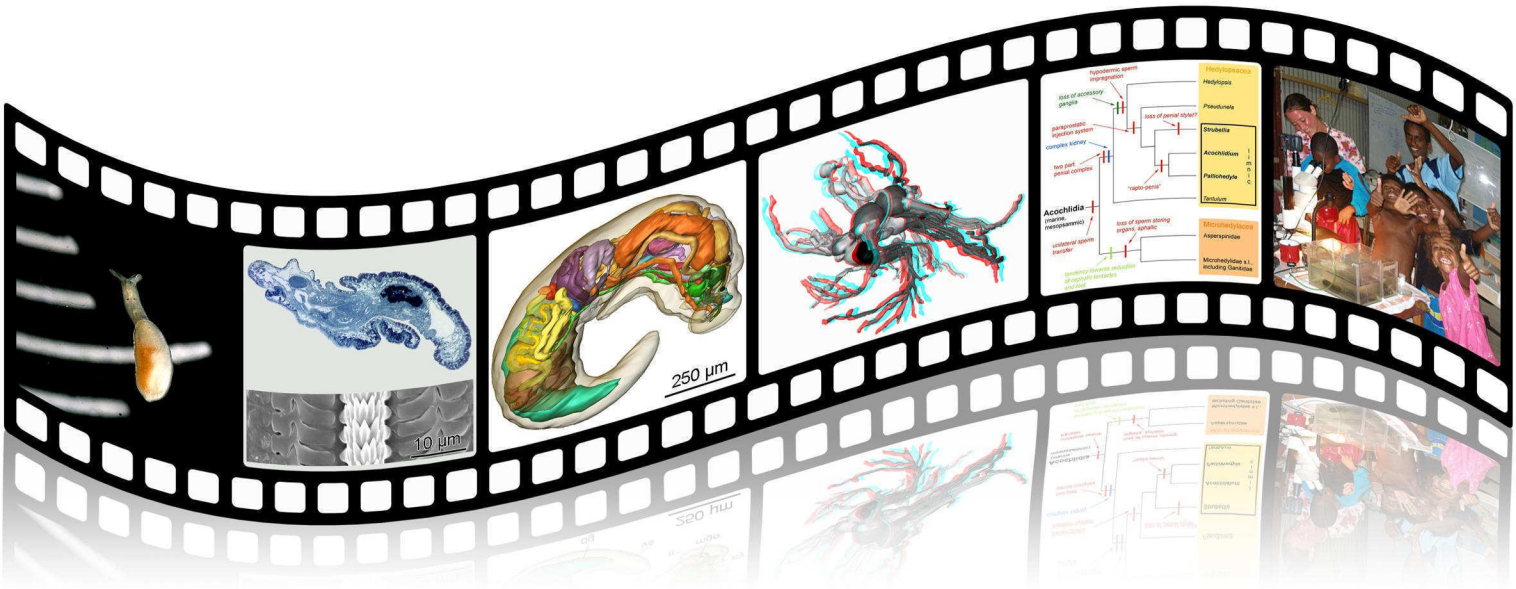
Systematics and evolution of the Acochlidia (Gastropoda, Euthyneura)
-
a microanatomical approach by means of computer-based 3D reconstruction using Amira®



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To
my parents Erzsi & Rudi
who always supported me when my dreams brought me far from home
and who showed me the beauty of nature and life

and

to
my beloved family Ted, Marisol & Eliel
for their love and patience

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1 SUMMARY

Only a small fraction of the estimated species diversity on Earth already has been discovered, and expected high extinction rates force biologists to rapid surveys. Molecular barcoding techniques meet such goals, but taken alone they can hardly connect genetic discoveries with the large morphology-based body of taxonomic knowledge that accumulated during centuries. Also, the study of organismic evolution requires reliable information on phenotypes. Morphological and biological knowledge on formally described species can be, however, very heterogeneous regarding both quality and quantity. Especially problematic are meiofaunal taxa – biodiversity generally is poorly explored, and species are small, hard to collect, externally quite uniform and difficult to distinguish by means of traditional taxonomic techniques. Old species descriptions often are fragmentary and information may or may not be reliable. Novel microanatomical imaging techniques raised the hope to combine the rapid examinations with the obligatory accuracy and desired comprehensiveness of structural information obtainable.

Among the most successful interstitial gastropod taxa are the Acochlidia, combining extremely high morphological and biological diversity with modest species diversity. The state of research at the beginning of my PhD thesis considered the Acochlidia as poorly known, enigmatic and morphologically and biologically aberrant Opisthobranchia, comprising only 27 valid species. Most of the acochlidian species are marine mesopsammic and distributed along the coasts of the world's oceans, but some species succeeded to invade freshwater systems on tropical islands. Uniquely among the otherwise hermaphroditic euthyneurans, some acochlidians have separate sexes. Previous sampling efforts were biased to European waters and a few other places that had been visited by experts. Original descriptions of the acochlidian species were often limited to the external morphology, the structure of calcareous spicules and the examination of the radula by light microscopy; furthermore, some anatomical data were traditionally obtained from gross-morphological dissection or from paraffin-based histology. Inner acochlidian classification was controversial and neither morphology-based nor molecular phylogenetic studies resolved the origin of this traditional "order" among euthyneuran heterobranch gastropods.

In a case study for Mollusca, and for the first time for heterobranch gastropods, I comparatively explored the microanatomy of a representative sampling of known acochlidian taxonomic diversity applying computer-aided 3D reconstructions with

Amira® based on serial semi-thin histological sections. My dissertation aimed (1) to revise the morphology and taxonomy of acochlidians, including the most dubiously and incompletely described species, (2) to generate detailed microanatomical data sets for comparative purposes, (3) to reconstruct global acochlidian phylogeny and major traits of their evolution, and (4) to explore the power and the limits of modern microanatomy against traditional taxonomy and molecular approaches, and to develop integrative approaches.

Original type material was traced in museums and institutions according to the literature and loaned for re-examination whenever possible. Most of the acochlidian species were re-collected at the type localities. Seven acochlidian species covering seven of eight families were re-examined in full detail; other species were studied to the level necessary and possible considering time constraints; additionally five species were introduced new to science. The microanatomical part of my dissertation clearly demonstrates that traditional acochlidian taxonomy did not provide sufficiently detailed and reliable anatomical information. In contrast, computer-based 3D reconstructions with the software Amira® are an efficient, powerful tool for microanatomy, providing a wealth of new data on all major organ systems of the Acochlidia. Transforming specimens into serial histological sections is "invasive", but generates vouchers that carry testable information. Semithin-sectioning (1-2 μm) and staining as applied herein provide resolution adequate to trace relevant organs, ducts and tissues; limits of this method refer to quantitative detection of fine nerves. The process of preparing complete 3D models is time consuming, but greatly supports accurateness of finding and identifying structures and includes several steps of internal quality control. 3D models, especially when interactive, are attractive and instructive, comprise verifiable high-quality data, and revealed considerable amounts of erroneous data within original species descriptions. Former outliers – i.e. apparently aberrant and enigmatic species - fit well into the pattern of known acochlidian species after the correction of the original data. 3D modeling from serial sections as applied herein is discussed as the best currently available method for exploring complex soft part microanatomy in small invertebrate specimens.

Using the verified and supplemented morphological data, more than 100 morphological characters were defined and coded for all 27 acochlidian species considered valid at that time, and 11 euthyneuran outgroups. A cladistic analysis with PAUP recovered monophyletic Acochlidia originating from an unresolved basal opisthobranch level. The Acochlidia split into the Hedylopsacea (*Tantulum* (*Hedylopsis* (*Pseudunela* (*Strubellia*

(‘*Acochlidium*’, ‘*Palliohedyle*’))))) and Microhedylacea (*Asperspina* (*Pontohedyle*, ‘*Parhedyle*’, ‘*Microhedyle*’, (*Ganitus*, *Paraganitus*))). The formerly enigmatic Ganitidae, resembling sacoglossan opisthobranchs by having dagger-like rhachidian radular teeth, were recovered as highly derived microhedylids. This topology is largely well-supported, robust to modifications of outgroup taxon sampling, and in principles was supported by a recent multi-locus molecular analysis. In addition, molecular analyses revealed the formerly enigmatic, amphibious Aitengidae also clustering within hedylopsacean Acochlidia. Although my phylogenetic hypothesis is not considered definitive, the paraphyly of some of the traditionally recognized family level taxa induced a preliminary reclassification of the Acochlidia.

Rarely among invertebrates, morphology-based and molecular acochlidian topologies are compatible, and thus may closely reflect natural relationships. Major traits of the acochlidian evolutionary history were reconstructed tracing character state changes on the tree. The previous hypothesis of a general regression of morphological complexity in the Acochlidia applies only for microhedylacean species. Within Microhedylacea, we confirmed a tendency towards successive reductions, particularly in the reproductive system. Species are aphallic, sperm transfer occurs by spermatophores and dermal fertilisation and the secondary gonochorism evolved once in the ancestor of the Microhedylidae. In contrast, already basal hedylopsacean species show a complex excretory system adapted to a freshwater influenced environment. An evolutionary trait from a simple unarmed copulatory system towards complex hypodermal injection systems was recognised culminating in a large, trap-like spiny rapto-penis of several limnic Acochlidiidae.

In spite of a high level of convergence involved, precise microanatomical data sets on a vast (yet incomplete) ingroup sampling thus allowed reconstructing a novel, plausible and detailed hypothesis on acochlidian phylogeny and evolution. This approach may have considerable potential also in other groups with similarly small and rare members that are elusive to molecular studies. Limits of morphology-based phylogeny concern any subgroups with just limited information available, old and possibly rapid diversifications, such as the origin of Acochlidia among Euthyneura, and relatively recent subgroups with little phenotypical differences fixed. We show that traditional taxonomy fails to differentiate some genetically clearly distinct lineages. In *Pseudunela*, sophisticated microanatomy alone cannot reliably delimitate all of the evolutionary lineages, but may reveal diagnosable differences among pseudocryptic species once they have been delimited by molecular analyses. Integrative taxonomy combining

modern microanatomical data on acochlidians with molecular analyses thus is superior to individual approaches.

With all key species revised in microanatomical and testable detail, and many additional species compared to such standard, now the Acochlidia probably range among the best described heterobranch groups. There is, however, still a critical gap of knowledge regarding biological observations and ontogenetic stages. Future work also should focus on resolving the exact origin of Acochlidia among Panpulmonata and on generating comparative anatomical data from potential sister groups. In spite of the urgency for speed facing the biodiversity crisis, my dissertation showed the essential need for revisory work on acochlidians, and this may be true also for other poorly known micromolluscs. Integrative 3D microanatomical and molecular approaches as exemplified herein are efficient, and thus suitable to explore the diversity and evolution of neglected micromolluscs within overall reasonable time scales.

"Learn from yesterday, live for today, hope for tomorrow.

The important thing is to not stop questioning."

Albert Einstein

2 INTRODUCTION

2.1 General introduction

At the beginning of the 21th century, the biodiversity science, defined by CRACRAFT (1995) as "those disciplines that contribute to the conservation and sustainable use of the world`s species, primarily through a scientific understanding of whole-organism biology", is probably among the most fascinating, but also challenging disciplines of biology. Realistic estimations of actual diversity of eukaryots range from 4 to 15 million species (e.g. DIRZO & RAVEN 2003; MAY 2011; MORA *et al.* 2011; STORK 1997) with only 1.2 million (REUTERS 2009) to 1.8 million (REAKA-KUDLA 1997) described valid species. Recent expeditions exploring the marine biodiversity, e.g. in New Caledonia (BOUCHET *et al.* 2002), Panglao (BOUCHET 2006), Sulawesi (BURGHARDT *et al.* 2006) or Vanuatu (BOUCHET *et al.* 2011), make clear that new species are discovered day-to-day and we are far from exploring and understanding the complete biodiversity. In the last decades acceleration in irreversible, global biodiversity loss was recognised (e.g. CRACRAFT 1995; PIMM & RAVEN 2000; RAVEN 2002; WHEELER & CRACRAFT 1997; WILSON 1997) including, amongst others, species extinctions. But while species are currently disappearing at an extinction rate higher than expected from fossil records (BARNOSKY *et al.* 2011), there is a mismatch between the discovery of new biodiversity and the capacity to describe them, known as the taxonomic impediment (e.g. AGNARSSON & KUNTNER 2007; DE CARVALHO *et al.* 2007, 2008; EVENHUIS 2007; PADIAL *et al.* 2010; RODMAN 2007; RODMAN & CODY 2003). WHEELER (2004) pointed out that the present generation is the first to fully become aware of the menaces facing millions of species and may be the last one getting the opportunity to "explore, describe and classify life on Earth". The question arises which is the appropriate method for a successful outcome?

A speedy response to the biodiversity crisis may consist in molecular barcoding (HEBERT *et al.* 2003a, b), which has been proposed as an accurate and rapid species identification tool (MITCHELL 2011; TELETCHEA 2010). However, taxonomic expertise is fundamental for building and validating a DNA barcode reference library (MITCHELL 2011). Barcoding also may contribute to species discovery, but barcodes alone cannot reliably delineate species (e.g. JINBO *et al.* 2011). Barcoding or other types of DNA taxonomy or molecular systematics should be combined and crossvalidated using morphological and biological data (e.g. GIRIBET 2010). Morphology thus is crucial to propose stable hypotheses on species boundaries (e.g. WILL & RUBINOFF 2004) and remains essential to understand biological diversity. Any new species description - no matter whether morphological or molecular - is impracticable without the evaluation of yet valid species. Obviously, such a revisory process depends on accurate and reliable data. In particular, inadequate descriptions may adversely affect reconstructing the phylogeny and evolution of higher taxa (e.g. MARTYNOV & SCHRÖDL 2011). Even if the biodiversity crisis requires a rapid response, scientists should make the acquisition of accurate primary data a top priority as they represent the heart of good taxonomy and the basis for any meaningful phylogenetic and evolutionary research.

Several recent studies highlighted the important role of morphology in life sciences (e.g. SCHOLTZ 2010; WILL & RUBINOFF 2004) and promoted the "renaissance for evolutionary morphology" (BUDD & OLSSON 2007). However, in the "molecular millenium", the efficacy of morphology for phylogeny reconstruction was doubted in several studies (JENNER 2004). According to SCOTLAND *et al.* (2003), morphology (1) cannot resolve phylogeny at any taxonomic level, and (2) should be mainly limited to mapping selected morphological characters onto molecular phylogenetic trees. The first is not necessarily the case; carefully checked and comprehensive morphological data sets may reveal robust and plausible phylogenetic hypotheses even on "difficult", progenetic groups (see MARTYNOV & SCHRÖDL 2011). The second requires the same quantity and quality of morphological information, plus sufficient molecular information to build a densely sampled and reliable tree. Except for our case studies on acochlidians, with global sampling efforts and combining morphology-based (herein) and molecular (parallel dissertation project of K. Jörger) approaches, this is not given for most other small-sized marine invertebrate groups.

Reliable phylogenetic hypotheses are essential prerequisites for testing hypotheses about biogeography and evolution (REID 1989), but are not yet available for many marine invertebrate taxa (MARTYNOV & SCHRÖDL 2011). Approximately 90 % of

molluscan species collected in New Caledonia were categorised as micromolluscs including many yet undescribed species (BOUCHET *et al.* 2002). But how can we achieve comprehensive taxonomic knowledge on tiny and elusive species, and how to treat small gastropods that lack the shell, i.e. the most broadly and instantly (using scanning electron microscopy (SEM)) usable character complex? Novel imaging techniques, such as computer-based 3D reconstructions, confocal laser scanning microscopy (CLSM), micro-computed tomography (μCT) or magnetic resonance imaging (MRI), raised the hope to combine economically rapidness with the obligatory accuracy and desired comprehensiveness. Such kind of "golden bullet" is needed for efficient exploration of the internal anatomy, taxonomy, and biodiversity of small marine species e.g. all those inhabiting the mesopsammon.

