWP-1	ZSM Mol 20090171	HQ168434	HQ168447	HQ168421	KF709369	AB35081833
Asperspina MOTU2 Kamtschatka	NWP-2	ZSM Mol 20090175	KF709294	KF709336	KF709261	KF709370	AB35081755
Asperspina MOTU Belize	WAT-3	SI-CBC2010 KJ01-A08	KF709295	KF709337	no data	no data	AB34402056
Asperspina MOTU Panama	WAT-5	ZSM Mol 20110721	KF709296	KF709338	no data	KF709371	AB34500492
Asperspina MOTU Washington	NEP-1	ZSM Mol 20100585	KF709297	no data	KF709262	KF709372	AB34402007
Asperspina MOTU Moorea	EIP-1	AMC.476049.001	KF709298	KF709339	no data	KF709373	AB34402009
Microhedylidae (s.l.)							
Microhedyle glandulifera	NEA-3a	ZSM Mol 20081019	HQ168437	HQ168449	HQ168424	HQ168461	AB35081799
	NEA-10	ZSM Mol 20080136	no data	no data	JF819817	JF819778	AB34404283
	NEA-9	ZSM Mol 20080392	no data	no data	KF709263	JF819779	AB35081748
Microhedyle MOTU Ghana	EAT-1	ZSM Mol 20110715	KF709299	no data	KF709264	KF709374	AB34402046
Microhedyle MOTU West Papua	CIP-13	ZSM Mol 20100386	KF709300	KF709340	no data	KF709375	AB34401999
Microhedyle MOTU Peru	SEP-2	ZSM Mol 20080559	KF709301	KF709341	KF709265	KF709376	AB35081828
Microhedyle MOTU Chile	SEP-1	ZSM Mol 20090206	KF709302	KF709342	KF709266	KF709377	AB35081771
Microhedyle MOTU Egypt	WIP-1	ZSM Mol 20090464	KF709303	KF709343	no data	KF709378	AB35081773
Microhedyle	SWA-1	ZSM Mol	KF709304	KF709344	KF709267	no data	AB34404296

remanei		20071124					
Microhedyle MOTU St. Vincent	WAT-8	ZSM Mol 20090193	KF709305	JQ410989	JQ410988	JQ410923	AB35081767
Microhedyle MOTU Mexico	NEP-2	generated by NGW	KF709306	no data	KF709268	KF709379	AB34500083
Microhedyle MOTU Moorea	EIP-5	AMC.476061.001	KF709307	no data	KF709269	KF709380	AB34499237
Parhedyle cryptophthalma	NEA-6	ZSM Mol 20100584	KF709308	JF828041	JF828042	JF828033	AB34599403
Parhedyle odhneri	NEA-5	ZSM Mol 20090571	KF709309	JF819814	no data	JF819819	AB35081818
Parhedyle tyrtowii	NEA-1	ZSM Mol 20091369	KF709310	JF819813	no data	JF819818	AB35081774
Parhedyle nahantensis	NWA-1	ZSM Mol 20110022	KF709311	KF709345	no data	KF709381	AB34500501
Pontohedyle milaschewitchii	NEA-3b	ZSM Mol 20080054	HQ168435	JF828043	HQ168422	no data	AB34404241
	NEA-2	ZSM Mol 20071381	no data	JQ410926	JQ410925	JQ410897	AB34404214
	NEA-4	ZSM Mol 20080953	KC984282	no data	JQ410929	JQ410898	AB35081832
Pontohedyle brasilensis	WAT-9	ZSM Mol 20110722	KC984285	JQ410952	JQ410951	JQ410906	AB34402086
	WAT-5	ZSM Mol 20110723	KC984284	JQ410952	JQ410951	JQ410906	AB34402034
	WAT-1	SI-CBC2010 KJ01-E03	KC984283	JQ410941	JQ410940	no data	AB34500510
Pontohedyle verrucosa	CIP-19	ZSM Mol 20071820	KC984287	JQ410978	JQ410977	JQ410920	AB34404223
	CIP-13	ZSM Mol 20100390	no data	JQ410975	no data	JQ410918	AB34402070
	CIP-13	ZSM Mol 20100391	KC984289	no data	JQ410976	JQ410919	AB34500531
	CIP-7	ZSM Mol 20071135	KC984288	JQ410971	JQ410970	JQ410914	AB34404221
Pontohedyle kepii	CIP-8	ZSM Mol 20081013	KC984290	JQ410967	JQ410966	JQ410912	AB35081769
Pontohedyle joni	WAT-6	ZSM Mol 20090197	KC984291	JQ410934	JQ410933	JQ410901	AB34858164
	WAT-3	SI-CBC2010 KJ01-C08	no data	JQ410939	JQ410938	JQ410903	AB34402065
Pontohedyle neridae	EIP-5	AMC.476062.001	no data	JQ410986	JQ410985	JQ410922	AB34500497
Pontohedyle liliae	WIP-1	ZSM Mol 20090471	KC984293	JQ410954	JQ410953	no data	AB35081802
Pontohedyle wiggi	WIP-5	ZSM Mol 20100595	no data	JQ410960	JQ410959	JQ410908	AB34402059
Pontohedyle wenzli	WIP-6	ZSM Mol 20100592	KC984294	JQ410958	JQ410957	JQ410907	AB34402021
	EIP-1	AMC.476051.001	KC984295	JQ410982	JQ410981	no data	AB34402037

	CIP-6	ZSM Mol 20081014	KC984296	JQ410969	JQ410968	JQ410913	AB35081827
	CIP-4	ZSM Mol 20100379	KC984297	JQ410973	JQ410972	JQ410915	AB34500521
Pontohedyle peteryalli	EAT-2	ZSM Mol 20071133	KC984298	no data	JQ410930	JQ410899	AB34404268
Pontohedyle martynovi	EIP-2	AMC.476054.001	no data	JQ410984	JQ410983	no data	AB34402062
Pontohedyle yurihookeri	EPT-1	ZSM Mol 20080565	KC984299	JQ410987	no data	no data	AB34402000
Ganitus evelinae	SWA-2	ZSM Mol 20100328	KF709312	JF828044	JF828045	JF828034	AB34404225
Ganitus MOTU Panama	WAT-4	ZSM Mol 20110210	KF709313	KF709346	KF709270	KF709382	AB34858203
Paraganitus ellynnae	CIP-20	ZSM Mol 20080170	HQ168436	HQ168448	HQ168423	HQ168460	AB34404203
Paraganitus MOTU Vanuatu	CIP-24	ZSM Mol 20080173	KF709314	KF709347	KF709271	KF709383	AB34404204
	CIP-25	ZSM Mol 20100619	KF709315	no data	KF709272	KF709384	AB34402002
Paraganitus MOTU Fiji	CIP-27	ZSM Mol 20080065	KF709316	KF709348	KF709273	KF709385	AB34404280
Paraganitus MOTU West Papua	CIP-13	ZSM Mol 20100393	KF709317	KF709349	no data	KF709386	AB34500507
Paraganitus MOTU Thailand	WIP-4	ZSM Mol 20100586	KF709318	KF709350	KF709274	KF709387	AB34402080

Table 2: List of included panpulmonate outgroup taxa for phylogenetic analyses with GenBank accession numbers.

Higher taxon	**Species**	**18S rRNA**	**28S rRNA**	**16S rRNA**	**COI**
‚LOWER HETEROBRANCHIA‘					
	Orbitestella vera	FJ917207	FJ917239	FJ917250	FJ917268
EUOPISTHOBRANCHIA					
	Tylodina perversa	AY427496	AY427458	FJ917424	AF249809
	Akera bullata	AY427502	AY427466	AF156127	AF156143
	Aplysia californica	AY039804	AY026366	AF192295	AF077759
PANPULMONATA					
Sacoglossa					
Oxynoacea	*Oxynoe antillarum*	FJ917441	FJ917466	FJ917425	FJ917483
	Volvatella viridis	HQ168426	HQ168439	HQ168413	HQ168451
Plakobranchacea	*Gascoignella nukuli*	HQ168427	HQ168440	HQ168414	HQ168452
	Elysia viridis	AY427499	AY427462	AJ223398	DQ237994
	Bosellia mimetica	AY427498	AY427460	DQ480202	DQ471212
Siphonarioidea					
Siphonariidae	*Siphonaria pectinata*	HQ659934	DQ279993	AY377627	HQ660000
	Kerguelenella lateralis	HQ659931	-	HQ650565	HQ659997
Pyramidelloidea					
Pyramidellinae	*Pyramidella dolabrata*	-	-	AY345054.2	AY345054.2
	Otopleura nodicincta	HQ659929	-	HQ650563	HQ659995
Odostomiinae	*Boonea seminuda*	AY145367	AY145395	AF355163	-
	Hinemoa sp.	GU331936	GU331926	GU331946	GU331955
	Odostomia plicata	GU331938	GU331928	GU331948	GU331957
	Pyrgisculus sp.	GU331939	GU331929	GU331949	GU331958
Turbonillinae	*Turbonilla elegantissima*	GU331941	GU331931	GU331951	GU331960
	Eulimella ventricosa	FJ917213	FJ917235	FJ917255	FJ917274
	Cingulina sp.	GU331940	GU331930	GU331950	GU331959

Glacidorboidea					
Glacidorbidae	*Glacidorbis rusticus*	FJ917211	FJ917227	FJ917264	FJ917284
	Striadorbis spiralis	-	DQ256746	-	-
Amphiboloidea					
Amphibolidae	*Amphibola crenata*	EF489337	EF489356	EF489304	JF439216
	Salinator rhamphidia	HQ659937	-	HQ650571	HQ660003
	Salinator sp.	GU331942	GU331932	GU331952	GU331961
Phallomedusidae	*Phallomedusa solida*	DQ093440	DQ279991	DQ093484	DQ093528
Hygrophila					
Chilinidae	*Chilina* sp.	EF489338	EF489357	EF489305	EF489382
Latiidae	*Latia neritoides*	EF489339	EF489359	EF489307	EF489384
Acroloxidae	*Acroloxus lacustris*	AY282592	EF489364	EF489311	AY282581
Physidae	*Physa acuta*	AY282600	EF489368	AY651219	AY282589
	Aplexa elongata	-	AY465071	EU038330	EU038377
Lymnaeidae	*Galba truncatula*	HQ659965	-	HQ659899	HQ660031
	Lymnaea stagnalis	AY427525	AY427490	AY577461	AY227369
Planorbidae	*Biomphalaria glabrata*	BGU65223	AF435694	DQ084845	DQ084823
	Bulinus globosus	HM756311	HM756400	AY029546	FN546814
EUPULMONATA					
Systellommatophora					
Veronicellidae	*Sarasinula linguaeformis*	HQ659989	-	HQ659923	HQ660055
	Laevicaulis natalensis	HQ659985	-	-	HQ660051
	Veronicella cubensis	HQ659991	DQ897670	HQ659925	HQ660057
Rathouisiidae	*Atopos australis*	-	AY014152	-	-
Onchidiidae	*Onchidium vaigiense*	HQ659974	-	HQ659908	HQ660040
	Onchidella floridana	AY427521	AY427486	EF489317	EF489392
	Peronia peronii	HQ659975	-	HQ659909	HQ660041
Ellobioidea					
Otinidae	*Smeagol phillipensis*	FJ917210	FJ917229	FJ917263	FJ917283
	Otina ovata	EF489344	EF489363	EF489310	EF489389
Trimusculidae	*Trimusculus reticulatus*	HQ659935	-	HQ650569	HQ660001
Ellobiidae	*Ellobium pellucens*	-	AY465079	-	-
	Carychium minimum	EF489341	EF489361	EF489308	EF489386
	Melampus fasciatus	HQ659941	-	HQ659875	HQ660007
	Pedipes mirabilis	HQ659945	AY465074	HQ659879	HQ660011
	Ophicardelus ornatus	DQ093442	DQ279994	DQ093486	DQ093530
Stylommatophora					
Arionoidea	*Arion silvaticus*	AY145365	AY145392	DQ465822	AF513018
Limacoidea	*Deroceras reticulatum*	AY145373	AY145404	AF238045	AF239734
Elasmognatha	*Succinea putris*	HQ659993	AY014057	HQ659927	HQ660059
Orthalicoidea	*Placostylus ambagiosus*	-	AY014059	-	AY148560
Helicoidea	*Helicella obvia*	GU331943	GU331933	GU331953	GU331962
	Cerion striatellum	-	EU409909	-	-
Orthurethra	*Cochlicopa lubrica*	GU331944	GU331934	GU331954	GU331963

Figures

Figure 1: Sampling localities of Acochlidia. Triangles indicate type localities (when colored in red material from type locality was included in the study, yellow triangles: unsampled type localities), red dots represent collecting sites of material included in this study (for collectors, coordinates and details on the collecting sites, see Additional material 1). Yellow triangles: unsampled type localities. Biogeographic areas modified after system

of Marine Ecoregions of the world (MEOW) by Spalding et al (2007), using their original shape-file to generate the map in DIVA-GIS.

CIP – Central Indo-Pacific; EAT – tropical Eastern Atlantic; EIP – Eastern Indo-West Pacific; EPT – tropical Eastern Pacific; NEA – North-Eastern Atlantic (+Mediterranean+Black Sea); NEP – North-Eastern Pacific; NWA – North-Western Atlantic; NWP – North Western Pacific; SEP – South-East Pacific; SWA – South-Western Atlantic; WAT – tropical Western Atlantic; WIP – Western Indo-Pacific. White coastlines mark unsampled biogeographic regions.

Figure 2: Overview of the phylogenetic relationships of Acochlidia within Panpulmonata, based on maximum likelihood analyses of the concatenated four marker dataset (mitochondrial COI and 16S rRNA and nuclear 28S rRNA and 18S rRNA).

Figure 3: Phylogeny of Acochlidia shown to species level (outgroups collapsed for presentation purposes), based on maximum-likelihood analyses of the concatenated four marker dataset (mitochondrial COI and 16S rRNA and nuclear 28S rRNA and 18S rRNA).

Figure 4: Ancestral area chronogram of Acochlidia, outgroups collapsed for presentation purposes (for complete chronogram see Additional material 3). Divergence times obtained from BEAST v1.6.1 under a relaxed uncorrelated clock model, node ages indicated above nodes. Geological timescale is based on the International Stratigraphic Chart by the International Commission on Stratigraphy (2012). Colored dots at terminals indicate geographic areas occupied the sampled specimens, squares at nodes ancestral area which received highest relative probability (p) in DEC-analyses.

Figure 5: Analyses of shifts in diversification rate of Acochlidia. a) Lineage through time plot, b) birth-death model calculated with TreePar.

Figure 6: Ancestral state reconstruction of ecological traits (climate, habitat and life style) in Acochlidia, retrieved in Mesquite on the maximum-likelihood phylogeny shown in Fig. 3, outgroups collapsed.

Additional material

Additional material 1: Sampling localities of Acochlidia included in the present study (recollecting attempts at the same position are marked with a and b). Localities referring to type localities of valid acochlidian species are marked with *.

Collectors: AA – Andreas Altenöder, PB – Pat Boaden, LD – Ludwig Demharter, AD – Angela Dinapoli, BE –Barbara Eder, GH – Gerhard Haszprunar, MH – Martin Heß, KJ – Katharina Jörger, YK – Yasunori Kano, KK – Kevin Kocot, AM – Alexander Martynov, RM – Roland Meyer, TN – Timea Neusser, GR – Greg Rouse, JS – Julia Sigwart, MS – Michael Schrödl, ES – Enrico Schwabe, NW – Nerida Wilson

Additional material 2: Summary of the different molecular clock analyses performed in this study (and compared to Jörger et al. 2010) and the resulting estimated node ages for the major acochlidian taxa.

Additional material 3: Complete chronogram of Acochlidia, panpulmonate outgroups shown. Divergence times obtained from BEAST v1.6.1 under a relaxed uncorrelated clock model, node ages indicated at nodes, bars represent 95 % highest posterior densities (only presented for nodes with a PP > 0.5).

Figure 1

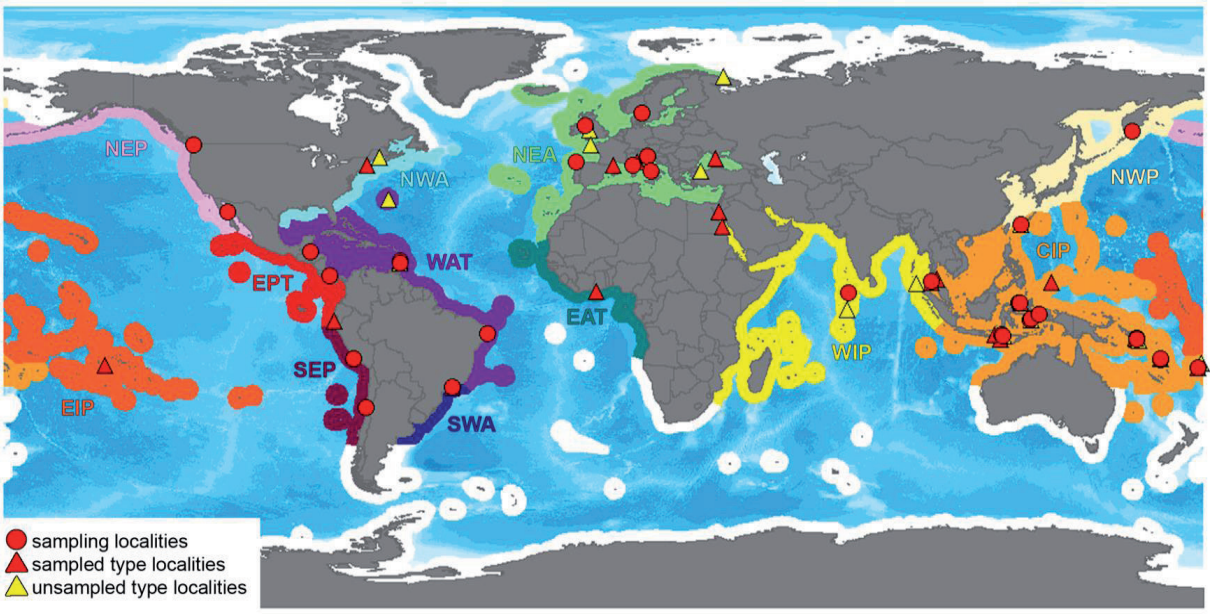
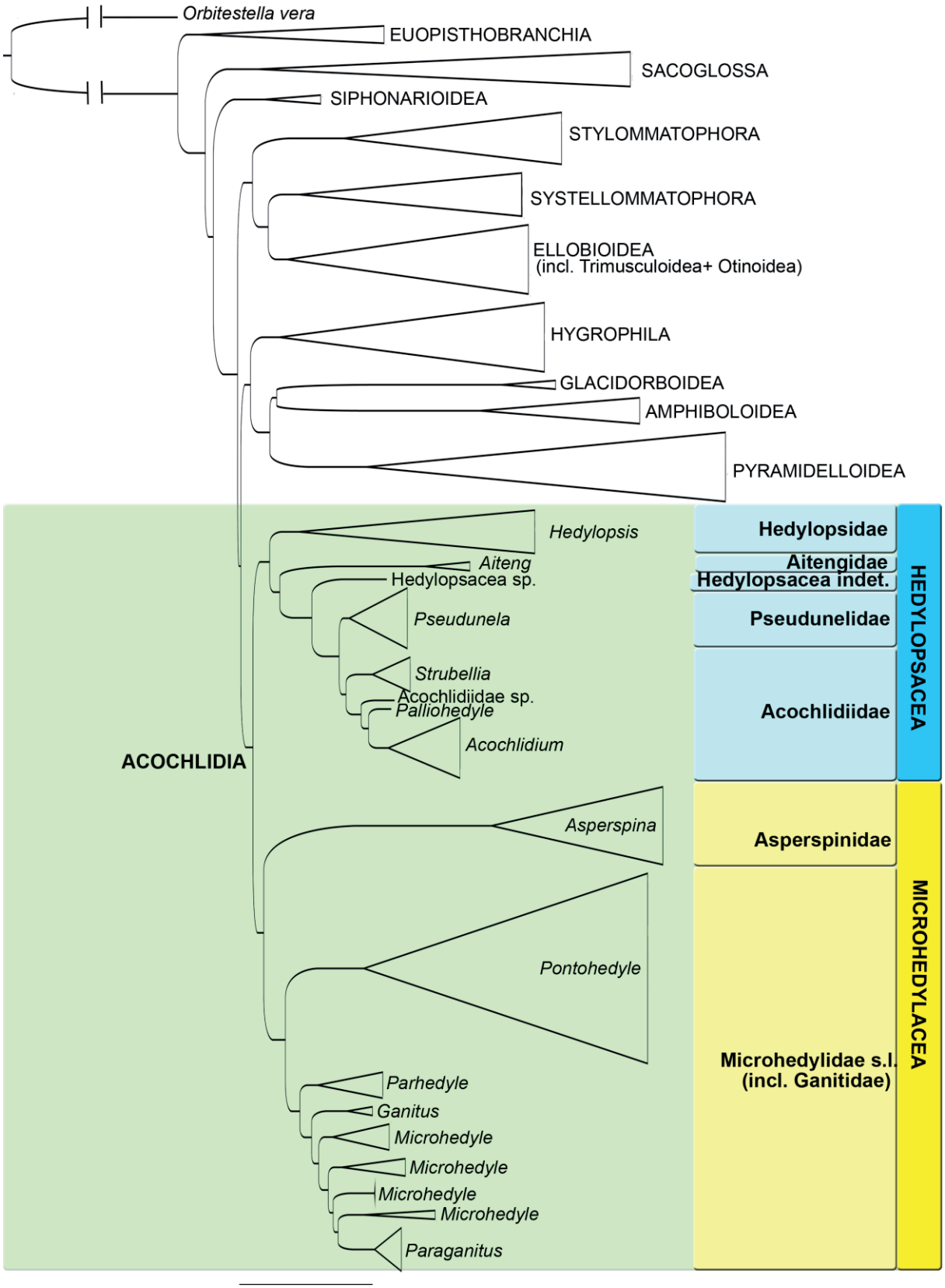

NEP
NWA
NEA
NWP
WAT
EPT
CIP
EAT
SEP
WIP
EIP
SWA
sampling localities
sampled type localities
unsampled type localities

Figure 2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EUOPISTHOBRANCHIA
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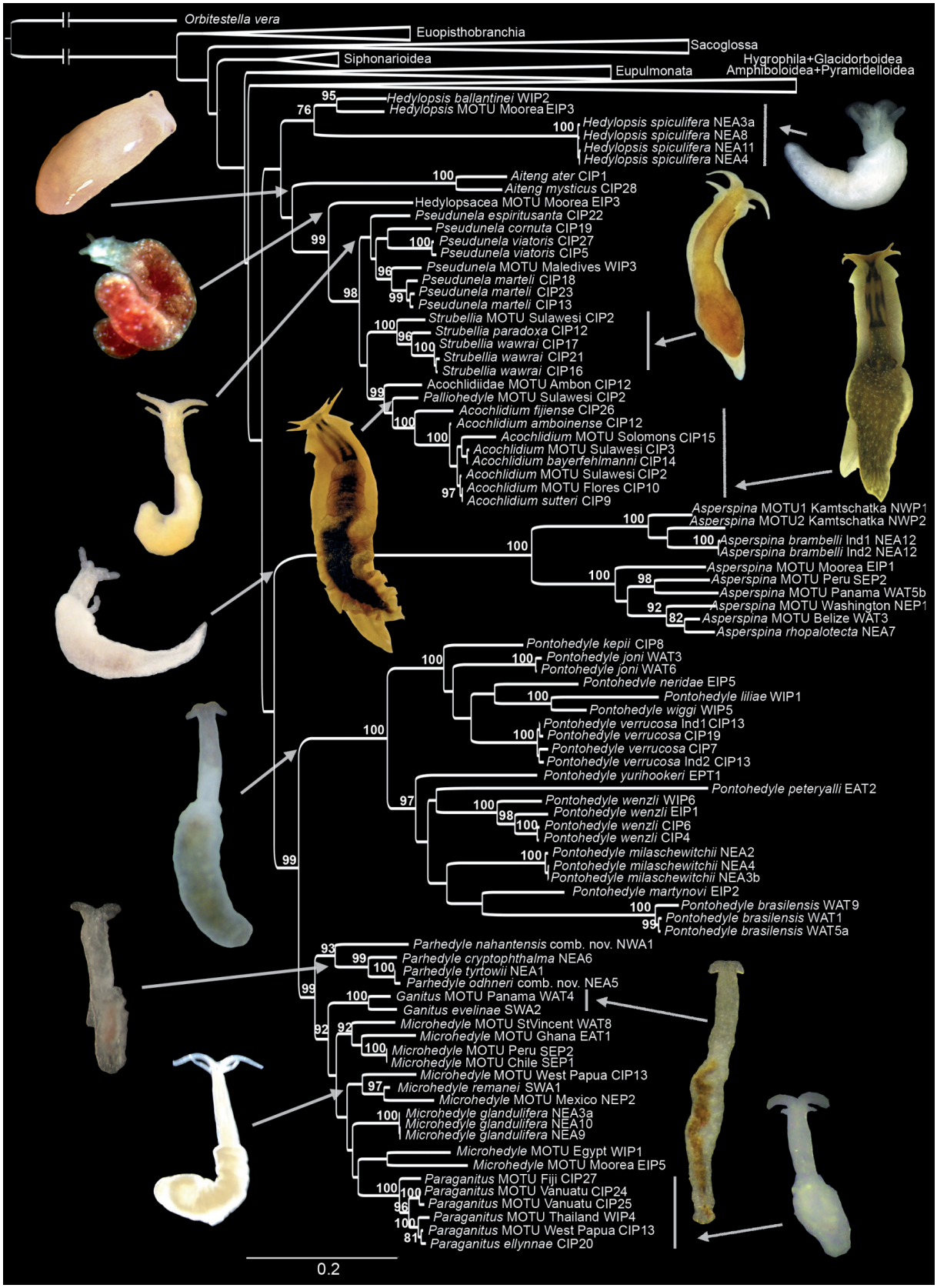
SIPHONARIOIDEA
STYLOMMATOPHORA
SYSTELLOMMATOPHORA
ELLOBIOIDEA
(incl. Trimusculoidea+ Otinoidea)
HYGROPHILA
GLACIDORBOIDEA
AMPHIBOLOIDEA
PYRAMIDELLOIDEA
ACOCHLIDIA
Hedylopsis
Aiteng
Hedylopsacea sp.
Pseudunela
Strubellia
Acochlidiidae sp.
Palliohedyle
Acochlidium
Asperspina
Pontohedyle
Parhedyle
Ganitus
Microhedyle
Microhedyle
Microhedyle
Microhedyle
Paraganitus
Hedylopsidae
Aitengidae
Hedylopsacea indet.
Pseudunelidae
Acochlidiidae
Asperspinidae
Microhedylidae s.l.
(incl. Ganitidae)
HEDYLOPSACEA
MICROHEDYLACEA

Figure 3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Sacoglossa
Siphonarioidea
Hygrophila+Glacidorboidea
Amphiboloidea+Pyramidelloidea
Eupulmonata
Hedylopsis ballantinei WIP2
Hedylopsis MOTU Moorea EIP3
Hedylopsis spiculifera NEA3a
Hedylopsis spiculifera NEA8
Hedylopsis spiculifera NEA11
Hedylopsis spiculifera NEA4
Aiteng ater CIP1
Aiteng mysticus CIP28
Hedylopsacea MOTU Moorea EIP3
Pseudunela espiritusanta CIP22
Pseudunela cornuta CIP19
Pseudunela viatoris CIP27
Pseudunela viatoris CIP5
Pseudunela MOTU Maldives WIP3
Pseudunela marteli CIP18
Pseudunela marteli CIP23
Pseudunela marteli CIP13
Strubellia MOTU Sulawesi CIP2
Strubellia paradoxa CIP12
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Palliohedyle MOTU Sulawesi CIP2
Acochlidium fijiense CIP26
Acochlidium amboinense CIP12
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Acochlidium bayerfehlmanni CIP14
Acochlidium MOTU Sulawesi CIP2
Acochlidium MOTU Flores CIP10
Acochlidium sutteri CIP9
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Asperspina MOTU2 Kamtschatka NWP2
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Asperspina brambelli Ind2 NEA12
Asperspina MOTU Moorea EIP1
Asperspina MOTU Peru SEP2
Asperspina MOTU Panama WAT5b
Asperspina MOTU Washington NEP1
Asperspina MOTU Belize WAT3
Asperspina rhopalotecta NEA7
Pontohedyle kepii CIP8
Pontohedyle joni WAT3
Pontohedyle joni WAT6
Pontohedyle neridae EIP5
Pontohedyle liliae WIP1
Pontohedyle wiggi WIP5
Pontohedyle verrucosa Ind1 CIP13
Pontohedyle verrucosa CIP19
Pontohedyle verrucosa CIP7
Pontohedyle verrucosa Ind2 CIP13
Pontohedyle yurihookeri EPT1
Pontohedyle peteryalli EAT2
Pontohedyle wenzli WIP6
Pontohedyle wenzli EIP1
Pontohedyle wenzli CIP6
Pontohedyle wenzli CIP4
Pontohedyle milaschewitchii NEA2
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Pontohedyle milaschewitchii NEA3b
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Pontohedyle brasilensis WAT1
Pontohedyle brasilensis WAT5a
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Parhedyle odhneri comb. nov. NEA5
Ganitus MOTU Panama WAT4
Ganitus evelinae SWA2
Microhedyle MOTU StVincent WAT8
Microhedyle MOTU Ghana EAT1
Microhedyle MOTU Peru SEP2
Microhedyle MOTU Chile SEP1
Microhedyle MOTU West Papua CIP13
Microhedyle remanei SWA1
Microhedyle MOTU Mexico NEP2
Microhedyle glandulifera NEA3a
Microhedyle glandulifera NEA10
Microhedyle glandulifera NEA9
Microhedyle MOTU Egypt WIP1
Microhedyle MOTU Moorea EIP5
Paraganitus MOTU Fiji CIP27
Paraganitus MOTU Vanuatu CIP24
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Paraganitus ellynnae CIP20
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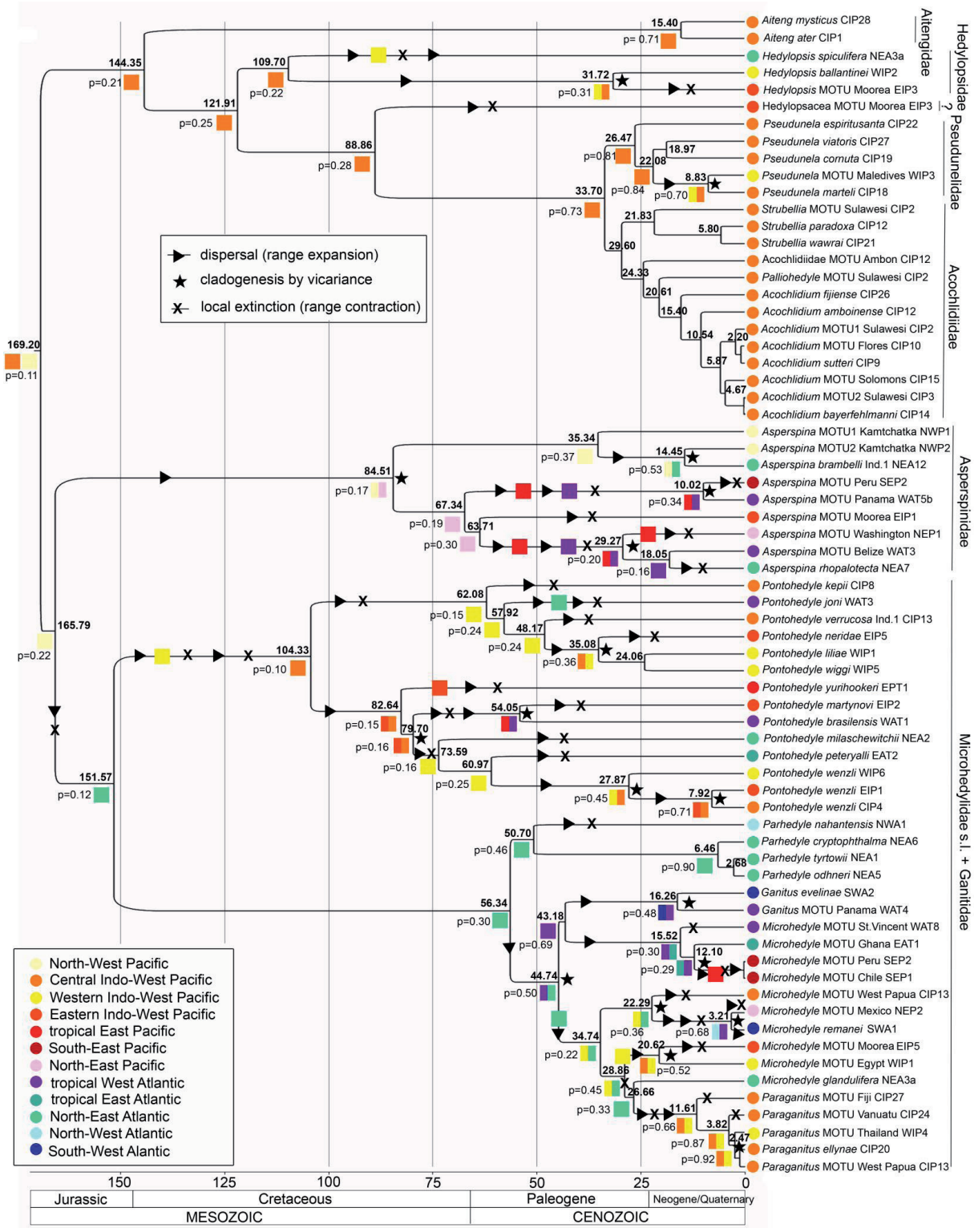
Figure 4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cladogenesis by vicariance
local extinction (range contraction)
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Central Indo-West Pacific
Western Indo-West Pacific
Eastern Indo-West Pacific
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South-East Pacific
North-East Pacific
tropical West Atlantic
tropical East Atlantic
North-East Atlantic
North-West Atlantic
South-West Alantic
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Aiteng ater CIP1
Hedylopsis spiculifera NEA3a
Hedylopsis ballantinei WIP2
Hedylopsis MOTU Moorea EIP3
Hedylopsacea MOTU Moorea EIP3
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Pseudunela viatoris CIP27
Pseudunela cornuta CIP19
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Strubellia paradoxa CIP12
Strubellia wawrai CIP21
Acochlidiidae MOTU Ambon CIP12
Palliohedyle MOTU Sulawesi CIP2
Acochlidium fijiense CIP26
Acochlidium amboinense CIP12
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Acochlidium MOTU Flores CIP10
Acochlidium sutteri CIP9
Acochlidium MOTU Solomons CIP15
Acochlidium MOTU2 Sulawesi CIP3
Acochlidium bayerfehlmanni CIP14
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Asperspina MOTU2 Kamtchatka NWP2
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Asperspina MOTU Peru SEP2
Asperspina MOTU Panama WAT5b
Asperspina MOTU Moorea EIP1
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Pontohedyle wiggi WIP5
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Pontohedyle wenzli CIP4
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Parhedyle odhneri NEA5
Ganitus evelinae SWA2
Ganitus MOTU Panama WAT4
Microhedyle MOTU St.Vincent WAT8
Microhedyle MOTU Ghana EAT1
Microhedyle MOTU Peru SEP2
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Microhedyle remanei SWA1
Microhedyle MOTU Moorea EIP5
Microhedyle MOTU Egypt WIP1
Microhedyle glandulifera NEA3a
Paraganitus MOTU Fiji CIP27
Paraganitus MOTU Vanuatu CIP24
Paraganitus MOTU Thailand WIP4
Paraganitus ellynae CIP20
Paraganitus MOTU West Papua CIP13
Aitengidae
Hedylopsidae
Pseudunelidae
Acochlididae
Asperspinidae
Microhedylidae s.l. + Ganitidae
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125
100
75
50
25
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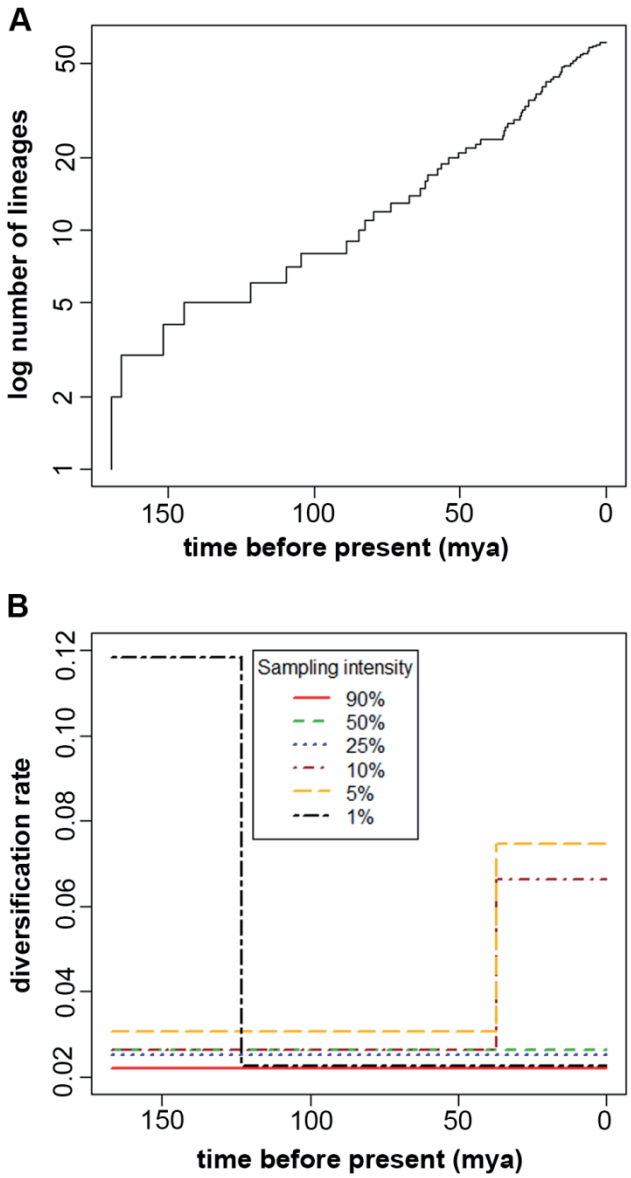
Cretaceous
Paleogene
Neogene/Quaternary
MESOZOIC
CENOZOIC

Figure 5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5
10
20
50
150
100
50
0
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B
diversification rate
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0.04
0.06
0.08
0.10
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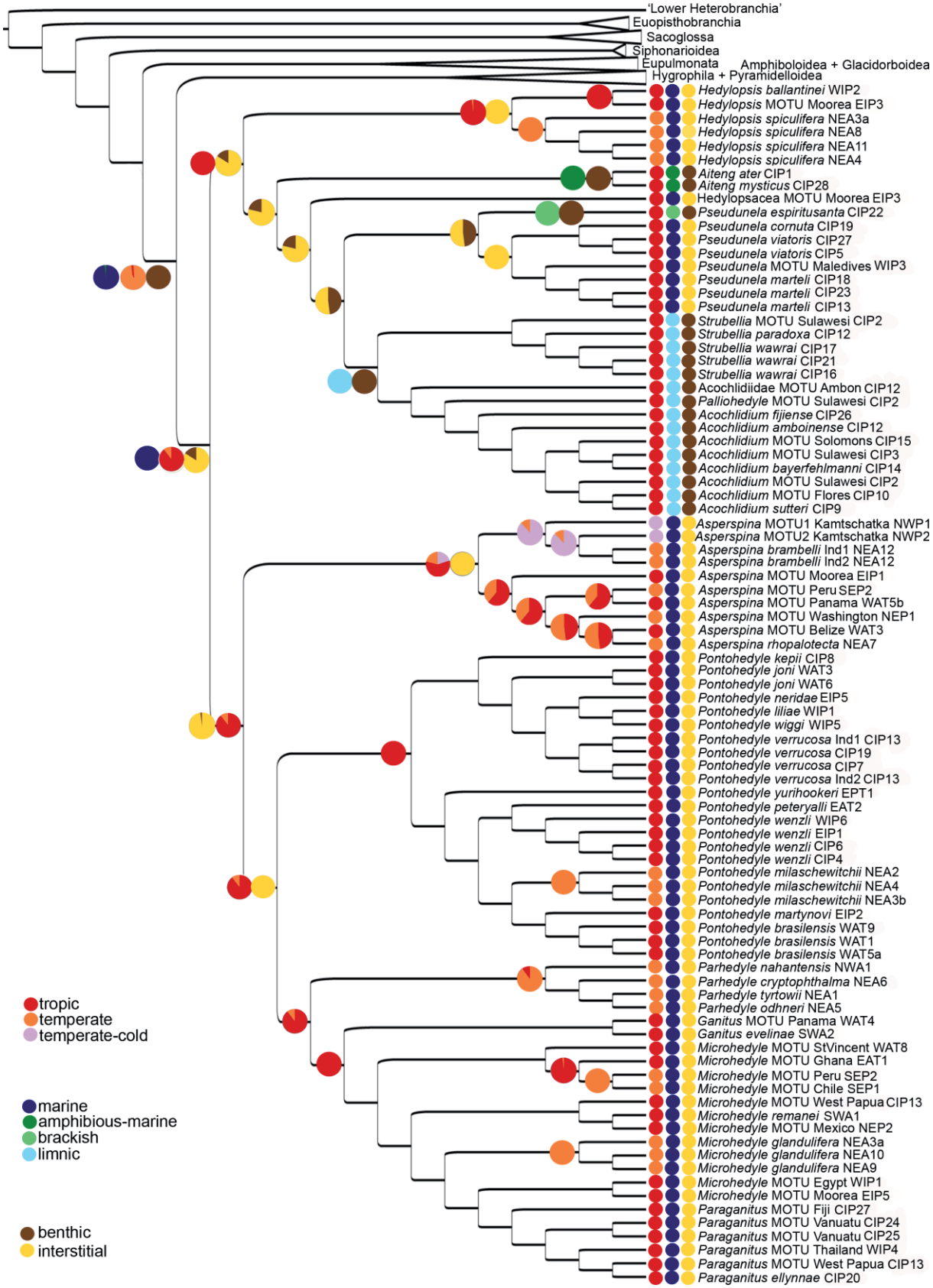
Figure 6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Eupulmonata
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Hygrophila + Pyramidelloidea
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Hedylopsis MOTU Moorea EIP3
Hedylopsis spiculifera NEA3a
Hedylopsis spiculifera NEA8
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Aiteng mysticus CIP28
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Palliohedyle MOTU Sulawesi CIP2
Acochlidium fijiense CIP26
Acochlidium amboinense CIP12
Acochlidium MOTU Solomons CIP15
Acochlidium MOTU Sulawesi CIP3
Acochlidium bayerfehlmanni CIP14
Acochlidium MOTU Sulawesi CIP2
Acochlidium MOTU Flores CIP10
Acochlidium sutteri CIP9
Asperspina MOTU1 Kamtschatka NWP1
Asperspina MOTU2 Kamtschatka NWP2
Asperspina brambelli Ind1 NEA12
Asperspina brambelli Ind2 NEA12
Asperspina MOTU Moorea EIP1
Asperspina MOTU Peru SEP2
Asperspina MOTU Panama WAT5b
Asperspina MOTU Washington NEP1
Asperspina MOTU Belize WAT3
Asperspina rhopalotecta NEA7
Pontohedyle kepii CIP8
Pontohedyle joni WAT3
Pontohedyle joni WAT6
Pontohedyle neridae EIP5
Pontohedyle liliae WIP1
Pontohedyle wiggi WIP5
Pontohedyle verrucosa Ind1 CIP13
Pontohedyle verrucosa CIP19
Pontohedyle verrucosa CIP7
Pontohedyle verrucosa Ind2 CIP13
Pontohedyle yurihookeri EPT1
Pontohedyle peteryalli EAT2
Pontohedyle wenzli WIP6
Pontohedyle wenzli EIP1
Pontohedyle wenzli CIP6
Pontohedyle wenzli CIP4
Pontohedyle milaschewitchii NEA2
Pontohedyle milaschewitchii NEA4
Pontohedyle milaschewitchii NEA3b
Pontohedyle martynovi EIP2
Pontohedyle brasilensis WAT9
Pontohedyle brasilensis WAT1
Pontohedyle brasilensis WAT5a
Parhedyle nahantensis NWA1
Parhedyle cryptophthalma NEA6
Parhedyle tyrtowii NEA1
Parhedyle odhneri NEA5
Ganitus MOTU Panama WAT4
Ganitus evelinae SWA2
Microhedyle MOTU StVincent WAT8
Microhedyle MOTU Ghana EAT1
Microhedyle MOTU Peru SEP2
Microhedyle MOTU Chile SEP1
Microhedyle MOTU West Papua CIP13
Microhedyle remanei SWA1
Microhedyle MOTU Mexico NEP2
Microhedyle glandulifera NEA3a
Microhedyle glandulifera NEA10
Microhedyle glandulifera NEA9
Microhedyle MOTU Egypt WIP1
Microhedyle MOTU Moorea EIP5
Paraganitus MOTU Fiji CIP27
Paraganitus MOTU Vanuatu CIP24
Paraganitus MOTU Vanuatu CIP25
Paraganitus MOTU Thailand WIP4
Paraganitus MOTU West Papua CIP13
Paraganitus ellynnae CIP20
tropic
temperate
temperate-cold
marine
amphibious-marine
brackish
limnic
benthic
interstitial

III. SPECIES DELINEATION AND INTEGRATIVE TAXONOMY

Integrative taxonomy

Chapter 11. Cryptic species in tropic sands - Interactive 3D anatomy, molecular phylogeny and evolution of meiofaunal Pseudunelidae (Gastropoda, Acochlidia)

Neusser TP, **Jörger KM**, Schrödl M (2011) Cryptic species in tropic sands - Interactive 3D anatomy, molecular phylogeny and evolution of meiofaunal Pseudunelidae (Gastropoda, Acochlidia). *PLoS ONE* 6: e23313.

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Cryptic Species in Tropic Sands - Interactive 3D Anatomy, Molecular Phylogeny and Evolution of Meiofaunal Pseudunelidae (Gastropoda, Acochlidia)

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Abstract

Background: Towards realistic estimations of the diversity of marine animals, tiny meiofaunal species usually are underrepresented. Since the biological species concept is hardly applicable on exotic and elusive animals, it is even more important to apply a morphospecies concept on the best level of information possible, using accurate and efficient methodology such as 3D modelling from histological sections. Molecular approaches such as sequence analyses may reveal further, cryptic species. This is the first case study on meiofaunal gastropods to test diversity estimations from traditional taxonomy against results from modern microanatomical methodology and molecular systematics.

Results: The examined meiofaunal *Pseudunela* specimens from several Indo-Pacific islands cannot be distinguished by external features. Their 3D microanatomy shows differences in the organ systems and allows for taxonomic separation in some cases. Additional molecular analyses based on partial mitochondrial cytochrome *c* oxidase subunit I (COI) and 16S rRNA markers revealed considerable genetic structure that is largely congruent with anatomical or geographical patterns. Two new species (*Pseudunela viatoris* and *P. marteli* spp. nov.) are formally described integrating morphological and genetic analyses. Phylogenetic analysis using partial 16S rRNA, COI and the nuclear 18S rRNA markers shows a clade of Pseudunelidae species as the sister group to limnic Acochlidiidae. Within *Pseudunela*, two subtypes of complex excretory systems occur. A complex kidney already evolved in the ancestor of Hedylopsacea. Several habitat shifts occurred during hedylopsacean evolution.

Conclusions: Cryptic species occur in tropical meiofaunal *Pseudunela* gastropods, and likely in other meiofaunal groups with poor dispersal abilities, boosting current diversity estimations. Only a combined 3D microanatomical and molecular approach revealed actual species diversity within *Pseudunela* reliably. Such integrative methods are recommended for all taxonomic approaches and biodiversity surveys on soft-bodied and small-sized invertebrates. With increasing taxon sampling and details studied, the evolution of acochlidian panpulmonates is even more complex than expected.

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Introduction

The study of cryptic species, i.e. two or more distinct species classified as a single species due to the lack of morphological differences, augmented during the last 20 years [1]. There is a consensus about the importance of our knowledge of cryptic diversity for, amongst others, animal diversity estimations, biological control, natural resource protection and conservation (e.g. [1,2]). However, the distribution of cryptic species among metazoan taxa and biogeographical regions is discussed controversially. Whereas Bickford et al. [1] proposed a non-random distribution across taxa and biomes, Pfenninger & Schwenk [3] suggested an almost even distribution among the major metazoan taxa and biogeographical regions. Trontelj & Fiser [2] emphasised that regularities of the cryptic diversity probably will be discovered only by means of genus- or species-level studies.

One area with an unexpectedly high level of cryptic speciation is the Antarctic Ocean. Molecular studies revealed flocks of cryptic rather than single widespread and variable species throughout benthic invertebrate groups examined, e.g. in crinoids, pycnogonids, crustaceans and molluscs [4,5,6,7]. Many, but not all of those organisms from high geographic latitudes are brooders or direct developers with low dispersal abilities, such as the nudibranch gastropod *Doris kerguelenensis* (Bergh, 1884) which ultimately was shown to have undergone an explosive cryptic radiation in the Southern Ocean [6]. According to Thorson's rule, direct developers in benthic organisms such as most molluscs are considered as scarce in subtropical or tropical waters [8]. Exceptions are members of taxa living in the mesopsammon which generally are assumed to be direct developers [9] or, as in case of acochlidian panpulmonate gastropods, may have planktonic larvae which remain in the interstitial spaces [10]. Thus, it can be assumed that their dispersal

ability in the larval stage is very low. Also, meiofaunal acochlidian gastropods appear to occur in coastal sands only, i.e. postlarval stages have virtually no potential for active migration or forming continuous populations across deeper waters. Given this level of supposed immobility and habitat restrictions as opposed to the vast coasts of the world's oceans and innumerable, highly isolated archipelagos and off-shore reefs we should expect that there are plenty of narrow ranged rather than a few wide-ranged acochlidian species. However, based on morphology, only 28 valid species, 20 of them mesopsammic, were described globally. Several of these species such as *Microhedyle remanei* (Marcus, 1953) were considered to be widespread throughout Western Atlantic warm water sands, i.e. in Brazil, Colombia and Bermuda [11,12,13,14], and *Pseudunela cornuta* (Challis, 1970) was recorded to occur on the Solomon Islands (Melanesia) and near Hong Kong (South China Sea) [15,16]. Recently, both species were re-described in considerable anatomical and histological detail [14,17]. However, until now, applying morphospecies concepts on tiny meiofaunal gastropods has never been tested by molecular analyses.

During several expeditions to different Indo-Pacific archipelagos and islands, specimens of the genus *Pseudunela* have been collected and preserved for comparative structural and molecular investigation. Externally, they show variation regarding the colour of the digestive gland shining through the epidermis and the external identification of the eyes, but both features do not allow an unambiguous discrimination from the well-described *P. cornuta* from the Solomon Islands. Within the Hedylopsacea the marine and brackish genus *Pseudunela* possesses a key position as sister group to the limnic Acochlidiidae [18]. For a better understanding of the invasion of freshwater systems and the evolution of involved organ systems in Acochlidia, it was thus indispensable to assess the organ and species diversity within *Pseudunela*, as well as their phylogeny and directions of evolution. *Pseudunela cornuta* from the Solomon Islands was first described by Challis [15]. Recently, these original data were complemented and corrected by Neusser et al. [17] including an interactive 3D-reconstruction. Hughes [16] reported of a second record of *P. cornuta* from Hong Kong. However, her species description is very brief and vague, so that a recollection at the same locality and a detailed re-description of this species is essential before including it in our comparative study of *Pseudunela*. The same situation applies to the description of *Pseudunela eirene* Wawra, 1988 [19] which needs a revision as well.

The present study gives an extensive anatomical description of all *Pseudunela* specimens available to us, including interactive 3D-reconstructions of *Pseudunela viatoris* sp. nov. from Fiji. Another new species involved is described in the same detail in the present study and is briefly compared with *P. viatoris* sp. nov.. The genetic diversity within *Pseudunela* is assessed using partial mitochondrial cytochrome *c* oxidase subunit I (COI) gene, which was proposed as standard DNA barcoding marker [20,21,22], and partial 16S rRNA gene sequences. The origin and the phylogenetic relationships of *Pseudunela* species are reconstructed by additionally using the nuclear 18S rRNA marker. The largely cryptic radiation of the different *Pseudunela* species is discussed. A possible scenario on the evolution of the excretory system in Acochlidia is given.

Methods

Sampling and semithin sectioning

Specimens of different *Pseudunela* species were collected during expeditions to various Indo-Pacific Islands, namely Fiji, Indonesia, Solomon Islands and Vanuatu. They were extracted from sand samples according to Schrödl [23] and subsequently relaxed by a solution of isotonic $MgCl_2$. Some specimens were preserved in 4% glutardialdehyde in 0.2 M sodium cacodylate buffer (0.1 M NaCl and 0.35 M sucrose, pH 7.2), followed by post-fixation in buffered 1% OsO_4 for 1.5 h in the dark. The specimens were decalcified in 1% ascorbic acid overnight and dehydrated in an acetone series (30, 50, 70, 90, 100%). For semithin sectioning specimens were embedded in Spurr's low viscosity resin [24]. Several series of ribboned serial semithin sections of 1.5 µm thickness were prepared using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) and contact cement on the lower cutting edge to form ribbons [25]. Sections finally were stained with methylene-azure II [26] and were deposited at the Mollusca Department, Bavarian State Collection of Zoology (ZSM), Munich, Germany. A list of the material examined including the museum numbers is shown in Table 1.

3D reconstruction

Digital photographs of every slice were taken with a CCD microscope camera (Spot Insight, Diagnostic Instruments, Sterling Heights, USA) mounted on a DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). Images were converted to 8bit greyscale format, contrast enhanced and unsharp masked with standard image editing software. A detailed computer-based 3D-reconstruction of all major organ systems was conducted with the software AMIRA 5.2 (Visage Imaging GmbH, Berlin, Germany) following basically the procedure explained by Ruthensteiner [25]. The presented 3D-reconstruction is based on series N° ZSM 20080492.

Interactive 3D-model

The interactive 3D-model for the supporting information was prepared according to Ruthensteiner & Heß [27], but using different software, i.e. the 3D tools of Deep Exploration 5.5 (Right Hemisphere EMEA, Germany) and Adobe Acrobat 9.0 Professional Extended (Adobe Systems GmbH, Germany). The reconstructed surfaces were saved as *.obj format in Amira and one by one opened in Deep Exploration. The display settings were adjusted (solid, no grid, CAD optimized illumination, smoothing 180°) and each surface was reduced to 10–30%. The surfaces were saved as *.u3d format. Finally, a complex *.u3d model including all surfaces was generated. For that purpose each surface was given a name and colour and the model was set up using the function 'merge file'. The surfaces were arranged according to organ systems using the function 'create group'. The *.u3d model was imported in a pdf in Adobe Acrobat 9.0 Professional Extended and different views of the organ systems were prefabricated to standard views allowing the reader to get rapidly a general idea of the model. The 3D-model is accessible by clicking onto the figure in the supporting information figure S1 (Adobe Reader Version 7 or higher required).

Analysis by scanning electron microscopy (SEM)

Specimens preserved in 75% and 96% EtOH were used for the examination of the radulae by SEM. They were macerated in 10% KOH overnight to separate the radula from the surrounding tissue. Remaining tissue was manually removed with fine dissection pins. The radulae were mounted on specimen stubs, sputter coated with gold for 135 sec. (SEM-Coating-System, Polaron) and analysed using a LEO 1430 VP (Leo Elektronenmikroskopie GmbH, Oberkochen, Germany) at 15 kV.

DNA extraction, polymerase chain reaction and sequencing

DNA was extracted from entire specimens using QIAGEN DNeasy Tissue Kit according to the manufacture's instructions. Three different gene regions were amplified: approximately 650 bp of the mitochondrial cytochrome *c* oxidase subunit I

Table 1. Material examined in the present study.

Species	Locality	Museum N°	Preparation type	Accession number of DNA voucher (ZSM)	GenBank Accession N°		
					COI	16S	18S
Pseudunela viatoris sp. nov.	Fiji, Viti Levu, Laucala Bay, Nukumbutho Island	20080492	sections				
		20080493	sections				
		20062048	SEM				
		20080020	mol	AB34404247	JF819766	JF819741	JF819751
		20080021	mol	AB34404265	JF819767	JF819742	-
		20080057	mol	AB34404281	JF819768	JF819743	-
Pseudunela viatoris sp. nov.	Indonesia, bay of Gili Lawa Laut Island	20090422	sections				
		20090423	sections				
		20071120	SEM				
		20071120	mol	AB34404285	JF819769	JF819744	JF819752
		20070953	mol	AB34404276	JF819770	JF819745	-
Pseudunela marteli sp. nov.	Solomon Islands, Guadalcanal, Honiara, beach of "Art Gallery"	20071851	sections				
		20071864	sections				
		20071865	sections				
		20071826	SEM				
		20080022	mol	AB34404252	JF819771	JF819746	JF819753
		20080023	mol	AB34404298	JF819772	-	-
		20080024	mol	AB34404218	JF819773	JF819747	-
Pseudunela marteli sp. nov.	Vanuatu, Oyster Island	20071061	sections				
		20090416	sections				
		20080105	SEM				
		20080393	GenBank	AB35081809	HQ168456	HQ168418	HQ168431
Pseudunela cornuta	Solomon Islands, Guadalcanal, Komimbo Bay	20071809	mol	AB34404215	JF819774	JF819748	JF819754
Pseudunela espiritusanta	Vanuatu, Espiritu Santo	20080117	mol	AB34404289	JF819775	JF819749	JF819755
		20071118	mol	AB34404210	JF819776	JF819750	-
Hedylopsis ballantinei	Egypt, Dahab, Red Sea	20090244	GenBank	AB34858170	HQ168454	HQ168416	HQ168429
Strubellia paradoxa	Indonesia, Ambon, Maluku Utara	193944 (Natural History Museum, Berlin)	GenBank	AB34858174	HQ168457	HQ168419	HQ168432
Acochlidium fijiense	Fiji, Viti Levu, Lami River	20080063	GenBank	AB34404244	HQ168458	HQ168420	HQ168433
Microhedyle glandulifera	Croatia, Istria, Kap Kamenjak	20081019	GenBank	AB35081799	HQ168461	HQ168424	HQ168437
Aitengidae sp.	Japan, Okinawa, Miyako Island	-	GenBank	-	HQ168453	HQ168415	HQ168428

Museums numbers refer to the Bavarian State Collection of Zoology, Germany (**ZSM**), if not indicated otherwise; **GenBank**, molecular data retrieved from GenBank; **mol**, molecular data generated within this study; **sections**, semithin serial sections for histology; **SEM**, scanning electron microscopy.

doi:10.1371/journal.pone.0023313.t001

(COI) gene; partial mitochondrial 16S rRNA gene sequence (around 420 bp) and approximately 1800 bp of the nuclear 18S rRNA gene (for PCR protocols and primers used see Table 2). Successful PCR products were cleaned up using ExoSapIT (USB, Affymetrix, Inc.). Cycle sequencing and the sequencing reaction was performed by the sequencing service of the Department of Biology Genomic Service Unit (GSU) of the Ludwig-Maximilians-University Munich using Big Dye 3.1 kit and an ABI 3730 capillary sequencer. All fragments were sequenced in both directions using the PCR primers as specified in Table 2.

For 16S rRNA gene and COI one to three individual(s) of each *Pseudunela* species were sequenced and analysed, for 18S rRNA gene and outgroup species only one specimen was analysed. Outgroup sequences were retrieved from GenBank (see Table 1) and selected based on the latest phylogenetic hypotheses of the Acochlidia [18,28]. All sequences generated within this study are deposited to GenBank and DNA aliquots are stored at DNAbank at the ZSM (http://www.dnabank-network.org) (see Table 1 for accession numbers).

Sequence alignment and phylogenetic analyses

All sequences generated were checked for contaminations with BLAST searches [29], implemented in the GenBank database. Sequences were edited using BioEdit 7.0.9 and Sequencher 4.8 (Gene Codes Corporation). The alignment was performed with MAFFT v6 [30] using the default settings. The alignment of the protein-coding COI data was corrected manually according to amino acids. Poorly

Table 2. Primer sequences and PCR protocols used for each of the amplified gene regions.

Gene region	Primer	Sequence 5′ - 3′	Reference	PCR program
18S	18A1	CCT ACT TCT GGT TGA TCC TGC CAG T	[70]	98°C 30 sec (98°C 5 sec, 48–65°C 5 sec, 72°C 20–25 sec)×28–40, 72°C 60 sec (Phire polymerase, New England Biolabs)
	700R	CGC GGC TGC TGG CAC CAG AC	[71]	
	470F	CAG CAG GCA CGC AAA TTA CCC	[71]	
	1500R	CAT CTA GGG CAT CAC AGA CC	[71]	
	1155F	CTG AAA CTT AAA GGA ATT GAC GG	[71]	
	1800	TAA TGA TCC TTC CGC AGG TT	[70]	
16S	16S-H	CGC CTG TTT ATC AAA AAC AT	[72]	98°C 30 sec (98°C 5 sec, 48–55°C 5 sec, 72°C 25 sec)×35–40, 72°C 60 sec (Phire polymerase, New England Biolabs)
	16S-R	CCG GTC TGA ACT CAG ATC ACG T	[72]	
	16Sf-50	GGC CGC AGT ACC TTG ACT GT	present study	
	16Sr-380	TCC ACC ATC GAG GTC ACA AG	present study	
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	[73]	94°C 3 min (94°C 60 sec, 48–52°C 60 sec, 72°C 90 sec)×35–40, 72°C 3 min (Taq polymerase, Sigma)
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	[73]	

doi:10.1371/journal.pone.0023313.t002

aligned positions and divergent regions in the 18S rRNA gene and 16S rRNA gene alignment were excluded using the standard options for a less stringent selection in Gblocks [31].

The combined data set comprised of the 18S, 16S and COI was subject to phylogenetic analyses using maximum likelihood in RAxML 7.0.4 [32]. Data were analysed in four partitions (18S; 16S; COI 1st and 2nd codon position and 3rd separately) under the G+Γ+I model selected with jModeltest [33]. The microhedylacean *Microhedyle glandulifera* was defined as outgroup, following recent phylogenetic approaches based on morphology [18] and molecular data [28]. The program parameters were adapted to the alignment as described in the manual ("hard and slow way" – with ten parsimony starting trees and six different rate categories). Additionally 200 multiple interferences were executed on the alignment and 1000 bootstrap replicates were generated.

For species delineation based on our molecular dataset, we additionally used Species Identifier (obtained from TaxonDNA [34]) to group sequences into clusters based on pairwise distances of both mitochondrial markers (testing thresholds from 1–10%) and to evaluate intra- and interspecific variation. Haplotype networks of *Pseudunela* based on the partial mitochondrial COI sequences were inferred using statistical parsimony as implemented in TCS 1.21 [35] under the default settings (95% confidence criterion) for both mitochondrial markers. Using a maximum likelihood approach, the general mixed Yule-coalescent (GMYC) model is able to discriminate between population and speciation patterns based on a phylogenetic tree (for detailed description of the methodology see [36,37]). We performed GMYC using the R package SPLITS (http://r-forge.r-project.org/projects/splits/). The input tree was generated with RAxML 7.0.4 [32] as described above, based on the concatenated mitochondrial dataset (COI+16S). Our RAxML tree was converted into an ultrametric tree using the package 'ape' in R (chronopl function [38]) and an analysis allowing multiple thresholds [36] was performed.

Nomenclatural acts

The electronic version of this document does not represent a published work according to the International Code of Zoological Nomenclature (ICZN), and hence the nomenclatural acts contained in the electronic version are not available under that Code from the electronic edition. Therefore, a separate edition of this document was produced by a method that assures numerous identical and durable copies, and those copies were simultaneously obtainable (from the publication date noted on the first page of this article) for the purpose of providing a public and permanent scientific record, in accordance with Article 8.1 of the Code. The separate print-only edition is available on request from PLoS by sending a request to PLoS ONE, Public Library of Science, 1160 Battery Street, Suite 100, San Francisco, CA 94111, USA along with a check for $10 (to cover printing and postage) payable to "Public Library of Science".

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The online version of this work is archived via PubMed Central and LOCKSS and also available at http://www.zsm.mwn.de/mol/pub_schroedl.htm.

Results

Species description of *Pseudunela viatoris* sp. nov. from Fiji and Indonesia

Systematics. Family Pseudunelidae Rankin, 1979

Genus *Pseudunela* Salvini-Plawen, 1973

Pseudunela viatoris sp. nov.

urn:lsid:zoobank.org:act: 9A559BA2-4EEE-4F3B-A1D2-A72ECB92096B.

Type Material—Holotype: ZSM Mol 20061954, stored in 75% EtOH; collected in Fiji, Viti Levu, Laucala Bay, Nukumbutho Island. GPS: 18°10.47′S, 178°28.34′E. Paratypes: ZSM Mol 20061945, 20 specimens stored in 75% EtOH; all paratypes collected together with holotype.

Etymology—*Pseudunela viatoris* sp. nov. is named after the latin word "viator" (engl. pilgrim/voyager) according to its supposed ability to travel over long distances.

Distribution—Known from Viti Levu, Fiji and Gili Lawa Laut, Indonesia.

In addition to the 3D plates please see also the supporting information (Fig S1): Interactive 3D-model of *Pseudunela viatoris* sp. nov. from Fiji.

External morphology. The body of *Pseudunela viatoris* sp. nov. is divided into an anterior head-foot complex (hf) and a posterior elongated visceral hump (vh) (Fig. 1A). The paired labial tentacles (lt) are broad at the base and taper to the end. The rhinophores (rh) are tapered and shorter and thinner than the labial tentacles (Fig. 1A). The densely ciliated foot (f) is as broad as the anterior head-foot complex and extends about one third of the elongated visceral hump (Fig. 1B). The heart bulb (hb) (Fig. 1A) is visible externally in the anterior part of the visceral hump on the right body side. Subepidermal, needle-shaped calcareous spicules are sparsely distributed in the cephalic tentacles, the foot and the visceral hump; in the anterior part of the latter they are larger than in the posterior part. The body colour is whitish translucent, the digestive gland (dg) (Fig. 1A) is brownish coloured (in specimens from Indonesia: orange-brownish (Fig. 2A)) shining through the epidermis. Epidermal glands (eg) (Fig. 3E) are distributed

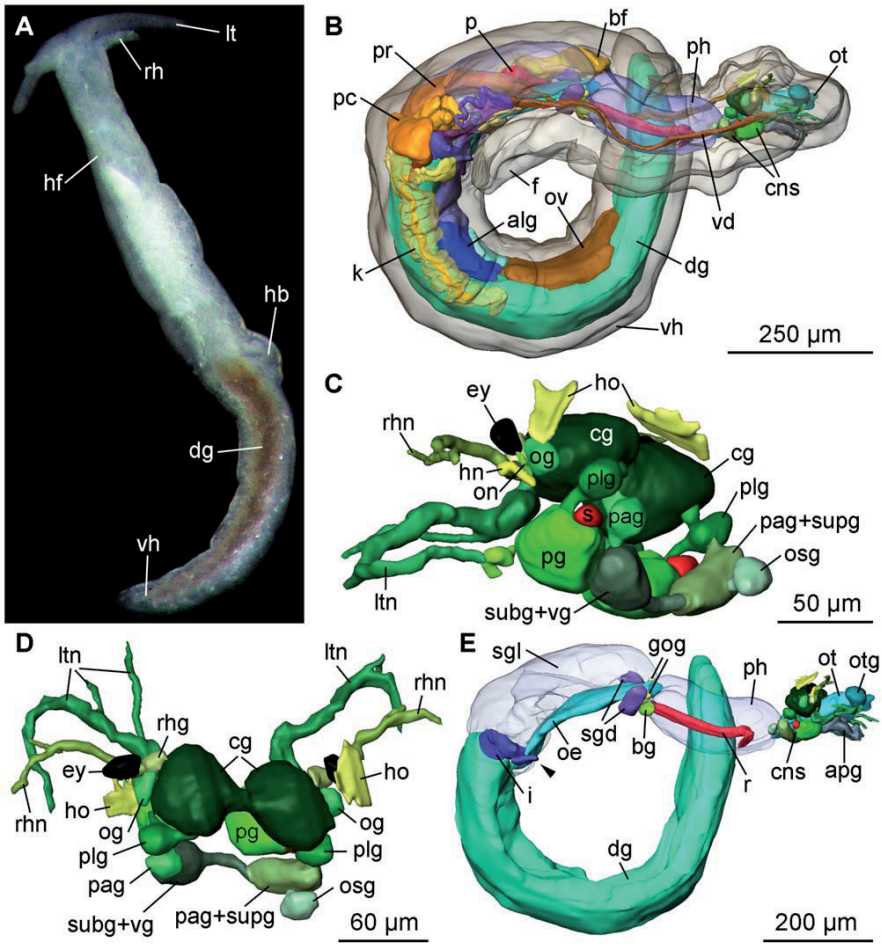

Figure 1. Photograph of a living specimen and 3D reconstruction of *P. viatoris* sp. nov. from Fiji. A: external morphology of a living specimen (body size 3 mm), dorsal view. B: general anatomy, right view. C: CNS, left view. D: CNS, dorsal view. E: digestive system with CNS, right view. Abbreviations: **alg**, albumen gland; **apg**, anterior pedal gland; **bf**, basal finger; **bg**, buccal ganglion; **cg**, cerebral ganglion; **cns**, central nervous system; **dg**, digestive gland; **ey**, eye; **f**, foot; **gog**, gastro-oesophageal ganglion; **hb**, heart bulb; **hf**, head-foot complex; **hn**, Hancock's nerve; **ho**, Hancock's organ; **i**, intestine; **k**, kidney; **lt**, labial tentacle; **ltn**, labial tentacle nerve; **oe**, oesophagus; **og**, optic ganglion; **on**, optic nerve; **osg**, osphradial ganglion; **ot**, oral tube; **otg**, oral tube gland; **ov**, ovotestis; **p**, penis; **pag**, parietal ganglion; **pc**, pericardium; **pg**, pedal ganglion; **ph**, pharynx; **plg**, pleural ganglion; **pr**, prostate; **r**, radula; **rh**, rhinophore; **rhg**, rhinophoral ganglion; **rhn**, rhinophoral nerve; **s**, statocyst; **sgd**, salivary gland duct; **sgl**, salivary gland; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **vd**, vas deferens; **vg**, visceral ganglion; **vh**, visceral hump; **arrowhead**, common opening of digestive and excretory systems. **The interactive 3D-model** of *P. viatoris* sp. nov. can be accessed by clicking onto the figure in the supporting information figure S1 (Adobe Reader Version 7 or higher required). Rotate model by dragging with left mouse button pressed, shift model: same action+ctrl (or change default action for left mouse button), zoom: use mouse wheel. Select or deselect (or change transparency of) components in the model tree, switch between prefab views or change surface visualization (e.g. lightning, render mode, crop etc.).

doi:10.1371/journal.pone.0023313.g001

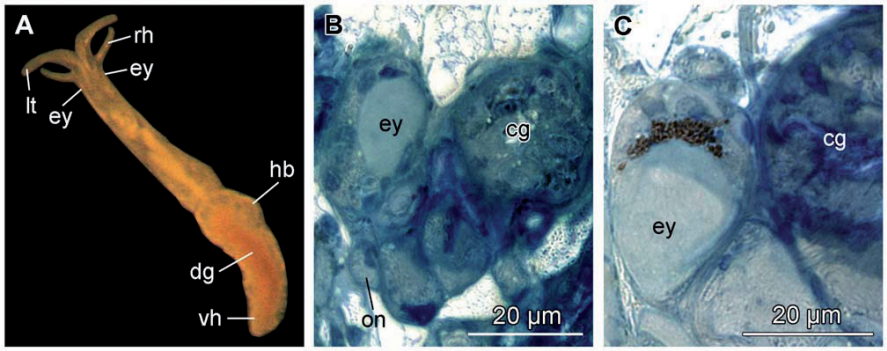

Figure 2. Photograph of a living specimen and histological cross-sections of *P. viatoris* sp. nov. from Indonesia. A: external morphology of a living specimen (body size 3 mm). B: unpigmented eye. C: pigmented eye. Abbreviations: **cg**, cerebral ganglion; **dg**, digestive gland; **ey**, eye; **hb**, heart bulb; **lt**, labial tentacle; **on**, optic nerve; **rh**, rhinophore; **vh**, visceral hump.
doi:10.1371/journal.pone.0023313.g002

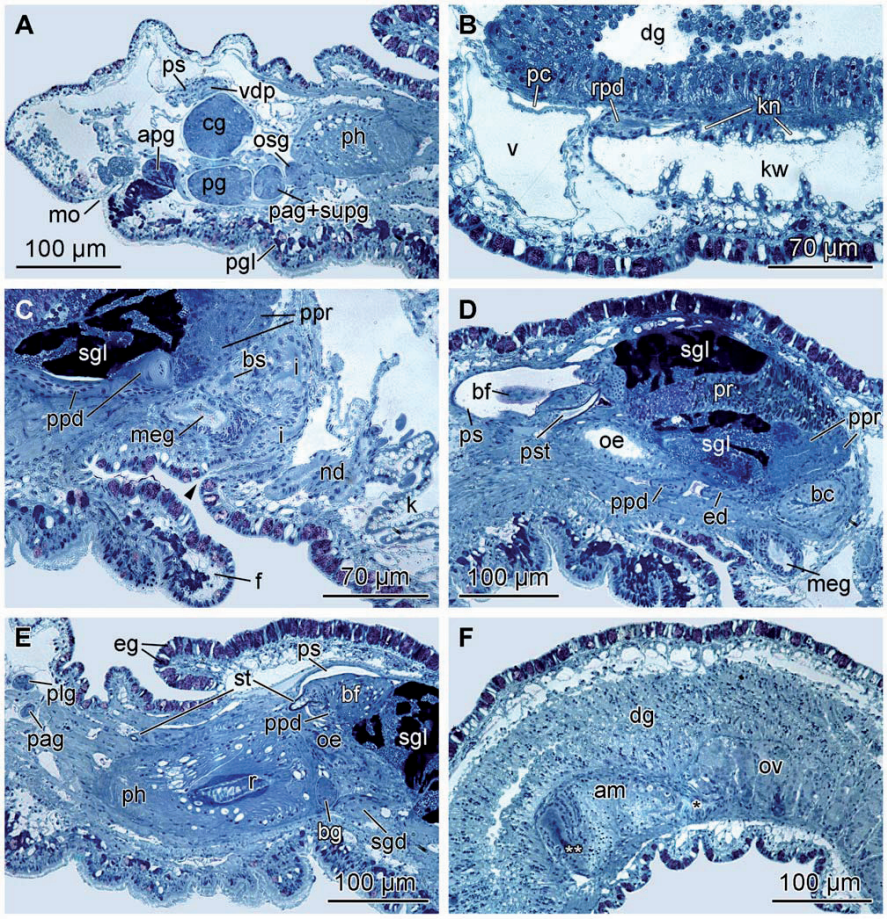

Figure 3. Histological cross-sections of *P. viatoris* sp. nov. from Fiji. A: anterior pedal gland and ganglia. B: circulatory and excretory systems. C: common opening of digestive and excretory systems. D: penial stylet and prostate. E: basal finger and pharynx. F: ampulla and ovotestis. Abbreviations: **am**, ampulla; **apg**, anterior pedal gland; **bc**, bursa copulatrix; **bf**, basal finger; **bg**, buccal ganglion; **bs**, bursa stalk; **cg**, cerebral ganglion; **dg**, digestive gland; **ed**, ejaculatory duct; **eg**, epidermal gland; **f**, foot; **i**, intestine; **k**, kidney; **kn**, narrow lumen of kidney; **kw**, wide lumen of kidney; **meg**, membrane gland; **mo**, mouth opening; **nd**, nephroduct; **oe**, oesophagus; **osg**, osphradial ganglion; **ov**, ovotestis; **pag**, parietal ganglion; **pc**, pericardium; **pg**, pedal ganglion; **pgl**, pedal gland; **ph**, pharynx; **plg**, pleural ganglion; **ppd**, paraprostatic duct; **ppr**, paraprostate; **pr**, prostate; **ps**, penial sheath; **pst**, penial stylet; **r**, radula; **rpd**, renopericardioduct; **sgd**, salivary gland duct; **sgl**, salivary gland; **st**, stylet of basal finger; **supg**, supraintestinal ganglion; **v**, ventricle; **vdp**, posterior-leading vas deferens; *, pre-ampullary gonoduct; **, post-ampullary gonoduct; **arrowhead**, common opening of digestive and excretory systems.
doi:10.1371/journal.pone.0023313.g003

particularly over the visceral hump. The body size of living specimens is about 3 mm. Whereas eyes are not visible externally in specimens from Fiji (Fig. 1A), eyes (ey) are weakly visible in some specimens from Indonesia (Fig. 2A).