2.2 The marine interstitial

The marine interstitial environment, called the mesopsammon, belongs to one of the most ancient ecosystems of our planet (RUNDELL & LEANDER 2010). It harbours an amazing diversity of coexisting taxa, together named the meiofauna. By the mid 19th and at the beginning of the 20th century, scientists discovered the water-filled interstitial space between the grains of coastal marine sands as a habitat for organisms (GIARD 1904; KOWALEVSKY 1901; LOVÉN 1844). Considerable progress has been made in different areas of meiofaunal research (e.g. AL-RASHEID 2001; AX 1969; DELAMARE-DEBOUTTEVILLE 1960; GIERE 2009; GOLEMANSKY & TODOROV 2004; MCINTYRE 1969; NORENBURG 1988; REMANE 1933; STOCK & VONK 1992; SWEDMARK 1964). However, our knowledge of meiofaunal biodiversity, ecology and evolution is still limited and RUNDELL & LEANDER (2010) emphasised that the exploration of the meiofauna "remains among the most challenging, the most neglected and potentially the most enlightening frontiers of discovery in biology".

The interstitial milieu is characterised by extreme ecological conditions, such as faint light and limited amount of space (SWEDMARK 1968a), which restricts the body size and limits the meiofauna to minute, vermiform organisms suited to a lacunar environment (SWEDMARK 1964). Currents, wind and wave action transform the interstitial biotope by permanent restratification of the surface layer of the sand (SWEDMARK 1964). The continuous rearrangement of the particles contributes to a dynamic environment and avoids the colonisation by plants (SWEDMARK 1968a). Furthermore, the living conditions in the intertidal zone or shallow water are complicated by diverse physical factors: the temperature is reliant on the daytimes, seasons and the rhythm of tides and, thus,

fluctuates significantly in the surface sand layers; on the other hand, the salinity increases by evaporation and otherwise decreases by rainfall or by the inflow of coastal freshwater (SWEDMARK 1964). Organisms, which successfully colonise the marine interstitial, therefore often develop special morphological and biological adaptations: body sizes are typically very small ranging from 0.5 mm to approx. 3 mm. Flat and broad or vermiform elongated body shapes are commonly favoured. The body wall is often reinforced by subepidermal spicules or cuticle for mechanical protection. Habitually, members of the meiofauna have a good contractibility and a high adhesive capability by epidermal glands to avoid being washed away (SWEDMARK 1964, 1968a). Consequently, the study of meiofaunal taxa is challenging – species are small, hard to collect, difficult to distinguish externally and to describe by means of traditional techniques.

Nearly all major metazoan taxa are represented in the marine meiofauna, e.g. Cnidaria, Echinodermata, Platyhelminthes, Nemertea, Ectoprocta, Entoprocta, Gnathostomulida, Rotifera, Mollusca, Annelida, Priapulida, Loricifera, Kinorhyncha and Crustacea (e.g. BOTOSANEANU 1986; HIGGINS & THIEL 1988; RUNDELL & LEANDER 2010; SWEDMARK 1964, 1968a). While many molluscan taxa occur in the mesopsammon during early ontogenetic stages, only some are adapted to the extreme environment of the mesopsammon as adults: besides e.g. a few Solenogastres (see e.g. GARCÍA-ÁLVAREZ *et al.* 2000; VON SALVINI-PLAWEN 1988, 2008), there exist mainly members of the Gastropoda (ARNAUD *et al.* 1986). Interstitial gastropod taxa comprise amongst others the prosobranch Caecidae, and the heterobranch Cephalaspidea (some Philinidae; Philinoglossidae), Sacoglossa (*Platyhedyle*), Nudibranchia (*Embletonia* and *Pseudovermis*), Rhodopemorpha (*Rhodope* and *Helminthope*) and Acochlidia. The most successful interstitial gastropod taxa are the euthyneuran Acochlidia combining extremely high morphological and biological diversity with modest species diversity.

2.3 Historical survey of the Acochlidia

According to the state of research at the beginning of my PhD thesis, the Acochlidia were considered as "fascinating" (DAYRAT & TILLIER 2003), i.e. poorly known, enigmatic and morphologically and biologically extremely aberrant Opisthobranchia, comprising only 27 valid species (WAWRA 1987). The shell-less Acochlidia are characterised by a worm-like, symmetric body shape and the division into a head-foot complex and an elongated visceral sac in which the head-foot complex can be (at least partly) retracted (e.g. KOWALEVSKY 1901; SWEDMARK 1968a; WAWRA 1987). Most of the acochlidian

species have one or two pairs of cephalic tentacles. Mosaic-like reductions concern the rhinophores, the eyes, the foot and the pigmentation (e.g. CHALLIS 1970; KOWALEVSKY 1901; MARCUS 1953; SWEDMARK 1968a). Most Acochlidia species are marine mesopsammic inhabiting coastal sands worldwide and, thus, they form part of the interstitial opisthobranch assemblages. The latter are subject to seasonal variations and comprise rheophilous species most of them living in clean and oxygenated waters. A long-term study in the Mediterranean Sea (e.g. Poizat 1983, 1984) demonstrated that they are particularly sensitive to any clogging of their habitat, either by man-made coastal pollution or by decrease of marine hydrodynamism, resulting in an impoverishment or even disappearing of the opisthobranch species. Therefore, they have been proposed as biological indicator organisms in the past (POIZAT 1985). However, while the interstitial acochlidian fauna along the European coast has been more extensively sampled (HERTLING 1930; MARCUS & MARCUS 1954, 1955; ODHNER 1937, 1952; POIZAT 1980, 1981, 1983, 1984, 1986, 1991; SWEDMARK 1968b; WAWRA 1974, 1978, 1989; WESTHEIDE & WAWRA 1974), the interstitial acochlidian species of North America (DOE 1974) and of tropical waters (Challis 1968, 1970; Kirsteuer 1973; Marcus 1953; Wawra 1988a) were almost unexplored. Uniquely among the otherwise marine Opisthobranchia, some acochlidian species succeeded to colonise freshwater systems: on the one hand the small (2 mm) *Tantulum elegans* Rankin, 1979 inhabiting muddy interstices of a mountain spring swamp on the Caribbean St. Vincent Island (RANKIN 1979). On the other hand there is a radiation of several large-sized species of up to 3.5 cm living benthically in coastal rivers on different Indo-Pacific Islands (Bergh 1895; Bücking 1933; Haynes & Kenchington 1991; Küthe 1935; Wawra 1979a, 1980, 1988b). Acochlidian species have a

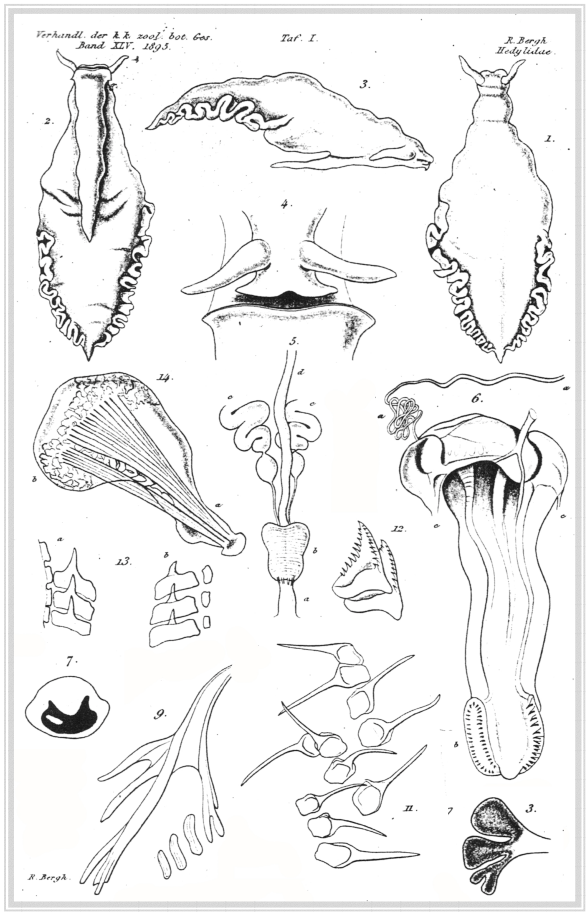

Figure 1 – Early anatomical description of an acochlidian species: *Hedyle weberi* (modified after BERGH (1895)).

variety of special reproductive features (MORSE 1994; SWEDMARK 1968a; WAWRA 1992): the sperm transfer can be realised by hypodermal injection via a hollow penial stylet, by spermatophores or by copulation. While euthyneuran gastropods generally possess male copulatory organs (DAYRAT & TILLIER 2003), some acochlidian species lack any. Of the latter, several species are gonochoristic, i.e. they have separate sexes - while most of the acochlidian species are hermaphrodites as usual for euthyneurans (e.g. HELLER 1993).

Early original descriptions of the acochlidian species were often limited to the external morphology, the structure of spicules and the examination of the radula by light microscopy. Furthermore, descriptions were traditionally based on morphological data obtained by classical dissection (e.g. BAYER & FEHLMANN 1960), squeezed whole mounts (KIRSTEUER 1973), whole mount or crush preparation of the radula (e.g. DOE 1974; WAWRA 1980, 1988b) and/or the examination of histological sections of up to 10 µm thickness (e.g. BÜCKING 1933; CHALLIS 1968, 1970; HAYNES & KENCHINGTON 1991; KÜTHE 1935; MARCUS 1953; MORSE 1976; ODHNER 1937; RANKIN 1979; WAWRA 1979a, 1980, 1988b), and are not always reliable.

The inner-acochlidian phylogeny remained unresolved, resulting in a controversial discussion of the acochlidian classification. RANKIN (1979) included in her description of *Tantulum elegans* a revision of the Acochlidia culminating in a nomenclatorial inflation: a total of only 25 nominal acochlidian species was assigned to five new suborders with two new superfamilies, 13 families (10 of them new) and 19 genera (11 of them new). Her morphological revision was based mainly on literature and failed due to erroneous interpretations of the original data. STAROBOGATOV (1983) created an own genus *Minicheviella* and a monotypic family Minicheviellidae for the arctic *Asperspina murmanica* (Kudinskaya & Minichev, 1978) based on the description of a, for acochlidian species unusual, well-developed mantle cavity. The pioneering work of the Austrian naturalist Erhard Wawra contributed considerably to the knowledge of the biology and systematics of the Acochlidia (for his list of publications see PAGET 1995). WAWRA (1987) introduced a new, much simpler classification based on a first phylogenetic model, which was first incorporated in the revision of interstitial Gastropoda by ARNAUD *et al*. (1986). The order Acochlidia was subdivided in the two superfamilies Hedylopsacea (with Hedylopsidae, Acochlidiidae, Tantulidae) and Microhedylacea (with Microhedylidae, Asperspinidae, Ganitidae). However, SOMMERFELDT & SCHRÖDL (2005) argued that at least the Hedylopsacea and Hedylopsidae *sensu* Wawra may be paraphyletic at best. The latest classification by BOUCHET & ROCROI (2005) is based on

different references and the authors followed tentativly STAROBOGATOV (1983). In summary, up to now several contradictory classification systems have been used at the same time.

Several hypotheses existed concerning the systematic position of the Acochlidia. BERGH (1895) considered the Acochlidia as cladobranch Nudibranchia due to the more or less branched digestive gland of some limnic species; but all marine acochlidian species possess a sac-like, holohepatic digestive gland. ODHNER (1937) positioned the Acochlidia in an own order because of their prepharyngeal central nervous system (CNS). ZILCH`S (1959) assumption of a close relationship to the Diaphanidae (Cephalaspidea s.l.) was accepted by VON SALVINI-PLAWEN (1973) due to similarities in the radula structure and the genital system. Hypotheses considering the Acochlidia to be related to the sacoglossan *Platyhedyle* (see RANKIN 1979; VON SALVINI-PLAWEN 1973) were mainly based on misinterpretations of central nervous and reproductive features of *Platyhedyle* (see WAWRA 1987, 1988c, 1991). WAWRA (1979b) showed that *Platyhedyle* has a sacoglossan ascus and therefore belongs to the Sacoglossa. JENSEN (1996) proposed that *Platyhedyle* is the sister group of *Gascoignella aprica* Jensen, 1985, a benthic elysioid sacoglossan that feeds on intertidal algae. RÜCKERT *et al.* (2006) confirmed close morphological similarities between *Platyhedyle* and *Gascoignella*; a unique muscular septum dividing the digestive gland medially into two rami was considered as a synapomorphy of both genera (RÜCKERT *et al.* 2008). GOSLINER (1994) assumed the monophyly of Acochlidia, Diaphanidae and Sacoglossa due to the similar radula structure; according to him, the Acochlidia were not monophyletic, because he considered the Ganitidae being Sacoglossa. However, JENSEN (1996) excluded the sacoglossan affinity of Ganitidae. In the morphological cladistic analysis of VON SALVINI-PLAWEN & STEINER (1996) the Acochlidia were regarded as sister group to the enigmatic, small-sized, and, in part, interstitial Rhodopemorpha (Rhodopidae and *Helminthope*) due to the presence of calcareous spicules and a monaulic genital system in both taxa. However, spicules are also present in the Nudipleura and a monaulic genital system was regarded as plesiomorphic within the Opisthobranchia (GOSLINER 1994; WÄGELE & WILLAN 2000). Lately, SOMMERFELDT & SCHRÖDL (2005) attempted to reconstruct the phylogeny of Acochlidia using apomorphy-based systematics and concluded, amongst others, that the Acochlidia is a monophyletic group originating from a basal opisthobranch level.

The traditionally assumed monophyly of Acochlidia was confirmed recently by cladistic studies on euthyneuran and opisthobranch phylogeny in which acochlidian species

were included, by both using morphological characters (DAYRAT & TILLIER 2002; WÄGELE & KLUSSMANN-KOLB 2005) and molecular markers (KLUSSMANN-KOLB *et al.* 2008; VONNEMANN *et al.* 2005). DAYRAT & TILLIER (2002) could not clarify the position of the Acochlidia within the Euthyneura, possibly due to the quite poor taxon sampling including only one *Hedylopsis* species. The cladistic morphological and histological analysis of opisthobranchs by WÄGELE & KLUSSMANN-KOLB (2005) showed acochlidians (represented by *Hedylopsis spiculifera*, *Microhedyle glandulifera*, *Pontohedyle milaschewitchii* (all Kowalevsky, 1901)) nested within a clade composed of similarly enigmatic and poorly explored taxa with small-sized members, such as Runcinidae, tiny Rhodopidae and mesopsammic Philinoglossidae, all forming basal opisthobranch offshoots resulting in a polytomy. However, as in the case of tiny Rhodopemorpha, such an assemblage might easily result from convergent organ reductions and adaptations to extreme interstitial environments impeding the discovery of the acochlidian origin based on morphological characters only. According to molecular analyses by VONNEMANN *et al.* (2005), Acochlidia (represented by *H. spiculifera*, *M. glandulifera*, *P. milaschewitchii*) are monophyletic, but depending of the gene sequences used, their position varies from being members of a clade of Cephalaspidea and Anaspidea (18S rRNA genes) to being a basal euthyneuran group (28S rRNA genes). In their combined analysis the Acochlidia were shown to be basal opisthobranchs in proximity to pulmonates, but no resolution was obtained. KLUSSMANN-KOLB *et al.*'s (2008) molecular analyses with multiple markers were challenging and revealed the Acochlidia (represented by *H. spiculifera*, *M. glandulifera* and *P. milaschewitchii*) forming part of a clade composed of opisthobranch Sacoglossa, pulmonates, and Pyramidelloidea. These results questioned the traditionally acknowledged monophyly of Opisthobranchia and Pulmonata (WÄGELE *et al.* 2008), but see HASZPRUNAR (1985b), and were recently supported by several molecular studies based on multi-locus markers (JÖRGER *et al.* 2010a; SCHRÖDL *et al.* 2011a). Lately, two phylogenomic approaches revealing the molluscan phylogeny (KOCOT *et al.* 2011; SMITH *et al.* 2011) contradicted the monophyly of the Opisthobranchia and Pulmonata, but are compatible with the phylogenetic hypothesis proposed by JÖRGER *et al.* (2010a). All modern analyses using nuclear rather than mitochondrial data (see SCHRÖDL *et al.* 2011b) thus support the backbone topology of JÖRGER *et al.* (2010a), validating their fundamental reclassification of euthyneuran gastropods. Acochlidia, rather than opisthobranchs, now are integrated in the Panpulmonata (together with Siphonarioidea, Sacoglossa, Glacidorboidea, Amphiboloidea, Pyramidelloidea, Hygrophila, and Eupulmonata).

MARTYNOV & SCHRÖDL (2011) emphasised that molecular approaches up to now are unsuccessful in providing 'all-species approaches' on the very tiny, hidden and often rare mesopsammic taxa, basically due to the restricted availability of properly fixed material suitable for sequence analyses. Probably this is the reason why (1) the above mentioned studies on opisthobranch and euthyneuran phylogenies (published before or at the beginning of my dissertation) suffered either from a limited taxon sampling included, or from using a generalised bauplan that does not automatically reflect the basal conditions within the heterogeneous Acochlidia; and (2) several recent molecular studies on opisthobranch and euthyneuran phylogeny did not even include Acochlidia (e.g. DAYRAT *et al.* 2001, 2011; GRANDE *et al.* 2004a, b; HOLZNAGEL *et al.* 2010; MEDINA & WALSH 2000; WÄGELE *et al.* 2003; WOLLSCHEID-LENGELING *et al.* 2001). Until molecular studies count with a reasonable acochlidian taxon sampling, the inner-acochlidian phylogeny can be resolved by a cladistic analysis based on morphological data and applying an 'all-species approach', once the original data were revised, corrected and/or supplemented. Reliable phylogenetic trees are the prerequisite to reconstruct the evolution (MARTYNOV & SCHRÖDL 2011) and to understand the species morphological and biological diversity.

In summary, the state of the art at the beginning of my PhD thesis showed a biased acochlidian taxon sampling with main focus around the Mediterranean Sea and nearly unexplored in tropic waters in combination with numerous incomplete and hardly reliable species descriptions. Consequently, a deficient knowledge of the marine and limnic acochlidian species diversity, their morphology, phylogeny and evolution had to be assumed.

2.4 Material and methods

At the beginning of my research I created a detailed list including all type material (see Appendix: Table 1) supposedly stored in museums or institutions according to the original literature. The research turned out that original type material for re-examination was hardly available, especially for the tiny, marine mesopsammic species. In several cases no type material at all was deposited of some species or it has been lost during the years and/or is untraceable until today. For example, holotypes/paratypes of *Pontohedyle verrucosa* (Challis, 1970), *Pseudunela cornuta* (Challis, 1970) und *Paraganitus ellynnae* Challis, 1968 should be deposited in the Natural History Museum, London. But the material is not present and there is no evidence that it ever arrived. Further paratypes of these three species should be deposited in the Museum of New Zealand Te

Papa Tongarewa and of *P. ellynnae* in Bernice Bishop Museum, Honolulu, Hawaii - again no material is present. Additionally, the type material of the marine species *Parhedyle tyrtowii* (Kowalevsky, 1900), *Asperspina brambelli* and *A. loricata* (both Swedmark, 1968), *Microhedyle glandulifera*, *Microhedyle odhneri* Marcus & Marcus, 1955, *Pontohedyle milaschewitchii* and of the limnic species *Acochlidium weberi* (Bergh, 1895) is not traceable. At the final stage of my PhD thesis I completed the Table 1 now including information on the type material of all acochlidian species described formally up to now.

Some (type) material could be loaned for re-examination from different museums (see Appendix: Table 2). But very soon it became evident that most acochlidian species, particularly the tiny marine ones, had to be recollected at the type localities, due to the very limited material available at the museums which is suitable for a detailed anatomical examination and 3D reconstruction. Most of the 27 valid acochlidian species could be recollected at the type localities during the last years by the workgroup of Michael Schrödl (ZSM) or obtained by collaborators. Additionally, a lot of supposedly new, yet undescribed acochlidian species were found together with the valid species at their type localities or during expeditions to other localities worldwide. A list of collection localities with acochlidian species found is given in Table 3 (see Appendix). An overview of all section series prepared and examined in the present dissertation including sampling localities and museum numbers is given in Table 4 (see Appendix). The acochlidian species were examined by a multimodal approach including, amongst others, 3D reconstructions based on histological sections using Amira® software, SEM, transmission electron microscopy (TEM) and phylogenetic analyses. For a detailed explanation of the different methods applied refer to the material and methods sections in the individual publications (see Chapter 3).

2.5 Aims of the dissertation

The aims of my dissertation were (1) revising the morphology and taxonomy of representatives of all major acochlidian subtaxa, including known and newly described species, (2) generating detailed microanatomical data sets for comparative purposes, (3) reconstructing global acochlidian phylogeny based on (at least partly) reliable and detailed morphological data, (4) reconstructing major traits of acochlidian evolution and (5) exploring the power and the limits of Amira®-based microanatomy against traditional taxonomy and molecular approaches, and developing integrative

approaches. The latter became possible by collaborative work with Katharina Jörger who used a molecular approach in parallel to my morphology-based one.

The taxonomic focus of my dissertation was on the Hedylopsacea, but also included members of the Asperspinidae and Microhedylidae s.l.. The acquisition of detailed morphological and histological data of representatives of major acochlidian subtaxa was expected to be of paramount importance. Old literature data should be corrected and supplemented and the morphological diversity determined. For that reason, acochlidian key species with most dubious or incomplete original descriptions had to be re-examined by means of modern microanatomy. Novel 3D reconstructions achieved by using Amira® software were recently shown (in the course of my diploma thesis) to be an efficient tool for describing morphological structures in Acochlidia (NEUSSER *et al.* 2006) and seemed to be promising to get accurate and comprehensive (micro)anatomical data. Exploring the potential of using high-quality data and all valid acochlidian species for phylogenetic purposes was both timely and viable. Examples from morphological and integrative approaches are given, trying to resolve some of the most interesting aspects of the acochlidian evolution, such as the invasion into the interstitial and freshwater systems, the evolution of asymmetric radulae, complex excretory systems and the wealth of morphological aberrant reproductive features.

3 PROJECTS AND RESULTS

The following publications are not arranged chronologically, but according to topics. First there are anatomical studies on Microhedylacea (3.1-3.4) and Hedylopsacea (3.5-3.9), followed by publications dealing with possible character sets for cladistic analysis (3.10), and the phylogeny and evolution of the Acochlidia (3.11). Finally publications with integrative approaches (3.12-3.14) are included.

3.1 Neusser TP, Martynov AV & Schrödl M 2009. **Heart-less and primitive? 3D-reconstruction of the polar acochlidian gastropod *Asperspina murmanica*.** *Acta Zoologica* **90**(3): 228-245.

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Heartless and primitive? 3D reconstruction of the polar acochlidian gastropod *Asperspina murmanica*

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Abstract

Neusser, T. P., Martynov, A. V. and Schrödl, M. 2009. Heartless and primitive? 3D reconstruction of the polar acochlidian gastropod *Asperspina murmanica*. — *Acta Zoologica* (Stockholm) **90**: 228–245

This study re-examines in detail the microanatomy of the Arctic opisthobranch *Asperspina murmanica*, the only acochlidian that was described as retaining a well-developed mantle cavity, and evaluates its supposedly basal position within the Acochlidia. Several specimens were recollected at the type locality in Russia. Spicules and radulae were studied by scanning electron microscopy. Semithin sections were prepared and a computer-based three-dimensional reconstruction of all major organ systems was made using AMIRA software. Our results show significant differences from the original description, e.g. the nervous system shows paired rhinophoral and gastro-oesophageal ganglia and large aggregations of precerebral accessory ganglia, whereas the presence of a postulated posterior genital ganglion can be excluded; the radula is asymmetric; the circulatory system includes a small heart; and the reproductive system comprises a sac-like ampulla and three female glands. The most surprising discrepancy to the original description refers to the complete absence of any mantle cavity. The gonopore, anus and nephropore open separately to the exterior. Instead of being aberrant or basal, *A. murmanica* fits well with other *Asperspina* species and comes closest to the Mediterranean *Asperspina rhopalotecta*. The monotypic genus/family *Minicheviella*/Minicheviellidae Starobogatov (1983) is confirmed as a junior synonym of *Asperspina*/Asperspinidae Rankin (1979).

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Introduction

The Acochlidia, shell-less slugs with their head-foot at least partly retractable into their elongated visceral hump, could be a suitable model system (Schrödl and Neusser 2007) for phylogenetic studies. Acochlidians comprise a manageable species number with an exceptional biological and morphological diversity and, therefore, an interesting evolutionary history. Some morphological information on large-sized limnic species can be obtained from dissections. The small body size of marine interstitial species allows for the preparation and analysis of entire specimens via serial semithin histological sections. The successive three-dimensional reconstruction of organ systems using AMIRA software is a reproducible and very powerful method (DaCosta *et al*. 2007). It enabled us to reveal an unsuspected degree of deficiency and misinterpretation in the extensive original description of the small limnic acochlidian *Tantulum elegans* Rankin (1979) (Neusser and Schrödl 2007). Since all phylogenetic analyses crucially depend on the quality of the primary data, the in-depth re-examination of dubious structures and species is mandatory.

One of the most intriguing acochlidian species is *Hedylopsis murmanica* Kudinskaya and Minichev (1978). First, it is the only known polar acochlidian species. Second, according to its original description by Kudinskaya and Minichev (1978), it shows a well-developed, tube-like elongated mantle cavity with a longitudinal bipartition in which anus, nephropore and genital duct open. As the single acochlidian species retaining a mantle cavity *H. murmanica* was already described as being an especially 'primitive' species by Kudinskaya and Minichev (1978; p. 83). Later, Fahrner and Haszprunar (2002) showed by ultrastructural investigation that the Red

Sea species *Hedylopsis ballantinei* Sommerfeldt and Schrödl (2005) (as *Hedylopsis* sp.) possesses a distinct though vestigial mantle cavity. Relying on the detailed results and histological drawings of Kudinskaya and Minichev (1978), they suggested *H. murmanica* and the Hedylopsidae as a whole to be basal within the Acochlidia. While Starobogatov (1983) established a separate genus *Minicheviella* and family Minicheviellidae for *H. murmanica*, Arnaud *et al.* (1986) and Wawra (1987) placed the species in the genus *Asperspina* Rankin (1979). According to the latter authors, the Asperspinidae comprises five *Asperspina* species which are all characterized by being hermaphroditic and aphallic, and by the presence of a visceral hump with a more or less dense 'secondary shell' of dermal fusiform calcareous spicules and two pairs of blunt and barely movable cephalic tentacles. The descriptions of the European *Asperspina brambelli* (Swedmark 1968) (as *Hedylopsis*), *Asperspina loricata* (Swedmark 1968) (as *Hedylopsis*) and *Asperspina rhopalotecta* (Salvini-Plawen 1973) (as *Hedylopsis*) offer little more detail, while Morse (1976) gave a comprehensive histological report on the north-western Atlantic *Asperspina riseri* (Morse 1976) (as *Hedylopsis*).

This study for the first time re-examines in detail the microanatomy of *Asperspina murmanica*. Special focus is put on the absence or presence of a true mantle cavity and its potential implication to acochlidian phylogeny.

Materials and Methods

According to the original description by Kudinskaya and Minichev (1978), the holotype and paratypes of *A. murmanica* (type locality: Dalniye Zelentsy, Russia (Fig. 1A)) were deposited in the Zoological Institute of the Russian Academy of Sciences (ZIN RAS), St Petersburg. The stored material was not marked as holotype and paratype, and therefore the material is now considered as syntypes. The ZIN RAS provided us with one section series of these syntypes for re-examination. Unfortunately, the section series was not fully adequate to carry out a reconstruction of major organ systems according to modern standards.

Additionally, we received one specimen collected by A. V. Smirnov at the type locality in August 1981 for semithin sectioning. The specimen was decalcified with Bouin's solution, dehydrated in a graded series of acetone dilutions and embedded, stained and sectioned as described below. The series is deposited at the ZIN RAS.

For a detailed re-examination, several specimens of *A. murmanica* were collected at the type locality in Yarnyshnaya Bay (Fig. 1B) near Dalniye Zelentsy settlement, Barents Sea, Russia, in August 2005 (Martynov *et al.* 2006). Sampling took place in the same habitat as originally described. No additional acochlidian species were found at the type locality. The specimens were extracted from sand samples (coarse sand from the lower and middle intertidal) and relaxed by a solution of isotonic $MgCl_2$. Some specimens were fixed in 4% glutardialdehyde buffered in 0.2 M sodium cacodylate (0.1 M NaCl and 0.35 M sucrose, pH 7.2), followed by postfixation in buffered 1% OsO_4 for 1.5 h. The specimens were decalcified with ascorbic acid, dehydrated in a graded series of acetone dilutions and embedded in Spurr's low-viscosity resin (Spurr 1969) for semithin sectioning. Four ribboned serial semithin section series of 1.5 m thickness were prepared using 'Ralph' glass knives or a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) and contact cement at the lower cutting edge (Henry 1977; Ruthensteiner *et al.* 2007), and finally stained with methylene-blue-azure II (Richardson *et al.* 1960). Computer-based three-dimensional reconstructions of all organ systems were carried out using the software AMIRA 3.1 (TGS Europe, Mercury Computer Systems, Merignac Cedex, France). The procedure of reconstruction basically followed the method described by Ruthensteiner *et al.* (2007). The sections were deposited at the Zoologische Staatssammlung München (ZSM), Mollusca Section (N° 20062163, 20062164, 20062165 and 20062167). Five ethanol-fixed specimens were macerated in 10% KOH and used for analysis of the radula and spicules by scanning electron microscopy (SEM). They were coated with gold for 120 s (SEM-Coating-System, Polaron) and analysed using a LEO 1430 VP SEM (15 kV). Preliminary illustrations have been published in Neusser *et al.* (2007b).

Results

Habitat

Asperspina murmanica inhabits patches of coarse gravel and sand between large rocks covered with algae (Fig. 1C,D) in the middle and lower intertidal at approximately 69°N (Fig. 1A).