Microanatomy: Central nervous system (CNS). The euthyneurous CNS of *Pseudunela viatoris* sp. nov. consists of the paired cerebral (cg), rhinophoral (rhg), optic (og), pedal (pg), pleural (plg), buccal (bg) and gastro-oesophageal ganglia (gog) and three distinct ganglia on the visceral nerve cord, plus an osphradial ganglion (osg) (Fig. 4). All ganglia excluding the buccal and gastro-oesophageal ganglia are located pre-pharyngeally (Fig. 1E). The cerebral, pedal and pleural ganglia are linked by short connectives forming the pre-pharyngeal nerve ring. The strong labiotentacular nerve (ltn) (Figs. 1C, D; 4) emerges from the cerebral ganglion innervating the labial tentacle. A rhinophoral ganglion (Figs. 1 D; 4) is connected anterodorsally to each cerebral ganglion by a short, single cerebro-rhinophoral connective. A nerve arises from the rhinophoral ganglion and bifurcates at its base. The rhinophoral nerve (rhn) (Figs. 1C, D; 4) innervates the rhinophore and the Hancock's nerve (hn) (Figs. 1C; 4) extends to the paired Hancock's organ (ho) (Figs. 1C, D; 4). The latter is a ciliated groove just behind the rhinophore. An optic ganglion (Figs. 1C, D; 4) is connected laterally to each cerebral ganglion by a thin nerve. The optic nerve (on) (Figs. 1C; 4) emerges from the optic ganglion innervating the unpigmented eye (ey) (Figs. 1C, D; 4) of 30–35 μm. In specimens from Indonesia unpigmented (Fig. 2B) and pigmented (Fig. 2C) eyes are present. Precerebral accessory ganglia are absent. The pedal commissure is slightly longer than the cerebral commissure. A statocyst (Figs. 1C; 4) is attached dorsally to each pedal ganglion. The pleural ganglia (Figs. 1C, D; 4) are connected by very short connectives to the visceral nerve cord, thus the latter is arranged anterior to the pharynx. There are three separate ganglia on the visceral nerve cord: the left parietal ganglion (pag), the fused subintestinal/visceral ganglion (subg+vg) and the fused right parietal/supraintestinal ganglion (pag+supg) (Figs. 1C, D; 4). Only the subintestinal/visceral-parietal/supraintestinal connective is long. An osphradial ganglion (Figs. 1C, D; 3A; 4) is connected to the fused parietal/supraintestinal ganglion. No histologically differentiated osphradium could be detected. The buccal ganglia (Figs. 1E; 3E; 4) are located posterior to the pharynx and the short buccal commissure runs ventrally to the oesophagus. A small gastro-oesophageal ganglion (Figs. 1E; 4) is connected dorsally to each buccal ganglion.

Microanatomy: Digestive system. The mouth opening (mo) (Fig. 3A) is situated ventrally between the labial tentacles. The paired anterior pedal glands (apg) (Figs. 1E; 3A) discharge ventrally of the mouth opening to the exterior. The oral tube (ot) (Fig. 1E) is long and flanked by paired oral tube glands (otg) (Fig. 1E) which discharge in its anterior part. The hook-shaped radula (r) (Figs. 1E; 3E) is approx. 180 μm long and embedded within the muscular pharynx (ph) (Figs. 1E; 3E). The radula formula is 44–50×1.1.2 with 32–37 teeth on the upper ramus and 12–17 teeth on the lower one. The triangular rhachidian tooth (Fig. 5B) bears one projecting central cusp (cc) with 3–4 lateral denticles (d) on each side. The first pair of lateral denticles shows almost the same size as the central cusp, the other denticles are smaller. The left lateral tooth (ltl) (Fig. 5A, D) is plate-like and has a well-developed, pointed denticle on their anterior margin and a prominent notch (n) on the posterior one, in which the denticle of the anterior lateral tooth matches. The right lateral teeth (ltr) (Fig. 5A, C) consist of two plates; the first inner one shows also a denticle on its anterior margin and a small emargination (Fig. 5C) next to the notch, the second outer lateral tooth lacks any denticle. The inner margins of the first lateral plates are always rounded;

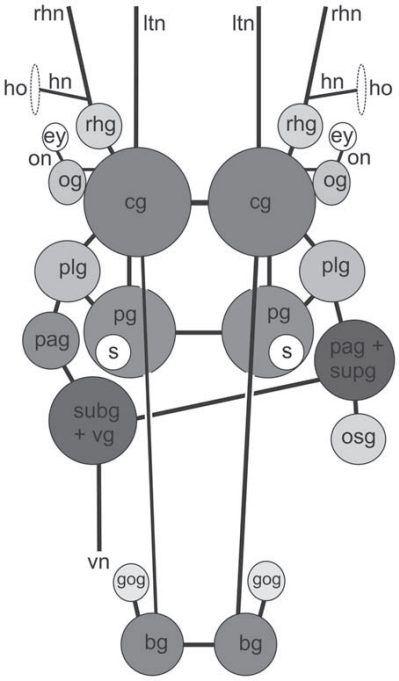

Figure 4. CNS of *P. viatoris* sp. nov. from Fiji (schematic overview, dorsal view). Abbreviations: **bg**, buccal ganglion; **cg**, cerebral ganglion; **ey**, eye; **gog**, gastro-oesophageal ganglion; **hn**, Hancock's nerve; **ho**, Hancock's organ; **ltn**, labial tentacle nerve; **og**, optic ganglion; **on**, optic nerve; **osg**, osphradial ganglion; **pag**, parietal ganglion; **pg**, pedal ganglion; **plg**, pleural ganglion; **rhg**, rhinophoral ganglion; **rhn**, rhinophoral nerve; **s**, statocyst; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **vg**, visceral ganglion; **vn**, visceral nerve. Not to scale.
doi:10.1371/journal.pone.0023313.g004

the outer margin of the left lateral tooth is rounded as well, whereas strait in the right lateral tooth. In the specimens from Indonesia the rhachidian tooth shows 2–4 denticles per side. The presence or absence of a second lateral tooth on the right side cannot be confirmed here; however, there is an emargination present and the outer margin of the first right lateral tooth is strait as in the Fijian specimens. These features may indicate a second lateral tooth in the specimen from Indonesia, as well. Jaws are absent. The oesophagus (oe) (Figs. 1E; 3D, E) is long and ciliated. In the anterior part one pair of large salivary glands (sgl) (Figs. 1E; 3C, D) is connected via salivary gland ducts (sgd) (Figs. 1E; 3E). The sac-like digestive gland (dg) (Figs. 1E; 3F) extends to the posterior end of the visceral hump (Fig. 1A, B). The intestine (i) (Figs. 1E; 3C) is densely ciliated and short. It receives the nephroduct (nd) before opening as a common duct (Figs. 3C; 6B) ventrolaterally on the right side of the visceral hump and posterior to the female gonopore to the exterior.

Microanatomy: Circulatory and excretory systems. The circulatory and excretory systems are situated at the beginning of the visceral hump at the right side of the body (Fig. 1B). The circulatory system comprises a thin-walled pericardium (pc) (Figs. 6A, B; 7) surrounding a large one-chambered heart (v) (Figs. 3B; 7). The aorta could not be detected. The reno-pericardioduct (rpd) (Figs. 3B; 6A; 7) is a well-developed, densely ciliated funnel. The kidney (k) is an elongated sac (Fig. 1B) that extends over the anterior half of the visceral hump. Internally it is subdivided into two histologically distinct sections: a narrow lumen

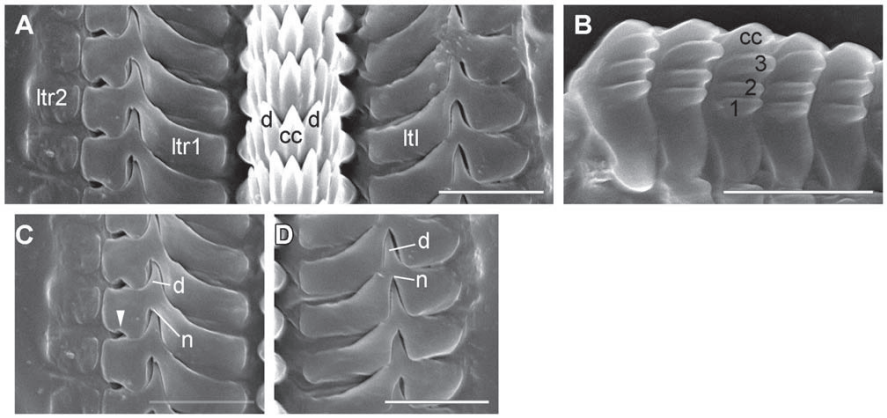

Figure 5. SEM micrographs of the radula of *P. viatoris* sp. nov. from Fiji. A: row of radular teeth. B: rhachidian tooth. C: right lateral teeth. D: left lateral tooth. Abbreviations: **cc**, central cusp; **d**, denticle; **ltl**, left lateral tooth; **ltr1**, first right lateral tooth; **ltr2**, second right lateral tooth; **n**, notch; **rh**, rhachidian tooth; **1**,**2**,**3**, lateral denticle on rhachidian tooth; **arrowhead**, emargination.
doi:10.1371/journal.pone.0023313.g005

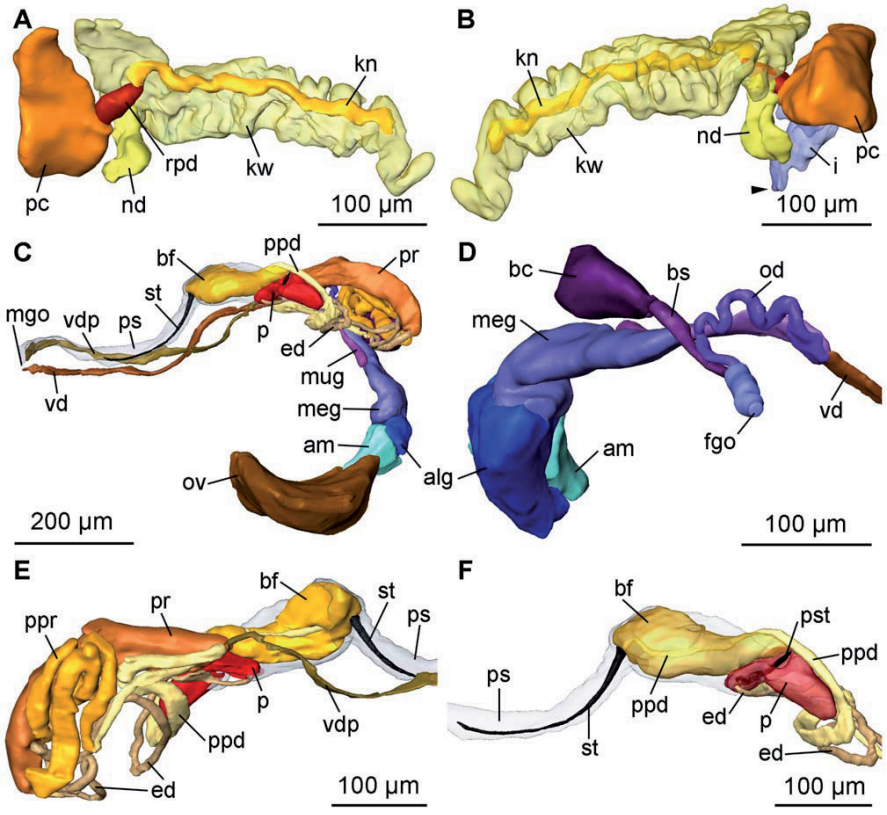

Figure 6. 3D reconstruction of the excretory and reproductive systems of *P. viatoris* sp. nov. from Fiji. A: circulatory and excretory systems, left view. B: circulatory and excretory systems, right view. C: complete reproductive system, left view. D: nidamental glands and sperm storing receptacles, right view. E: anterior male copulatory organs, right view. F: penis and basal finger, left view. Abbreviations: **alg**, albumen gland; **am**, ampulla; **bc**, bursa copulatrix; **bf**, basal finger; **bs**, bursa stalk; **ed**, ejaculatory duct; **fgo**, female gonopore; **i**, intestine; **kn**, narrow lumen of kidney; **kw**, wide lumen of kidney; **meg**, membrane gland; **mgo**, male gonopore; **mug**, mucus gland; **nd**, nephroduct; **od**, oviduct; **ov**, ovotestis; **p**, penis; **pc**, pericardium; **ppd**, paraprostatic duct; **ppr**, paraprostate; **pr**, prostate; **ps**, penial sheath; **pst**, penial stylet; **rpd**, renopericardioduct; **st**, stylet of basal finger; **vd**, vas deferens; **vdp**, posterior-leading vas deferens; **arrowhead**, common opening of digestive and excretory systems.
doi:10.1371/journal.pone.0023313.g006

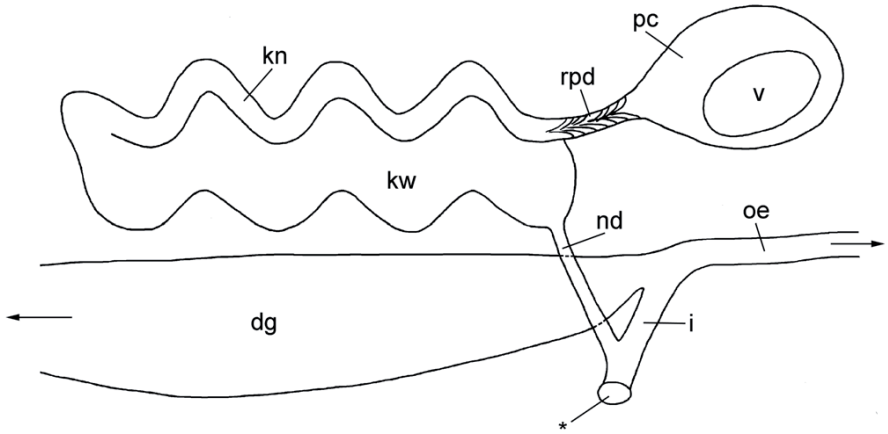

Figure 7. Circulatory and excretory systems of *P. viatoris* sp. nov. from Fiji (schematic drawing, right view). Abbreviations: **dg**, digestive gland; **i**, intestine; **kn**, narrow lumen of kidney; **kw**, wide lumen of kidney; **nd**, nephroduct; **oe**, oesophagus; **pc**, pericardium; **rpd**, renopericardioduct; **v**, ventricle; *, common opening of excretory and digestive systems. Drawing not to scale.
doi:10.1371/journal.pone.0023313.g007

(kn) bordered by tissue with small vacuoles, and a wide lumen (kw) limited by tissue with large vacuoles (Figs. 3B; 6A, B; 7). The renopericardioduct connects to the excretory system in the anterior part of the kidney to its narrow lumen (Fig. 3B). The latter joins the wide lumen in the posterior part of the kidney (Fig. 7). The transition of the kidney and the nephroduct is narrow and ciliated. The nephroduct (Figs. 6A, B; 7) is short and empties into the distal part of the intestine just before the opening to the exterior (Figs. 3C; 7).

Microanatomy: Reproductive system. The terminology used below follows basically Ghiselin [39], Klussmann-Kolb [40] and Haase & Wawra [41].

Specimens of *Pseudunela viatoris* sp. nov. have a hermaphroditic and special androdiaulic reproductive system. The sac-like ovotestis (ov) (Figs. 1B; 6C; 8) extends over the half of the visceral hump and is separated into follicles (Fig. 3F). No yolky oocytes are developed in the examined specimen. Anterior to the ovotestis there is a tubular ampulla (am) (Figs. 3F; 6C, D; 8) filled with autosperm lying in disorder. Sperm heads are short (Fig. 3F). A receptaculum seminis (rs) is absent or not developed in the examined specimen. Three nidamental glands (Figs. 6C, D; 8) can be distinguished from proximal to distal: the sac-like blue-stained albumen gland (alg), the tubular purple-stained membrane gland (meg) and the sac-like purple-stained mucus gland (mug). The distal part of the mucus gland runs to the right side of the body where the hermaphroditic duct bifurcates into the vas deferens (vd) and the highly undulated oviduct (od) (Figs. 6D; 8). The bursa stalk (bs) (Figs. 3C; 6D; 8) connects to the large bursa copulatrix (bc) (Figs. 3D; 6D; 8) the content of which is stained dark blue. The oviduct and the bursa stalk join to a common duct just before opening through the female gonopore (fgo) (Figs. 6D; 8) laterally at the right side of the visceral hump to the exterior. The female gonopore is situated considerably anterior to the common opening of the digestive and the excretory systems. The internal vas deferens (Fig. 8) extends subepidermally up to the right rhinophore connecting the posterior reproductive system to the anterior male copulatory organs (Fig. 6E). The posterior-leading vas deferens (vdp) (Figs. 6E; 8) joins the tubular prostate gland (pr) (Figs. 3D; 6E; 8). The long, coiled and muscular ejaculatory duct (ed) (Figs. 3D; 6E, F) arises from the prostate and discharges at the top of the penis (p) through a hollow penial stylet (pst) (Figs. 3D; 6F; 8) of approx. 70 μm length (125 μm in a specimen from Indonesia). The blind ending and highly coiled glandular paraprostate (ppr) (Figs. 3D; 6E; 8) is longer and thinner than the prostate. The paraprostatic duct (ppd) (Figs. 3C, D; 6E, F) connects the paraprostate with the muscular basal finger (bf) (Fig. 6E, F), which is united to the penial muscle mass at its base. It enters the basal finger approx. in the upper half of the muscle (Fig. 6F) and discharges terminally via a hollow curved stylet (st) (Figs. 3E; 6F; 8) of about 200 μm length (30 μm in a specimen from Indonesia). Both stylets can be somewhat retracted into the muscles. Parts of the penis and the basal finger are surrounded by a thin-walled penial sheath (ps) (Figs. 3D; 6F; 8).

Note: Morse [42] reported on a *Pseudunela* species from Fiji. However, at present stage of knowledge we would not like to assign her specimens to our species *P. viatoris* sp. nov. from Fiji. Due to a different collecting site in Morse [42] we cannot exclude that there are two different *Pseudunela* species on different Fijian islands. On the Solomon Islands we found two distinct species on the same island, at neighbouring beaches. Furthermore, Morse's drawing ([42] fig. 4A) indicates the presence of externally visible eyes which is definitely not applicable for our species. Nevertheless, there are pigmented and externally visible eyes in at least one specimen of *P. viatoris* sp. nov. from Indonesia, but our molecular results show great similarities even on the fast evolving mitochondrial markers, despite of the large geographic distance.

Species description of *Pseudunela marteli* sp. nov. from the Solomon Islands and Vanuatu

Systematics. ***Pseudunela marteli*** sp. nov.

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Type Material—Holotype: ZSM Mol 20071803, stored in 99% EtOH; collected in Solomon Islands, Guadalcanal, Honiara, beach of "Art Gallery". Paratypes: ZSM Mol 20090418, two specimens stored in 99% EtOH; ZSM Mol 20071851 (one serially sectioned specimen); all paratypes collected together with holotype.

Etymology—*Pseudunela marteli* sp. nov. with its large heart-bulb, is named in honour of our big-hearted friend and colleague Martin "Martl" Heß.

Distribution—Known from Guadalcanal, Solomon Islands and Oyster Island, Vanuatu.

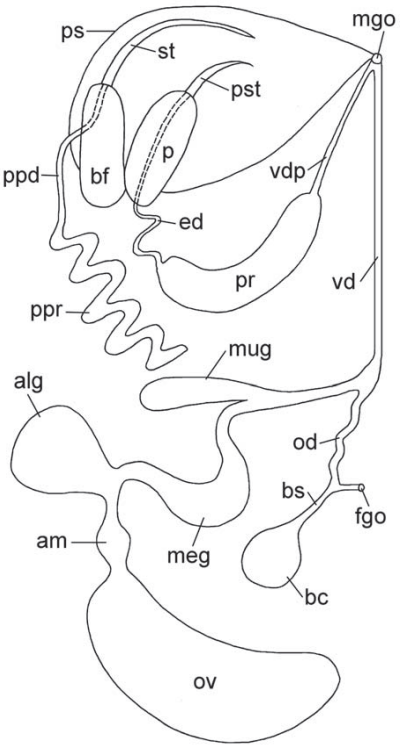

Figure 8. Reproductive system of *P. viatoris* sp. nov. from Fiji (schematic drawing, dorsal view). Abbreviations: **alg**, albumen gland; **am**, ampulla; **bc**, bursa copulatrix; **bf**, basal finger; **bs**, bursa stalk; **ed**, ejaculatory duct; **fgo**, female gonopore; **meg**, membrane gland; **mgo**, male gonopore; **mug**, mucus gland; **od**, oviduct; **ov**, ovotestis; **p**, penis; **ppd**, paraprostatic duct; **ppr**, paraprostate; **pr**, prostate; **ps**, penial sheath; **pst**, penial stylet; **st**, stylet of basal finger; **vd**, vas deferens; **vdp**, posterior-leading vas deferens. Not to scale.
doi:10.1371/journal.pone.0023313.g008

Species diagnosis. External morphology and anatomy as in *P. viatoris* sp. nov. from Fiji.

Exceptions. Colour of digestive gland greenish or orange-brownish (Fig. 9A); eyes (30–35 µm) pigmented (Fig. 9B) and well visible externally (Fig. 9A); foot length up to half of the visceral hump (Fig. 9A); subepidermal spicules more abundant in cephalic tentacles, foot and visceral hump. The radula formula is 57–59×1.1.?; rhachidian tooth with 3–4 denticles per side. The hollow curved penial stylet measures 130 µm in length, the stylet of basal finger is 30 µm long. The ampulla is sac-like; allosperm receptacles are absent in the examined specimen. The albumen and mucus glands are tubular; the membrane gland is sac-like.

Note: Specimens of *P. marteli* sp. nov. collected in Vanuatu (Fig. 10) differ from those collected on the Solomon Islands in some details: the pigmented eyes are slightly smaller (25–30 µm) and only weakly visible externally (Fig. 10A); subepidermal spicules are situated additionally around the CNS (Fig. 10D); the hollow curved penial stylet is longer measuring 180–200 µm in length; the ampulla (Fig. 10F) is tubular; the albumen and the mucus glands (Fig. 10E) are sac-like, the membrane gland (Fig. 10F) is tubular. Based on these anatomical differences both populations could, however, not satisfyingly be delimited due to potential intraspecific variation (see discussion). Future comparative analyses dedicated to evaluate the degree of intraspecific variation might, however, lead to a delineation of both populations.

Molecular results

The result of the maximum likelihood analysis of the concatenated dataset analysed in four partitions is shown in Fig. 11. The genus *Pseudunela* results monophyletic, but with low support (bootstrap value (BS) 56%). The sister group relationship of *Pseudunela* (i.e. Pseudunelidae) with limnic Acochlidiidae is well supported (BS 91%). The internal phylogeny of *Pseudunela* is fully resolved, but the sister group relationships within the genus do not gather support. All morphologically defined *Pseudunela* lineages are recovered as monophyletic. The topological species delimitation based on the available molecular dataset (combining nuclear and mitochondrial markers) results in four different clades within the genus *Pseudunela*, supporting the morphological descriptions of *P. viatoris* and *P. marteli* spp. nov..

Pairwise genetic differences and values of intraspecific variation were generated based on partial mitochondrial COI and 16S rRNA using Species Identifier. The largest variation within the different populations of *Pseudunela* species is relatively low (0.15–0.45% on partial COI and 0.0–0.69% on partial 16S rRNA). The largest intraspecific uncorrected p-distances among *P. viatoris* sp. nov. are 1.67% on COI and 1.39% on 16S rRNA (n = 5), in *P. marteli* sp. nov. the largest distance between individuals of Solomon Island and Vanuatu populations is comparably high with 5.49% on COI and 3.24% on 16S rRNA. Between species, the smallest interspecific distances within *Pseudunela* were considerably larger with 14.04–16.48% on COI and 8.82–14.85% on 16S rRNA; smallest interspecific distances occurred between the morphologically clearly distinct *P. espiritusanta* and *P. marteli* sp. nov. (see Tables 3, 4, 5, 6).

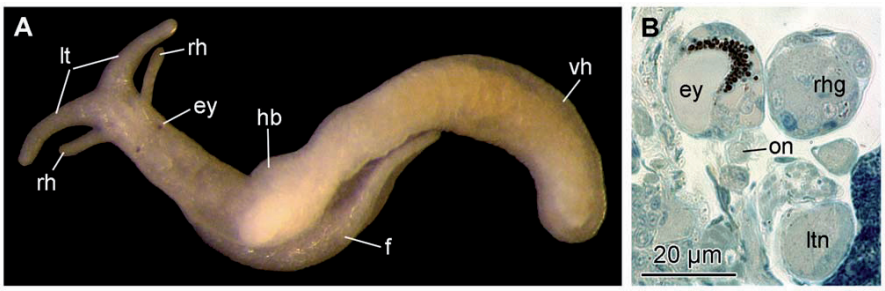

Figure 9. Photograph of a living specimen and histological cross-section of *P. marteli* sp. nov. (Solomon Islands). A: external morphology of a living specimen (body size 3 mm). B: pigmented eye. Abbreviations: **ey**, eye; **f**, foot; **hb**, heart bulb; **lt**, labial tentacle; **ltn**, labial tentacle nerve; **on**, optic nerve; **rh**, rhinophore; **rhg**, rhinophoral ganglion; **vh**, visceral hump.
doi:10.1371/journal.pone.0023313.g009

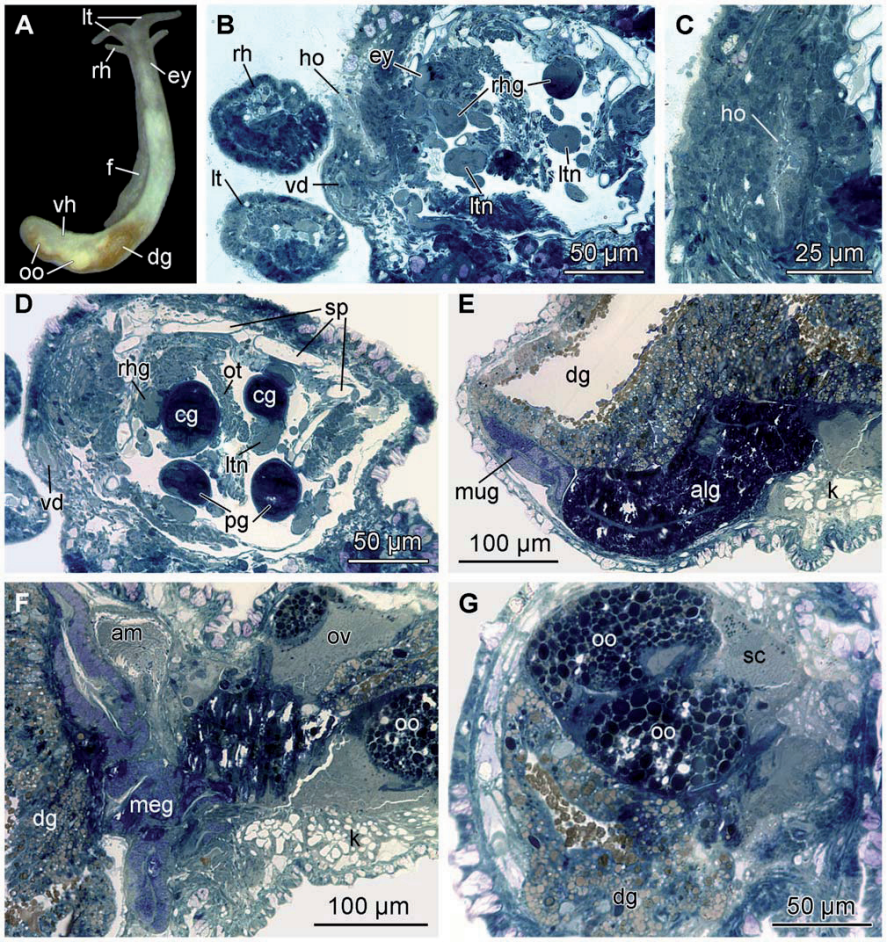

Figure 10. Histological cross-sections of *P. marteli* sp. nov. from Vanuatu. A: external morphology of a living specimen (body size 3 mm). B: Hancock's organ and eye. C: Hancock's organ. D: spicule cavities. E: albumen and mucus glands. F: ampulla and membrane gland. G: oocytes and spermatocytes. Abbreviations: **alg**, albumen gland; **am**, ampulla; **cg**, cerebral ganglion; **dg**, digestive gland; **ey**, eye; **f**, foot; **ho**, Hancock's organ; **k**, kidney; **lt**, labial tentacle; **ltn**, labial tentacle nerve; **meg**, membrane gland; **mug**, mucus gland; **oo**, oocyte; **ot**, oral tube; **ov**, ovotestis; **pg**, pedal ganglion; **rh**, rhinophore; **rhg**, rhinophoral ganglion; **sc**, spermatocytes; **sp**, spicule cavity; **vd**, vas deferens; **vh**, visceral hump.
doi:10.1371/journal.pone.0023313.g010

Statistical parsimony analyses in TCS 1.21 of each mitochondrial marker (COI and 16S rRNA) congruently produce unconnected haplotype networks (not shown) for each of the herein morphologically defined *Pseudunela* species (i.e. *P. cornuta*, *P. espiritusanta*, *P. viatoris* sp. nov. (uniting populations from Fiji and Indonesia) and *P. marteli* sp. nov.). Moreover, the haplotype of *P. marteli* sp. nov. from Vanuatu is unconnected to the haplotypes from the Solomon population in both markers.

As an additional method of species delineation we applied GMYC to our molecular dataset, using a RAxML starting tree generated from the concatenated mitochondrial dataset (COI+16S). Under the multiple threshold option, GMYC recovers four entities, representing the above morphologically distinguished species: *P. cornuta*, *P. espiritusanta*, *P. marteli* sp. nov. and *P. viatoris* sp. nov.

Discussion

Morphology-based taxonomy

The *Pseudunela* specimens from different Indo-Pacific islands examined herein are compared according to their external morphology, microanatomy, and molecular markers. Externally, only the larger, recently discovered *Pseudunela espiritusanta* from Vanuatu [43] can be clearly distinguished from congeners by its much larger body size, the foot width and the shape of the visceral hump, as well as its unique brackish-water habitat (Table 3). In contrast, the herein examined, fully marine *Pseudunela* species all resemble externally *P. cornuta* from the Solomon Islands which was recently re-examined by Neusser et al. [17]. The body size and colour, the foot length and width, as well as the presence of subepidermal spicules do not differ between the species (Table 3). Only the visibility of the eyes through the body integument greatly varies among - and partly within - the marine *Pseudunela* species. In contrast to external features, our detailed anatomical examinations enable the discrimination of *P. cornuta* from the remaining marine *Pseudunela* species. Differences are related to all organ systems (Tables 4, 5, 6). The eyes are unpigmented and considerably smaller in *P. cornuta* than in the other *Pseudunela* species and they are not innervated by the optic ganglion, but the optic nerve emerges from the rhinophoral nerve [17]. The common opening of the excretory and digestive systems is absent in *P. cornuta* and the

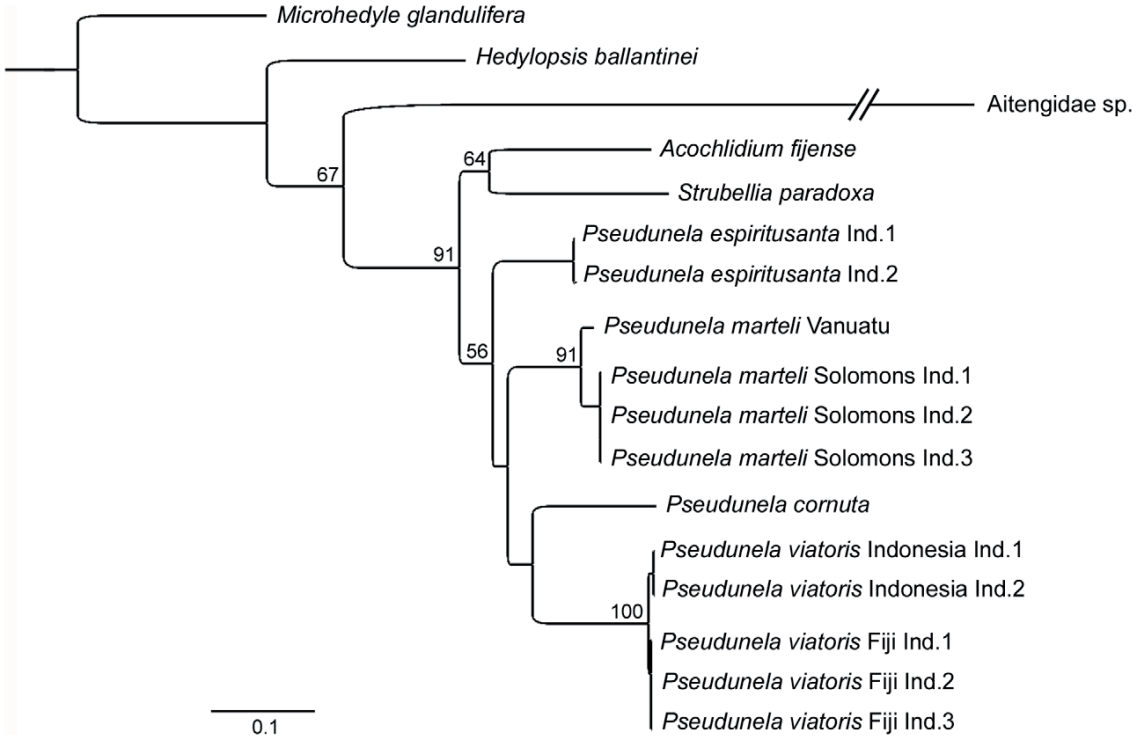

Figure 11. Molecular phylogeny of the genus *Pseudunela*. RAxML analysis of concatenated sequences of partial 18S rRNA, 16S rRNA and COI markers, analysed in four partitions. Bootstrap values (>50%) given at nodes. Sister group relationship between Pseudunelidae and limnic Acochlidiidae receives strong support. Within *Pseudunela*, brackish *P. espiritusanta* is basal to the remaining species, but sister group relationships within *Pseudunela* do not gather any bootstrap support.
doi:10.1371/journal.pone.0023313.g011

Table 3. Comparison of the external morphology within the genus *Pseudunela*.

	***P. espiritusanta* Neusser & Schrödl, 2009**	***P. cornuta* (Challis, 1970)**	***P. eirene* (Wawra, 1988)**	***Pseudunela viatoris* sp. nov.**	***Pseudunela viatoris* sp. nov.**	***Pseudunela marteli* sp. nov.**	***Pseudunela marteli* sp. nov.**
Collection site	Espiritu Santo, Vanuatu	Guadalcanal, Solomon Islands	Andaman Islands, India	Viti Levu, Fiji	Gili Lawa Laut, Indondesia	Guadalcanal, Solomon Islands	Espiritu Santo, Vanuatu
Data source	Neusser & Schrödl 2009	Challis 1970; **Neusser et al. 2009**	Wawra 1988	present study	present study	present study	present study
Habitat	brackish	marine; *	marine	marine	marine	marine	marine
Body size (mm)	9	3 ; *	4 (fixed specimen)	3	3–4	3	3
Colour of body	translucent-whitish	translucent-whitish; *	?	translucent-whitish	translucent-whitish	translucent-whitish	translucent-whitish
Colour of digestive gland	yellowish	?; **orange-brownish**	?	brownish	orange-brownish	greenish or orange-brownish	orange-brownish
Eyes visible externally	well	no; *	?	no	weakly	well	weakly
Foot width	broader than body	as broad as head; *	as broad as body	as broad as body	as broad as body	as broad as body	as broad as body
Foot length	2/3 of vh	slightly longer than anterior body; **1/2 of vh**	?	1/3 to 1/2 of vh	1/3 to 1/2 of vh	1/2 of vh	1/2 of vh
Visceral hump	bent, recurved	elongated; *	?	elongated	elongated	elongated	elongated
Heart bulb visible	yes	?; **yes**	?	yes	yes	yes	yes
Subepidermal calcareous spicules	bean-shaped; in cephalic tentacles, foot, vh, around CNS	absent; **few in vh**	?	in cephalic tentacles, foot and vh	in cephalic tentacles, foot and vh	in cephalic tentacles, foot, vh,	in cephalic tentacles, foot, vh, around CNS

CNS, central nervous system; **vh**, visceral hump; **?**, no data available; revised data in **bold**, * = confirmed.
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Table 4. Comparison of the central nervous system and the radula within the genus *Pseudunela*.

	P. espiritusanta **Neusser & Schrödl, 2009**	*P. cornuta* **(Challis, 1970)**	*P. eirene* **(Wawra, 1988)**	*Pseudunela viatoris* **sp. nov.**	*Pseudunela viatoris* **sp. nov.**	*Pseudunela marteli* **sp. nov.**	*Pseudunela marteli* **sp. nov.**
Collection site	Espiritu Santo, Vanuatu	Guadalcanal, Solomon Islands	Andaman Islands, India	Viti Levu, Fiji	Gili Lawa Laut, Indondesia	Guadalcanal, Solomon Islands	Espiritu Santo, Vanuatu
Data source	Neusser & Schrödl 2009	Challis 1970; **Neusser et al. 2009**	Wawra 1988	present study	present study	present study	present study
Accessory ganglia	absent	present; **absent**	present	absent	absent	absent	absent
Optic ganglion	present	absent; **present**	?	present	present	present	present
Origin of optic nerve	optic ganglion	?; **rhinophoral nerve**	?	optic ganglion	optic ganglion	optic ganglion	optic ganglion
Eye pigment	present	?; **absent**	?	absent	absent/present	present	present
Eye diameter (µm)	45	?; **20**	?	30–35	30–35	30–35	25–30
Hancock's organ	present	?; **?**	?	present	?	present	present
Osphradial ganglion	present	absent; **present**	present	present	present	present	present
Gastro-oesophageal ganglion	present	absent; **present**	absent	present	?	present	present
Radula formula	67×1.1.2	50×1.1.1; **?**	52×1.1.2	44–50×1.1.2	38×1.1.?	57–59×1.1.?	57×?
Rhachidian cusp	projecting	projecting; **?**	?	projecting	projecting	projecting	projecting
Rhachidian tooth denticles/side	4–7	3–4; **?**	3–4	3–4	2–4	3–4	3–4

?, no data available; revised data in **bold**.
doi:10.1371/journal.pone.0023313.t004

brackish-water *P. espiritusanta* [17,43] and the anus and the nephropore open separately to the exterior. The most surprising feature concerns the excretory system with a complex kidney and a long, looped nephroduct consisting of two branches in *P. cornuta*. This kind of excretory system is characteristic for the brackish *P. espiritusanta* [43] and other limnic acochlidians studied in detail [44,45]. In contrast, all marine *Pseudunela* species examined herein (i.e. *P. viatoris* and *P. marteli* spp. nov.) show a complex kidney as well, but have a short nephroduct as characteristic for other marine acochlidian species. Peculiar is the very long (600 µm) and curled, hollow penial stylet in *P. cornuta*, whereas the penial stylet in the other *Pseudunela* species is slightly curved but not curled and does not exceed 200 µm of length. The remaining *Pseudunela* species show several anatomical differences (mainly concerning the length of the copulatory stylets, and the shape of the ampulla and of the female glands; Table 6), which can be used for species delimitation. Such features, however, may depend on reproductive maturity and are not well explored yet. In summary, morphology-

Table 5. Comparison of the circulatory and excretory systems within the genus *Pseudunela*.

	P. espiritusanta **Neusser & Schrödl, 2009**	*P. cornuta* **(Challis, 1970)**	*P. eirene* **(Wawra, 1988)**	*Pseudunela viatoris* **sp. nov.**	*Pseudunela viatoris* **sp. nov.**	*Pseudunela marteli* **sp. nov.**	*Pseudunela marteli* **sp. nov.**
Collection site	Espiritu Santo, Vanuatu	Guadalcanal, Solomon Islands	Andaman Islands, India	Viti Levu, Fiji	Gili Lawa Laut, Indondesia	Guadalcanal, Solomon Islands	Espiritu Santo, Vanuatu
Data source	Neusser & Schrödl 2009	Challis 1970; **Neusser et al. 2009**	Wawra 1988	present study	present study	present study	present study
Anal-genital cloaca	absent	present; **absent**	?	absent	absent	absent	absent
Common opening of digestive and excretory system (a/np)	absent	absent; *	?	present	present	present	present
Heart	ventricle	ventricle; **atrium and ventricle**	?	ventricle	ventricle	ventricle	ventricle
Renopericardioduct	long, ciliated funnel	present; **long, ciliated funnel**	?	long, ciliated funnel	long, ciliated funnel	long, ciliated funnel	long, ciliated funnel
Kidney	long, internally divided	large, unfolded sac; **long, internally divided**	?	long, internally divided	long, internally divided	long, internally divided	long, internally divided
Nephroduct	long with two branches	?; **long with two branches**	?	short	short	short	short

?, no data available; revised data in **bold**, * = confirmed.
doi:10.1371/journal.pone.0023313.t005

Table 6. Comparison of the reproductive system within the genus *Pseudunela*.

	P. espiritusanta **Neusser & Schrödl, 2009**	***P. cornuta*** **(Challis, 1970)**	***P. eirene*** **(Wawra, 1988)**	***Pseudunela viatoris*** **sp. nov.**	***Pseudunela viatoris*** **sp. nov.**	***Pseudunela marteli*** **sp. nov.**	***Pseudunela marteli*** **sp. nov.**
Collection site	Espiritu Santo, Vanuatu	Guadalcanal, Solomon Islands	Andaman Islands, India	Viti Levu, Fiji	Gili Lawa Laut, Indondesia	Guadalcanal, Solomon Islands	Espiritu Santo, Vanuatu
Data source	Neusser & Schrödl 2009	Challis 1970; **Neusser et al. 2009**	Wawra 1988	present study	present study	present study	present study
Hollow curved penial stylet (µm)	80	100 ; **600 (coiled 1.5 spirals)**	200	70	125	130	180–200
Solid basal thorn (µm)	absent	absent; *	30	absent	absent	absent	absent
Hollow curved stylet on basal finger (µm)	340	absent; **110**	?	200	30	30	30
Glands associated with copulatory organs	prostate, paraprostate	prostate, penial gland; **prostate, paraprostate**	?	prostate, paraprostate	prostate, paraprostate	prostate, paraprostate	prostate, paraprostate
Yolky oocytes developed	present	present; *	?	absent	?	absent	present
Ampulla	sac-like	?; **sac-like**	?	tubular	?	sac-like	tubular
Receptaculum seminis	present	?; **present**	?	absent	?	absent	absent
Bursa copulatrix	present	present; *	?	present	?	absent	absent
Albumen gland	tubular	?; tubular	?	sac-like	?	tubular	sac-like
Membrane gland	tubular	?; **tubular**		tubular		sac-like	tubular
Mucus gland	sac-like	sac-like	?	sac-like	?	tubular	sac-like

?, no data available; revised data in **bold**, * = confirmed.
doi:10.1371/journal.pone.0023313.t006

based taxonomy and even sophisticated 3D modelling of anatomical details as applied herein can only reveal parts of the actual species diversity of *Pseudunela* unambiguously; diagnosable microanatomical units found need to be tested by molecular phylogenetic analyses.

Cryptic species?

The present molecular dataset is limited due to the low amount of individuals sampled, thus not allowing population genetic approaches and in depth comparison between intraspecific versus interspecific variation justifying molecularly based species delineation. Still, there are several lines of evidence supporting the defined microanatomical units as genetically separated partially cryptic lineages: 1) our maximum likelihood analyses based on a concatenated molecular dataset (combining nuclear and mitochondrial markers) recovers all microanatomical units as monophyla (Fig. 11). In our phylogenetic hypothesis *P. cornuta* separates cryptic *P. marteli* sp. nov. and *P. viatoris* sp. nov. 2) In contrast to earlier approaches relying on thresholds of divergence for the barcoding marker COI in molluscs [6,21,46], several recent studies showed that there is no universal threshold and that rates of intraspecific variation can outnumber supposedly 'high' rates of interspecific variation [34,47]. Our limited dataset shows low rates of intraspecific variation, even when comparing far distant populations of *P. viatoris* sp. nov. from Fiji and Indonesia (n = 5; largest p-distance: 1.67% on partial COI, 1.39% on 16S rRNA). Then again interspecific variation among the microanatomically defined units is comparably high (14.04–16.48% on COI and 8.82–14.85% on 16S RNA) and the distances between the morphologically cryptic species are in the same range as to the morphologically clearly distinct *P. espiritusanta*. 3) In addition to ML tree-based methods and the comparison of pairwise distances, we generated haplotype networks applying 95% parsimony criterion, which resulted in unconnected haplotype networks for the described microanatomical units on both markers. Additionally, the *P. marteli* sp. nov. from Vanuatu (n = 1) is unconnected to the haplotype network of *P. marteli* sp. nov. from the Solomon Islands (n = 3) on both mitochondrial markers. 4) GMYC recovers all four microanatomical units; however, the performance and accuracy of GMYC to our knowledge has never been tested on such a small dataset, as ours. These independent molecular approaches are in congruence with our microanatomical units and thus, in our opinion, justify a separation in two formal new species.

There are several microanatomical differences between the two populations of *P. marteli* sp. nov. (e.g. size of eyes, length of penial stylet, see Tables 4, 5, 6), but intraspecific variation of these characters cannot be evaluated at present stage of knowledge and results from molecular data are incongruent (e.g. unconnected haplotype networks vs. one entity in GMYC). Moreover, the genetic distance between the two populations is low compared to the distances present in the closely related *Pseudunela* species. More data is needed to evaluate intraspecific variation and test conspecifity of the two *P. marteli* populations. Within specimens of *Pseudunela viatoris* sp. nov. from Fiji and Indonesia there are slight differences concerning the eye visibility and the length of stylets on the penial papilla, while stylets on the basal finger are remarkably different-sized. Specimens from Indonesia and Fiji cluster on different clades (Fig. 11). However, the genetic similarity between these specimens is very high (approx. 98–99% on COI and 16S rRNA) and intrapopulation variation is low. Thus, we do not consider these lineages to be specifically distinct, despite the distant geographic localities. More specimens are needed to explore morphological variability and genetic structure of these populations.

We conclude that we discovered morphologically cryptic species within the genus *Pseudunela*. External morphological, microanatomical and genetic evidences for recognizing species are congruent, and a combined approach of 3D-microanatomy and

genetic markers can reliably distinguish and delineate all of the four species. Surprisingly, far distant geographic populations of specimens with slightly differing anatomy and presumably poor dispersive ability do not necessarily indicate different species, as revealed by highly similar mitochondrial sequences in *P. viatoris* sp. nov.. An integrative taxonomic approach combining morphological, 3D-microanatomical and molecular markers, like demonstrated here for *Pseudunela* species, thus is a powerful tool to independent structural or genetic approaches.

Overall, our results might be indicative for a still unknown diversity within mesopsammic gastropods. Recent studies on cryptic speciation within Meiofauna across taxa, has often revealed formerly considered wide-spread or even cosmopolitan species as flock of cryptic species (e.g. in proseriate flatworms [48,49], polychaete annelids [50,51] and gastrotrichs [52,53]). Leading to the assumption that especially within this habitat, which is generally known for taxa with low dispersal abilities, there might be a high degree of cryptic speciation and the contribution of Meiofauna to marine biodiversity might be currently seriously underestimated [49]. However, some studies supported the presence of truly amphi-atlantic or cosmopolitan meiofaunal taxa, with the distribution and genetic interaction across Oceans in the absence of pelagic larvae still to be explained [50,54].

Distribution

The distribution of the four different *Pseudunela* species (*P. eirene* from Andaman Islands is not considered in this discussion as there exist only inadequate data and no material is available for detailed study) on the Indo-Pacific islands raises questions: 1) How can two different, genetically isolated *Pseudunela* species inhabit nearby beaches on one island with continuous coastline and 2) how can we explain the occurrence of *P. viatoris* sp. nov. on two far distant islands?

Considering that all Hedylopsacea occur in warm or tropical waters (except of *Hedylopsis spiculifera*, which inhabits temperate waters), we can assume that the common ancestor of the Pseudunelidae and Acochlidiidae s.l. has its origin in warm tropical waters as well. Recently, Jörger et al. [28] calibrated a molecular clock estimating divergence times for shell-less, and hence fossil-lacking Heterobranchia. In this study the origin of Acochlidia was estimated to the Mesozoic Triassic or Jurassic. According to the authors, the major diversification of Acochlidia took place in Jurassic, but the split between Pseudunelidae and Acochlidiidae was estimated to the Palaeogene. Even though this is a very rough estimation, it indicates that the diversification and distribution of the genus *Pseudunela* might have started over 35 mya, a long timeframe for a long-distance distribution, even for marine meiofaunal acochlidian species, which are regarded as poor dispersers. The hedylopsacean species *Pseudunela cornuta* [17] and *P. marteli* sp. nov. from Vanuatu, as well as the microhedylacean species, such as *Microhedyle remanei* (Marcus, 1953), *M. nahantensis* (Doe, 1974), *Parhedyle cryptophthalma* (Westheide & Wawra, 1974) and *Asperspina murmanica* (Kudinskaya & Minichev, 1978) [14,55,56,57] have only a small number of large, yolky oocytes indicating a low reproductive output and a lecithotrophic development within a capsule rather than a planktotrophic larval development [10,57]. Therefore, the distribution of larval and adult stages is expected only within a small radius step by step. Natural disasters (such as volcanic eruptions, earthquakes, heavy storms or erosion) or settlement by humans may disturb or even destroy sandy beaches [42]. This might result in genetically isolated populations or even local extinctions, which can explain the co-occurrence of two distinct *Pseudunela* species on nearby beaches. Another explication may be the adaptation to diverse, but subtle ecological conditions in the habitat, such as different currents, grain size, freshwater influx or food resources, which finally might result in separation of species.

The extensive distribution of *P. viatoris* sp. nov. is surprising. Due to aforementioned reasons a distribution of larvae via water currents is not likely. An accidentally distribution of different ontogenetic stages after heavy (sub-)tropical storms is not very probable due to the large distances. We cannot exclude a man-made dispersal, where small patches of sand of neighboured populations were displaced e.g. by ships. More likely, however, there exist intermediate populations between those from Fiji and Indonesia that have not been discovered yet – or already got extinct. Missing intermediates and restricted gene flow across these stepping stones might also explain the slight anatomical differences between the Fijian specimens and those from Indonesia, such as the variation in the length of the copulatory stylets or the pigmentation of the eye. Possibly, small genetic distances observed between these distant populations also may reflect a stage of ongoing allopatric speciation. Finally, another aspect should be considered: juveniles of the amphidromous nerite snail *Neritina asperulata* Recluz, 1842 show a "hitchhiking" behaviour by attaching to the shell of the congeneric *N. pulligera* Linnaeus, 1758. In this way young specimens travel upstream for growth and reproduction [58]. We can imagine that eggs and accordingly larval or adult acochlidians stick to e.g. benthic living organisms when the living conditions in the sand are changing for the worse and thus, may be displaced into another habitat [45].

Phylogeny and evolution

Our molecular analysis (see Fig. 11) shows the marine and brackish-water *Pseudunela* as the sister group to the limnic Acochlidiidae s.l. and supports herein the results of recent morphological analysis [18] and previous molecular analysis [28]. Again, Aitengidae sp. clusters within the Hedylopsacea, as sister to Pseudunelidae plus Acochlidiidae [59]. The relationships between the *Pseudunela* species are fully resolved but with no robust support. As suspected by Neusser & Schrödl [43], the brackish *Pseudunela espiritusanta* from Vanuatu is the most basal *Pseudunela* species forming the sister group to all marine and temporary brackish *Pseudunela* species. The fully marine *P. marteli* sp. nov. from the Solomon Islands and Vanuatu form the sister group to the temporary brackish *P. cornuta* (also from the Solomon Islands) and the marine *P. viatoris* sp. nov. from Fiji and Indonesia. This tree topology (Fig. 11), however, does not clearly support previous ideas [18], i.e. that evolution within acochlidians was directed from marine to limnic habitats, possibly via brackish water. Instead, the ancestor of *Pseudunela* plus Acochlidiidae might have been already limnic or brackish water associated, with marine species evolving secondarily within *Pseudunela*.

To visualise patterns and reconstruct evolution in a more comprehensive context, habitats were plotted on a consensus tree (Fig. 12) combining all relevant acochlidian clades from morphology-based and molecular analyses. While the ancestral acochlidian [28] and all microhedylacean species are marine, the Hedylopsacea clade includes a mosaic of limnic, marine and brackish water associated taxa, implying several independent incidents of habitat shifts from marine to limnic and brackish water systems and/or vice versa. In contrast to previous assumptions [17,18], the hedylopsacean ancestor could have been either still marine or already limnic.

In order to decide on a preferred scenario, we explored different characteristics and organ systems that are most closely linked to osmolarity changes. The first one is the body volume as a whole. Since all acochlidians, including all marine species and the basal

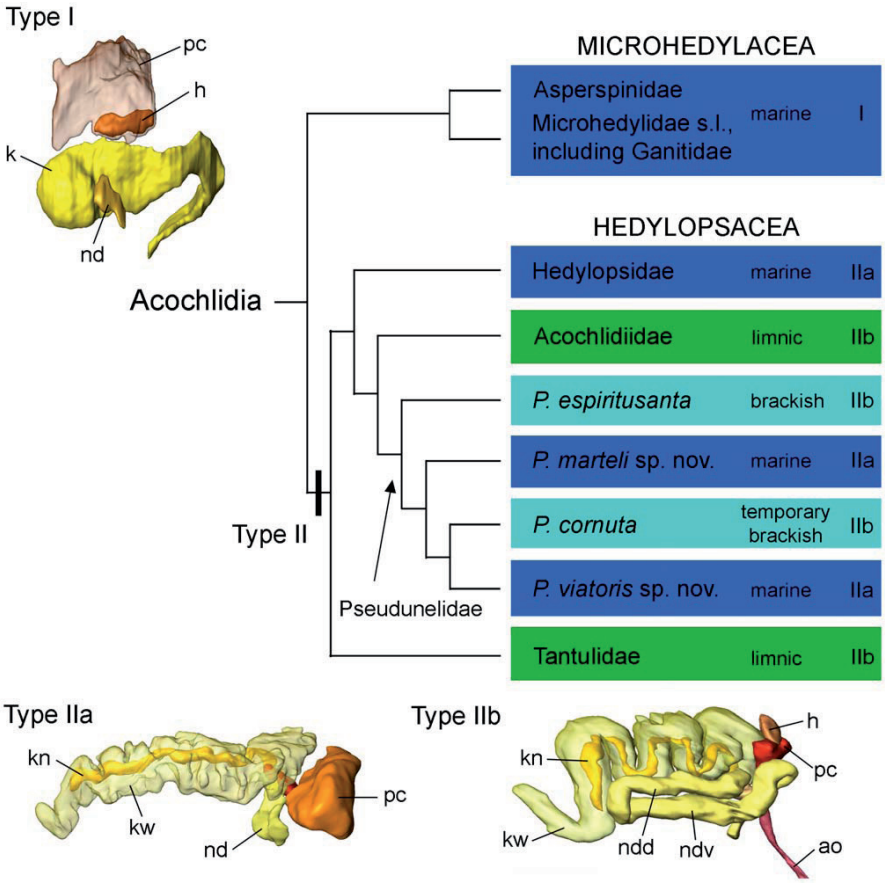

Figure 12. Evolution of excretory systems and habitat in acochlidian lineages. The habitat of the different acochlidian lineages and their types of excretory systems are plotted on a consensus tree (topology combined from Schrödl & Neusser [18] and molecular results herein; the enigmatic Aitengidae are not shown due to the uncertain position within Hedylopsacea and the different and special excretory system [59]). While Microhedylacea present a simple excretory system with a small, sac-like kidney (type I), hedylopsacean taxa evolved a complex excretory system with a large, internally divided kidney (type II): type IIa is characterised by a short nephroduct, type IIb by a long, looped nephroduct. The complex kidney already evolved in the ancestor of the Hedylopsacea. The mosaic-like distribution of habitat and excretory system types within Hedylopsacea implies an evolutionary scenario with multiple habitat shifts and adaptations. Abbreviations: **ao**, aorta; **h**, heart; **k**, kidney; **kn**, narrow lumen of kidney; **kw**, wide lumen of kidney; **nd**, nephroduct; **ndd**, dorsal branch of nephroduct; **ndv**, ventral branch of nephroduct; **pc**, pericardium. Not to scale.
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limnic *Tantulum elegans* are small sized meiofaunal forms, there is no doubt that the large adult size of limnic, benthic Acochlidiidae is an adaptive apomorphy of this clade. The brackish water *Pseudunela espiritusanta* that is no more mesopsammic but living under stones either independently increased to an intermediate size or, alternatively, the common ancestor of *Pseudunela* plus Acochlidiidae already was large, with secondary reduction in mesopsammic *Pseudunela* species. Summing up, increasing body size alone may be advantageous but not strictly necessary for acochlidians invading freshwater or brackish water systems.

The second feature that is crucial for dealing with osmotic stress, especially in small species and juveniles, is the excretory system. Neusser & Schrödl [43] emphasised that the acochlidian excretory system varies considerably between marine and limnic species. The different types are illustrated in Fig. 12 and, based on our results, mapped on the consensus tree. All microhedylacean Acochlidia known in detail (e.g. *Microhedyle remanei*, *Pontohedyle milaschewitchii* (Kowalevsky, 1901) or *Asperspina murmanica*) have a quite simple excretory system of type I consisting of a small, sac-like kidney and a short nephroduct (Fig. 12) [14,55,60]. This simple type of sac-like kidney corresponds to almost all marine euthyneurans, including marine Panpulmonata, such as Siphonarioidea [61], the sacoglossan *Platyhedyle* [62], Amphiboloidea [63], and marine eupulmonates. In contrast, the acochlidian excretory system type II comprises a complex, internally divided kidney with a narrow and a wide lumen. All fully marine hedylopsacean species (such as the newly described *Pseudunela* species) have an excretory system of type II (Fig. 12), i.e. with a complex kidney, and with a short nephroduct (type IIa). *Hedylopsis ballantinei* Sommerfeldt & Schrödl, 2005 was described with a long, sac-like kidney and a nephropore opening into a mantle cavity [64,65]. However, a brief re-examination of the original sections revealed this species to possess a complex, internally divided kidney (own unpubl. data). The most complex excretory system type IIb consists of a large, divided kidney as in type IIa, and additionally a long looped nephroduct with two branches. This type is present in all limnic acochlidian species, i.e. the small Caribbean limnic *Tantulum elegans* [66] and the large Indo-Pacific Acochlidiidae [44], in the brackish *Pseudunela espiritusanta* [43] and the at least temporary

brackish *P. cornuta* [17]. Thus, the type of the excretory system in acochlidians is not strictly correlated with the habitat in acochlidian species: marine acochlidian species have either a type I or IIa excretory system with a simple or a complex kidney, respectivly.

Interestingly, all (marine) microhedylacean species have the simple, supposedly ancestral type I system. In contrast, all hedylopsacean species have the complex type II excretory system, even the marine species. We therefore conclude that the ancestral hedylopsacean species already had a complex kidney, which is an apomorphy of the clade. The presence of complex kidneys can be seen as a preadaptation to brackish water or limnic life, or much more likely, evolved as an adaptation to invading such habitats. Thus, considering evidence from excretory systems, we favour a scenario with hedylopsaceans originating in a freshwater, or at least freshwater influenced, habitat.

Considering the still poorly known and enigmatic Aitengidae [59] aberrant amphibious hedylopsacean offshoot (Fig. 11) would fit with and further extend the ecological tolerance and evolutionary plasticity observed within the hedylopsacean lineage.

Finally, the question arises if the complex type II kidney has already evolved in the – then supposedly brackish water or even limnic - ancestor of the Acochlidia. A recent multi-locus molecular study including six out of seven acochlidian families in a comprehensive euthyneuran taxon sampling [28] fundamentally changed our understanding of euthyneuran systematics. Surprisingly, this study confirms the Acochlidia in a well-supported (pan)pulmonate rather than opisthobranch relationship, as sister of basally still marine Eupulmonata. However, there is an alternative, though less likely topology suggesting that Acochlidia are the sister of – limnic – Hygrophila. In this scenario, a common ancestor could have been limnic as well, with a simple or complex kidney as both conditions occur apparently among different hygrophilan subgroups [61,67,68].

Conclusions

Our study on mesopsammic Acochlidia testing the power of traditional taxonomy (i.e. examination of the external morphology and the radula) against results from in-depth micro-anatomical and molecular data clearly shows: 1) Traditional taxonomy fails to reveal the cryptic diversity within the genus *Pseudunela* in tropical sands, and thus is likely to generally underestimate biodiversity of meiofaunal invertebrates; 2) labour intensive and sophisticated 3D-modelling of micro-morphology is more suitable to delineate species, i.e. diagnosable units within *Pseudunela* are congruent with genetic lineages, and show relatively high genetic divergence; 3) only the combined evidence of microanatomical and molecular data enabled us to uncover and describe the full range of cryptic speciation in our material; low genetic distances of anatomically distinguishable genetic lineages of *P. viatoris* sp. nov. suggest there could be some gene flow between geographically distant populations, preventing us from establishing separate species; 4) patterns of distribution of *Pseudunela* species are discovered that cannot, however, be satisfyingly explained in the absence of sound biological knowledge on tiny meiofaunal species. We thus agree with Cook et al. [69] and advocate that taxonomy should integrate and consider all relevant types of data. Our exploration of the genus *Pseudunela* in older studies [17,43] and herein also showed considerable ecological and structural diversity, i.e. of fully marine species, and those steadily or temporarily exposed to freshwater, having complex excretory systems. The combination of molecular phylogenetic and detailed micromorphological studies will shed further light on the origin of acochlidians, their much more frequent than expected habitat shifts, and their evolutionary adaptations to an extraordinarily wide range of completely different habitats.

Supporting Information

Figure S1 Interactive 3D-model of *Pseudunela viatoris* sp. nov. from Fiji. To activate the 3D-model of *P. viatoris* sp. nov. for interactive manipulation click into figure. Rotate model by dragging with left mouse button pressed, shift model: same action+ctrl (or change default action for left mouse button), zoom: use mouse wheel. Select or deselect (or change transparency of) components in the model tree, switch between prefab views or change surface visualization (e.g. lightning, render mode, crop etc.). Interactive manipulation requires Adobe Reader 7 or higher. (PDF)

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Author Contributions

Conceived and designed the experiments: TPN KJM MS. Performed the experiments: TPN KJM. Analyzed the data: TPN KJM. Collected and contributed materials: TPN KJM MS. Realised morphological analyses and interactive 3D-model: TPN. Carried out molecular studies: KJM. Planned and supervised the study: MS. Read, approved and wrote the final manuscript: TPN KMJ MS.

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Chapter 12. Systematics and redescription of the european meiofaunal slug *Microhedyle glandulifera* (Kowalevsky, 1901) (Heterobranchia: Acochlidia): evidence from molecules and morphology.

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SYSTEMATICS AND REDESCRIPTION OF THE EUROPEAN MEIOFAUNAL SLUG *MICROHEDYLE GLANDULIFERA* (KOWALEVSKY, 1901) (HETEROBRANCHIA: ACOCHLIDIA): EVIDENCE FROM MOLECULES AND MORPHOLOGY

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ABSTRACT

Despite the long history of meiofaunal research in Europe our knowledge of its Acochlidia—the most diverse, abundant and widespread group of interstitial slugs—is still fragmentary. Distribution ranges and modes of dispersal are unknown and taxonomic hypotheses based on traditional light-microscopical examination have never been tested against a modern integrative approach combining microanatomical techniques with molecular analyses. This study redescribes *Microhedyle glandulifera* (Kowalevsky, 1901), a key species for microhedylid taxonomy and focus of taxonomic disorder. Three-dimensional reconstructions from histological semithin serial sections reveal several previously unknown characters, in particular concerning the nervous system (e.g. presence of gastro-oesophageal ganglia). There are no jaws, but a 'cuticular element' is attached anteriorly to the radula cushion. Scanning electron microscopic examination shows a radula with the formula 34–38 × 1.1.1. *Microhedyle glandulifera* can be distinguished from other Microhedylidae by a combination of external and radular features, and the unique presence of triaxonic spicules. Population genetic analyses based on mitochondrial markers support *M. glandulifera* as a widespread European species known to range from the North Sea to the Sea of Marmara (eastern Mediterranean). Accordingly, northern Atlantic '*M. lactea*' and Mediterranean '*M. glomerans*' are confirmed as junior synonyms of *M. glandulifera*. Molecular data indicate a recent radiation of *M. glandulifera* in European waters and potential means of dispersal in meiofaunal slugs with low reproductive output and no pelagic larval stages are discussed. Based on our molecular phylogeny and revision of distinguishing morphological characters, four valid Microhedylidae species occur in European waters: *Pontohedyle milaschewitchii*, *Parhedyle tyrtowii*, *Parhedyle cryptophthalma* and *Microhedyle glandulifera*. Morphological and molecular evidence indicate that *Microhedyle odhneri* is a member of the genus *Parhedyle*, and possibly a junior synonym of *Parhedyle tyrtowii*.

INTRODUCTION

European waters have a long tradition of meiofaunal research and several of the first descriptions of meiofaunal taxa were made at marine research centres at Kristineberg (Sweden), Heligoland (Germany), Roscoff, Banyuls-sur-Mer (both France) or Sebastopol (Ukraine) (see Coull & Giere, 1988 on history of meiofauna research). Despite having the best-studied meiofauna in the world, our knowledge of certain European groups is still fragmentary concerning number of species, their phylogenetic relationships, distributional ranges and modes of dispersal.

In the meiofauna Acochlidia are the most successful group of heterobranch gastropods in regard to diversity and local densities (Swedmark, 1968; Poizat, 1986, 1991). Due to their vulnerability to degradation of their habitat (e.g. due to increasing pollution), they have been shown to be valuable indicator organisms for clean and well-oxygenated sediments (Poizat, 1984, 1985). A solid taxonomic framework is needed for marine biodiversity estimations, conservational efforts and ecological approaches. However, for Acochlidia in general—and European Microhedylidae in particular—systematics are complicated by (1) original descriptions that lack detail; (2) poor understanding of some distinguishing characters and their

intraspecific variation; (3) frequent loss of type material; and (4) imprecise type localities. *Pontohedyle milaschewitchii* (Kowalevsky, 1901) has recently been redescribed in detail (Jörger *et al*., 2008, 2009) and is well characterized by the presence of only one pair of bow-shaped head appendages. *Pontohedyle milaschewitchii* is thus considered a taxonomically unambiguous member of the European meiofauna, with reported collecting localities throughout the Mediterranean and Black Sea (see e.g. Kowalevsky, 1901; Arnaud, Poizat & Salvini-Plawen, 1986; Wawra, 1986; Poizat, 1991). In contrast, the taxonomic validity and distribution range of slender microhedylids with two pairs of head appendages is still uncertain. In one of his pioneering studies on meiofaunal gastropods, Kowalevsky (1901) described *Parhedyle tyrtowii* (Kowalevsky, 1901) and *Microhedyle glandulifera* (Kowalevsky, 1901) (both as *Hedyle*) from the Black Sea, Sea of Marmara and eastern Mediterranean, but unfortunately no type material remains from his studies (Wawra, 1974, 1978). While the original description of *P. tyrtowii* is detailed given the resources available at that time, *M. glandulifera* was only briefly described in comparison to *P. tyrtowii*. Later, *Microhedyle lactea* Hertling, 1930 was described from Heligoland (North Sea, Germany) as a geographic subspecies of Mediterranean and Black Sea *M. glandulifera* (see Hertling, 1930). Odhner (1937) elevated it (without further comment) to the rank of species and further sampling localities were reported from Banyuls-sur-Mer (France, Mediterranean) (Odhner, 1952) and Arcachon (France, Atlantic) (Marcus & Marcus, 1955). Additionally, Salvini-Plawen (1973) described *M. glomerans* Salvini-Plawen, 1973 from Secche della Meloria (Livorno, Italy). Without revising any material or providing any additional data, Rankin (1979) created the new species *M. napolitana* (Rankin, 1979) (as *Stellaspina*), referring only to a brief description of *M. glandulifera* found in Naples (Italy) by Marcus (1954). To resolve taxonomic issues, Wawra (1974) recollected *M. glandulifera* from its type locality in Greece. In a detailed taxonomic revision Wawra (1978) corrected the radula formula of *M. glandulifera* to 1.1.1. Comparing morphology of *Microhedyle* populations from Lesbos (Greece, type locality of *M. glandulifera*), Rovinj (Croatia), Livorno (Italy), Banyuls (France) and Heligoland (type locality of *M. lactea*), Wawra (1978) demonstrated the coloration of the digestive gland and numbers of rows of radula teeth to vary within and among populations rather than distinguishing the two species; thus, he considered *M. lactea* as junior synonym of a widespread Mediterranean and Atlantic *M. glandulifera*. In his later classification of Acochlidia, Wawra (1987) also synonymized *M. glomerans* and *M. napolitana* with *M. glandulifera*, although without detailed discussion. Here we give a morphological redescription of the key species *Microhedyle glandulifera* using modern technologies [i.e. scanning electron microscopy (SEM) of the radula and three-dimensional (3D) reconstruction from histological semithin sections] as a basis for a taxonomic revision of European Acochlidia.

Recent integrative taxonomic approaches testing traditional taxonomy against molecular data have revealed flocks of cryptic species across different meiofaunal taxa, e.g. polychaete annelids (Schmidt & Westheide, 1999; Schmidt & Westheide, 2000), proseriate flatworms (Casu & Curini-Galletti, 2004; Casu *et al*., 2009), gastrotrichs (Todaro *et al*., 1996; Leasi & Todaro, 2009) and acochlidian gastropods (Neusser, Jörger & Schrödl, 2011b). Minute body size, low reproductive output and the frequent absence of pelagic larvae (Swedmark, 1959, 1964) make meiofaunal taxa prone to reproductive isolation and potential cryptic speciation. Wawra's (1987) taxonomic hypothesis based on morphological characters (synonymizing Northern Sea and Atlantic *M. lactea*, Mediterranean *M. glomerans* and *M. napolitana* with *M. glandulifera*) is here tested with molecular markers.

MATERIAL AND METHODS

Sampling

Microhedylid Acochlidia were collected from nine different localities along the European coast, including the North Sea, Atlantic Ocean, Mediterranean Sea and Black Sea. Wherever possible, we collected at type localities (or additional localities reported in the original literature) and also covered some sites in between (Fig. 1, Table 1). For morphological redescription, *M. glandulifera* was collected near Rovinj, Croatia (Mediterranean), a locality where populations had been previously collected and exhaustively compared morphologically to *M. glandulifera* from the type locality on Lesbos, Greece (Wawra, 1978). Specimens were extracted from sand samples following the method described by Schrödl (2006). Living specimens were investigated under the light microscope, mainly for the presence and types of spicules. For molecular purposes and for radula preparation specimens were fixed in 96% ethanol. For histological work specimens were slowly anesthetized using $MgCl_2$ to prevent them from retracting into their visceral hump and subsequently fixed in 4% glutaraldehyde (buffered in cacodylate).

Morphological analysis of Microhedyle glandulifera

For histological work, specimens were embedded in Spurr's low-viscosity epoxy resin (Spurr, 1969), following the protocol previously used for micromolluscs (e.g. Neusser *et al*., 2006). Semithin serial sections (1.5 μm) of eight individuals (all subadult juveniles) were prepared using a Histo Jumbo diamond knife (Diatome, Biel, Switzerland) with a rotation microtome (HM 360, Zeiss, Germany) and glue on the lower cutting edge, after the method described by Ruthensteiner (2008). Sections were stained with a 1:1 dilution of Richardson's Blue for 20–25 s (Richardson, Jarett & Finke, 1960). Every section was photographed through a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany) with mounted Spot CCD camera (Spot Insight, Diagnostic Instruments, Sterling Heights, MI, USA). Photographs were then edited (i.e. downsized, converted to greyscale, un-sharp masked and contrast enhanced) with standard picture-editing software. A computer-

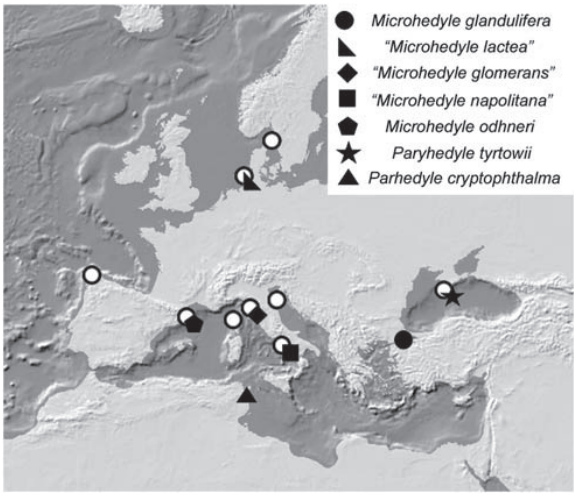

Figure 1. Type localities of European Microhedylidae species (solid symbols) and sampling localities for the present study (open circles, see also Table 1).

Table 1. Collecting sites and remarks on the habitat of European Microhedylidae sampled.

Collecting site	Location	GPS (retrieved from Google Earth)	Habitat description
Kristineberg	Bonden Island, Bohuslän, Sweden, North Sea	—	Subtidal, 20 m, coarse sand and shell gravel
Ferrol	La Coruna, Galicia, Spain, Atlantic Ocean	43°16′12″N, 08°12′11″E	Subtidal, 41 m, medium-grained sand
Canet-Plage	Languedoc-Roussillon, France, Mediterranean Sea	42°39′55″N, 03°02′06″E	Subtidal, 1 m, fine sand
Calvi	Bay of Revellata, Corsica, France, Mediterranean Sea	42°33′57″N, 8°44′15″E	Subtidal, 22 m, coarse sand and shell gravel
Livorno	Secche della Meloria, Tuscany, Italy, Mediterranean Sea	43°33′01″N, 10°13′08″E	Subtidal, 3–4 m, coarse sand
Cape Kamenjak	Premantura, Istria, Croatia, Mediterranean Sea	44°46′03″N 13°54′58″E	Subtidal, 6–9 m, coarse sand
Rovinj	Istria, Croatia, Mediterranean Sea	45°04′05″N, 13°02′14″E	Subtidal, 2–3 m, coarse sand
Sebastopol	Cape Fiolent, Crimea, Ukraine, Black Sea	—	Subtidal, 15 m
Heligoland	Germany, North Sea	—	Subtidal, coarse sand

Table 2. Acochlidian specimens used for phylogenetic analysis of European Microhedylidae, with sampling localities, museums voucher numbers (ZSM, Bavarian State Collection of Zoology), DNA voucher accession numbers and GenBank accession numbers.

Species	Collecting sites	ZSM number	DNA Bank accession number	GenBank accession number		
				28S rRNA	16S rRNA	COI
Hedylopsis spiculifera	Rovinj	20080951	AB35081816	HQ168443	HQ168417	HQ168455
Pontohedyle milaschewitchii	Cape Kamenjak	20080054	AB34404241	JF828043	HQ168422	HQ168459
Parhedyle cryptophthalma	Naples	20100584	AB34599403	JF828041	JF828042	JF828033
Parhedyle tyrtowii	Sebastopol	20091369	AB35081774	JF819813*	—	JF819818*
Microhedyle odhneri	Canet-Plage	20090571	AB35081818	JF819814*	—	JF819819*
Microhedyle glandulifera	Cape Kamenjak	20081019	AB35081799	HQ168449	HQ168424	HQ168461
Microhedyle glandulifera	Rovinj	20080056	AB34404242	—	JF819815*	JF819777*
"*Microhedyle glomerans*"	Livorno	20080413	AB35081799	—	JF819816*	JF819780*
"*Microhedyle lactea*"	Kristineberg	20080136	AB34404283	—	JF819817*	JF819778*

Sequences generated for the present study are marked with *.

based 3D reconstruction of the nervous and digestive systems of *M. glandulifera* was created (based on ZSM Mol 20090600) using Amira v.4.1 software (Visage Imaging GmbH, Germany), following the method described by Ruthensteiner (2008). All section series are deposited in the Mollusca Department of the Bavarian State Collection for Zoology (museums numbers: ZSM Mol 20090600, 20100610, 20100612–615).

For examination of the radulae by SEM, five specimens of *M. glandulifera* were dissolved in a proteinase K solution (90 µl ATL buffer + 10 µl proteinase derived from the Qiagen DNeasy Blood and Tissue Kit). Subsequently, radulae were rinsed several times in ultrapure water and placed onto SEM stubs with self-adhesive carbon stickers. The stubs were coated with gold for 120 s in a Polaron Sputter Coater and viewed with a LEO 1430 VP SEM (15 kV).

DNA extraction, PCR and sequencing

DNA was extracted from entire specimens using the Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's protocol. For phylogenetic analyses portions of three markers were amplified with PCR using the protocols and primers described by Jörger *et al.* (2010a): nuclear 28S rRNA and mitochondrial 16S rRNA and cytochrome oxidase *c* subunit I (COI). PCR products were cleaned with ExoSAP-IT (Affymetrix) and sequenced using the PCR primers in both directions by the Genomic Service Unit (GSU) of the Department of Biology of the Ludwig-Maximilians-University Munich (Big Dye v.3.1; ABI 3730 capillary sequencer). Sequence data of microhedylacean *Parhedyle cryptophthalma*, *Pontohedyle milaschewitchii* and the hedylopsacean *Hedylopsis spiculifera* were retrieved from GenBank (Table 2).

For population genetic studies on Mediterranean *Microhedyle glandulifera* (including '*M. lactea*' and '*M. glomerans*') the partial COI (654 bp) was sequenced as described above from 36 individuals belonging to seven populations.

All sequences generated were deposited in GenBank (see Tables 2, 3). DNA vouchers are available from the DNA Bank of the Bavarian State Collection of Zoology (ZSM) and (if available) voucher specimens were deposited in ZSM.

Phylogenetic analyses

Sequences were edited with Geneious Pro v.5.2 (Biomatters) and checked with BLAST searches (Altschul *et al.*, 1990) against potential contaminations via the NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments for each marker were obtained with MUSCLE v.3.8 (Edgar, 2004) and the COI alignment was afterwards checked manually according to amino acid translation. We concatenated the resulting alignments using BioEdit (Hall, 1999). Phylogenetic analyses of the combined dataset (28S + 16S + COI) were conducted using RAxML v.7.0.4 (Stamatakis, 2006). Data were analysed in three partitions (according to each marker) under the GTR + Γ + I model, selected as best-fitting model of nucleotide substitution with jModeltest (Posada, 2008). Analyses were conducted following the RAxML manual ("hard and slow way"), with hedylopsacean *Hedylopsis spiculifera* defined as outgroup. Statistical support for each node was estimated via multiple nonparametric bootstrapping (1,000 replicates).

Table 3. Mitochondrial COI sequences generated within this study, for population genetics on *Microhedyle glandulifera* (including '*M. glomerans*' and '*M. lactea*').

Species	Collecting sites	ZSM number	DNA Bank voucher	GenBank accession number (COI)
M. glandulifera	Cape	20080056	AB34404242	JF819777
	Kamenjak	20081019	AB35081799	HQ168461§
		20091332	AB35081756	JF819791
		20091168	AB34858195	JF819786*
		20091169	AB34404297	JF819787
		20100411	AB34402384	JF819792
		20100412	AB35081778	JF819793
		20100413	AB34858243	JF819794
		20100414	AB34858183	JF819795
		20100415	AB34858186	JF819796
		20100416	—	JF819797
		20100417	AB35081836	JF819798
		20110027	AB35081779	JF819811
		20110028	AB34499233	JF819812
	Rovinj	20100419	AB35081749	JF819799
		20100420	AB34858209	JF819800
		20100421	AB34858194	JF819801
		20100422	AB34404240	JF819802
		20100423	AB35081781	JF819803
		20100424	AB34599359	JF819804
		20100425	AB34404230	JF819805
	Calvi	20080959	AB35081807	JF819781
		20080960	AB35081815	JF819782
		20091178	AB34858185	JF819790
'*M. glomerans*'	Livorno	20080413	AB34858172	JF819780
'*M. lactea*'	Ferrol	20080392	AB35081748	JF819779
	Kristineberg	20080136	AB34404283	JF819778
		20091170	AB35081831	JF819788*
		20091171	AB35081820	JF819789
		20081017	AB35081825	JF819783
		20081018	AB35081761	JF819784
	Heligoland	20100426	AB34599395	JF819806
		20100427	AB34858168	JF819807
		20100428	AB34858180	JF819808
		20100429	AB34858189	JF819809
		20100430	AB35081763	JF819810

§marks sequence retrieved from GenBank. * marks two sequences that were not included in population genetic analyses due to missing data.

Population genetic analyses

Haplotype networks of *M. glandulifera* based on mitochondrial COI sequences were inferred using statistical parsimony as implemented in TCS v.1.21 (Clement, Posada & Crandall, 2000) under the default settings (95% confidence criterion). Population genetic analyses were conducted in Arlequin v.3.5 (Excoffier & Lischer, 2010): to describe the genetic diversity of each sample, the number of haplotypes, haplotype and nucleotide diversity, and mean number of pairwise differences between populations were estimated. Additionally, pairwise F_{st} values between populations were calculated (with 1,000 permutations) and a hierarchical analysis of molecular variance (AMOVA) was conducted comparing Atlantic (Ferrol, Kristineberg and Heligoland) and Mediterranean populations (Rovinj, Cap Kamenjak, Livorno and Calvi).

SYSTEMATIC DESCRIPTION

MICROHEDYLIDAE Odhner, 1937

Microhedyle Hertling, 1930

Microhedyle glandulifera (Kowalevsky, 1901)

(Figs 2–6)

Hedyle glandulifera Kowalevsky, 1901: 1–32, pl. IV, figs 52–55.
Microhedyle glandulifera lactea Hertling, 1930: 1–11.
Microhedyle lactea Odhner, 1937: 51–64.
Microhedyle glomerans Salvini-Plawen, 1973: 123–125.
Stellaspina napolitana Rankin, 1979: 96–97.

Description: a slender, minute interstitial microhedylid (Fig. 2A), 1.5–2.5 mm in length. Head bears two pairs of thin appendages (oral tentacles and rhinophores) which are roundish in section and slightly tapering. Four main types of calcareous spicules: (1) monaxonic spicules (30–70 μm) in headfoot and visceral sac (Fig. 2D); (2) large triaxonic spicules (30–60 μm) in visceral sac (Fig. 2B, D); (3) short bean-shaped or oval spicules (15–20 μm) near posterior end of radula (Fig. 2C); (4) tiny structures like strings of beads (S-, C- or ring-shaped, 5–15 μm) distributed over entire body (Fig. 2D). Transitional forms between monaxonic and triaxonic, and occasional tetraxonic or pentaxonic, spicules were observed in some individuals. Absence of monaxonic/triaxonic spicules was recorded in specimens held in captivity for several months (see Discussion for interpretation).

Digestive system consists of oral tube, pharynx (containing radula), oesophagus, paired salivary glands, digestive gland and intestine, and follows general body plan described for other Microhedylacea (Neusser *et al.*, 2006; Jörger *et al.*, 2008; Neusser, Martynov & Schrödl, 2009b). Bulbous muscular pharynx 140 μm with two joined cavities, containing both rami of hook-shaped radula (Fig. 4A). Radula 100 μm (formula 34–38 × 1.1.1); rhachidian tooth triangular with central cusp and two small denticles on either side; lateral teeth rectangular with one rounded denticle at anterior margin and a corresponding notch in posterior margin (Fig. 3A–C). V-shaped 'cuticular element' (30 μm long, 6–18 μm wide) with two symmetrical sides and central groove is located in anterior part of pharyngeal cavity, attached by posterior end to lower muscle of radula cushion where oldest teeth of ventral radula ramus terminate (Fig. 4A, B). 'Cuticular element' consists of noncellular material with same properties and appearance as chitinous cuticle under light microscope; visible in histological sections but difficult to detect on whole mounts.

Central nervous system (CNS) euthyneurous, slightly epiathroid, following typical acochlidian plan: paired cerebral, rhinophoral, pedal, pleural, buccal and gastro-oesophageal ganglia; three separated, single ganglia on visceral nerve cord; single osphradial ganglion (Figs 5, 6A, C). Cerebral, pedal and pleural ganglia form prepharyngeal nerve ring; ganglia of visceral cord in posterior part of pharynx; only buccal and gastro-oesophageal ganglia are postpharyngeal.

In anterior head region (i.e. cephalic tentacles up to posterior end of pedal ganglia, total length of 175 μm) a mass of accessory ganglia (defined as ganglia-like aggregations of neuronal tissue without subdivision into cortex and medulla, according to Neusser *et al.*, 2006) (Figs 5, 6B) forming two complexes on right and left sides of body, connected to cerebral ganglia by cerebral nerves. Form and size of accessory ganglia differ slightly between both sides of body, but are separated into same main portions: large anterior part connected to cerebral ganglia by rhinophoral and labiotentacular nerves; other

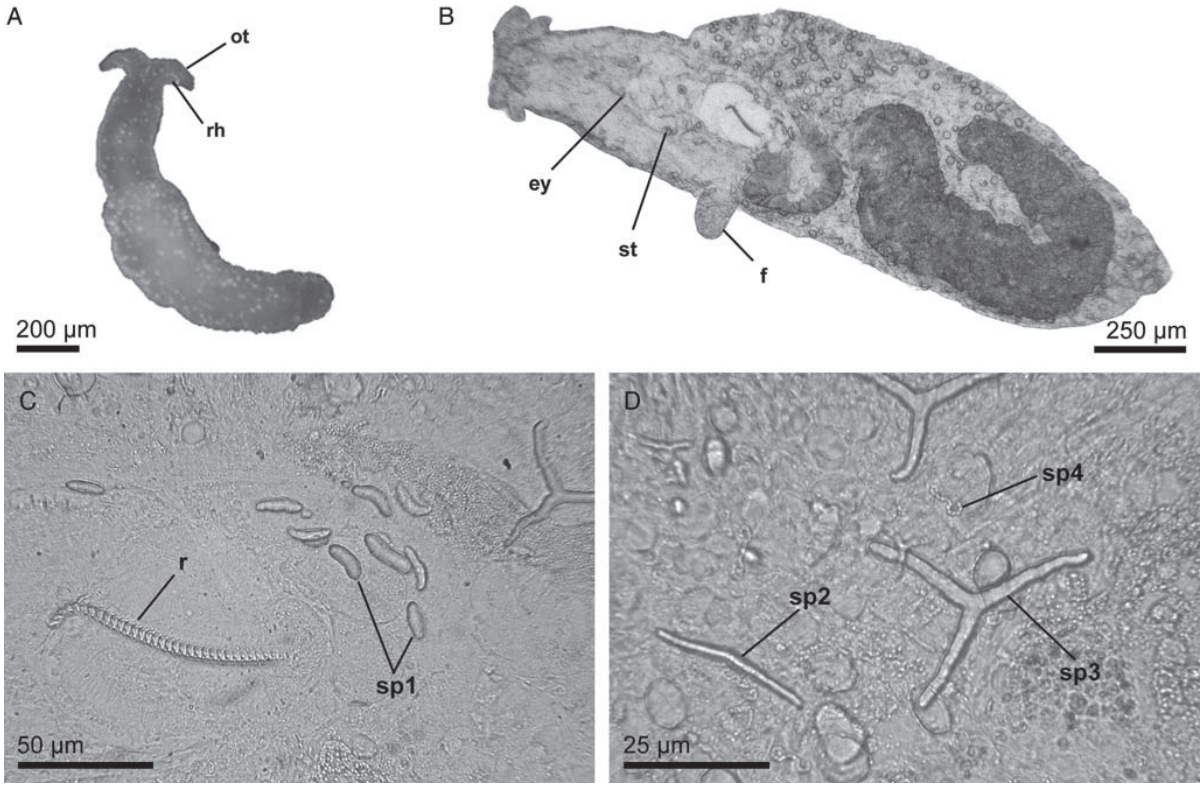

Figure 2. External morphology and spicules in *Microhedyle glandulifera*. **A.** Living specimen under dissecting microscope. **B.** Overview of an entire juvenile specimen. **C.** Accumulation of oval to bean-shaped spicules posterior to the radula. **D.** Monaxonic, triaxonic and bead string-like spicules. Abbreviations: ey, eye; f , foot; ot, oral tentacle; r, radula; rh, rhinophore; sp1, oval to bean-shaped spicules; sp2, monaxonic spicule; sp3, triaxonic spicule; sp4, bead string-like spicule; st, statocyst.

portion extends posteriorly on outer side of cerebral and pleural ganglia, and is innervated by a cerebral nerve (interpreted as Hancock's nerve). Additional pair of small accessory ganglia in foot near anterior end of pedal ganglia; no connection between these and remaining accessory ganglia. Spherical cerebral ganglia (65 µm diameter) connected via thick commissure. Slightly ventrally the strong labiotentacular nerve emerges from cerebral ganglion; rhinophoral nerve emerges dorsally. At base of rhinophoral nerve Hancock's nerve leads posterolaterally to flanking accessory ganglia. Anterolateral to cerebral ganglia Hancock's nerve leaves outer side of accessory ganglia and extends to barely visible groove in epidermis regarded as Hancock's organ (Fig. 6E). From posterior part of cerebral ganglia the thin static nerve emerges, innervating statocysts. Pigmented eyes (15 µm diameter) anteroventral to cerebral ganglia (Fig. 6D). Posterior to eyes, small rhinophoral ganglia (25 µm) close to cerebral ganglia, connected to latter via thin connective. Pedal ganglia (50 µm) bear short strong commissure; three nerves emerge ventrally from each ganglion, innervating anterior and posterior part of foot. Paired statocysts (20 µm) with one statolith each, attached posterodorsally to pedal ganglia; innervated by thin cerebral static nerve. Pleural ganglia (30 µm) posterior to cerebral and dorsal to pedal ganglia, with very short connective to cerebral ganglia and longer one to pedal ganglia (i.e. epiathroid condition of CNS). Three ganglia on visceral nerve cord: left parietal ganglion (25 µm), fused subintestinal/visceral ganglion (40 µm) and fused supraintestinal/parietal ganglion (40 µm), with smaller osphradial ganglion (25 µm) attached posteriorly via short connective. Thick visceral nerve emerges from large subintestinal/visceral ganglion and leads posteriorly to visceral sac. Buccal ganglia (35 µm) connected by commissure; slightly smaller elongated gastro-oesophageal ganglia (30 µm) nestle dorsally on buccal ganglia, connected to latter by thin connectives.

Phylogenetic analysis

In our maximum-likelihood analyses of European Microhedylidae *Pontohedyle milaschewitchii* forms the sister group to remaining Microhedylidae (Fig. 7), uniting the genera *Parhedyle* and *Microhedyle* (bootstrap probability BS = 72%). The species collected at the type locality of *M. odhneri* clusters among species of *Parhedyle* (BS = 94%), sister to *Parhedyle tyrtowii* (BS = 97%). Direct comparison of mitochondrial COI sequences show 98.25% identity between Black Sea *Parhedyle tyrtowii* and the microhedylid collected as '*M. odhneri*'. Atlantic '*M. lactea*' and Mediterranean '*M. glomerans*' form a clade with Mediterranean *M. glandulifera* (BS = 90%).

Population genetic and demographic analyses

Network analysis shows one connected haplotype network for populations of *M. glandulifera* throughout the Mediterranean and along the Atlantic Coast to the North Sea (as '*M. lactea*') (Fig. 8). The specimen collected at the type locality of *M. glomerans* (Livorno, Italy) nests within the Mediterranean *M. glandulifera*, sharing a common haplotype with two

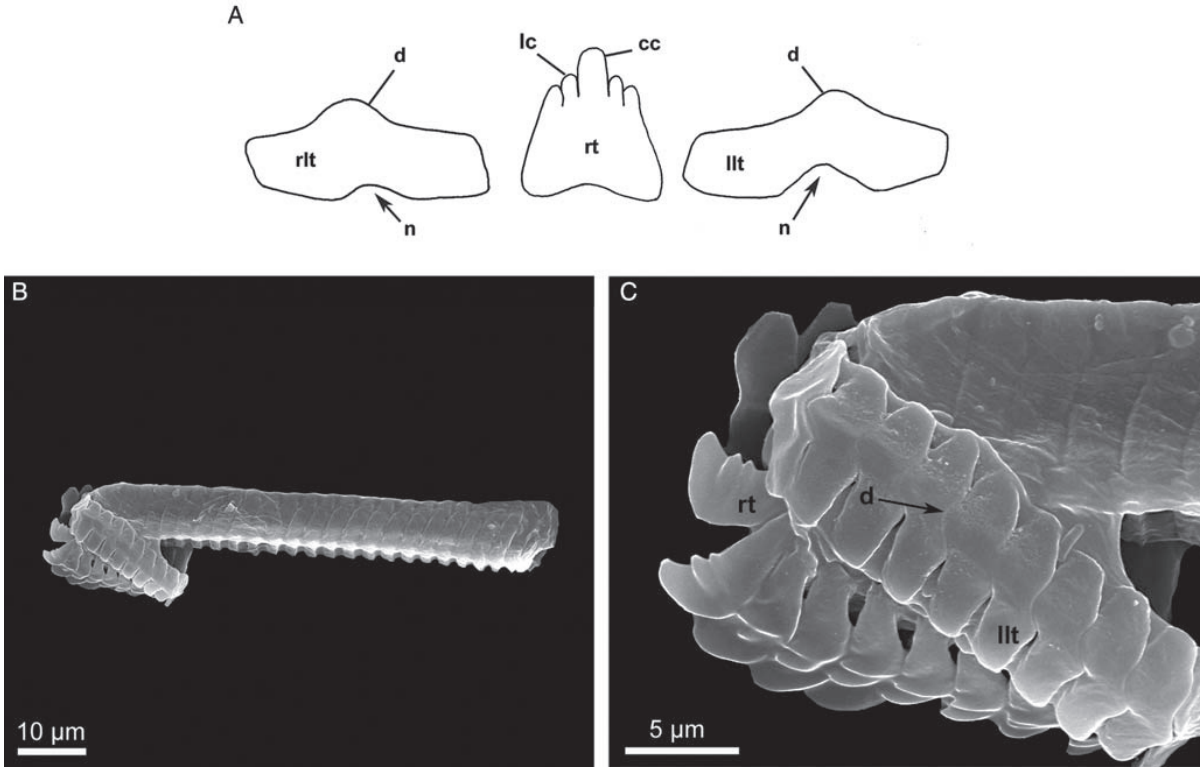

Figure 3. Radula of *Microhedyle glandulifera*. **A.** Schematic drawing of rhachidian and lateral teeth. **B, C.** Scanning electron micrographs. Abbreviations: cc, central cusp; d, denticle; lc, lateral cusp; llt, left lateral tooth; n, notch; rlt, right lateral tooth; rt, rhachidian tooth.

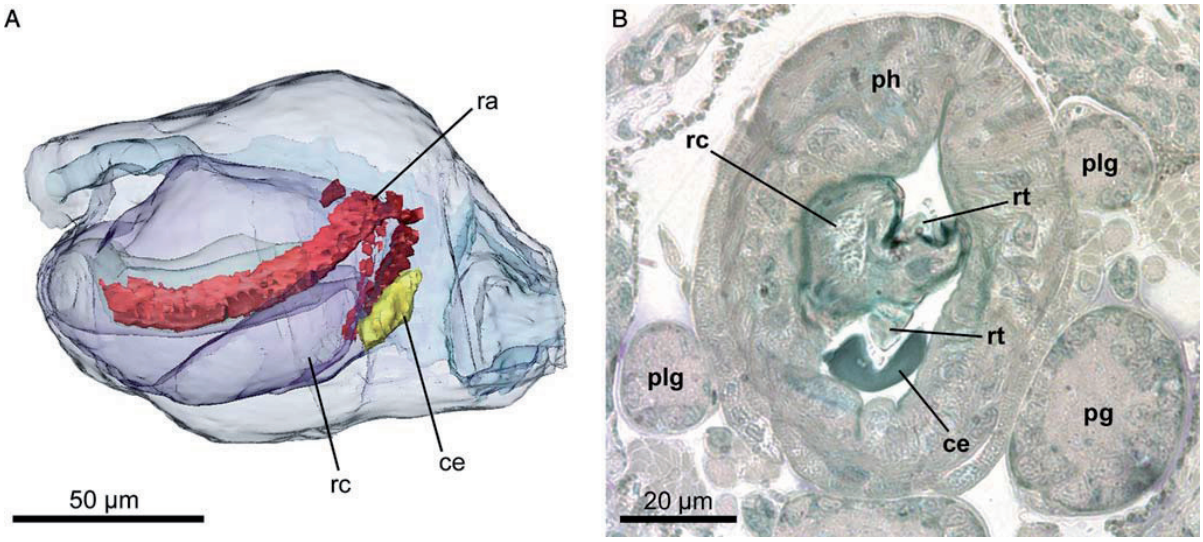

Figure 4. Three-dimensional reconstruction (**A**) and semithin cross-section (**B**) of the pharynx of *Microhedyle glandulifera* showing the position of the radula, radular cushion and 'cuticular element'. Abbreviations: ce, cuticular element; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; ra, radula; rc, radular cushion; rt, rhachidian tooth.

specimens of *M. glandulifera* collected at Rovinj and Cape Kamenjak (Croatia).

Sequences of 34 specimens (two specimens were excluded from the analyses due to missing data; Table 3) yielded 17 different mitochondrial haplotypes, 10 of which are represented by single individuals only. Populations from Heligoland ($n = 5$), Corsica ($n = 3$), Rovinj ($n = 7$) and Cape Kamenjak ($n = 13$) show relatively high haplotype diversities, ranging from

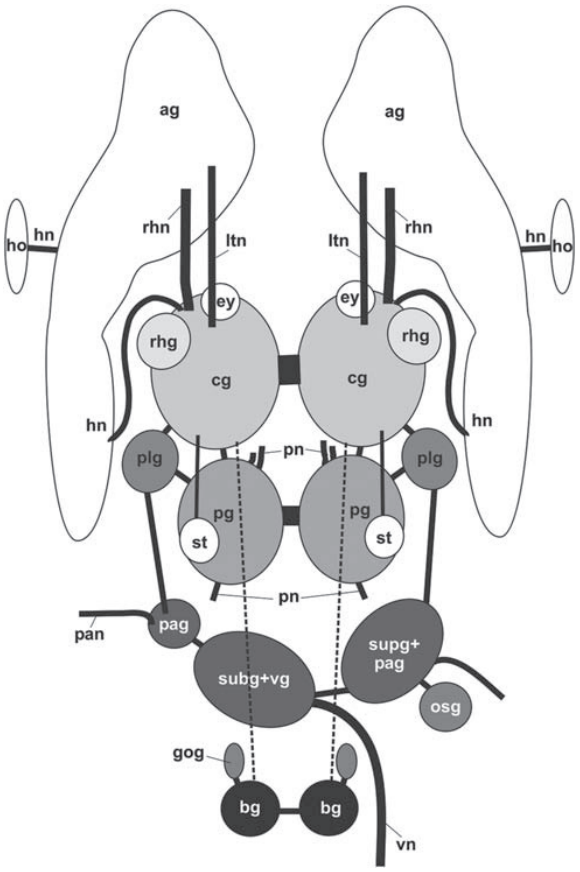

Figure 5. Schematic overview over the CNS of *Microhedyle glandulifera* (not to scale). Abbreviations: ag, accessory ganglion; bg, buccal ganglion; cg, cerebral ganglion; ey, eye; gog, gastro-oesophageal ganglion; hn, Hancock's nerve; ho, Hancock's organ; ltn, labiotentacular nerve; osg, osphradial ganglion; pag, parietal ganglion; pan, parietal nerve; pg, pedal ganglion; plg, pleural ganglion; pn, pedal nerve; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; st, statocyst; subg + vg, subintestinal/visceral ganglion; supg + pag, supraintestinal/parietal ganglion; vn, visceral nerve.

0.89 to 1.00. Among the same populations, nucleotide diversity is very low (0.0036–0.0062). Average pairwise nucleotide differences were 2.41–4.09. Haplotype diversity in the Kristineberg population ($n = 4$) is comparably low (0.50) and also has low nucleotide diversity (0.0022, corresponding to 1.50 pairwise nucleotide differences). 'Populations' from Ferrol and Livorno were not considered, because they were each represented by only one individual. AMOVA analyses based on grouped datasets comparing Atlantic with Mediterranean populations showed considerably higher variation within than between these two groups (Table 4); only the F_{st} value comparing the Kristineberg to Cape Kamenjak populations is significant, but very low (0.009).

DISCUSSION

Morphology of Microhedyle glandulifera

Since the original description of *Microhedyle glandulifera* by Kowalevsky (1901), four more microhedylid species have been described in comparison to *M. glandulifera*, which has therefore become a key species for taxonomic descriptions in Microhedylidae (Hertling, 1930; Odhner, 1952; Marcus, 1953; Marcus & Marcus, 1955; Westheide & Wawra, 1974). The original description of *M. glandulifera* was, however, brief and fragmentary (e.g. lacking details on radula morphology), until it was revised by Wawra (1978) who added valuable details on spicules, radula morphology and sperm structure. Using SEM we confirm Wawra's (1978) description of the radula with a formula of 1.1.1, correcting the earlier description by Marcus (1954), who probably interpreted the denticle on the lateral teeth and the corresponding notch as cleavage in the teeth, resulting in a radula formula 2.1.2 (see Salvini-Plawen, 1973; Wawra, 1978). The radula (Fig. 3) is highly similar to the one described for Western Atlantic *M. remanei* (Marcus, 1953) apart from the absence of the second potential denticle on the lateral tooth and less prominent lateral cusps on the rhachidian tooth (Neusser *et al.*, 2006: fig. 4D). The radula of the microhedylid *Pontohedyle milaschewitchii* has the same formula ($n \times 1.1.1$), but can be clearly differentiated by the presence of three lateral cusps on the rhachidian tooth and a pointed (*vs* round) denticle on the lateral tooth (Jörger *et al.*, 2008: fig. 7C). The present study redescribes *M. glandulifera* in microanatomical detail, confirming some unusual features such as the 'cuticular element' in the pharynx and presenting novel data on the nervous system (e.g. paired gastro-oesophageal ganglia attached to the buccal ganglia). The presence of Hancock's organs as described by Edlinger (1980) was confirmed, such as the presence of an osphradial ganglion as illustrated (but not described) in a comparative study of heterobranch nervous systems (Huber, 1993: fig. 13; as *Unela*).

The 'cuticular element' in *M. glandulifera* was first described by Wawra (1978) as a bilateral separated structure fused only at its base. In our populations of *M. glandulifera* this is a V-shaped structure with a central groove surrounding the oldest portion of the radula. Its function is still a matter of speculation, but since it is only attached at its base to the radula cushion, and otherwise hanging loosely within the pharynx cavity, we consider it unlikely that it forms a jaw-like counterpart of the radula as suggested by Wawra (1978). It might instead be interpreted as protective sheet for the surrounding tissue or a sort of chute for the radula. Due to the attachment site on the radula cushion we do not consider the (paired or fused) 'cuticular element' to be homologous with paired jaws reported for microhedylacean Ganitidae or other Euthyneura.

Microhedyle glandulifera can be distinguished from other Microhedylidae by the unique presence of large triaxonic spicules (Fig. 2C), while the other types of spicules (i.e. monaxonic, bead-string-like, and oval or bean shaped) can also be found in other microhedylids. Triaxonic spicules have so far only been reported from an undescribed *Asperspina* (Asperspinidae) from North Carolina, USA (Rieger & Sterrer, 1975; as *Hedylopsis*). On one occasion we noticed the absence of triaxonic and monaxonic spicules in specimens of *M. glandulifera* (from Cape Kamenjak, with identical COI sequences to the remaining material) after being held in captivity for several months. Thus, while the presence of triaxonic spicules is characteristic for *M. glandulifera*, their absence is insufficient for species delineation. Rieger & Sterrer (1975) suggested that spicules—characteristic for many meiofaunal organisms—might be a by-product of metabolic processes. Long-time captivity and absence of natural food resources might hinder these metabolic processes, or slightly acidic conditions could potentially lead to dissolution of calcareous spicules. Spicules were also suggested to have a stabilizing effect for the surrounding tissue when moving through the interstitial habitat (Rieger & Sterrer, 1975). A conspicuous accumulation of monaxonic spicules in *Pontohedyle milaschewitchii* for example

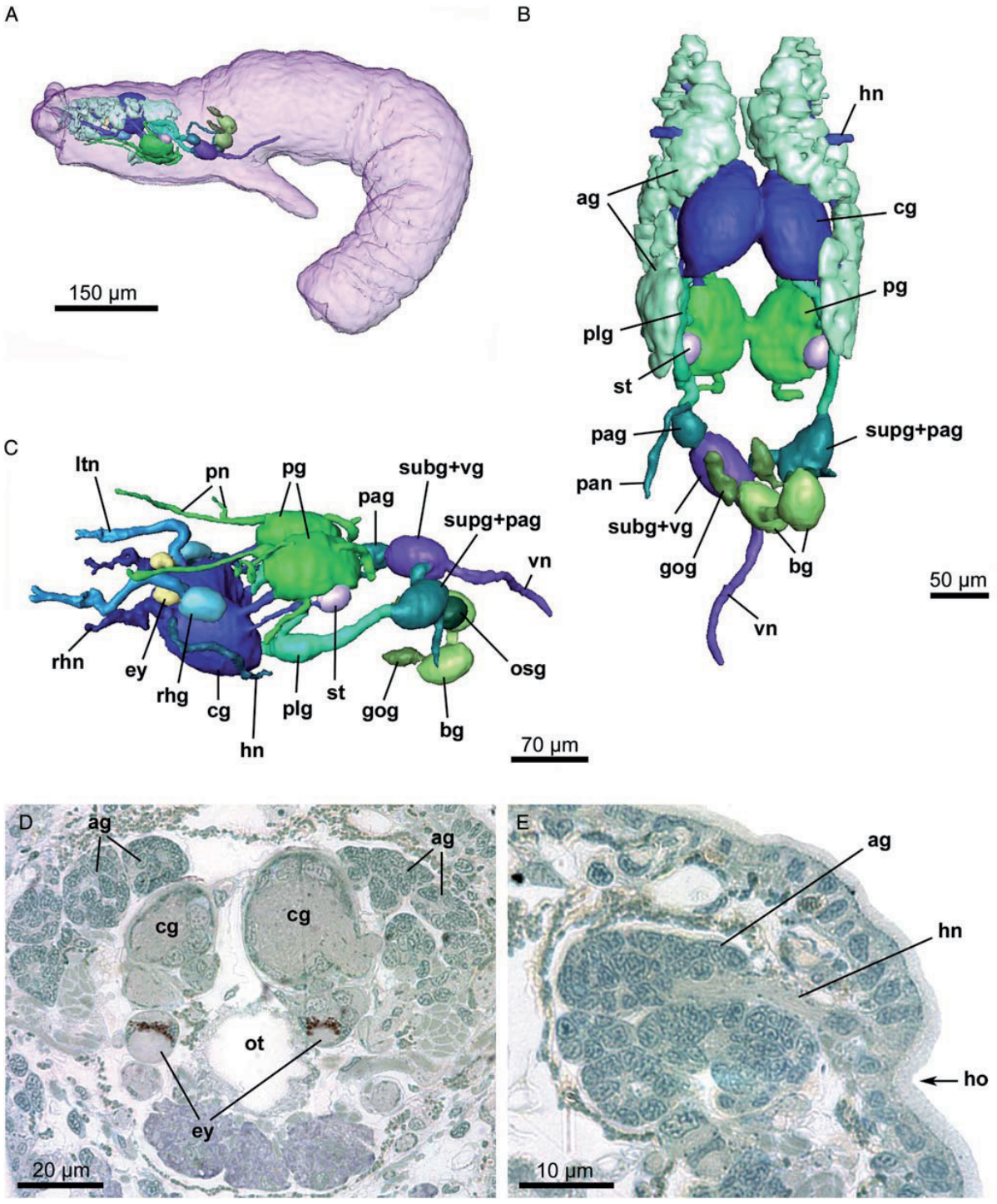

Figure 6. CNS of *Microhedyle glandulifera*. **A–C.** 3D reconstructions of CNS. **A.** Position of CNS in body. **B.** Dorsal view with accessory ganglia. **C.** Lateroventral view (accessory ganglia omitted). **D–E.** Semithin cross-sections. **D.** Cerebral ganglia with eyes. **E.** Accessory ganglia, Hancock's nerve and Hancock's organ. Abbreviations: ag, accessory ganglion; bg, buccal ganglion; cg, cerebral ganglion; ey, eye; gog, gastro-oesophageal ganglion; hn, Hancock's nerve; ho, Hancock's organ; ltn, labiotentacular nerve; osg, osphradial ganglion; ot, oral tube; pag, parietal ganglion; pan, parietal nerve; pg, pedal ganglion; plg, pleural ganglion; pn, pedal nerve; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; st, statocyst; subg + vg, subintestinal/visceral ganglion; supg + pag, supraintestinal/parietal ganglion; vn, visceral nerve.

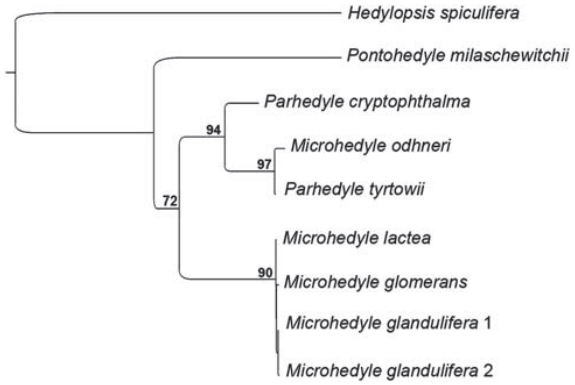

Figure 7. Phylogenetic analysis of European Microhedylidae. Maximum-likelihood tree generated with RAxML based on the concatenated dataset of 28S rRNA, 16S rRNA and COI. Bootstrap values >50% given above nodes.

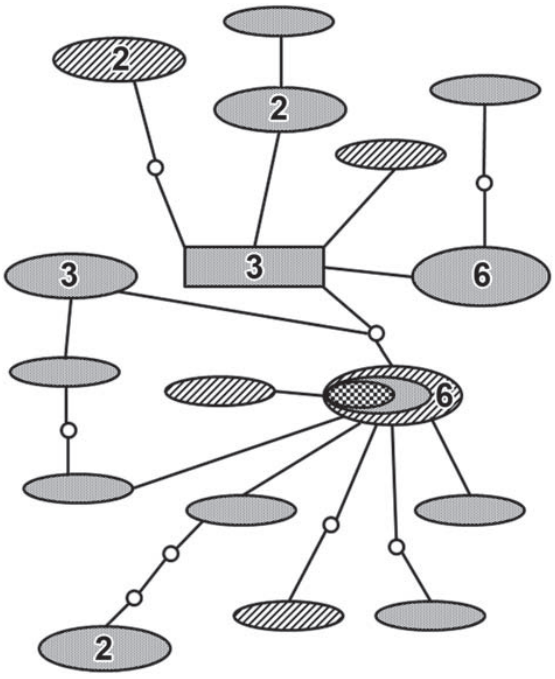

Figure 8. Statistical parsimony haplotype network of *Microhedyle glandulifera* (including '*M. lactea*' and '*M. glomerans*') based on mitochondrial COI (654 bp), generated with TCS 1.21 (Clement *et al.*, 2000). Square indicates haplotypes likely to be ancestral in this network; small, open circles represent unsampled haplotypes; numbers indicate frequency of haplotypes occurrence (if higher than one). Shading: dotted, Mediterranean; hatched, North Sea; chequered, European Atlantic.

was interpreted as a stabilizer of the head region (Jörger *et al.*, 2008). Due to their triaxonic shape, spicules of *M. glandulifera* might have an even better 3D stabilizing effect, e.g. as squeeze protection of certain organs.

Synonymy of Microhedyle glandulifera

Microhedyle lactea from Heligoland was distinguished from *M. glandulifera* by the lack of coloration in salivary and digestive glands and more rows of radula teeth (38–44 *vs* 34–35) (Hertling, 1930). Wawra (1978) demonstrated that the number of rows of teeth depends on the developmental stage and varies considerably within and among populations; this is confirmed by our observations. In several Acochlidia the coloration of the digestive glands is variable, probably depending on type and availability of food resources (Jörger *et al.*, 2008; Neusser *et al.*, 2009b; personal observations) and must thus as be treated with caution as a taxonomic character.

Table 4. AMOVA of *Microhedyle glandulifera*, Atlantic populations (Heligoland, Kristineberg and Ferrol) *vs* Mediterranean populations (Cape Kamenjak, Rovinj, Livorno and Calvi).

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among groups	1	4.421	0.26457 Va	16.60
Among populations within groups	5	4.578	−0.13511 Vb	−8.48
Within populations	27	39.531	1.46410 Vc	91.88

There are several indications that the description of *M. glomerans* from Secche della Meloria (Livorno, Italy) by Salvini-Plawen (1973) was only based on one fixed individual, e.g. due to the lack of spicules, which is not commented on as a characteristic feature, and the descriptions of external morphology "in fixed material". Salvini-Plawen (1973) mentioned that *M. glandulifera* occasionally curls up, instead of contracting, and we support Wawra's (1987) interpretation of the eponymous curling up of *M. glomerans* as a fixation artefact. The unusual "subpharyngeal position of the visceral cord" can also be interpreted as an artefact, since the relative position of ganglia varies with the stage of contraction (personal observations) and the supposedly distinguishing "folded digestive gland" depends on the (non)stretched stage of the visceral hump and has been frequently observed in Microhedylidae (personal observation). Salvini-Plawen (1973) described *M. glomerans* without eyes, but had only a whole mount of the head region available, on which barely pigmented eyes can easily be overlooked. Reinvestigation of the type material by Wawra (1987) led to the synonymization of *M. glomerans* with *M. glandulifera*, and is supported by our observations.

Microhedyle napolitana was named by Rankin (1979) based on a literature record containing a brief description by Marcus (1954) of '*M. glandulifera*' found in Naples (Italy). Wawra (1987) considered it against good taxonomic practice to establish new species based on other authors' short notes only. In the absence of any reliable distinguishing characters we follow Wawra (1987) in synonymizing *M. napolitana* with *M. glandulifera*. Morphological evidence thus supports Wawra (1978) in considering *M. lactea*, *M. glomerans* and *M. napolitana* as junior synonyms of the widespread Mediterranean and Atlantic *M. glandulifera*.

Though poorly known and only reported from its type locality at Canet-Plage (France, Mediterranean), '*Microhedyle odhneri*' has not yet been proposed as synonym of *M. glandulifera*. The original description of *M. odhneri* was based on fixed material; it briefly mentions a microhedylid body shape with two pairs of head tentacles and radula with a formula 39–48 × 1.1.1 (Marcus & Marcus, 1955). Information on spicules is lacking, but empty spaces in the epidermis are mentioned that resemble cavities of dissolved spicules. Unfortunately, the type material of *M. odhneri* could not be found in the Marcuses' collection in São Paulo and might be lost (C. Magenta Cuñha, personal communication). We collected new material at the type locality at Canet-Plage and light-microscopic investigation revealed the presence of plate-

like spicules with holes and fine bead string-like spicules. While the latter have been reported for the genera *Parhedyle* and *Microhedyle*, plate-like spicules are characteristic of *Parhedyle* (Wawra, 1987; Jörger *et al*., 2010b). Light-microscopic and SEM examination of the radula reveals the unusual asymmetric 1.1.2 formula (own unpublished data) known for *P. cryptophthalma* and suspected for *P. tyrtowii* (Westheide & Wawra, 1974; Jörger *et al*., 2010b), rather than a radula with the formula 1.1.1, as reported by Marcus & Marcus (1955); the small inner lateral tooth was obviously overlooked by the original authors due to inadequate methodology. We conclude that *M. odhneri* generically differs from *M. glandulifera*, since spicule types and radula shape indicate inclusion in *Parhedyle*.

Worldwide *Microhedyle* species have been compared morphologically by Neusser *et al*. (2006). *Microhedyle glandulifera* can be clearly distinguished from its Western Atlantic congener *M. remanei* by details of radula morphology (see Discussion above), the presence of large triaxonic spicules and commonly pigmented eyes (*vs* lack of eyes) (Kirsteuer, 1973; Neusser *et al*., 2006). However, the latter two features need to be treated with caution. The eyes (traditionally considered a reliable character for species delineation in Acochlidia; e.g. Odhner, 1938, 1952; Marcus, 1954; Salvini-Plawen, 1973) are a rather unreliable character since intensity of pigmentation can be variable between and within populations and barely pigmented eyes can easily be overlooked (Jörger *et al*., 2010b; Neusser *et al*., 2011b). Differences from Western Atlantic *M. nahantensis* (Doe, 1974) also refer to the difference in spicule types (Doe, 1974). Having roundish plate-like spicules (Doe, 1974) that are elsewhere only present in the genus *Parhedyle* raises doubts on its placement within *Microhedyle*; re-examination of the radula morphology by SEM is needed to clarify the generic affiliation of *M. nahantensis*.

With present knowledge, *Microhedyle glandulifera* seems morphologically characterized by a unique combination of characters: slender microhedylid with two head appendages; radula formula 1.1.1 with the rhachidian tooth bearing one central and two lateral cusps and lateral tooth with one central denticle; presence of monaxonic, triaxonic, bean-shaped and bead-string-like spicules.

More comparative data are needed to evaluate the value of additional microanatomical characters for species delineation in Acochlidia. In contrast to Hedylopsacean taxa, Microhedylacea have a reduced, aphallic reproductive system providing little comparable characters, and excretory and digestive systems (with the exception of the radula) also offer little distinguishing details (see e.g. Jörger *et al*., 2008; Neusser *et al*., 2009b; Schrödl & Neusser, 2010). The acochlidian nervous system and sensory organs have been discussed as valuable characters for phylogenetic analyses (Neusser, Jörger & Schrödl, 2007) and microanatomical redescriptions have indeed revealed a variety of previously unknown features, e.g. an osphradium in limnic *Strubellia* (Brenzinger *et al*., 2011b) and the detection of an unpaired osphradial ganglion in *Parhedyle crypthophthalma* (Jörger *et al*., 2010b) and *M. glandulifera* (Huber, 1993; present study). However, detailed studies on acochlidian nervous systems have also shown a high variety and partial inconsistency of some nervous features e.g. position of cerebral nerves and the difficulties of detection of all parts of the nervous system in single individuals (Sommerfeldt & Schrödl, 2005; Neusser *et al*., 2006, 2007, 2009b, 2011b; Neusser & Schrödl, 2007, 2009; Jörger *et al*., 2008, 2010b; Neusser, Heß & Schrödl, 2009a; Brenzinger *et al*., 2011a). Homologies of the cerebral nerves are still unclear and comparative studies at the population level and across different ontogenetic stages are needed to evaluate the degree of intraspecific variation in nervous features and thus their value for phylogenetic purposes.

In summary, the distinction and classif Microhedylidae by morphological characters can due to their generally small sizes and regressive (Neusser *et al*., 2009a; Schrödl & Neusser, 2010); tl lar data are needed for independent and ecological assessment.

Molecular systematics

We used molecular markers to test morphology-bas eses on the identification of *M. glandulifera*. Phyloge sis of molecular markers (nuclear 28S rI mitochondrial 16S rRNA and COI) showed two n (1) All specimens of *M. glandulifera* form a clad includes specimens of '*M. lactea*' and a potential '*M* confirming morphology-based taxonomic assun Wawra (1978, 1987) and herein. (2) In contrast, m specimens collected at the type locality of *M. odh* with a specimen of *Parhedyle tyrtowii* from the type the Black Sea. As also indicated by radular and tures, '*Microhedyle*' *odhneri* differs from *M. glanc* should be transferred to the genus *Parhedyle*; the seq larity with *Parhedyle tyrtowii* is high enough (98.25% COI) to suggest conspecificity. At the present stag edge the genus *Parhedyle* (including the two va *P. cryptophthalma* and *P. tyrtowii*) seems to be well based on molecular (Fig. 7) and morphological d formula 1-1-2, presence of plate-like spicules) (K 1901; Westheide & Wawra, 1974; Wawra, 1987; J 2010b).

Microhedyle glandulifera has a derived position in o netic analyses although taxon sampling was li improved sampling of Microhedylidae (includir Atlantic *M. remanei* and Northwestern Atlantic *M.* is needed to clarify the relationships within Mic and between the genera *Microhedyle*, *Parhedyle* and Cladistic analyses based on morphological charac Microhedylidae paraphyletic due to the inclusion c cal family Ganitidae (characterized by uniserate ra dagger-shaped teeth) and relationships within Mic remain unresolved due to a lack of reliable characte servative coding procedures (see Schrödl & Neusse accordance with the present study, previous approaches showed *Pontohedyle* as a basal offsh Microhedylidae but, as in morphological analyse the family paraphyletic due to the inclusion of (Jörger *et al*., 2010a; Neusser *et al*., 2011a).

Cryptic species vs *a single widely distributed species*

Microhedyle glandulifera specimens (including those synonymous species) form a clade in the phylogene indicating their monophyletic origin fro Microhedylidae *s. l.* The question is whether or n genetic structure within this clade indicating limite among populations and/or cryptic speciation. *Micr dulifera* extends from the Black Sea through the Me to the North Sea, covering an area of several tho metres of coastline and different hydrographic Wide distributions of tiny meiofaunal taxa with low dispersal abilities (the "meiofaunal paradox", C remain controversial (Schmidt & Westheide, 2000 *et al*., 2003; Boeckner, Sharma & Proctor, 2009) molecular data have supported the existence of widespread and even amphi-atlantic species, e.g. a chaete annelids (Schmidt & Westheide, 2000; West 2003), several studies across different meiofaunal revealed that species formerly considered to be cosm

at least amphi-atlantic are flocks of cryptic species (Todaro *et al.*, 1996; Schmidt & Westheide, 1999; Schmidt & Westheide, 2000; Casu & Curini-Galletti, 2004; Casu *et al.*, 2009; Leasi & Todaro, 2009). Based on the reported collecting sites some species of Acochlidia have wide distributional ranges, e.g. *M. remanei* in the Western Atlantic and *Pseudunela cornuta* from Solomon Islands and Hong Kong (Marcus, 1953; Challis, 1970; Kirsteuer, 1973; Hughes, 1991; Neusser *et al.*, 2006). Reported populations have however never been compared in microanatomical detail or with molecular approaches; their ranges thus still need to be confirmed. In a first integrative taxonomic approach combining data from detailed 3D reconstructions on the anatomy and molecular mitochondrial markers, Neusser *et al.* (2011b) on the one hand revealed the presence of cryptic species inhabiting nearby beaches and on the other hand geographically distant populations of one species of *Pseudunela* (from Indonesia and Fiji).

For Mediterranean *M. glandulifera*, molecular data from mitochondrial COI and 16S rRNA sequences show no or only minor differences from Atlantic '*M. lactea*' and Mediterranean '*M. glomerans*'. In network analyses, using the barcoding marker COI, which is known to be rather fast-evolving and shows good resolution for the separation of species (Hebert *et al.*, 2003a; Hebert, Ratnasingham & deWaard, 2003b), all sampled populations of *M. glandulifera* are united in one haplotype network with mixed haplotypes, which are interconnected by only a few changes in nucleotides (Fig. 8). These similarities support the wide-ranging distribution of a single species *M. glandulifera* and rejects cryptic speciation. The present study provides a first glimpse of population genetic structure in a mesopsammic gastropod. However, the dataset is still limited in specimens per population, populations sampled and choice of genetic markers. Based on COI no genetic structure differentiating Atlantic and Mediterranean populations of *M. glandulifera* could be detected. Only populations from Kristineberg (Sweden) and Cape Kamenjak (Croatia) show a significant but low F_{st} value. These populations represent the most distant areas within our dataset and the presence of some genetic structure between the two might indicate some emerging genetic differentiation between Mediterranean and Atlantic populations. Faster evolving molecular markers such as AFLPs or genomic microsatellites applied to larger sample sets are needed for future population genetic studies of European microhedylids.

A molecular clock analysis calibrated with fossils from different heterobranch outgroups dated the origin of Acochlidia to the early Mesozoic and the main radiations events within Acochlidia were estimated to have taken place in the Jurassic, with diversification of Microhedylidae in the late Jurassic or Cretaceous (Jörger *et al.*, 2010a). The establishment of molecular clocks for more recent acochlid radiations (genus or species level) is hindered by the lack of a fossil record and likely incomplete knowledge of extant diversity of the group.

Our preliminary data could suggest gene flow between populations in *M. glandulifera*. Owing to the low number (maximum 35) of yolk-rich eggs (Wawra, 1978) which indicates lecithotrophic development (Swedmark, 1959, 1968), it is unlikely that larvae play a major role in long-distance dispersal. Studies on dispersal of meiofaunal taxa, however, suggest that adults are the main dispersal stage (Palmer, 1986, 1988; Boeckner *et al.*, 2009). While e.g. copepods and some polychaetes are considered to be capable of actively dispersing through the water column to colonize new areas (Boeckner *et al.*, 2009), based on their external morphology acochlidian slugs seem less prone to active dispersal in the water column. Boeckner *et al.* (2009) observed meiofaunal taxa high in the water column even at calm, low-energy sites, concluding that even slight turbulence might be sufficient to suspend meiofaunal organisms and allow their dispersal b While occasional dispersal by accidentally suspend uals should be considered, Acochlidia actively e water column to facilitate dispersal (e.g. from ove or nutrient-poor habitats) remains pure speculati knowledge no acochlids have ever been found i samples. Soft-bodied slugs floating in the wat without protection are probably at high risk of pre more secure means of adult dispersal e.g. by raft pended sand grains (as reported for other meiof Hicks, 1988; Jokiel, 1990) or even by hitchhiking benthic animals as speculated by Neusser *et al.* (Brenzinger *et al* (2011b) might be more likely.

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Molecular species delineation and DNA taxonomy

Chapter 13. Barcoding against a paradox? Combined molecular species delineations reveal multiple cryptic lineages in elusive meiofaunal sea slugs

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RESEARCH ARTICLE Open Access

Barcoding against a paradox? Combined molecular species delineations reveal multiple cryptic lineages in elusive meiofaunal sea slugs

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Abstract

Background: Many marine meiofaunal species are reported to have wide distributions, which creates a paradox considering their hypothesized low dispersal abilities. Correlated with this paradox is an especially high taxonomic deficit for meiofauna, partly related to a lower taxonomic effort and partly to a high number of putative cryptic species. Molecular-based species delineation and barcoding approaches have been advocated for meiofaunal biodiversity assessments to speed up description processes and uncover cryptic lineages. However, these approaches show sensitivity to sampling coverage (taxonomic and geographic) and the success rate has never been explored on mesopsammic Mollusca.

Results: We collected the meiofaunal sea-slug *Pontohedyle* (Acochlidia, Heterobranchia) from 28 localities worldwide. With a traditional morphological approach, all specimens fall into two morphospecies. However, with a multi-marker genetic approach, we reveal multiple lineages that are reciprocally monophyletic on single and concatenated gene trees in phylogenetic analyses. These lineages are largely concordant with geographical and oceanographic parameters, leading to our primary species hypothesis (PSH). In parallel, we apply four independent methods of molecular based species delineation: General Mixed Yule Coalescent model (GMYC), statistical parsimony, Bayesian Species Delineation (BPP) and Automatic Barcode Gap Discovery (ABGD). The secondary species hypothesis (SSH) is gained by relying only on uncontradicted results of the different approaches ('minimum consensus approach'), resulting in the discovery of a radiation of (at least) 12 mainly cryptic species, 9 of them new to science, some sympatric and some allopatric with respect to ocean boundaries. However, the meiofaunal paradox still persists in some *Pontohedyle* species identified here with wide coastal and trans-archipelago distributions.

Conclusions: Our study confirms extensive, morphologically cryptic diversity among meiofauna and accentuates the taxonomic deficit that characterizes meiofauna research. We observe for *Pontohedyle* slugs a high degree of morphological simplicity and uniformity, which we expect might be a general rule for meiofauna. To tackle cryptic diversity in little explored and hard-to-sample invertebrate taxa, at present, a combined approach seems most promising, such as multi-marker-barcoding (i.e., molecular systematics using mitochondrial and nuclear markers and the criterion of reciprocal monophyly) combined with a minimum consensus approach across independent methods of molecular species delineation to define candidate species.

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Background

Sediment-associated marine meiofaunal organisms inhabit one of the largest ecosystems on earth – sediment-covered ocean floors and beaches – and comprise a major part of marine biodiversity [1]. However, only a small fraction of the predicted species richness currently is known to science [1-4] and recent surveys have shown a high number of new, undescribed species even in well-studied areas (see [4]). Minute body sizes often prohibit direct visual identification in the field; instead, morphological identification generally requires time-consuming, technologically sophisticated anatomical studies. Additionally, taxonomy frequently is complicated by morphological convergence and/or pronounced intraspecific variation (e.g., [3,5]). In Acochlidia, the most diverse group of meiofaunal slugs, the Microhedylacea, shows 'regressive evolution' [6], exhibiting highly simplified organ systems and little morphological diversity even at higher taxonomic levels [7]. Thus, it is challenging to use only morphology to delimit species boundaries in meiofaunal slugs. In consequence of the fragmentary knowledge of meiofaunal taxonomy, this fauna is frequently neglected in conservation and biogeography, and ecological analyses remain superficial despite the undoubted importance of meiofauna; e.g., in the food chain [8].

For taxon-specific analyses, DNA-barcoding has been advocated as a fast and efficient way to reduce the taxonomic deficit and automate taxon determination for ecological research [3,5,8]. DNA-barcoding in its simple, similarity-based form of species identification [9] is not predictive; it fails if no identical sequence has yet been determined and deposited in a voucher database, or if no limit in species boundaries has been established [10,11]. In well-known taxa with good sampling coverage, identification rates via DNA-barcoding can be quite successful (e.g.,[12,13]), but in case of meiofauna finding identical sequences in public databases for a newly collected mollusk or other under-investigated taxon is not expected to become the rule for decades to come. The application of the typical barcoding approach for species delineation - COI in conjunction with a comparison of pairwise distances - in Mollusca has resulted in mixed reports: although the identification success with known taxa was generally high (e.g., [12,14,15]), the determination of a 'barcoding gap' (i.e., significant difference between inter- and intraspecific variation) and thus a delimiting threshold has been problematic, especially above local-scale approaches and in undersampled phylogenies [12]. Doubts have also arisen concerning species identification and delimitation based on single-locus DNA sequences, which frequently result in problematic under- or overestimation of species [16-18]. Mitochondrial markers, especially, came under criticism due to possible inadvertent inclusion of nuclear mitochondrial pseudogenes (= nonfunctional copies of mtDNA in the nucleus, or numts) [19], and other mitochondria-specific pitfalls such as reduced effective population size or inconsistent recombination [20]. The risk of incorrect species delineation due to incomplete lineage sorting or introgression can be reduced by analyzing independent loci [21], which is generally considered superior to single-gene approaches [22]. We chose a barcoding approach based on three molecular markers that have been demonstrated to provide good resolution for species delineation in some Mollusca [23-25]. We included, in addition to mitochondrial Cytochrome *c* Oxidase subunit I (COI) and 16S rRNA, nuclear 28S rRNA (large ribosomal subunit – LSU), which has performed well for species separation and was suggested as a signature gene fragment for a DNA taxonomy system for meiobenthos [8,26].

Any method of species delineation is sensitive to sampling [27], and rarity is almost universal when dealing with invertebrates [28]. Rarity is not only a theoretical problem in species delineation methods, but hinders assessment of genetic variability [16,28]; if populations with intermediate haplotype composition are left unsampled, barcoding and molecular-based species delineation approaches tend to overestimate species [18,27]. With large parts of the worldwide meiofauna still unexplored, and patchy, discrete distributions being characteristic for meiofaunal taxa [29], the present-day knowledge of this fauna is prone to incomplete sampling. The rapidly spreading biodiversity crisis with the destruction of habitats and high extinction rates calls for quick surveys and realistic data for efficient conservation strategies (e.g., [16], and references therein). Currently, most molecular species delineation approaches have been conducted on large datasets with dense sampling coverage or on local scales (e.g., [30-35]), with few exceptions using small datasets in integrative approaches (e.g., [36]). Barcoding approaches using COI trees for defining species clusters and revealing gaps between intra- and intercluster distance; multi-locus tree-based methods with or without using diagnostic characters; and a couple of newly developed, tree-independent methods for species delineation, all serve as methods for DNA taxonomy. However, as a solution to address the challenges of the taxonomic impediment in problematic taxa the power of these methods is still largely untested.

Here, we performed thorough phylogenetic analyses of all three molecular markers and integrate available additional data from morphology and geography. In parallel, we applied four different methods of species delineation: 1) The General Mixed Yule Coalescent model (GMYC) [32,33] is a maximum likelihood approach, able to discriminate between population and speciation patterns on a given ultrametric tree; 2) Statistical parsimony [37]

is designed to present intraspecific relationships in haplotype networks, but can also be reversed and used to detect species boundaries [33]; 3) Bayesian Species Delineation (BPP) is a method which accounts for uncertainties in gene trees and is promoted as especially useful for delineation of cryptic species in sympatry [22,38]; 4) Automatic Barcoding Gap Discovery (ABGD) [39] is an exploratory tool based on pairwise distances to detect automatically significant difference in intra- and interspecific variation (i.e., barcoding gap), without an *a priori* species hypothesis. Results are compared to a simple single-gene COI barcoding approach in conjunction with pairwise distances.

Our study organism, *Pontohedyle*, is a morphologically well-defined genus of meiofaunal slugs (Acochlidia, Heterobranchia) with two valid species: the well described and abundant *P. milaschewitchii* (Kowalevsky, 1901) from the Black Sea and Mediterranean [40] and the poorly known Western Pacific *P. verrucosa* (Challis, 1970) from the Solomon Islands. In absence of distinguishing morphological characters Jörger *et al.* [41] synonymized the tropical Western Atlantic *'P. brasilensis'* with its temperate congener *P. milaschewitchii*. This resulted in the only meiofaunal slug with amphi-Atlantic distribution, and the authors pointed out the need to subsequently test this morphological hypothesis with molecular markers to detect possible cryptic species [41]. Sampling efforts in the course of this study revealed a worldwide distribution of the genus. In applying traditional morphological characters for species delineation (external morphology, radula and spicules) all collected material resolved into two morphospecies represented by the currently recognized species (see further details in discussion on species delineation in *Pontohedyle*). Wide-range distributions, as e.g., in *P. milaschewitchii*, are commonly reported for other meiofauna as well, but are treated with suspicion and known as the 'meiofauna paradox'[42]: Most meiofauna have low reproductive output and lack recognized dispersal stages, such as planktonic larvae [43,44]. Thus, their dispersal abilities and levels of gene flow are assumed to be low [45]. Recent molecular and advanced morphological approaches have revealed putative amphi-Atlantic or even cosmopolitan meiofaunal taxa to be radiations of cryptic species (e.g., [46-52]). Uncovering putative cryptic lineages is fundamental not only for our advances in understanding speciation processes in meiofaunal taxa, but also to understanding historical biogeography.

We present the first species-level phylogenetic analysis in meiofaunal Mollusca to have a world-wide sampling, and aim to 1) establish a workflow of molecular species delineation in rare (or rarely sampled) taxa; 2) test for the presence of putative cryptic species by applying several independent approaches of molecular-based species delineations; 3) test putative wide-range distributions in a meiofaunal slug; and 4) explore the origin and diversification of *Pontohedyle*. Resulting insights into allopatric and sympatric speciation, morphological stasis and distribution are discussed for a better understanding of meiofaunal biogeography and evolution.

Results

Phylogenetic analyses and primary species hypotheses

We used a phylogenetic approach to determine molecular operational taxonomic units (MOTUs), i.e. preliminary molecular units unaffected by existing nomenclature serving as a starting point for further species delineation approaches. Our phylogenetic analyses resulted in a stable topology with only minor changes among different analyses with individual or concatenated markers, revealing a complex picture of diversification in *Pontohedyle*. The topology of the maximum-likelihood analyses of the concatenated three-marker dataset analyzed in three partitions is shown in Figure 1A. This topology was quite stable regardless of the partitioning scheme of the dataset or the phylogenetic approach chosen (likelihood, parsimony or bayesian analysis) (see Figures 1A, 2A and Additional file 1). Differences in the topology referred to poorly supported sister group relationships, frequently involving singletons (e.g. MOTUs VII or X).

The genus *Pontohedyle* was monophyletic with high bootstrap support (BS 91; BS values derived from concatenated three-marker ML analyses, see Figure 1A). It was divided in two sister clades, one included *P. verrucosa* from the type locality (BS 99) and the other *P. milaschewitchii* from the type locality (BS 80, see 1A). Both major clades consisted of six lineages each and represented a geographical mixture across ocean boundaries (see Figure 1A). However, relationships among lineages within the major clades were not supported (i.e. BS <50) in many cases.

Distinguishing features traditionally used for morphological species delineation and ecological traits such as hydrographic conditions, geography and habitat were plotted onto the phylogeny (see Figure 1A). Specimens of *Pontohedyle* are externally uniform and easily distinguishable from other acochlids by the lack of rhinophores and the bow-shaped oral tentacles (Figure 1B). No diagnostic differences in external morphology or spicules could be detected between the collected populations apart from eyes externally visible or not (see Figure 1A). Comparative SEM-examination of the available radulae revealed two types of the typically hook-shaped radula (Figure 1C): a lateral tooth without a denticle (*P. verrucosa*, Figure 1D) or with a denticle (*P. milaschewitchii*, Figure 1E).

We identified our MOTUs according to the criterion of reciprocal monophyly across different phylogenetic approaches and between single gene trees and concatenated datasets (partially missing data however resulted

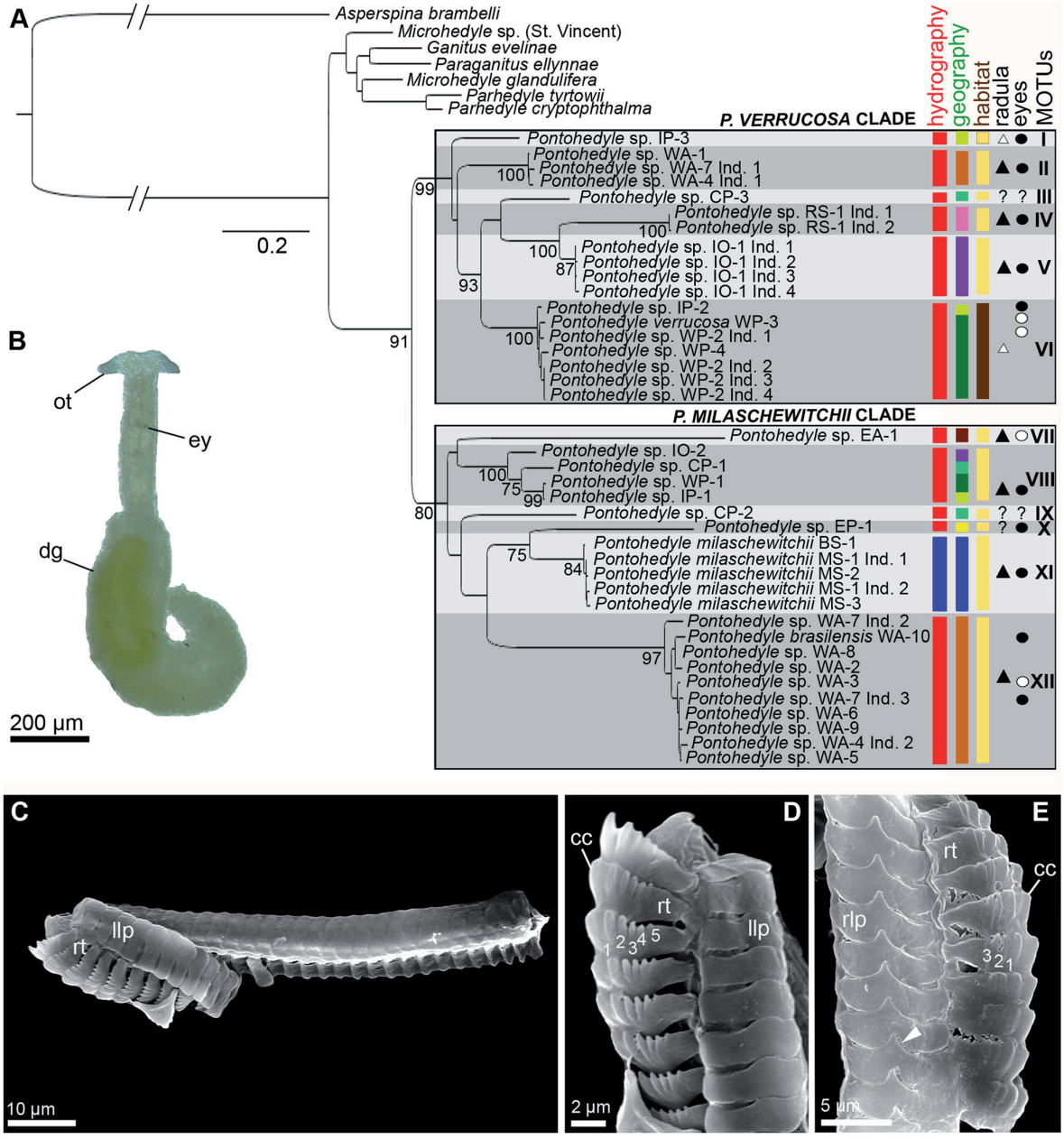

Figure 1 Molecular phylogeny of *Pontohedyle* with morphological and ecological data plotted. A. Maximum-likelihood tree of the genus *Pontohedyle* generated with RAxML for the concatenated dataset (28S rRNA, 16S rRNA, COI) analyzed in three partitions. Bootstrap support (BS) above 75 given below nodes (BS within MOTUs shown only for VIII). ● eyes externally visible (as in Figure 1B); ○ eyes not visible externally; ▲ lateral radula tooth with denticle (see Figure 1E); Δ lateral radula tooth without denticle (see Figure 1C,D); Hydrography: red = tropical, blue = temperate. Geography: East-Pacific = yellow, Central Indo-Pacific = light-green, Central-Pacific = turquoise, West-Pacific = dark green, Mediterranean and Black Sea = blue, Red Sea = pink, Indian Ocean = purple, West Atlantic = dark brown, East Atlantic = light brown, Habitat: intertidal = brown, subtidal = beige. **B**. Living *Pontohedyle milaschewitchii*. **C**.-**E**. Scanning electron microscopy of *Pontohedyle* radulae, arrowhead indicates denticle in lateral plate of radula, numbers mark lateral cusps of rachidian tooth. **C**.- **D**. Radula of *P. verrucosa*. **E**. Radula of *P. milaschewitchii*. cc = central cusp of rachidian tooth, dg = digestive gland, ey = eyes, llp = left lateral plate, ot = oral tentacles, rlp = right lateral plate, rt = rachidian tooth.

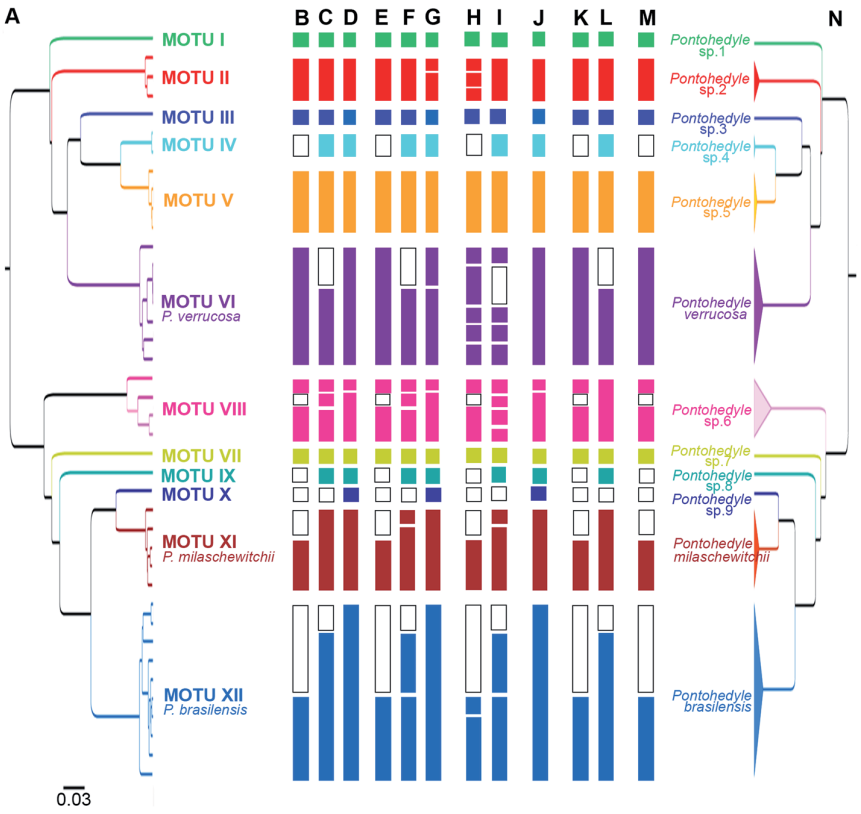

Figure 2 Molecular based species delineation of the genus *Pontohedyle*. **A**. Ultrametric tree generated with BEAST from the concatenated three-marker dataset, with the PSH derived from phylogeny coded by color. **B**-**D**. GMYC single threshold analyses: **B**. COI. **C**. 16S rRNA. **D**. concatenated three-marker dataset. **E**-**G**. GMYC multiple threshold analyses: **E**. COI. **F**. 16S rRNA. **G**. concatenated three-marker dataset. **H**-**I**. Statistical parsimony haplotype networks analyzed with TCS under the 95% parsimony criterion. **H**. COI. **I**. 16S rRNA. **J**. Summary of the Bayesian Species delineation approach, recognizing entities with posterior probability values ≥ 0.95. **K**-**L**. ABGD analyses. **K**. COI. **L**. 16S rRNA. **M**. Fixed delineating pairwise-distance threshold of 11%. **N**. Candidate species (secondary species hypothesis – SSH) under a minimum consensus approach across methods. (Empty squares represent missing data.).

in a lack of some MOTUs in single gene trees, see Additional file 1B-D). A combination of the plotted morphological and ecological data led to diagnosable entities. We detected seven terminal clades, which are reciprocally monophyletic with moderate (BS > 75) to strong support (BS > 95) (see Figure 1A and Additional file 1), and also showed short intra- vs. longer interspecific branch lengths. Additionally, 5 singletons were identified as MOTUs based on similar relative branch lengths when compared to the reciprocally monophyletic entities. Parsimony analysis conducted with PAUP v. 4.10 showed lower resolution among clades, which results in MOTU X and XI collapsing to form a single clade and MOTU VII being recovered outside *Pontohedyle* both are considered an artifact, due to long branch attraction and/or respectively missing data (see Additional file 1A). Monophyly and relative branch lengths of the identified MOTUs were unaffected by masking ambiguous parts of the 16S and 28S rRNA alignment. Our phylogenetic analyses in combination with the plotted morphological and ecological data led to a primary species hypothesis, which was subjected to the following species delineation approaches.