External morphology

The body of *A. murmanica* is worm-like and shows an anterior head-foot complex that is clearly separated from the posterior elongate shell-less visceral sac (Fig. 1B) into which the specimens can retract. The body length of living specimens is up to 3.0 mm. The head bears one pair of cylindrical labial tentacles (Fig. 1B) and, posterior to these, one pair of cylindrical rhinophores. These are slightly longer than the labial tentacles in some specimens but most often are of the same length. The densely ciliated foot of *A. murmanica* is as broad as the anterior body, extending as a well-developed free tail about one-third of the length of the visceral sac. The tail shows a blunt end. The visceral sac is subepidermally densely covered by calcareous spindle-shaped spicules up to 120 m in length (Figs 1B and 2B). The spicules are orientated obliquely to the median dorsal line of the visceral sac and are distributed irregularly without forming rows. The posterior portion of the visceral sac is laterally compressed and forms a dorsal keel. Small spicules of approx. 30–50 m length are situated between the labial tentacles and rhinophores (Fig. 2A). In addition, small, spherical and refractive structures can be

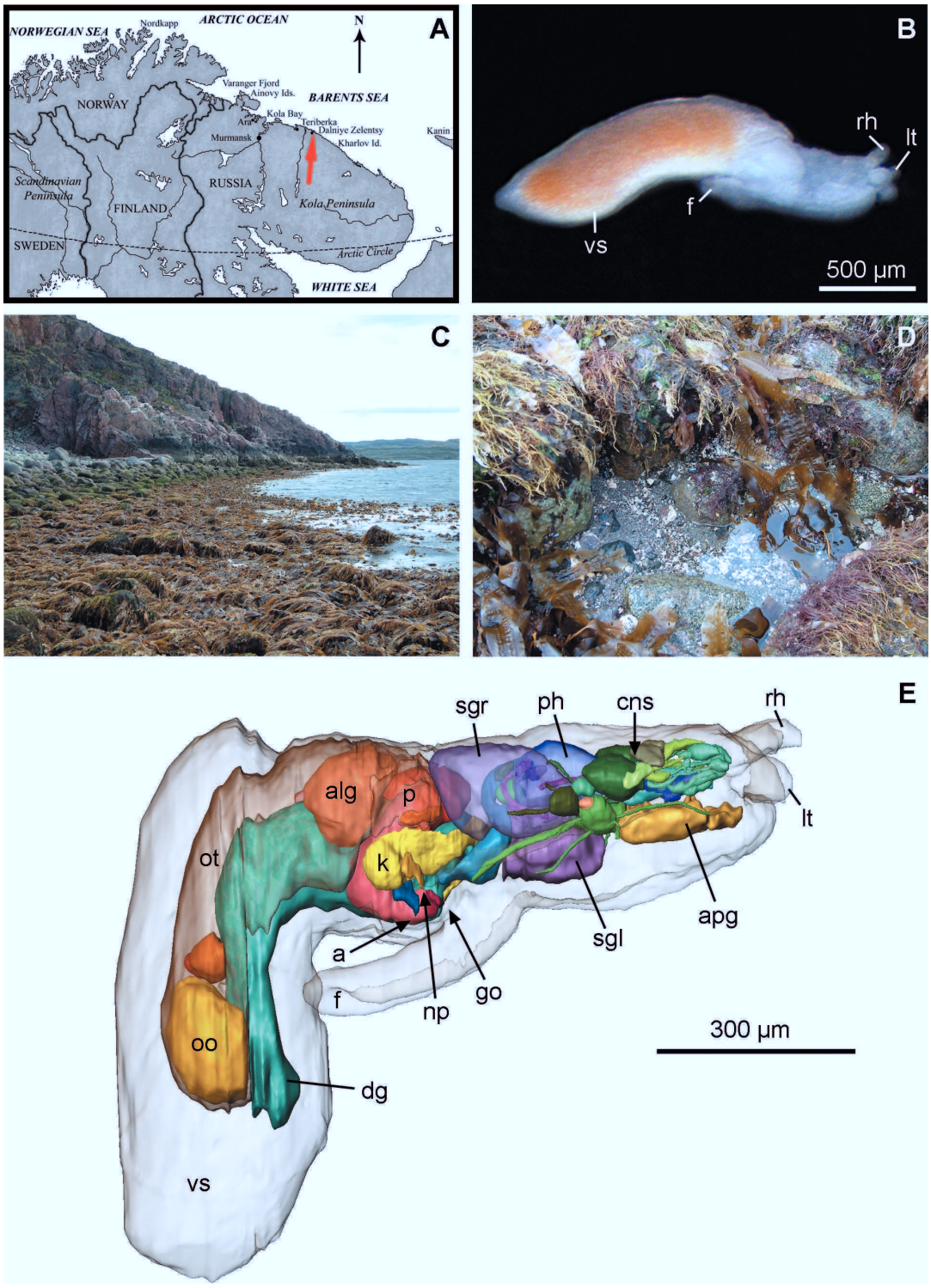

Fig. 1—Habitat, external morphology and general anatomy of *Asperspina murmanica*. —**A**. Type locality: Dalniye Zelentsy, near Murmansk, Russia. —**B**. Photograph of a living specimen. —**C**. Sample station: Yarnyshnaya Bay. —**D**. Habitat of *A. murmanica*: patches of coarse sand between rocks covered with algae. —**E**. Three-dimensional reconstruction, position of internal organs: green, central nervous system; blue, digestive system; yellow, circulatory and excretory systems; red/brownish, reproductive system. a, anus; alg, albumen gland; apg, anterior pedal gland; cns, central nervous system; dg, digestive gland; f, foot; go, gonopore; k, kidney; lt, labial tentacle; np, nephropore; oo, oocyte; ot, ovotestis; p, pericardium; ph, pharynx; rh, rhinophore; sgl, left salivary gland; sgr, right salivary gland; vs, visceral sac.

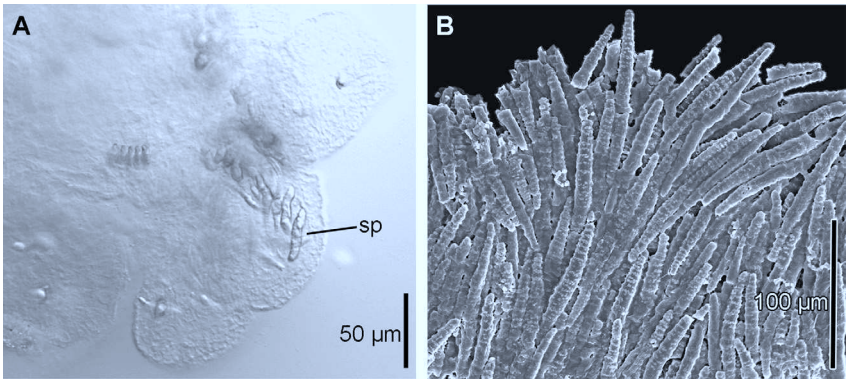

Fig. 2—Pattern of spicules in *Asperspina murmanica*. —**A**. Observation by light microscopy, small spicules between the labial tentacles. —**B**. Scanning electron micrograph, large spicules covering the visceral sac. sp., spicules.

found in the labial tentacles. It is not clear if these structures are calcareous or glandular. The body colour of living specimens is whitish, often with an orange-coloured digestive gland visible (Fig. 1B).

General anatomy

The head-foot complex contains the central nervous system (CNS) and the anterior part of the digestive system (oral tube, pharynx with radula, salivary glands and oesophagus) (Figs 1E, 4A and 6A). The (probably bilobed) anterior pedal gland lies ventral to the oral tube and opens to the exterior ventral to the mouth opening (Fig. 1E) forming a ciliated patch. It extends to the level of the pedal ganglia and is stained dark blue (Fig. 5A,B) like the small pedal glands (Fig. 5A) that are distributed all over the foot. A second glandular mass (Fig. 5A) with the same staining properties is situated dorsal to the oral tube. It is much smaller than the anterior pedal gland and seems to be connected with the oral tube. The excretory and circulatory systems are placed on the right side in the anterior part of the visceral sac (Figs 1E and 8A). The digestive gland is situated on the left side of the visceral sac (Figs 1E and 6A), whereas the reproductive system is dorsal and on the right side (Figs 1E and 10A). The gonopore, nephropore and anus (from anterior to posterior, respectively) are located ventrolaterally on the right side of the visceral sac. They lie next to each other, but open separately and directly to the exterior (Fig. 1E).

Central nervous system

The CNS of *A. murmanica* is euthyneurous and epiathroid, i.e. the pleural ganglion lie closer to the cerebral ganglion than to the pedal ganglion. It consists of paired rhinophoral, cerebral, pedal, pleural, buccal and gastro-oesophageal ganglia and three distinct, separated ganglia on the visceral nerve cord (Figs 3 and 4C). All ganglia are arranged around the anterior part of the pharynx (Fig. 1E), only the buccal and gastro-oesophageal ganglia are located postpharyngeally. Terms used for ganglia and nerves are according to Haszprunar (1985) and Huber (1993).

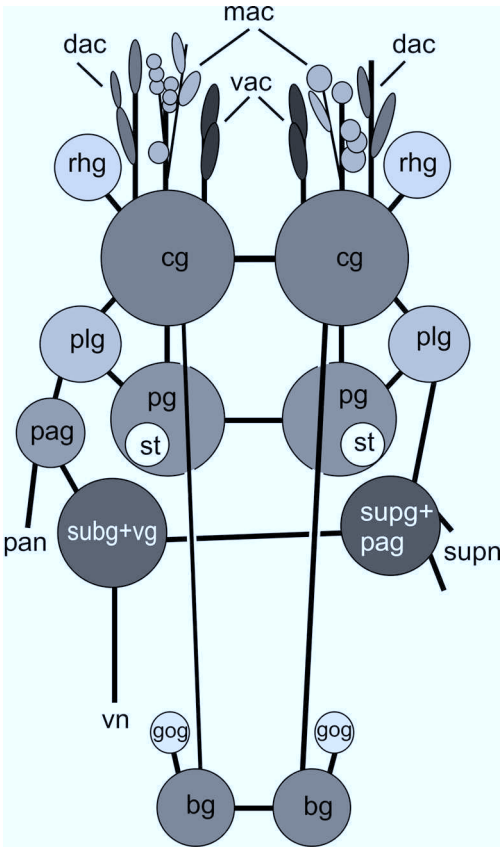

Fig. 3—Central nervous system of *Asperspina murmanica* (schematic, dorsal view). bg, buccal ganglion; cg, cerebral ganglion; dac, dorsal accessory ganglia complex; gog, gastro-oesophageal ganglion; mac, median accessory ganglia complex; pag, parietal ganglion; pan, parietal nerve; pg, pedal ganglion; plg, pleural ganglion; rhg, rhinophoral ganglion; st, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; supn, supraintestinal nerve; vac, ventral accessory ganglia complex; vg, visceral ganglion; vn, visceral nerve. Not to scale.

Large aggregations of accessory ganglia are situated in the anterior part of the CNS (Figs 3 and 4B,C). These cell aggregations of neuronal tissue are surrounded by a thin layer of connective tissue and are similar to ganglia, but lack

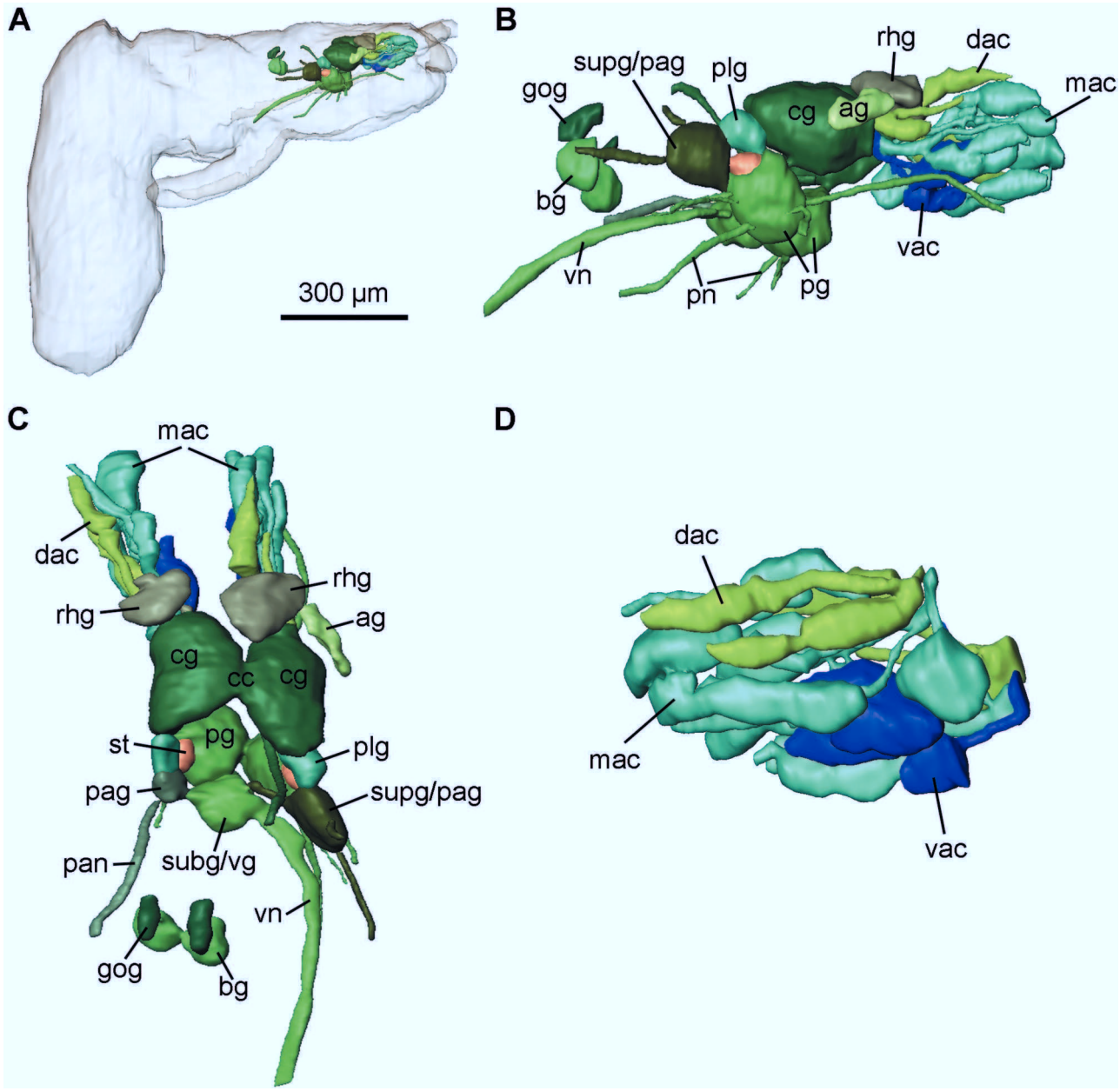

Fig. 4—Three-dimensional reconstruction of the central nervous system of *Asperspina murmanica*. —**A**. Position of the organ system in the specimen (right view). —**B**. Right view. —**C**. Dorsal view. —**D**. Accessory ganglia complexes (left view). ag, accessory ganglion; bg, buccal ganglion; cc, cerebral commissure; cg, cerebral ganglion; dac, dorsal accessory ganglia complex; gog, gastro-oesophageal ganglion; mac, median accessory ganglia complex; pag, parietal ganglion; pan, parietal nerve; pg, pedal ganglion; plg, pleural ganglion; pn, pedal nerve; rhg, rhinophoral ganglion; st, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vac, ventral accessory ganglia complex; vg, visceral ganglion; vn, visceral nerve.

the characteristic separation into cortex and medulla (Fig. 5A). The nuclei are distributed all over the accessory ganglion. The number and size of the accessory ganglia differ among specimens, and sometimes even in the same specimen between the right and the left side of the CNS. The accessory ganglia in *A. murmanica* are attached to cerebral nerves and usually arranged in three paired main complexes: the dorsal, the ventral and the median accessory ganglia complexes (Figs 3 and 4B–D). The dorsal accessory ganglia complex (DAC) consists of few accessory ganglia (Fig. 4D) and is attached to a bifurcated cerebral nerve that arises anterodorsally from the cerebral ganglion. The ventral accessory ganglia