Molecular-based species delineation and secondary species hypotheses

Maximum-likelihood (GMYC)

Discriminating between population and speciation patterns, under single-threshold analyses, GMYC identified all MOTUs as separate species, regardless whether based on COI (Figure 2B), 16S rRNA (Figure 2C) or the concatenated three-marker dataset including nuclear 28S rRNA (Figure 2D). Additionally, MOTU VIII was divided into two species (incomplete COI dataset and concatenated dataset), or three species (16S rRNA). In multiple-threshold analyses (Figure 2E-G), GMYC based on 16S rRNA further divided *P. milaschewitchii* (MOTU XI) and *P. brasilensis* (MOTU XII) into two species each (Figure 2F). In the multiple-threshold GMYC-analyses of

the concatenated dataset these MOTUs formed one entity, but *P. verrucosa* and MOTU II were divided in two species each (Figure 2G).

Statistical parsimony

Haplotype networks were generated via statistical parsimony implemented in TCS 1.21. With the 95% parsimony criterion (default setting) applied to the single-marker alignments of the mitochondrial datasets, TCS generated 17 networks on COI and 19 unconnected haplotype networks based on 16S rRNA (Figure 2H, I). Statistical parsimony was in agreement with our PSH described above and recovered all the identified MOTUs as unconnected networks. Additionally, 16S rRNA analysis split populations identified above as *P. milaschewitchii* and *P. verrucosa* into unconnected haplotypes (Figure 2I). In COI analyses *P. milaschewitchii* formed one entity but populations of *P. verrucosa* showed unconnected networks (Figure 2H). COI results also showed MOTU II and XII (*P. brasilensis*) each including multiple unconnected networks and the ambiguous MOTU VIII (recovered as two or three putative species in GMYC) resulted in two (COI), or four (16S) unconnected haplotypes under statistical parsimony. The nuclear 28S rRNA haplotype network resulted in connected haplotype networks for representatives of two different (morphologically well-supported) outgroup genera (*Microhedyle* and *Paraganitus*). We thus considered this approach problematic for species delineation in *Pontohedyle* and excluded it from our consensus approach.

Bayesian species delineation (BPP)

We ran two sets of Bayesian species delineation analyses: 1) testing the support of the MOTUs retrieved in our primary species hypothesis (PSH) and 2) checking for putative additional species by calculating the speciation probabilities for each population (separating putative sympatric cryptic species uncovered in the phylogenetic approach into separate populations). To evaluate the effect of user-incorporated prior values we tested four different prior combinations allowing for large vs. small ancestral population sizes and deep vs. shallow divergence times (see Methods for details). When using the twelve MOTUs from our PSH as a guide tree, most nodes were supported by posterior probabilities (PP) of 1.0 (i.e., 100% of the applied speciation models supported the two lineages of the specific node as species) (Figure 3A). We consider a speciation probability value of ≥ 0.95 as strong support for a speciation event, which is recovered for all identified MOTUs except for the speciation event between MOTU X and XI (PP 0.90-0.96, Figure 3A). The latter event however received consistent support ≥ 0.95 in the second set of analyses in which each population was treated separately (Figure 3B). BPP also indicated high support for a split within MOTU XI (*P. milaschewitchii*) between populations from the Mediterranean and Black Sea; however these results were ambiguous among analyses. In general, assumed small ancestral population size and long divergence times resulted in the highest speciation support values (Figure 3 in green), while large ancestral population sizes and long

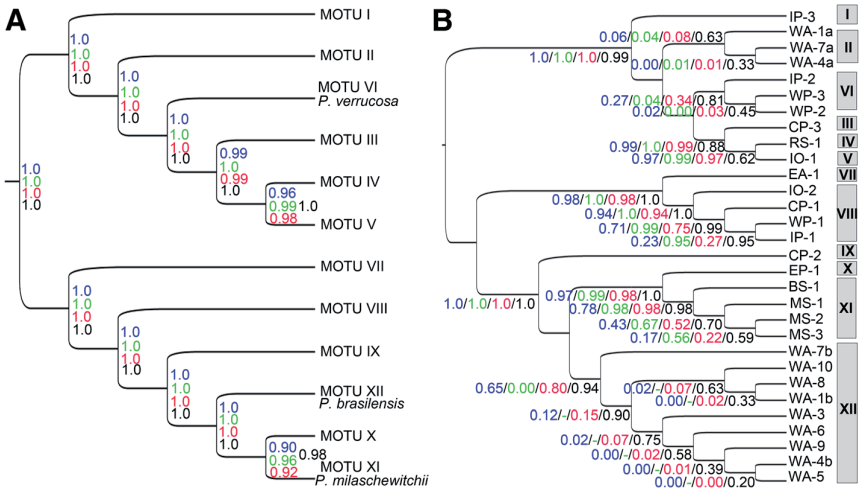

Figure 3 Bayesian species delimitation for *Pontohedyle*. **A**. Results assuming our primary species hypothesis guide tree (12 MOTUs). **B**. Results assuming a guide tree, in which each population is treated as separate species (30 populations – MOTUs are indicated at the left side of the graph). Speciation probability limits are provided for each node under different prior estimates on ancestral population size (θ) and divergence times (τ): 1) prior means 0.1 (blue), 2) prior mean θ = 0.001, τ = 0. 1 (green), 3) prior mean θ = 0.1, τ = 0.001 (red), 4) prior means 0.001 (black). Posterior probabilities are calculated as mean values from repeated analyses. We applied different algorithms and starting seeds, as recommended in the BP&P manual [38].

divergence times resulted in the lowest PPs (Figure 3 in blue). Shallow divergence times also provided better support values (Figure 3 in red) but are considered unlikely based on molecular clock data of *Pontohedyle* [53]. In summary, BPP resulted in 13 MOTUs with PP ≥ 0.95 (see Figure 2J).

Pairwise distances and Automatic Barcoding Gap Discovery (ABGD)

Applying the ABGD with the standard settings resulted in one partition (i.e., no barcoding gap) in both our COI and 16S dataset. When lowering the X-value (relative width of barcoding gap) to 1.2, ABGD clustered the sequences into 10 MOTUs for COI (incomplete dataset, see Figure 2K) with a prior of intraspecific divergence up to 0.0359, and 11 MOTUs for 16S rRNA (Figure 2L) with a prior of up to 0.0599, which was congruent with the PSH. The 16S rRNA results, however, contradicted all other approaches applied here in not splitting the ambiguous MOTU VIII into two separate entities (which was strongly supported in BPP analyses). ABGD results were independent from the chosen model (Jukes-Cantor and Kimura) and unaffected by changes of prior limit of intraspecific variation.

Species Identifier was then used to calculate the intra- and interspecific variability within the MOTUs. Choosing our PSH as *a priori* species input for pairwise distance calculation, clusters were in agreement at a threshold from 14.7% - 18.5% for COI and 11.2% - 18.9% for 16S rRNA. Repeating the analyses and subdividing the ambiguous MOTU VIII into two putative candidate species clusters were in full agreement starting at a threshold of 8.8% for COI and 6.3% for 16S rRNA. Within the MOTUs, the largest intraspecific uncorrected p-distances occurred within the ambiguous MOTU VIII with 14.65% for COI (n = 3) and 14.47% for 16S rRNA (n = 4), followed by '*P. brasilensis*' (MOTU XII) with 8.7% for COI (n = 4), and *P. verrucosa* (MOTU VI) with 7.1% for 16S rRNA (n = 4). Among the other clades, the largest uncorrected intraspecific p-distances were lower, ranging from 1.83 - 5.03% for COI and 0.22 - 4.45% for 16S rRNA. Between MOTUs, the smallest interspecific p-distances were larger than the intraspecific variation; i.e., 18.32% for COI (between Western Atlantic MOTU II and Indo-Western Pacific *P. verrucosa* (MOTU VI)) and 14.69% for 16S rRNA (between Red Sea MOTU IV and Indian Ocean MOTU V), whereas the smallest mean interspecific p-distances within our dataset were 24.68% for COI and 28.58% for 16S rRNA. With a fixed threshold of 11% – recorded as mean sequence divergence for COI in congeneric species pairs in Mollusca [54] – applied to our (incomplete) COI dataset, Species Identifier recovered 10 clusters in comparison to the other species delineation approaches (Figure 2M).

Secondary species hypothesis (SSH)

Our SSH is based on a minimum consensus approach (see Figure 2N, Material and Methods and detailed discussion below) across species delineation approaches. It was identical to our PSH and suggested at least 12 mainly cryptic candidate species, three of which correspond to existing names in nomenclature. *Pontohedyle* sp. 6 (corresponding to MOTU VIII), however, remains problematic, since nearly all molecular species delineation approaches split this unit into a minimum of two independent lineages (with high support, see e.g., Figure 3B); only the ABGD analysis based on 16S rRNA did not support this split.

Discussion

Molecular species delineation in elusive taxa

Our study demonstrates that traditional taxonomic treatment is not efficient for uncovering the true diversity in meiofaunal *Pontohedyle* slugs. It is essential to have an operational molecular-based concept for species delineation (DNA taxonomy) and species re-identification that informs future ecological, biogeographical or conservation approaches. The methods should be cost-efficient and fast, but in the first place they need to be reliable, and able to deal with rare (or rarely sampled) meiofaunal species elusive to population genetics.

Puillandre et al. [55] proposed a workflow for large-scale species delineation in hyperdiverse groups, starting with a COI barcoding dataset analyzed with ABGD and GMYC which leads to the primary species hypothesis (PSH). Independent information (from other molecular markers, morphology and ecological traits) is subsequently added to lead to the secondary species hypothesis (SSH) [55]. This formalized strategy [55] is linear, starting with pre-selecting samples according to a PSH that depends on a single mitochondrial marker, before further information is added that might expand or contradict the PSH. What is an efficient workflow for large-datasets with dense sampling coverage and thus high-quality COI barcoding output, may be inapplicable for datasets in little known and putatively undersampled taxa. The latter would benefit from full consideration of all information already available for a PSH, and a parallel, combined approach of multiple markers and multiple delimitation methods. Especially when it is unfeasible to sample multiple specimens, multiple loci lead to more reliable results [22]. Multi-marker barcoding provides an *a posteriori* double-check for contamination, sequencing errors or mitochondria-specific pitfalls (e.g., the presence of numts or mitochondrial introgression), and the idiosyncrasies of individual markers [16,56]. Our study shows that COI analyses perform well on our dataset but due to amplification problems applying universal COI barcoding primers, three candidate species would have remained unconsidered. Multi-marker barcoding makes better use of rare specimens.

Our global sampling is sparse rather than comprehensive, including a few singletons from distant populations. Nevertheless, we are able to propose a primary species hypothesis on the evidence of a molecular phylogeny and concordance in reciprocally monophyletic entities (Figure 1A). We use concordance to mean two things: 1) in agreement among different phylogenetic analyses (ML, parsimony, Bayes), to account for the risk in false species delimitation due to errors in phylogenetic reconstruction [22,38]; and 2) in agreement among single-gene trees (two mitochondrial markers, one nuclear) and the concatenated dataset, to avoid false signals due to recent population genetic processes [16,22].

In this phylogenetic approach as starting point for further analyses, we consider relative branch lengths as proxies for evolutionary distance. Reciprocal monophyly as a criterion for species delineation is being criticized as too stringent [22], since monophyly of species in gene trees is not assured if lineages are not fully sorted [16,57,58]. Although the absence of reciprocal monophyly might not be sufficient for lumping species, its presence with several independent markers indicates compatibility of gene and species trees and can thus be used for a PSH, especially when combined with approaches capable of detecting recent speciation processes (like BPP [22,38]). As an example for molecular species delineation in poorly known taxa, our study benefits from divergence times, which in *Pontohedyle* is estimated to have started in a late Mesozoic timeframe ([53], KMJ own unpublished data). Thus, recent radiations, which present a major pitfall for molecular delineation approaches [12,16,34,57], are unlikely to hamper overall delineation success in our study; exceptions and problematic cases are discussed below. Plotting available data from morphology, biogeography and hydrographic features at least partially allows the diagnosis of the MOTUs beyond their molecular characteristics.

To date, the analyses of pairwise distances, with application of generalized universal thresholds (e.g., [9,54]) or relative thresholds (e.g., 10× rules, requiring the interspecific variability to be at least ten times the variability of intraspecific variation [59]) is the most commonly used form of molecular species delineation. This approach has however been widely criticized as arbitrary due to high variation and frequent overlap of intra- and interspecific variation [12,14,16,17,34,60]. This criticism also affects the concept of a 'barcoding gap', i.e., a significant gap in the distribution of intra- vs. interspecific variation [12]. Intrapopulation variation might, in fact, exceed divergence between species [61], which has been demonstrated in well-sampled groups with reliable independent datasets for species delineation such as karyology [13]. It is evident that intra- and interspecific variation are biased by sampling coverage [17] and there is a high risk of misidentification, especially in undersampled phylogenies [12]. Applying a fixed threshold of 11%, which has been determined as the mean sequence differentiation between species pairs in Mollusca [54], yields the same clusters as our complex delineation approach. However, we consider the good performance of a fixed threshold as random and due to the fact that this applies a mean distance. Using the smallest interspecific distances (as recommended by Meier et al.[62]) might-logically smaller than 11% -lead to an overestimation of species richness in our dataset. Moreover, these pairwise distance approaches do not serve as an independent tool for species delineation, but depend highly on pre-defined species limits. Using our PSH as the *a priori* species hypothesis we detect a barcoding gap, which, however, shifts considerably when the e.g., MOTU VIII is additionally split into two entities, demonstrating the circularity of the approach. The ABGD method [39] still suffers from limitations based on the genetic distance concept and barcoding gap discussed above, but presents a major advantage since it is applicable as an independent tool without an *a priori* species hypothesis. ABGD analyses may be problematic on small datasets with less than three sequences per species [39]. When the standard settings of ABGD were applied to our dataset, it failed to partition our dataset based on pairwise distances. Lowering the user-defined relative-gaps width (X) enabled ABGD to recover clusters that are congruent with the other delineation approaches for both mitochondrial markers. Although we present the first study on meiofaunal slugs with representative worldwide taxon sampling, we know our dataset is likely to represent only a fragment of the hidden diversity in the genus because a) tropical sands still are largely unsampled, b) suitable habitats are patchy and disjunct, and c) the indication of accumulated diversity in geographically small areas (e.g., three distinct genetic *Pontohedyle* lineages on the island of Moorea). Thus, the discovered 'barcoding gap' may be an artifact of limited sampling.

A series of independent tools of molecular species delineation have been developed recently [21,22,32,33,38], but only few studies have tested these comparatively. In a thorough comparison, Sauer & Hausdorf [16] report that Gaussian Clustering [21] yielded the best performance in relation to morphological species delineation, but several sequences per population are needed to recognize reliably a separate cluster (i.e., candidate species) [16]. In contrast, the GMYC-model has shown, in a series of studies, ability to discriminate effectively between coalescent and speciation patterns. These matched species identified via independent criteria (e.g., independent molecular markers, morphology, geography) [32,33,35,55,63], even in groups with little sampling coverage [36]. In our study GMYC congruently recognizes the same MOTUs as separate entities as our PSH and thus does not tend to oversplit data, as suspected previously [18]. Sauer & Hausdorf [16] demonstrated

considerable differences in GMYC results depending on the method of reconstruction of the ultrametric input tree. While this stresses the need to test the effect of different input trees, we consider the risk in our dataset as minor because all our phylogenetic approaches recover similar topologies. We conclude that GMYC in general is a delineation tool capable of dealing with data from poorly-sampled groups.

Even though the performance of BPP dramatically improves when sampling at least five sequences per population, it achieves correct assignment of models also in small datasets (simulated, empirical and including singletons) when a high number of loci is used [38]. It is especially useful to detect cryptic species in sympatry [22,38,64]. Our BPP analyses supported all MOTUs identified in our PSH and, additionally, split the ambiguous MOTU VIII into two entities, in agreement with GMYC analyses. Given the possibility of testing the speciation support for each of the sampled populations by incorporating prior information on population size and divergence times, BPP is especially useful to avoid lumping of species. However, in its present form it is limited to dealing with small datasets (max. 20 species).

In the present study statistical parsimony congruently recovered the same MOTUs of the PSH, but considerably oversplit the data in comparison to the other methods (see Figure 2H-I). Whereas GMYC also resulted in additional splits and some populations in BPP resulted in ambiguous PPs (see Figure 2B-G, Figure 3B) that are potentially related to recent speciation processes, the comparative oversplitting in TCS might rather be an artifact of high substitution rates on mitochondrial markers [16], as reported from several other molluscan taxa (e.g., [14]).

Although all MOTUs based on singletons (but with the complete dataset of all three markers available) are clearly separated into independent lineages in all different approaches (see Figure 2), inconsistencies arose within the dataset for MOTUs containing 5–10 specimens (see e.g., MOTU VI, XI, XII in Figure 2), which clustered into one or more entities in different analyses. However, speciation is a continuous process [65,66] and delineation approaches offer only a snapshot of this process, so we expect to encounter various stages of differentiation. Recent radiations leading to incomplete lineage sorting might explain ambiguous results on different mitochondrial markers (see e.g., Figure 2H-I MOTU VI and XII) and more data and population genetic approaches are needed to reveal the genetic structure within these entities.

Most of the molecular species delineation methods currently available are not designed to incorporate the common phenomenon of rarity (i.e., species only represented by singletons or few sequences) [28]. Sampling efforts by us and colleagues confirm that *Pontohedyle* and many other meiofauna taxa truly are rare and can be expected to have small effective population sizes. Thus, we consider a integrative approach as most suitable for molecular species delineation in little known, putatively widespread and notoriously under-sampled taxa such as meiofauna in remote areas. Available methods of species delineation should be applied in parallel on different suitable barcoding markers (mitochondrial and nuclear markers) and combined with phylogenetic analyses that allow mapping of additional information from morphological and ecological traits. We chose a minimum consensus approach across all methods, conservatively relying only on fully corroborated entities. Sauer & Hausdorf [16,38] noted an oversplitting in all different species delineation approaches when these are compared to morphological analyses that include characters directly involved in speciation patters (i.e., morphology of genitalia). We aim to minimize the risk of oversplitting (i.e., inclusion of false positives), and rather put up with the risk of false negatives (i.e., lumping multiple species into one) and not detecting, yet, the entire diversity present in our dataset. Moritz [67] argued that false positives, because they are highly divergent genetically, might still present important components of biodiversity. We agree but their inclusion causes an incalculable taxonomic inflation and might lead to misinterpretations of meiofaunal biogeography and evolution. In our study, the minimum consensus approach is feasible, since results are not contradictory in recovering different entities (Figure 2N), probably due to long periods of reproductive isolation. Our scheme, however, is not applicable to studies with ambiguous results, which would call for further lines of evidence and thorough evaluation of the contradictions before delineation of candidate species could be achieved.

Species delineation in *Pontohedyle*

Our results revealed a secondary species hypothesis of twelve distinct species, diagnosable by multiple methods. Morphological characters traditionally used for species delineation in Acochlidia, split the worldwide sampled *Pontohedyle* populations into only two morphospecies: *P. milaschewitchii* (lateral radula tooth with denticle, Figure 1E) and *P. verrucosa* (lateral radula tooth smooth, Figure 1D). Previously used external morphological characters such as the shape of oral tentacles, body length and width, or color of the digestive gland (e.g., [68]) depend highly on the stage of contraction and nutrition, and are variable through time for each individual [40,41] and therefore inappropriate for species delineation. *Pontohedyle* typically bear monaxone, rodlet-like spicules distributed randomly and frequently accumulated between the oral tentacles [40,69,70], which is

confirmed here for members of both major clades of *Pontohedyle*. Although the presence of certain types of spicules is diagnostic for acochlidian genera or species, their absence (e.g., as in MOTU VII) is not, because it can be caused by environmental influences [71]. Using the presence of externally clearly visible eyes as a delineating character would lead to the identification of two more morphospecies (Figure 1A): one with smooth lateral radula tooth and externally clearly visible eyes (corresponding to MOTU I, distinguished from *P. verrucosa,* which lacks visible eyes) and one without externally visible eyes and with a denticle on the lateral radula tooth (in our phylogeny clustering among *P. brasilensis* with visible eyes). However, the presence of externally visible eyes depends on the degree of pigmentation, and was shown to be highly variable intraspecifically in other acochlids [72]. This is confirmed by our phylogenetic and molecular analyses, which clustered both 'eyeless' and eye-bearing individuals in '*P. brasilensis*' and *P. verrucosa* (see Figure 1A).

In the light of our phylogenetic hypothesis, a convergent modification of the lateral radula tooth has taken place within the *P. verrucosa* clade in the two intertidal MOTUs I and VI. The power of morphological species delineation is the potential to include characters directly involved in the speciation process, e.g., from the reproductive system [16]. Based on previous histological comparisons, Jörger *et al.* [41] failed to find any morphological characters justifying discrimination between the closely related western Atlantic '*P. brasilensis*' (MOTU XII) and its Mediterranean congener *P. milaschewitchii* (MOTU XI). Details on the reproductive system of *P. verrucosa* are missing in the original description [73], but own histological comparisons using 3D reconstruction based on serial semi-thin sections from material collected at the type locality revealed no major differences (KMJ, own unpublished data). Thus, even sophisticated microanatomical comparisons seem unpromising for species delineation in these highly simplified and uniform slugs.

In general, morphology in meiofaunal organisms is characterized by extensive parallelism and convergent adaptations to the mesopsammic environment [44,74], which frequently results in low interspecific morphological variability [7]. This is true of the microhedylacean Acochlidia, which are exclusively found in interstitial spaces in sediment, and show a tendency toward reduction of complexity in major organ systems [7]. In contrast, hedylopsacean Acochlidia, whose evolution involves several habitat shifts from marine interstitial to amphibious or freshwater benthic habitats, subsequently possess complex excretory and reproductive systems (e.g., [75-78]). Generally, there is little morphological variation in all major organ systems even at family- and genus-level see [7], but the morphological uniformity in global *Pontohedyle* is most striking. With its vermiform body, a putatively multi-functional radula, 'simplified' organ systems and a special fast and imprecise mode of sperm transfer [79], *Pontohedyle* reflects a meiofaunal slug lineage highly adapted to its interstitial habitat (see discussion below).

Integrating available data on morphology and ecology to the most conservative of our molecular results, the minimum consensus approach (see Figure 2N), suggests that *Pontohedyle* represents a newly-discovered radiation of cryptic species. This radiation consists of at least nine candidate species plus the confirmed valid species *P. milaschewitchii* (Kowalevsky, 1901) and *P. verrucosa* (Challis, 1970), plus *P. brasilensis* (Rankin, 1979), which is here reestablished as a valid taxon. In accord with recent findings for other microscopic taxa (e.g., [29,35,47,80]), our data indicates that expanded meiofaunal sampling in the future will likely uncover even more cryptic lineages. We agree with earlier authors [52] in the practical benefit and importance (e.g., for biodiversity assessments, and conservational and ecological concerns) of describing cryptic species to give them formal taxonomic validity, rather than retaining them as numbered candidate species. A formal description based on diagnostic molecular characters (DNA taxonomy in a strict sense) of all herein discovered candidate species is, however, beyond the scope of the present paper and will be documented in a subsequent taxonomic publication.

Does the unveiling of cryptic species solve the meiofauna paradox?

For centuries taxonomy has depended on morphological distinctiveness. In the absence of distinguishing morphological characters many taxa (particularly meiofauna) were described as amphi-Atlantic or even cosmopolitan see (e.g., [51,52]). Due to the predicted low dispersal abilities and limited reproductive output, long-range distribution in meiofauna is known as the 'meiofauna paradox' [42]. In fact, recent re-examination has uncovered a series of radiations of cryptic species across different meiofaunal taxa (see e.g., [35,46-51,72]). Our molecular analyses show considerable geographic structure within global *Pontohedyle* and demonstrate that the putatively amphi-Atlantic meiofaunal slug, *P. milaschewitchii* (from the Mediterranean and including its Western Atlantic synonym *P. brasilensis*) represent cryptic sibling species, including the Eastern Pacific *Pontohedyle* sp. 9 (see Figure 1A, as MOTU X). Meanwhile, our data also confirms surprisingly wide ranges in distribution: in *P. brasilensis* (MOTU XII), with a range from southern Brazil to Belize (over 6500 km linear distance), or in *P. verrucosa* (MOTU VI) from the Pacific Solomon Islands to Indo-Pacific Indonesia (approx. 5000 km linear distance). The same scenario of long-distance dispersal on the one hand and clear spatial structuring on

the other have also been recorded in other meiofaunal taxa; e.g., Nematoda [81], Nemertea [29,82] and Rotifera [45]. The widespread MOTUs in the present study span predicted barriers of gene flow for minute meiofaunal taxa, such as the Amazon freshwater and sediment plume or deep-sea regions between islands. With a typically low reproductive output in Acochlidia (max. of 40 eggs in *P. milaschewitchii*, KMJ pers. obs.), free veliger larvae are assumed to stay in the interstices of the sand grains rather than entering the water column [74] thereby avoiding long distance dispersal. Fertilized eggs are attached to sand grains (KMJ, pers. obs.) and might promote dispersal via current driven sediment transport along shorelines [42]. Data from other meiofaunal taxa suggest that the adult rather than larva serve as the main dispersal stage [83-85]. Dispersal by actively entering the water column as observed, e.g., in copepods [85] is considered improbable in soft-bodied acochlidian slugs [71], but accidental suspension (e.g., caused by waves, currents or tropical storms) and transport in the water column could allow a step-wise distribution along continuous coastlines and thus explain large range distribution [83] as observed in *P. brasilensis*. Neither our phylogenetic analyses (Figure 1A) nor Bayesian Species Delineation (Figure 3B) offered evidence that geographical barriers (as e.g., the Amazonas basin) constitute a distribution barrier for these meiofaunal slugs, as the Brazilian *P. brasilensis* clustered among individuals from the Caribbean (BPP only split Black Sea and Mediterranean populations of *P. milaschewitchii* into two entities, however with ambiguous support between analyses). Comparably high genetic distances from mitochondrial markers between the geographically separated populations especially in *P. verrucosa* and *P. brasilensis* and unconnected haplotype networks (Figure 2H-I) might indicate recent reproductive isolation and genetic diversification. More sensitive molecular markers (e.g., AFLPs) and more material are needed for thorough population genetic approaches to reveal the genetic structure in widespread meiofaunal slugs.

In the absence of a fossil record for meiofaunal slugs the only available estimate for divergence times derives from a molecular clock approach calibrated with shelled heterobranch fossils. Jörger *et al.* [53] estimated the origin of the genus *Pontohedyle* to the late Cretaceous, 84 mya (95% confidence interval ranging from 160–60 mya), providing a rough estimation of how much time was available for diversification and circum-global dispersal of *Pontohedyle* slugs.

Origin and diversification of *Pontohedyle*

The genus *Pontohedyle* shows a circum-tropical distribution with a single derived species (Mediterranean/ Black Sea *P. milaschewitchii*) inhabiting temperate waters (see Figure 1A), confirming general trends of highest species diversity in tropical sediments [1]. Although the distribution of co-existing Mediterranean acochlids like *Microhedyle glandulifera* or *Hedylopsis spiculifera* extends northwards on the European Atlantic Coast, recorded up to 59° latitude ([71], own unpublished data), *Pontohedyle* has never been found in colder waters despite a well-studied meiofauna and hydrographic conditions similar to the Mediterranean. The distribution of *Pontohedyle* might be constrained by ancestry from warm-water adapted animals.

Considering the estimated mid to late Mesozoic origin [53] and the recent primarily tropical distribution pattern in *Pontohedyle,* it is most likely that this meiofaunal slug clade originated in Tethyan waters. The tropical radiation in both *Pontohedyle* clades (see Figure 1A) reveals a mixture of Western Atlantic and Indo-Western Pacific entities with single Eastern Atlantic or Eastern Pacific lineages. Such a complex distributional pattern points to a complex historic biogeographic scenario: large-scale geological events, such as the separation of the Atlantic Ocean and the Indo-West Pacific province, sealed in the closure of the Tethys seaway in the early Miocene [86] followed by a series of vicariant events (of tectonic and climatic origin) during the Cenozoic that affected the global tropical ocean [87]. All of these likely contributed to allopatric speciation in *Pontohedyle*. Due to the predicted low dispersal abilities in meiofaunal taxa, relatively small-scale geological disruptive events (via landslides or formation of rivers) might form a (temporary) barrier for gene flow, proving time for ecological diversification and reproductive boundaries to evolve. Two species (*Pontohedyle* sp. 2 and *P. brasilensis*) were collected at the same localities (WA-1, WA-4, WA-7, see Table 1). Sympatric speciation might be common in the marine environment [88] and especially in the mesopsammic habitat, which is highly structured by gradients in chemistry, type and quantity of food resources or predation, thereby forming numerous ecological micro-niches within small areas (see e.g., [89]). Differences in the histology of the digestive gland content (KMJ, pers. obs.), potentially correlated with the lack of denticle on the lateral radula tooth, indicate putative different food sources and ecological micro-niches in *Pontohedyle* (e.g. in *P. verrucosa* and *Pontohedyle* sp. 1).

It remains stunning that circum-tropical dispersal and speciation processes in *Pontohedyle* over a long evolutionary timeframe (i.e., Mesozoic [53]) are not reflected in morphological differentiation. This extreme case of morphological stasis and similar reports from other meiobenthic groups (e.g. [90]) might be explained in the light of the main physical constraints of the interstitial environment: This habitat is unstable at very short timescales (e.g., due to wave action, currents or storms) and

Table 1 Details on sampling localities and habitat description for *Pontohedyle* analysed in the present study

Field code	Locality	Water body	GPS	Depth	Habitat remarks
BS-1*	Sebastopol, Ukraine, Europe	Black Sea	-	8 m	subtidal, coarse sand
MS-1	Cape Kamenjak, Istria, Croatia, Europe	Mediterranean Sea	N 44°46'04" E 13°54'58"	6-9 m	subtidal, between rocks, exposed, coarse sand
MS-2	Rovinj, Istria, Croatia, Europe	Mediterranean Sea	N 45°07'05" E 13°36'58"	3-4 m	subtidal, sand patches between rocks and sea grass, coarse sand
MS-3	Calvi, Corse, France, Europe	Mediterranean Sea	N 42°33'57" E 08°44'15"	22 m	subtidal, sand patches between seagras, coarse sand/ shell grid
EA-1	MiaMia, Ghana, Africa	Gulf of Guinea, East Atlantic Ocean	N 04°47'46" W 02° 10'06"	2-3 m	subtidal, fine sand
WA-1	near Castries, St. Lucia, Central America	Caribbean Sea, West Atlantic Ocean	N 14°3'34.56" W 60°58' 18.24"	2-3 m	subtidal, sand patches between seagras, coarse sand
WA-2	Soufriere Bay, St. Lucia, Central America	Caribbean Sea, West Atlantic Ocean	N 13°51'24" W 61° 03'58"	8-9 m	subtidal, sand patches between coral blocks, coarse sand
WA-3	Carrie Bow Cay, Belize, Central America	Caribbean Sea, West Atlantic Ocean	N 16°48'13.44" W 88° 4'36.9"	31 m	subtidal, exposed sand plain, relatively fine sand
WA-4	Carrie Bow Cay, Belize, Central America	Caribbean Sea, West Atlantic Ocean	N 16°48'13.44" W 88° 4'36.9"	15 m	subtidal, sand patches between corals, coarse sand
WA-5	Carrie Bow Cay, Belize, Central America	Caribbean Sea, West Atlantic Ocean	N 16°48' 8.94" W 88°4' 47.1"	3-5 m	subtidal, exposed, coarse sand
WA-6	Curlew Reef, Belize, Central America	Caribbean Sea, West Atlantic Ocean	N 16°47'24.96" W 88° 4'43.38"	2 m	subtidal, sand patches between corals exposed to waves, coarse sand
WA-7	Carrie Bow Cay, Belize, Central America	Caribbean Sea, West Atlantic Ocean	N 16°48'7.62" W 88° 4'36.42"	14-15 m	subtidal, sand patches on ridge, coarse sand
WA-8	Carrie Bow Cay, Belize, Central America	Caribbean Sea, West Atlantic Ocean	N 16°48'7.62" W 88° 4'36.42"	31 m	subtidal, protected trough inside ridge, coarse sand
WA-9	Bocas del Toro, Panama, Central America	Caribbean Sea, West Atlantic Ocean	N 9° 21' 2.34" W 82° 10' 20.7"	5-8 m	subtidal, protected, coarse sand
WA-10	off Recife, Brazil, South America	South West Atlantic Ocean	S 8° 3' 17.34" W34° 47' 40.38"	20 m	subtidal, relatively fine coral sand
RS-1	Sha'abMalahi, Egypt, Africa	Red Sea	++) N 24°11'50" E 35° 38'26"	20 m	subtidal, relatively fine coral sand
IO-1	KoRacchaYai, Phuket, Thailand, Asia	Andaman Sea, Indian Ocean	N 7°36'15" E 98°22'37"	6-7 m	subtidal, relatively fine coral sand
IO-2	KoRacchaYai, Phuket, Thailand, Asia	Andaman Sea, Indian Ocean	N 7°36'15" E 98°22'37"	20-22 m	subtidal, coarse sand, exposed
IP-1	Pulau Moyo, Nusa Tengarra, Indonesia	Flores Sea, Indian/ PacificOcean	S 8°13'59" E 117°28'32"	5-6 m	subtidal, coarse coral sand
IP-2	Pulau Banta, Nusa Tengarra, Indonesia	Flores Sea, Indian/ PacificOcean	S 8°23'58" E 119°18'56"	5-6 m	subtidal, coarse coral sand
IP-3	Pulau Banta, Nusa Tengarra, Indonesia	Flores Sea, Indian/ PacificOcean	S 8°23'58" E 119°19'01"	0-1 m	intertidal, coarse coral sand
WP-1	Lembeh Strait, Sulawesi, Indonesia	Banda Sea, West Pacific Ocean	N 1°27'53" E 125°13'48"	3-5 m	subtidal, between coral blocks, exposed, coarse sand
WP-2	Misool, Raja Ampat, Indonesia, Asia	Ceram Sea, West Pacific Ocean	S 2°14'53.46" E 130° 33'18.42"	0-1 m	intertidal, protected beach, coarse, coral sand
WP-3*	Komimbo Bay, Guadalcanal, Solomon Islands, Oceania	West Pacific Ocean	S 9°15'50.58" E 159° 40'5.82"	0-1 m	intertidal, protected beach, coarse, coral sand
WP-4	Honiara, Guadalcanal, Solomon Islands, Oceania	West Pacific Ocean	S 9°25'43.29'' E 159° 56'57.24''	0-1 m	intertidal, protected beach, coarse, coral sand
CP-1	E of Cook's Bay Pass, Moorea, Oceania	Central Pacific Ocean	S 17°28'33.96" W 149° 49'51.6"	10-11m	subtidal, coarse sand, shell grit and rubble
CP-2	E of Cook's Bay Pass, Moorea, Oceania	Central Pacific Ocean	S 17°28'17" W149° 48'42"	18-20 m	subtidal, coarse sand, shell grit and rubble
CP-3	Motu Iti, Moorea, Oceania	Central Pacific Ocean	S 17°32'50.172" W 149° 46'35.4"	3-4 m	subtidal, fine to medium coral sand
EP-1	Punta Sal, Peru, South America	East Pacific Ocean	S 3°58'55" W 80° 59'10"	8 m	subtidal, coarse sand

++) approximation from Google Earth.
* marks the localities, which correspond to the type localities of valid *Pontohedyle* species.

can be split into numerous micro-niches, allowing for changes in ecological, physiological and behavioral traits. However, these are not necessarily reflected in morphological changes and the mesopsammon might be highly stable in an evolutionary perspective. Our results on *Pontohedyle* slugs show that a well-adapted body plan can be conserved for millions of years in a worldwide evolutionary success story.

Conclusions

Combining traditional taxonomic, hydrographic and geographic evidence with multi-marker phylogenetic and multiple species delineation approaches herein allowed us to refute a cosmopolitan and amphi-oceanic distribution of *Pontohedyle* species. Uncovering a radiation of cryptic species partially solves the meiofaunal paradox. Remaining long-range distributions in some *Pontohedyle* species might indicate that the dispersal abilities of meiofaunal slugs are currently underestimated, or that our approach is unsuitable of detecting an even higher degree of cryptic radiation in recent times. Overall, *Pontohedyle* presents a stunning example of extreme morphological stasis and uniformity over long evolutionary timeframes, probably constrained by their simplified bodyplan and by the requirements of the meiofaunal habitat.

Our study boosts diversity in *Pontohedyle* sea slugs from 3 nominal to 12 (candidate) species, and confirms the taxonomic deficit in the mesopsammic fauna. It suggests an unexpected magnitude of diversification and cryptic speciation still exists in other small-sized, neglected taxa. Our workflow of delineating minute and highly cryptic *Pontohedyle* species included integrating phylogenetic, traditional taxonomic and any other relevant evidence towards a primary species hypothesis. This was then evaluated and refined by the consensus evidence from a selection of molecular species delineation methods, including ABGD, statistical parsimony, GMYC and Bayesian species delineation. Both latter methods can deal with rarity as is also confirmed herein. In the age of the biodiversity crisis, we need an efficient and reliable way of addressing species diversity in rare and elusive species. Our workflow still only provides a conservative estimation on species diversity and tolerates the risk of false negatives; we still hope it can serve as a starting point to uncover the hidden diversity of elusive taxa, regardless whether coastal, mesopsammic, deep sea or terrestrial.

Methods

Sampling and fixation

The sampling effort for *Pontohedyle* was conducted worldwide, resulting in specimens from 28 collecting sites in temperate and tropical zones. Samples include re-collection from the type localities of valid *Pontohedyle* species for taxonomy see [7,91]: *P. milaschewitchii* [69] and *P. verrucosa* [73]. '*P. brasilensis*' was considered a junior synonym of *P. milaschewitchii* based on morphological data [41] and was recollected near the original locality (see Figure 4). For detailed data on localities, depth and habitat descriptions see Table 1. Subtidal sands were collected via snorkeling or SCUBA diving. *Pontohedyle* were extracted from intertidal and subtidal sand samples following the method described by Schrödl [92] using a $MgCl_2$/ seawater solution for anesthetization. For molecular work, specimens were fixed in 96–99% ethanol. Voucher specimens were preserved in FSW or 4% glutaraldehyde after relaxation in $MgCl_2$ solution to prevent retraction.

Morphological comparison

All specimens were documented alive under a dissecting microscope and whenever possible analyzed under a light-microscope for spicules and radula characteristics prior to fixation. Radulae of groups defined by molecular data were analyzed by light- and scanning

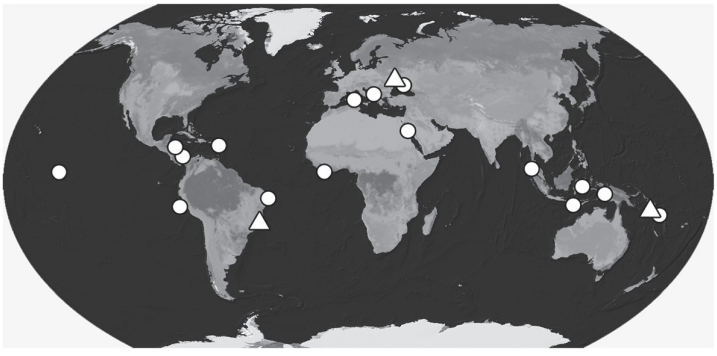

Figure 4 Map of sampling sites for *Pontohedyle*. Type localities of described *Pontohedyle* species (white triangle) and own collecting sites (white dots). For details on localities and habitat description see Table 1).

electron microscopy (SEM). Radulae from specimens from EP-1, CP-2 and CP-3 could unfortunately not be recovered from DNA extraction and were unavailable for further study. To separate the radulae from the surrounding tissue, entire specimens were dissolved in a solution of 45 µl ATL (tissue lysis) buffer and 5 µl Proteinase K (derived from the Qiagen DNeasy Blood and Tissue Kit) overnight at 56°C. Radulae were rinsed in Millipore-purified water, studied with a Leica DMB-RBE microscope (Leica Microsystems, Germany) and photographed with a SPOT CCD camera (Spot Insight, Diagnostic Instruments, Inc., USA). Following light-microscopical examination, radulae were transferred onto SEM stubs with self-adhesive carbon stickers and coated in gold with a Polaron Sputter Coater E5100 for 120 sec. SEM examination was carried out using a LEO 1430VP SEM at 15 kV.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from entire specimens using the DNeasy Blood and Tissue Kit (Qiagen) or the NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co), following the manufacturer's instructions. DNA vouchers are stored at the DNA bank of the Bavarian State Collection for Zoology (ZSM; http://www.dnabank-network.org, see Additional file 2 for accession numbers). Three markers were amplified using polymerase chain reaction (PCR): partial nuclear 28S rRNA (approx. 950 bp) and partial mitochondrial 16S rRNA (approx. 440 bp) and Cytochrome *c* Oxidase subunit I (COI) (approx. 655 bp), using primers and PCR protocols listed in Jörger *et al.* [53]. Successful PCR products were purified using ExoSap IT (USB, Affimetrix Inc.; for 16S rRNA and COI) and the NucleoSpin Extract II (Macherey-Nagel GmbH & Co, for 28S rRNA). Cycle sequencing using Big Dye 3.1 and the PCR primers was conducted by the Genomic Service Unit of the Department of Biology, Ludwig-Maximilians-University Munich, as well as the sequencing reaction on an ABI 3730 capillary sequencer.

Phylogenetic analyses

Consensus sequences from forward and reverse strands were created and edited using Geneious Pro 5.4.2 [93]. All sequences generated in this study were checked for potential contamination using BLAST searches [94] in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments for each marker were generated with Muscle [95] using the default settings. To avoid misleading signal from hard to align regions, ambiguous parts of the 28S and 16S rRNA alignments were removed using Gblocks [96] with settings for a less stringent selection and analyzed comparatively. The removed parts of the alignments (94 bp out of 471 in the 16S rRNA alignment, 49 bp out of 1036 in the 28S rRNA alignment) were carefully checked manually for putative diagnostic signal such as insertions. The COI alignment was checked manually according to amino acids for stop codons and potential shifts in reading frame. Maximum likelihood single-gene trees of each marker (28S rRNA, 16S rRNA, COI) and multi-gene trees of the concatenated dataset were generated using RAxML v. 7.2.6 [97]. Models for nucleotide substitution were chosen with jModeltest [98], with five substitution schemes; i.e., choosing from 40 different models (GTR + G for 28S rRNA and COI and GTR + G + I for 16S rRNA). The RAxML analyses were conducted following the 'hard and slow-way' described in the RAxML 7.0.4 manual (using five parsimony starting trees, six different rate categories and generating 200 multiple inferences and 1000 bootstrap replicates). Additionally, we applied the '-d'-option (generating random starting trees) recommended for small datasets. The concatenated dataset was analyzed 1) without partitioning, 2) in two partitions (nuclear 28S rRNA and mitochondrial 16S rRNA + COI) and 3) in three partitions (corresponding to each marker) and topologies are compared. Outgroups (see Additional file 2) were selected based on the latest phylogenetic hypothesis for Acochlidia [7,53] and include members of all microhedylacean genera; *Asperspina brambelli* (Microhedylacea, Asperspinidae) was defined as the outgroup in phylogenetic analyses. For topological comparison we additionally generated a consensus tree with PAUP v 4.10 [99] applying maximum parsimony to the concatenated three marker dataset. All alignments and trees generated within this study are deposited to TreeBASE under project number 13633 (http://purl.org/phylo/treebase/phylows/study/TB2:S13633).

Molecular based species delineation

We applied four different methods of molecular-based species delineation:

General Mixed Yule-Coalescent Model (GMYC) - a maximum likelihood approach as implemented in the 'General Mixed Yule-Coalescent' model (GMYC) was applied to discriminate between population and speciation processes and to identify species see [32,33]. Therefore, we generated ultrametric starting trees using BEAST 1.5.3 [100,101] from the COI and masked 16S rRNA alignments. Even though tested and designed for mitochondrial markers, for comparison we additionally calculated an ultrametric tree from the concatenated three-marker alignment (COI + 16S rRNA + 28S rRNA) which was also used for phylogenetic comparison. For the starting trees we performed relaxed lognormal clock analyses using the Yule prior and models for each marker specified above. We ran five independent Monte Carlo Markov Chains (MCMC) for 50 $\times 10^6$ generations each, sampling every 5000 steps. Single runs were combined with Log-Combiner 1.5.3 and checked for

sufficient effective sampling size (ESS) in Tracer 1.5.3. Trees were combined using TreeAnnotator 1.5.3 with the first 10% of the trees discharged as burn-in. GMYC was performed in R using the SPLITS package (http://r-forge.r-project.org/projects/splits/) and analyses allowing single and multiple thresholds were performed.

Statistical parsimony - generating haplotype networks using statistical parsimony [37] is a common method derived from population genetics to visualize possible intraspecific relationships. Sequences are assigned to networks connected by changes, which are non-homoplastic with a certain probability. Even though this is not equivalent to defining species boundaries, statistical parsimony has also been applied successfully to delimit candidate species [16,33]. We generated haplotype networks with TCS 1.21 [102] applying a 95% parsimony criterion to both mitochondrial markers (COI and 16S rRNA) and nuclear 28S rRNA.

Bayesian species delineation – analysis was conducted using the program BP&P (BPP v2.1) [38,103] on the full three marker dataset. We ran two sets of BP&P analyses: 1) using our PSH as user-specified guide tree to evaluate the support of different speciation models for the identified MOTUs; 2) to test whether our PSH is too conservative and lumps species, we used a guide tree testing each population from different collecting sites as putative species. Putative sympatric cryptic species resulting in different MOTUs in our PSH were also separated into different populations. As prior information on ancestral population size (θ) and divergence times (τ) can affect posterior probabilities for speciation models [38,64], we tested 4 different prior combinations for each set: a) large ancestral population size, assigned gamma prior G(1,10) and deep divergences, root of the tree (τ) is assigned the gamma prior G(1,10), while the other divergence time parameters are assigned the Dirichlet prior ([38]: equation 2); b) small ancestral population size G(2,2000) and deep divergences G(1,10); c) large ancestral population size G(1,10) and shallow divergences G(2,2000); d) small ancestral population size G(2,2000) and shallow divergences G(2,2000). The latter cases are, however, considered evolutionary unlikely based on molecular clock estimates [53] of *Pontohedyle*. Since BP&P can currently only deal with up to 20 species, the population approach had to be conducted in several subsets. Each single analyses was run at least twice to confirm consistency between runs, run with two different algorithms and two different fine-tuning parameters. Since no biological data exists on ancestral population size in *Pontohedyle*, we consider it most objective to calculate the mean from different approaches and consider species with PP ≥ 0.95 as strongly supported by Bayesian species delineation.

Automatic Barcode Gap Discovery (ABGD) and pairwise distances – ABGD is an automated procedure that clusters sequences into candidate species based on pairwise distances by detecting differences between intra- and interspecific variation (i.e., barcoding gap) without *a priori* species hypothesis [39,55]. We used the web-server of ABGD http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html and analyzed both mitochondrial markers using the two available models: Jukes-Cantor (JC69) and the Kimura K80 model. The program requires two user-specified values: P (prior limit to intraspecific diversity) and X (proxy for minimum gap width). To evaluate the effect on our dataset we tested X values from 0.1 to 5 and extended the maximum P value from 0.10 to 0.20.

Fixed thresholds – to calculate intra- and interspecific divergence among our detected MOTUs we used Species Identifier (obtained from Taxon DNA [17]) for both mitochondrial markers (COI and 16S rRNA), using the raw (unmasked) sequences. For comparison we tested the application of a fixed threshold of 11% for Mollusca suggested by Hebert et al. [54].

Minimum consensus approach - For our secondary species hypothesis (SSH, i.e., defining candidate species), we chose a conservative minimum consensus approach relying only on uncontradicted positive species identification based on the methods described above. Entities that were identified only by some of the approaches are thus ignored, giving equal priority to the applied methods.

Additional files

Additional file 1: Additional phylogenetic analyses of the concatenated and single-gene dataset (bootstrap values ≥ 50 given above nodes). A. Maximum parsimony analyses conducted with PAUP on the concatenated three marker dataset. B. Maximum likelihood (ML) single-gene tree of nuclear 28S rRNA. C. ML single-gene tree of mitochondrial 16S rRNA (ambiguous parts in the alignment masked with GBlocks). D. ML single-gene tree of mitochondrial COI (due to extremely long branches *Asperspina brambelli* was considered as too distant and excluded from the analysis).

Additional file 2: Molecular data analyzed in the present study. Museum numbers (ZSM – Bavarian State Collection of Zoology, SI – Smithsonian Institute (numbers refer to plate coordinates), AM – Australian Museum), DNA vouchers (all at ZSM) and GenBank accession numbers. Sequences retrieved from GenBank are marked with *.

Competing interests

The authors declare that they do not have competing interests.

Authors' contributions

KMJ collected material, conducted molecular work and phylogenetic and species delineation analyses and drafted the manuscript. JLN organized sampling trips and supported molecular work. NGW dedicated valuable material to the study. All authors contributed to the discussion of the results and the final version of the manuscript. MS collected material, and planned and supervised the study. All authors read and approved the final version of the manuscript.

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Chapter 14. How to describe a cryptic species? Practical challenges of molecular taxonomy

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Note: The erroneous formatting of molecular diagnostic characters in the presented species descriptions as tables resulted in their misplacement in the final version of the manuscript. The misplacement occurred in the final stage of the BMC post-production process and was not intended by the authors. Unfortunately, *Frontiers in Zoology* so far still refuses to correct their mistake.

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How to describe a cryptic species? Practical challenges of molecular taxonomy

Katharina M Jörger[1,2*] and Michael Schrödl[1,2]

Abstract

Background: Molecular methods of species delineation are rapidly developing and widely considered as fast and efficient means to discover species and face the 'taxonomic impediment' in times of biodiversity crisis. So far, however, this form of DNA taxonomy frequently remains incomplete, lacking the final step of formal species description, thus enhancing rather than reducing impediments in taxonomy. DNA sequence information contributes valuable diagnostic characters and –at least for cryptic species – could even serve as the backbone of a taxonomic description. To this end solutions for a number of practical problems must be found, including a way in which molecular data can be presented to fulfill the formal requirements every description must meet. Multi-gene barcoding and a combined molecular species delineation approach recently revealed a radiation of at least 12 more or less cryptic species in the marine meiofaunal slug genus *Pontohedyle* (Acochlidia, Heterobranchia). All identified candidate species are well delimited by a consensus across different methods based on mitochondrial and nuclear markers.

Results: The detailed microanatomical redescription of *Pontohedyle verrucosa* provided in the present paper does not reveal reliable characters for diagnosing even the two major clades identified within the genus on molecular data. We thus characterize three previously valid *Pontohedyle* species based on four genetic markers (mitochondrial cytochrome c oxidase subunit I, 16S rRNA, nuclear 28S and 18S rRNA) and formally describe nine cryptic new species (*P. kepii* sp. nov., *P. joni* sp. nov., *P. neridae* sp. nov., *P. liliae* sp. nov., *P. wiggi* sp. nov., *P. wenzli* sp. nov., *P. peteryalli* sp. nov., *P. martynovi* sp. nov., *P. yurihookeri* sp. nov.) applying molecular taxonomy, based on diagnostic nucleotides in DNA sequences of the four markers. Due to the minute size of the animals, entire specimens were used for extraction, consequently the holotype is a voucher of extracted DNA ('DNA-type'). We used the Character Attribute Organization System (CAOS) to determine diagnostic nucleotides, explore the dependence on input data and data processing, and aim for maximum traceability in our diagnoses for future research. Challenges, pitfalls and necessary considerations for applied DNA taxonomy are critically evaluated.

Conclusions: To describe cryptic species traditional lines of evidence in taxonomy need to be modified. DNA sequence information, for example, could even serve as the backbone of a taxonomic description. The present contribution demonstrates that few adaptations are needed to integrate into traditional taxonomy novel diagnoses based on molecular data. The taxonomic community is encouraged to join the discussion and develop a quality standard for molecular taxonomy, ideally in the form of an automated final step in molecular species delineation procedures.

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Background

Species boundaries are frequently hard to delimit based on morphology only, a fact which has called for integrative taxonomy, including additional sources of information such as molecular data, biogeography, behavior and ecology [1,2]. Founding a species description on a variety of characters from different, independent datasets is generally regarded as best practice [3]. When species are considered as independently evolving lineages [4], different lines of evidence (e.g., from morphology, molecules, ecology or distribution) are additive to each other and no line is necessarily exclusive nor need different lines obligatory be used in combination [3,5]. Taxonomists are urged to discriminate characters according to their quality and suitability for species delineation, rather than to just add more and more data [5]. The specifics of the taxon in question will guide the way to the respective set(s) of characters that will provide the best backbone for the diagnosis. In cases of pseudo-cryptic species (among which morphological differences can be detected upon re-examining lineages separated e.g. on molecular data) or of fully cryptic species (that morphology fails to delimit), the traditional lines of evidence have to be modified by using, e.g., molecular information to break out of the 'taxonomic circle' [6,7].

Cryptic species are a common phenomenon throughout the metazoan taxa, and can be found in all sorts of habitats and biogeographic zones [8-10]. Groups characterized by poor dispersal abilities (e.g., most meiofaunal organisms or animals inhabiting special regions where direct developers predominate, such as Antarctica), are especially prone to cryptic speciation [11,12]. Uncovering these cryptic species is fundamental for the understanding of evolutionary processes, historical biogeography, ecology, and also to conservation approaches, as distribution ranges that are smaller than initially assumed mean a higher risk of local extinction [8,10]. The lack of morphological characters to distinguish cryptic species should not lead to considerable parts of biological diversity remaining unaddressed.

The utility of DNA barcoding and molecular species delineation approaches to uncover cryptic lineages has been demonstrated by numerous studies (e.g., [11,13-19]). Unfortunately, inconsistencies in terminology associated with the interface between sequence data and taxonomy have led to confusion and various criticisms [6,20]. First of all, one needs to distinguish between species identification via molecular data (DNA barcoding in its strict sense) and species discovery [6,21,22]. While species identification is a primary technical application, species delimitation requires means of molecular species delineation that is either distance, tree or character based [6,23]. Under ideal circumstances sufficient material is collected from different populations across the entire distribution area of a putative group of cryptic species. Using population genetics the distribution of haplotypes can be analyzed and different, genetically isolated lineages can be detected [24]. Population genetic approaches are, however, not always feasible with animals that are rare or hard to collect, which might actually be a common phenomenon across faunas of most marine ecosystems [25-28]. Derived from barcoding initiatives, threshold based species delimitation became the method of choice, aiming for the detection of a 'barcoding gap' between intra- and interspecific variation [29-31]. This approach has been criticized, however, due to its sensitivity to the degree of sampling, the general arbitrariness of fixed or relative thresholds, and to frequent overlap between intra- and interspecific variation [6,32,33]. In the recently developed Automatic Barcode Gap Discovery (ABGD) [34], progress has been made in avoiding the dependence of *a priori* defined species hypotheses in threshold based approaches, but reservations remain concerning the concept of a barcoding gap [25]. Several independent delineation tools exist, e.g. using haplotype networks based on statistical parsimony [35], maximum likelihood approaches applying the General Mixed Yule-Coalescent model [36,37], or Bayesian species delineation [38,39]. Empirical research currently compares the powers of these different tools on real datasets [25,32,40]. The effect of the inclusion of singletons in analyses is considered as most problematic [25]. At the present stage of knowledge, independent approaches allowing cross-validation between the different methods of molecular species delineation and other sources of information (morphology, biogeography, behavioral traits) seem the most reliable way of delimiting cryptic species [25].

The second inconsistency in terminology concerns usages of 'DNA taxonomy'. Originally, DNA taxonomy was proposed to revolutionize taxonomy by generally founding descriptions on sequence data and overthrowing the Linnaean binominal system [41]. Alternatively, it was suggested as a concept of clustering DNA barcodes into MOTUs [42]. Since then, however, it has been applied as an umbrella term for barcoding, molecular species delineation, and including molecular data in species descriptions (see e.g., [13,14,20,36,43,44]). In a strict sense, one cannot speak of molecular taxonomy if the process of species discovery is not followed by formal species description (i.e. there are two steps to a taxonomic process: species discovery (delimitation) and attributing them with formal diagnoses and names.) Taxonomy remains incomplete if species hypotheses new to science are flagged as merely putative by provisional rather than fully established scientific names. For practical reasons and journal requirements, most studies on molecular species delineation postpone formal descriptions of the discovered species (e.g., [13,14,25,33,36,40,43-46]), and then rarely carry them

out later. DNA barcoding and molecular species delineation are promoted as fast and efficient ways to face the 'taxonomic impediment', i.e. the shortage of time and personnel capable of working through the undescribed species richness in the middle of a biodiversity crisis [7,47,48]. However, keeping discovered entities formally unrecognized does not solve the taxonomic challenges but adds to them by creating parallel worlds populated by numbered MOTUs, OTUs or candidate species. In many cases the discovered taxa remain inapplicable to future research, thus denying the scientific community this taxonomic service, e.g. for species inventories or conservation attempts. Without formal description or a testable hypothesis, i.e. a differential diagnosis, 1) the discovered species might not be properly documented or vouchered by specimens deposited at Natural History Museums; and 2) their reproducibility can be hindered and confusion caused by different numbering systems. A deterrent example of the proliferation of informal epithets circulating as '*nomina nuda*' (i.e. species which lack formal diagnoses and deposited vouchers) in the literature is given by the 'ten species in one' *Astraptes fulgerator* complex [31,49]. Thus, we consider it as all but indispensable for DNA taxonomy to take the final step and formalize the successfully discovered molecular lineages.

The transition from species delimitation to species description is the major task to achieve. Nearly ten years after the original proposal of DNA taxonomy [41], revolutionizing traditional taxonomy has found little acceptance in the taxonomic community, as most authors agree that there is no need for overthrowing the Linnaean System. Consequently, the challenge is to integrate DNA sequence information in the current taxonomic system. Several studies have attempted to include DNA data in taxonomic descriptions, albeit in various non-standardized ways; see the review by Goldstein and DeSalle ([21]; box 3): In some cases, DNA sequence information is simply added to the taxonomic description (in the form of GenBank numbers or pure sequence data), without evaluating and reporting diagnostic features [21]. Others rely on sequence information for the description, either reporting results of species delineation approaches, e.g. raw distance measurements or model based assumptions, or extracting diagnostic characters from their molecular datasets. There still is a consensus that species descriptions should be character based [50] (but see the Discussion below for attempts at model based taxonomy), and that tree or distance based methods fail to extract diagnostic characters [6]. Character based approaches, like the Characteristic Attribute Organization System (CAOS), are suggested as an efficient and reliable way of defining species barcodes based on discrete nucleotide substitution, and these established diagnostics from DNA sequences can be used directly for species descriptions as molecular taxonomic characters [51,52]. Yet, the application of CAOS or similar tools requires an evaluation of how to select and present molecular synapomorphies and how to formalize procedures to create a 'best practice' linking DNA sequence information to existing taxonomy [20].

In the present study, we formally describe the candidate species of minute mesopsammic sea slugs in the genus *Pontohedyle* Golikov & Starobogatov (Acochlidia, Heterobranchia) discovered by Jörger et al. [25]. This cryptic radiation was uncovered in a global sampling approach with multi-gene and multiple-method molecular species delineation [25]. The initially identified 12 MOTUs, nine of which do not correspond to described species, are considered as species [following 4] resulting from a conservative minimum consensus approach applying different methods of molecular species delineation [25]. The authors demonstrated that traditional taxonomic characters (external morphology, spicules and radula features) are insufficient to delineate cryptic *Pontohedyle* species [25]. To evaluate the power of more advanced histological and microanatomical data, we first provide a detailed computer based 3D redescription of the anatomy of *Pontohedyle verrucosa* (Challis, 1970) and additional histological semithin sections of *P. kepii* sp. nov. In the absence of reliable diagnostic characters from morphology and microanatomy, we then rely on DNA sequence data as the backbone for our species descriptions. For the three previously valid *Pontohedyle* species we extract diagnostic characters using the Character Attribute Organization System (CAOS) based on four standard markers (mitochondrial cytochrome c oxidase subunit I, 16S rRNA, and nuclear 18S rRNA and 28S rRNA). In addition, nine new species are formally described on molecular characteristics and evidence from other data sources. Various approaches to the practical challenges for molecular driven taxonomy – such as critical consideration of the quality of the alignment, detection of diagnostic nucleotides and their presentation aiming for maximum traceability in future studies – are tested and critically evaluated.

Results

Evaluation of putative morphological characters

The diversity within *Pontohedyle* revealed by molecular data cannot be distinguished externally: the body shows the typical subdivision into the anterior head-foot complex and the posterior visceral hump. Bodies are whitish-translucent, digestive glands are frequently bright green to olive green. Rhinophores are lacking, labial tentacles are bow-shaped and tapered towards the ends (see Figures 1 and 2). Monaxone rodlet-like spicules distributed all over the body and frequently found in an accumulation between the oral tentacles are characteristic for *Pontohedyle*. These spicules can be confirmed for *P. wenzli* sp. nov., for

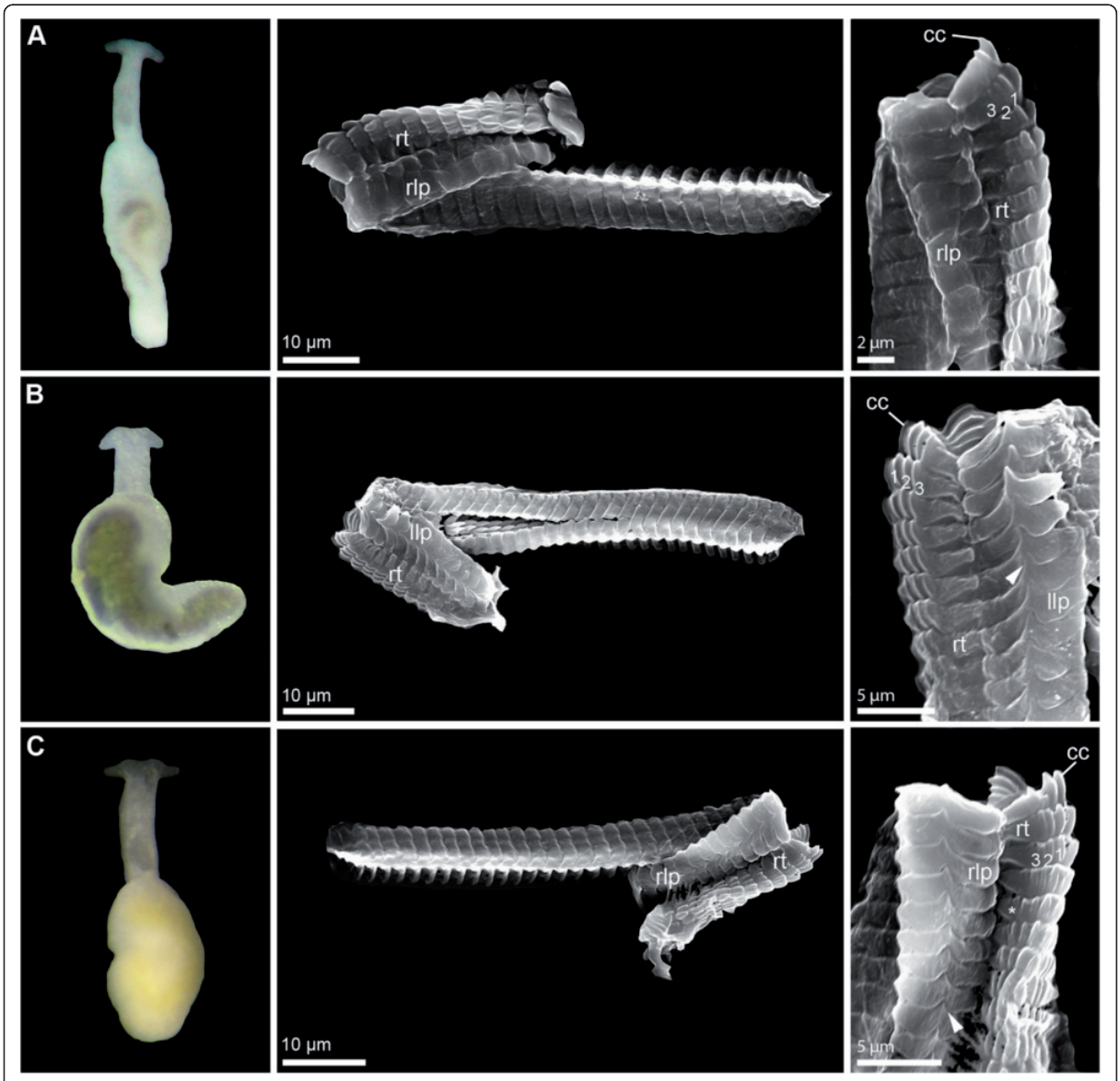

Figure 1 External morphology (living specimens) and radula characteristics (SEM micrographs) in *Pontohedyle* species (part 1). **A)** *Pontohedyle kepii* sp. nov. (*Pontohedyle* sp. 1 in [25]); **B)** *Pontohedyle joni* sp. nov. (*Pontohedyle* sp. 2 from WA-5 (Belize) in [25]); **C)** *Pontohedyle liliae* sp. nov. (*Pontohedyle* sp. 4 in [25]), * marks putative 4th cusp on rhachidian tooth. cc = central cusp of rhachidian tooth, llp = left lateral plate, rlp = right lateral plate, rt = rhachidian tooth.

P. yurihookeri sp. nov., *P. milaschewitchii* (Kowalevsky, 1901) and *P. brasilensis* (Rankin, 1979), and, in contrast to the original description [53], also in *P. verrucosa*. No spicules could be detected in *P. peteryalli* sp. nov. from Ghana. The absence of spicules is insufficient, however, to delineate microhedylid species, since their presence can vary under environmental influence [54].

The radulae of eight species were investigated using SEM (see Figures 1 and 2). Radulae of *P. neridae* sp. nov., *P. martynovi* sp. nov. and *P. yurihookeri* sp. nov. were not recovered whole from molecular preparations, and thus were unavailable for further examination [25]. The radula of *P. wiggi* sp. nov. could only be observed under the light-microscope, but not successfully transferred to a SEM stub. All radulae are hook-shaped with a longer dorsal and a shorter ventral ramus, typical for Acochlidia. Radula formulas are 38–53 × 1.1.1, lateral plates are curved rectangular, and the rhachidian tooth is triangular and bears a central cusp and typically three smaller lateral denticles. Most radulae bear one pointed denticle centrally

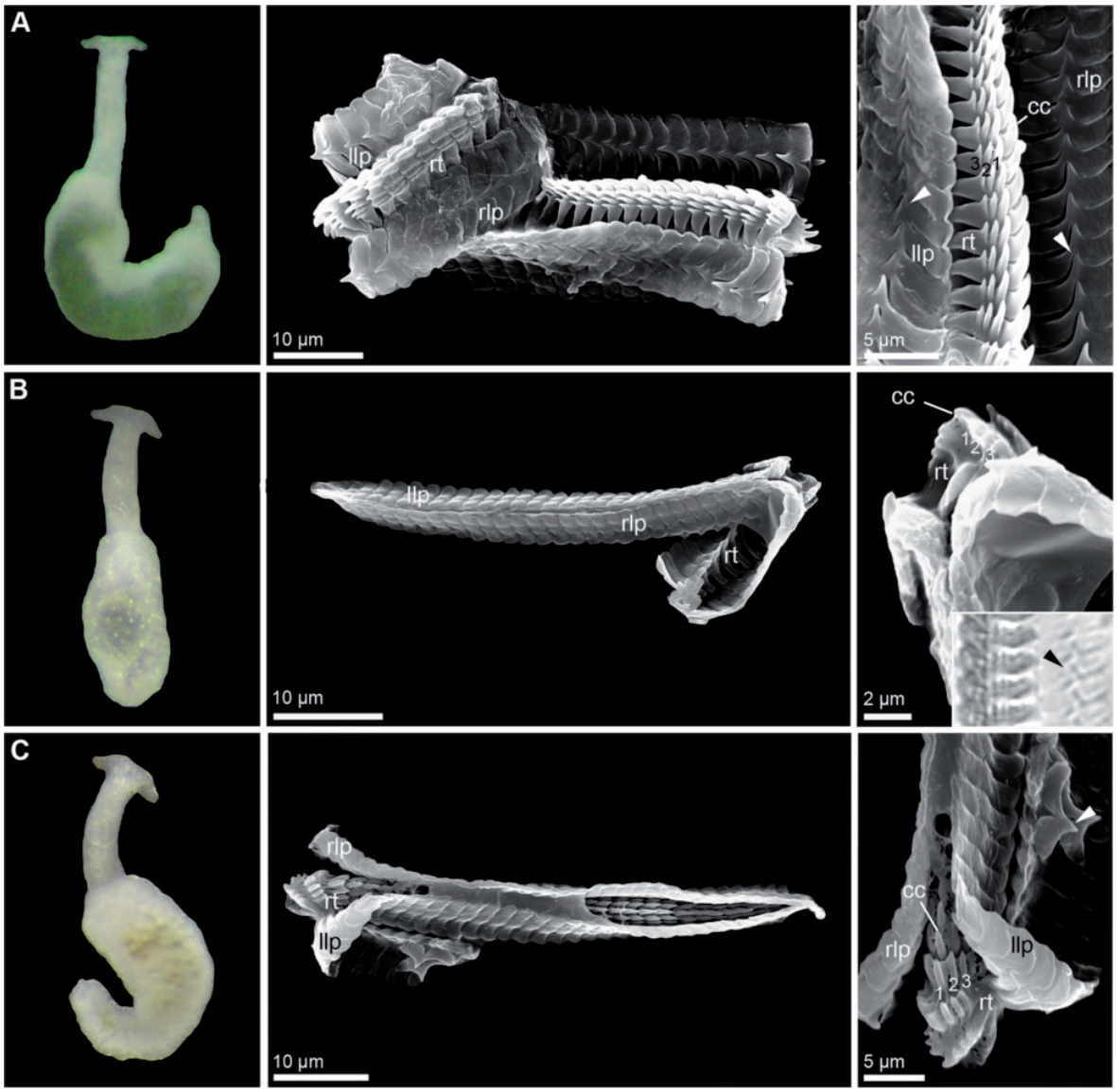

Figure 2 External morphology (living specimens) and radula characteristics (SEM micrographs) in *Pontohedyle* species (part 2). **A)** *Pontohedyle peteryalli* sp. nov. (*Pontohedyle* sp. 7 in [25]); **B)** *Pontohedyle wenzli* sp. nov. (*Pontohedyle* sp. 6, picture of living animal from WP-1 (holotype), radula from IP-2, see [25]); **C)** *P. brasilensis* (living animal from WA-3 (Belize), radula from WA-10 (Brazil), see [25]). cc = central cusp of rhachidian tooth, llp = left lateral plate, rlp = right lateral plate, rt = rhachidian tooth.

on the anterior margin of each lateral plate and a corresponding notch on the posterior side. Only the radula of *P. kepii* sp. nov. and *P. verrucosa* can be clearly distinguished from the others by the absence of this denticle and the more curved lateral teeth (see Figure 1A and [25], Figure 1D,E). Uniquely, *P. verrucosa* bears five lateral denticles next to the central cusp of the rhachidian tooth [25]; in *P. liliae* sp. nov. a tiny fourth denticle borders the central cusp (see * in Figure 1C).

Previous phylogenetic analyses [25] recovered a deep split into two *Pontohedyle* clades: the *P. milaschewitchii* clade and the *P. verrucosa* clade. This is supported by novel analyses in a larger phylogenetic framework and additionally including a second nuclear marker (18S rRNA) (own unpublished data). Since no detailed histological account exists of any representative from the large *P. verrucosa* clade, we redescribe *P. verrucosa* (based on ZSM Mol-20071833, 20071837 and 20100548), supplementing

the original description with detailed information of the previously undescribed nervous and reproductive systems. The central nervous system (cns) of *P. verrucosa* lies prepharyngeal and shows an epiathroid condition. It consists of paired rhinophoral, cerebral, pleural, pedal and buccal ganglia and three unpaired ganglia on the visceral nerve cord, tentatively identified as left parietal ganglion, median fused visceral and subintestinal ganglion and right fused parietal and supraintestinal ganglion (Figure 3A). An osphradial ganglion or gastro-oesophagial ganglia were not detected. Anterior and lateral to the cerebral ganglia are masses of accessory ganglia. Due to the retracted condition of all examined specimens, tissues are highly condensed and no separation in different complexes of accessory ganglia could be detected. Attached to the pedal ganglia are large monostatolith statocysts. Oval, unpigmented globules are located in an anteroventral position of the cerebral ganglia, interpreted as the remainder of eyes (see Figure 3B).

P. verrucosa is a gonochoristic species. The three sectioned specimens include two males and one female. The male reproductive system is comprised of gonad, ampulla, postampullary sperm duct, prostatic vas deferens, ciliated (non-glandular) vas deferens, genital opening and a small ciliated 'subepidermal' duct leading to a second genital opening anterodorsally of the mouth opening (Figure 3C). The sac-like gonad is relatively small and bears few irregular distributed spermatozoa. The large tubular ampulla emerges from the gonad without a detectable preampullary sperm duct; it is loosely filled with irregularly distributed spermatozoa (Figure 3D). The ampulla leads into a short, narrow ciliated post-ampullary duct widening into the large tubular prostatic vas deferens (staining pink in methylene-blue sections, Figure 3D). Close to the male genital opening, the duct loses its glandular appearance and bears cilia. The primary genital opening is located on the right side of the body at the visceral hump and close to the transition with the head-foot complex. Next to the genital opening, the anterior vas deferens splits off as an inconspicuous subepithelial ciliated duct that leads anteriorly on the right side of the head foot complex. It terminates in a second genital opening between the oral tentacles anterodorsally from the mouth opening.

The female reproductive system consists of gonad, nidamental glands and oviduct (Figure 3E) and a genital opening located on the right side, in the posterior part of the visceral hump (not visible in Figure 3E, due to the retracted stage of the individual). The gonad is sac-like and bears one large vitellogenic egg (see Figure 3F) and several developing oocytes. Three histologically differentiated tube-like nidamental glands could be detected with a supposedly continuous lumen and with an epithelium bearing cilia. From proximal to distal these glands are identified as albumen gland (cells filled with dark blue stained granules), membrane gland (pinkish, vacuolated secretory cells) and winding mucus gland (secretory cells stained pink-purple). In its proximal part the distal oviduct shows a similar histology as the mucous gland, but then loses its glandular appearance. The epithelium of the distal oviduct bears long, densely arranged cilia.

Additional notable histological features are numerous dark-blue-stained epidermal gland cells (see e.g., arrowhead in Figure 3D) and refracting fusiform structures in the digestive gland (see Figure 3B). An additional series of histological semi-thin sections of *Pontohedyle kepii* sp. nov. was sectioned and brief investigation revealed no variation in the major organization of the organ systems in *Pontohedyle* as described herein and in previous studies [55,56].

Remarks on the presentation of molecular characters

Diagnostic characters for each species of *Pontohedyle* were extracted using the 'Characteristic Attribute Organization System' (CAOS) [51,57,58]. We define diagnostic characters as single pure characters, i.e. unique character states that respectively occur in all investigated specimens in a single *Pontohedyle* species but in none of the specimens of its congeners. As additional information single heterogeneous pure characters (i.e., different character states present within the species but absent from the congeners) are reported (for further details on the chosen approach see the Material and methods and Discussion sections). Positions refer to the position of the diagnostic nucleotide within the respective alignment (see Additional files 1, 2, 3, 4, 5 and 6). Where alignment positions differ from those in the deposited sequences, positions within the sequence of the holotype or in another reference sequence are also provided.

Taxonomy of *Pontohedyle*

Family: Microhedylidae Odhner, 1938 [59]
Genus: *Pontohedyle* Golikov & Starobogatov, 1972 [60]
Synonymy: *Mancohedyle* Rankin, 1979; *Gastrohedyle* Rankin, 1979; *Maraunibina* Rankin, 1979
Type species (by subsequent designation): *Pontohedyle milaschewitchii* (Kowalevsky, 1901) [61]

Phylogenetic analyses of the genus *Pontohedyle* [25] confirmed earlier assumptions, that the three genera established by Rankin [62] (see above) present junior synonyms of *Pontohedyle*.

Morphological characteristics of genus *Pontohedyle*: Minute (0.7–6 mm) marine interstitial microhedylacean acochlid. Body divided into anterior head-foot complex and posterior visceral hump. In case of disturbance head-foot complex can be entirely retracted into visceral

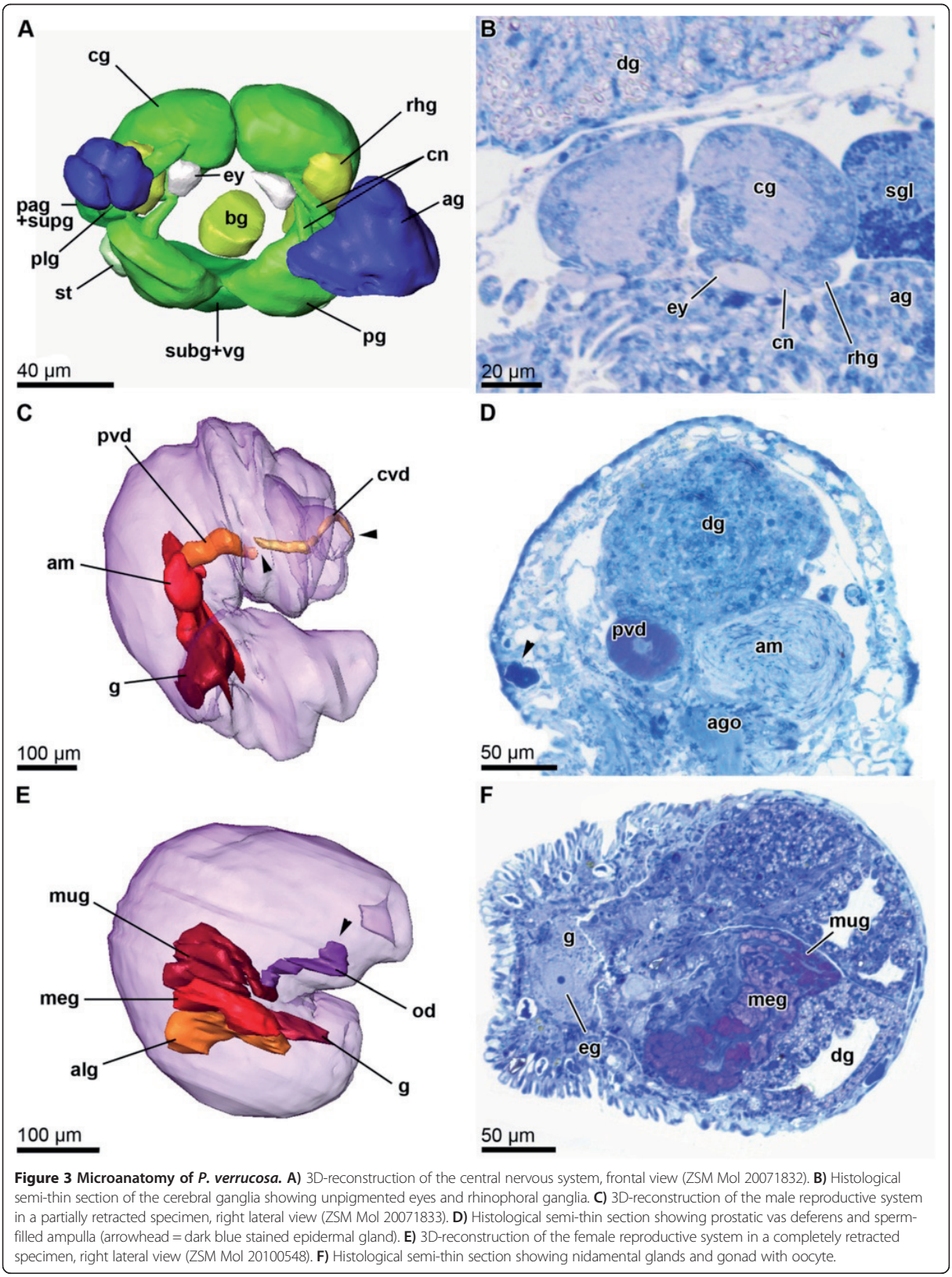

Figure 3 Microanatomy of *P. verrucosa*. **A)** 3D-reconstruction of the central nervous system, frontal view (ZSM Mol 20071832). **B)** Histological semi-thin section of the cerebral ganglia showing unpigmented eyes and rhinophoral ganglia. **C)** 3D-reconstruction of the male reproductive system in a partially retracted specimen, right lateral view (ZSM Mol 20071833). **D)** Histological semi-thin section showing prostatic vas deferens and sperm-filled ampulla (arrowhead = dark blue stained epidermal gland). **E)** 3D-reconstruction of the female reproductive system in a completely retracted specimen, right lateral view (ZSM Mol 20100548). **F)** Histological semi-thin section showing nidamental glands and gonad with oocyte.

hump. Body whithish translucent. Foot with short rounded free posterior end. Head bears one pair of bow-shaped dorso-ventrally flattened oral tentacles. Rhinophores lacking. Monaxone, calcareous spicules irregularly distributed over head-foot complex and visceral hump. Radula hook-shaped band (lateral view), formula 1-1-1, lateral plates curved or with one pointed denticle, rhachidian tooth triangular with one central cusp and 2–4 lateral cusps on each side. Nervous system with accessory ganglia at cerebral nerves anterior to the cns. Sexes separate, male reproductive system aphallic, sperm transferred via spermatophores.

Molecular diagnosis of the genus *Pontohedyle*, based on the sequences analyzed herein (Table 1) and on sequences from a set of outgroups including all acochlidian genera for which data are available [63,64]. Positions refer to the alignments in Additional files 1 and 2, and to the reference sequences of *P. milaschewitchii*, ZSM Mol 20080054 (GenBank HQ168435 and JF828043) from Croatia, Mediterranean Sea (confirmed to be conspecific with material collected at the type locality in molecular species delineation approaches [25]). Molecular diagnosis is given in Table 2.

Pontohedyle milaschewitchii (Kowalevsky, 1901) [61]

Hedyle milaschewitchii Kowalevsky, 1901: p. 19–20 [61]
Pontohedyle milaschewitchii (Kowalevsky) – Golikov & Starobogatov [60]
Mancohedyle milaschewitchii (Kowalevsky) – Rankin (1979: p. 100) [62]
Pontohedyle milatchevitchi (Kowalevsky) – Vonnemann et al. (2005: p. 3) [65]; Göbbeler & Klussmann-Kolb (2011: p. 122) [66].

Type locality: Black Sea, bay of St George monastery near Sevastopol, Crimean Peninsula, Ukraine.

Type material: To our knowledge no type material remains. Nevertheless we refrain from designating a neotype, as there is no taxonomic need, i.e. no possibility of confusion in the species' area of distribution.

Distribution and habitat: Reported from the Black Sea and numerous collecting sites throughout the Mediterranean e.g. [55,61,67,68]; marine, interstitial, subtidal 1–30 m, coarse sand.

Molecular diagnosis is given in Table 3.

ZSM Mol 20071381 (recollected at the type locality, see Figure 4) serves as the reference sequence, unless the sequence could not be successfully amplified. Then sequences (indicated below) from material from the Mediterranean serve as reference sequences (conspecifity was confirmed in a previous molecular species delineation approach 25]). Diagnostic characters in 18S rRNA were determined based on ZSM Mol 20080054 (GenBank HQ168435 = reference sequence) and ZSM Mol 20080953 (GenBank KC984282); in nuclear 28S rRNA based on ZSM Mol 20071381 (GenBank JQ410926) and ZSM Mol 20080054 (GenBank JF828043 = reference sequence), in mitochondrial 16S rRNA based on ZSM Mol 20071381 (GenBank JQ410925), ZSM Mol 20080054 (GenBank HQ168422), ZSM Mol 20080055 (GenBank JQ410927), ZSM Mol 20080925 (GenBank JQ410928) and ZSM Mol 20080953 (GenBank JQ410929), in mitochondrial COI based on ZSM Mol 20071381 (GenBank JQ410827), ZSM Mol 20080925 (GenBank HQ168459) and ZSM Mol 20080953 (GenBank JQ410898).

Pontohedyle verrucosa (Challis, 1970) [53]

Microhedyle verrucosa Challis, 1970: pp. 37–38 [53]
Pontohedyle verrucosa (Challis) – Wawra (1987: p. 139) [69]
Maraunibina verrucosa (Challis) – Rankin (1979: p. 102) [62]

Type locality: Coarse, clean shell sand, a little above low water at neap tide, near southern end of Maraunibina Island, Marau Sound, East Guadalcanal, Solomon Islands.

Type material: According to Challis [53] in the Natural History Museum, London, and the Dominion Museum, Wellington, New Zealand. Own investigations revealed that the type material of Challis never arrived at the Natural History Museum, London and visiting the Museum of New Zealand Te Papa Tongarewa (former Dominion Museum), we were unable to locate any of her types. Thus, at current stage of knowledge, type material might only remain in her private collection. We refrain from designating a neotype because we were unable to recollect at the type locality (see below).

Distribution and habitat: Reported from Indonesia and the Solomon Islands [25,53]; marine, interstitial, intertidal, coarse sand.

Sequenced material: In a collecting trip to the Solomon Islands, we were unfortunately unable to recollect at the type locality (Maraunibina Island, East Guadalcanal), but successfully recollected in Komimbo Bay (West Guadalcanal), a locality, from which the describing author noted similar ecological parameters and recorded several meiofaunal slug species occurring at both sites [53,70] Additional material was collected at different collecting sites in Indonesia (see Figure 4).

Molecular diagnosis is given in Table 4.

ZSM Mol 20071820 (from Komimbo Bay, East Guadalcanal, Solomon Islands) serves as the reference sequence. Diagnostic characters in nuclear 18S rRNA were determined based on ZSM Mol 20071820 (GenBank KC984287), ZSM Mol 20071135 (GenBank KC984288) and ZSM Mol 20100391 (GenBank KC984289), in nuclear

Table 1 DNA sequence data analyzed in the present study to determine diagnostic nucleotides in *Pontohedyle*

Species	Museums number	DNA voucher	GenBank accession numbers			
			18S rRNA	28S rRNA	16S rRNA	COI
P. milaschewitchii	ZSM Mol 20071381	AB34404214	-	JQ410926	JQ410925	JQ410897
	ZSM Mol 20080054	AB34404241	HQ168435	JF828043	HQ168422	-
	ZSM Mol 20080055	AB34404239	-	-	JQ410927	-
	ZSM Mol 20080925	-	-	-	JQ410928	HQ168459
	ZSM Mol 20080953	AB35081832	KC984282	-	JQ410929	JQ410898
P. brasilensis	SI-CBC20 10KJ01-E03	AB34500510	KC984283	JQ410941	JQ410940	-
	SI-CBC20 10KJ01-B07	AB34402082	-	JQ410943	JQ410942	-
	SI-CBC20 10KJ01-D07	AB34500513	-	JQ410944	-	-
	SI-CBC20 10KJ01-B09	AB34402031	-	JQ410946	JQ410945	JQ410904
	SI-CBC20 10KJ01-C09	AB34500576	-	JQ410948	JQ410947	JQ410905
	SI-CBC20 10KJ01-A10	AB34402026	-	-	JQ410949	-
	SI-CBC20 10KJ02-E01	AB34402030	-	JQ410950	-	-
	ZSM Mol 20110723	AB34402034	KC984284	JQ410952	JQ410951	JQ410906
	ZSM Mol 20110722	AB34402086	KC984285	JQ410932	JQ410931	JQ410900
	ZSM Mol 20090198	AB35081813	KC984286	JQ410936	JQ410935	-
P. verrucosa	ZSM Mol 20071820	AB34404223	KC984287	JQ410978	JQ410977	JQ410920
	ZSM Mol 20080176	AB34404286	-	JQ410980	JQ410979	JQ410921
	ZSM Mol 20071135	AB34404221	KC984288	JQ410971	JQ410970	JQ410914
	ZSM Mol 20100388	AB34500547	-	-	-	JQ410916
	ZSM Mol 20100389	AB34402044	-	JQ410974	-	JQ410917
	ZSM Mol 20100390	AB34402070	-	JQ410975	-	JQ410918
	ZSM Mol 20100391	AB34500531	KC984289	-	JQ410976	JQ410919
Pontohedyle kepii sp. nov.	ZSM Mol 20081013	AB35081769	KC984290	JQ410967	JQ410966	JQ410912
Pontohedyle joni sp. nov.	ZSM Mol 20090197	AB34858164	KC984291	JQ410934	JQ410933	JQ410901
	SI-CBC20 10KJ01-D05	AB34402049	KC984292	-	JQ410937	JQ410902
	SI-CBC20 10KJ01-C08	AB34402065	-	JQ410939	JQ410938	JQ410903
Pontohedyle neridae sp.nov.	AM C. 476062.001	AB34500497	-	JQ410986	JQ410985	JQ410922
Pontohedyle liliae sp.nov.	ZSM Mol 20090471	AB35081802	KC984293	JQ410954	JQ410953	-
	ZSM Mol 20090472	AB35081838	-	JQ410956	JQ410955	-
Pontohedyle wiggi sp.nov.	ZSM Mol 20100595	AB34402059	-	JQ410960	JQ410959	JQ410908
	ZSM Mol 20100596	AB34402001	-	-	JQ410961	JQ410909
	ZSM Mol 20100597	AB34500571	-	JQ410963	JQ410962	JQ410910
	ZSM Mol 20100603	AB34402020	-	JQ410965	JQ410964	JQ410911
Pontohedyle wenzli sp.nov.	ZSM Mol 20100592	AB34402021	KC984294	JQ410958	JQ410957	JQ410907
	AM C. 476051.001	AB34402037	KC984295	JQ410982	JQ410981	-
	ZSM Mol 20081014	AB35081827	KC984296	JQ410969	JQ410968	JQ410913
	ZSM Mol 20100379	AB34500521	KC984297	JQ410973	JQ410972	JQ410915
Pontohedyle peteryalli sp. nov.	ZSM Mol 20071133	AB34404268	KC984298	-	JQ410930	JQ410899
Pontohedyle martynovi sp. nov.	AM C. 476054.001	AB34402062	-	JQ410984	JQ410983	-
Pontohedyle yurihookeri sp. nov.	ZSM Mol 20080565	AB34402000	KC984299	JQ410987	-	-

Museum numbers (ZSM – Bavarian State Collection of Zoology, SI – Smithsonian Institute, AM - Australian Museum), DNA vouchers (at ZSM) and GenBank accession numbers. 18S rRNA sequences generated in this study marked with *, all remaining sequences retrieved from GenBank.

Table 2 Molecular diagnostic characters of *Pontohedyle*

Marker	Diagnostic characters with position in alignment (in reference sequence)
18S rRNA	165 (168), G; 1358 (1365), A; 1360 (1367), T; 1371 (1378), T; 1514 (1521), T
28S rRNA	260, C; 576, T; 622, T

28S rRNA based on ZSM Mol 20071820 (GenBank JQ410978), ZSM Mol 20080176 (GenBank JQ410980), ZSM Mol 20071135 (GenBank JQ410971), ZSM Mol 20100389 (GenBank JQ410974) and ZSM Mol 20100390 (GenBank JQ410975), in mitochondrial 16S rRNA based on ZSM Mol 20071820 (GenBank JQ410977), ZSM Mol 20080176 (GenBank JQ410979), ZSM Mol 20071135 (GenBank JQ410970) and ZSM Mol 20100391 (GenBank JQ410976) and in mitochondrial COIbased on ZSM Mol 20071820 (GenBank JQ410920), ZSM Mol 20080176 (GenBank JQ410921), ZSM Mol 20071135 (GenBank JQ410914), ZSM Mol 20100388 (GenBank JQ410916), ZSM Mol 20100389 (GenBank JQ410917), ZSM Mol 20100390 (GenBank JQ410918) and ZSM Mol 20100391 (GenBank JQ410919).

Pontohedyle brasilensis (Rankin, 1979)

Microhedyle milaschewitchii (Kowalevsky) – *sensu* Marcus (1953: pp. 219–220) [71]
Gastrohedyle brasilensis Rankin, 1979: p. 101 [62]
Pontohedyle milaschewitchii (Kowalevsky) – *sensu* Jörger et al. (2007) [56], *partim*: all Western Atlantic specimens.

Type locality: Shell gravel, intertidal, Vila, Ilhabela, São Paulo, Brazil.

Type material: No type material remaining in Marcus' collection (pers. comm. Luiz Simone). We nevertheless refrain from designating a neotype, since we lack material from the type locality.

Distribution and habitat: Caribbean Sea to southern Brazil [25,72]; marine, interstitial, intertidal to subtidal, coarse sand and shell gravel.

Sequenced material: Despite a series of recollecting attempts at the type locality and its vicinity in the past five years, we were unable to recollect any specimen of *Pontohedyle* in Southern Brazil. Our reference sequence refers to the southern-most specimen of a Western Atlantic *Pontohedyle* clade (see Figure 4), herein assigned to *P. brasilensis* (see Discussion). Additional material was collected at different collecting sites in the Caribbean (see Figure 4 for collecting sites and Figure 2C for photograph of a living specimen and SEM of radula).

Molecular diagnosis is given in Table 5.

Diagnostic characters in nuclear 18S rRNA were determined based on ZSM Mol 20110722 from Pernambuco, Brazil (GenBank KC984285 = reference sequence), ZSM Mol 20110723 (GenBank KC984284), SI-CBC2010KJ01-E03 (GenBank KC984283), ZSM Mol 20080198 (Gen Bank KC984286), in nuclear 28S rRNA based on ZSM Mol 20110722 (GenBank JQ410932); ZSM Mol 20090198 from St. Lucia Caribbean (GenBank JQ410936 = reference sequence); SI-CBC2010KJ01-E03 (GenBank JQ410941); SI-CBC2010KJ01-B07 (GenBank JQ410943), SI-CBC2010 KJ01-D07 (GenBank JQ410944); SI-CBC2010KJ01-B09 (GenBank JQ410946), SI-CBC2010KJ01-C09 (GenBank JQ410948), SI-CBC2010KJ02-E01(GenBank JQ410950), ZSM Mol 20110723 (GenBank JQ410952); in mitochondrial 16S rRNA based on ZSM Mol 20110722 (GenBank JQ410931 = reference sequence); ZSM Mol 20090198 (GenBank JQ410935); SI-CBC2010KJ01-E03 (GenBank JQ410940); SI-CBC2010KJ01-B07 (GenBank JQ410942), SI-CBC2010KJ01-B09 (GenBank JQ410945), SI-CBC2010 KJ01-C09 (GenBank JQ410947), SI-CBC2010KJ01-A10 (GenBank JQ410949), ZSM Mol 20110723 (GenBank JQ410951) and in mitochondrial COI based on ZSM Mol 20110722 (GenBank JQ410900 = reference sequence); SI-CBC2010KJ01-B09 (GenBank JQ410904); SI-CBC2010KJ01-C09 (GenBank JQ410905); ZSM Mol 20110723 (GenBank JQ410906).

Descriptions of new *Pontohedyle* species

Pontohedyle kepii sp. nov.

Pontohedyle sp. 1 (MOTU I) in [25]

Table 3 Molecular diagnostic characters of *Pontohedyle milaschewitchii*

Marker	Diagnostic characters with position in alignment (in reference sequence)	Heterogeneous single pure positions
18S rRNA	159, C; 164 (165), G	-
28S rRNA	329 (324), T	-
16S rRNA	8, G; 26, A; 145 (146), C; 203 (209), A; 243 (274), G; 275 (306), T; 290 (321), T; 333 (363), A; 352 (382), T	351 (381), T (G in ZSM Mol 20080953, position 381)
COI	11, C; 25, C; 58, T; 160, C; 272, A; 273,G; 319, T; 352, G; 371, G; 376, G; 397, A; 451, A; 476, C; 495, G; 496, G; 520, C	-
COI (AA)	4, L; 124, A; 159, L; 165, S	-

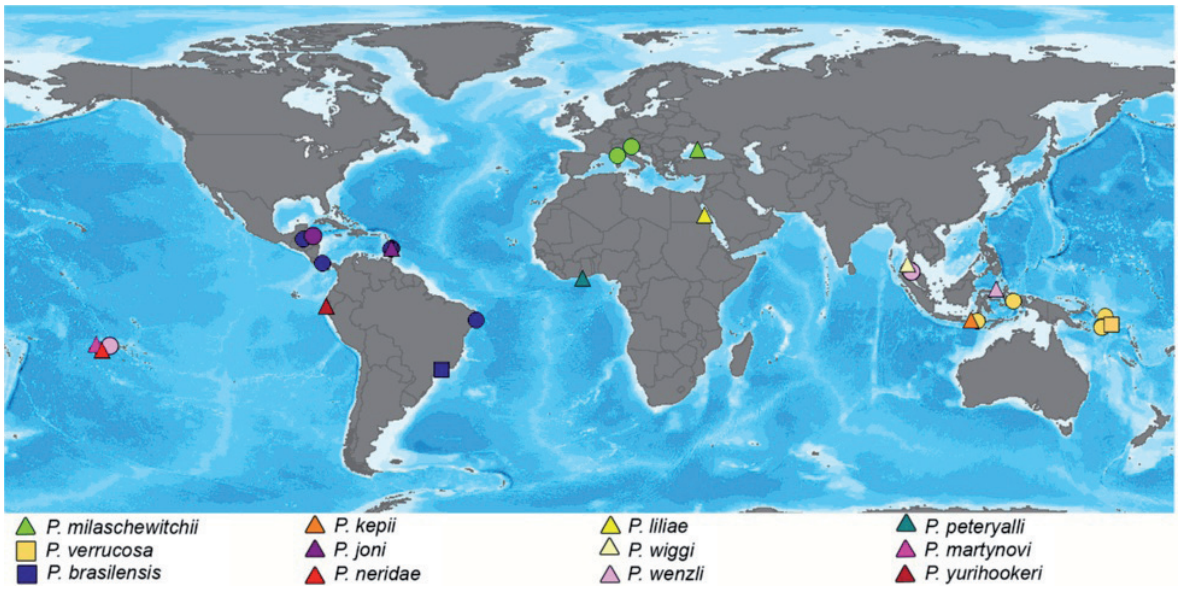

Figure 4 World map showing the sampling sites and type localities of *Pontohedyle* species (modified after [25]). Type localities with material included in this study are marked by triangles. Unsampled type localities are resembled by squares. Additional collecting sites are marked with dots.

Types: Holotype: DNA voucher (extracted DNA in buffer, stored deep frozen at -80°C) ZSM Mol 20081013 (DNA bank accession number AB35081769). Paratypes: two specimens fixed in 96% ethanol were lost during DNA extraction. Two specimens fixed in glutaraldehyde and embedded in epoxy resin (ZSM 20080877 and 20080977). ZSM 20080877 sectioned at 1 μm. One additional specimen dissolved for radula preparation, SEM stub with radula available (ZSM Mol 20131101). All material collected at type locality.

Type locality: S 8°13′59“, E 117°28′32“; Pulau Moyo, Nusa Tengarra, Indonesia, Flores Sea, Indo Pacific (see Figure 4).

ZooBank registration: urn:lsid:zoobank.org:act:694022A2-BE21-4082-8CFD-A66094740A95

Etymology: Named after our good friend and long-time diving companion, Klaus-Peter (‘Kepi’) Schaaf, who assisted us in collecting sand samples during diving in Indonesia.

Distribution and habitat: Currently known from type locality only; marine, interstitial, subtidal 5–6 m, coarse coral sand.

Description: morphologically with diagnostic characters of the genus *Pontohedyle* (see Figure 1A). Radula formula 1-1-1, rhachidian tooth with three lateral cusps, lateral plate smooth without denticle (Figure 1A).

Molecular diagnosis is given in Table 6.

Positions of the diagnostic characters refer to the sequence of the holotype. Diagnostic characters in nuclear 18S rRNA were determined based on GenBank KC984290, in 28S rRNA based on GenBank JQ410967, in mitochondrial 16S rRNA based on GenBank JQ410966, and in mitochondrial COI based on GenBank JQ410912.

Pontohedyle joni sp. nov.

Pontohedyle sp. 2 (MOTU II) in [25]

Table 4 Molecular diagnostic characters of *Pontohedyle verrucosa*

Marker	Diagnostic characters with position in alignment (in reference sequence)	Heterogeneous single pure positions
18S rRNA	-	-
28S rRNA	597 (605), T; 604 (612), G	-
16S rRNA	235, deletion; 243 (266), C; 249 (272), T; 330 (352), C	-
COI	118, A; 343, G; 367, C; 421, A; 451, C	541, T (C in ZSM 20080176, position 541)

Table 5 Molecular diagnostic characters of *Pontohedyle brasilensis*

Marker	Diagnostic characters with position in alignment (in reference sequence)	Heterogeneous single pure positions
18S rRNA	164, T; 213 (225), G; 1693 (1706), T	-
28S rRNA	648 (654), A; 653 (659), T; 678, deletion, 679 (684), T; 683 (688), T; 704 (709), C; 801 (806), T	564 (570), T (in SI-CBC2010KJ01-B09 and ZSM 20090198: A); 793 (798) , C (in SI-CBC2010KJ02-E01: T, position 682)
16S rRNA	1, T; 11, deletion; 18 (17), A ; 80 (81), T; 102 (103), G; 107 (108), T; 131, G; 142, C; 172 (173), C; 182 (184), A; 210 (212), A; 214, deletion; 288 (306), G; 308 (325), C; 359 (376), C; 369 (386), G	-
COI	4, G; 16, C; 40, C; 44, G; 46, G; 68, G; 97, C; 101, C; 102, C; 167, G; 169, C; 170, T; 197, A; 202, G; 217, A; 227, G; 228, C; 239, T; 272, G; 287, A; 295, G; 310, C; 332, T; 351, deletion; 352, deletion; 353, deletion; 357 (354), A; 358(355), G; 365 (362), T; 372 (369), T; 387 (384), C; 434 (431), G; 456 (453), G; 457 (454), G; 467 (464), G; 482 (479), T; 483 (480), G; 497(494), C; 499 (496), T; 512 (509), T; 518 (515), A; 529 (526), A; 535 (532), G; 542 (539), T; 543 (540), C; 566 (563), C; 619 (616), G; 635 (632), G	70, A (in ZSM Mol 20110722, G); 205, T (in ZSM Mol 20110722, C); 517, T (in ZSM Mol 20110722, C);
COI (AA)	4, I; 15, A; 23, V; 32, T; 34, P; 56, V; 57, L; 66, I; 76, A; 80, L; 91, A; 96, M; 111, L; 118, E; 119, deletion; 124 (123), F; 129 (128), A; 145 (144), V; 152 (151), W; 156 (155), A; 161 (160), W; 171 (170), L; 173 (172), I; 176 (175), L; 189 (188), L; 212 (211), V	-

Types: Holotype: DNA voucher (extracted DNA in buffer) ZSM Mol 20090197 (DNA bank accession number AB34858164). Paratype: one specimen fixed in 96% ethanol, collected with the holotype.

Type locality: N 14°3′34.56", W 60°58′18.24"; near Castries, St. Lucia, Central America, Caribbean Sea, West Atlantic Ocean (see Figure 4).

Additional material: DNA voucher (extracted DNA in buffer) SI-CBC2010KJ01-D05 (DNAbank at ZSM AB34402049) and SEM preparation of radula (ZSM Mol 20131102) from N 16°48′13.44", W 88°4′36.9", and DNA voucher (extracted DNA in buffer) SI-CBC2010KJ01-C08 (DNAbank AB34402065) from N 16°48′7.62", W 88°4′ 36.42" both Carrie Bow Cay, Belize, Central America, Caribbean Sea, West Atlantic Ocean.

ZooBank registration: urn:lsid:zoobank.org:act:73AAC79D-5A43-40E4-B0D6-0329CAAA2AA0

Etymology: Named after Dr. Jon Norenburg to honor his efforts and enthusiasm for meiofaunal research and to thank him for his support for uncovering the largely unknown Caribbean meiofauna.

Distribution and habitat: Currently known from the Caribbean Sea (St. Vincent and Belize), type locality subtidal, 2–3 m depth, sand patches between seagrass, coarse sand. Additional material also subtidal, 14–15 m, sand patches between corals, coarse sand.

Description: morphologically with diagnostic characters of the genus *Pontohedyle*. Radula formula 48 × 1-1-1, rhachidian tooth with 3 lateral cusps, lateral plate with one pointed denticle (see Figure 1B).

Molecular diagnosis is given in Table 7.

The sequences retrieved from the holotype ZSM Mol 20090197 serve as reference sequences. Diagnostic characters in nuclear 18S rRNA were determined based on ZSM Mol 20090197 (GenBank KC984291) and SI-CBC2010KJ01-D05 (GenBank KC984292), in nuclear 28S rRNAbased on ZSM Mol 20090197 (GenBank JQ410934) and SI-CBC2010KJ01-C08 (GenBank JQ410939), in mitochondrial 16S rRNA based on ZSM Mol 20090197 (GenBank JQ410933), SI-CBC2010KJ01-D05 (GenBank JQ410937) and SI-CBC2010KJ01-C08 (GenBank JQ41 0938), and in mitochondrial COI based on ZSM Mol 20090197 (GenBank JQ410901), SI-CBC2010KJ01-D05 (GenBank JQ410902) and SI-CBC2010KJ01-C08 (GenBank JQ410903).

Table 6 Molecular diagnostic characters of *Pontohedyle kepii* sp. nov.

Marker	Diagnostic characters with position in alignment (in reference sequence)
18S rRNA	199 (182), G; 202 (185), C; 203, deletion; 204, deletion; 206, deletion; 254 (244), T; 707 (697), T; 1355 (1345), A; 1356 (1346), C
28S rRNA	410 (439), T; 419 (448), C; 719 (754), G; 867 (902), C
16S rRNA	11, T; 184 (189), A; 187 (192), C; 239 (267), A; 242, deletion; 243, deletion; 244, deletion; 294 (324), G; 302 (328), G
COI	49, A; 79, T; 118, C; 148, C; 160, A; 193, G; 292, G; 331, G; 466, T; 494, G; 583, G; 628, A; 638, C
COI (AA)	165, D

Pontohedyle neridae sp. nov.

Pontohedyle sp. 3 (MOTU III) in [25]

Types: Holotype: DNA voucher (extracted DNA in buffer, stored deep frozen at -80°C) AM C. 476062.001 (DNA bank accession number at ZSM AB34500497).

Table 7 Molecular diagnostic characters of *Pontohedyle joni* sp. nov.

Marker	Diagnostic characters with position in alignment (in reference sequence)	Heterogeneous single pure positions
18S rRNA	207 (215), T; 209 (217), T; 256 (263), A	-
28S rRNA	443 (446), A; 547 (556), T; 868 (873), A	
16S rRNA	44 (47), C; 122 (125), T; 141 (144), A; 142 (145), G; 143 (146), G; 146, G; 152 (157), A; 182 (188), T; 236 (252), A; 259 (284), C	181 (187), T (in SI-CBC20 10KJ01-C08, C at position 187)
COI	31, A; 85, G; 160, G; 283, G; 298, G; 451, G; 523, C; 526, A; 578, C; 580, T	

Paratype: one specimen fixed in 5% formalin and embedded in epoxy resin (AM C.476063.001), collected with the holotype.

Type locality: S 17°32′50.172", W 149°46′35.4"; Motu Iti, Moorea, Oceania, Central Pacific Ocean (see Figure 4).

ZooBank registration: urn:lsid:zoobank.org:act:BE3E7920-5451-429D-95E4-C8D2F859C7CB

Etymology: Named after our friend and colleague, Dr. Nerida Wilson, with a big 'thank you' for actively sharing with us the fascination for interstitial Acochlidia.

Distribution and habitat: Known from type locality only; subtidal 3-4 m, fine to medium coral sand.

Description: Morphologically with diagnostic characters of the genus *Pontohedyle*. Radula characteristics unknown.

Molecular diagnosis is given in Table 8.

The sequences retrieved from the holotype serve as reference sequences. Diagnostic characters in nuclear 28S rRNA were determined based onAM C. 476062.001 (GenBank JQ410986), in mitochondrial 16S rRNA based on AM C. 476062.001 (GenBank JQ410985), and in mitochondrial COI based on AM C. 476062.001 (GenBank JQ410922).

Pontohedyle liliae sp. nov.

Pontohedyle sp. 4 (MOTU IV) in [25]

Types: Holotype: DNA voucher (extracted DNA in buffer, stored deep frozen at -80°C) ZSM Mol 20090471 (DNA bank accession number AB35081802). Paratypes (all collected with the holotype): DNA voucher (extracted DNA in buffer) ZSM Mol 20090472 (DNA bank accession number AB35081838), one additional specimen used for radula preparation, SEM stub with radula available (ZSM Mol 20131103).

Table 8 Molecular diagnostic characters of *Pontohedyle neridae* sp. nov.

Marker	Diagnostic characters with position in alignment (in reference sequence)
28S rRNA	61 (57), G; 522 (518), A
16S rRNA	11, G; 121 (123), T; 145 (147), T; 147 (149), G; 252 (276), C; 263 (286), T; 330 (352), G; 336 (358), G
COI	46, C; 151, C; 169, G; 220, A; 277, C; 278, T; 289, T; 391, C; 397, G; 421, C; 479, T; 505, A; 601, C

Type locality: N 24°11′50", E 35°38′26" (approximation from Google Earth), Sha'ab Malahi, Egypt, Africa, Red Sea (see Figure 4).

ZooBank registration: urn:lsid:zoobank.org:act:2711E3E5-1D1D-41B0-B919-7D7E690FD525

Etymology: Named after Reinhilde ('Lili') Schmid, our friend and diving companion, who assisted us during sand collecting in Egypt and shares our fascination for this world of little creatures.

Distribution and habitat: Known from type locality only; subtidal 20 m, relatively fine coral sand.

Description: Morphologically with diagnostic characters of the genus *Pontohedyle*. Radula formula 45 × 1-1-1, rhachidian tooth with three (to four) lateral cusps, lateral plate with one pointed denticle (Figure 1C). Eyes clearly visibly externally, monaxone spicules in accumulation between oral tentacles and irregular all over the body.

Molecular diagnosis is given in Table 9.

The sequences retrieved from the holotype (ZSM Mol 20100471) serve as reference sequences. Diagnostic characters in nuclear 18S rRNA were determined based on ZSM Mol 20100471 (GenBank KC984293), in nuclear 28S rRNA based on ZSM Mol 20100471 (GenBank JQ410954) and ZSM Mol 20100472 (GenBank JQ410956), and in mitochondrial 16S rRNA based on ZSM Mol 20100471 (GenBank JQ410953) and ZSM Mol 20100472 (GenBank JQ410955).

Pontohedyle wiggi sp. nov.

Pontohedyle sp. 5 (MOTU V) in [25]

Types: Holotype: DNA voucher (extracted DNA in buffer) ZSM Mol-20100595 (DNA bank accession number AB34402059). Paratypes (all collected with the holotype): DNA voucher (extracted DNA in buffer) ZSM Mol-20100596 (DNA bank AB34402001), ZSM Mol 20100597 (DNA bank AB34500571), ZSM Mol 20100603 (DNA bank AB34402020); one specimen fixed in glutaraldehyde and embedded in epoxy resin (ZSM Mol 20100598).

Table 9 Molecular diagnostic characters of *Pontohedyle liliae* sp. nov.

Marker	Diagnostic characters with position in alignment (in reference sequence)
18S rRNA	33, C; 40, C; 54, G; 117, T; 129, T; 146 (147), C; 149 (150), T; 186 (187), C; 214 (223), A; 215 (224), C; 623 (631), T; 663 (673), T; 677 (687), C; 841 (853), G; 959 (971), G; 1028 (1040), T; 1030 (1042), C; 1348 (1360), A; 1363 (1375), T
28S rRNA	34 (30), C; 63 (59), C; 536 (532), T; 537 (533), G; 542, deletion; 555 (554), G; 590 (589), T; 642 (641), C; 643 (642), T; 658 (657), A; 671 (670), C; 696 (695), A; 827, G; 837, C; 902 (904), C
16S rRNA	10, C; 211 (222), C; 246 (277), C; 330 (359), T; 336 (365), C; 357 (386), C

Type locality: N 7°36′15“, E 98°22′37“, Ko Raccha Yai, Phuket, Thailand, Andaman Sea, Indian Ocean (see Figure 4).

ZooBank registration: urn:lsid:zoobank.org:act:808E562E-0E1A-4D79-BB2C-1377B3734F86

Etymology: Named in memory of Ludwig (‘Wigg’) Demharter, a malacologist friend, passionate diver, ‘fun researcher’, and for many years a supporter of the ZSM and the second author's working group.

Distribution and habitat: Known from the type locality only; marine, interstitial between sand grains, relatively fine coral sand, subtidal 6–7 m depth, sandy slope among patches of corals.

Description: Morphologically with diagnostic characters of the genus *Pontohedyle*. Radula formula 1-1-1, lateral plate with one pointed denticle (as in *P. milas chewitchii*). Eyes visibly externally, monaxone spicules present.

Molecular diagnosis is given in Table 10.

The sequences retrieved from the holotype (ZSM Mol 20090595) serve as reference sequences. Diagnostic characters in nuclear 28S rRNA were determined based on ZSM Mol 20100595 (GenBank: JQ410960), ZSM Mol 20100597 (GenBank: JQ410963), ZSM Mol 20100603 (GenBank: JQ410965), in mitochondrial 16S rRNA based on ZSM Mol 20100595 (GenBank: JQ410959), ZSM Mol 20100596 (GenBank: JQ410961), ZSM Mol 20100597 (GenBank: JQ410962), ZSM Mol 20100603 (GenBank: JQ410964), and in mitochondrial COI based on ZSM Mol 20100595 (GenBank: JQ410908), ZSM Mol 20100596 (GenBank: JQ410909), ZSM Mol 20100597 (GenBank: JQ410910), ZSM Mol 20100603 (GenBank: JQ410911).

Table 10 Molecular diagnostic characters of *Pontohedyle wiggi* sp. nov.

Marker	Diagnostic characters with position in alignment (in reference sequence)
28S rRNA	483 (472), T; 508 (497), T; 536, deletion; 537, deletion; 538, deletion; 699 (687), A
16S rRNA	180 (188), C; 374 (406), T
COI	127, C; 325, A; 583, C
COI (AA)	29, T

***Pontohedyle wenzli* sp. nov.**

Pontohedyle sp. 6 (MOTU VIII) in [25]

Types: Holotype: DNA voucher (extracted DNA in buffer) ZSM Mol 20100379 (DNA bank accession number AB34500521).

Type locality: N 1°27′53“, E 125°13′48“, Lembeh Strait, Sulawesi, Indonesia, Banda Sea, West Pacific Ocean (see Figure 4).

Additional material DNA voucher (extracted DNA in buffer) ZSM Mol 20081014 (DNA bank accession number AB35081827) and one specimen used for SEM preparation of radula (available at ZSM Mol 20131105), locality S 8°23′58“, E 119°18′56“, Pulau Banta, Nusa Tengarra, Indonesia Flores Sea, Indo-Pacific. DNA voucher (extracted DNA in buffer) ZSM 20100592 (DNA bank AB34402021), locality N 7°36′15“, E 98°22′37“, Ko Raccha Yai, Phuket, Thailand, Andaman Sea, Indian Ocean. DNA voucher (extracted DNA in buffer) AM C. 476051.001 (DNA bank AB34402037) and one specimen fixed in 5% formalin and embedded in epoxy resin (AM C.476050.001), locality S 17°28′33.96”, W 149°49′51.6”, E of Cook’s Bay Pass, Moorea, Oceania, Central Pacific.

Note: Most species delineation approaches suggested ZSM 20100592, and some also AM C. 476051.001, as an independently evolving lineage [25]. Due to the conservative consensus approach, these specimens were included in the described species. Future analyses might show that their separation as independent species is warranted.

ZooBank registration: urn:lsid:zoobank.org:act:558EC548-1FB3-4B00-B248-4424CA7B098C

Etymology: Named after Alexander Wenzl, for his support during the development of this manuscript and his interest for meiofaunal research.

Distribution and habitat: Known from Indonesia, with putative distribution across the Indo-Pacific and Central Pacific; marine, subtidal (3–22 m), interstitial, coarse sand and shell grid.

Description: Morphologically with diagnostic characters of the genus *Pontohedyle*, eyes clearly visible externally (see Figure 2B, picture of living holotype). Radula 43 × 1-1-1, rhachidian tooth with three lateral cusps, lateral plate with pointed denticle (like in *P. milaschewitchii*).

Molecular diagnosis is given in Table 11.

The sequences retrieved from the holotype (ZSM Mol 20100379) serve as reference sequences. Diagnostic characters in nuclear 18S rRNA were determined based on ZSM Mol 20100379 (GenBank KC984297), ZSM Mol 20081014 (GenBank KC984296), ZSM Mol 20100592 (GenBank KC984294), AM C. 476051.001 (GenBank

Table 11 Molecular diagnostic characters of *Pontohedyle wenzli* sp. nov.

Marker	Diagnostic characters with position in alignment (in reference sequence)	Heterogeneous single pure positions
18S rRNA	771 (791), T; 772 (792), T	-
28S rRNA	449 (455), C; 539 (545), A	-
16S rRNA	36, G; 41, T; 84 (88), A; 143 (147), A; 144 (148), A; 161 (167), T; 176 (182), A; 194 (201), T; 207 (214), A; 256 (296), C; 258 (298), A; 269 (309), T; 295, deletion; 331 (369), A; 340 (378), A	332 (370), A (ZSM Mol 20081014, G at position 370)
COI	181, A; 218, G; 219, T; 296, T; 383, C; 430, T; 593, A	-
COI (AA)	73, V; 94, F; 122, A; 198, I	-

KC984295), in nuclear 28S rRNA based on ZSM Mol 20100379 (GenBank JQ410973), ZSM Mol 20081014 (GenBank JQ410969), ZSM Mol 20100592 (GenBank JQ410958), AM C. 476051.001 (GenBank JQ410982), in mitochondrial 16S rRNA based ZSM Mol 20100379 (GenBank JQ410972), ZSM Mol 20081014 (GenBank JQ410968), ZSM Mol 20100592 (GenBank JQ410957), AM C. 476051.001 (GenBank JQ410981), and in mitochondrial COI based on ZSM Mol 20100379 (GenBank JQ410915), ZSM Mol 20081014 (GenBank JQ410913), ZSM Mol 20100592 (GenBank JQ410907).

Pontohedyle peteryalli sp. nov.

Pontohedyle sp. 7 (MOTU VII) in [25]

Types: Holotype: DNA voucher (extracted DNA in buffer) ZSM Mol 20071133 (DNA bank accession number AB34404268). Paratypes (all collected with the holotype): eight specimens preserved in 96% ethanol (ZSM Mol 20070827); four in 75% ethanol (ZSM Mol 20070827), sixteen specimens fixed in glutaraldehyde, post-fixed in osmium and embedded in epoxy resin (ZSM Mol 20080453–60; ZSM Mol 20080462–69). SEM stub with radula available (ZSM Mol 20131104).

Type locality: N 04°47′46”, W 02°10′06”, MiaMia, Ghana, Africa, Gulf of Guinea, East Atlantic Ocean (see Figure 4).

Additional material: six specimens in 75% Ethanol collected at Nzema Cape, Ghana, Africa, Gulf of Guinea, East Atlantic Ocean; conspecifity still needs to be confirmed via barcoding.

ZooBank registration: urn:lsid:zoobank.org:act:B25E50F7-F0D2-4842-B6C3-5A79EA784A0C

Etymology: Named for our friend and malacologist, Peter (‘Pete’) Ryall, who invited us to explore sea slugs right in front of his MiaMia home.

Distribution and habitat: Currently only known from the Ghana West Coast around MiaMia, marine, interstitial, subtidal 2-3 m, fine sand.

Description: Morphologically with diagnostic characters of the genus *Pontohedyle*. Radula 42 × 1-1-1, rhachidian tooth with three lateral cusps, lateral plate with pointed denticle (like in *P. milaschewitchii*), see Figure 2A.

Molecular diagnosis is given in Table 12.

The sequences retrieved from the holotype (ZSM Mol 20071133) serve as reference sequences. Diagnostic characters in nuclear 18S rRNA were determined based on GenBank KC984298, in mitochondrial 16S rRNA based GenBank JQ410930 and in mitochondrial COI based on GenBank JQ410899.

Pontohedyle martynovi sp. nov.

Pontohedyle sp. 8 (MOTU IX) in [25]

Types: Holotype: DNA voucher (extracted DNA in buffer) AM C. 476054.001 (DNA bank accession number at ZSM AB34402062). Paratype: one specimen fixed in 5% formalin embedded in epoxy resin (AM C.476053.001), collected together with the holotype.

Type locality: S 17°28′17”, W 149°48′42”, E of Cook’s Bay Pass, Moorea, Oceania, Central Pacific Ocean (see Figure 4).

ZooBank registration: urn:lsid:zoobank.org:act:9431E4B8-EAF3-4E29-9993-BCD7C52928C6

Etymology: Named to thank our Russian friend and taxonomist, Alexander (‘Sasha’) Martynov, for collecting acochlidians for us in many places, including *Pontohedyle milaschewitchii* at its type locality.

Distribution and habitat: Known from type locality only; marine, interstitial, subtidal 18–20 m, coarse sand, shell grid and rubble.

Description: Morphologically with diagnostic characters of the genus *Pontohedyle*. Radula characteristics unknown.

Molecular diagnosis is given in Table 13.

The sequences retrieved from the holotype (AM C. 476054.001) serve as reference sequences. Diagnostic characters in nuclear 28S rRNA were determined based on GenBank JQ410984, and in mitochondrial 16S rRNA based on GenBank JQ410983.

Pontohedyle yurihookeri sp. nov.

Pontohedyle sp. 9 (MOTU X) in [25]

Table 12 Molecular diagnostic characters of *Pontohedyle peteryalli* sp. nov.

Marker	Diagnostic characters with position in alignment (in reference sequence)
18S rRNA	160, C; 164, C
COI	14, T; 23, A; 48, C; 68, A; 76, C; 81, T; 83, A; 95, T; 101, A; 102, G; 140, A; 141, C; 167, A; 187, C; 209, C; 232, C; 280, A; 286, C; 293, A; 294, G; 357, C; 358, A; 361, A; 365, A; 373, A; 433, C; 448, G; 467, A; 468, T; 487, T; 503, T; 504, G; 512, A; 535, C; 556, C; 574, A; 586, C; 628, C; 634, C
COI (AA)	5, L; 8, I; 16, A; 23, I; 27, V; 28, T; 32, S; 34, S; 47, T; 56, I; 70, L; 119, T; 156, I; 162, D; 168, C; 171, I

Types: Holotype: DNA voucher (extracted DNA in buffer) ZSM Mol 20080565 (DNA bank accession number AB34402000).

Type locality: S 3°58′55”, W 80° 59′10”, Punta Sal, Peru, South America, East Pacific Ocean (see Figure 4).

ZooBank registration: urn:lsid:zoobank.org:act:9B858AA5-59FA-4505-AE94-FB2EA27FBEF6

Etymology: Named for our Peruvian friend and marine biologist, Yuri Hooker, who joined us during a great diving expedition to explore the Peruvian sea slug fauna.

Distribution and habitat: Known from type locality only; marine, interstitial, subtidal (8 m), coarse sand.

Description: Morphologically with diagnostic characters of the genus *Pontohedyle*. Radula characteristics unkown.

Molecular diagnosis is given in Table 14.

The sequences retrieved from the holotype (ZSM Mol 20080565) serve as reference sequences. Diagnostic characters in nuclear 18S rRNA were determined based on GenBank KC984299, and in nuclear 28S rRNA based on GenBank JQ410987.

Discussion

Cryptic species challenging traditional taxonomy

Largely due to the development of molecular methods, research on cryptic species has increased over the past two decades [8,9], demonstrating their commonness across Metazoan taxa, though with random or non-random distribution among taxa and biomes still to be investigated [9,10]. Several recent studies have underlined that there is a large deficit in alpha taxonomy and that the diversity of marine invertebrates and especially meiofaunal animals might be much higher than expected, partly caused by high proportions of cryptic species e.g., [11,13,14,25,73-75]. Rather than global, amphi-Oceanic, circum-tropical or otherwise wide ranging, the distribution areas of the biological meiofaunal species involved may be regional and their ecology more specialized [12,25,76]. At an initial stage of molecular and ecological exploration, cryptic meiofauna is potentially threatened by global change and cannot effectively be included in conservation approaches.

Table 13 Molecular diagnostic characters of *Pontohedyle martynovi* sp. nov.

Marker	Diagnostic characters with position in alignment (in reference sequence)
28S rRNA	539 (541), C; 623 (629), A
16S rRNA	8, deletion; 33 (32), T; 130 (131), C; 144, deletion; 151 (155), G; 168 (172), G; 171 (175), A; 218 (232), A; 230, T; 232 (244), G; 235 (258), C; 242 (274), C; 332 (365), C; 334 (367), G; 353 (386), G; 373 (408), G

Table 14 Molecular diagnostic characters of *Pontohedyle yurihookeri* sp. nov.

Marker	Diagnostic characters with position in alignment (in reference sequence)
18S rRNA	163 (156), T; 200 (193), A; 213 (225), A; 770 (783), T; 810 (823), T
28S rRNA	110 (139), A; 398 (427), T; 399 (428), T; 403 (432), T; 409 (438), A; 410, deletion; 413 (441), G; 436 (464), T; 445, deletion; 446, deletion; 447 (473), C; 449 (475), A; 451 (477), A; 452 (478), A; 457 (483), A; 460 (486), T; 477 (503), C; 563 (593), T

In traditional taxonomy, most species descriptions are based on morphological and anatomical characters. Morphological species delineation, however, can fail to adequately address the diversity of life on Earth by leaving cryptic species unrevealed. Many taxonomists agree that the future of taxonomic descriptions should be integrative, embracing all available data sources (morphology, molecular sequences, biogeography, behavioral traits...) that can contribute to species delineation [1-3]. Previous authors have argued that ‘integrative taxonomy’ does not necessarily call for a maximum of different character sets, but rather requires the taxonomist to select character sets adequate for species delineation in the particular group of taxa [3,5]. Thus, there should be no obligation in taxonomic practice to stick to morphology as the primary source [77], and there are no official requirements by the International Code of Zoological Nomenclature to do so [78,79].

The results of Jörger et al. [25] indicate that the members of *Pontohedyle* slug lineages are so extremely uniform that conventional taxonomic characters (i.e. external morphology, radula characteristics, spicules) fail to delineate species. A series of studies have demonstrated the generally high potential of advanced 3D-microanatomy for character mining in Acochlidia (e.g., [80-82]). However, the exclusively mesopsammic microhedylacean Acochlidia form an exception, as they show reduced complexity in all organ systems and uniformity that leaves few anatomical features for species delineation even on higher taxonomic levels [83]. Based on previous histological comparisons, Jörger et al. [56] were unable to find any morphological characters justifying discrimination between the closely related western Atlantic *P. brasilensis* and its Mediterranean congener, *P. milaschewitchii*. Here, we provided a detailed histological (re-)description using 3D-reconstruction

based on serial semi-thin sections of *P. verrucosa*, to evaluate whether advanced 3D-microanatomy provides distinguishing morphological characters for the two generally accepted species, *P. milaschewitchii* and *P. verrucosa*, as representatives of the two major *Pontohedyle* clades (see [25], Figure 1). Indeed, we revealed some putative distinguishing features in the reproductive and digestive systems (see Table 15). However, the encountered (minor) morphological differences are problematic to evaluate in the absence of data on ontogenetic and intraspecific variation, and on potential overlap with interspecific differences. For example, slight differences in the reproductive system could be due to different ontogenetic stages, therefore presently they cannot be used to discriminate species. Comparatively investigated serial semi-thin sections of *Pontohedyle kepii* sp. nov. also confirmed the similarity in all major organ systems reported previously [55,56]. We thus conclude that in *Pontohedyle* even advanced microanatomy is inefficient or even inadequate for species diagnoses. Molecular character sets currently offer the only chances for unambiguous discrimination between the different evolutionary lineages. Proponents of morphology based alpha taxonomy [84] might argue that we have not attempted a fully integrative approach since we have not performed 3D-microanatomy on all proposed new species, including enough material for intra-specific comparisons, ultrastructural data on, e.g., cilia, sperm morphology or specific gland types, to reveal whether these forms indeed represent cryptic species. However, in light of the biodiversity crisis and the corresponding challenges to taxonomy, we consider it as little effective to dedicate several years of a taxonomist's life to the search for morphological characters, when there is little to expect, while molecular characters enable straightforward species delineation. This is not a plea to speed up description processes at the expense of accuracy and quality, or by allowing ignorance of morphology, but for a change in taxonomic practice to give molecular characters similar weight as morphological ones, in cases in which this is more informative or practical.

Still debated is the way how the traditional Linnaean System needs to be adapted to incorporate different character sets, in the first place the growing amount of molecular data. Probably the most radical way ignores the character-based requirements of the International Code of Zoological Nomenclature [78,79] and proposes to base descriptions of new species directly on support values under species delineation models [85,86]. Aside from the paradigm shift this would bring, far away from long-standing taxonomic practice, opponents criticize that unambiguous allocation of newly collected material is impossible in the absence of definitions and descriptors and requires repetition of the species delineation approach applied [50]. As a method of species delineation, coalescent based approaches are objective and grounded on evolutionary history and population genetics [86,87]; thus it is indeed tempting to use results derived from molecular species delineations approaches directly as species descriptions ('model-based species descriptions' [87]). This would clearly facilitate descriptions, thus reduce the taxonomic impediment and the risk of an endless number of discovered but undescribed candidate species. Every species description should aim for differentiation from previously described species; therefore, diagnostic characters are usually derived from comparisons to other, closely related species. Nevertheless, the species description itself has to be self-explanatory and should not rely on comparative measurements which are only valid in comparison to a special set of other species used for a certain analysis, i.e. on a complex construct that may not be reproducible when new data are added. In contrast to Fujita & Leaché [87], we believe that each species, i.e. separately evolving lineage [4], will present – in the current snap-shot of evolutionary processes – fixed diagnostic characters of some sort (e.g., from morphology, DNA sequence information, behavioral, karyology…), and we consider it the task of modern taxonomy to detect the most reliable and efficient set of characters on which to found species descriptions.

The Characteristic Attribute Organization System (CAOS) [51,57,58] is a character based method proposed

Table 15 Putative distinguishing features between *P. milaschewitchii* and *P. verrucosa* (intraspecific variation not evaluated)

	***P. milaschewitchii* (Kowalevsky, 1901)**	***P. verrucosa* (Challis, 1970)**
Data source	Jörger et al. 2008 [55]	Present study
Epidermal glands	Predominantly whitish, blue stained only in one small row	Predominantly whitish and numerous dark blue stained ones
Nervous system	Eyes pigmented and externally visible	Eyes unpigmented
Reproductive system	Only one cephalic male genital opening detected	Two male genital openings (cephalic and visceral)
Digestive system/ putatively different feeding habits	Lateral radula teeth with central denticle	Lateral radula teeth without denticle
	Lipid-like droplets in digestive gland	Refracting fusiform structures

for uniting species discovery and description [88]. As an approach to species delineation, we consider it inferior to coalescent based approaches (e.g., GMYC and BP&P); CAOS successfully determines putative diagnostic nucleotides, but is not predictive, i.e. lacks objective criteria with which to delimit a threshold number of distinguishing nucleotides that would indicate a species boundary. One has to distinguish between diagnosability of entities and the delimitation of species. Diagnostic characters of whatever sort can be found for all levels in the hierarchical classification, but there is no objective criterion for determining a number of characters needed to characterize a (new) species, e.g. versus a population. Nevertheless, for the purpose of species description, we think that character based approaches like CAOS are highly valuable and should complement molecular species delineation procedures, thus enabling the transition from species discovery to description.

Requirements of molecular taxonomy

While calls for replacing the Linnaean system by a DNA sequence based one [41] have trailed away, we still lack a common procedure on how to include molecular data into the Linnaean system [21]. Like any other source of data, molecular data is not explicitly treated by the International Code of Zoological Nomenclature, there are no provisions dictating the choice of characters [78,79]. Currently, molecular data are included in species descriptions in various mutually inconsistent ways [21]. If DNA sequence data are only used as additive to, e.g., morphology based species descriptions or molecular species delineation approaches to confirm pre-identified entities, the addition is straightforward and requires no specific considerations. But if molecular sequence information is to be used as the partial or even sole content of a species description, a discussion of the corresponding best practice is needed.

Type material for species based on molecular data

Previous authors highlighted the need for voucher material in molecular studies [89]. Ideally, DNA is extracted from (a subsample of) a name-bearing type specimen (holotype, syntype, lectotype or neotype); if no such specimen is available for molecular studies, an attempt should be made to collect fresh material at the type locality. If parts of larger animals belonging to putative new species are used for DNA extraction, DNA and remaining specimen can both become part of the type material under nomenclatural rules. However, where the members of a putatively new species, e.g. of meiofauna, are so small that molecular extraction from only part of an individual is impossible, taxonomists may be confronted with the critical decision to either have DNA without a morphological type specimen or a type without DNA. In taxonomically unproblematic groups one can add new material or use paratypes for DNA (or other) analyses, relying on specimens to be conspecific if they were collected from 'the same population', i.e. from a place (and time) close enough to the type locality to assume gene flow. But what if, as has been shown for *Pontohedyle* slugs [25], there is a possibility of cryptic species occurring sympatrically and at the same time? Would it be better (A) to sacrifice a (single available) type specimen to obtain molecular data for species delineation or (B) to save the type and use a secondary specimen, taking the risk that the latter might not be conspecific with the former? In a group like our *Pontohedyle* slugs in which DNA sequence data are much more promising for species delineation than morphological approaches, and considering the wealth of potential DNA sequence characters, we prefer to sacrifice even single specimens to DNA extraction. In absence of a term referring to vouchers exclusively consisting of extracted DNA, we term this type material: 'DNA types'. However, prior to this, researchers should attempt an optimization of microscopical documentation (for details see [90]) and recovery of hard parts (e.g. radulae) from the spin columns used for extraction [91]. In the case of DNA aliquots serving as type material, natural history collections are urged to create long term DNA storage facilities [41,42] like the DNA bank network (http://www.dnabank-network.org/), and should apply the same caution and requirements (i.e. documentation of collection details) as for any morphological type.

Risk of two parallel taxonomies?

Old type material often does not allow molecular analyses [84,92], and searching for fresh material at a type locality can be unsuccessful. Future technical advances are likely to enable DNA acquisition from some old type material, as there has been considerable progress in dealing with degenerated DNA [93]. Nevertheless, there are the potential risks that two parallel taxonomic systems could develop, and that the one based on molecular characters could duplicate, under separate names, some taxa already established on morphological grounds [77]. Similar concerns have arisen previously when the taxonomy of certain taxa was based on a character set other than morphology (e.g. cytotaxonomy based on data from chromosomes) and the investigation of one character set hindered the exploration of the other. It clearly remains the duty of taxonomists to carefully check type material of closely related taxa before describing new species [77]. To keep molecule driven taxonomy 'workable' [94] and connected to traditional morphology based taxonomy, authors should include a brief morphological diagnosis of the (cryptic) species [77], even in the absence of species-diagnostic characters, in order to make the species recognizable as belonging to a certain group of (cryptic) species.

Trouble with names

Any specimen identified from molecular data only can belong to a previously established species or to one new to science. If unambiguous identification with a single existing species name is possible then, of course, the latter should be used. In our cases in *Pontohedyle*, we call those Indo-Pacific specimens collected near the type locality of *P. verrucosa* (Challis, 1970) on the Solomon Islands by this single available name for Indo-Pacific *Pontohedyle*. Concerning Atlantic *Pontohedyle*, the name *P. brasilensis* (Rankin, 1979), proposed for Brazilian specimens, was treated as a junior synonym of the older name, *P. milaschewitschii* (Kowalevsky, 1901). Since we have shown that *P. milaschewitschii* refers to Mediterranean and Black Sea specimens only [25], we resurrected the name *P. brasilensis* for Western Atlantic *Pontohedyle*, and now apply it to the only species in of two cryptic ones that has been collected from Brazil. In doing so we accept the risk resulting from the fact that these specimens were collected at some distance from the type locality of *P. brasilensis* (see Figure 4), as the latter has not yielded any *Pontohedyle* specimens for more than the last 50 years, despite considerable and repeated collecting efforts, including our own. These assignments of previously established species names left at least nine additional, clearly separate *Pontohedyle* species for which available names did not exist. In cases of microscopic animals such as *Pontohedyle*, molecular taxonomy thus may benefit from morphology based taxonomy having missed them in the past.

Species descriptions based on singletons

Species descriptions based on singleton specimens cannot reflect intraspecific variation, and Dayrat [1] even proposed a guideline to restrict species descriptions to well-sampled taxa. However, there is no objective way to determine any sample size at which intraspecific variation would be covered sufficiently. Moreover, excluding taxa described from singletons would lead to considerably lower, and effectively false, estimates of the scientifically known biodiversity [5,26-28]. The present study on *Pontohedyle* includes five species descriptions based on DNA sequence information from one individual only. Usually, this is done when such a singleton presents a combination of characters so discrete that it is considered highly unlikely to fall within the variational range of another species [28]. In a complex molecular species delineation approach Jörger et al. [25] recognized our five singletons as independently evolving lineages. Approximations with molecular clock analyses estimate the diversification of these species from their respective sister groups to have occurred 54–83 mya (own unpublished data), which indicates significant timespans of genetic isolation. In light of our general revision of the genus *Pontohedyle*, we consider it as less productive to keep these entities on the formally unrecognized level of candidate species than to run the risk that our species hypotheses may have to be modified due to future additional material. Nevertheless, we are well aware of the fact that taxon sampling and data acquisition (i.e. incomplete molecular data sets) are not yet ideal for some of our newly described species (e.g., *P.martynovi* sp. nov., *P. yurihookeri* sp. nov.).

What is a diagnostic character in molecular taxonomy?

In character based taxonomy, descriptions of new taxa are, or should be, based on diagnostic differences from previously known taxa. In a phenetic framework (key systematics), similarity based distinction relies on sufficient sampling and detectable degrees of difference, whereas phylogenetic taxonomy additionally presumes knowledge of character homologies and sister group relationships. In an ideal phylogenetic framework diagnoses are based on apomorphic (i.e. derived) versus homologous but plesiomorphic (ancestral) states of a given character. In molecular taxonomy, the detection of homologies and apomorphic conditions among the four character states (bases) is handicapped by the high chance of convergent multiple transformations causing homoplasy. Reconstruction of ancestral sequences to support homology and differentiate between apomorphic and plesiomorphic character states for each node is possible [95]. However, unfortunately, robust phylogenetic hypotheses with strong support values for all sister group relationships are the exception rather than the rule. Since the evaluation of a state as apomorphic highly depends on the topology, and reconstruction of ancestral nucleotides is constrained by sampling coverage, we suggest more conservative approaches for cases of unclear phylogenetic relationships, as in our study. We use diagnostic nucleotides as unique character attributes (which may be apomorphic or plesiomorphic or convergent) within a certain entity, i.e. a monophylum with strong support values. This is clearly a trade off between the number and phylogenetic significance of diagnostic characters and the degree of dependence of these characters on a certain topology, as with increasing size and diversity of the selected entity, the likelihood of homoplasy also rises [96]. To enhance the stability of our molecular taxonomic characters we chose to determine diagnostic characters of each *Pontohedyle* species in relation to all its congeners, rather than just to the respective sister taxon as is the default in CAOS. Equal character states in non-*Pontohedyle* outgroups are left unconsidered, however, due to the larger evolutionary distances and the correspondingly increased risk of homoplasies. It will be one of the major challenges for molecule driven taxonomy to select the appropriate monophylum in which all included taxa are evaluated

against each other. Rach et al. [88] addressed homoplasy within the selected ingroup by applying an 80% rule to so-called single private characters (see below). *Pontohedyle* species recognized here offered enough single pure diagnostic bases to avoid using single private characters and some further, more equivocal attributes provided by CAOS.

The Characteristic Attribute Organization System (CAOS) [51,57,58] can be used to identify diagnostic nucleotides for pre-defined taxonomic units [51]. The program offers discrimination between four types of 'character attributes' (CAs): simple (single nucleotide position) vs. compound (set of character states) and pure vs. private [51]. Pure CAs are nucleotides present in all members of a clade and absent from members of other clades; private CAs are only present in some members of the clade, but absent from others [51]. We consider only single pure CAs as eligible for diagnostic characters in DNA taxonomy, i.e. as supporting new species proposals. In our diagnoses of the new *Pontohedyle* species we emphasize those single pure CAs, which in protein coding genes code for a different amino acid. The probability of single pure CAs referring to fixed genetic differences increases exponentially with their number [88]. In our dataset, all *Pontohedyle* species have between 12 and 36 single pure CAs on independently evolving markers, which supports their treatment as genetically isolated lineages. Additionally, the CAOS program distinguishes between homogeneous pure CAs (shared by all members of the taxon under study, and not present in the outgroups) and heterogeneous pure CAs (with two or three different characters present in the taxon but absent from the outgroups). The latter characters can be treated as diagnostic, but are problematic as they may refer to convergently evolved character states. Therefore, we report them as additional information. In contrast, compound CAs can be unique for certain species, but they may have evolved from several independent mutation events. Consequently, compound CAs as an entity have low probabilities of homology; in analogy to morpho-anatomical key systematics, these compound CAs can serve for re-identification of well-sampled species, but they are not diagnostic characters in a phylogenetic sense and thus should be avoided in DNA taxonomy.

CAOS identifies discrete nucleotide substitutions at every node of a given tree and has been complemented to find diagnostic bases in a 'phylogenetic-free context' [97], referring to the difference between CAs and true apomorphies. This notion can be misleading, however, as the results provided by CAOS are one hundred percent topology dependent in only comparing sister pairs at each node. To overcome this topology dependence, we ran several analyses placing each species at the root of the ingroup, which we defined as the most inclusive secure and taxonomically relevant monophylum, in our case the genus *Pontohedyle* (see Material and Methods). This procedure of a manually iterative, exhaustive intrageneric comparison of base conditions makes the recognized single pure CAs less numerous but more rigorous than with CAOS default parameters, i.e. by decreasing the chances of homoplasy and increasing the chances of single pure CAs representing apomorphies in our wider taxon comparison.

Towards a 'best practice' in molecular taxonomy

Considering stability and traceability in future research, the presentation of the identified diagnostic nucleotides is not trivial. Some recent studies just reported the number of differing nucleotides without specifying the position and character state e.g., [98]. This is equivalent to a morphological species description that would merely refer to, e.g., 'diagnostic differences in the reproductive system' without offering any descriptive details. Other studies present part of an alignment without identifying positions, and underline putative diagnostic nucleotides e.g., [99] without explanation what determined these bases as diagnostic. This practice leaves it to future researchers to identify the proposed bases, which is highly time consuming and error-prone, especially when the original alignment is not deposited in a public database. Reporting the positions within the alignment is a step towards reproducibility and traceability of molecular diagnostic characters e.g., [94,100-102], but when new material is added that was generated with different primers or includes insertions or deletions, the critical positions are still difficult to trace. Yassin et al. [103] included the positions within a reference genome, which probably provides the greatest clarity for future research. Unfortunately, for non-model taxa closely related reference genomes which allow for unambiguous alignment of even fast evolving markers are usually unavailable. We thus suggest the following procedure for reporting positions in an alignment. (1) Clearly report primers and alignment programs, and clarify what determined position 1 (e.g., first base after the primer sequence); (2) deposit alignments in public databases or as additional material accompanying the publication's online edition. To make a diagnostic position in a sequence traceable independently from a specific alignment, we additionally recommend to (3) report the corresponding position in a deposited reference sequence (ideally generated from type material). Technically, the necessary values are easily retrievable from sequence editing programs such as Geneious [104]. To evaluate intraspecific variation, sequences from all specimens assigned to a certain species were included in our analyses of diagnostic characters. In new species descriptions the provided reference sequences should be generated from type material. In cases where the molecular data

retrieved from the type are, however, incomplete, we consider it little problematic to additionally include data from other specimens, if there is justification on conspecifity (e.g. via other molecular markers). If future research rejects conspecifity, the respective characters can be easily excluded from the original description. We refrain from adopting the term 'genetype', however, as label for sequences data from type material [105], as it might be easily misunderstood: sequences themselves are not types but amplified copies of certain parts of type material.

Since an alignment presents the positional homology assumptions that are crucial for the determination of diagnostic nucleotides, we consider the quality of the alignment as essential for the success of molecular taxonomy. Therefore, we sincerely recommend to critically compare the output of different alignment programs, as in the present study. While coding mitochondrial markers (such as COI) can be checked via reading frames and translation into amino acids, and are generally less problematic, non-coding fast evolving markers (e.g. 16S rRNA) can be difficult to align even among closely related species. Obviously, undetected misalignments can result in tremendous overestimation of diagnostic characters. For example, a misalignment occurred in the ClustalW approach to our 28S rRNA dataset, which increased the number of characters diagnostic for a sister clade within *Pontohedyle wenzli* sp. nov. on this marker from 0 to 34 compared to the MUSCLE [106] alignment. And even without obvious misalignments, the use of different alignment programs can result in a differing number of diagnostic nucleotides (e.g. 9 vs. 13 diagnostic nucleotides in *P. milaschewitchii* comparing the MUSCLE and ClustalW alignment). By removing ambiguous parts of the alignment, one reduces the number of diagnostic characters considerably (e.g. from 19 to 13 diagnostic nucleotides on 16S rRNA in *P. milaschewitchii* when masking ClustalW alignments with Gblocks [107]). However, those diagnostic characters that remain can be considered as more stable and reliable for species identification. Based on our comparative analyses, we decided to choose the most conservative approach (alignment conducted with MUSCLE [106] and masked with GBlocks [107]), and based on the above mentioned examples stress the need to dedicate time to alignment issues when performing molecular taxonomy.