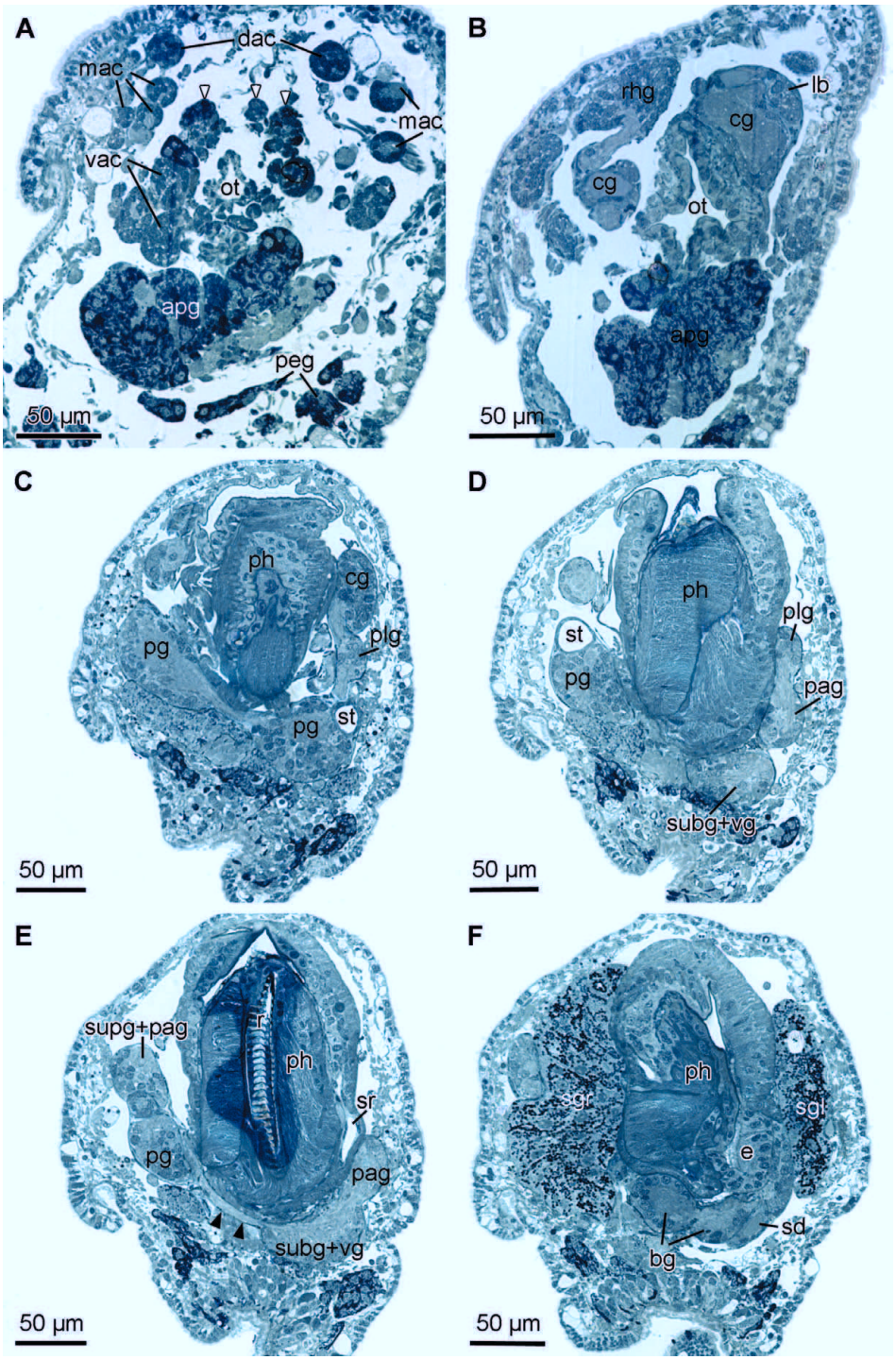

Fig. 5—Transverse sections of the central nervous system of *Asperspina murmanica*. —**A**. Precerebral accessory ganglia. —**B**. Cerebral and rhinophoral ganglion. —**C**. Pedal and pleural ganglion, statocyst. —**D**. Parietal and fused subintestinal/visceral ganglion. —**E**. Fused supraintestinal/parietal ganglion. —**F**. Buccal ganglion. apg, anterior pedal gland; bg, buccal ganglion; cg, cerebral ganglion; dac, dorsal accessory ganglia complex; e, oesophagus; lb, lateral body; mac, median accessory ganglia complex; ot, oral tube; pag, parietal ganglion; peg, pedal gland; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; r, radula; rhg, rhinophoral ganglion; sd, salivary duct; sgl, left salivary gland; sgr, right salivary gland; sr, salivary reservoir; subg, subintestinal ganglion; supg, supraintestinal ganglion; st, statocyst; vac, ventral accessory ganglia complex; vg, visceral ganglion; black arrow heads, subintestinal/visceral-supraintestinal/parietal-connective; white arrow heads, glandular mass flanking the oral tube dorsally.

complex (VAC) flanks the oral tube (Fig. 5A). It is composed of two or three accessory ganglia (Figs 4B,D) and is innervated by the strong labiotentacular nerve emerging anteroventrally from the cerebral ganglion. The median accessory ganglia complex (MAC) is located between the dorsal and the ventral complexes (Fig. 4D). It comprises numerous accessory ganglia innervated by a dorsal nerve originating also from the cerebral ganglion and running up to the base of the rhinophore.

All ganglia are surrounded by a layer of connective tissue and separated into an outer cortex containing the nuclei and an inner medulla. The medulla, nerves, commissures and connectives lack any nuclei and are stained slightly blue-greyish. The cerebral ganglia (Figs 3, 4B,C and 5B) lie dorsolaterally at the anterior end of the pharynx and are approximately 100–120 m in diameter. The cerebral commissure (Figs 3 and 4C) is short and thick. A group of cells is dispersed in the connective tissue above the cerebral commissure. Lateral bodies (Fig. 5B) on the cerebral ganglia, as described by Neusser *et al.* (2007a), are present. Neither Hancock's organs nor eyes could be detected.

The rhinophoral ganglion (Figs 3, 4B,C and 5B) is located anterodorsally of the cerebral ganglion. The cerebro-rhinophoral connective (Fig. 5B) emerges anterodorsally from the cerebral ganglion, very close to the cerebral nerve bearing the MAC. There is no additional nerve arising from the rhinophoral ganglion.

The pedal ganglia (Figs 3 and 4B,C) are located posteroventrally to the cerebral ganglia. They are connected by a short commissure (Fig. 5C) and are smaller than the cerebral ganglia (approx. 85–100 m in diameter). Five nerves emerge from each pedal ganglion (Fig. 4B) innervating the foot: two arise anteroventrally and lead to the anterior part of the foot. Posteriorly, one nerve arises ventrally and two additional nerves dorsally. There is a statocyst (Figs 3, 4C and 5C,D) with one statolith attached to each of the pedal ganglia.

The pleural ganglia (50 m in diameter) lie posterior to the cerebral ganglia and dorsal to the pedal ganglia (Figs 3, 4B and 5C). Cerebro-pleural connectives are very short as are the pleuro-pedal connectives (Figs 4B and 5C).

On the visceral nerve cord (Fig. 3) there are three separate ganglia, which lie ventral to the pharynx. The left parietal ganglion (Figs 3, 4C and 5D) shows almost the same size as the pleural ganglion. The fused subintestinal/visceral ganglion (Figs 3, 4C and 5E) bears the thick visceral nerve that runs through the visceral hump and is approximately as large as the fused supraintestinal/parietal ganglion (approx. 100 m in diameter) (Figs 3, 4B,C and 5E). The pleuro-parietal connective (Fig. 4C), parietal-subintestinal/visceral connective (Fig. 5D) and the pleuro-supraintestinal/parietal connective (Fig. 4B) are very short but the subintestinal/visceral-supraintestinal/parietal connective (Figs 3 and 5E) is longer, being approximately 80 m in length. There is no additional ganglion attached to the fused supraintestinal/right parietal ganglion. A genital ganglion is absent.

The buccal ganglia (approx. 50 m in diameter) (Figs 3 and 4B,C) are located postpharyngeally and are connected by a thin commissure ventral to the oesophagus (Fig. 5F). Each buccal ganglion is linked by a thin, vertical connective with the smaller gastro-oesophageal ganglion (Figs 3 and 4B,C). The latter are located dorsal to the buccal ganglia and are flanking the oesophagus.

Digestive system

The oral tube of *A. murmanica* starts at the mouth opening (Fig. 6B,C) ventrally between the labial tentacles and is not ciliated (Fig. 5A,B). The bulbous pharynx (Figs 5C,D and 6D) is a complex system of longitudinal muscles in the outer layers and circular muscles in the inner ones and contains the asymmetric radula (Figs 5E, 6B,C and 7E). The latter is hook-shaped and characterized by the formula 42–48 1.1.2, with 28–33 teeth on the dorsal ramus and 14–15 teeth on the ventral ramus (Fig. 7A). The dorsal ramus is slightly curved and up to 137 m long, the ventral ramus is up to 58 m. The rhachidian tooth is triangular (Fig. 7B,E) and bears one large central cusp with five to seven lateral denticles on each side. The first pair of lateral denticles flanking the central cusp shows the same size as the latter, the other lateral denticles are considerably smaller. The left lateral tooth is plate-like, rectangular (Fig. 7C,E) and has a prominent denticle in the middle of the anterior margin. Each plate has a notch on the posterior margin in which the denticle of the anterior lateral tooth matches. The right lateral teeth consist of two rectangular plates (Fig. 7D,E). The first plate bears a prominent denticle on the anterior margin. The second plate lacks any denticle. Dimensions of the teeth are given in Table 1. Jaws are absent. The paired and well-developed salivary glands (Fig. 6B,C) are located posterior to the pharynx. The secretory cells of the salivary glands are characterized by vesicles that stain light and dark blue (Figs 5F and 6D,E). The thin salivary duct (Figs 5F and 6C) connects the salivary gland to the food channel at the posterior end of the pharynx forming a small salivary reservoir (Figs 5E and 6C) close to the pharynx. Large salivary pumps at the transition between the salivary gland and the salivary duct are absent. The ciliated oesophagus (Figs 5F and 6B,C) emerges from the pharynx posterodorsally. In some specimens the oesophagus widens posteriorly (Fig. 6E), but, histologically, it cannot be distinguished from the anterior part; the dilated part may be an artefact. The oesophagus connects to a sac-like expansion ('stomach') that is continuous with the anterior cavity of the digestive gland. This cavity is separated from the posterior portion of the digestive gland only by a deep fold (Fig. 8D). The epithelia of the digestive gland and of the stomach have the same staining properties, but show a different ciliation pattern. No cilia are found in the epithelium of the digestive gland, whereas the epithelium cells of the stomach are ciliated. The voluminous holohepatic digestive gland is placed on the left side of the visceral sac (Figs 1E and 6A).

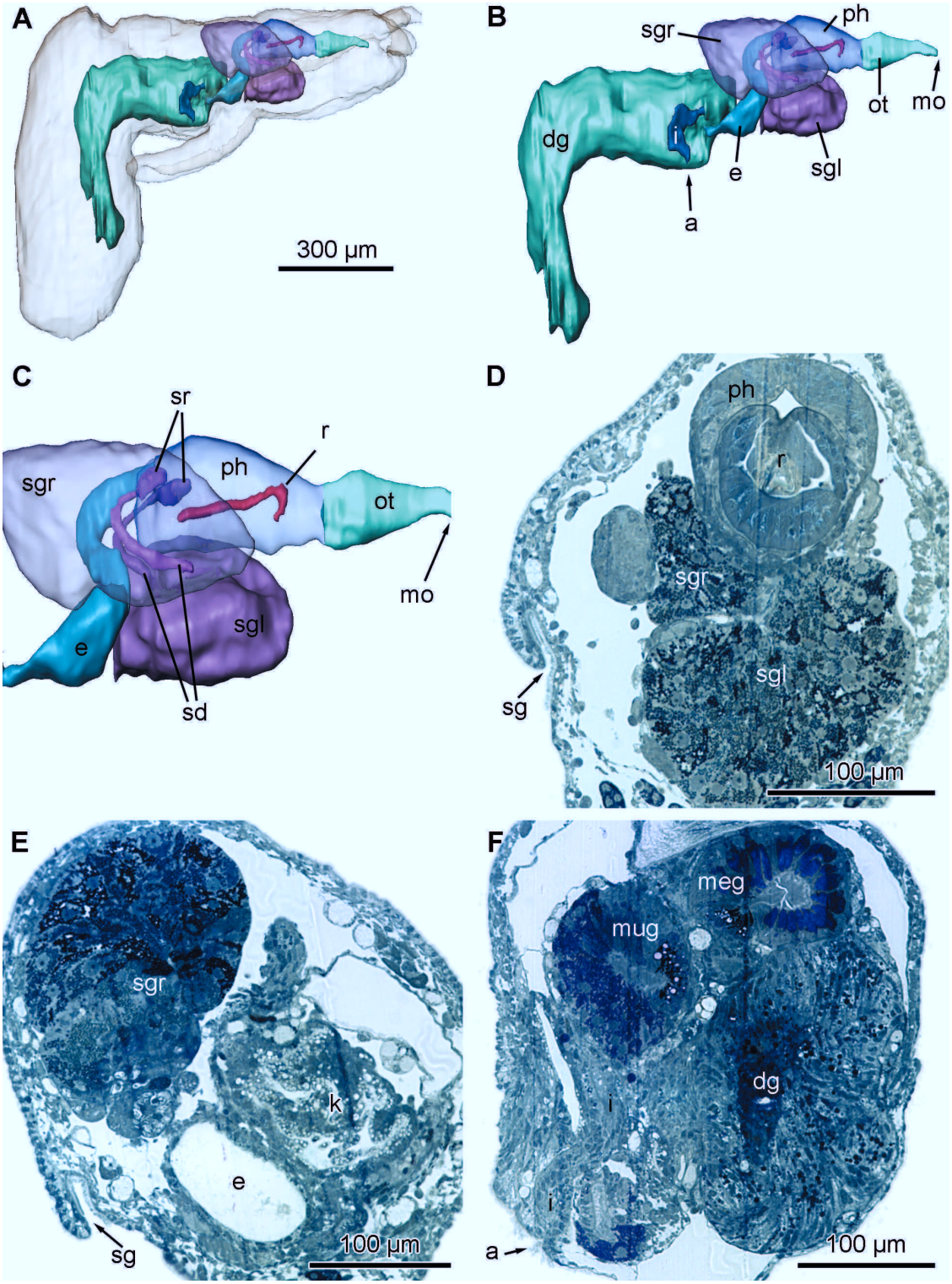

Fig. 6—Digestive system of *Asperspina murmanica*. —**A**–**C**. Three-dimensional reconstructions. —**A**. Position of the organ system in the specimen (right view). —**B**. Digestive system (right view). —**C**. Salivary gland system (right view). —**D**–**F**. Transverse sections. —**D**. Pharynx and salivary glands. —**E**. Oesophagus. —**F**. Anus and digestive gland. a, anus; dg, digestive gland; e, oesophagus; i, intestine; ph, pharynx; k, kidney; meg, membrane gland; mo, mouth opening; mug, mucus gland; ot, oral tube; r, radula; sd, salivary duct; sg, sperm groove; sgl, left salivary gland; sgr, right salivary gland; sr, salivary reservoir.