Several potential sources of error unique to taxonomy from molecular data have been pointed out [23]. (1) contamination and chimeric sequences, (2) faulty alignments resulting in comparisons of non-homologous nucleotides, and (3) the risk of dealing with paralogs. Authors of species descriptions based on molecular data should bear these pitfalls in mind. The risk of chimeric sequences can be reduced by carefully conducting BLAST searches [108] for each amplified fragment; misidentifications of diagnostic characters due to non-homologous alignments can be avoided by applying the considerations discussed above. The quality and stability of molecular taxonomic results considerably increase when several independent loci support the species delineation. To avoid idiosyncrasies of individual markers, misidentifications due to sequencing errors, or the pitfalls of paralogs, we strongly recommend not to base molecular species delineation and subsequent species description on single markers. Otherwise, if subsequent results negate the diagnostic value of nucleotides on that marker, the species description loses its entire foundation. Furthermore, the use of single pure CAs rather than of other types of CAs, and especially the use of genus-level compared CAs as discussed above, increases the chances of establishing and diagnosing new species on apomorphies rather than on homoplasies.

We acknowledge the risk that species descriptions based on molecular data might contain errors in the form of incorrectly assumed apomorphies, especially when working in sparsely sampled groups. Moreover, putative molecular apomorphies of described species may have to be reconsidered as plesiomorphies when new species with the same characteristics are added, or they may vanish in intraspecific variation. The more potentially apomorphic nucleotides are found across independently evolving markers, the higher the chances that at least some of them truly refer to unique mutations accumulated due to the absence of gene exchange. But in all this, molecular characters do not differ from morphological or other sets of characters. Species descriptions are complex hypotheses on several levels: novelty of taxon, placement within systematic context, and hypothesis of homology applying descriptive terms [5,109,110]. Species descriptions based on molecular characters are founded on the well-established hypothesis that character differences reflect lineage independence [50] and that mutations accumulate in the absence of gene exchange. It is the task of the taxonomist to evaluate whether the observed differences in character states can be explained by a historical process causing lineage divergence [3]. According to rough time estimations by molecular clock analyses, the radiation of *Pontohedyle* species included in the present study took place 100–25 mya (own unpublished data). Therefore we are confident that many of the bases recognized as diagnostic within our sampling truly refer to evolutionary novelties and unique attributes of species-level entities. However, even in cases of more recent divergences it should be possible to detect at least some diagnostic bases. Regardless of which character set a species description is based on, species descriptions are hypotheses, which means that they need to be re-evaluated, i.e. confirmed, falsified or modified when new data, material or methods of analysis become available.

Conclusions

This contribution issues a plea to follow up discoveries of cryptic species by molecular species delineation with the steps necessary to establish formal scientific names for these species. This can be achieved by selection of diagnostic characters, e.g., via the CAOS software. Depending on the robustness of the underlying phylogenetic hypothesis, taxonomists need to evaluate the optimal balance between the number of diagnostic bases and their stability subject to the topology. In general, pure diagnostic bases rather than private or combined ones should be selected, and such single pure CAs should be compared against all the potentially closely related lineages, not only against the direct sister in a predefined tree entered in CAOS as is the default procedure. We also wish to highlight the following considerations. 1) When basing a species description on molecular data the same rules as in traditional taxonomy should be applied considering deposition and accessibility of data; DNA aliquots and additional type material should be deposited in long term storage facilities, and sequences in public databases (GenBank). As with morphological type specimens, special attention should be given to the storage and availability of molecular types. 2) Due to the underlying homology assumption, we consider the quality of the alignment as critical to determining and extracting diagnostic bases. Thus, we recommend exploring changes to the alignment and, thus, the identified diagnostic characters by applying different alignment programs and masking options. 3) Alignments may change when new data is added, especially concerning non-coding markers. For better traceability, we regard it as beneficial to report not only the alignment position but also refer to a closely related reference genome (if applicable) and report the position in a deposited reference sequence (ideally generated from type material). In its current stage of development, the extraction of diagnostic characters for molecular taxonomy is not yet ready for inclusion in automated species delimitation procedures, as it still requires time-consuming manual steps. However, little adaptation of existing programs would be needed to make them serve molecular taxonomy in its entirety, to overcome the current gap between species discovery and species description.

Methods

Type localities and collecting sites

The collecting sites of material included in the present study are shown in Figure 4 (modified after Jörger et al. [25]). Of the three valid species, we were able to recollect *P. milaschewitchii* from its type locality. *P. verrucosa* was collected in vicinity of the type locality on Guadalcanal, Solomon Islands. Despite several attempts, we were unsuccessful in recollecting *P. brasilensis* at the type locality (see Discussion for assignment of specimens to this species).

Morphology and microanatomy

Jörger et al. [25] analyzed the radulae of most of the species described above. Unfortunately, for *Pontohedyle neridae* sp. nov., *P. martynovi* sp. nov. and *P. yurihookeri* sp. nov. radulae could not be recovered from the specimens used for DNA extraction. The radula of *P. wiggi* sp. nov. could only be studied under the light microscope, but was lost when attempting to transfer it to a SEM-stub.

Phylogenetic analyses by Jörger et al. [25] revealed two major clades within *Pontohedyle*. One includes *P. milas chewitchii*, for which detailed microanatomical and ultrastructural data is available [55,111]. The other clade is morphologically poorly characterized, since the original description of *P. verrucosa* lacks details on major organ systems like the reproductive system and the nervous system. For detailed histological comparison of the two major *Pontohedyle* clades, glutaraldehyde fixed specimens of *P. verrucosa* (from near the type locality WP-3 and WP-2 see [25]) were post-fixed in buffered 1% osmium tetroxide, decalcified using ascorbic acid and embedded in Spurr low-viscosity epoxy resin [112] or Epon epoxy resin (for detailed protocols see [113,114]). Serial semi-thin sections (1 and 1.5 μm) of three specimens were prepared using a diamond knife (Histo Jumbo, Diatome, Switzerland) with contact cement on the lower cutting edge to form ribbons [115]. Ribbons were stained using methylene-blue azur II [116] and sealed with Araldit resin under cover slips. Sectioned series are deposited at the Bavarian State Collection of Zoology, Mollusca section (ZSM Mol-20071833, 20071837 and 20100548). Additionally, histological series of *Pontohedyle kepii* sp. nov. were sectioned as described above.

Digital photographs of each section were taken using a ProgRes C3 camera (Jenoptik, Germany) mounted on a Leica DMB-RBE microscope (Leica Microsystems, Germany). Subsequently, photographs were edited (i.e., grey-scale converted, contrast enhanced and reduced in size) using standard imaging software, then loaded into AMIRA 5.2 (Visage Imaging Software, Germany) for 3D reconstruction of the major organ systems. Alignment, labeling of the organ systems and surface rendering followed in principle the method described by Ruthensteiner [115].

Acquisition of molecular data

This study aims to characterize the genus *Pontohedyle* (Acochlidia, Microhedylacea) based on molecular standard markers, i.e., nuclear 18S and 28S rRNA and mitochondrial COI and 16S rRNA. We included the three previously valid *Pontohedyle* species (for taxonomy see [69,83]):

P. milaschewitchii (Kowalewsky, 1901), *P. verrucosa* (Challis, 1970) and recently re-established *P. brasilensis* (Rankin, 1979) [25]. The nine additional species earlier identified as candidates in the genus *Pontohedyle* [25] are subject to molecular taxonomy. 28S rRNA, 16S rRNA and COI sequences analyzed by Jörger et al. [25] were retrieved from GenBank (see Table 1 for accession numbers). Additionally, we amplified nuclear 18S rRNA (approx. 1800 bp) for at least one individual per species. 18S rRNA was amplified in three parts using the primers for euthyneuran gastropods by Vonnemann et al. [65] and Wollscheid & Wägele [117]: 18A1 (5' - CCT ACT TCT GGT TGA TCC TGC CAG T – 3′), 700R (5′ - CGC GGC TGC TGG CAC CAG AC – 3′), 470 F (5′ - CAG CAG GCA CGC AAA TTA CCC – 3′), 1500R (5′ - CAT CTA GGG CAT CAC AGA CC – 3′), 1155 F (5′ - CTG AAA CTT AAA GGA ATT GAC GG – 3′), 1800 (5′ - TAA TGA TCC TTC CGC AGG TT – 3′). Polymerase chain reactions were conducted using Phire polymerase (New England Biolabs) following this protocol: 98°C 30 sec, 30-35x (98°C 5 sec, 55-65°C 5 sec, 72°C 20-25 sec), 72°C 60 sec. Successful PCR products were cleaned up with ExoSap IT. Cycle sequencing such as sequencing reactions was performed by the Genomic Service Unit (GSU) of the Department of Biology, Ludwig-Maximilians-University Munich, using Big Dye 3.1 kit and an ABI 3730 capillary sequencer. Sequences were edited (forward and reverse strands), concatenated and checked for potential contamination via BLAST searches [108] against the GenBank database via Geneious 5.5.2 [104].

Detection of diagnostic molecular characters

We used the Characteristic Attribute Organization System (CAOS) [51,57,58] to detect discrete nucleotide substitutions on our previously determined candidate species [25]. The program distinguishes single (single nucleotide) vs. compound (set of nucleotides) 'character attributes' (CA) [51]. Both, single and compound CAs can be further divided into pure (present in all members of a clade but absent from all members of another clade) and private CAs (only present in some members of the clade, but absent in members of other clades) [51]. For taxonomic purposes at this stage we consider only 'single pure characters' (sPu) as diagnostic characters for species descriptions (see Discussion). Since some sister group relationships among *Pontohedyle* species are not well supported (see [25], Figure 1), we chose our diagnostic molecular characters in the sense of unique within the genus *Pontohedyle*, rather than assigning plesiomorphic or apomorphic polarity to character states of one species in relation to its direct sister species.

As discussed above, the homology assumption presented in the alignment is crucial for the correct detection of diagnostic characters. For quality control, we performed data input into CAOS with alignments derived from three commonly applied alignment programs and critically compared the resulting differences concerning amounts and positions of the sPus. Alignments were generated for each marker individually using MUSCLE [106], Mafft [118,119] and CLUSTAL W [120]. The COI alignment was checked manually, supported by translation into amino acids. Due to difficulties in aligning highly variable parts of rRNA markers, we removed ambiguous parts of the alignment with two different masking programs, Aliscore [121] and GBlocks [107], and compared the respective effects on character selection. After comparison of the various results we chose MUSCLE [106] in combination with GBlocks [107] as the most conservative approach that results in fewer but more reliable diagnostic characters than the other approaches.

Alignments were analyzed and converted between different formats using Geneious 5.6 (Biomatters) [104]. We performed a phylogenetic analysis under a maximum-likelihood approach with RAxML 7.2.8 on each individual marker, applying the 'easy and fast way' described in the RAxML 7.0.4 manual to obtain an input tree. For our present study the phylogenetic hypothesis on sister group relationships of the different *Pontohedyle* species, however, is not relevant: We manipulated the resulting trees in Mesquite [122], generating a single starting file for CAOS for each species and for each marker, with each of the analyzed species successively being sister to all remaining *Pontohedyle* species. This iterative procedure retrieves diagnostic characters for the node that compares each single species to all its congeners.

The single gene alignments which formed the basis for the selection of diagnostic nucleotides are available in fasta format as Additional material 3–6. Diagnostic nucleotides are reported with positions in the reference alignment. Position 1 of each alignment refers to position 1 after the primer region, which was removed in the alignment. For better traceability, and in the absence of a closely related reference genome, we additionally report the positions within a reference sequence for each species (deposited in GenBank; see Table 1). In the description of our new species these reference sequences are retrieved from the holotype. Diagnostic molecular characters of the genus *Pontohedyle* in 18S and 28S rRNA are diagnosed based on alignments including all available *Pontohedyle* sequences (Table 1) and representatives of all other acochlidian genera currently available in public databases (see Additional files 1 and 2 for the original alignments in fasta format).

To meet the requirements by the International Code of Zoological Nomenclature (ICZN) [78,79], this article was registered at ZooBank (www.zoobank.org) under the ZooBank Life Science Identifiers (LSIDs):

urn:lsid:zoobank.org:pub:4AE75E9C-4303-42CB-AED2-77266C8F6601.

Additional files

Additional file 1: 18S rRNA alignment of *Pontohedyle* with outgroups to determine diagnostic nucleotides for the genus (fasta format). The alignment was generated with MUSCLE [107] and ambiguous parts of the alignment were masked with Gblocks [108] (settings for a less stringent selection).

Additional file 2: 28S rRNA alignment of *Pontohedyle* with outgroups to determine diagnostic nucleotides for the genus (fasta format). The alignment was generated with MUSCLE [107] and ambiguous parts of the alignment were masked with Gblocks [108] (settings for a less stringent selection).

Additional file 3: 18S rRNA alignment of *Pontohedyle* (fasta format). The alignment was generated with MUSCLE [107] and ambiguous parts of the alignment were masked with Gblocks [108] (settings for a less stringent selection).

Additional file 4: 28S rRNA alignment of *Pontohedyle* (fasta format). The alignment was generated with MUSCLE [107] and ambiguous parts of the alignment were masked with Gblocks [108] (settings for a less stringent selection).

Additional file 5: 16S rRNA alignment of *Pontohedyle* (fasta format). The alignment was generated with MUSCLE [107] and ambiguous parts of the alignment were masked with Gblocks [108] (settings for a less stringent selection).

Additional file 6: COI alignment of *Pontohedyle* (fasta format). The alignment was generated with MUSCLE [107].

Competing interests
Both authors declare that they have no competing interests.

Authors' contributions
KMJ generated the morphological and molecular data and drafted the manuscript. MS planned and supervised the study. The material was collected jointly and in cooperation with a series of collaborators (see Acknowledgements). All authors read and approved the final manuscript.

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DISCUSSION

The impact of meiofaunal slugs on the systematics of Heterobranchia

Origin of meiofaunal slugs

In our molecular phylogenetic analyses of the origin of Acochlidia with a targeted taxon sampling of Euthyneura, including most previously hypothesized sister groups and other meiofaunal slug lineages, acochlidian slugs cluster among pulmonate taxa (Jörger et al. 2010b). This supports and refines the results of initial multi-locus molecular studies of Opisthobranchia/Euthyneura, which had already indicated the surprising placement of formerly opisthobranch Acochlidia in the vicinity or amidst pulmonates (Vonnemann et al. 2005, Klussmann-Kolb et al. 2008). Proposed relationships with the opisthobranch cephalaspid *Toledonia* based on shared morphological features such as radula characteristics (Sommerfeldt & Schrödl 2005) were rejected based on cladistic analyses (Schrödl & Neusser 2010) as well as molecular approaches (Klussmann-Kolb et al. 2008, Jörger et al. 2010b). In morphology-based analyses Acochlidia usually cluster with equally minute Runcinacea or other meiofaunal lineages of Cephalaspidea or Rhodopemorpha (Wägele & Klussmann-Kolb 2005, Schrödl & Neusser 2010). Schrödl & Neusser (2010), however, have demonstrated the susceptibility of topological placement to the intrusion of other meiofaunal slugs such as *Rhodope* or sacoglossan *Platyhedyle*, which always resulted in direct sister group relationships of all included meiofaunal taxa. Our molecular study supports the authors' assumption that a series of convergent adaptations to the mesopsammic habitat (recently summarized as 'meiofaunal syndrome' (Brenzinger et al. 2013a)) masks the true phylogenetic signal in cladistics analyses of morphological characters (Jörger et al. 2010b, Schrödl & Neusser 2010). Our established multi-locus molecular phylogeny of heterobranch slugs and snails clearly refutes the notion that meiofaunal slugs have a common origin, indicating a minimum of five independent pathways in the evolution of Heterobranchia leading into the mesopsammon: in Rhodopemorpha, Nudibranchia, Cephalaspidea, Sacoglossa and Acochlidia (Jörger et al. 2010b, Wilson et al. 2010a, Schrödl et al. 2011a). The absence of a cuticularized gizzard in Philinoglossidae points to their independent origin from equally meiofaunal representatives among *Philine* (Brenzinger et al. 2013b) and thus constitutes a potential sixth invasion into the interstitial habitat (i.e., two independent ones occurring among Cephalaspidea) as indicated by molecular data (Jörger et al. 2010b). Whether the meiofaunal nudibranchs *Embletonia* and *Pseudovermis* originated from a common meiofaunal ancestor or might yet split into two independently evolved meiofaunal lineages remains to be investigated in future studies.

The 'new phylogenetic hypothesis' on Euthyneura

The placement of Acochlidia and other formerly opisthobranch clades such as Sacoglossa or 'lower heterobranch' taxa (i.e. Pyramidelloidea and Glacidorboidea) in pulmonate relationships has considerable consequences for the systematics of heterobranch Euthyneura: it clearly renders the long-standing traditional systematic entities of 'Opisthobranchia' and 'Pulmonata', established by Milne Edwards (1848) non-monophyletic. Monophyletic 'Opisthobranchia' have never been well supported by morphological features (Haszprunar 1985, Dayrat & Tillier 2002). The only putative apomorphy of 'Opisthobranchia' – the presence of a rhinophoral nerve with basal swelling (i.e. a rhinophoral ganglion) – has long been discussed as being homologous to the pulmonate procerebrum (Haszprunar 1988, Ruthensteiner 1998, Jörger et al. 2010b). The presence of rhinophores innervated by the rhinophoral nerve (= *nervus rhinophoralis* = N3) is currently regarded as an apomorphy of Euthyneura based on revised data of basal heterobranchs including Rhodopemorpha (Brenzinger et al. 2013a). Reinvestigation of the three major morphological apomorphies of 'Pulmonata' – i.e. pallial cavity with pneumostome, presence of a procerebrum (with cerebral gland and double cerebro-connectives) and the existence of medio-dorsal (cerebral) bodies (see e.g., Dayrat & Tillier 2002, Mordan & Wade 2008) – in light of the new hypothesis, Jörger et al (2010b) revealed them to be either homoplastic or plesiomorphic. Currently, morphological characters neither favor alternative sister group relationships nor do they contradict the presented phylogenetic hypothesis (Jörger et al. 2010b, Schrödl et al. 2011a). Our study revealed 'Opisthobranchia' and 'Pulmonata' as artificial groupings and urged the need for a new classification which reflects the state-of-the-art in the knowledge on their evolutionary relationships (Jörger et al. 2010b). Aiming for continuity in terminology, we proposed a new classificatory system of euthyneuran slugs terming the major clades Euopisthobranchia and Panpulmonata (Jörger et al. 2010b), together forming the sister clade of Nudipleura. The combined clade of Euopisthobranchia + Panpulmonata was later termed Tectipleura (Schrödl et al. 2011a). Euopisthobranchia comprise Umbraculoidea, Runcinacea, Cephalaspidea s.s., Anaspidea and Pteropoda, and are supported morphologically by the apomorphic presence of a cuticularized gizzard (i.e., muscular oesophagial crop lined with cuticula) (Jörger et al. 2010b). Panpulmonata expand the groups traditionally classified as 'Pulmonata' (including previously disputed Glacidorboidea and Amphiboloidea) to include Sacoglossa, Siphonarioidea, Pyramidelloidea and Acochlidia (Jörger et al. 2010b). Currently, the double rooted rhinophoral ganglion, or the homologous double rooted procerebrum presents the only putative morphological apomorphy for this highly diverse panpulmonate clade. This implies, however, that this nervous condition developed convergently in the basal Rhodopemorpha (Brenzinger et

al. 2013a). No other morphological characters unambiguously supporting the monophyly of Panpulmonata could be detected so far, impeded by ecological flexibility and according morphological diversity of this hyper-diverse clade combined with the infamous high degree of homoplasies of euthyneurans (see e.g., Dayrat & Tillier 2002, 2003, Wägele et al. 2013) due to convergent developments across all organ systems.

The traditional classification of Euthyneura had already been challenged by single marker molecular approaches (see e.g., Thollesson 1999, Dayrat et al. 2001) and based on mitochondrial sequences data (Grande et al. 2004a, b). The first multi-locus studies on Euthyneura combining mitochondrial and nuclear markers (Klussmann-Kolb et al. 2008, Dinapoli & Klussmann-Kolb 2010) also rendered 'Opisthobranchia' and 'Pulmonata' as non-monophyletic with similar topologies as in Jörger et al. (2010b). Subsequent molecular phylogenetic studies using a heterobranch dataset to clarify the origin and evolution of certain euthyneuran subclades equally revealed similar relationships (Göbbeler & Klussmann-Kolb 2010, Dayrat et al. 2011, Dinapoli et al. 2011, Göbbeler & Klussmann-Kolb 2011). Therefore, with increased taxon sampling in different euthyneuran lineages and improved data quality (with regard to eliminating contaminated sequences, testing different alignment programs and comparing different phylogenetic approaches), studies are converging in their support for the classification of Euopisthobranchia and Panpulmonata (Schrödl et al. 2011a, Wägele et al. 2013). All these studies (Klussmann-Kolb et al. 2008, Dinapoli & Klussmann-Kolb 2010, Jörger et al. 2010b, Dayrat et al. 2011, Dinapoli et al. 2011, Göbbeler & Klussmann-Kolb 2011) rely on the same (sub)set of genetic 'standard markers' (nuclear 18S rRNA, 28S rRNA and mitochondrial 16S rRNA and COI), however, and present a similarly inferred 'concatenated gene tree'. Therefore, all studies would likewise be affected by conflicting signals between genes or noisy signal due to saturation effects in deep nodes.

Phylogenetic relationships of Acochlidia

While most major clades (orders or super-families) of Panpulmonata and Euopisthobranchia are supported with high bootstrap values, their deep sister group relationships especially within panpulmonates lack decent support values across all recent studies (Klussmann-Kolb et al. 2008, Dinapoli & Klussmann-Kolb 2010, Jörger et al. 2010b, Dayrat et al. 2011). Acochlidia always cluster among pulmonate taxa, but their precise sister group relationship remains ambiguous (e.g., as sister to (Pyramidelloidea+ Amphiboloidea)+ Eupulmonata (Klussmann-Kolb et al. 2008) or Eupulmonata (Jörger et al. 2010b) or a clade comprised of Hygrophila+ (Pyramidelloidea+ (Amphiboloidea+ Glacidorboidea)) (Jörger et al. in review)) and poorly

supported, despite an increasingly denser outgroup and ingroup taxon sampling. The recent molecular approaches covering panpulmonate diversity point to a rapid radiation into the major panpulmonate taxa (indicated by short branches at the base of the higher taxa in relation to terminal branches) (see e.g., Klussmann-Kolb et al. 2008, Jörger et al. 2010b, Dayrat et al. 2011, Jörger et al. in review). Molecular clock analyses on Euthyneura calibrated with a broad set of heterobranch fossils (see Jörger et al. in review, for sensitivity analyses on different calibration points) dated the origin of Euthyneura to the late Paleozoic and the radiation of the major panpulmonate lineages to the early to mid-Mesozoic (Klussmann-Kolb et al. 2008, Jörger et al. 2010b, Jörger et al. in review). These time trees contradict the assignment of putative terrestrial Paleozoic gastropod fossils to pulmonate taxa (Solem & Yochelson 1979, Solem 1985) and support critics who suggest that the few detectable characters of these fossil shells might also refer to prosobranch lineages (Mordan & Wade 2008, Dayrat et al. 2011). The Mesozoic origin combined with a relatively rapid diversification into the major panpulmonate taxa likely impedes current phylogenetic approaches. Currently, the 'standard marker' set (i.e. nuclear 18S rRNA and 28S rRNA and partial mitochondrial COI and 16S rRNA) still outperforms all competing approaches by the availability of the broadest taxon sampling for gastropods and Mollusca in general (Stöger et al. 2013). Despite the dense taxon sampling at the present stage, this set of 'standard markers' seems incapable of reliably solving the relationships among the major panpulmonate taxa.

Euthyneuran relationships inferred from other markers

In contrast to the nevertheless converging topologies of 'standard markers' analyses, studies based on mitochondrial genomes support the monophyly of 'Opisthobranchia' (Grande et al. 2002, Grande et al. 2008, Medina et al. 2011). Their taxon sampling has been criticized, however, as inadequate and under-representative, lacking key heterobranch lineages, which artificially favors opisthobranch monophyly (Schrödl et al. 2011b). White et al. (2011) supplemented additional pulmonate mitochondrial genomes; their study rejects the traditional classification, but also the Panpulmonata and Euopisthobranchia, with pulmonate taxa forming a basal grade. In recent reanalyses covering all available mitochondrial genomes on Mollusca in a broad metazoan framework, heterobranch gastropods form a well-supported but long-branched clade which clustered away from their supposed caenogastropod sister basally in the lophotrochozoan tree (Bernt et al. 2013, Stöger & Schrödl 2013). The most comprehensive analyses on heterobranch mitogenoms confirm the 'standard marker' hypothesis in several aspects, especially when it comes to traditional classifications of 'Opisthobranchia' and

'Pulmonata' and including formerly 'lower heterobranch' Pyramidelloidea (excluding Murchisonellidae) and opisthobranch Sacoglossa within pulmonate relationships (White et al. 2011, Stöger & Schrödl 2013). However, the proposed classification into Tectipleura (Nudipleura + (Euopisthobranchia + Panpulmonata) is contradicted by mitogenomics, which instead engender unconventional topologies with e.g., Stylommatophora that form a basal euthyneuran offshoot (e.g., Grande et al. 2008) or reject well-supported clades such as Nudibranchia (Grande et al. 2004a, b). Based on the reversed topology in mitogenomic analyses compared to standard marker topologies, Stöger & Schrödl (2013) support earlier assumptions (Schrödl et al. 2011b) that the mitogenomic trees currently available are misrooted suffering from long branch effects. In the present stage, therefore, mitogenomics do not seem suitable to resolving basal euthyneuran relationships (Bernt et al. 2013, Stöger & Schrödl, 2013).

A first phylogenomic approach confirms our new classification of Euthyneura, with Nudipleura forming a basal offshoot to Euopisthobranchia and Panpulmonata (Kocot et al. 2011). This raises confidence that combining the four standard markers may be capable of resolving the 'true' euthyneuran relationships. However, the taxon sampling designed to address deep molluscan relationships in phylogenomic studies is poor with regard to Euthyneura (only four (Smith et al. 2011) or nine (Kocot et al. 2011) taxa included); it still lacks most major subgroups (e.g. Cephalaspidea, Pteropoda, Amphiboloidea, Systellommatophora and others). Kocot et al. (2013), however, have expanded the phylogenomic taxon sampling for Euthyneura and recovered Sacoglossa among pulmonates, and have also rendered Nudipleura paraphyletic. Although the number of genetic markers found in phylogenomic analyses is impressive, they are still limited with regard to taxon sampling coverage. New molecular datasets combining the character wealth of phylogenomic approaches with the density in taxon sampling available for standard markers are needed in the future.

Wägele et al. (2013) reviewed in detail the history of the classification of opisthobranch sea slugs and presented a consensus tree on the 'new phylogenetic hypothesis' of Heterobranchia based on standard marker analyses (Dinapoli & Klussmann-Kolb 2010, Jörger et al. 2010b, Schrödl et al. 2011a), adding limited morphological evidence from Brenzinger et al. (2013a). Future studies making use of new molecular markers with potential phylogenetic signal for deep euthyneuran splits are needed to provide better resolution and test whether this phylogenetic hypothesis adequately depicts evolutionary relationships of heterobranch slugs and snails. This new classification of Euthyneura highlights the need to unite pulmonate and opisthobranch research communities and join efforts in the scientific exploration of the

diversity and evolution of Heterobranchia. The fact that scientific progress in the understanding of the evolution of heterobranchs sometimes proceeds at a slug's pace might have at least partially been due to artificially separating of what can only be understood as an evolutionary unity.

On the evolution of meiofaunal slugs

The role of progenesis

The 'new phylogenetic hypothesis' on Euthyneura discussed above clearly shows that meiofaunal slugs have inhabited the marine mesopsammon at least five separate times (i.e., within Acochlidia, Rhodopemorpha, Nudibranchia, Cephalaspidea s.s. and Sacoglossa) (Jörger et al. 2010b, Schrödl et al. 2011a). The colonization process of the mesopsammic habitat evidently requires morphological and behavioral adaptations to cope with the limited space available (Swedmark 1964, Westheide 1987, Worsaae et al. 2012, Brenzinger et al. 2013a).The spatial restrictions of the habitat tend to favor vermiform body shapes with reduced head and body appendages (Swedmark 1968a). Convergent adaptations to the interstitial habitat among meiofaunal slugs were summarized as 'meiofaunal syndrome'; in addition to the vermiform external morphology, this frequently entails loss of pigmentation, reduction of eyes, development of intracellular, calcareous spicules and different modes of rapid adhesion to the substrate (Brenzinger et al. 2013a). Jörger et al. (2010b) suggested that the aberrant external morphology of Acochlidia with their visceral hump separated from the head-foot complex presents a paedomorphic feature retained via progenesis from an abnormal larval development, as reported by Tardy (1970) in his studies on the ontogeny of the nudibranch *Aeolidiella alderi* (Cocks, 1852) and observations on pulmonate slugs. Based on the current phylogeny of Acochlidia and a reconstruction of ancestral habitats, the ancestor of Acochlidia putatively evolved from a temporary mesopsammic juvenile of a benthic panpulmonate sea slug via progenesis and switched to a permanent mesopsammic lifestyle (Jörger et al. in review). Meiofaunal Acochlidia still resemble benthic slugs due to the presence of head appendages and an externally separated foot, which serves as a gliding sole (see e.g., Wawra 1987). Both features show regressive tendencies, however, among different acochlidian lineages, e.g., the independent reduction of rhinophores in microhedylacean *Pontohedyle* and *Ganitus* (Schrödl & Neusser 2010, Jörger et al. in review). In most mesopsammic genera of Acochlidia the visceral hump is only slightly elongated in comparison to their benthic sister species; it thus lacks the typical vermiform appearance characteristic for other meiofaunal lineages. Two recently

discovered yet undescribed forms of mesopsammic Acochlidia, however, present character states of an evolutionary adaptational elongation of their visceral humps (one is Hedylopsacea sp. from Moorea (see Jörger et al. (in review)) and another one putatively resembles a novel microhedylacean lineage from Papua and is the most elongated vermiform acochlid ever encountered (B. Brenzinger, T. Neusser, pers. comm.)). Vermiform body shapes are characteristic for the nudibranch *Pseudovermis*, which also has reduced body appendages (cerata), a reduced foot without separation from the body mass and an acorn-shaped head lacking appendages (Salvini-Plawen & Sterrer 1968). A similarly shaped head is also present in meiofaunal *Platyhedyle* (Salvini-Plawen 1973, Rückert et al. 2008). Members of Rhodopemorpha carried the reduction and modification of the external morphology to an extreme in the worm-shaped body, a complete lack of appendages and the presence of strong overall body ciliation, which likely replaces the foot in a three-dimensional habitat (Salvini-Plawen 1991b, Haszprunar & Heß 2005, Brenzinger et al. 2011c, Brenzinger et al. 2013a). In *Pseudovermis* and rhodopemorphs the aberrant morphology was identified as potential paedomorphic feature (Brenzinger et al. 2013a, my own unpublished data on *Pseudovermis*), retaining larval features via progenesis (for detailed studies on the ontogeny of closely related rhodopemorphs respectively aeolidioid nudibranchs, see Riedl (1960) and Tardy (1970)). In general, progenesis is discussed as a driving force of the evolution of meiofaunal taxa (Westheide 1987) and the resulting characters states have been observed in various taxonomic groups (see e.g., Worsaae et al. (2008) on annelids). The alternative evolutionary scenario of adapting to the mesopsammic world is formed by a step-wise miniaturization from a benthic or sand-dwelling macrofaunal ancestor driven by the selective pressure in favor of small body size (Westheide 1987, Hanken & Wake 1993). In the absence of obvious paedomorphic features in the philinoglossid *Pluscula cuica* Marcus, 1953, the streamlined detorted body with reduced shell and mantle cavity of this minute meiofaunal lineage more likely evolved via a gradual miniaturization rather than progenesis (Brenzinger et al. 2013b).

Apart from the external morphology, the microanatomy of Acochlidia provides heterogeneous evidence of progenetic effects, with regard to subtaxa and organ systems. In contrast to Rhodopemorpha, there are usually three ganglia on the acochlidian visceral nerve cord (see e.g., Neusser et al. 2006, Jörger et al. 2008, Neusser et al. 2009a, Neusser et al. 2009b, Eder et al. 2011), which might present a derived fused stage based on the pentaganglionate hypothesis (Haszprunar 1985), depending on the outgroup condition. The rhodopemorph *Helminthope psammobionta* Salvini-Plawen, 1991 – the most worm-like, interstitial gastropod – presents a concentrated central nervous system but with a pentaganglionate stage of the visceral loop. This suggests that this typical larval character might have been retained in the evolution of

Rhodopemorpha involving progenesis (Brenzinger et al. 2013a). Neusser & Schrödl (2007) noted a variation of four vs. three ganglia on the visceral cord of mature specimens of limnic, meiofaunal *Tantulum elegans* Rankin,1979. This is not the only account of intraspecific or even intraindividual (from left to right body side) variation of nervous features in meiofaunal slugs which frequently affects accessory ganglia or precerebral ganglia such as rhinophoral and optic ganglia and the associated nerves (see e.g., Jörger et al. 2008, Brenzinger et al. 2013b). Characters of the nervous system traditionally served as key features for classifying Heterobranchia (Haszprunar 1985, Huber 1993) and were optimistically investigated in depth for species delineation in Acochlidia (Neusser et al. 2007). The intraspecific variation of nervous features (especially major features such as the number of visceral loop ganglia) weakens the reliability of this character set for systematic and taxonomic purposes. Potentially high intraspecific variation demands cautious application and highlights the need for thorough comparison of several individuals (ideally belonging to different populations), as intraspecific variability may exceed interspecific variability. The conspicuous intraspecific variability of the nervous system of meiofaunal slugs might be a consequence of miniaturization and paedomorphosis, which is known to frequently induce variation in late-forming structures truncated individually during development (Hanken and Wake, 1993). Concerning the circulatory and excretory systems, the heart is reported to form late in the development of heterobranch slugs, viz. right before metamorphosis (LaForge & Page 2007); some meiofaunal slugs were even described lacking a heart (see e.g., Kowalevsky 1901b, Kudinskaya & Minichev 1978). 3D-supported microanatomy, however, has revealed that a minute heart is present in Acochlidia, and had only been previously overlooked due to its small size and putatively collapsed stage (e.g., Jörger et al. 2008, Neusser et al. 2009b). Rhodopemorphs truly lack any trace of a heart and pericardium, and possess a protonephridial excretory system resembling larval protonephridia (Salvini-Plawen 1991b, Haszprunar 1997, Brenzinger et al. 2013a). The paedomorphic stage of the kidney is a unique feature of Rhodopemorpha among meiofaunal slugs, which despite the supposed absence of a heart possess a pericardium and usually a simple to rather complex metanephridial-stage kidney (see e.g., Challis 1969, Bartolomaeus 1997, Rückert et al. 2008, Neusser et al. 2009a).

Convergent adaptations to the mesopsammon

Next to the aberrant external morphology and paedomorphic traces in e.g., the nervous system, potentially resulting from progenetic effects, meiofaunal slugs show novel anatomical features such as calcareous spicules, accessory ganglia, adhesive glands enabling rapid adhesion to the

substrate, as well as different adaptations in the reproductive system allowing for rapid, unilateral sperm transfer. As suspected previously (Schrödl & Neusser 2010), our phylogenetic analyses confirmed these novel morphological inventions as convergent adaptations to the mesopsammic habitat (Jörger et al. 2010b).

Spicules – Rieger & Sterrer (1975) defined 'spicular formation' from a structural point of view as an 'individual biogenic element made up primarily of inorganic substance' and the presence of such 'spicular skeletal structures' are recognized as common phenomenon across meiofaunal taxa (Swedmark 1964, Rieger & Sterrer 1975). In meiofaunal slugs spicules are usually calcareous structures of uni- and intracellular origin which are embedded in the subepidermal connective tissue (see e.g., Rieger & Sterrer 1975, Arnaud et al. 1986, Jörger et al. 2008, Jörger et al. 2013). They occur in various simple to highly complex forms with the most remarkable structural developments in a yet undescribed lineage of Rhodopemorpha, which closely resembles the cross-shaped to snow-flake like spicules reported for '*Rhodope crucispiculata*' Salvini-Plawen, 1991 *nomen nudum*. Spicules are characteristic for Acochlidia, Rhodopemorpha and *Platyhedyle,* but are absent in some philinoglossids, and in most meiofaunal nudibranchs with the exception of *Pseudovermis mortoni* Challis, 1969 (see e.g., Salvini-Plawen & Sterrer 1968, Challis 1969, Salvini-Plawen 1973, Wawra 1987, Urgorri et al. 1991). In some meiofaunal acochlidians (*Asperspina* and *Hedylopsis*) large rodlet-like spicules form dense aggregation covering the entire visceral hump and serving as a 'secondary shell' for the head-foot complex which can be retracted into the protected visceral sac (Swedmark 1968a, Sommerfeldt & Schrödl 2005, Schrödl & Neusser 2010). The 'secondary spicule shell' might serve as effective protection for essential body parts against bites of (small-sized) meiofaunal predators and expand the body diameter to avoid being swallowed whole (Jörger et al. in review). Accumulations of spicules on a smaller scale – likely ineffective as a protective device against predators – have been discussed as a stabilizing feature of certain body parts or as mechanical protection of delicate internal organs (Rieger & Sterrer 1975, Jörger et al. 2008). Rieger & Sterrer (1975) have discussed spicules as a potential by-product of metabolic processes, which might explain the fact that they can disappear in captivity under laboratory conditions (Eder et al. 2011).

Spicules are characteristic but not unique to meiofaunal slugs, but also present in e.g., benthic doridiid or phyllidiid Nudibranchia (see e.g., Chang et al. 2013, Sánchez-Tocino et al. 2013). Based on their intracellular origin, a homology on the cellular level of slug spicules with gastropod shells is unlikely (Jörger et al. 2013). Screening acochlidian transcriptomes for the expression of genes considered to be involved in biomineralization, we detected an expression

of several biomineralization genes, two of which had been previously recorded as directly involved in shell deposition in gastropods and bivalves (the 'shell-forming' genes *nacrein* and *perlustrin*) (Jörger et al. 2013). To date, the lack of a broad comparative dataset on the genetic processes of shell deposition hinders sound interpretation of the preliminary transcriptomic data on spicule-bearing slugs. Nevertheless, 'shell-forming' genes – e.g., those involved in the formation of the larval shell – might be plesiomorphic for heterobranch slugs and reactivated later in their evolutionary history for the formation of spicules (Jörger et al. 2013).

Accessory ganglia – Accessory ganglia are histologically characterized as conspicuous clusters of nervous tissue usually associated with cerebral nerves and sensory structures and differ from 'true' ganglia by the lack of separation into cortex and medulla, i.e. showing a homogeneous distribution of nuclei (see e.g., Neusser et al. 2006). The presence of accessory ganglia is characteristic for many meiofaunal slugs such as Rhodopemorpha, some *Pseudovermis*, *Platyhedyle*, Philinoglossidae and microhedylacean Acochlidia. Surprisingly, they are absent from hedylopsacean Acochlidia (including the meiofaunal *Hedylopsis* and *Pseudunela*), with the exception of limnic meiofaunal *Tantulum elegans* (see e.g., Wawra 1987, Salvini-Plawen 1991a, Neusser & Schrödl 2007, Rückert et al. 2008, Neusser et al. 2009a, Neusser et al. 2011b, Brenzinger et al. 2013a, Brenzinger et al. 2013b). Accessory ganglia develop later in ontogeny than the main ganglia of the central nervous system (Jörger et al. 2010a) and are highly variable intraspecifically in shape and number (own observations). The presence of accessory ganglia cannot (exclusively) be related to small body sizes, which might require an outsourcing of neuronal tissue (Haszprunar & Huber 1990) as they are absent in equally small but benthic slugs (Jörger et al. 2008). Since the accessory ganglia form swellings associated with the cerebral sensory nerves (labiotentacular, rhinophoral, oral and optic nerves), they were suspected to serve as an enhancement of sensory abilities and to enable the processing of stimuli of a three-dimensional habitat (Brenzinger et al. 2013b). The extremely elongated rhodopid *Helminthope psammobionta* uniquely presents accessory ganglia not only in the anterior head region but also posterior to the cerebral nerve ring, which are partially innervated not only by cerebral but also by pedal nerves. This has been interpreted as a further adaptation for faster signal processing in an extremely elongated body (Brenzinger et al. 2013a). In an examination of the immunocytochemical reactivity of the nervous system in the microhedylid *Parhedyle cryptophthalma* Westheide & Warwa, 1974, we were unable to detect any expression of neurotransmitters such as FMRFamide and Tyrosine Hydroxylase (TH) in the accessory ganglia (positively confirmed in 'true' ganglia and nerves) (Jörger et al. 2010a). TH-expression was observed in cerebral ganglia, sensory nerves and drew attention to an accumulation of TH-expressing neurons near the mouth opening (Jörger et al. 2010a). It is thus

remarkable that no catecholamines expression, which is likely related to chemo- and mechanoreception (Croll et al. 2003, Faller et al. 2008) was observed in the associated accessory ganglia. Reexamination is necessary to clarify whether the absence of neurotransmitter expression was due to the not fully developed stage of accessory ganglia in subadult *Parhedyle* (Jörger et al. 2010a); furthermore, additional comparative studies combining immunocytochemistry and ultrastructural investigation on other meiofaunal slugs are needed to clarify the function and origin of accessory ganglia.

Sperm transfer – Life in between grains of sand is accompanied by several unusual reproductive traits in meiofaunal taxa. The minute body sizes typically result in a small number of gametes and a consequently low reproductive output (Swedmark 1959, 1968a, Ax 1969). Methods of direct sperm transfer that economize reproduction (i.e., via copulation, hypodermic injection or direct application of spermatophores) are therefore predominant (Ax 1969). Sperm transfer via spermatophores is especially common across meiofauna; due to several independent evolutionary origins, spermatophores are discussed as typical adaptation to the mesopsammic environment (Swedmark 1959, 1964, 1968a, Ax 1969). The plesiomorphic condition of sperm transfer in hermaphroditic heterobranch slugs and snails is reciprocal copulation (Schrödl & Neusser 2010), which has been modified considerably, however, among all lineages of meiofaunal slugs. Among Acochlidia copulation likely resembles the ancestral state of sperm transfer (see Schrödl & Neusser 2010), with remarkably different modifications of reproductive features in the evolution of the two major clades: Hedylopsacea have evolved complex copulatory apparatuses and predominantly transfer sperm via hypodermic injection, while Microhedylacea are aphallic and transfer sperm via spermatophores (Wawra 1992, Schrödl & Neusser 2010). Sperm transfer via spermatophores differs among meiofaunal taxa in terms of whether the spermatophores are attached either precisely to the female gonopore or imprecisely somewhere to the body wall. In cases of imprecise placement, sperm migrates epidermally to the female gonopore or directly penetrates the body wall at the attachment site travelling in the body cavity toward the site of fertilization (Jörger et al. 2009 and references therein). In microhedylacean Acochlidia, spermatophores are attached imprecisely all over the head-foot complex and the visceral hump, with slightly higher frequencies recorded in the posterior region of the visceral hump (Swedmark 1968b, Poizat 1986). At the attachment site of the spermatophores, the tissue is then lysed and sperm intrudes into the body cavity (Swedmark 1968a, Morse 1994, Jörger et al. 2009). The cork-screw shaped nucleus of microhedylacean spermatozoa is potentially capable of penetrating epithelia, tissues and eggs via drilling (Jörger et al. 2009). Surprisingly, no taxis of spermatozoa towards the gonad could be observed in *P. milaschewitchii* and histological reports of other Microhedylacea also show 'misdirected'

sperm penetrating all kinds of organ systems including ganglia (Marcus 1953, Jörger et al. 2009, own observations). The evolutionary disadvantages of this likely harmful mode of fertilization are apparently outweighed by the advantages of a rapid and imprecise, yet still secure mode of sperm transfer, with the above listed difference in spermatophores placement in meiofaunal taxa highlighting this trend. This cutaneous fertilization is probably the reproductive mode for all aphallic meiofaunal Acochlidia (Jörger et al. 2009, Schrödl & Neusser 2010) and presumably also for some equally aphallic meiofaunal *Rhodope, Helminthope* and *Pseudovermis*, which lack sperm storing organs such as a *receptaculum seminis* or a *bursa copulatrix* (Brenzinger et al. 2011c, Brenzinger et al. 2013a, own unpublished data on *Pseudovermis*). In both lineages (Rhodopemorpha and Pseudovermidae) copulation is probably the plesiomorphic state with a tendency towards reduction of copulatory organs in an unstable and spatially limited environment favoring unilateral, rapid and imprecise modes of sperm transfer. Independent of the reduction of copulatory organs, in meiofaunal slugs the male genital opening tends to shift toward the anteriormost tip of the slug head, in extreme cases leading to a genital opening right above the mouth opening in the male microhedylacean acochlids *Pontohedyle milaschewitchii* (Wawra 1986, Jörger et al. 2008, Jörger et al. 2009) or the penis opening through the oral tube in the philinoglossidean cephalaspid *Pluscula cuica* (Marcus 1953, Brenzinger et al. 2013b). This is interpreted as further adaptation to the restrictions of the environment favoring a head-to-tail sperm transfer over lateral transfer.

While randomly, externally attached spermatophores seem to be a beneficial mode of sperm transfer in the interstitial habitat, they potentially present a competitive disadvantage in benthic habitats, especially those with different osmotic conditions or the potential risk of desiccation (Jörger et al. in review). All hedylopsacean Acochlidia rely on alternative modes of sperm transfer, i.e. copulation and hypodermic injection via penial stylets or armed penises (Schrödl & Neusser 2010). These modes of insemination, which lack any device for storing and transferring sperm externally, was likely advantageous for the transition out of the fully marine environment. With the evolution of a putatively harmful cutaneous insemination via a penis equipped with a hollow apical stylet in the hedylopsacean ancestor (excluding enigmatic *Tantulum elegans* which probably transfers sperm via regular copulation), the reproductive complexity in Hedylopsacea includes evolving complex copulatory structures and additional glands with dermal injection systems (Schrödl & Neusser 2010). This has been interpreted as an evolutionary 'arms race' (Schrödl & Neusser 2010) due to 'traumatic mating' (for terminology see Lange et al. 2012, Lange et al. 2013). The remarkable re-establishment of reproductive organs (such as *bursa copulatrix* and *receptaculum seminis*) in limnic derived

Acochlidiidae, which are absent in basal Hedylopsacea but present in panpulmonate sister clades, may indicate that the genetic source of these reproductive features persists and can be reactivated later in evolution (Jörger et al. in review). A secondary origin of mesopsammic lineages from benthic ancestors within Hedylopsacea is weakly supported in all current analyses (Jörger et al. in review), but the fact that meiofaunal Hedylopsacea differ from other meiofaunal slugs in their resistance against regressive tendencies in evolution remains troubling (see Discussion below).

In summary, most meiofaunal slugs show traces of paedomorphosis in their external morphology, which indicates that progenesis might have played a major role in their acquisition of minute body sizes and the evolutionary shift into the interstitial habitat (Jörger et al. 2010b, Brenzinger et al. 2013a, Jörger et al. in review). Cephalapidean Philinoglossidae form an exception, potentially originating via a gradual miniaturization from a benthic or sand-dwelling cephalaspid ancestor (Brenzinger et al. 2013b). Among meiofaunal molluscs Rhodopemorpha have carried the 'meiofaunal syndrome' to an extreme and adapted beyond recognition, reflected in the century-long debate on their molluscan vs. turbellarian affinities. Several features count as characteristic adaptations of meiofaunal slugs to the mesopsammon (i.e., form part of the 'meiofaunal syndrome') and have been confirmed as convergent developments with thus probably major functional implications. These implications and the functional contribution of e.g. accessory ganglia and spicules to life in between sand grains are still poorly understood, however, and require further comparative investigation on ultrastructural and gene or protein expression level.

Out of the mesopsammon: reversing the 'meiofaunal syndrome'?

The regressive evolution in meiofaunal taxa leading to simplified organ systems is generally considered a dead-end road to the ecological diversity of a clade, and very few examples exist which present habitat shifts out of the mesopsammon. To my knowledge, the hesionid annelid *Microphthalmus hamosus* Westheide, 1982 and the psammodrilid annelid *Psammodrilus aedificator* Kristensen & Norrevang, 1982 might be the only examples in which the phylogenetic hypothesis and morphological data indicate a secondary increase in body size and re-acquisition of a benthic life-style from a mesopsammic ancestor (Westheide 1982, Worsaae & Kristensen 2005). In *M. hamosus*, morphological features that were reduced as a result of a previous adaptation to interstitial life (e.g. setae) did not return with increasing body size (Westheide 1982) pointing to the irreversibility of the regressive evolution in these meiofaunal taxa. In meiofaunal slugs, the relationships between meiofaunal *Philine* and philiniglossids and

their macrobenthic sand-dwelling relatives among Philinidae have not been sufficiently studied to be able to address potential habitat switches; the same is true for internal relationships of rhodopemorphs. Our comprehensive molecular phylogeny of Acochlidia, which covers roughly 85% of the described species and adds another 50% of yet undescribed diversity, is largely concordant with previous morphology-based topologies and can therefore be considered a robust framework for tracing habitat shifts of these slugs in and out of the mesopsammon (Schrödl & Neusser 2010, Jörger et al. in review). Based on ancestral state reconstruction the most likely scenario is that the mesopsammic habitat had already been invaded by the ancestor of Acochlidia (Jörger et al. in review), which probably involved progenetic processes leading to a minute, aberrant body-shape (see Discussion above), loss of shell and detortion of the body, resulting in a largely symmetric acochlidian body (Schrödl & Neusser 2010). Plotting excretory and circulatory systems onto the phylogenetic hypothesis of Acochlidia, a simple sac-shaped kidney, typical for marine Euthyneura, probably resembled the ancestral state of Acochlidia (Neusser et al. 2011b). Once the acochlidian stemline split into the two major acochlidian clades in the Jurassic, their evolutionary history followed remarkably different roads.

The microhedylacean clade presents a line of regressive evolution and lacks novel morphological inventions (Jörger et al. in review). All organ systems are highly simplified and few interspecific variations occur even at the genus level: all Microhedylacea studied in sufficient detail possess a simple sac-like kidney and a simple aphallic genital system, and sperm is transferred via spermatophores (see e.g., Challis 1970, Morse 1976, Neusser et al. 2006, Jörger et al. 2007, Jörger et al. 2008, Jörger et al. 2009, Neusser et al. 2009b, Eder et al. 2011). Microhedylacean acochlids are circum-tropically distributed and also have successfully colonized temperate and even polar waters (with *Asperspina murmanica* Kudinskaya & Minichev, 1978 being the only known meiofaunal slug from polar regions (Kudinskaya & Minichev 1978, Neusser et al. 2009b)) (Jörger et al. in review). Their evolutionary history dates back to the Jurassic and, based on ancestral area chronograms on recent species, they likely survived major extinction events in the Western Atlantic or successfully recolonized the area. Nowadays they form the most successful clade of meiofaunal slugs in terms of global distribution and high local species densities (Jörger et al. in review). With regard to morphology and anatomy, they represent a stunning case of morphological stasis with hardly any variation in their regressive and highly adapted body plan (for details see Jörger et al. 2012, Jörger et al. in review). While some physiological flexibility of Microhedylacea is reflected in adaptation to cooler waters, their morphological stasis correlates with ecological stasis; no habitat shifts are known from Microhedylacea. Their morphology, which is highly adapted to

the mesopsammic world, seems to present an irreversible dead-end road to ecological diversification (Jörger et al. in review).

By contrast, a remarkable habitat flexibility is displayed by their hedylopsacean sister clade, which inhabits the marine mesopsammon and has also successfully colonized brackish waters in Pseudunelidae (see Neusser & Schrödl 2009), limnic systems in Acochlidiidae (see e.g., Odhner 1937, Wawra 1974, 1979, Haynes & Kenchington 1991, Brenzinger et al. 2011b) and semi-terrestrial habitats in the intertidal zone in Aitengidae (Swennen & Buatip 2009, Neusser et al. 2011a). Moreover, members of Aitengidae have been recently discovered in truly terrestrial habitats (Y. Kano, T. Neusser, pers. comm.) and an additional undescribed benthic lineage with acochlidian affinities has been reported from the deep sea (T. Neusser, pers. comm.). Based on our ancestral state reconstruction of Acochlidia, the most likely scenario indicates independent habitat shifts out of the fully marine mesopsammic habitat in each lineage, reversing the ancestrally minute to a secondary larger body size ('secondary gigantism') (Jörger et al. in review). In stark contrast to the morphological stasis and regressive evolution in Microhedylacea, Hedylopsacea evolved a series of morphological novelties from the rather simplified progenetic morphology of the hedylopsacean ancestor onward. The development of a complex kidney at the base of Hedylopsacea – already present in meiofaunal representatives (see e.g., Wawra 1989, Sommerfeldt & Schrödl 2005) – is discussed as a key feature of the ecological diversification of the clade, probably serving as a precursor to habitat shifts to osmotically challenging habitats (Neusser et al. 2009a, Neusser & Schrödl 2009, Brenzinger et al. 2011b, Neusser et al. 2011b). The hermaphroditic genital system shows complex developments with stylets used for sperm transfer via hypodermic injection, the development of additional paraprostatic injection systems in Pseudunelidae and peaks in the evolution of a complex 'rapto-penis' in limnic lineages (Schrödl & Neusser 2010). In this morphological complexity meiofaunal Hedylopsacea oppose the general trends of regressive evolution in meiofaunal taxa; furthermore, the complex meiofaunal forms such as *Hedylopsis* and *Pseudunela* lack characteristic adaptations to the mesopsammon such as accessory ganglia. Resisting the 'meiofauna syndrome' they retained or regained their morphological and anatomical diversity, which enabled them to rise from the mesopsammon and invade benthic marine, limnic and even terrestrial habitats, presenting a case of extraordinary ecological flexibility originating from a meiofaunal ancestor.

Diversity in Acochlidia and other meiofaunal slugs

Our world-wide sampling efforts over the past several years have revealed a wealth of undiscovered diversity in Acochlidia, as well as other lineages of meiofaunal slugs whose diversity needs to be addressed in future studies (e.g., novel lineages of Rhodopemorpha lineages (Wilson et al. 2010a)). With regard to Acochlidia the new material partly relates to novel morphological and ecological forms: 1) Aitengidae in (semi-) terrestrial habitats (Swennen & Buatip 2009, Neusser et al. 2011a), 2) a novel lineage at genus level in limnic habitats (see Acochlidiidae in Jörger et al. (in review)) and 3) a novel yet undescribed family-level lineage which (re-)establish a marine benthic lifestyle in the deep sea (T. Neusser pers. comm.). The marine mesopsammon also bore novel forms, which differed unequivocally from all described acochlidian lineages by characters of the external morphology – which is usually rather conserved due to constraints of the habitat (see e.g., the novel family-level Hedylopsacea sp. in Jörger et al. (in review)). On the other hand, the new material from the mesopsammon largely comprises cryptic lineages (especially within morphologically static Microhedylacea), which could only be revealed as novel evolutionary entities through the use of integrative approaches employing 3D-microanatomical descriptions and molecular data (Neusser et al. 2011b, Jörger et al. 2012, Jörger & Schrödl 2013). A comparative microanatomical approach with their sister groups in combination with molecular species delineation is still needed for the 30 presented molecular operational taxonomic units (MOTUs) discovered by Jörger et al. (in review).

The low reproductive output and dispersal abilities of meiofaunal slugs point to a high degree of endemism, supported by rather narrow ranges of distribution of many meiofaunal slugs and deep genetic divergence in globally distributed lineages (Jörger et al. 2012). Moreover, the patchy occurrence typical for meiofaunal animals (e.g., Andrade et al. 2011) can easily cause species to go undiscovered even in densely sampled areas (see Curini-Galletti et al. 2012). Considering the fact that the vast majority of marine sands, worldwide and in all varying depth ranges, are still virgin soil to meiofaunal research, the currently known diversity of Acochlidia and other meiofaunal slugs very likely underrepresents the still hidden diversity by several magnitudes. The contribution of meiofauna to marine biodiversity surveys has doubtlessly been underestimated, leaving this important ecosystem largely neglected in conservation approaches. Still needed to face the taxonomic deficit are fast, efficient and reliable means of species delineation in meiofaunal taxa – means that are capable of dealing with the putatively high degree of cryptic speciation likely to be the rule for meiofauna (Jörger et al. 2012).

Towards integrative species delineation in elusive taxa

The past and the present of species delineation in meiofaunal slugs

Traditionally, species delineation in gastropods largely relies on external morphology, i.e., characteristics of the shell, combining the advantages of unproblematic preservation in natural history collections and *post mortem* identification potential (Bouchet & Strong 2010). However, several largely molecular driven studies have demonstrated the potentially high intraspecific variability of both shell morphology (e.g., Hauswald et al. 2008, Bouchet & Strong 2010, Puillandre et al. 2012b) and external characters in general, such as color variation in slugs (e.g., Nitz et al. 2009). When representatives of meiofaunal slug lineages were first discovered in the late 19th century, their aberrant external morphology was in many cases sufficient for species delimitation (see e.g., Kowalevsky 1901a, b). This changed with every new discovery of other closely related meiofaunal gastropods, making further characteristics of radulae and spicules obligatory for species delineation within clades (see e.g., Salvini-Plawen 1973, Arnaud et al. 1986, Wawra 1987, Salvini-Plawen 1991a). In restricted geographic areas, a combination of these characters might still be sufficient to diagnose mesopsammic slugs (Eder et al. 2011), but on a broader scale these characters become insufficient (Neusser et al. 2011b, Jörger et al. 2012). External features in mesopsammic slugs are heavily constrained by the requirements of the spatially restricted habitat and provide little variation. Other features – such as the presence of externally visible eyes and, potentially, details of radula morphology – show high intraspecific plasticity (see e.g., Neusser et al. 2011b, Jörger et al. 2012, Brenzinger et al. 2013b). Redescriptions of all major meiofaunal slug lineages based on advanced 3D-microanatomy in conjunction with ultrastructural data from e.g., radulae have contributed microanatomical characters across all organ systems, and these characters have proven reliable for taxonomic purposes (Neusser et al. 2006, Neusser & Schrödl 2007, Jörger et al. 2008, Rückert et al. 2008, Neusser et al. 2009a, Neusser et al. 2009b, Jörger et al. 2010a, Martin et al. 2010, Brenzinger et al. 2011c, Eder et al. 2011, Kohnert et al. 2011, Brenzinger et al. 2013a). These studies demonstrated the high quality of modern morphological approaches, which provide reliable, highly detailed diagnostic characters for taxonomic and systematic studies. But even high-end morphology ran up against its limits when confronted with the extraordinary degree of convergent adaptation notorious for gastropods (Ponder & Lindberg 1997, Dayrat & Tillier 2002, Wägele et al. 2013); and this adaptation is carried to an extreme in taxa that inhabit environments such as the mesopsammon, which selects for certain morphological and anatomical adaptations. Moreover, 3D-microanatomical approaches are very time-consuming, facing taxonomists with a trade-off between detailed accounts on a small number of specimens

and estimations of the intraspecific vs. interspecific variability of characters. Intraspecific and even intraindividual variation seems to be prominent in meiofaunal lineages, potentially induced by progenesis in their evolution. In fatal combination with regressive tendencies resulting in low interspecific variability in small, complex organ systems, this clearly impedes the delineation success of approaches restricted to morphology. Therefore, only integrative approaches that combine evidence from morphology and molecules represent a viable method for tackling the diversity of meiofaunal slugs (Neusser et al. 2011b, Jörger et al. 2012). Given the putative high degree of cryptic speciation in meiofaunal taxa with supposedly low dispersal abilities (see e.g., Westheide & Schmidt 2003, Casu et al. 2009, Fontaneto et al. 2009, Leasi & Todaro 2009, Andrade et al. 2011, Jörger et al. 2012, Tulchinsky et al. 2012), it currently seems most efficient to reverse the traditional taxonomic workflows and initiate species delineation in meiofauna with barcoding and molecular species delineation approaches.

Molecular species delineation

DNA barcoding and molecular species delineation have been broadly advocated as fast and efficient means for dealing with the taxonomic impediment in times of biodiversity crisis (Blaxter 2004, Blaxter et al. 2005, Hebert & Gregory 2005, Markmann & Tautz 2005, Hajibabaei et al. 2007). However, DNA-barcoding in its similarity-based form, which uses genetic distances, is a tool of species identification and not species discovery (DeSalle et al. 2005, DeSalle 2006). Lacking a predictive component, DNA-barcoding fails when no identical sequences are deposited in public databases (like Barcode of Life Data System http://www.boldsystems.org/ or GenBank http://www.ncbi.nlm.nih.gov/genbank/). With the vast majority of marine meiofauna that have yet to be explored (Curini-Galletti et al. 2012), not to mention identified and sequenced, identical matches of newly collected material with deposited sequences will be the exception for meiofaunal taxa for decades to come (Jörger et al. 2012). Meiofaunal biodiversity assessments, therefore, require advanced methods of molecular species discovery beyond typical DNA barcoding identification approaches.

Most of the numerous emerging programs and algorithms that have recently become available for molecular species delineation either rely on the comparison of genetic distances or use phylogenetic trees to estimate support under different model assumptions. To cluster sequences based on genetic distances, programs either use fixed or relative thresholds between intraspecific and interspecific variation (e.g., Hebert et al. 2004a, Jones et al. 2011, Ratnasingham & Hebert 2013) or aim to detect breaks in patterns of distance distribution (i.e., a 'barcoding gap' (Meyer & Paulay 2005)) (Puillandre et al. 2012a). Distance-based approaches

are usually based on mitochondrial cytochrome *c* oxidase subunit I (COI); this standard barcoding marker presents unique species-specific diagnostics in approximately 95% of all tested species. Moreover, the interspecific variability clearly exceeds the intraspecific one in most of these cases (Hebert et al. 2003a, Hebert et al. 2003b, Ratnasingham & Hebert 2013). Despite high success rates in praxis, the use of thresholds as proxy of species delimitation has been criticized for their arbitrariness; and criticism has been underscored by empirical studies demonstrating the disappearance or absence of a 'barcoding gap' (i.e., intraspecific exceeds interspecific variability) with increased sampling rates (Moritz & Cicero 2004, Meyer & Paulay 2005, Wiemers & Fiedler 2007, Astrin et al. 2012). Model-based approaches such as the popular General-Mixed-Yule-Coalescent (GMYC) model infer evolutionary entities by evaluating likelihood values under speciation vs. population genetic processes on phylogenetic trees (Pons et al. 2006, Monaghan et al. 2009). But the accuracy of this and other model-based approaches also relies on sampling coverage (Lohse 2009). A dense sampling coverage is usually utopic when it comes to elusive taxa, whose sampling records frequently include a high degree of singletons. The rarity of taxa in undersampled datasets hampers reliable estimation of intraspecific vs. interspecific variation, and constitutes the primary obstacle of successful molecular species delineation in elusive taxa. Currently, only a Bayesian approach evaluating for differences among gene trees is potentially capable of dealing with singletons, provided that data from several independent markers is combined (Yang & Rannala 2010, Zhang et al. 2011). In the absence of a 'gold standard' for evaluating the performance of different species delineation approaches, and in view of high degrees of rarity in putatively undersampled datasets, an integrative approach of species delineation is needed for elusive taxa, one which allows for thorough cross-validation between approaches.

Workflow of integrative species delineation

In times of biodiversity crisis, fast and efficient means of species delineation and re-identification are needed (see e.g., Wiens 2007, Puillandre et al. 2012b, Riedel et al. 2013a), but just as important are approaches targeted to delimit elusive taxa, if we are to avoid missing this certainly significant part of biodiversity. Herein, I aim to develop a workflow capable of dealing with the above-mentioned problems, which are likely symptomatic for many meiofaunal taxa and other little explored and rare taxa such as, e.g., many deep-sea clades. Due to the putative high degree of cryptic speciation and intraspecific variability on morphological character sets, the workflow is founded on molecular data. Faced with incomplete datasets and rarity, however, it is designed to make best use of the taxonomic information scattered across

different lines of evidence. The proposed workflow (see Fig. 2) develops and revises the approach described in Jörger et al. (2012).

Step 1: Optimize taxon sampling and character sampling

In concordance with previous species delineation workflows (e.g., Puillandre et al. 2012b), this approach emphasizes the importance of dense taxon sampling to ensure reliable species delineation and to avoid overestimating interspecific variability (Hebert et al. 2004b). In elusive taxa, taxonomists frequently lack knowledge on biology, dispersal abilities and geographic distribution, which requires an even stronger emphasis on targeted taxon sampling with regard to both geography and phylogenetic relationships. This includes collecting and analyzing several individuals from populations covering the potential geographic range of a taxon. Hence this workflow requires an *a priori* survey of the described species of a lineage; and whenever possible, material of valid species derived from type material or specimens re-collected at type localities should be included.

Under the unified species concept, species are defined as independently evolving metapopulation lineages, with the former secondary species criteria of competing concepts serving as equal operational criteria, i.e., lines of evidence (de Queiroz 2005b, 2007). As central operational criteria serve intrinsic reproductive isolation, monophyly, exclusive coalescence, diagnosibility and deficits of genetic intermediates; the reliability of proposed species hypotheses increases with the number of supporting lines of evidence (de Queiroz 2005b, 2007). Entities discovered in molecular species delineation approaches should, therefore, be supported by a minimum of one line of evidence. Consequently, the workflow requires gathering all characters sets evaluated and selected by the taxonomist as contributions to one of these operational criteria (e.g., molecular data, morphological and anatomical characters with special emphasis on reproductive features, geographic distribution, behavioral data, ecological niches).

Step 2: 'All-in' – plotting of data onto a molecular phylogeny.

Studies in species delineation of elusive taxa should aim to gather as much putative relevant information from different sources as possible. Despite all efforts to ensure complete data matrices, in reality some populations will provide exhaustive information, others will be resembled by singletons only, amplification success may vary among individuals, immature specimens can prevent the exploration of reproductive features, etc. In typical broad-scale barcoding approaches for biodiversity assessments, amplification problems of the COI-barcoding marker can result in incomplete, ambiguous sequences that are unable to pass quality

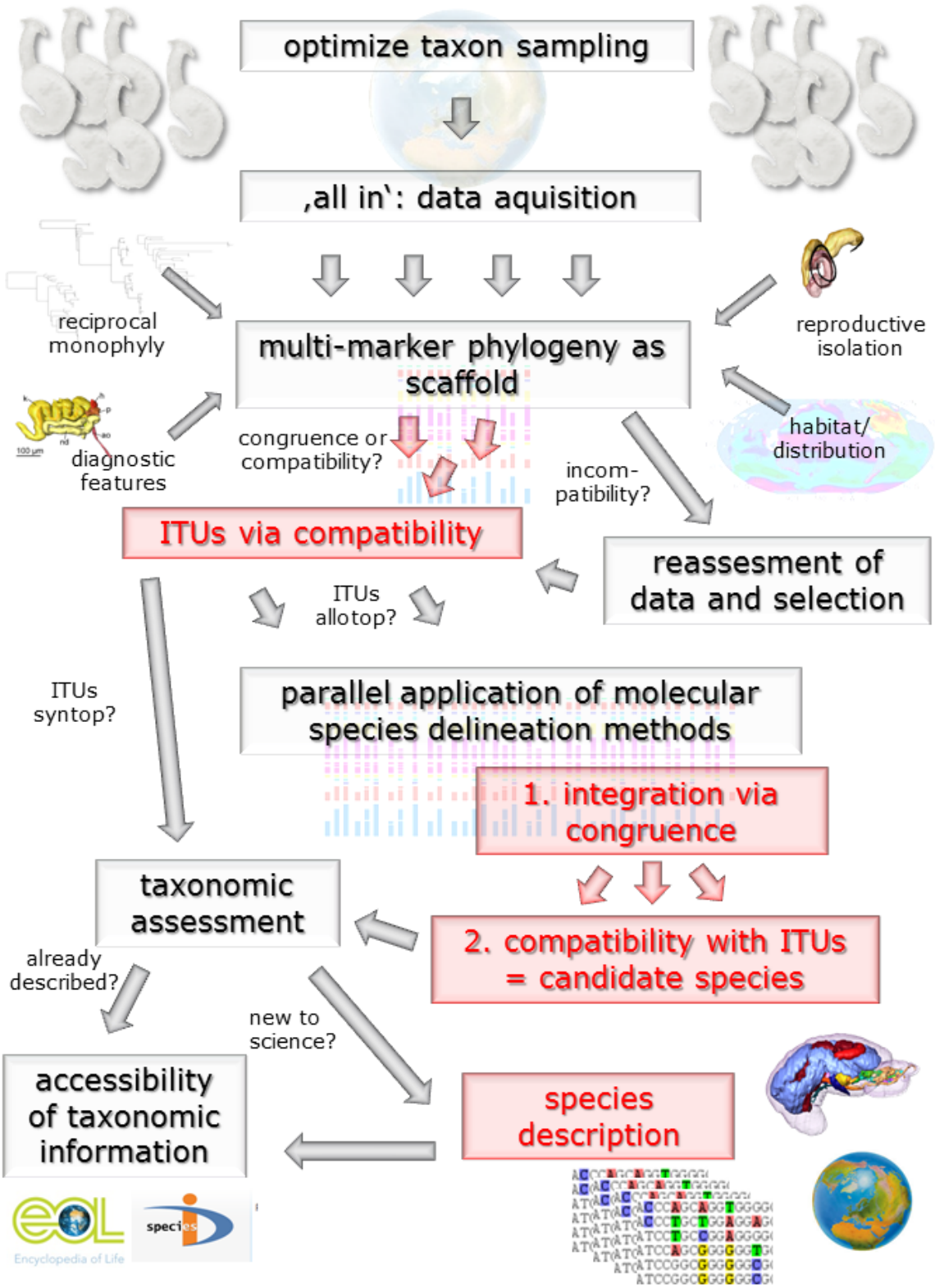

Figure 2: Flowchart on the proposed workflow on species delineation in elusive taxa, modified after the approach by Jörger et. al. (2012)

filters on automated OTU-pipelines on BOLD (Ratnasingham & Hebert 2013), thus causing the lineage to be irretrievably lost for further assessments. To ensure the inclusion of all available lineages in this species delineation approach in spite of missing data, the scattered information on individual lines of evidence must be compensated by the amount of (self-contained) lines of evidence. Therefore, and to account for problems of incompatibility between species and gene trees (caused mainly by incomplete lineage sorting, pseudogenes or introgression (Bensasson et al. 2001, Funk & Omland 2003, Song et al. 2008)), this approach is based on 'multi-barcoding', including independently evolving markers (ideally from mitochondria and nucleus) (Jörger et al. 2012). As this approach is based on phylogenetic theory via the criterion of monophyly, single gene trees are calculated from each individual marker, while a concatenated all-marker phylogeny serves as scaffold for plotting other sources of data (focusing on those that putatively serve as operational criteria). There are two major advantages of this unfortunately time-consuming initial step: 1) The unvalued objective plotting of the available characters of all terminals (or at least populations, if there is no doubt on conspecifity) in the dataset without biased pre-selection based either on initial (single-gene) molecular data or taxonomic intuition relying on, e.g., morphological criteria. A prefiltering of available data into morphotypes (Riedel et al. 2013a) leaves potential cryptic species undiscovered and is therefore not advisable for meiofaunal taxa. 2) The potential for quality checks and cross validation between different lines of evidence. This critical reassessment of the primary data can help to reveal problematic molecular markers or potential homoplasies on morphological characters.

Step 3: 'Wild cards' and selection of integrative taxonomic units (ITUs) via compatibility.

Based on the plotted data, integrative taxonomic units (ITUs) are defined: Integrative taxonomy is commonly considered best practice (Dayrat 2005, Will et al. 2005, Valdecasas et al. 2008, Padial & De La Riva 2010, Padial et al. 2010, Astrin et al. 2012, Riedel et al. 2013a), but approaches differ considerably in how they interpret integration, with severe consequences for the resulting species hypothesis. Typical large-scale barcoding workflows cluster COI sequences into operational taxonomic units (OTUs) based on genetic distances via different algorithms, and they encourage the addition of accessory data from other molecular markers or e.g., morphology (Jones et al. 2011, Puillandre et al. 2012a, Ratnasingham & Hebert 2013). When additional data is added, the species diagnosis becomes integrative, whereas species delineation, which has led to the discovery of the OUT, remains single-lined and is not questioned or critically revised by additional data.

When truly integrating data into the process of species delineation, there is debate on the degree of congruence that different characters need to provide (Padial et al. 2010). Integrating via

congruence require a minimum of two selected lines of evidence to support the proposed species hypothesis, while in 'integration via cumulation' approaches the divergence of any character can justify the designation of species (Padial et al. 2010). The former approach promotes taxonomic stability, but implies the risk of underestimating species numbers. Integrative taxonomy via cumulation, on the other hand, tends to overestimate species, but is thereby likely best suited to discovering recent lineages (Padial et al. 2010). Ideally, ITUs can be selected across the integrative scaffold established in this workflow via congruence across all lines of evidence i.e., reciprocal monophyly supported by morphological features and geographic and habitat boundaries. On small datasets, concordance between operational criteria can be evaluated by eye, but especially on larger datasets, the support of statistical methods as developed by Cardoso et al. (2009) is advisable. The presented workflow suggests a less stringent application of congruence, promoting integration via compatibility, i.e. allowing for entities supported by some and uncontradicted by other lines of evidence (see the 'minimum consensus' approach in Jörger et al. (2012)). This accommodates the fact that the process of speciation does not necessarily implement changes on all different levels of characters (Padial & De La Riva 2010, Padial et al. 2010), but remains conservative in relying solely on uncontradicted support for species hypothesis. The effects of speciation patterns may, however, result in incongruent datasets (Padial & De La Riva 2010, Padial et al. 2010). Integrative taxonomy must not be misunderstood as simply adding more and more data, rather it urges cautious selection of the appropriate character set for the species under investigation (Valdecasas et al. 2008). Faced with incongruence, the debate on which character set is best suited to species delineation cannot be solved universally, but has to be decided in each individual case with regard to the specifics of each taxon.

At this point the posed workflow offers a potential short-cut to species assignment: reciprocally monophyletic clades occurring in syntopy can be considered species under the unifying species concept, combining the operational criteria of intrinsic reproductive isolation with reciprocal monophyly (de Queiroz 2005b, 2007). The evaluation of syntopy in meiofauna is problematic, however, due to the largely unknown ecological interactions and potentially small-scale microhabitats.