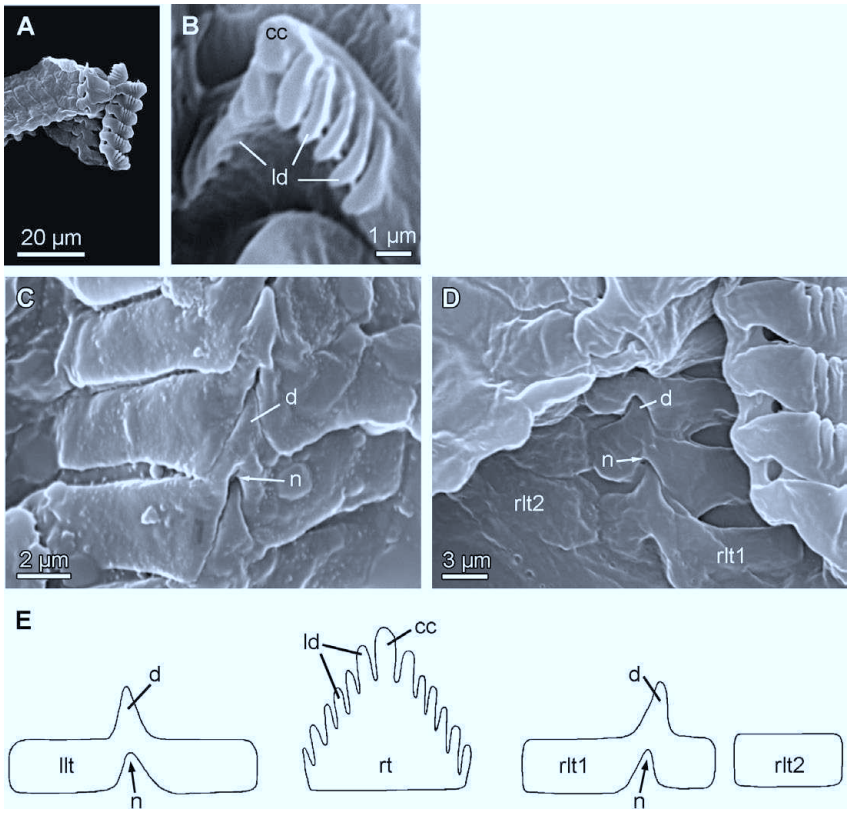

Fig. 7—Radula of *Asperspina murmanica*. —**A**–**D**. Scanning electron micrographs. —**A**. Radula (right view). —**B**. Triangular rhachidian tooth. —**C**. One lateral tooth of the left side. —**D**. Two lateral teeth of the right side. —**E**. Teeth of one row (schematic drawing, not to scale). cc, central cusp; d, denticle; ld, lateral denticle; llt, left lateral tooth; n, notch; rlt1, first right lateral tooth; rlt2, second right lateral tooth; rt, rhachidian tooth.

Table 1 Comparison of the radula within the Asperspinidae

	Asperspina murmanica	*Asperspina rhopalotecta*	*Asperspina riseri*	*Asperspina brambelli*	*Asperspina loricata*
Data source	present study	Salvini-Plawen (1973)	Morse (1976)	Swedmark (1968)	Swedmark (1968)
Radula formula	42–48 1.1.2	38–42 1.1.2	47 1.1.1	38–45 2.1.2	60 1.1.1
Rhachidian tooth; No. of denticles/side	6–9 5 m; 5–7	10 8 m; 4–6	?; 5–6	19 18; 8	?; 4–5
1° left lateral plate	14 3 m	14 2.5 m	?	22 ? m	?
2° left lateral plate	absent	absent	absent	?	absent
1° right lateral plate	11 3 m	10 2.5 m	?	22 ? m	?
2° right lateral plate	6 3 m	3 2.5 m	absent	?	absent

? indicates no data available.

It is a straight, elongated sac that forms neither curves nor loops (Figs 6B,F and 8D). Posteriorly, the oesophagus joins the short and densely ciliated intestine (Figs 6F and 8D). The anus opens ventrolaterally at the right side of the visceral sac (Fig. 6F), slightly posterior to, but separated from, the nephropore.

Circulatory and excretory systems

The circulatory and excretory systems of *A. murmanica* are situated dextrally in the anterior part of the visceral sac (Fig. 8A). The thin-walled pericardium is located dorsal to the kidney (Fig. 8B) and ventrally encloses the heart. The latter is small, thin-walled and comprises only one chamber (Fig. 8C). The short, non-muscular renopericardial duct arises ventrally from the pericardium and opens dorsally into the kidney. The kidney is sac-like with the proximal end bent backwards (Fig. 8B) and is characterized by highly vacuolated cells (Figs 6E and 8D). In the posterior part of the kidney the ciliated nephroduct emerges (Fig. 8B). It is short and the lumen is narrow. It opens ventrolaterally on the right side by the nephropore (Fig. 8C) forming a ciliated patch. The nephropore is situated posterior to the gonopore and just anterior to the anus, and slightly dorsal of both.

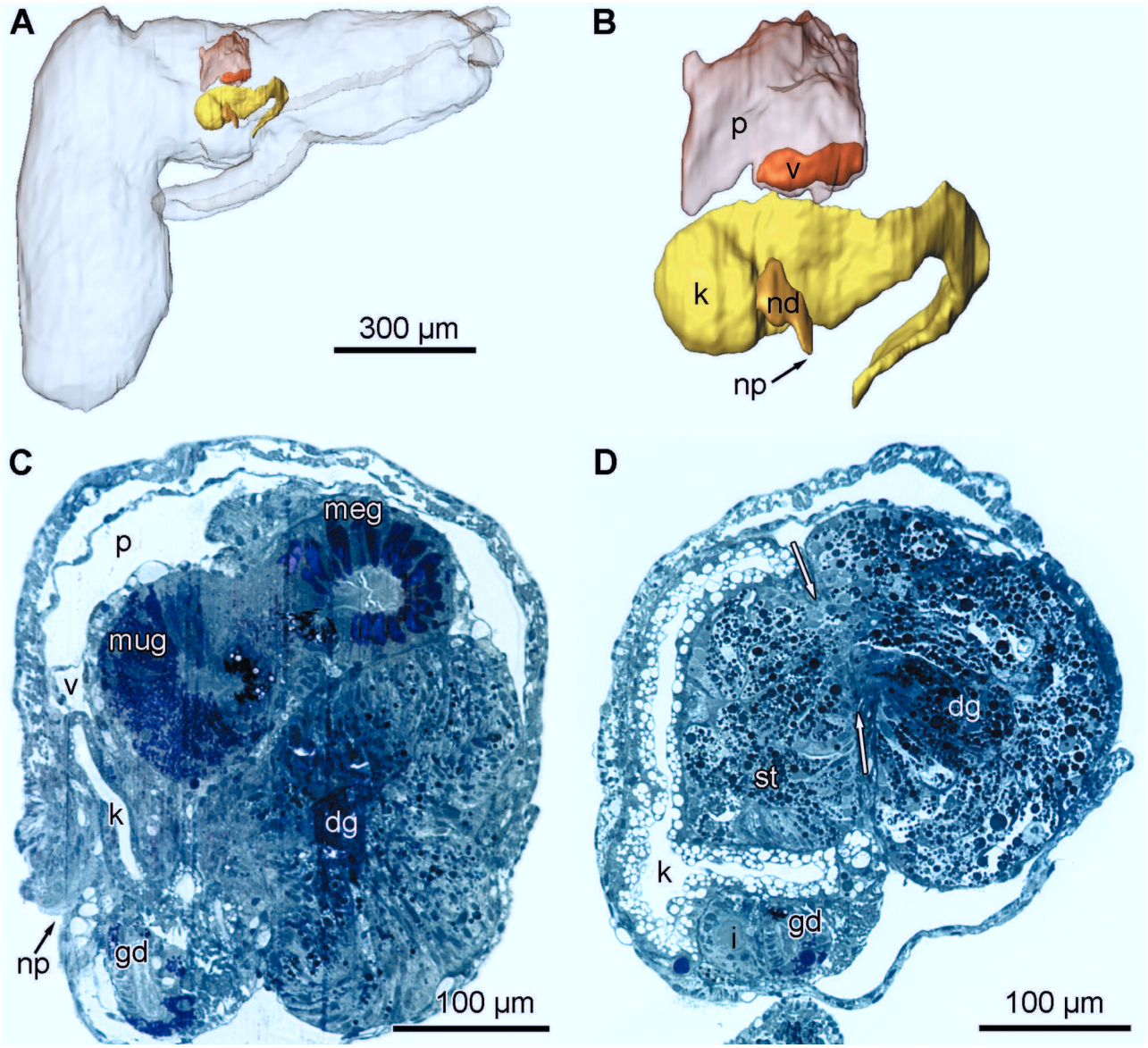

Fig. 8—Excretory and circulatory systems of *Asperspina murmanica*. —**A**, **B**. Three-dimensional reconstructions. —**A**. Position of the organ system in the specimen (right view). —**B**. Excretory and circulatory systems (right view). —**C**, **D**. Transverse sections. —**C**. Pericardium, ventricle and nephropore. —**D**. Kidney. dg, digestive gland; gd, distal gonoduct; i, intestine; k, kidney; meg, membrane gland; mug, mucus gland; nd, nephroduct; np, nephropore; p, pericardium; st, stomach; v, ventricle; white arrows, transition groove between digestive gland and stomach.

Reproductive system

The terminology used for the description of the reproductive system follows Ghiselin (1965) and the terminology of the nidamental glands is according to Klussmann-Kolb (2001). *Asperspina murmanica* is a simultaneous hermaphrodite and develops a monaulic reproductive system. The ovotestis is sac-like (Fig. 9), extends over approximately two-thirds of the visceral sac and lies on the right side of the latter (Figs 1E and 10A). Spermatocytes and oocytes occur at the same time in the ovotestis (Fig. 10F) and are not arranged in separate follicles. Dark-blue-stained spermatocytes are elongate and spiral (Fig. 10G) and can be found especially dorsally and in the anterior part of the ovotestis. In the reconstructed specimen only three oocytes containing yolk material are present (Fig. 10B,G). They are at different stages of development and located ventrally in the posterior part of the ovotestis. The largest oocyte measures approximately 225 m in diameter. The short, ciliated preampullary gonoduct (Fig. 9) emerges anteriorly from the ovotestis. It leads to the ciliated,

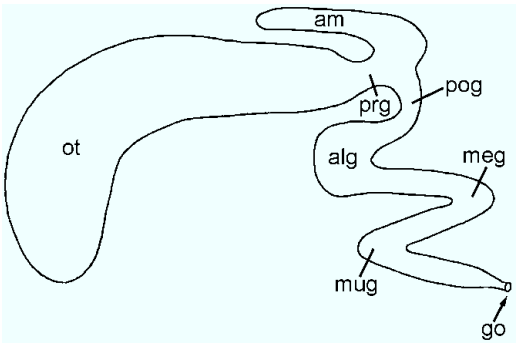

Fig. 9—Reproductive system of *Asperspina murmanica* (schematic drawing). alg, albumen gland; am, ampulla; go, gonopore; meg, membrane gland; mug, mucus gland; ot, ovotestis; pog, postampullary gonoduct; prg, preampullary gonoduct. Not to scale.

sac-like ampulla (Figs 9 and 10B,C), which is filled with autosperm (Fig. 10F) lying in disorder in all specimens examined. The ciliated postampullary gonoduct (Fig. 9) connects to the nidamental gland mass. According to their position in the pallial gonoduct from proximal to distal, the nidamental glands are identified as albumen, membrane and mucus glands (Fig. 10B,C). The epithelium of these three glands consists of alternating glandular and supporting cells. Histologically, each gland shows characteristic staining properties and ciliation patterns. The albumen gland is the largest among the nidamental glands. It is tubular and thick-walled with a narrow lumen. Its glandular cells are characterized by dark-blue-stained vesicles (Fig. 10E). The supporting cells bear long cilia. The membrane gland is tubular with a wide lumen. The glandular cells are filled with homogeneous violet-stained or pink-stained secretions (Figs 6F and 10D,E). Long cilia are present. There is a smooth transition to the tubular mucus gland, which shows small lilac-stained vesicles (Figs 6F and 10E). The distal portion shows long cilia. Both a receptaculum seminis and a bursa copulatrix are absent. The nidamental glands connect to the most distal part of the gonoduct (Figs 8C and 10C) that is short and densely ciliated but only slightly glandular. The gonopore (Fig. 10D) opens ventrolaterally on the right side of the body to the exterior. It is situated at the beginning of the visceral sac, slightly anterior to the nephropore and the anus. A deep and densely ciliated external sperm groove (Figs 6D, E and 10A) runs from the gonopore to the base of the right rhinophore. Anterior male copulatory organs are absent.

Discussion

Habitat

The habitat of *A. murmanica* is remarkable in several aspects. *Asperspina murmanica* is the only known acochlidian species inhabiting the polar region. The water temperature during summer (mean of 8 °C in August; Martynov *et al.* 2006) is, however, not truly arctic. All other nominal species are found in temperate or tropical regions. Furthermore, *A. murmanica* is one of two acochlidian species that inhabit patches of coarse sand between large rocks in the middle intertidal. Only the habitat of *A. riseri* was described as similar to that of *A. murmanica* ('... there are a number of seaweed covered boulders with coarse sand in between', Morse 1976; p. 229). In contrast, all other asperspinid species appear to inhabit deeper, subtidal waters (Table 2). The deepest record of an acochlidian ever found is 58 m and refers to an (undescribed) *Asperspina* sp. from San Juan Island, WA, USA (Morse 1994).

External morphology

The external morphology of *A. murmanica* was well described by Kudinskaya and Minichev (1978). The vermiform shell-less body corresponds to the body shape of all other marine acochlidian species (Arnaud *et al.* 1986) as well as the ability to retract the head-foot complex into the anterior portion of the visceral sac when the animal is disturbed. A comparison of the external morphology of all asperspinid species is given in Table 2. The relatively short, cylindrical labial tentacles and rhinophores are characteristic for species of *Asperspina*, the only genus within the family Asperspinidae (Wawra 1987). The rhinophores of *A. murmanica* are slightly longer than the labial tentacles, as reported by Salvini-Plawen (1973) for *A. rhopalotecta*. The foot of *A. murmanica* is as broad as the anterior body showing a cephalo-pedal groove as in *A. rhopalotecta*, *A. brambelli*, *Pseudunela*, *Hedylopsis*, *Palliohedyle weberi* (Bergh 1895) (as *Acochlidium*) and *T. elegans* (Bergh 1895; Challis 1970; Salvini-Plawen 1973; Wawra 1989; Sommerfeldt and Schrödl 2005; Neusser and Schrödl 2007). In contrast, the foot is broader than the body in the limnic *Palliohedyle sutteri* (Wawra 1979) (as *Acochlidium*), *Acochlidium* and *Strubellia* (Bücking 1933; Küthe 1935; Wawra 1979; Haynes and Kenchington 1991), whereas it is narrow without showing a cephalo-pedal groove in *Asperspina loricata*, *A. riseri*, Microhedylidae and Ganitidae (Challis 1968; Marcus 1953; Swedmark 1968; Morse 1976; Neusser *et al.* 2006; Jörger *et al.* 2008). The tail is well developed but considerably shorter than the visceral sac in all asperspinid species, just as in *Hedylopsis spiculifera* (Kowalevsky 1901) (as *Hedyle*), *Pseudunela* and *T. elegans* (Challis 1970; Swedmark 1968; Salvini-Plawen 1973; Morse 1976; Wawra 1989; Neusser and Schrödl 2007). Our Fig. 2B shows the visceral sac densely covered with needle-like spicules that are not arranged in rows as illustrated by Kudinskaya and Minichev (1978: Fig. 1). The spicule pattern in the visceral sac of *A. murmanica* closely resembles that of *A. rhopalotecta* and *A. loricata*: all show the visceral sac more or less densely covered with spicules that are directed obliquely to the dorsal mid-line and in its posterior, laterally compressed portion forming a keel (Salvini-Plawen 1973;