Although less conservative than integrative taxonomy via congruence, the compatibility approach will nevertheless tend to lump species. Because it relies on the criterion of reciprocal monophyly, this initial step is likely not suited for detecting recent lineages (Knowles & Carstens 2007, Sauer & Hausdorf 2012). It therefore needs to be refined in Step 5 in order to uncover any potential lumping of species.

Step 5: Parallel application of available methods of molecular species delineation

As discussed above, the accuracy of all available algorithms of molecular species delineation suffers from undersampled datasets and the inclusion of singletons (Jörger et al. 2012), also tending to oversplit datasets in empirical studies (e.g., Sauer & Hausdorf 2012). In the absence of a 'gold-standard' for comparing the performance of each analysis on the respective dataset, the workflow suggests an unbiased parallel application of available molecular species delineation methods across all markers. Special emphasis should be given to model-based approaches such as GMYC (Pons et al. 2006, Monaghan et al. 2009) – provided that minimum requirements, e.g., on sampling density are fulfilled – and algorithms capable of dealing with rarity (such as Bayesian Species delineation when combining multiple markers (Yang & Rannala 2010, Zhang et al. 2011)). The inference of genetic connectivity via haplotype networks applying statistical parsimony (Clement et al. 2000) is merely an indirect method of estimating species boundaries (Pons et al. 2006); nevertheless, it visualizes the genetic structure in the dataset, which is valuable for cross-validating molecular entities revealed by other approaches. Even though the performance of distance-based approaches is conceptually disputed and practically hindered in lineages that putatively suffer from incomplete sampling (Meyer & Paulay 2005, Hickerson et al. 2006, Meier et al. 2006, Wiemers & Fiedler 2007, Meier et al. 2008), the parallel application of Refined Single Linkage analysis (RESL) (Ratnasingham & Hebert 2013) or ABGD (Puillandre et al. 2012a) contributes to empirically evaluating the efficiency of standard barcoding approaches on elusive taxa.

Step 6: Congruence in molecular species delineation and compatibility of ITUs to determine candidate species

To exploit the potential of molecular species delineation methods for revealing prior lumping of species, integration via compatibility is inapplicable in this step, as it would directly transfer over-splitting of each individual method to the identification of the candidate species. The workflow aims for a cross-validation between the different approaches achieved by integrating the results via congruence. Only molecular entities supported by all molecular species delineation approaches are then further integrated via compatibility to the formerly identified ITUs in order to lead to the final determination of the candidate species.

Step 7: Assessment of the taxonomic history

Advantageous compared to hyperdiverse clades in the focus of e.g., biodiversity assessments, elusive taxa usually lack an exhaustive history of available descriptions and putative synonyms,

the majority of lineages being still undescribed. To avoid the creation of synonyms, it is inevitable to clarify whether the discovered candidate species already bears a valid name.

Step 8: Species description

Independently evolving lineages discovered as candidate species, but which cannot be assigned to valid species, should be described to receive formal recognition. Molecular species delineation approaches frequently terminate efforts with the discovery independently evolving lineages (e.g., Fontaneto et al. 2009, Monaghan et al. 2009, Astrin et al. 2012). The BIN system (Barcode Index Numbering on BOLD) even propagates the use of OTUs as an alternative taxonomic reference system (Ratnasingham & Hebert 2013), drawing on initial proposals on DNA taxonomy (Tautz et al. 2003). The amount of deposited sequences, which are unidentified at the species level and bear other unique identifiers, has grown tremendously in the course of the barcoding endeavor over the past few years (see http://iphylo.blogspot.de/2011/04/dark-taxa-genbank-in-post-taxonomic.html). Clustering sequences into OTUs may be sufficient for many further applications such as biodiversity assessments, while unidentified sequences can still contribute valuable information in the absence of species assignment. The name of a species is entirely extrinsic to its nature, it could thus be replaced by any alternative identifier such as a BIN. However, the use of OTUs not merely as a source of taxonomic characters, but also as a taxonomic reference system, can be problematic when it comes to establishing novel unique identifiers, as they create parallel taxonomies flagged with new acronyms and classification systems in competition with traditional taxonomy (Jörger & Schrödl 2013). The Linnaean name anchors the species to its taxonomic history and all available biological and morphological data (Polaszek et al. 2008, Patterson et al. 2010). Moreover, the genus name includes a hypothesis on phylogenetic relationships. A species name can be linked to life science identifiers via Zoobank (http://zoobank.org/), capable of uniquely linking content on this specie through different computational platforms (Polaszek et al. 2008). In order to reduce and not enhance impediments in taxonomy by parallel inconsistent identifiers, discovered lineages should be connected to the taxonomic history of a clade by providing formal descriptions (Jörger & Schrödl 2013). Depending on the chosen operational criteria of species delimitation, this set of characters will form the basis for the diagnoses of species. Jörger & Schrödl (2013) illustrated how molecular diagnostic characters can be extracted via character-based barcoding approaches (Sarkar et al. 2008, Bergmann et al. 2009) and used for taxonomic description. Future efforts should aim to automate the extraction of diagnostic molecular characters to facilitate and accelerate species description, as has already been achieved in 'turbo-taxonomic' approaches for morphological data (Butcher et al. 2012, Riedel et al. 2013a).

Step 9: Ensure accessibility of all data

Digital technologies provide powerful methods of making taxonomic data more accessible to the research communities via, e.g., virtual access to museums collections, digitalized biodiversity libraries, online registration systems for zoological names and infrastructure for biogeographic assessments (see e.g., Wheeler 2008, Padial & De La Riva 2010, Padial et al. 2010). Next to the obligatory voucher deposition in museum collections (including vouchers of extracted DNA) and of genetic sequences in public databases, data from species descriptions can now be deposited in online platforms (wiki databases such as the Species ID portal (http://species-id.net/wiki/))(Hendrich & Balke 2011). This increases the accessibility of taxonomic knowledge and allows for dynamic expansion of species diagnoses through future studies (Riedel et al. 2013a), ideal for gradually augmenting knowledge on enigmatic taxa.

The future of taxonomy: deep descriptions in times of turbo pipelines?

The calls for a renewal of taxonomy – frequently coined with the term 'new taxonomy' – have increased over the past decade, reflecting taxonomists' efforts to shrug off their stuffy image and fight for the survival of an 'endangered science' (see e.g., Wilson 2004, Wheeler 2008, Wägele et al. 2011). Discussions on modern forms of taxonomy frequently comprise three major aspects: 1) modern taxonomy should be integrative. Today, as discussed above, integrative taxonomy, which especially unites molecular and morphological data but also embraces other lines of evidence, is generally considered best practice (Dayrat 2005, Will et al. 2005, Valdecasas et al. 2008, Padial & De La Riva 2010, Padial et al. 2010, Riedel et al. 2013a), while several approaches to integration of data on different levels of species delineation and description still need to converge. 2) Modern taxonomy should be accessible ('cybertaxonomy') and thus dynamic (Wheeler & Valdecasas 2010, Hendrich & Balke 2011, Riedel et al. 2013a). It is evident that taxonomy has transformed in the digital age and can only be of benefit through the use of online resources that enable better interaction among research communities and increase accessibility of taxonomic content and literature to its users (Wilson 2003, 2004, Wheeler 2008, Wheeler & Valdecasas 2010). 3) Modern taxonomy should be highly effective and fast. In times of biodiversity crisis, the taxonomic community and its end-users alike call for an acceleration of taxonomic descriptions to face the 'taxonomic impediment' enhanced by substantial shortage of funding for taxonomic training and labor (de Carvalho et al. 2005, de Carvalho et al. 2007, Ebach et al. 2011). Advances in 'turbo-taxonomy' have impressively demonstrated the acceleration species descriptions via automated taxonomic procedures, nevertheless broadening initial hypotheses based on molecular

barcoding data into integrative fast-track taxonomic descriptions including morphological characters (Butcher et al. 2012, Riedel et al. 2013a, Riedel et al. 2013b). Turbo-taxonomic approaches – provided they are not pursued at the back of accuracy – contribute significantly to known biodiversity and perform a great service to biodiversity assessments. Neglected, rare and elusive taxa, however, are usually of little interest to large-scale biodiversity surveys; and the mode of taxonomy in these taxa is influenced by its major application, such as in the study of evolutionary processes. Taxonomy serves as a pioneering exploration of life on Earth and constitutes the foundation for most other disciplines within biology, such as ecology, phylogenetics or conservation (Wilson 2004). Taxonomic in-depth exploration of microanatomy and morphology are just as justified as turbo-taxonomy in focusing on different applications of taxonomic knowledge, e.g., as a backbone for studying the evolutionary processes which have formed present day biodiversity.

Clearly, modern taxonomy should and will integrate novel sources of information as well as advanced technologies, thus enhancing the efficiency and transparency of taxonomic work. However, these are merely tools. The scientific hypotheses driving a taxonomist's research, as well as the species-specific distinctiveness of every discovered novel lineage that reflects its evolutionary history, will continue to determine workflows in taxonomy and pave a way between turbo-taxonomy and deep descriptions.

CONCLUSIONS AND OUTLOOK

Over the course of my thesis, minute mesopsammic slugs have managed to overthrow the long-standing classification of one of the most diverse clades of gastropods. The proposed 'new phylogenetic hypothesis on Euthyneura' is in concordance with initial large scale phylogenomic approaches, though the latter still need to be improved with regard to taxon sampling before the hypothesis can claim to be supported. In order to take the dense taxon sampling on heterobranch gastropods gathered over the past years by various research groups and raise it to the next level of Next Generation Sequencing – which would allow an augmentation of molecular markers by magnitudes – novel DNA-based phylogenomic approaches will need to be tested for phylogenetic analyses. My thesis has highlighted the importance of dealing with euthyneuran slugs and snails as an evolutionary entity, avoiding pre-selections based on systematic biases that might result in artificial groupings and hinder the reconstruction of their true phylogenetic relationships. It has been shown how the inclusion of enigmatic, neglected taxa can significantly contribute to the understanding of the evolutionary history of a clade. My thesis therefore reinforces the need to fill in the gaps in existing taxon samplings with remaining elusive taxa, in order to supplement the complex picture of heterobranch evolution step by step.

What started with a small enigmatic group of poorly known mesopsammic sea slugs with few limnic relatives has developed via an extensive morphological and molecular framework into a well-explored basal panpulmonate clade that presents a complex evolutionary history. Over the course of their evolution, Acochlidia have successfully transitioned between interstitial and epibenthic habitats as well as diverse aquatic systems and terrestrial grounds. Given their striking habitat flexibility, these small slugs show how far a gastropod can get even without a shell. They might therefore serve as a model taxon in future studies that seek to explore the morphological, physiological and biochemical properties that accompany habitat transitions and the evolutionary processes driving these changes.

Based on my data, the shift to a permanent inhabiting of the mesopsammon occurred along the acochlidian stemline in the Mesozoic Jurassic; it likely involved progenesis, shaping the aberrant acochlidian external morphology and leading to the minute body size. Progenesis is likely involved in the colonization process of the interstitial habitat in most meiofaunal slug lineages, many of which exhibit a combination of regressive tendencies and novel inventions in their body plans. Past and ongoing 3D-enhanced microanatomical (re-) descriptions of all major meiofaunal slug lineages serve as foundation for comparative morphological studies on the convergent adaptations to a challenging – because spatially restricted and dynamic – habitat.

Studies on ultrastructural as well as gene and protein expression level are now needed to further investigate the functional aspects of these convergent adaptations, such as accessory nervous features or the biochemical properties of the slugs striking adhesive abilities, to contribute to a better understanding of life between sand grains.

Almost every single meiofauna sampling trip conducted in the course of this thesis resulted in several lineages new to science, once more emphasizing the taxonomic deficit in meiofaunal research and the high degree of still hidden diversity in meiofaunal slugs. In establishing several new clades and reorganizing acochlidian systematics via molecular phylogenies, my thesis calls for a taxonomic revision of Acochlidia to incorporate the results of the past years into a revised classificatory system. The species status of discovered entities flagged as MOTUs in the present work needs to be determined via species delineation methods, which in the case of most meiofaunal slugs presents a struggle with rarity and uniformity. The developed species delineation workflow for elusive taxa is designed to make best use of scattered data available on different lines of evidence and consequently presents a time-consuming form of 'deep-taxonomy'. Because morphologically static meiofaunal slugs with low dispersal abilities are prone to cryptic speciation, molecular analyses serve as a backbone for species delineation and description. Formal species description should be routinely included as a step in automated species delineation workflows in order to eliminate the risk of parallel taxonomies. In cryptic lineages, automatically extracted molecular diagnostic characters can be smoothly integrated into the traditional Linnaean system as demonstrated in my thesis. Last but not least, it is time to make meiofauna more visible both to other researchers and the interested public via online depository systems such as the 'Encyclopedia of Life'.

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SUPPLEMENTARY MATERIAL

Supplementary material 1: Sampling localities of Acochlidia included in this thesis

Recollecting attempts at the same position are marked with a and b. Collectors: AA – Andreas Altenöder, PB – Pat Boaden, LD – Ludwig Demharter, AD – Angela Dinapoli, BE –Barbara Eder, GH – Gerhard Haszprunar, MH – Martin Heß, KJ – Katharina Jörger, YK – Yasunori Kano, KK – Kevin Kocot, AM – Alexander Martynov, RM – Roland Meyer, TN – Timea Neusser, GR – Greg Rouse, JS – Julia Sigwart, MS – Michael Schrödl, ES – Enrico Schwabe, NW – Nerida Wilson

Material from Indonesia was made available as permanent loan through Fontje Kaligis and Gustaf Mamangkey (University of Manado). Sampling in Panama and Belize was conducted under a permit to Jon Norenburg.

Field code	Locality	Water body	GPS	Collector/ date	Depth	Habitat remarks
North East Atlantic (including Mediterranean and Black Sea)						
NEA-1	Cape Fiolent, Sevastopol, Krim, Ukraine	Black Sea	44°29.00'N; 33°29.00'E	AM/TK 07.09	15 m	marine, interstital
NEA-2	Cape Fiolent, Sevastopol, Krim, Ukraine	Black Sea	44°29.00'N; 33°29.00'E	AM/TK, 08.07	7 m	marine, interstital
NEA-3a	Little Saline-Bay, Rovinj, Istria, Croatia	Mediterranean Sea	45°7.06'N; 013°3.99'E	MS, 06.08	2-3 m	marine, interstitial, coarse sand
NEA-3b	Little Saline-Bay, Rovinj, Istria, Croatia	Mediterranean Sea	45°7.06'N; 013°3.99'E	KJ/RM, 07.05	3-4 m	marine, interstitial, coarse sand
NEA-3c	Little Saline-Bay, Rovinj, Istria, Croatia	Mediterranean Sea	45°7.06'N; 013°3.99'E	ES, 08.08	2-3 m	marine, interstitial, coarse sand
NEA-3d	Little Saline-Bay, Rovinj, Istria, Croatia	Mediterranean Sea	45°7.06'N; 013°3.99'E	MS, 06.10	2-3 m	marine, interstitial, coarse sand
NEA-4	Revellata, Calvi, Corse, France	Mediterranean Sea	42°33.95'N; 008°44.25'E	MH/GH, 09.08	22 m	marine, interstitial, coarse sand and shell grid
NEA-5	Canet-Plage, Languedoc-Roussillon, France	Mediterranean Sea	42°39.92'N; 003°2.10'E	MH, 07.09	intertidal	marine, interstital, fine dark sand
NEA-6a	Bacoli, Campania, Italy	Mediterranean Sea	40°47.32'N; 014°3.90'E	MS, 09.09	intertidal	marine, interstitial, fine vulcanic sand
NEA-6b	Bacoli, Campania, Italy	Mediterranean Sea	40°47.32'N; 014°3.90'E	MS, 10.06	intertidal	marine, interstitial, fine vulcanic sand
NEA-7	Secche della Meloria, Livorno, Italy	Mediterranean Sea	43°32.75'N; 010°13.20'E	MS, 09.05	0-3 m	marine, interstital, coarse sand
NEA-8	Rua de Ares, Ferrol, Galicia, Spain	Atlantic Ocean	43°25.66'N; 008°18.48'W	MS, 03.08	35 m	marine, interstitial, coarse sand
NEA-9	Pieiro pequeno, Ferrol, Galicia, Spain	Atlantic Ocean	43°27.55'N; 008°20.20'W	MS, 03.08	8.5 m	marine, interstital
NEA-10	Fiskabäckskil, Bohuslän, Sweden	Northern Sea	58°12.78'N; 011°19.40'E	AD, 06.07	23 m	marine, interstitial
NEA-11	Bonden Island, Bohuslän, Sweden	Northern Sea	no data	MS/TN/ KJ, 08.08	20 m	marine, interstitial, coarse sand and shell gravel

NEA-12	Portaferry, Northern Ireland	Irish Sea	54°22.84'N; 5°33.04'W	JS/PB, 10.09	0-1 m	marine, interstitial, coarse sand and shell gravel
NEA-13a	Cape Kamenjak, Premantura, Istria, Croatia	Mediterranean Sea	44°46.00'N; 013°55.30'E	MS, 09.09	3-4 m	marine, interstital, coarse sand and shell gravel
NEA-13b	Cape Kamenjak, Premantura, Istria, Croatia	Mediterranean Sea	44°46.066'N, 13°54.948'E	BB, 06.10	7-8 m	marine, interstital, coarse sand
NEA-13c	Cape Kamenjak, Premantura, Istria, Croatia	Mediterranean Sea	44°46.00'N; 013°55.30'E	BB, 10.10	3-4 m	marine, interstital, coarse sand
NEA-13d	Cape Kamenjak, Premantura, Istria, Croatia	Mediterranean Sea	44°46.00'N; 013°55.30'E	KJ/RM, 05.07	6-9 m	marine, interstitial, coarse sand and shell gravel
NEA-14	"Amphioxous Grund", Heligoland, Germany	Northern Sea	no data	MS, 06.10		marine, interstital, coarse sand
NEA-15	Secche della Meloria, Livorno, Italy	Mediterranean Sea	43°33.02'N; 010°13.13'E	MS, 09.09	3-4 m	marine, interstital, coarse sand
NEA-16	Campese, Gilio, Italy	Mediterranean Sea	42°21.96'N; 10°52.64'E	TL, 06.10	1-2 m	marine, interstital, coarse sand
NEA-17	S. Andrea, Elba	Mediterranean Sea	42°48.578 N, 10°08.480 E	MS, 99	10-20 m	marine, interstital, coarse sand
NEA-18	Punta Corrente, Rovinj, Istria, Croatia	Mediterranen Sea	45°3.994'N; 13°37.670'E	DE, 06.05		marine, interstitial, coarse sand
NEA-19	Dalniye Zelentsy, Murmanskaya obl., Russia	Barents Sea	69°07.00'N; 36°04.00'E	AM/TK, 06.06	intertidal	marine, interstitial, coarse sand
NEA-20	Islote Mesa do Con, Ría de Arousa, Galicia, Spain	Atlantic Ocean	42°31.50'N; 008°55.00'W	VU,		marine, interstital
NEA-21	Ria de Ferrol, Ferrol, Galicia, Spain	Atlantic Ocean	43°27.03'N; 008°20.39'W	MS, 03.08	41 m	marine, interstitial, medium grain size
NEA-22	Chanteiro, Ria de Ferrol, Galicia, Spain	Atlantic Ocean	43°26.95'N; 008°18.98'W	MS, 03.08	21 m	marine, interstitial, coarse sand and shell gravel
NEA-23	Roscoff, Bretagne, France	Atlantic Ocean	48°43.53'N; 03°50.71'W	GH, 06.07	20-25 m	marine, interstitial, coarse sand and shell gravel
NEA-24	Olenjovka, Kap Tarankut, Krim, Ukraine	Black Sea	45°20.53'N; 32°30.48'E	JH, 06.09	14 m	marine, interstital, coarse sand and shell gravel
North West Atlantic						
NWA-1	Canoe Beach, Nahant, Massachusetts, USA	Atlantic Ocean	42°25.20'N; 070°54.45'W	KJ, 10.10	low intertidal	marine, interstital, fine black sand with rubble
Eastern Atlantic (tropical)						
EAT-1	Mia Mia, Ghana	Atlantic Ocean	04°47.77'N; 002°10.10'W	TN/MS, 02.07	intertidal	marine, interstital, coarse sand
EAT-2	Mia Mia, Ghana	Atlantic Ocean	04°47.77'N; 002°10.10'W	TN/MS, 02.07	2-3 m	marine, interstital, coarse sand
EAT-3	Nzema Cape, Ghana	Atlantic Ocean	04°46.27'N; 002°6.50'W	TN/MS, 02.07	7 m	marine, interstital, coarse sand

Western Atlantic (tropical)						
WAT-1	"CBC-Station 3", Carrie Bow Cay, Belize	Caribbean Sea	16°48.224'N; 88°04.615'W	KJ, 01.10	31 m	marine, interstital, fine sand
WAT-2	"CBC-Station 8", Curlew Reef, Carrie Bow Cay, Belize	Caribbean Sea	16°47.416'N, 88°04.723'W	KJ, 01.10	2 m	marine, interstitial, coarse, exposed sand
WAT-3	"CBC-Station 9", Carrie Bow Cay, Belize	Caribbean Sea	16°48.127'N, 88°04.607'W	KJ, 01.10	14 m	marine, interstitial, coarse sand
WAT-4	Wild Cane Key, Bastimentos, Bocas del Toro, Panama	Caribbean Sea	09°21.102'N; 082°9.720'W	KJ/BE, 06.10	6.5 m	marine, interstital, coarse sand
WAT-5a	Wild Cane Reef, Bastimentos, Bocas del Toro, Panama	Caribbean Sea	09°21.039'N; 82°10.345'W	KJ/BE, 06.10	3 m	marine, interstital, coarse sand
WAT-5b	Wild Cane Reef, Bastimentos, Bocas del Toro, Panama	Caribbean Sea	09°21.039'N; 82°10.345'W	KJ/BE, 06.10	3 m	marine, interstital, coarse sand
WAT-6	Bay of 'Windjammer Landing', St. Lucia	Caribbean Sea	14°3.58'N; 060°58.30'W	KJ, 02.09	2-3 m	marine, interstital, coarse sand
WAT-7	Soufriere Bay, St. Lucia	Caribbean Sea	N13°51.4'; W61°3.96'	KJ, 02.09	8-9 m	marine, interstital, coarse sand
WAT-8	Villa Bay, St. Vincent	Caribbean Sea	13°7.83'N; 061°12.42'W	KJ, 02.09	intertidal	marine, interstital, coarse sand
WAT-9a	near wreck "Vapor Baixo", Pernambuco, Brazil	Atlantic Ocean	08°3.29'S; 034°47.67'W	MS, 03.10	21m	marine, interstitial, coarse sand
WAT-9b	near wreck "Vapor Baixo", Pernambuco, Brazil	Atlantic Ocean	08°3.29'S; 034°47.67'W	MS, 01.10	21m	marine, interstitial, coarse sand
WAT-10	off Castle Roads, Bermuda	Atlantic Ocean	32°20.266'N, 64°39.843'W	SF/MS, 08.99	6.5m	marine, interstitial, coarse sand
WAT-11	"CBC-Station 4", Carrie Bow Cay, Belize	Caribbean Sea	16°48.224'N, 88°04.615'W	KJ, 01.10	15 m	marine, interstitial, coarse sand
WAT-12	"CBC-Station 7", Carrie Bow Cay, Belize	Caribbean Sea	16°48.149'N, 88°04.785'W	KJ, 01.10	3-5 m	marine, interstitial, coarse, exposed sand
WAT-13	"CBC-Station 11", Carrie Bow Cay, Belize	Caribbean Sea	16°48.127'N, 88°04.607'W	KJ, 01.10	15 m	marine, interstitial, coarse sand
WAT-14	"CBC-Station 15", Carrie Bow Cay, Belize	Caribbean Sea	16°48.127'N, 88°04.607'W	KJ, 01.10	31 m	marine, interstitial, coarse sand
WAT-15	"CBC-Station 19", Carrie Bow Cay, Belize	Caribbean Sea	16°48.127'N, 88°04.607'W	KJ, 01.10	15 m	marine, interstitial, coarse sand
WAT-16	"CBC-Station 22", Curlew Reef, Carrie Bow Cay, Belize	Caribbean Sea	16°47.466'N, 88°04.568'W	KJ, 01.10	15 m	marine, interstitial, coarse sand
WAT-17	Pirapama, Pernambuco, Brazil	Atlantic Ocean	08°03.383'S, 34°46.967'W	MS, 02.10	24m	marine, interstital, coarse sand
South West Atlantic (tropical)						
SWA-1	Vila, Ilhabela, Sao Paolo, Brazil	Atlantic Ocean	23°46.33'S; 045°21.25'W	MS, 12.05	intertidal	marine, interstital, coarse sand
SWA-2	Pedra do Sino, Ilahbela, Sao Paolo, Brazil	Atlantic Ocean	23°46.72'S; 045°21.55'W	MS, 03.10	intertidal	marine, interstital, coarse sand

South East Pacific (temperate)						
SEP-1	Totoralillo, south of Coquimbo, Chile	Pacific Ocean	30°4.383'S; 71°22.639'W	TN, 10.08	intertidal	marine, interstital
SEP-2	San Juan de Marcona, Ica, Peru	Pacific Ocean	15°21.47'S; 075°11.42'W	MS, 11.06	10 m	marine, interstital, coarse sand
SEP-3	Bahia Inglesa, Región de Coquimbo, Chile	PacificOcean	27°6.264'S; 70°51.390'W	TN, 10.08	intertidal	marine, interstital
East Pacific (tropical)						
EPT-1	Punta Sal, Tumbes, Peru	Pacific Ocean	03°58.92'S; 080°59.17'W	MS, 12.06	8 m	marine, interstital, coarse sand
Eastern Indo-West Pacific						
EIP-1	channel MPA marker, Moorea, French Polynesia	Central Pacific	17°28.57'S; 149°49.86'W	NW/GR, 11.10	10-11 m	marine, interstital
EIP-2	Cook's bay pass, Moorea, French Polynesia	Central Pacific	17°29.43'S; 149°49.60'W	NW/GR, 11.10	18-20 m	marine, interstital
EIP-3	Temae lighthouse, Moorea, French Polynesia	Central Pacific	17°28.41'S; 149°46.49'W	NW/GR, 11.10	0.5 - 1m	marine, interstital
EIP-4	south end of Moorea, Moorea, French Polynesia	Central Pacific	17°36.05'S; 149°50.48'W	NW/GR, 11.10	10-20 m	marine, interstital
EIP-5	Motu Iti, Moorea, French Polynesia	Central Pacific	17°32.84'S; 149°46.59'W	NW/GR, 11.10	3-4 m	marine, interstital
North East Pacific						
NEP-1	near Friday Harbour Laboratories, San Juan, Washington, USA	Pacific Ocean	48°32.44'N; 122°58.88'W	KK, 05.10	60 m	marine, interstital
NEP-2	Punta San Francisquito, Baja California, Mexico	Gulf of California	28°24.53'N; 112°51.46'W	NW,	0-1 m	marine, interstital, coarse sand
North West Pacific						
NWP-1	Starichkov Island, Avachinsky Bay, Kamtchatka, Russia	Bering Sea	52°46.00'N; 158°36.00'E	AM/TK, 10.08	21-23 m	marine, interstital, coarse sand
NWP-2	Starichkov Island, Avachinsky Bay, Kamtchatka, Russia	Bering Sea	52°46.00'N; 158°36.00'E	AM/TK, 11.08	16-18 m	marine, interstital, coarse sand
Western Indo-Pacific						
WIP-1	Sha'ab Malahi, Egypt	Red Sea	24°11.83'N; 35°38.43'E	KJ, 07.09	20 m	marine, interstitial, fine coral sand, Amphioxous fauna
WIP-2a	Dahab, Sinai, Egypt	Red Sea	28°29.29'N; 34°30.97'E	KJ, 04.09	15 m	marine, interstitial, coarse coral sand
WIP-2b	Dahab, Sinai, Egypt	Red Sea	28°29.29'N; 34°30.97'E	MS, 10.99	15 m	marine, interstitial, coarse coral sand
WIP-3	Thulhagiri Island, North Malé Atoll, Maledives	Indian Ocean	no data	LD, 12.10	intertidal	marine, interstitial, coarse coral sand
WIP-4	Ao Yon, Phuket, Thailand	Andaman Sea	07°48.85'N; 098°23.65'E	KJ/MS, 07.10	intertidal	marine, interstitial, coarse coral sand

WIP-5	Raja Island, Phuket, Thailand	Andaman Sea	07°36.25'N; 098°22.62'E	KJ/MS, 07.10	6-7 m	marine, interstitial, fine coral sand
WIP-6	Raja Island, Phuket, Thailand	Andaman Sea	07°36.25'N; 098°22.62'E	KJ/MS, 07.10	20-22 m	marine, interstitial, coarse coral sand
WIP-7	south of Hamata, Egypt	Red Sea	24°11.83'N; 035°26.30'E	KJ, 07.09	13 m	marine, interstitial, coarse coral sand
WIP-8	Raja Island, Phuket, Thailand	Andaman Sea	07°36.53'N; 098°22.68'E	KJ/MS, 07.10	5-6 m	marine, interstitial, coarse coral sand
Central Indo-Pacific						
CIP-1	Pak Phanang Bay, Thailand	Gulf of Thailand	8°29.3'N; 100°10.92'E	CS, 09.07		semiterrestrial, mangrove swamp, benthic on mud
CIP-2	Tambala River, near Manado, Sulawesi, Indonesia	Celebes Sea	01°24.18'N; 124°41.13'E	KJ, 11.09	0.3-0.5 m	limnic, benthic, under stones
CIP-3	Malalayang River, Manado, Sulawesi, Indonesia	Celebes Sea	01°27.50'N; 124°49.30'E	KJ, 11.09	0.1-0.4 m	limnic, benthic, under stones
CIP-4	Lembeh Strait, Sulawesi, Indonesia	Celebes Sea	1°27.88'N; 125°13.8'E	KJ, 11.09	3-5 m	marine, interstitial, coarse coral sand, Amphioxous fauna
CIP-5	Gili Lawa Laut, Komodo, Indonesia	Flores Sea	8°26.98'S; 119°34.03'E	KJ, 08.07	0-3m	marine, interstitial, fine to coarse coral sand
CIP-6	Pulau Banta, Nusa Tenggara, Indonesia	Flores Sea	8°23.96'S; 119°18.93'E	KJ, 09.08	5-6 m	marine, interstitial, coarse sand
CIP-7	Pulau Banta, Nusa Tenggara, Indonesia	Flores Sea	8°23.96'S; 119°19.01'E	KJ, 08.07	0-3m	marine, interstitial, fine to coarse coral sand
CIP-8	Pulau Moyo, Nusa Tenggara, Indonesia	Flores Sea	8°13.98'S; 117°28.53'E	KJ, 09.08	5-6 m	marine, interstitial, coarse coral sand, Amphioxous fauna
CIP-9	Bondokodi, Sumba, Indonesia	Indian Ocean	9°35.57'S; 118°59.82'E	KJ/RM, 09.08	0.1-0.4 m	limnic, benthic, under stones
CIP-10	Capi, Flores, Indonesia	Flores Sea	8°32.92'S; 119°53.92'E	KJ/RM, 09.08	0.1-0.4 m	limnic, benthic, under stones
CIP-11	Kemeri, Leitihu, Ambon, Indonesia	Banda Sea	3°40.4'S; 128°8.57'E	MG, 10.08		limnic, benthic, under stones
CIP-12	Watatiri, Leitihu, Ambon, Indonesia	Banda Sea	9°37.05'S; 128°16.26'E	MG, 10.08		limnic, benthic, under stones
CIP-13	Batbitiem, Misool, West Papua, Indonesia	Ceram Sea	02°14.89'S; 130°33.31'E	KJ, 11.09	intertidal	marine, interstital, fine coral sand
CIP-14	Ngetpang Waterfall, Tabeding River, Babelthuap Island, Palau	Philippine Sea	7°27.166'N; 134°31.733'E	YK, 11.97		limnic, benthic, under stones
CIP-15	Mamara river, Guadalcanal, Solomon Islands	South Pacific	09°24.15'S; 159°53.40'E	KJ/YK, 10.07	0.1-1 m	limnic, benthic, under stones
CIP-16	Kohove river, Guadalcanal, Solomon Islands	South Pacific	09°25.33'S; 159°54.16'E	KJ/YK, 10.07	0.1-0.2 m	limnic, benthic, under stones
CIP-17	Lungga river, Guadalcanal, Solomon Islands	South Pacific	09°26.92'S; 160°2.45'E	KJ/YK, 10.07	0.1-0.2 m	limnic, benthic, under stones
CIP-18	Honiara, Guadalcanal, Solomon Islands	South Pacific	09°25.72'S; 159°56.95'E	KJ, 10.07	intertidal	marine, interstitial, coarse coral sand

CIP-19	Komimbo Bay, Tambea, Guadalcanal, Solomons	South Pacific	09°15.84'S; 159°40.10'E	KJ, 10.07	intertidal	marine, interstitial, coarse coral sand
CIP-20	Komimbo Bay, Tambea, Guadalcanal, Solomons	South Pacific	09°15.84'S; 159°40.10'E	KJ, 10.07	1-2 m	marine, interstitial, fine sand
CIP-21	VM43, Wenoi River, Espiritu Santo, Vanuatu	South Pacific	15°34.83'S; 167°2.88'E	TN, 09.06		limnic, benthic, under stones
CIP-22	VM53, Luganville, Espiritu Santo, Vanuato	South Pacific	15°30.92'S; 167°11.83'E	TN, 10.06	intertidal	brackish, interstital
CIP-23a	VM59, Mounparap Island,Vanuatu	South Pacific	15°22.59'S; 167°11.62'E	TN, 10.06	low intertidal	marine, interstital
CIP-23b	VM39, Mounparap Island,Vanuatu	South Pacific	15°22.59'S; 167°11.62'E	TN, 09.06	low intertidal	marine, interstital
CIP-24	VM17, Nandioudi, Malo Island, Vanuatu	South Pacific	15°37.68'S; 167°10.32'E	TN, 09.06	intertidal	marine, interstital
CIP-25	FR36, Mavéa Island, Vaucluse Passage, Vanuatu	South Pacific	15°22.455'S; 167°12.985'E	TN, 09.06	40-45 m	marine, interstital, coarse sand and rubbel
CIP-26	Lami District, Viti Levu, Fiji	Lami River, South Pacific	18°6.27'S; 178°24.08'E	MS/ES/ AA 08.06	0.5 m	limnic, under stones, calm current
CIP-27	Laucala Bay, Nukumbutho Island, Viti Levu, Fiji	South Pacific	18°10.47'S; 178°28.34'E	MS/ES/ AA 08.06	0.5-1 m	marine, interstitial
CIP-28	Matsubara, Miyako, Okinawa, Japan	East China Sea	24°47.02'N; 125°16.08'E	HF/YT, 05.08	intertidal	marine, semiterrestrial, benthic on rocks
CIP-29	Rungkam, Boleng Bay, Flores, Indonesia	Flores Sea	8°28.09'S; 119°59.44'E	KJ/RM 09.08	0.1-0.4 m	limnic, benthic, under stones
CIP-30	Merombok, Flores, Indonesia	Flores Sea	8°32.37'S; 119°53.71'E	KJ/RM 09.08	0.1-0.8 m	limnic, benthic, under stones
CIP-31	Mataniko river, Guadalcanal, Solomon Islands	South Pacific	09°27.52'S; 159°57.49'E	KJ/YK, 10.07	0.2-0.5 m	limnic, benthic, under stones
CIP-32	VM37, Port Beniér, Aoré Island, Vanuatu	South Pacific	15°34.50'S; 167°12.62'E	TN, 09.06	intertidal	marine, interstital
CIP-33	VM16, Malo Island, near Asuloka Island, Vanuatu	South Pacific	15°37.68'S; 167°10.99'E	TN, 09.06	intertidal	marine, interstital
CIP-34	VM5, Tapuntari Waterfall, Wounaouss River, Espiritu Santo, Vanuatu	South Pacific	15°34.32'S; 167°0.16'E	TN, 09.06		limnic, benthic, under stones
CIP-35	La Marona river Efate Island, Vanuatu	South Pacific	17°34.028'S; 168°17.170'E	AH, 11.95		limnic, benthic, under stones
CIP-36	Vaisala lagoon, Savaii Island, Samoa	South Pacific	13°30.42'S; 172°39.94'W	MS, 08.05	0.5 -2 m	marine, interstitial, fine coral sand

Supplementary material 2: Type localities of valid acochlidian species

Type localities of acochlidian species retrieved from literature, successful re-collection attempts or type localities of species established in the course of this PhD thesis and the one by T. Neusser are linked to the field codes of Supplementary material 1 (see also Chapter 10 on biogeography). 'u' - unsuccessful re-collection attempts at the type locality, * species status disputed (see Schrödl & Neusser 2010)

Species	**Type -Locality**	**Water body**	**Collected**
Hedylopsis spiculifera	Prince Islands, Turkey	Sea of Marmara	-
Hedylopsis ballantinei	Dahab, Sinai, Egypt	Red Sea	WIP-2
*Pseudunela eirenae**	Nicobar Islands, India	Andaman Sea	-
Pseudunela cornuta	Maraunibina Island, Solomon Islands	Cental Indo-West Pacific	-
Pseudunela viatoris	Laucala Bay, Nukumbutho Island, Viti Levu, Fiji	South Pacific	CIP-27
Pseudunela marteli	Honiara, Guadalcanal, Solomon Islands	South Pacific	CIP-18
Pseudunela espiritusanta	Luganville, Espiritu Santo, Vanuato	South Pacific	CIP-22
Strubellia paradoxa	Batu Gatja River, Ambon Island, Maluku Utara, Indonesia	Banda Sea	CIP-12
Strubellia wawrai	Mataniko River, Guadalcanal, Solomon Islands	South Pacific	CIP-31
Acochlidium fijense	Nasekawa River, Vanua Levu, Fiji	South Pacific	-
Acochlidium amboinense	Batu gatja river, Ambon Island, Maluku Utara, Indonesia	Banda Sea	CIP-11
Acochlidium bayerfehlmanni	Ngetpang Waterfall, Tabeding River, Babelthuap Island, Palau	Philippine Sea	CIP-14
Palliohedyle weberi	near Bari, Flores Island, Indonesia	Flores Sea	u
Tantulum elegans	Golden Grove, St. Vincent, West Indies	Caribbean Sea	u
Acochlidium sutteri	Bondokodi, Sumba, East Nusa Tengarra, Indonesia	Indian Ocean	CIP-9
Aiteng ater	Pak Phanang Bay, Thailand	Gulf of Thailand	CIP-1
Aiteng mysticus	Shimozaki, Nikadori, Hirara, Miyako Island, Okinawa, Japan	East China Sea	CIP-28
Asperspina brambelli	Menai Strait, Anglesey, Wales, United Kindom	Irish Sea	-
Asperspina riseri	Crowe Neck, North Trescott, Maine, USA	Bay of Fundy, North-West Atlantic	u
Asperspina loricata	Trezen ar Skoden, near Roscoff, France	North-East Atlantic	NEA-20
Asperspina murmanica	Dalniye Zelentsy, Murmanskaya obl., Russia	Barents Sea	NEA-19
Asperspina rhopalothecta	Secche della Meloria, Livorno, Italy	Mediterranean Sea	NEA-7
Microhedyle glandulifera	Prince Islands, Turkey	Sea of Marmara	-
Microhedyle remanei	off Castle Roads, Bermuda	Atlantic Ocean	WAT-10

Parhedyle cryptophthalma	Nabeul, Tunisia	Mediterranen Sea	-
Parhedyle odhneri	Canet-Plage, Languedoc-Roussillon, France	Mediterranean Sea	NEA-5
*Parhedyle gerlachi**	Addu Atoll, Gan Channel, Maldives	Indian Ocean	-
Parhedyle tyrtowii	Cape Fiolent, Sebastopol, Krim, Ukraine	Black Sea	NEA-1
Parhedyle nahantensis	Canoe Beach, Nahant, Massachusetts, USA	Atlantic Ocean	NWA-1
Pontohedyle milaschewitchii	Chersones, Cape Fiolent, Sebastopol, Krim, Ukraine	Black Sea	NEA-2
Pontohedyle brasilensis	Vila, Ilhabela, Brazil	Western Atlantic	u
Pontohedyle verrucosa	Maraunibina Island, Solomon Islands	Cental Indo-West Pacific	-
Pontohedyle kepii	Pulau Moyo, Nusa Tenggara, Indonesia	Flores Sea	CIP-8
Pontohedyle joni	Bay of 'Windjammer Landing', St. Lucia	Caribbean Sea	WAT-6
Pontohedyle neridae	Motu Iti, Moorea, French Polynesia	Central Pacific	EIP-5
Pontohedyle liliae	Sha'ab Malahi, Egypt	Red Sea	WIP-1
Pontohedyle wiggi	Raja Island, Phuket, Thailand	Andaman Sea	WIP-5
Pontohedyle wenzli	Lembeh Strait, Sulawesi, Indonesia	Celebes Sea	CIP-4
Pontohedyle peteryalli	Mia Mia, Ghana	Atlantic Ocean	EAT-2
Pontohedyle martynovi	Cook's bay pass, Moorea, French Polynesia	Central Pacific	EIP-2
Pontohedyle yurihookeri	Punta Sal, Tumbes, Peru	Pacific Ocean	EPT-1
Ganitus evelinae	Vila, Ilhabela, Brazil	Western Atlantic	SWA-1
Paraganitus ellynnae	Komimbo Bay, Tambea, Guadalcanal, Solomons	South Pacific	CIP-20

Supplementary file 3: Molecular data generated within this study: DNA Bank vouchers and GenBank accession numbers

Museums numbers and accession numbers of DNA aliquots and deposited sequences generated within this thesis. The table lists all material used for molecular work, independent of the extraction/ amplification success within the present study. Future studies with e.g., more elaborated primers may make use of the material. However, some extractions might have failed entirely due to inappropriate fixation or long imperfect storage. Institutions: AM – Australian Muesum, SI – Smithsonian Institute, ZSM – Bavarian State Collection of Zoology. Involved scientists: KH – Katharina Händeler, YK – Yasunori Kano, IS – Isabella Stöger, NW – Nerida Wilson.

Taxon / **HEDYLOPSACEA**	**Locality**	**Museums number**	**DNA extraction (extraction kit, dilution, date)**	**DNA concen-tration ng/µl**	**GenBank accession numbers of generated sequences**				**DNA Bank master**
Hedylopsidae					**18S rRNA**	**28S rRNA**	**16S rRNA**	**COI**	
Hedylopsis spiculifera	NEA-18	ZSM Mol 20071116	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	4.97			unpublished (only 300 bp)	unpublished	AB34404269 25 µl
	NEA-3a	ZSM Mol 20080951	CTAB-extraction (50 µl) 9.10.08	13.60	HQ168430	HQ168443	no PCR product	HQ168455	AB35081816 15 µl
	NEA-13a	ZSM Mol 20091331	Quiagen Blood & Tissue Kit (100 µl) 24.9.08					unpublished	AB34858198 20 µl
	NEA-3a	ZSM Mol 20080952	CTAB-extraction (50 µl) 9.10.08	4.10				unpublished	AB35081793 15 µl
	NEA-8	ZSM Mol 20080389	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	10.50		KF709319	KF709245	KF709351	AB35081764 25 µl
	NEA-7	ZSM Mol 20080415	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	8.80			unpublished (only 300 bp)	unpublished	AB35081768 25 µl
	NEA-4	ZSM Mol 20080955	CTAB-extraction (50 µl) 9.10.08	3.40			HQ168417 (only 300bp)	KF709352	AB35081817 25 µl
	NEA-4	ZSM Mol 20080956	CTAB-extraction (50 µl) 9.10.08	9.00				unpublished	AB35081801 25 µl
Hedylopsis spiculifera ("suecica")	NEA-11	ZSM Mol 200801015	Quiagen Blood & Tissue Kit (200 µl) 7.11.08	11.30	no or only contaminated PCR products				AB34402029 150 µl
	NEA-11	ZSM Mol 20081016	Quiagen Blood & Tissue Kit (200 µl) 7.11.08	6.60	KF709275	no PCR product	KF709246	KF709353	AB34404246 25 µl

Hedylopsis ballantinei	WIP-2b	ZSM Mol 20040551	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	1.08	no or only contaminated PCR products			AB34404213 25 µl
	WIP-2a	ZSM Mol 20090243	Quiagen Blood & Tissue Kit (200 µl) 23.4.08	2.00			unpublished (only 300 bp)	unpublished	AB35081760 25 µl
	WIP-2a	ZSM Mol 20090244	Quiagen Blood & Tissue Kit (200 µl) 23.4.08	8.20	HQ168429	HQ168442	HQ168416 (only 300bp)	HQ168454	AB34858170 25 µl
Hedylopsis MOTU Ghana	EAT-3	ZSM Mol 20070812	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	3.02	no or only contaminated PCR products				AB34404206 25 µl
	EAT-3	ZSM Mol 20070823	Quiagen Blood & Tissue Kit (200 µl) 23.4.08	5.40	no or only contaminated PCR products				AB34402084 170 µl
Hedylopsis MOTU Moorea	EIP-3	AMC. 476056.001	Machery + Nagel (100 µl) 28.01.11		KF709276	no PCR product	KF709247	KF709354	AB34402008 50 µl
Hedylopsacea indet.									
Hedylopsacea MOTU Moorea	EIP-4	AMC. 476059.001	Machery + Nagel (100 µl) 28.01.11		KF709277	KF709320	no PCR product	KF709355	AB34402051 50 µl
Pseudunelidae									
Pseudunela cornuta	CIP-19	ZSM Mol 20071809	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	2.81	JF819754	KF709321	JF819748	JF819774	AB34404215 25 µl
Pseudunela viatoris	CIP-27	ZSM Mol 20080057	Quiagen Blood & Tissue Kit (200 µl) 15.9.06	4,7			JF819743	JF819768	AB34404281 25 µl
	CIP-27	ZSM Mol 20080020	CTAB-extraction (50 µl) 25.2.08	6.70	JF819751	unpublished	JF819741	JF819766	AB34404247 10 µl
	CIP-27	ZSM Mol 20080021	CTAB-extraction (50 µl) 25.2.08	11.20			JF819742	JF819767	AB34404265 20 µl
	CIP-5	ZSM Mol 20071120	CTAB-extraction (50 µl) 25.2.08	3.40	JF819752		JF819744	JF819769	AB34404285 10 µl
	CIP-5	ZSM Mol 20070953	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	9.90		KF709322	JF819745	JF819770	AB34404276 25 µl
Pseudunela marteli	CIP-18	ZSM Mol 20080022	CTAB-extraction (50 µl) 25.2.08	4.10	JF819753	unpublished	JF819746	JF819771	AB34404252 - µl
	CIP-18	ZSM Mol 20080023	CTAB-extraction (50 µl) 25.2.08	2.90			no PCR product	JF819772	AB34404298 20 µl
	CIP-18	ZSM Mol 20080024	CTAB-extraction (50 µl) 25.2.08	2.20			JF819747	JF819773	AB34404218 20 µl
	CIP-23a	ZSM Mol 20080393	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	14.10	HQ168431	HQ168444	HQ168418	HQ168456	AB35081809 25 µl
	CIP-13	ZSM Mol	Quiagen Blood & Tissue		KF709278	KF709323	KF709248	KF709356	AB34402060

		20100381	Kit (200 µl) 11.1.10						180 µl
	CIP-13	ZSM Mol 20100384	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.30			no PCR product		AB34500512 180 µl
	CIP-13	ZSM Mol 20100385	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	1.20			no PCR product		AB34500508 180 µl
Pseudunela MOTU Maledives	WIP-3	ZSM Mol 20110029	Machery + Nagel (100 µl) 28.01.11		KF709279	KF709324	KF709249	KF709357	AB34402077 50 µl
Pseudunela espiritusanta	CIP-22	ZSM Mol 20080117	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	26.72	JF819755	KF709325	JF819749	JF819775	AB34404289 25 µl
	CIP-22	ZSM Mol 20071118	CTAB-extraction (50 µl) 25.2.08	2.60			JF819750	JF819776	AB34404210 20 µl
Acochlidiidae									
Strubellia paradoxa	CIP-11	ZMB Moll. 193.943	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	22.30			JF819739	no PCR product	AB35081823 25 µl
	CIP-12	ZMB Moll. 193.944	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	59.60	HQ168432	HQ168445	HQ168419	HQ168457	AB34858174 25 µl
Strubellia wawrai	CIP-16	ZSM Mol 20080014	CTAB-extraction (50 µl) 25.2.08	7.80			JF819728	JF819756 (COI long)	AB34404271 20 µl
	CIP-16	ZSM Mol 20080015	CTAB-extraction (50 µl) 25.2.08	51.80			JF819729	JF819757 (COI long)	AB34404208 20 µl
	CIP-16	ZSM Mol 20080016	CTAB-extraction (50 µl) 25.2.08	51.60	KF709280		JF819730	JF819758	AB34404250 10 µl
	CIP-31	ZSM Mol 20080017	CTAB-extraction (50 µl) 25.2.08	5.40			JF819731	JF819759	AB34404264 20 µl
	CIP-31	ZSM Mol 20080018	CTAB-extraction (50 µl) 25.2.08	27.80			JF819732	JF819760 (COI long)	AB34404255 20 µl
	CIP-31	ZSM Mol 20080019	CTAB-extraction (50 µl) 25.2.08	6.50			JF819733	JF819761	AB34404256 20 µl
	CIP-17	ZSM Mol 20071810	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	25.19		KF709326	JF819734	JF819762	AB34404212 25 µl
	CIP-35	ZSM Mol 20080152	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	1.84			no PCR product		AB34404237 25 µl
	CIP-35	ZSM Mol 20080064	Quiagen Blood & Tissue Kit (200 µl) 15.9.06	3.00			no PCR product		AB34404245 25 µl
	CIP-21	ZSM Mol 20080150	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	36.52	KF709281	KF709327	JF819736	JF819764 (COI long)	AB34404205 25 µl

	CIP-21	ZSM Mol 20080151	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	47.49			no PCR product		AB34404238 25 µl
	CIP-21	ZSM Mol 20071117	Quiagen Genomic Tip Kit 20.9.07 IS	Eluat 1: 1.54;			JF819735	JF819763	AB34404234 25 µl (Eluat1)
	CIP-34	ZSM Mol 20080148	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	9.83			JF819738	no PCR product	AB34404251 25 µl
	CIP-34	ZSM Mol 20080072	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	11.08			JF819737	no PCR product	AB34404207 25 µl
Strubellia MOTU Sulawesi	CIP-2	ZSM Mol 20100339	Quiagen Blood & Tissue Kit (200 µl) 11.1.10		KF709282	KF709328	JF819740	JF819765	AB35081762 25 µl
Acochlidiidae MOTU Ambon	CIP-12	ZMB Moll. 193.966	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	15.10	KF709283	KF709329	KF709250	KF709358	AB35081841 25 µl
Palliohedyle MOTU Sulawesi	CIP-2	ZSM Mol 20100356	Quiagen Blood & Tissue Kit (200 µl) 11.1.10		KF709284	JF828039	JF828040	JF828032	AB35081794 25 µl
	CIP-2	ZSM Mol 20100357	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				unpublished	unpublished	AB34500523 150 µl
Acochlidium fijense	CIP-26	ZSM Mol 20080063	Quiagen Blood & Tissue Kit (200 µl) 15.9.06	8.90	HQ168433	HQ168446	HQ168420	HQ168458	AB34404244 25 µl
Acochlidium amboinense	CIP-12	ZMB Moll. 193.942a	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	27.40	KF709285	KF709330	KF709251	KF709359	AB35081759 25 µl
	CIP-12	ZMB Moll. 193.942b	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	16.10			unpublished	unpublished	AB35081837 25 µl
Acochlidium bayerfehlmanni	CIP-14	ZSM Mol 20080384	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	12.40	KF709286	no PCR product	KF709252	KF709360	AB35081800 25 µl
Acochlidium sutteri	CIP-9	ZSM Mol 20080911	Quiagen Blood & Tissue Kit (200 µl) 22.10.08	8.10	KF709287	KF709331	KF709253	KF709361	AB35081785 25 µl
	CIP-9	ZSM Mol 20080909	Quiagen Blood & Tissue Kit (200 µl) 22.10.08	3.60	unpublished (partial)		unpublished	unpublished	AB35081826 25 µl
	CIP-9	ZSM Mol 20080910	Quiagen Blood & Tissue Kit (200 µl) 22.10.08	3.90			unpublished	no PRC product	AB35081776 25 µl
Acochlidium MOTU Flores	CIP-30	ZSM Mol 20080892	Quiagen Blood & Tissue Kit (200 µl) 22.10.08	7.90			unpublished	unpublished	AB35081808 25 µl
	CIP-30	ZSM Mol 20080891	Quiagen Blood & Tissue Kit (200 µl) 22.10.08	8.50			unpublished	unpublished	AB35081824 25 µl
	CIP-10	ZSM Mol 20080897	Quiagen Blood & Tissue Kit (200 µl) 22.10.08	18.40	KF709288	KF709332	KF709254	KF709362	AB35081777 25 µl

	CIP-29	ZSM Mol 20080899	Quiagen Blood & Tissue Kit (200 µl) 22.10.08	2.60			no PCR product	unpublished	AB35081784 25 µl
	CIP-29	ZSM Mol 20080900	Quiagen Blood & Tissue Kit (200 µl) 22.10.08	7.10			unpublished	unpublished	AB35081792 25 µl
Acochlidium MOTU Sulawesi	CIP-2	ZSM Mol 20100341	Quiagen Blood & Tissue Kit (200 µl) 11.1.10		KF709289	KF709333	KF709255	KF709363	AB34500502 150 µl
	CIP-2	ZSM Mol 20100352	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				unpublished	unpublished	AB34402039 150 µl
	CIP-2	ZSM Mol 20100355	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				unpublished	unpublished	AB34402068 150 µl
	CIP-3	ZSM Mol 20100359	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				KF709256	KF709364	AB34402017 150 µl
Acochlidium MOTU Solomons	CIP-15	ZSM Mol 20080159	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	94.68	KF709290	KF709334	KF709257	KF709365	AB34404232 25 µl
	CIP-15	ZSM Mol 20080161	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	111.50			unpublished	unpublished	AB34404226 25 µl
Aitengidae									
Aiteng ater	CIP-1	KH, University Bonn			JF828036	JF828037	JF828038	JF828031	AB34858187 25 µl
	CIP-1	KH, University Bonn							AB34599425 25 µl
Aiteng mysticus	CIP-28	YK, University of Tokyo			HQ168428	HQ168441	HQ168415	HQ168453	-
MICROHEDYLACEA									
Asperspinidae									
Asperspina brambelli	NEA-12	ZSM Mol 20100575	Quiagen Blood & Tissue Kit (200 µl) 11.1.10		unpublished (partial)		no PCR product		AB34402033 180 µl
	NEA-12	ZSM Mol 20100576	Quiagen Blood & Tissue Kit (200 µl) 11.1.10			JQ410991	JQ410990	JQ410924	AB34402042 180 µl
	NEA-12	ZSM Mol 20100577	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				no PCR product		AB34402052 180 µl
	NEA-12	ZSM Mol 20100573	Quiagen Blood & Tissue Kit (200 µl) 11.1.10		KF709291	no PCR product	KF709258	KF709366	AB34402038 180 µl
	NEA-12	ZSM Mol 20100572	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				no PCR product		AB34500562 180 µl

Asperspina loricata	NEA-20	ZSM Mol 20080406	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	6.40	no or only contaminated PCR products				AB34402055 170 µl
Asperspina murmanica	NEA-19	ZSM Mol 20071123	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	4.55	no or only contaminated PCR products				AB34404209 25 µl
	NEA-19	ZSM Mol 20070132	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	7.20	no or only contaminated PCR products				AB34402087 150 µl
Asperspina rhopalotecta	NEA-15	ZSM Mol 20100581	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				unpublished	unpublished	-
	NEA-7	ZSM Mol 20080409	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	16.70	KF709292	KF709335	KF709259	KF709367	AB35081814 25 µl
	NEA-7	ZSM Mol 20080410	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	7.50			no or only contaminated PCR products		AB34402048 150 µl
Asperspina MOTU Peru	SEP-2	ZSM Mol 20080560	Quiagen Blood & Tissue Kit (200 µl) 19.5.08		KF709293	no PCR product	KF709260	KF709368	AB35081775 25 µl
Asperspina MOTU1 Kamtchatka	NWP-1	ZSM Mol 20090171	Quiagen Blood & Tissue Kit (200 µl) 7.11.08	5.00	HQ168434	HQ168447	HQ168421	KF709369	AB35081833 25 µl
Asperspina MOTU2 Kamtschatka	NWP-2	ZSM Mol 20090175	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	10.60	KF709294	KF709336	KF709261	KF709370	AB35081755 25 µl
Asperspina MOTU Belize	WAT-3	SI-CBC2010 KJ01-A08	Smithsonian Institute, Washington		KF709295	KF709337	no or only contaminated PCR product		AB34402056 10µl
Asperspina MOTU Brazil	WAT-17	ZSM Mol 20100334	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.60	no or only contaminated PCR products				AB34500514 180 µl
Asperspina MOTU Panama	WAT-5b	ZSM Mol 20110721	Machery + Nagel (200 µl) 01.07.10	0.90	KF709296	KF709338	no or only contaminated PCR products	KF709371	AB34500492 80 µl
Asperspina MOTU Washington	NEP-1	ZSM Mol 20100585	Machery + Nagel (100 µl) 27.08.10		KF709297	no or only contaminated PCR products	KF709262	KF709372	AB34402007 80 µl
Asperspina MOTU Ghana	EAT-3	ZSM Mol 20070826	Machery + Nagel (100 µl) 27.08.10		no or only contaminated PCR products				AB34500500 80 µl
Asperspina MOTU Moorea	EIP-1	AM C. 476049.001	Machery + Nagel (100 µl) 28.01.11		KF709298	KF709339	no or only contaminated PCR products	KF709373	AB34402009 50 µl

Microhedylidae (s.l.)									
Microhedyle glandulifera	NEA-13d	ZSM Mol 20080056	Quiagen Blood & Tissue Kit (200 µl) 15.9.06	7.70			JF819815	JF819777	AB34404242 25 µl
	NEA-3a	ZSM Mol 20081019	Quiagen Blood & Tissue Kit (200 µl) 7.11.08	2.60	HQ168437	HQ168449	HQ168424	HQ168461	AB35081799 25 µl
	NEA-13a	ZSM Mol 20091332	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819791	AB35081756 20 µl
	NEA-3a	ZSM Mol 20091167	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	1.20				no PCR product	AB34402047 80 µl
	NEA-3a	ZSM Mol 20091168	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	2.20				JF819786	AB34858195 20 µl
	NEA-3a	ZSM Mol 20091169	Quiagen Blood & Tissue Kit (100 µl) 24.9.08					JF819787	AB34404297 50 µl
	NEA-13b	ZSM Mol 20100411	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819792	AB34402384 50 µl
	NEA-13b	ZSM Mol 20100412	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819793	AB35081778 50 µl
	NEA-13b	ZSM Mol 20100413	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819794	AB34858243 50 µl
	NEA-13b	ZSM Mol 20100414	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819795	AB34858183 50 µl
	NEA-13b	ZSM Mol 20100415	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819796	AB34858186 50 µl
	NEA-13b	ZSM Mol 20100416	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819797	AB34402041 150 µl
	NEA-13b	ZSM Mol 20100417	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819798	AB35081836 50 µl
	NEA-13c	ZSM Mol 20110027	Machery + Nagel (100 µl) 28.01.11					JF819811	AB35081779 50 µl
	NEA-13c	ZSM Mol 20110028	Machery + Nagel (100 µl) 28.01.11					JF819812	AB34499233 50 µl
	NEA-13a	ZSM Mol 20091454	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	1.30				no PCR product	AB34858169 20 µl
	NEA-3d	ZSM Mol 20100419	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819799	AB35081749 50 µl
	NEA-3a	ZSM Mol 20100420	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819800	AB34858209 50 µl

	NEA-3a	ZSM Mol 20100421	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819801	AB34858194 50 µl
	NEA-3d	ZSM Mol 20100422	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819802	AB34404240 50 µl
	NEA-3d	ZSM Mol 20100423	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819803	AB35081781 50 µl
	NEA-3d	ZSM Mol 20100424	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819804	AB34599359 50 µl
	NEA-3d	ZSM Mol 20100425	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819805	AB34404230 50 µl
	NEA-7	ZSM Mol 20080413	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	8.70			JF819816	JF819780	AB34858172 25 µl
	NEA-11	ZSM Mol 20091170	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	2.70				JF819788	AB35081831 25 µl
	NEA-11	ZSM Mol 20091171	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	2.00				JF819789	AB35081820 25 µl
	NEA-10	ZSM Mol 20080136	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	10.11			JF819817	JF819778	AB34404283 25 µl
	NEA-11	ZSM Mol 20081017	Quiagen Blood & Tissue Kit (200 µl) 7.11.08	3.70			unpublished	JF819783	AB35081825 25 µl
	NEA-11	ZSM Mol 20081018	Quiagen Blood & Tissue Kit (200 µl) 7.11.08	3.90			unpublished	JF819784	AB35081761 25 µl
	NEA-14	ZSM Mol 20100426	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819806	AB34599395 50 µl
	NEA-14	ZSM Mol 20100427	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819807	AB34858168 50 µl
	NEA-14	ZSM Mol 20100428	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819808	AB34858180 50 µl
	NEA-14	ZSM Mol 20100429	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819809	AB34858189 50 µl
	NEA-14	ZSM Mol 20100430	Quiagen Blood & Tissue Kit (200 µl) 07.10					JF819810	AB35081763 50 µl
	NEA-22	ZSM Mol 20091172	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	2.20				no PCR product	AB35081840 25 µl
	NEA-21	ZSM Mol 20091173	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	0.00				no PCR product	AB34402050 80 µl
	NEA-21	ZSM Mol 20091174	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	11.5 (?)				no PCR product	AB34500499 80 µl

	NEA-21	ZSM Mol 20091175	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	0.40				no PCR product	AB34500509 80 µl
	NEA-9	ZSM Mol 20080392	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	8.80			KF709263	JF819779	AB35081748 25 µl
	NEA-4	ZSM Mol 20080959	CTAB-extraction (50 µl) 9.10.08	3.00			unpublished	JF819781	AB35081807 25 µl
	NEA-4	ZSM Mol 20080960	CTAB-extraction (50 µl) 9.10.08	4.10			unpublished	JF819782	AB35081815 25 µl
	NEA-4	ZSM Mol 20091176	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	0.60				no PCR product	AB34402018 80 µl
	NEA-4	ZSM Mol 20091177	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	1.20				no PCR product	AB34402063 80 µl
	NEA-4	ZSM Mol 20091178	Quiagen Blood & Tissue Kit (100 µl) 24.9.08	29.7 (?)				JF819790	AB34858185 50 µl
	NEA-23	ZSM Mol 20090049	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	18.10			no PCR product	no PCR product	AB34402069 150 µl
Microhedyle MOTU Ghana	EAT-1	ZSM Mol 20080146	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	1.84	no or only contaminated PCR products				AB34404222 25 µl
	EAT-1	ZSM Mol 20071119	Macherey & N. Tissue XS (20 µl) 4.9.07 IS	3.04	no or only contaminated PCR products				-
	EAT-1	ZSM Mol 20110715	Macherey & Nagel 25.8.10 (100µl)		KF709299	no PCR product	KF709264	KF709374	AB34402046 50 µl
	EAT-1	ZSM Mol 20090191	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	10.60	no or only contaminated PCR products				AB34402058 150 µl
Microhedyle MOTU Vanuatu	CIP-32	ZSM Mol 20080166	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	2.05	no or only contaminated PCR products				AB34404219 25 µl
	CIP-23b	ZSM Mol 20080168	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	3.65	no PCR product	unpublished	no PCR product	no PCR product	AB34404235 25 µl
Microhedyle MOTU West Papua	CIP-13	ZSM Mol 20100386	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.70	KF709300	KF709340	no or only contaminated PCR product	KF709375	AB34401999 150 µl
Microhedyle MOTU Peru	SEP-2	ZSM Mol 20080559	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	14.30	KF709301	KF709341	KF709265	KF709376	AB35081828 25 µl
Microhedyle MOTU Chile	SEP-1	ZSM Mol 20090202	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	5.30			unpublished	unpublished	AB35081789 25 µl

	SEP-1	ZSM Mol 20090206	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	11.60	KF709302	KF709342	KF709266	KF709377	AB35081771 25 µl
	SEP-3	ZSM Mol 20090209	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	12.90			unpublished	unpublished	AB35081819 25 µl
Microhedyle MOTU Egypt	WIP-1	ZSM Mol 20090464	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	5.10	KF709303	KF709343	no PCR product	KF709378	AB35081773 25 µl
	WIP-1	ZSM Mol 20090465	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	1.90				unpublished	AB35081803 25 µl
	WIP-1	ZSM Mol 20090466	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	3.50				unpublished	AB35081766 25 µl
	WIP-7	ZSM Mol 20090469	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	2.20				no PCR product	-
Microhedyle remanei	WAT-10	ZSM Mol 20080164	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	1.42	no or only contaminated PCR products				AB34404216 25 µl
	WAT-10	ZSM Mol 20100617	Machery + Nagel (100 µl) 27.08.10		no or only contaminated PCR products				AB34402081 80 µl
	WAT-10	ZSM Mol 20100618	Machery + Nagel (100 µl) 27.08.10		no or only contaminated PCR products				AB34500505 80 µl
	SWA-2	ZSM Mol 20100330	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	2.80			no or only contaminated PCR products		AB34500493 180 µl
	SWA-2	ZSM Mol 20100331	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.90			no or only contaminated PCR products		AB34402085 180 µl
	SWA-2	ZSM Mol 20100332	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.90		unpublished	no or only contaminated PCR products		AB34402071 150 µl
	SWA-1	ZSM Mol 20071124	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	7.4	KF709304	KF709344	KF709267	no PCR product	AB34404296 25 µl
Microhedyle MOTU St. Vincent	WAT-8	ZSM Mol 20090193	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	6.90	KF709305	JQ410989	JQ410988	JQ410923	AB35081767 25 µl
Microhedyle MOTU Mexico	NEP-2	NW, Australian Museum			KF709306		KF709268	KF709379	AB34500083 20 µl
Microhedyle MOTU Moorea	EIP-5	AMC.4760 61.001	Machery + Nagel (100 µl) 28.01.11		KF709307	no PCR product	KF709269	KF709380	AB34499237 50 µl

Parhedyle cryptophthalma	NEA-6b	ZSM Mol 20071125	Omega Mollusc (150 µl) 4.9.07 *	0.66	no or only contaminated PCR products				AB34402083 150 µl
	NEA-6b	ZSM Mol 20071126	Macherey & N. Tissue XS (20 µl) 4.9.07 IS	7.18				unpublished	-
	NEA-6b	ZSM Mol 20090164	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	16.10			no or only contaminated PCR products		AB34500496 170 µl
	NEA-6b	ZSM Mol 20090166	Quiagen Blood & Tissue Kit (200 µl) 1.4.09	5.90			no or only contaminated PCR products		AB34402073 150 µl
	NEA-6a	ZSM Mol 20100582	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				no PCR product	unpublished	AB34402066 180 µl
	NEA-6a	ZSM Mol 20100583	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				no PCR product	unpublished	AB34500524 180 µl
	NEA-6a	ZSM Mol 20100584	Quiagen Blood & Tissue Kit (200 µl) 11.1.10		KF709308	JF828041	JF828042	JF828033	AB34599403 25 µl
Parhedyle odhneri	NEA-5	ZSM Mol 20090571	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	0.60	KF709309	JF819814	no PCR product	JF819819	AB35081818 25 µl
	NEA-5	ZSM Mol 20090572	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	1.90				unpublished	AB35081805 25 µl
	NEA-5	ZSM Mol 20090573	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	3.30				unpublished	AB35081746 25 µl
Parhedyle tyrtowii	NEA-1	ZSM Mol 20091369	Quiagen Blood & Tissue Kit (200 µl) 24.9.08	2.10	KF709310	JF819813	no PCR product	JF819818	AB35081774 AB35081829 25 µl
	NEA-16	ZSM Mol 20100431	Quiagen Blood & Tissue Kit (200 µl) 07.10					unpublished	AB34402064 180 µl
Parhedyle nahantensis	NWA-1	ZSM Mol 20110022	Machery + Nagel (100 µl) 28.01.11		KF709311	KF709345	no PCR product	KF709381	AB34500501 50 µl
	NWA-1	ZSM Mol 20110023	Machery + Nagel (100 µl) 28.01.11			unpublished	no PCR product	unpublished	AB34500504 50 µl
	NWA-1	ZSM Mol 20110024	Machery + Nagel (100 µl) 28.01.11					unpublished	AB34500515 50 µl
Pontohedyle milaschewitchii	NEA-3b	ZSM Mol 20080054	Quiagen Blood & Tissue Kit (200 µl) 15.9.06	7.90	HQ168435	JF828043	HQ168422	no PCR product	AB34404241 25 µl

	NEA-3b	ZSM Mol 20080055	Quiagen Blood & Tissue Kit (200 µl) 15.9.06	1.70			JQ410927	no PCR product	AB34404239 25 µl
	NEA-3b	ZSM Mol 20071130	Quiagen Tissue Kit (200 µl) 6.9.07 Isi	2.56		no PCR product			AB34404243 25 µl
	NEA-3b	ZSM Mol 20091131	Qiagen Blood & Tissue Kit (100µl) 24.9.09						AB35081804 20 µl
	NEA-3b	ZSM Mol 20071129	Omega Mollusc (150 µl) 4.9.07 IS	0.20				no PCR product	AB34402032 130 µl
	NEA-3c	ZSM Mol 20080925	CTAB-extraction (50 µl) 09.10.08	0.70			JQ410928	HQ168459	-
	NEA-2	ZSM Mol 20071381	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	14.14		JQ410926	JQ410925	JQ410897	AB34404214 25 µl
	NEA-24	ZSM Mol 20090475	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	2.30			no PCR product		-
	NEA-17	ZSM Mol 20080175	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	1.08			no PCR product		AB34404259 25 µl
	NEA-4	ZSM Mol 20080953	CTAB-extraction (50 µl) 09.10.08	3.10	KC984282		JQ410929	JQ410898	AB35081832 25 µl
Pontohedyle brasilensis	WAT-9b	ZSM Mol 20100337	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.60			no PCR product		AB34500516 180 µl
	WAT-9b	ZSM Mol 20100338	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.50			no PCR product		-
	WAT-9a	ZSM Mol 20110722	Machery + Nagel (200 µl) 01.07.10	1.30	KC984285	JQ410952	JQ410951	JQ410906	AB34402086 150 µl
	WAT-7	ZSM Mol 20090198	Quiagen Blood & Tissue Kit (200 µl) 1.4.09		KC984286	JQ410936	JQ410935	no PCR product	AB35081813 20 µl
	WAT-5a	ZSM Mol 20110723	Machery + Nagel (200 µl) 01.07.10	4.10	KC984284	JQ410952	JQ410951	JQ410906	AB34402034 180 µl
	WAT-1	SI-CBC2010 KJ01-E03	Smithsonian Institute, Washington		KC984283	JQ410941	JQ410940	no PCR product	AB34500510 10 µl
	WAT-2	SI-CBC2010 KJ01-B07	Smithsonian Institute, Washington			JQ410943	JQ410942	no PCR product	AB34402082 10 µl
	WAT-11	SI-CBC2010 KJ01-D07	Smithsonian Institute, Washington			JQ410944	no PCR product		AB34500513 10 µl
	WAT-13	SI-CBC2010 KJ01-B09	Smithsonian Institute, Washington			JQ410946	JQ410945	JQ410904	AB34402031 10 µl

	WAT-12	SI-CBC2010 KJ01-C09	Smithsonian Institute, Washington			JQ410948	JQ410947	JQ410905	AB34500576 10 µl
	WAT-14	SI-CBC2010 KJ01-A10	Smithsonian Institute, Washington				JQ410949	no PCR product	AB34402026 10 µl
	WAT-15	SI-CBC2010 KJ02-E01	Smithsonian Institute, Washington			JQ410950	no PCR product		AB34402030 10 µl
	WAT-16	SI-CBC2010 KJ02-B02	Smithsonian Institute, Washington				no PCR product		AB34402078 10 µl
Pontohedyle verrucosa	CIP-19	ZSM Mol 20071820	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	1.91	KC984287	JQ410978	JQ410977	JQ410920	AB34404223 (1/E4) 25 µl
	CIP-18	ZSM Mol 20080176	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	2.88		JQ410980	JQ410979	JQ410921	AB34404286 25 µl
	CIP-13	ZSM Mol 20100388	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				no PCR product	JQ410916	AB34500547 180 µl
	CIP-13	ZSM Mol 20100389	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.50		JQ410974	no PCR product	JQ410917	AB34402044 180 µl
	CIP-13	ZSM Mol 20100390	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.10	unpublished (partial)	JQ410975	no PCR product	JQ410918	AB34402070 180 µl
	CIP-13	ZSM Mol 20100391	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.80	KC984289	no PCR product	JQ410976	JQ410919	AB34500531 180 µl
	CIP-7	ZSM Mol 20071135	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	3.44	KC984288	JQ410971	JQ410970	JQ410914	AB34404221 25 µl
Pontohedyle kepii	CIP-8	ZSM Mol 20081013	Quiagen Blood & Tissue Kit (200 µl) 7.11.08	2.20	KC984290	JQ410967	JQ410966	JQ410912	AB35081769 25 µl
Pontohedyle joni	WAT-6	ZSM Mol 20090197	Quiagen Blood & Tissue Kit (200 µl) 1.4.09		KC984291	JQ410934	JQ410933	JQ410901	AB34858164 25 µl
	WAT-11	SI-CBC2010 KJ01-D05	Smithsonian Institute, Washington		KC984292		JQ410937	JQ410902	AB34402049 10 µl
	WAT-3	SI-CBC2010 KJ01-C08	Smithsonian Institute, Washington			JQ410939	JQ410938	JQ410903	AB34402065 10 µl
Pontohedyle neridae	EIP-5	AMC. 476062.001	Machery + Nagel (100 µl) 28.01.11			JQ410986	JQ410985	JQ410922	AB34500497 50 µl

Pontohedyle liliae	WIP-1	ZSM Mol 20090471	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	2.40	KC984293	JQ410954	JQ410953	no PCR product	AB35081802 25 µl
	WIP-1	ZSM Mol 20090472	Quiagen Blood & Tissue Kit (200 µl) 21.7.09	1.80		JQ410956	JQ410955	no PCR product	AB35081838 25 µl
Pontohedyle wiggi	WIP-5	ZSM Mol 20100595	Machery + Nagel (100 µl) 27.08.10			JQ410960	JQ410959	JQ410908	AB34402059 80 µl
	WIP-5	ZSM Mol 20100596	Machery + Nagel (100 µl) 27.08.10			no PCR product	JQ410961	JQ410909	AB34402001 80 µl
	WIP-5	ZSM Mol 20100597	Machery + Nagel (100 µl) 27.08.10			JQ410963	JQ410962	JQ410910	AB34500571 80 µl
	WIP-8	ZSM Mol 20100603	Machery + Nagel (100 µl) 27.08.10			JQ410965	JQ410964	JQ410911	AB34402020 80 µl
Pontohedyle wenzli	WIP-6	ZSM Mol 20100592	Machery + Nagel (100 µl) 27.08.10		KC984294	JQ410958	JQ410957	JQ410907	AB34402021 80 µl
	EIP-1	AMC. 476051.001	Machery + Nagel (100 µl) 28.01.11		KC984295	JQ410982	JQ410981	no PCR product	AB34402037 50 µl
	CIP-6	ZSM Mol 20081014	Quiagen Blood & Tissue Kit (200 µl) 7.11.08	3.10	KC984296	JQ410969	JQ410968	JQ410913	AB35081827 20 µl
	CIP-4	ZSM Mol 20100379	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	1.50	KC984297	JQ410973	JQ410972	JQ410915	AB34500521 180 µl
Pontohedyle peteryalli	EAT-2	ZSM Mol 20071133	Quiagen Tissue Kit (200 µl) 6.9.07 IS	2.59	KC984298	no PCR product	JQ410930	JQ410899	AB34404268 25 µl
Pontohedyle martynovi	EIP-2	AMC. 476054.001	Machery + Nagel (100 µl) 28.01.11		no PCR product	JQ410984	JQ410983	no PCR product	AB34402062 50 µl
Pontohedyle yurihookeri	EPT-1	ZSM Mol 20080565	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	8.70	KC984299	JQ410987	no PCR product		AB34402000 130 µl
Ganitus evelinae	SWA-2	ZSM Mol 20100327	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	1.30	no or only contaminated PCR products				AB34500495 150 µl
	SWA-2	ZSM Mol 20100328	Quiagen Blood & Tissue Kit (200 µl) 11.1.10	0.00	KF709312	JF828044	JF828045	JF828034	AB34404225 25 µl
	SWA-1	ZSM Mol 20070860	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	2.05	no or only contaminated PCR products				AB34404220 25 µl
Ganitus MOTU Panama	WAT-4	ZSM Mol 20110210	Machery + Nagel (200 µl) 01.07.10	1.70	KF709313	KF709346	KF709270	KF709382	AB34858203 25 µl
	WAT-4	ZSM Mol 20110211	Machery + Nagel (200 µl) 01.07.10	1.50			unpublished	no PCR product	AB35081806 25 µl

	WAT-4	ZSM Mol 20110212	Machery + Nagel (200 µl) 01.07.10	0.90			unpublished	no PCR product	AB34404391 25 µl
	WAT-4	ZSM Mol 20110213	Machery + Nagel (200 µl) 01.07.10	0.80			unpublished	no PCR product	AB34858218 25 µl
Paraganitus ellynnae	CIP-20	ZSM Mol 20080170	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	2.81	HQ168436	HQ168448	HQ168423	HQ168460	AB34404203 25 µl
Paraganitus MOTU Vanuatu	CIP-24	ZSM Mol 20080173	Quiagen Blood & Tissue Kit (200 µl) 6.3.08	3.58	KF709314	KF709347	KF709271	KF709383	AB34404204 25 µl
	CIP-25	ZSM Mol 20100619	Machery + Nagel (100 µl) 27.08.10		KF709315	no PCR product	KF709272	KF709384	AB34402002 80 µl
	CIP-33	ZSM Mol 20100620	Machery + Nagel (100 µl) 27.08.10		unpublished (partial)	no PCR product	unpublished	unpublished	AB34402043 80 µl
Paraganitus MOTU Fiji	CIP-27	ZSM Mol 20080065	Quiagen Blood & Tissue Kit (200 µl) 15.9.06	2.90	KF709316	KF709348	KF709273	KF709385	AB34404280 25 µl
Paraganitus MOTU Samoa	CIP-36	ZSM Mol 20080407	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	4.30	no or only contaminated PCR products				AB34402053 150 µl
	CIP-36	ZSM Mol 20080408	Quiagen Blood & Tissue Kit (200 µl) 19.5.08	6.50	unpublished (partial)	no or only contaminated PCR products			AB34500498 150 µl
Paraganitus MOTU West Papua	CIP-13	ZSM Mol 20100393	Quiagen Blood & Tissue Kit (200 µl) 11.1.10		KF709317	KF709349	no PCR product	KF709386	AB34500507 180 µl
	CIP-13	ZSM Mol 20100394	Quiagen Blood & Tissue Kit (200 µl) 11.1.10				no PCR product	unpublished	AB34402074 180 µl
Paraganitus MOTU Thailand	WIP-4	ZSM Mol 20100586	Machery + Nagel (100 µl) 27.08.10		KF709318	KF709350	KF709274	KF709387	AB34402080 80 µl
	WIP-4	ZSM Mol 20100587	Machery + Nagel (100 µl) 27.08.10					unpublished	AB34402014 80 µl
	WIP-4	ZSM Mol 20100588	Machery + Nagel (100 µl) 27.08.10						AB34402061 80 µl

Additional file 4: Key for the identification of meiofaunal slugs in the field

Key for identification of meiofaunal slugs to family and genus level (in the field)

(still in a preliminary stage and testing phase…)

Specimen found in marine sand sample (presumably belonging to meiofauna), not exceeding a few mm in body length, lack of shell in which the body can be at least partially retracted (internal shell or minute external shell might be present)

try this key!

Not found in marine sand sample and/ or larger than a few mm and/ or with external shell in which the body can be at least partially retracted

please find another key!

I. Body dorso-ventrally flattened, notum undivided, notum overhangs the subpallial groove, a minute reduced shell may be present at the posterior end of the notum (externally or internally), foot usually broad and longer than notum, no parapodia (Fig. 1A)

Runcinacea

II. Body cylindrical or dorsally flattened, divided into a large cephalic shield and a visceral hump, lacking shell or with fragile internal shell (ovate to elongate in shape, of very few whorls and with greatly dilated aperture), large foot but not longer than body, parapodia elevated, between shields (Fig. 1B)

Cephalaspidea: Philinidae: *Philine*

III. Body more or less rectangular shaped (three to six times longer than wide), broad foot with lateral groove along the anterior part of the body (sometimes extending over the entire length of the animal) separating foot and notum, parapodia reduced, limited to anterior head, radula 3-0-3 (Fig. 1C)

Cephalaspidea: Philinoglossidae*

a) with internal shell, lateral groove between foot and notum extending along the entire length of the animal

Pluscula

b) without internal shell, notum without separation between cephalic shield and visceral hump

Philinoglossa/ Sapha

c) without internal shell, cephalic shield indicated by lateral cuts

Abavopsis

IV. Typical aeolid body slightly tapered towards the ends, head roundish with flattened lateral lobes and one pair of rhinophores, body with several elongated cerata (Fig. 1D)

Nudibranchia: Embletoniidae: *Embletonia***

V. Body vermiform, slightly tapered towards the end, lacking head appendages, often with some cerata (Fig. 1E)

Nudibranchia: Pseudovermidae: *Pseudovermis*

VI. Body vermiform, not tapered towards the end, without separations or grooves, lacking head - and other appendages, lacking a radula, with fusiform spicules and eyes (Fig. 1F)

Rhodopemorpha

a) short “flatworm-style”, body whitish often with distinct orange/ reddish or purple colour pattern

Rhodope

b) elongated “nemertine-style”, body whitish translucent

Helminthope

VII. Body separated into an anterior head-foot complex and an elongated posterior visceral hump (Fig. 1G, H)

a) Head rounded, lacking any head appendages, broad head-foot, short, rounded free end of the foot, curls up in case of disturbance

Sacoglossa: Platyhedylidae: *Platyhedyle*

b) Head with one or two head appendages, in case of disturbance head-foot complex can (at least partially) be retracted into visceral hump

Acochlidia

Key to the genera of Acochlidia

a) Head with one pair of appendages (oral/ labial tentacles)

1.

b) Head with two pairs of appendages (oral/ labial tentacles and rhinophores)

2.

1.

a) Head with bow-shaped, flattened oral tentacles tapered towards the ends (see Fig. 2A), radula 1-1-1, with monaxone spicules (see Figure 3 for different spicule types), digestive gland frequently with green colour, very short free foot end (tail)

Pontohedyle

b) Head with flattened, broad oral tentacles not tapered towards the ends (Fig. 2B), short foot/tail, tip of foot pointed, dagger-shaped radula 0-1-0 (see Figure 4 for different radulae types), only very small “perl chain spicules” (see Fig. 3D), digestive gland frequently orange coloured

Ganitus

2.

a) Head with flattened broad oral tentacles, clearly larger than finger-like rhinophores (Fig. 2C), fusiform spicules especially concentrated in visceral hump forming a secondary “spicule shell”

Hedylopsis

b) Oral tentacles not flattened

3.

3.

a) Oral tentacles and rhinophores thick and round in diameter more or less of equal length and quite immobile, visceral hump with densely arranged large, monoaxone spicules forming a secondary "spicule shell"

Asperspina

b) Head appendages rather thin and slender, spicules (if present) only randomly distributed in visceral hump and head-foot complex, not forming a secondary "spicule shell"

4.

4.

a) Oral tentacles slightly thicker and longer than rhinophores both tapering towards the ends and rounded in sections, radula 1-1-1 (Fig. 4C), spicules lacking or scattered fusiform ones. hermaphrodite, penis with penial stylet(s)

Pseudunela

b) Head appendages similar than in a) but species with separate sexes, lack of copulatory organs and sperm transfer occurs via spermatophores

5.

5.

a) Oral tentacles slightly curved, thicker and longer than rhinophores; radula 1-1-1; variety of different spicules: monaxome, triaxial (Fig. 3A), "perl-chain" or lacking

Microhedyle

b) General body shape and head appendages like in *Microhedyle*, but with dagger-shapes radula 0-1-0 (Fig. 4B), bean shaped or thick curved spicules (Fig. 3C)

Paraganitus

c) General body shape and head appendages similar to *Microhedyle*, comparably thin and slender oral tentacles and rhinophores, oral tentacles cylindrical, only very small "perl-chain spicules" (Fig. 3D), radula formula 1-1-2 (Fig. 4D)***

Parhedyle

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*) Classification of genera within Philinoglossidae is doubtful and the entire group urgently requires revision.

**) Next to Pseudovermis, *Embletonia* is up to current knowledge the only genus with truly meiofaunal representatives described for aeolidian nudibranchs, however juveniles of other groups are frequently encountered in sand samples.

***) The small inner lateral tooth might be very difficult to detect using light microscopy.

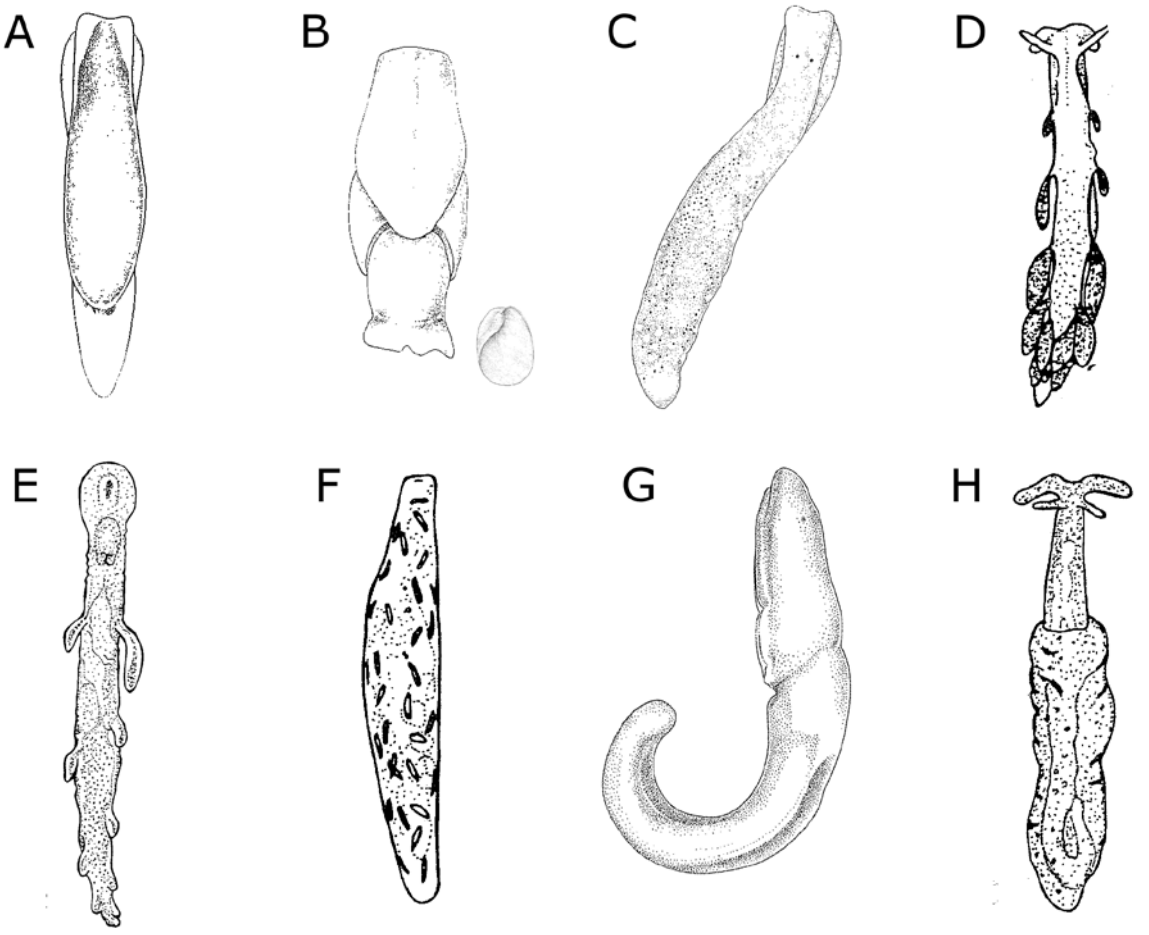

Fig. 1: General body shape of meiofaunal slugs. A) Runcinacea (from Beesley/ Wells (eds): Mollusca – Southern Synthesis). B) *Philine*, with fragile internal shell (from Beesley/ Wells (eds): Mollusca – Southern Synthesis). C) *Philinoglossa* (from Beesley/ Wells (eds): Mollusca – Southern Synthesis). D) *Embletonia* (from Botosaneanu (ed) Stygofauna Mundi). E) *Pseudovermis* (from Botosaneanu (ed) Stygofauna Mundi). F) *Rhodope* (from Botosaneanu (ed) Stygofauna Mundi). G) *Platyhedyle* (from Beesley/ Wells (eds): Mollusca – Southern Synthesis) H) Acochlidia (from Botosaneanu (ed) Stygofauna Mundi).

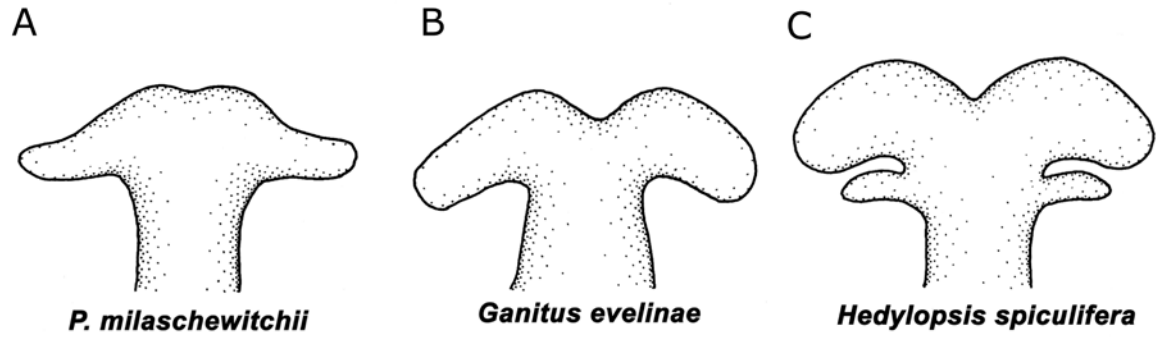

Fig. 2: Head appendages in Acochlidia (from Jörger et al. 2007).

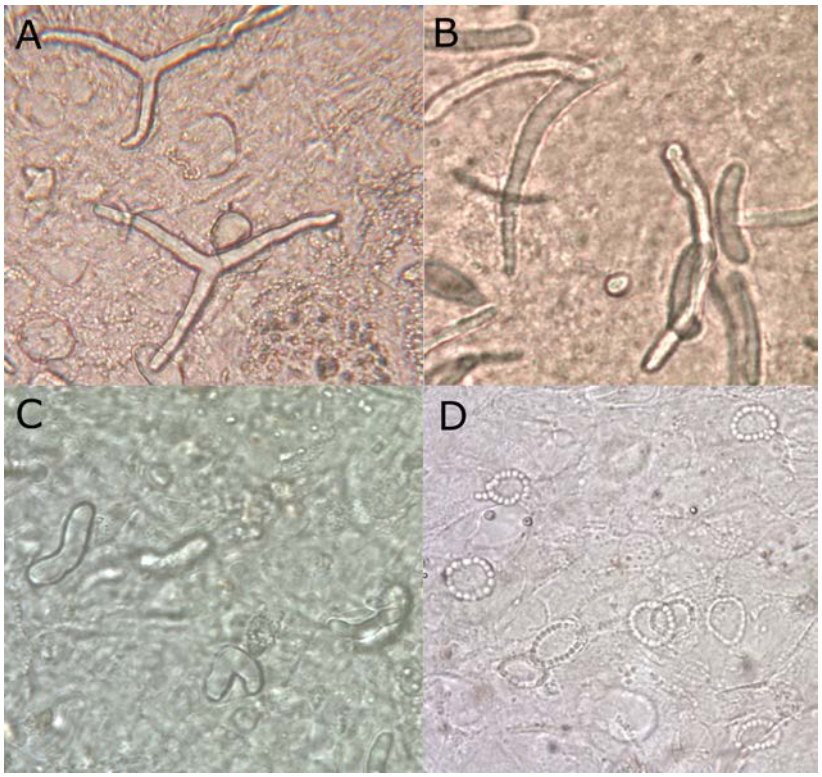

Fig. 3: Different types of spicules in Acochlidia. A) triaxial spicules. B) monaxone spicules. C) bean-shaped spicules. D) 'pearl chain' spicules.

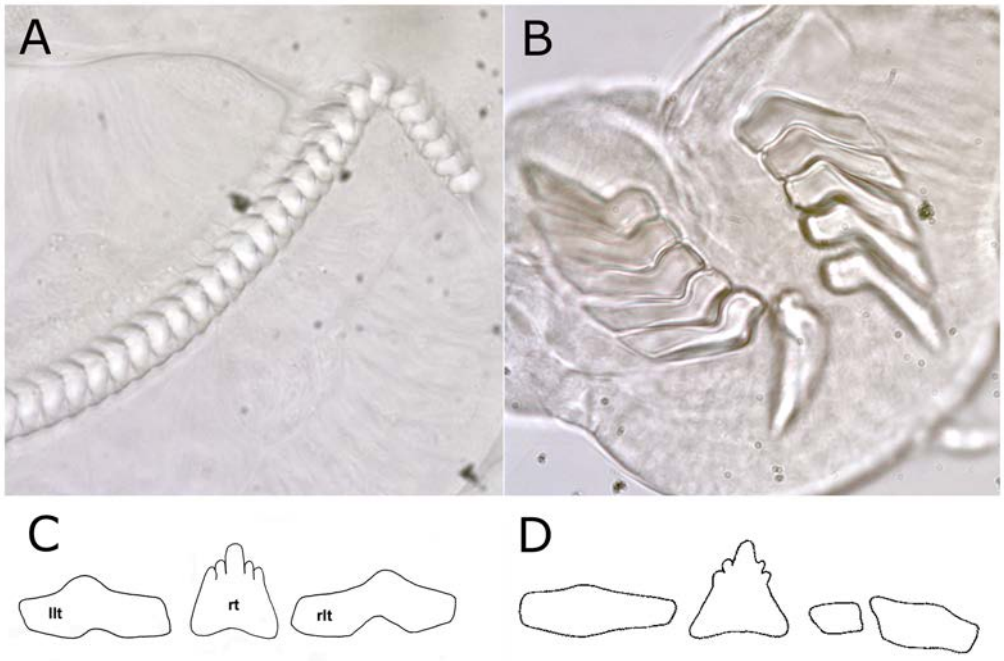

Fig. 4: Different types of radulae in Acochlidia. A) Typical U-shape acochlidian radula. B) Dagger-shaped radula of Ganitidae. C) Acochlidian radula with the formula 1-1-1. D) Radula of *Parhedyle* 1-1-2 (from Westheide and Wawra, 1974). llt – left lateral tooth, rlt – right lateral tooth, rt – rhachidian tooth.

APPENDIX

Own contributions to each publication

Chapter 1. On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia

I conducted the major part of the molecular lab work (with exception of the extraction and amplification of two species) in the beginning with technical assistance of Isabella Stöger and Thomas Knebelsberger and performed the RAxML, SplitsTree and BEAST analyses. I designed the figures and drafted the first version of the manuscript, which was then improved in cooperation between all authors.

Chapter 2. Bye bye "Opisthobranchia"! A review on the contribution of mesopsammic sea slugs to euthyneuran systematics

I contributed to the data collection for this review and the discussion and improvement of the final version of the manuscript, which was written by Dr. Michael Schrödl.

Chapter 3. A reply to Medina et al. (2011): Crawling through time: Transition of snails to slugs dating back to the Paleozoic based on mitochondrial phylogenomics

Medina et al. (2011) was discussed, evaluated and criticized jointly. Dr. Michael Schrödl drafted the manuscript; I contributed to the final version of the letter.

Chapter 4. Exploring cerebral features in Acochlidia (Gastropoda: Opisthobranchia)

I contributed comparative histological data on *Pontohedyle* and *Microhedyle* and joined the discussion of the final version of the manuscript.

Chapter 5. Redescription of the meiofaunal gastropod *Parhedyle cryptophthalma*, with focus on nervous system and sensory organs (Acochlidia, Panpulmonata)

I conducted the histological work and performed the immunocytochemistry and confocal laser scanning microscopy (with instruction and assistance by Alen Kristof, in the lab of Dr. Annette Klussmann-Kolb). I designed the figures and drafted the manuscript, which was subsequently improved in collaboration with all authors.

Chapter 6. Sex in the beach: spermatophores, dermal insemination and 3D sperm ultrastructure of the aphallic mesopsammic *Pontohedyle milaschewitchii* (Acochlidia, Opisthobranchia, Gastropoda)

Material, TEM sections and histology within this publication are based on data generated within my diploma thesis. 3D sperm reconstructions were mainly generated by Dr. Martin Heß for this study. I designed the figures and drafted the manuscript.

Chapter 7. Time for sex change! 3D-reconstruction of the copulatory system of the 'aphallic' *Hedylopsis ballantinei* (Gastropoda, Acochlidia)

I instructed and assisted the 3D-reconstructions and contributed to the discussion and improvement of the final version of the manuscript.

Chapter 8. Integrating 3D microanatomy and molecules: natural history of the Pacific freshwater slug *Strubellia* Odhner, 1937 (Heterobranchia, Acochlidia) with description of a new species

I collected the material of *Strubellia wawrai* from the Solomon Islands, performed the molecular work and phylogenetic analyses and contributed the referring sections to the manuscript. The final version of the manuscript was discussed and improved jointly.

Chapter 9. Sacoglossa or Acochlidia? 3D-reconstruction, molecular phylogeny and evolution of Aitengidae (Gastropoda: Heterobranchia)

I performed the phylogenetic analyses of the study and drafted the corresponding parts of the manuscript (including the design of the related figures). The final version of the manuscript was discussed and improved in cooperation between all authors.

Chapter 10. Panpulmonate habitat transitions: tracing the evolution of Acochlidia (Heterobranchia, Gastropoda)

I conducted the molecular work, all included analyses and drafted the manuscript. Results were discussed among all authors, who all contributed to improve the final version of the manuscript. Material for the study was collected jointly by all authors and several collaborators.

Chapter 11. Cryptic species in tropic sands - Interactive 3D anatomy, molecular phylogeny and evolution of meiofaunal Pseudunelidae (Gastropoda, Acochlidia)

I collected parts of the material, conducted the molecular lab work, performed the phylogenetic and species delineation approaches and wrote the corresponding paragraphs of the manuscript (including design of the related figures). The final version of the manuscript was discussed and improved in cooperation between all authors.

Chapter 12. Systematics and redescription of the european meiofaunal slug *Microhedyle glandulifera* (Kowalevsky, 1901) (Heterobranchia: Acochlidia): evidence from molecules and morphology.

I designed the study and conducted the molecular lab work and phylogenetic such as population genetic analyses. I supervised and instructed histological work and 3D-reconstructions and revised the manuscript together with Dr. M. Schrödl.

Chapter 13. Barcoding against a paradox? Combined molecular species delineations reveal multiple cryptic lineages in elusive meiofaunal sea slugs

I conducted major part of the molecular work (with the exception of the material from Belize which was extracted in the molecular labs of the Smithsonian Institute), performed all species delineation analyses and drafted the manuscript. Collection of material, discussion of results and the final version of the manuscript were achieved in cooperation with the co-authors.

Chapter 14. How to describe a cryptic species? Practical challenges of molecular taxonomy.

I conducted the molecular, SEM and micro-anatomical work, performed the character-based species descriptions and drafted the manuscript, which was discussed with and improved by Dr. Michael Schrödl.

I hereby confirm the above statement.

(Katharina Jörger) (PD Dr. Michael Schrödl)

Congress Contributions

In the following, the congress contributions (abstracts of talks and posters) in which I functioned as presenting author and which are directly related to my PhD thesis are listed chronologically (for entire list of congress contributions, see Curriculum Vitae).

Talks

Jörger KM, Schrödl M (2009) Tracing evolution: Molecular phylogeny of the Acochlidia (Gastropoda, Opisthobranchia). Systematics 2009, Leiden, Netherlands.

The Acochlidia are a morphological aberrant group of opisthobranch gastropods. They inhabit mainly the marine mesopsammon, however some species conquered true limnic systems. A robust phylogenetic hypothesis will be the basis to explain this and other major events in acochlidian evolution (e.g. the development of special reproductive features and strategies). While morphology-based phylogenetic analysis faces the problem of a high degree of convergent development due to adaptations to the similar (interstitial) habitat, molecular markers provide a dataset independent from direct ecological pressures. This study presents a first molecular phylogeny of the Acochlidia based on different gene regions (nuclear 18S, 28S-sequence and mitochondrial 16S and CoI), leading to a good resolution at all taxonomic levels. Fast-evolving mitochondrial markers uncovered a high degree of cryptic species within similar habitats and little geographic distance and on the other hand confirmed species with large-range distribution; potential explanations are discussed.

Jörger KM, Schrödl M (2010) Uncovering enigmas from the sand: Molecular phylogeny and evolution of Acochlidia (Heterobranchia, Gastropoda). 2nd VW Status Symposium Evolutionary Biology, Frauenchiemsee, Germany.

The Acochlidia are an enigmatic group of heterobranch gastropods, mainly inhabiting marine sands worldwide. Being a small groups with only 28 valid species, these slugs are morphologically highly aberrant and divers, comprising a series of unusual characters (e.g. occurrence secondary gonochorism, lack of copulatory organs, dagger-shaped radulae). While most inhabit the marine mesopsammon, some show adaptations to brackish waters or even truly conquered limnic systems. To explain this and other major events in acochlidian evolution a robust phylogenetic hypothesis is needed. While morphological analyses face the problem of a high degree of convergent development due to adaptations to the similar (interstitial) habitat, molecular markers provide a dataset independent from direct ecological pressures. This study presents a first molecular phylogeny of the Acochlidia based on four markers (nuclear 18S,

28S-sequence and mitochondrial 16S and CoI), leading to a good resolution at all taxonomic levels. Fast evolving mitochondrial markers uncovered a high degree of cryptic species within similar habitats and little geographic distance and on the other hand confirmed species with large-range distribution. To major lineages (Microhedylacea and Hedylopsacea) developed two opposite trends of reproduction, both interpreted as special adaptations to a spatially limited and unstable habitat. Based on the presented phylogenetic hypothesis the invasion of limnic systems in Acochlidia is discussed.

Jörger KM, Schrödl M (2010) The end of the guessing game? Origin and evolution of Acochlidia (Gastropoda, Heterobranchia). World Congress of Malacology, Phuket, Thailand. (Awarded as best student talk by Unitas Malacologica)

As traditional order of the “Opisthobranchia” the enigmatic, mainly mesopsammic Acochlidia look back on a long history of controversial taxonomic placements, among others influenced by convergent adaptation to the meiofaunal habitats. Analyzing specimens of 11 of 12 acochlidian genera (representing 6 of 7 acochlidian families) in a comprehensive euthyneuran taxon sampling with special focus on minute aberrant slugs, our multi-locus molecular study places Acochlidia in a pulmonate relationship, as sister to Eupulmonata. Previous hypotheses of “opisthobranch" relationships of Acochlidia or a common origin with other meiofaunal Euthyneura are clearly rejected by our data. Potential morphological synapomorphies are critically evaluated, currently neither contradicting nor supporting molecular results. Evolutionary pathways leading to the aberrant acochlidian morphology are discussed.

The robust phylogenetic hypothesis on the phylogeny of Acochlidia based on molecular markers and in congruence with previous morphological analyses is the base to trace the evolution of this formerly enigmatic taxon. The position of the amphibious and insectivorous Aitengidae *incerta sedis* in relation to Acochlidia is discussed. Flexibility in habitat choice (from marine meiofaunal, to limnic benthic and semi-terrestrial) within Acochlidia is highlighted and the evolutionary key features are discussed.

Acochlidian divergence times are estimated to Mesozoic Jurassic using a relaxed molecular clock approach. These relatively old splits and recent findings of a high level of cryptic speciation might indicate that known acochlidian species form just the tip of the iceberg concerning acochlidian diversity.

Jörger KM, Brenzinger B, Schrödl M (2011). Sneaking into the meiofaunal world – evolution and adaptations in microslugs. 2nd International Congress on Invertebrate Morphology, Boston, USA.

Within gastropod molluscs, truly meiofaunal groups occur mainly within Heterobranchia and comprise a series of previously enigmatic taxa with controversially debated phylogenetic affinities. Poor morphological knowledge and unclear sister group relationships used to hinder comparative and evolutionary approaches. Our molecular phylogenetic hypothesis, based on a concatenated 'standard-marker' dataset in a comprehensive euthyneuran taxon sampling, indicates at least five independent pathways into the Mesopsammon within heterobranch gastropods: in Rhodopemorpha, Nudibranchia, Cephalaspidea s.s., Sacoglossa and Acochlidia. We supplement original morphological descriptions traditionally based on light microscopy with data from modern methodology such as 3D-microanatomy, redescribing representatives from the main meiofaunal groups. Major convergent adaptations to the Mesopsammon are revisited and compared in histological detail, e.g. the characteristic reduction of a shell in combination with the formation of spicules, presence of additional 'accessory' ganglia' and the development of adhesive mechanisms. Different modes of sperm transfer are compared across microslug taxa and a general trend towards rapid, yet imprecise modes of sperm transfer is discussed. At the current state of knowledge, Acochlidia form the most successful group among meiofaunal slugs with regard to species diversity and local abundances. Adaptations in their excretory system that confer tolerance towards freshwater impact widen the range of potential habitats and might have thus play a key role for the success of Acochlidia in (and, uniquely, again out of) the meiofaunal habitat. Recent sampling efforts have augmented estimations of species diversity and their mostly worldwide distribution, while molecular data provide evidence for a high degree of cryptic speciation on the one hand and wide-spread species on the other. With biology and ontogeny of most clades being still little explored, this implies that our current knowledge is, however, likely to be still just a glimpse into current microslug diversity and evolution.

Jörger KM, Schrödl M (2013). Struggling with uniformity and rarity – molecular species delineation and DNA taxonomy in elusive meiofaunal slugs. BioSys EU 2013, Vienna, Austria.

Due to frequently low dispersal abilities, many meiofaunal taxa are prone to cryptic speciation. Barcoding and molecular species delineation have been advocated to uncover theses cryptic species. But in addition to the striking uniformity in meiofaunal slugs, these groups are rare and hard to sample and sampling efforts frequently result in singletons from distant localities. To our knowledge, none of the proposed methods of molecular species delineation is specifically designed to deal with the common phenomenon of rarity. We struggled with a workflow to nevertheless receive reliable diversity estimates and species delineations hypothesis for elusive taxa as a basis for e.g. biogeography or conservation biology approaches. Our workflow was performed on a worldwide sampling Pontohedyle (Acochlidia, Heterobranchia). Combining sequence data from three gene regions (mitochondrial COI and 16S rRNA and nuclear 28S rRNA), we constructed a molecular phylogeny of the genus Pontohedyle and determined preliminary molecular operational taxonomic units (MOTUs) based on the criterion of reciprocal monophyly in concatenated and single gene trees. We performed four independent methods of molecular species delineation: General Mixed Yule Coalescent model (GMYC), statistical parsimony, Bayesian Species Delineation (BP&P) and Automatic Barcode Gap Discovery (ABGD). To choose the conservative approach in species delineation, we rely only on uncontradicted results from the different approaches resulting in a minimum of twelve candidate species (compared to two valid species described on morphology). In our opinion, DNA taxonomy remains incomplete with the discovery of species only. An increasing amount of detected but unnamed candidate species, which lack minimum voucher information, float around in taxonomic no-man's land. In the present study, all identified candidate species were objected to morphological analyses of characters traditionally used for species delineation (e.g. SEM of radulae) and combined with micro-anatomical comparison based on histological semithin sections of the major clades. In absence of any reliable diagnostic characters, species descriptions were based on diagnostic molecular characters, retrieved using the Character Attribute Organization System (CAOS). Necessary consideration and putative pitfalls when integrating DNA taxonomy into the Linnaean system are evaluated.

Jörger KM, Neusser TP, Brenzinger B, Schrödl M (2013) Out of the marine mesopsammon, into the (pan-) pulmonate world: historical biogeography and habitat shifts in the evolution of acochlidian slugs. World Congress of Malacology Azores, Portugal.

Molecular phylogenetics have challenged our conventional classification of euthyneuran gastropods and placed traditional opisthobranch or 'lower heterobranch' clades (such as Sacoglossa, Acochlidia and Pyramidelloidea) into sistergroup relationships with established pulmonate taxa. We present a comprehensive molecular phylogeny on acochlidian slugs based on a global sampling that covers 82.5% of the described diversity and additionally nearly doubles known diversity by undescribed taxa. Our phylogenetic hypothesis is largely congruent with previous morphological analyses and provides a robust framework to trace the evolutionary history of Acochlidia. To do so, we conducted molecular clock analyses and reconstructed ancestral areas as well as the ancestral states of major ecological traits. Based on our data, the Acochlidia originated from a marine, mesopsammic ancestor adapted to tropical waters in the Mesozoic Jurassic. Early acochlidian radiation might have been triggered by isolation events due the breakup of the Panthalassa Ocean and the formation of the shallow early Atlantic and Tethys oceans. A series of vicariance events influenced the history of the two major clades with remarkably different evolutionary history. The Microhedylacea remained in stunning morphological and ecological stasis for approximately 150 million years, and are restricted to the marine mesopsammon. Molecular data reveals that the majority of microhedylacean diversity belongs to (pseudo-)cryptic species with deep genetic divergences, indicating long reproductive isolation. In contrast, the evolution of Hedylopsacea is characterized by a series of independent habitat shifts out of the marine mesopsammon, conquering semi-terrestrial and limnic habitats while reestablishing a benthic lifestyle. The major radiations and habitat shifts in hedylopsacean families occur in the central Indo-West Pacific and can be related to tectonic events in the area.

This study on acochlidian phylogeny and biogeography adds another facet to the yet complex (pan-) pulmonate evolution and highlights the various parallel pathways in which these snails and slugs invaded non-marine habitats.

Jörger KM, Kristof A, Klussmann-Kolb A, Schrödl M (2008) Keeping nerves: central nervous system of the interstitial acochlid *Parhedyle cryptophthalma* (Gastropoda, Opisthobranchia). 1st International Congress on Invertebrate Morphology, Copenhagen, Denmark.

Keeping nerves – Central nervous system of the acochlid *Parhedyle cryptophthalma* (Gastropoda, Opisthobranchia)

Jörger, Katharina[1]; Kristof, Alen[2]; Klussmann-Kolb, Annette[3]; Schrödl, Michael[1]

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INTRODUCTION

Acochlidia are a small group of mainly minute interstitial opisthobranchs. Due to the small body size (i.e. 1-4 mm) older descriptions often lack detail and thus require redescription using modern technologies. Especially the highly complex nervous system offers a wide range of valuable morphological details (i.e. the presence of ganglia, innervation patterns and sensory structures). With an adult size reaching only 1.5 mm the microhedylid *Parhedyle cryptophthalma* (Westheide & Wawra, 1974) is one of the smallest described acochlids. It inhabits the interstitial with direct wave impact in the Mediterranean. *Parhedyle cryptophthalma* was recollected at the type locality in Naples, Italy. The CNS, peripheral nervous system and sensory structures were analysed using computer-based 3D reconstruction from serial sections and scanning electron microscopy (SEM). Histological results were supplemented by the examination of Tyrosine hydroxylase (TH) and FMRF-amid immunoreactivity – the minute size of sub-adult specimens enabled entire penetration of the laser scanner.

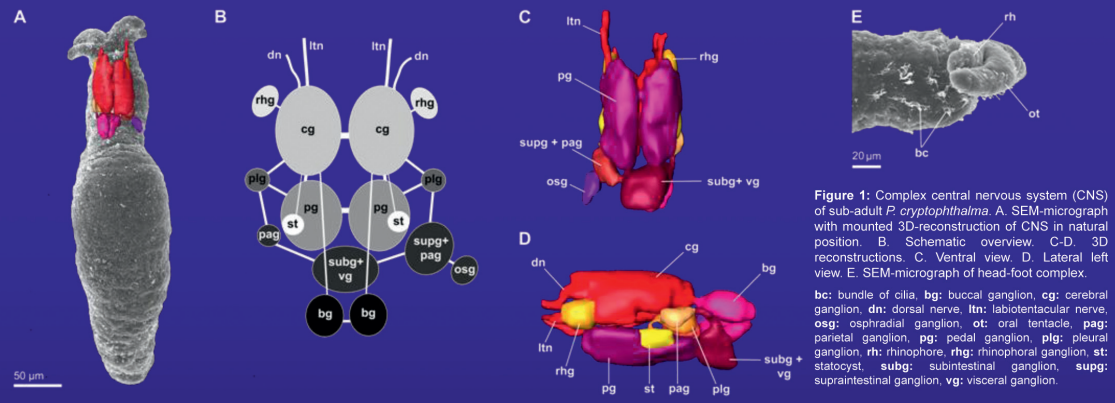

Figure 1: Complex central nervous system (CNS) of sub-adult *P. cryptophthalma*. A. SEM-micrograph with mounted 3D-reconstruction of CNS in natural position. B. Schematic overview. C-D. 3D reconstructions. C. Ventral view. D. Lateral left view. E. SEM-micrograph of head-foot complex.

bc: bundle of cilia, **bg:** buccal ganglion, **cg:** cerebral ganglion, **dn:** dorsal nerve, **ltn:** labiotentacular nerve, **osg:** osphradial ganglion, **ot:** oral tentacle, **pag:** parietal ganglion, **pg:** pedal ganglion, **plg:** pleural ganglion, **rh:** rhinophore, **rhg:** rhinophoral ganglion, **st:** statocyst, **subg:** subintestinal ganglion, **supg:** supraintestinal ganglion, **vg:** visceral ganglion.

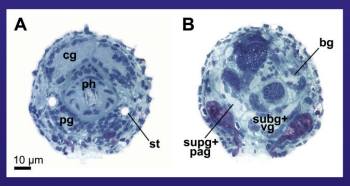

Figure 2: Semithin sections (1 µm) of *P. cryptophthalma*. A. Cerebral and pedal gaglia. B. Ganglia of visceral nerve cord.

bg: buccal ganglion, **cg:** cerebral ganglion, **pag:** parietal ganglion, **ph:** pharynx, **pg:** pedal ganglion, **st:** statocyst, **subg:** subintestinal ganglion, **supg:** supraintestinal ganglion, **vg:** visceral ganglion.

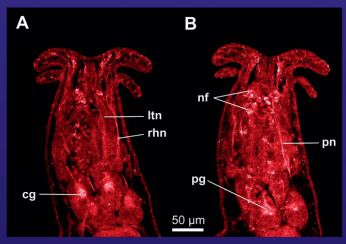

Figure 3: TH-immunoreactivity in the nervous system of *P. cryptophthalma*. A. Cerebral ganglia and nerves. B. Pedal ganglia and neurons bordering the foot sole.

cg: cerebral ganglion, **ltn:** labiotentacular nerve, **nf:** neurons bordering the foot sole, **pg:** pedal ganglion, **pn:** pedal nerve, **rhn:** rhinophoral nerve.

RESULTS AND CONCLUSIONS

- **CNS:** The prepharyngeal CNS of *Parhedyle cryptophthalma* consists of paired cerebral, rhinophoral, pedal, pleural and buccal ganglia, and three distinct unpaired ganglia on the visceral nerve cord. This confirms to the general setting within Acochlidia (see Neusser *et al.* 2008). An additional unpaired, putative osphradial, ganglion is attached to the right parietal/supraintestinal ganglion, detected for the first time in a microhedylid species.
- **Accessory ganglia:** Anterior to the cerebral ganglia a still undifferentiated mass of accessory ganglia is found. The presence of accessory ganglia can be considered as characteristic for microhedylids - and the enigmatic *Tantulum elegans*. In contrast to "true" ganglia, accessory ganglia do not show FMRF-amid expression, their function remains unknown.
- **Cerebral nerves:** Three cerebral nerves could be detected in sub-adult specimens: the ventrally emerging labiotentacular nerve; the dorsally emerging dorsal nerve, herein interpreted as rhinophoral nerve, and the static nerve.
- **Sensory structures:** surprisingly, the eponymous cryptic eyes could not be detected in sub-adult specimens. A pair of un-pigmented minute nervous globules are found nestling anterior on the cerebral ganglia. These structures might either develop to eyes in adult stages or can be interpreted as relicts of eyes. No Hancock's organ – as reported for some other Microhedylidae – could be detected. Some bundles of cilia, supposedly with sensoric function, are located in the anterior region of the head-foot-complex and frontal of the oral tentacles.
- **Retraction:** Protective complete retraction of the head-foot complex into the visceral hump is achieved by the action of strong retractor muscles: this condition leaves none of the above mentioned sensory structures exposed.
- **Keeping nerves:** Despite of the minute size the nervous system of *P. cryptophthalma* confirms with the general characteristics known of larger acochlids. Remarkable, at least in sub-adult stages, is the elongated shape of the cerebral and pedal ganglia.

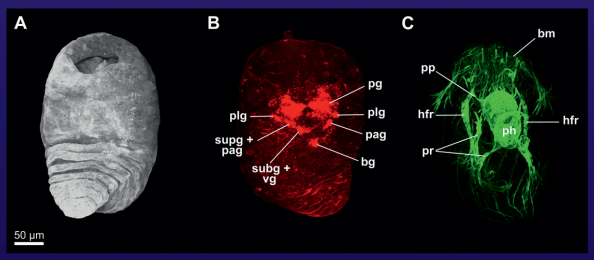

Figure 4: Retraction of the head-foot complex into the visceral hump and its effects on ganglia position and sensorics in *P. cryptophthalma*. A. Confocal laser scanning micrograph of a completely retracted specimen. B. FMRF-amid expression in ganglia (note: no FMRF-expression in accessory ganglia) C. Phalloidin-staining showing arrangement of (preliminarily identified) muscles in a retracted specimen.

bg: buccal ganglion, **hfr:** head-foot retractor, **bm:** body wall muscles, **pag:** parietal ganglion, **ph:** pharynx, **plg:** pleural ganglion, **pp:** pharyngeal protractor, **pr:** pharyngeal retractor, **subg:** subintestinal ganglion, **supg:** supraintestinal ganglion, **vg:** visceral ganglion.

ACKNOWLEDGEMENTS

This study was partially financed by a PhD-scholarship of the "Volkswagenstiftung" to KJ. The GeoBio Center (LMU) is thank for supporting AMIRA Software.

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Under (re-) construction: Evolution in Acochlidia (Gastropoda, Opisthobranchia)

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INTRODUCTION

The Acochlidia are a small group of opisthobranch gastropods in which a large variety of morphological and biological characters is combined. They are distributed worldwide and inhabit mainly the marine interstitial. A robust phylogenetic hypothesis will be the basis to explain the major events in acochlidian evolution such as the conquering of true limnic systems and the development of special reproductive features (i.e. secondary gonochorism – uniquely within the usually hermaphroditic opisthobranchs – and different methods of sperm transfer). Morphological analysis faces the problem of a high degree of convergent development due to adaptations to the similar (interstitial) habitat (such as i.e. the worm-shaped body and reduction of pigmentation and eyes). Thus, molecular markers – independent from ecological pressures – are needed to critically evaluate morphologically based phylogenetic hypothesis and enlighten the complex evolution of the Acochlidia. The present study aims to reconstruct the phylogeny of Acochlidia based on the nuclear 18S-sequence (complete) and the mitochondrial 16S and Col-sequences (partial) and presents a first preliminary molecular phylogeny.

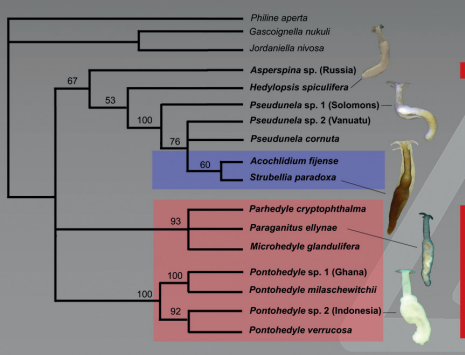

Figure 1: Strict consensus tree of the 10 most-parsimony trees based on the complete 18S sequence, bootstrap indices (50% majority rule) are given; showing the different acochlidian subgroups. Occurrence of secondary gonochorism highlighted in red; freshwater species shown in blue. 18S results confirm with the morphology-based hypothesis. But *Asperspina* is the sister clade to Hedylopsacea, rendering the basally unresolved Microhedylacea paraphyletic.

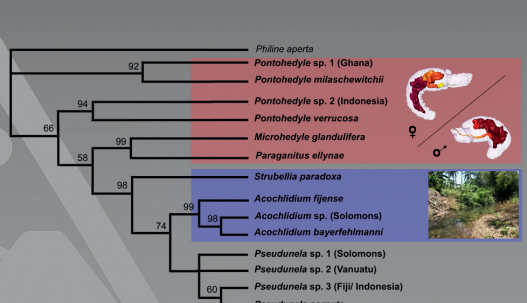

Figure 2: Strict consensus tree of the most-parsimony trees based on the combined 16S (partially) and Col-sequence (partially), bootstrap indices (50% majority rule) are given. Occurrence of secondary gonochorism highlighted in red; freshwater species shown in blue. The basal topology of this tree is incongruent with 18S and morphology-based phylogenies. Due to their high variability these markers seem to be inappropriate for the reconstruction of basal events in acochlidian evolution.

Conquering of limnic systems

In accordance with the morphology-based phylogeny the 18S results suggest that the invasion of freshwater occurred once in the Indopacific Acochlidiidae (see Fig. 1). Contradicting results based on 16S and Col markers (see Fig. 2) might be misleading due to the high variability of the markers. Results from morphology suggest a second independent colonisation of limnic systems in the enigmatic Caribbean *Tantulum elegans* (see Schrödl & Neusser, in press), not yet included in the present study.

Figure 3: Radiation in limnic Acochlidiidae.
A *Strubellia paradoxa*
B *Strubellia* sp. (Vanuatu)
C *Acochlidium* sp. (Solomons)
D *A. fijense*
E *Palliohedyle sutteri*

The strong similarity in Col and 16S sequences of different populations of *Strubellia paradoxa* and *Palliohedyle sutteri* from different rivers and even neighbouring islands indicate a genetic exchange between populations, i.e. via marine larvae or a very recent radiation in the limnic systems.

Evolution of reproductive features

Based on the preliminary 18S phylogeny secondary gonochorism might have evolved two times independently in two different Microhedylacean lineages. The seldom occurrence of secondary gonochorism in the animal kingdom, however, makes a unique evolution more likely. A combined analysis of the different genetic markers might lead to a better resolution in the basally unresolved Microhedylacea.

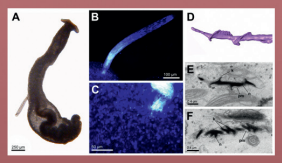

Figure 4: Sperm transfer via spermatophores in *Pontohedyle milaschewitchii*. **A** Specimen with attached spermatophore. **B** DAPI-staining of emptying spermatophore. **C** DAPI-staining of attachment site showing intruding sperm. **D** 3D reconstruction of the tip of the corkscrew-like sperm nucleus. **E,F** TEM-micrographs of sperm nucleus.

Microhedylacean species and supposedly the hedylopsacean *Hedylopsis ballantinei* are aphallic and transfer sperm via spermatophores combined with dermal fertilisation. The aphallic condition and this mode of sperm transfer might have evolved convergently as an adaptation to the, spaciously restricted, interstitial environment (see Jörger et al., in press).

DISSCUSSION and OUTLOOK

- The presented mitochondrial markers reveal recent radiations and help to uncover cryptic species such as *Pseudunela* sp. 1 from the Solomons and *Pseudunela* sp. 3 from Fiji and Indonesia or *Pontohedyle milaschewitchii* and *Pontohedyle* sp. 2 from Indonesia, being identical concerning their external morphology. However, these markers – like in other Opisthobranchs – seem to be to variable to solve the basal nodes.
- The 18S results largely confirm morphological data (see Schrödl and Neusser, in press). Current genera such as *Pontohedyle* and even basal hedylopsacean nodes show at least some to very good statistical support, indicating that the 18S is a suitable marker for reconstructing acochlidian phylogeny from its likely origin in the mesozoic to quite recent radiations. Accomplishing a better outgroup and ingroup taxon sampling, complete 18S, together with the, in parts, similarly conservative 28S, are expected to uncover the evolutionary history of this group.

ACKNOWLEDGEMENTS

This study is funded by a PhD-scholarship of the "Volkswagenstiftung" to KJ. The GeoBio Center (LMU) is thanked for supporting AMIRA Software and the LMU Mentoring program for funding computer equipment. Thanks to Timea Neusser for constant support and discussion on the project.

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From Tropical Waters to Northern Seas: Biogeography and Taxonomic Revision of the "*Microhedyle* Species Complex" in Europe

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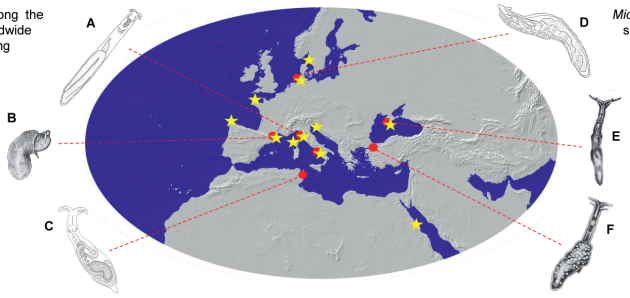
INTRODUCTION

Acochlidian Microhedylidae species are among the most common meiofaunal gastropods in worldwide intertidal and shallow subtidal sands, regarding densities and s pecies numbers. Acochlids were first discovered over a hundred years ago and despite of the comparably well described Mediterranean Meiofauna, the taxonomic validity of most European Microhedylidae species is unclear and their biogeography is thus little understood. The anatomy and taxonomy of *Pontohedyle milaschewitchii* is well explored and its distribution throughout the Mediterranean and Black Sea is well documented. However, species belonging to the genera *Microhedyle* and *Parhedyle* have been mixed up in literature and require reinvestigation. We recollected all Microhedylidae species described from the Mediterranean and adjacent Seas from type localities, collecting points mentioned in original descriptions or intermediate sites.

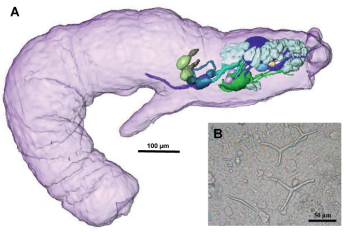


Type localities (●) and sampling sites (★) of the different Microhedylidae species from the Mediterranean and adjacent waters, with the drawings from the original descriptions: A) *Microhedyle glomerans* (Salvini-Plawen, 1973); B) *M. odhneri* (Marcus & Marcus, 1955); C) *Parhedyle cryptophthalma* (Westheide & Wawra, 1974); D) *M. lactea* (Hertling, 1930); E) *P. tyrtowii* (Kowalevsky, 1901); F) *M. glandulifera* (Kowalevsky, 1901).

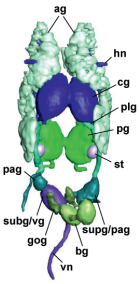
OBJECTIVES

Microhedyle glandulifera was made the key species of this unclear "*Microhedyle* species complex" in the Mediterranean to which all later described microhedylids were compared. We are the first to redescribe *M. glandulifera* with modern methodology (3D reconstructions based on histological semithin sectioning and scanning electron microscopy, SEM), and use it as a base for comparative anatomy. Additionally to morphological work, a molecular phylogeny (based on nuclear 18S rRNA and mitochondrial cytochrome "*c*" oxidase subunit I (COI) and 16S rRNA sequences) is done to solve relationships within Microhedylidae and gain insights into the biogeography of this clade. A haplotype network based on COI sequences of the different populations of *M. glandulifera* is generated to visualise the genetic structure of this species within European waters.

MORPHOLOGY OF *M. glandulifera*

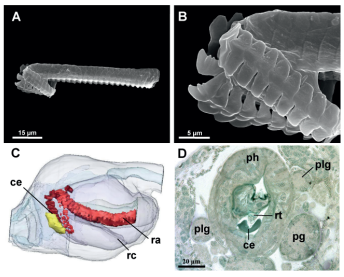


A) 3D reconstruction of a juvenile specimen showing the central nervous system (cns). B) Light microscopic picture of the three different types of spicules characteristic for *M. glandulifera*: monaxonic, triaxonic and „pearl-chain" type.



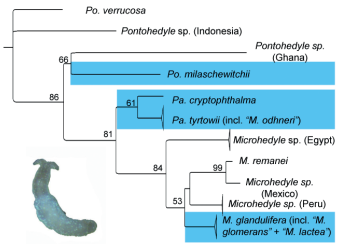
3D reconstructions of the cns with accessory ganglia (dorsal view)

ag: accessory ganglion, **bg:** buccal ganglion, **cg:** cerebral ganglion, **gog:** gastro-oesophageal ganglion, **hn:** Hancock´s nerve, **pag:** parietal ganglion, **pg:** pedal ganglion, **plg:** pleural ganglion, **st:** statocyst, **subg/ vg:** subintestinal/ visceral ganglion, **supg/ pag:** supraintestinal/ parietal ganglion, **vn:** visceral nerve.



Radula and "cuticular element". A) SEM-micrograph of the hooked-shaped radula. B) SEM close-up of rhachidian and left lateral teeth. C) 3D reconstruction of pharynx containing radula and "cuticular element". D) Semithin cross-section of pharynx.

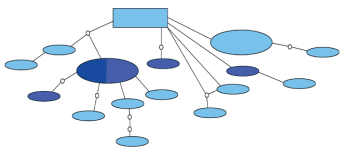
ce: cuticular element, **ph:** pharynx, **pg:** pedal ganglion, **plg:** pleural ganglion, **ra:** radula, **rc:** radular cushion, **rt:** rhachidian tooth.



Phylogeny of all available Microhedylidae including material from European type localities (extracted from a large acochlidian phylogeny) – RAxML analysis based on the concatenated 18S rRNA, 16S rRNA and COI dataset (2898 bp – bootstrap support given). "*M. glomerans*" and "*M. lactea*" clearly cluster within *M. glandulifera*, "*M. odhneri*" within *P. tyrtowii*. Taxa from European temperate waters in light blue.

CONCLUSIONS

- There are four different valid Microhedylidae species (see Table) in the Mediterranean, well separated morphologically and molecularly. All species share a wide range distribution throughout the Mediterranean and partially within the Black Sea and Atlantic.
- Based on our phylogenetic hypothesis, colonisation of European temperate waters and the Mediterranean has occurred three times independently within Microhedylidae originating from tropical forms.
- Within *M. glandulifera* the haplotype network analysis shows little genetic structure within and among the different populations in Atlantic, North Sea and Mediterranean. This points at an evolutionary young origin and/ or indicates gene transfer between populations. The latter would be surprising for a mesopsammic gastropod with supposedly little dispersal capabilities.



Parsimony haplotype network based on mitochondrial COI sequences of different *M. glandulifera* populations from the Mediterranean (●), North Sea (●) and European Atlantic (●) (n=35). The network analysis reveals little genetic structure in *M. glandulifera* and indicates a close relation between the populations from different Europeans Seas.

TAXONOMY OF THE MEDITERRANEAN MICROHEDYLIDAE

OLD	NEW	DIAGNOSTIC FEATURES
Pontohedyle milaschewitchii (Kowalevsky, 1901)	*Pontohedyle milaschewitchii* (Kowalevsky, 1901)	lack of rhinophores, oral tentacles flattened and bow shaped, radula 1-1-1, with eyes
Microhedyle glandulifera (Kowalevsky, 1901) *Microhedyle lactea* (Hertling, 1930) *„Microhedyle glomerans"* (Salvini-Plawen, 1972)	*Microhedyle glandulifera* (Kowalevsky, 1901)	two pairs of head appendages, triaxonic and "pearl-chain" spicules, radula 1-1-1, with eyes
Parhedyle tyrtowii (Kowalevsky, 1901) *Microhedyle odhneri* (Marcus & Marcus, 1955)	*Parhedyle tyrtowii* (Kowalevsky, 1901)	two pairs of head appendages, only "pearl-chain" spicules, radula 1-1-2, with eyes
Parhedyle cryptophthalma (Westheide & Wawra, 1974) *„Microhedyle napolitana"* (Rankin, 1979)	*Parhedyle cryptophthalma* (Westheide & Wawra, 1974)	like *P. tyrtowii* but without eyes

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Jörger KM, Schrödl M, Brenzinger B, Neusser TP (2013) Back to the sea? First ontogenetic data of limnic slugs (Acochlidia, Heterobranchia). BioSys EU 2013, Vienna, Austria.

Back to the sea? First ontogenetic data of limnic slugs (Acochlidia, Heterobranchia)

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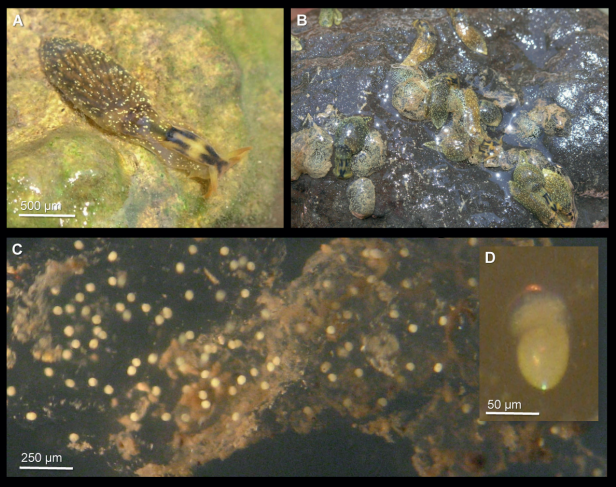
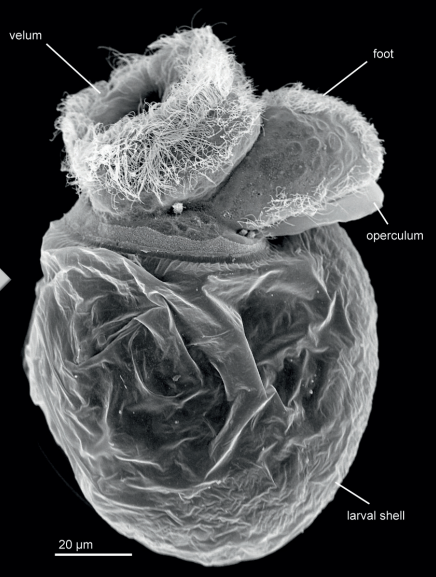
INTRODUCTION

To current knowledge, Acochlidia (Heterobranchia, Panpulmonata) are the only group of slugs with representatives in limnic systems. Based on our molecular phylogenetic hypotheses (Jörger *et al.* 2010), the transition to fresh water occurred at least twice independently within the evolution of Acochlidia. The major radiation refers to the Acochlidiidae, which are unique limnic slugs found in rivers and streams on Indo-Pacific islands. They have evolved a benthic life style on the underside of submerged stones and can occur upstream, even several kilometers inland from the river mouth. We observed Acochlidiidae feeding on eggs of syntopic neritid gastropods (Brenzinger *et al.* 2011). No reports on the ontogeny of acochlidiids exist, hindering conclusions on dispersal abilities and interpretation of biogeographic patterns.

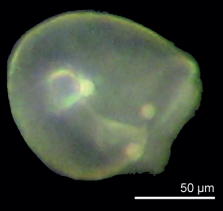
RESULTS



We collected *Acochlidium sutteri* Wawra, 1979 slugs (A) together with their egg masses (B) from streams, near Boleng Bay, Flores Island (Indonesia). Conspecifity with specimens collected from the type locality on Sumba Island and between egg masses and adults were confirmed via DNA- Barcoding. Egg masses are gelatinous and amorphous, having a varying amount of up to several hundred oval egg capsules loosely embedded (C,D).



Scanning electron micrograph of a free-swimming veliger larva of *A. sutteri* 24 hours after hatching in fresh water.



‚Adhesive larva' 36 days after hatching, after two days transferred to sea water.

Hatching veliger larvae were actively swimming for up to two days, but died after this period when kept in fresh water. Larvae are comparably small (approx. 95 µm), but lecithotrophy is likely, as suggested by histology, SEM and light-microscopy. Experimentally transferred to sea water, they stopped swimming, closed the larval shell and attached to the substratum. Externally inactive they survived for several weeks glued to the bottom of the petri-dish or a sand grain. When removed these 'adhesive larvae' reattached themselves to the substrate.

CONCLUSION

We conclude that after an initial limnic phase acochliid larvae need marine conditions for further development and that soon after hatching larvae are flushed into the sea. Here, after short pelagic phase, larvae will attach to a certain substrate where they metamorphose into a long term resting stage. On one hand, the observed long-lived 'adhesive larva' may inhibit uncontrolled drift into the open ocean, far away from potential adult habitats. On the other hand, it could serve as a dispersal stage by using larger mobile organisms as vectors. If we hypothesize that such vectors are neritids, acocchlidiid larvae would benefit from neritid migration up to the rivers and such transient epibiosis would even transport acochlidiids to suitable spawning and feeding habitats, i.e. those with neritid eggs. Acochlidiid amphidromy thus might have coevolved with neritid migration patterns.

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ACKNOWLEDGEMENTS

This study received financial support by a PhD-scholarship of the "Volkswagenstiftung" to KJ and by the "Universität Bayern" to BB. Dr. Gustav Mamangkey is thanked for support in field work and with arranging permits. Thanks to Heidi Gensler for sectioning embedded egg masses.

Jörger KM, Brenzinger B, Schrödl M, Andrade SCS, Giribet G, Riesgo A (2013) Slug spicules: adding insights from histology and transcriptome analyses to the already complex picture of biomineralization in Gastropoda. World Congress of Malacology Azores, Portugal.

Slug spicules: adding insights from histology and transcriptome analyses to the already complex picture of biomineralization in Gastropoda

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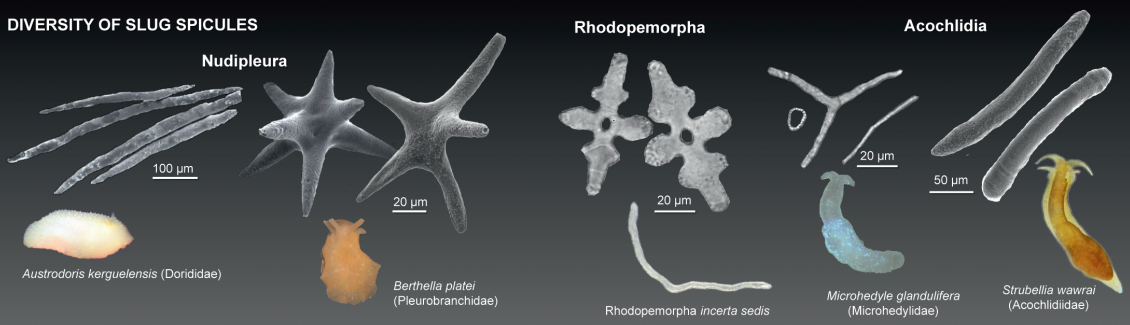
BACKGROUND

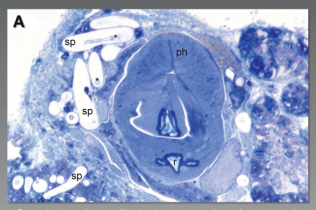
Molluscan shells are remarkable in their variety of forms and shapes, and appropriately receive the attention of biomineralization research. Beside shells, other biomineralization products occur among gastropods, although yet of unclear evolutionary origins. Among heterobranch gastropods, intra- or extracellular calcareous deposits are especially prominent in lineages of meiofaunal and nudipleuran slugs, a morphotype that evolved independently several times. It remains unclear, though, whether the formation of these calcareous deposits is homologous to shell formation at least on cellular or molecular level, or whether they develop independently and convergently.

DIVERSITY OF SLUG SPICULES

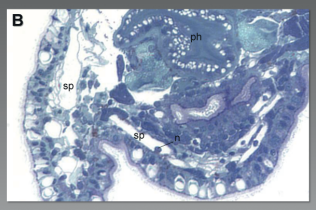


HISTOLOGY OF SPICULE CELLS

In the slug taxa (Acochlidia, Rhodopemorpha, Sacoglossa) studied in histological detail, spicules are formed intracellularly by one single spicule-bearing cell. Often some supposedly organic matrix (* in A) remains in the cells after decalcification. Notches or holes in spicules frequently correspond to the position of the nucleus of the spicule-bearing cell (see B).



Spicule-bearing cells (sp) next to the pharynx (ph) with radula (r) in *Strubellia wawrai*.

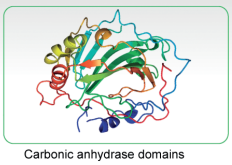


Spicule-bearing cell (sp) with nucleus (n) corresponding to a notch in the spicule of *Rhodope* sp.

FIRST TRANSCRIPTOME DATA

We generated and *de novo* assembled two adult transcriptomes for different species of Acochlidia (*Strubellia wawrai* and *Microhedyle glandulifera*), sequencing paired end (150bp) reads using the Illumina Genome Analyzer II. The transcriptomes were screened for a compiled list of proteins previously described as being involved in biomineralization in Mollusca (mainly Gastropoda and Bivalvia). Based on our data, we report the expression of some biomineralization genes in shell-less acochlidian slugs (see Table) with varying expression levels (in reads per kilo base per million RPKM). Special interest lies on proteins like *nacrein* and *perlustrin*, which were so far uniquely associated to shell deposition.

Strubellia wawrai - nacrein

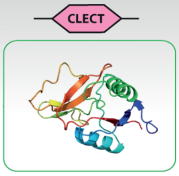


Carbonic anhydrase domains

		Strubellia wawrai			*Microhedyle glandulifera*		
protein name	function	presence	sequence length (bp)	RPKM	presence	sequence length (bp)	RPKM
BIOMINERALISATION PROTEINS							
nacrein	associated with aragonit	Yes	3606	13.2	Yes	290	5.3
dermatopontin	associated with aragonit	Yes*	2097	4.5	Yes	524	107.8
tyrosinase-like1	associated with calcite	Yes*	1121	300.4	Yes	209	0.1
perlucin	associated with aragonit	Yes	732	47.6	No	-	-
perlustrin	associated with aragonit	Yes	1264	13.7	No	-	-
CONTROL							
haemocyanin		Yes	495	257.0	Yes	1408	70.5

* tentative not fully confirmed

Strubellia wawrai - perlucin



C-type lectin or carbohydrate-recognition domain with carbonhydrate binding function

CONCLUSIONS + OUTLOOK

The distribution of spicules in the investigated taxa, and their intracellular nature and composition, do not support a homology with heterobranch shells on cellular level. They rather present independently evolved products for biomineralization, which highlights the complexity of biomineralization in Gastropoda. A putative homology between slug spicules and 'calcium-cells' of shell-bearing snails still needs to be investigated. To date, the lack of a broad comparative dataset on the genetic processes of shell deposition hinders sound interpretation of our transcriptomic data. Nevertheless, some putatively plesiomorphic 'shell-forming' genes – e.g., those involved in the formation of the larval shell – might be retained and reactivated later in the evolutionary history for the formation of spicules.

ACKNOWLEDGEMENTS

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Curriculum Vitae

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Language skills German (mother tongue), English (fluent), Spanish (fluent), French (basic)

Current position

2012-present Research assistant, Systematic Zoology, Prof. G. Haszprunar, Ludwig-Maximilians-University (LMU), Munich, Germany

Teaching

Basics in Zoology, LMU (winter term)
Animal diversity, LMU (summer term)
Malacology, LMU (winter term)
Marine biodiversity and systematics, LMU (field course, summer term)

Work experience

Private lecturer winter term 2005/2006 – winter tem 2007/2008
conceptual design and lecturing of the 'PROFIS' course (preparatory course in life sciences for foreign students), Department for International Studies, LMU and Deutschkursen e.V.

Student assistant involved in various research projects (sampling, data acquisition and data management) in the Departments for Pisces, Arthropoda and Mollusca, Bavarian State Collection of Zoology, Munich, Germany (2005-2006)

Higher Education

2008-2012 PhD thesis 'Tracing evolution: Molecular phylogeny of the Acochlidia (Heterobranchia, Gastropoda)', Bavarian State Collection of Zoology, Munich, Germany (funded by a PhD scholarship of the VW-Foundation), supervision Dr. M. Schrödl

2010 4-month research stay for the PhD thesis in the lab of Prof. G. Giribet, Museum for Comparative Zoology, Harvard University, USA

2003-2004 Study of tropical ecology at the University of Costa Rica (UCR), San José, Costa Rica (funded by a scholarship of the DAAD)

1999-2006 Diploma (with distinction) in Life Science, Ludwig-Maximilians-University, Munich, Germany. Major in Systematic Zoology

Grants and prizes

2008-2011	PhD scholarship of the Volkswagen Foundation, Initiative 'Evolutionary Biology'
2007-2010	Participant in the mentoring program of LMUexcellent for career support to highly qualified, emerging female academics
2010	Prize for best student talk at the World Congress for Malacology (Phuket, Thailand) awarded by Unitas Malacologica
2010	Scholarship of the Encyclopedia of Life to participate in the 'Meiofauna Diversity and Taxonomy' course, Smithsonian Institute, Panama
2003-2004	Scholarship of the DAAD for a study year abroad (Tropical Ecology in Costa Rica) within the ISAP-program of the University Ulm

Invited talks and workshop organization

2013	Invited scientific speaker of 'Evolutionsbiologisches Kolloquium' at Museum König, Bonn, Germany, October 2013
2013	Invited scientific speaker of the colloquium of the Institute for Ecology, Evolution and Diversity at the Goethe-University Frankfurt, Germany, January 2013
2012	Invited lecturer on meiofaunal molluscs at the workshop for 'Taxonomy and diversity of marine meiofauna', Centro de Biologia Marinha da USP, São Sebastião, Brasil, October 2012
2010	Organization of the 12. Annual Meeting of the 'Young Systematics' (GfBS) in Munich, Germany, August 2010
2010	Organization of the Young Systematics-Workshop on 3D-reconstructions at the Leibnitz-Rechenzentrum, Munich, Germany, August 2010

Expeditions (selection)

2012:	Participation in the deepsea benthos expedition SYSTCO II (SYSTem COupling in the deep Southern Ocean II) on board FS Polarstern
2010:	Participation in meiofauna workshops within the 'LAMPS'-Project (Latin American Meiofauna Phylogeography and Systematics) in Belize and Panama
2007:	Participation in the scientific diving expedition 'Huinay IV' in the fjords of Northern Patagonia (Chile)

Munich, December 2013

Katharina Jörger

Publications (peer-reviewed)

Brenzinger B, Neusser TP, **Jörger KM**, Schrödl M (2011) Integrating 3D-microanatomy and molecules: natural history of the Pacific freshwater slug *Strubellia* Odhner, 1937 (Heterobranchia, Acochlidia, Acochlidiidae). *Journal of Molluscan Studies* 77: 351-374.

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Eidesstattliche Versicherung und Erklärung

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

München, den 12.12.2013 .....

(Katharina Jörger)

Erklärung

Hiermit erkläre ich, dass die Dissertation **nicht** ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich **nicht** anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

München, den 12.12.2013 .....

(Katharina Jörger)