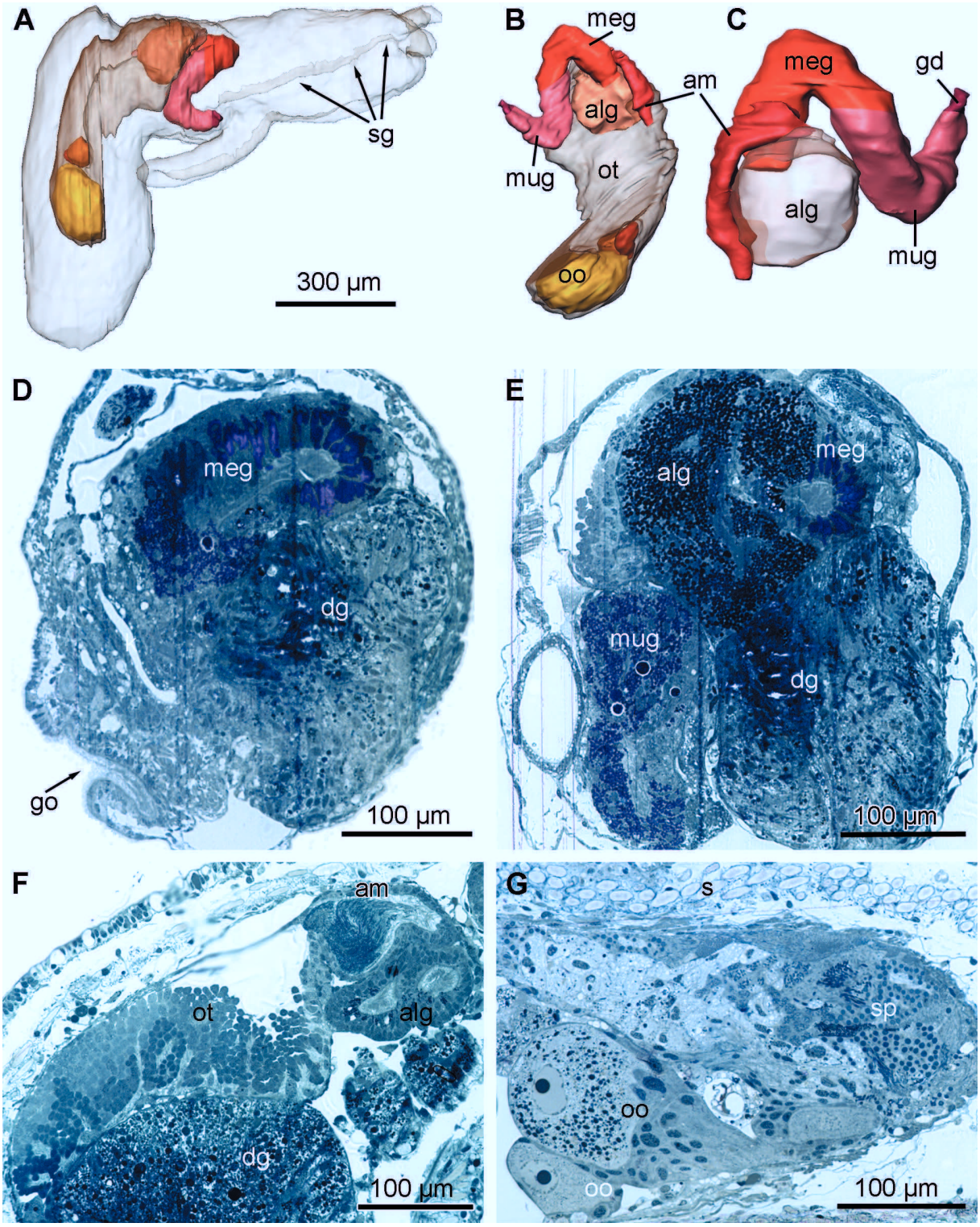

Fig. 10—Reproductive system of *Asperspina murmanica*. —**A**–**C**. Three-dimensional reconstructions. —**A**. Position of the organ system in the specimen, sperm groove (right view). —**B**. Reproductive system (ventral view). —**C**. Nidamental glands and ampulla (dorsal view). —**D**–**G**. Transverse sections. —**D**. Gonopore. —**E**. Nidamental glands. —**F**. Ovotestis and ampulla. —**G**. Oocytes and spermatocytes. alg, albumen gland; am, ampulla; dg, digestive gland; gd, distal gonoduct; go, gonopore; meg, membrane gland; mug, mucus gland; oo, oocyte; ot, ovotestis; s, spicule; sg, sperm groove; sp., spermatocyte.

Table 2 Comparison of the external morphology within the Asperspinidae

	Asperspina murmanica	*Asperspina rhopalotecta*	*Asperspina riseri*	*Asperspina brambelli*	*Asperspina loricata*
Data source	Kudinskaya and Minichev (1978); present study	Salvini-Plawen (1973)	Morse (1976)	Swedmark (1968)	Swedmark (1968)
Type locality	Dalniye Zelentsy, Yarnyshnaya Bay, Barents Sea, Russia; intertidal	Secche della Meloria, Livorno, Italy; 3 m	Crow Neck, North Trescott, Maine USA, intertidal	Church Island, Menai Bridge, UK; subtidal. Kristineberg, Skagerrak, Sweden; 15–20 m	Trezen ar Skoden, off Roscoff, France; 50 m
Size (mm)	3	2	2	2.5	0.9
Eyes	absent	absent	absent	absent	absent
Head tentacles	rhinophores > labial tentacles	rhinophores > labial tentacles	rhinophores < labial tentacles	rhinophores < labial tentacles	rhinophores = labial tentacles
Foot	as broad as the anterior body	as broad as the anterior body	narrower than the anterior body	as broad as the anterior body	narrower than the anterior body
Cephalo-pedal groove	present	present	absent	present	absent
Tail	< vs	< vs	< vs	< vs	< vs
Spicules (m) in the vs	120; vs densely covered	120–160; vs densely covered	100–120; sparsely distributed in vs	225; sparsely distributed in vs	180; vs densely covered
Smaller spicules between the head tentacles	present	present	present, variable in shape/number	present	present

vs, visceral sac; > larger than; < shorter than; = same length as.

Swedmark 1968). The length of the spicules in *A. murmanica* is similar to that of *A. rhopalotecta* and *A. riseri*. The smaller spicules between the head tentacles, as shown here for *A. murmanica*, were already reported for all other asperspinid species. Recently, Jörger *et al*. (2008) discussed the different types of spicules in Acochlidia and their probable function. The authors point to a possible correlation between the different types of spicules and the type of interstitial habitat and suggest further comparative investigations.

Within the Asperspinidae, *A. murmanica* shows the largest body length with up to 3 mm, whereas other congeners are smaller. While most acochlidian species have eyes, *Microhedyle nahantensis* (Doe 1974) (as *Unela*) and *T. elegans* show poorly developed unpigmented eyes (Doe 1974; Neusser and Schrödl 2007; Neusser *et al*. 2007a), but eyes are absent in all known asperspinid species (Table 2) and *Microhedyle remanei* (Marcus 1953) (Neusser *et al*. 2006).

General anatomy

The position of the organ systems in the body of *A. murmanica* corresponds to that of other acochlidian species known in detail (Sommerfeldt and Schrödl 2005; Neusser *et al*. 2006; Neusser and Schrödl 2007; Jörger *et al*. 2008). We consider the multicellular glands discharging into the ciliated groove of the buccal cavity described by Kudinskaya and Minichev (1978) to be the anterior pedal gland reported by Robinson and Morse (1979). Our results show that the anterior pedal gland opens to the exterior just ventral to the mouth opening. It resembles the bilobed anterior pedal gland described recently in *T. elegans* by Neusser and Schrödl (2007) and *Pontohedyle milaschewitchii* (Kowalevsky 1901) by Jörger *et al*. 2008).

Neusser and Schrödl (2007) discuss the relative position of gonopore, nephropore and anus as being of potential phylogenetic significance. The arrangement of these three openings (gonopore, nephropore, anus from anterior to posterior, respectively) in *A. murmanica* resembles that in *M. remanei* and *P. milaschewitchii*, but their relative distances differ: in *A. murmanica* the nephropore is closely associated with the anus, whereas in *M. remanei* all three openings are situated close to each other (Neusser *et al*. 2006), and in *P. milaschewitchii* the nephropore is closely associated to the female gonopore (Jörger *et al*. 2008).

Central nervous system

The nervous system of *A. murmanica* was described by Kudinskaya and Minichev (1978). In the present study we correct some discrepancies and provide further details on nervous features. The euthyneurous and epiathroid CNS of *A. murmanica* seems to be the general condition in acochlidian species. *Asperspina murmanica* shows numerous precerebral accessory ganglia. Such structures were reported from all asperspinid species except for *A. loricata* (Swedmark 1968;

Morse 1976; Wawra 1987) and from microhedylacean species of the families Microhedylidae and Ganitidae (Wawra 1987). According to Wawra (1987), the Hedylopsacea (Hedylopsidae, Acochlidiidae and Tantulidae) develop cerebral nerves without any accessory ganglia. *Hedylopsis spiculifera* and *H. ballantinei* are known to lack any accessory ganglia (Sommerfeldt and Schrödl 2005), but Challis (1970; p. 35) described 'anterior nerves in the form of two chains of ganglia ...' in *Pseudunela cornuta* (Challis 1970) (as *Hedylopsis*). Most recently, Neusser and Schrödl (2007) reported accessory ganglia in at least one specimen of *T. elegans*, a species that is still enigmatic. Further re-examination of presence or absence of accessory ganglia in *Strubellia* and members of the Acochlidiidae is essential. While older descriptions never included details of the cellular structure of the accessory ganglia, Neusser *et al.* (2006) recently described accessory ganglia in detail for *M. remanei*. Jörger *et al.* (2008) confirmed this structure for *P. milaschewitchii* and suggested immunocytochemical studies and labelling against different neurotransmitters to reveal the so far unknown function of such accessory ganglia. The accessory ganglia in *A. murmanica* seem to be smaller than in *P. milaschewitchii* and *M. remanei*, and they are not spherical in shape but more slender and elongate (Neusser *et al.* 2006; Jörger *et al.* 2008). The arrangement of three distinct complexes of accessory ganglia in *A. murmanica* resembles that of *P. milaschewitchii* reported recently by Jörger *et al.* (2008), but their placement and innervation are different. In *A. murmanica* we can distinguish the VAC, MAC and DAC. In contrast, *P. milaschewitchii* is reported to have a small VAC, the anterior accessory ganglia complex and the dorsolateral accessory ganglia complex (Jörger *et al.* 2008). The innervation of these complexes is still not completely resolved and the homology of the cerebral nerves remains problematic.

The rhinophoral ganglion in *A. murmanica* is located anterodorsally of the cerebral ganglion, as is usual in other acochlidian species (Sommerfeldt and Schrödl 2005; Neusser *et al.* 2006; Neusser and Schrödl 2007; Jörger *et al.* 2008). It is noticeable that there is no nerve leaving the rhinophoral ganglion and innervating the rhinophore in *A. murmanica*, which is in clear contrast to *M. remanei*, *T. elegans* and *H. ballantinei* (Sommerfeldt and Schrödl 2005; Neusser *et al.* 2006; Neusser and Schrödl 2007). The cerebro-rhinophoral connective lies very close to the dorsal cerebral nerve bearing the MAC that is leading to the base of the rhinophore. It is probable that the cerebro-rhinophoral connective and the cerebral nerve bearing the MAC emerge together from the cerebral ganglion and innervate the rhinophore. Jörger *et al.* (2008) reported a rhinophoral ganglion with a thin (reduced) rhinophoral nerve in *P. milaschewitchii*; but in contrast to the four species with rhinophoral ganglia mentioned above, *P. milaschewitchii* lacks any rhinophores. Instead, the rhinophoral ganglion in *P. milaschewitchii* is thought to be related to the innervation of the putative Hancock's organ (Jörger *et al.* 2008).

The cerebral, pedal and pleural ganglia are intimately attached to each other, but unfused, as is usual in Acochlidia (Huber 1993; Sommerfeldt and Schrödl 2005; Neusser *et al.* 2006; Neusser and Schrödl 2007). The 'lateral bodies' attached to the cerebral ganglia were described first as dorsal bodies for *H. ballantinei* by Sommerfeldt and Schrödl (2005), and were recently confirmed for *H. spiculifera* and *A. murmanica* by Neusser *et al.* (2007a). However, the function and homology of these structures is still unclear and further (immuno)histochemical and transmission electron microscopical studies are needed.

In *A. murmanica* there are three distinct ganglia located on the visceral nerve cord. As discussed by Sommerfeldt and Schrödl (2005), their identification is always problematic. Kudinskaya and Minichev (1978) described them as subintestinal, visceral and supraintestinal ganglia. In the present study, the ganglia on the visceral nerve cord were interpreted according to the pentaganglionate hypothesis proposed by Haszprunar (1985) as the left parietal, the fused subintestinal/visceral and the fused supraintestinal/right parietal ganglia (from the left to the right side, respectively). The visceral nerve cord of *A. murmanica* differs from that of *H. ballantinei* and *T. elegans* by lacking an additional ganglion attached to the fused supraintestinal/parietal ganglion (Sommerfeldt and Schrödl 2005; Neusser and Schrödl 2007). The left pleuro-parietal and the right pleuro-supraintestinal/parietal connectives of the visceral nerve cord are short in *A. murmanica*, *H. ballantinei* and *T. elegans*. Accordingly, the visceral nerve cord is short and the ganglia are located in the anterior part of the pharynx (present study; Sommerfeldt and Schrödl 2005; Neusser and Schrödl 2007). In contrast, the left pleuro-parietal and the right pleuro-supraintestinal/parietal connectives are longer in the microhedylid species, e.g. *M. remanei* and *P. milaschewitchii* (Neusser *et al.* 2006; Jörger *et al.* 2008). Therefore, the visceral nerve cord is longer and the position of the ganglia is more posterior than in *A. murmanica*, *H. ballantinei* and *T. elegans.*

Like *A. murmanica*, *A. rhopalotecta* has three different ganglia on the visceral nerve cord (Wawra 1987). Morse (1976) reported only two ganglia on the visceral nerve cord for *A. riseri* (subintestinal and supraintestinal), as did Swedmark (1968) for *A. brambelli* and *A. loricata*. Our previous investigations showed that the number of visceral cord ganglia given in older studies is not reliable (Sommerfeldt and Schrödl 2005; Neusser *et al.* 2006). Three ganglia on the cord seem to be the rule for acochlidians, although there might be some intraspecific and possibly ontogenetic variation, e.g. *T. elegans* is known to possess three or four distinct ganglia on the visceral nerve cord (Neusser and Schrödl 2007).

In several acochlidian species an additional ganglion attached to the supraintestinal ganglion has been described. Because of its position, this ganglion traditionally was identified as an osphradial ganglion by Huber (1993), but

the presence of an osphradium has never been confirmed in any acochlidian species. Wawra (1988, 1989) reported such an additional ganglion in the limnic *Strubellia paradoxa* (Strubell 1892) (as *Acochlidium paradoxum*) and the marine *H. spiculifera*, both of which are protandric hermaphrodites and have well-developed copulatory organs in their male phase (only). In the phallic sequential hermaphrodite *T. elegans*, Neusser and Schrödl (2007) proposed this ganglion to be involved in the control of copulatory functions. Such a function, however, would be difficult to explain for the apparently aphallic *H. ballantinei* (Sommerfeldt and Schrödl 2005). Neither *A. murmanica* that lacks copulatory organs nor any of the likewise aphallic microhedylid species possesses such an additional ganglion.

Kudinskaya and Minichev (1978) described a genital ganglion connected to the visceral ganglion by a long connective for *A. murmanica*. This is clearly contradicted by the present study; there are no posterior genital ganglia in any acochlidian species studied in detail. The authors might have misinterpreted parts of the thick, long and often undulated visceral nerve as an additional genital ganglion.

Gastro-oesophageal ganglia are present in *A. murmanica* and were reported by Wawra (1988, 1989) for *S. paradoxa* and *H. spiculifera*, and by Neusser and Schrödl (2007) for *T. elegans*.

Digestive system

The digestive system of *A. murmanica* conforms with the usual ground-pattern of the digestive system in acochlidian species (Sommerfeldt and Schrödl 2005). Our results show, however, major discrepancies from the original description concerning the radula and the stomach. According to the light microscope examination by Kudinskaya and Minichev (1978), the radula of *A. murmanica* is symmetric and characterized by the formula 17–19 2.1.2; there are two lateral teeth on each side with one denticle on the first tooth. This disagrees with our finding of an asymmetric radula by SEM examination.

The radula of *A. murmanica* closely resembles that of *A. rhopalotecta*, but the number of rows is slightly higher in *A. murmanica*. The rhachidian tooth in *A. murmanica* is slightly smaller than in *A. rhopalotecta*, but shows more denticles per side. On the right side, the second lateral tooth of *A. murmanica* is twice as large as in *A. rhopalotecta*. The supposedly symmetric radulae of all other asperspinid species with one lateral tooth in *A. riseri* and *A. loricata* or two laterals in *A. brambelli* should be re-examined. A comparison of the radula of all valid asperspinid species is given in Table 1.

The salivary reservoirs in *A. murmanica* are situated close to the pharynx where the salivary duct joins the food channel. Small salivary reservoirs were reported only for the limnic acochlidian species *T. elegans* by Rankin (1979). Neusser and Schrödl (2007) could not confirm the presence of this structure in their re-examination of *T. elegans*. Nevertheless, the small salivary reservoirs are difficult to detect when the tissue is very compressed. In contrast, the large salivary pumps reported by Neusser and Schrödl (2007) and Rankin (1979) for *T. elegans* cannot be overlooked and are definitely absent in *A. murmanica*.

Kudinskaya and Minichev (1978) described a small round stomach without glandular cells and externally covered by a thick layer of muscle fibres. Probably they interpreted the dilated part of the oesophagus, which is flanked by longitudinal muscle fibres, as stomach. The large Indo-Pacific limnic acochlidian species *Palliohedyle weberi* and *Acochlidium amboinense* (Strubell 1892) (as *Hedyle*) have been reported to possess a well-developed and differentiated stomach (Bergh 1895; Bücking 1933). In contrast, all small acochlidian species examined in detail, such as *M. remanei* or *P. milaschewitchii*, lack any separate stomach (Neusser *et al.* 2006; Jörger *et al.* 2008) or the stomach is almost or completely fused with the digestive gland, as in *A. riseri*, *Pseudunela cornuta* and *T. elegans* (Challis 1970; Morse 1976; Rankin 1979; Neusser and Schrödl 2007). Therefore, the description of a stomach in *A. brambelli* and *A. loricata* by Swedmark (1968) is questionable and should be re-examined carefully.

Circulatory and excretory systems

The original description of *A. murmanica* shows the kidney and the pericardium on the right side of the visceral sac, but lacks any information about the size and shape of the kidney and the presence or the absence of a heart. Our results match with data on other marine acochlidian species examined in detail. The thin-walled pericardium of *A. murmanica* encloses a small, one-chambered heart, as reported for *P. milaschewitchii* by Jörger *et al.* (2008). In the past, species of the genus *Hedylopsis* were considered as having only a one-chambered heart and the Microhedylacea (including *Asperspina*, *Microhedyle* and *Pontohedyle*) as lacking one (Rankin 1979). Recently, histological and ultrastructural re-examinations revealed that *H. ballantinei* and *M. remanei* possess a two-chambered heart (Fahrner and Haszprunar 2002; Sommerfeldt and Schrödl 2005; Neusser *et al.* 2006). No details about the circulatory system of other asperspinid species are known.

The short, sac-like kidney and the short nephroduct in *A. murmanica* are characteristic also for other marine acochlidian species, e.g. *M. remanei* or *P. milaschewitchii* (Neusser *et al.* 2006; Jörger *et al.* 2008). According to drawings in Morse (1976), *A. riseri* shows a short and sac-like kidney, too, lying on the right side of the visceral sac. Swedmark (1968) described the kidney of *A. loricata* as small, but gave no information about *A. brambelli*. Neither Salvini-Plawen (1973) nor Wawra (1987) provided data of the excretory system of *A. rhopalotecta*. The marine *H. ballantinei* has a simple sac-like kidney, too, but this extends over two-thirds of the visceral sac and is considerably longer than in other marine species (Fahrner and Haszprunar 2002; Sommerfeldt and Schrödl 2005).

Reproductive system

According to Ghiselin (1965), euthyneurous gastropods basally have a monaulic reproductive system with an undivided pallial gonoduct. This is also true for the simultaneous hermaphrodite *A. murmanica*. The original description of the reproductive system by Kudinskaya and Minichev (1978) is complemented herein. Kudinskaya and Minichev (1978) described an ovotestis with female lobes placed near the digestive gland on the left side and male lobes in the right side of the ovotestis. Our results show spermatocytes and oocytes not arranged into follicles and located principally anteroventrally and posteroventrally, respectively, in the ovotestis. Swedmark (1968) described the ovotestis of *A. brambelli* and *A. loricata* with oocytes in the anterior and spermatocytes in the posterior parts. In *A. rhopalotecta* spermatocytes were found mostly in the centre and oocytes in the periphery of the ovotestis (Wawra 1987). *Asperspina riseri* is the only asperspinid species that shows completely separated testis and ovary at the same time in a single specimen (Morse 1976). As the common characteristic in all asperspinid species, only a few oocytes mature at the same time.

Kudinskaya and Minichev (1978) described a 'narrow epithelial strip along the ventral side of the gonoduct', which might refer to the sac-like ampulla in *A. murmanica* appearing like a diverticulum of the gonoduct. A sac-like ampulla is also reported from the hermaphrodite *H. ballantinei* by Sommerfeldt and Schrödl (2005), whereas the gonochoristic species *P. milaschewitchii* and *M. remanei* show a tubular ampulla (Neusser *et al.* 2006; Jörger *et al.* 2008). According to Ghiselin (1965), the sac-like ampulla is a modification and improvement on the inefficient tubular ampulla of the ancestral opisthobranch hermaphroditic reproductive system. In this way the storage of spermatocytes in the ampulla does not interfere with the oocytes passing the gonoduct. According to Morse (1976), the hermaphrodite *A. riseri* shows a tubular ampulla, but also develops two separate gonads and a postampullary glandular sperm duct. The latter is regarded as being involved in the production of the spermatophores. Such a local separation of male organs in a hermaphroditic species contrasts with the temporal separation of male and female organs in the protandric *T. elegans* or in sequential hermaphrodites (e.g. *S. paradoxa* and *H. spiculifera*). Phylogenetic analyses will show whether or not one of these conditions may have been a precursor for secondary gonochorism as expressed in microhedylids and ganitids.

Klussmann-Kolb (2001) proposed the homology of the albumen, membrane and mucus glands throughout the opisthobranchs because of their identical relative position in the gonoduct, similar histology and ultrastructure, similar mode of secretion and their similar staining properties. The three nidamental glands in *A. murmanica* can only be identified by their relative position in the pallial gonoduct and their staining properties. The proximalmost gland in *A. murmanica* herein was recognized as an albumen gland from its position, the sac-like shape and the lack of internal folding. The albumen gland in *A. murmanica* is characterized by dark-blue-stained vesicles; the membrane and mucus glands show purple and violet staining. This is like the glands in *P. milaschewitchii* and *M. remanei* (Neusser *et al.* 2006; Jörger *et al.* 2008). No detailed and comparable data about the nidamental glands on other asperspinid species are available. Wawra (1987) did not name the s-shaped, ciliated female glands in *A. rhopalotecta*, but provided data about their staining properties: the first glandular portion shows blue-stained granules, the cells of the second were stained purple and in the third portion they were light blue. Morse (1994) reported distinct areas within the hermaphroditic duct that correspond to the albumen, membrane and mucus areas in the undescribed *Asperspina* sp. from San Juan Island, WA, USA. She confirmed a similar condition for *A. riseri*.

Asperspina murmanica lacks a receptaculum seminis and a bursa copulatrix. This agrees with most of the acochlidian species that generally do not develop any allosperm-storing receptacles. Only *S. paradoxa* is described as possessing a receptaculum seminis as well as a bursa copulatrix (Wawra 1988). Challis (1970) reported a bursa copulatrix for the marine species *Pseudunela cornuta*, which needs, however, reconfirmation, and recently, Neusser and Schrödl (2007) reported the same for the limnic *T. elegans*.

The deep, well-developed so-called sperm groove in *A. murmanica* is characteristic for all *Asperspina* species. The function of the sperm groove in basal opisthobranchs with anterior copulatory organs is the transportation of sperm (Ghiselin 1965). This can be reasonable assumed for those acochlidian species showing both an external sperm groove and anterior male copulatory organs at least in the male phase, too, e.g. *H. spiculifera* and *S. paradoxa* (Wawra 1987, 1989). However, the function of such a sperm groove in aphallic asperspinids is not evident.

The sperm transfer in the Asperspinidae occurs via spermatophores (Wawra 1987). Morse (1976, 1994) described spermatophores attached to the visceral sac in *Asperspina* sp. and *A. riseri*. Swedmark (1968) observed one or two spermatophores attached to both the visceral sac and the head in *A. brambelli*. However, in *A. murmanica* we could not detect where spermatophores might be produced; in fact living acochlidians have never been observed attaching spermatophores to another individual. It is thus unclear, whether the spermatophores are attached to the mate directly after leaving the gonoduct or if they are first transported via the external sperm groove to the head. The latter would not only explain the function of the asperspinid sperm groove, but would also foster a more targeted positioning of the spermatophores to the mate, as reported by Jörger *et al.* (2008) for *P. milaschewitchii*.

Absence of the mantle cavity and systematic implications

Kudinskaya and Minichev (1978) considered the presence of a very special mantle cavity as the characteristic feature of

A. murmanica. This mantle cavity was said to form a long and narrow channel that is divided into two branches by a transverse fold and that is placed at the base of the visceral sac. The nephroduct and anus were said to open into the right branch of the mantle cavity, while the genital opening was situated in the left, and the sperm groove was illustrated to start from somewhere within the mantle cavity.

However, the re-examination of the original sections did not reveal any long and narrow channel, but only a shallow invagination that is formed when the specimen is withdrawn into the visceral sac and that was misinterpreted by Kudinskaya and Minichev (1978) as mantle cavity. Additionally, our results show that there is no mantle cavity whatsoever in *A. murmanica*. The gonopore, anus and nephropore open close to one another, but separately to the exterior. Most of the discussion of Kudinskaya and Minichev (1978) about the systematic placement of *A. murmanica* (as *Hedylopsis*) was based on the apparent presence of a mantle cavity and, therefore, on fundamental errors.

In the course of his short review of the Acochlidia, Starobogatov (1983) created the new genus *Minicheviella* and the new family Minicheviellidae for *Hedylopsis murmanica* Kudinskaya and Minichev (1978). The main reason was the presence of a large mantle cavity. All other diagnostic characters of Minicheviellidae, such as the absence of copulatory organs, the absence of eyes and the visceral sac densely covered by spicules fitted well into the genus *Asperspina* and the family Asperspinidae, which were both established earlier by Rankin (1979). The family Asperspinidae was listed by Starobogatov (1983), but was nevertheless not compared with Minicheviellidae. According to the present study, *Hedylopsis murmanica* does not have any mantle cavity and thus *Minicheviella* and Minicheviellidae do not differ from the genus *Asperspina* and the family Asperspinidae by any characters. Consequently, *Minicheviella* and Minicheviellidae are junior synonyms of the genus *Asperspina* and family Asperspinidae, respectively.

There is no, or at least no well-developed mantle cavity, in the other asperspinid species either (Swedmark 1968; Morse 1976). Only Wawra (1988) reported the presence of a 'cloaca' in *A. rhopalotecta*. The anus and nephropore discharge into the gonoduct and then open together to the exterior. Further, Challis (1970) described a 'cloaca' in *Pseudunela cornuta*. The intestine and the gonoduct discharge together into a common cloaca. Both species should be re-examined carefully, since Fahrner and Haszprunar (2002) showed *H. ballantinei* to possess a small, but distinct mantle cavity. While the retention of such more or less rudimentary mantle cavities could well indicate basal positions within Acochlidia, there is no more indication for an especially basal position of *A. murmanica*. Instead, its head tentacles, the absence of eyes, the visceral spicule roof and the deep sperm groove confirm its placement together with other known *Asperspina* species (Wawra 1987). *Asperspina murmanica* in fact is quite similar to the Mediterranean *A. rhopalotecta* from which it is, however, distinguished by its larger size, a slightly different radula and, in case Wawra's (1987) observation was right, the presence of a cloaca.

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3.2 Jörger KM, **Neusser TP**, Haszprunar G & Schrödl M 2008. **Undersized and underestimated: 3D-visualization of the Mediterranean interstitial acochlidian gastropod *Pontohedyle milaschewitchii* (Kowalevsky, 1901).** *Organisms Diversity & Evolution* **8**(3): 194-214.

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Undersized and underestimated: 3D visualization of the Mediterranean interstitial acochlidian gastropod *Pontohedyle milaschewitchii* (Kowalevsky, 1901)

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Abstract

Pontohedyle milaschewitchii (Kowalevsky, 1901) is one of the most common mesopsammic opisthobranchs in the Mediterranean and Black Seas and has been considered as a comparably well-described acochlidian species. However, data on its complex internal anatomy were fragmentary and little detailed due to inadequate methodology available, and contradictory between different sources. The present study redescribes all major organ systems of *P. milaschewitchii* in full detail by three-dimensional reconstruction from serial semithin sections using AMIRA software. The prepharyngeal central nervous system (cns) of *P. milaschewitchii* is highly concentrated and shows a euthyneurous and epiathroid condition. Contrary to earlier reports, the cerebral and pleural ganglia are not fused. Aggregations of precerebral accessory ganglia can be grouped into three complexes supplied by distinct cerebral nerves. Rhinophoral ganglia with thin, double cerebro-rhinophoral connectives are described for the first time in acochlidians. A Hancock's organ is present in the form of a conspicuous, curved fold in the epidermis posterior to the oral tentacles. Cerebral nervous features and sensory structures are discussed comparatively. Our study confirms *P. milaschewitchii* as having the male genital opening in an unusual position above the mouth. Homology of the ciliated vas deferens of the gonochoristic and aphallic *P. milaschewitchii* with that of hermaphroditic acochlidian species with cephalic male genitals is discussed. The radula formula of *P. milaschewitchii* is 41–54 × 1-1-1, i.e. the single lateral teeth are broad and, contrary to previous descriptions, undivided. SEM examination of the body wall of entire specimens revealed a special and constant ciliary pattern. Providing a novel additional set of characters for taxonomic and phylogenetic purposes, external SEM examination is suggested as the standard method for describing acochlidian species in the future.

Keywords: Mollusca; Gastropoda; Opisthobranchia; 3D reconstruction; Anatomy; Histology

Introduction

Only few gastropods are able to colonize the marine interstitial, a habitat with extreme ecological conditions (Swedmark 1968b). Within the opisthobranchs the

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Acochlidia are the most successful mesopsammic group, with currently 27 valid species (Wawra 1987; Sommerfeldt and Schrödl 2005). Among the most common species in the shallow subtidal sands of the Mediterranean is *Pontohedyle milaschewitchii* (Kowalevsky, 1901), with densities of more than 200 individuals per m^2 (Poizat 1984), reported from numerous collecting sites throughout the Mediterranean (e.g. Swedmark 1968b; Salvini-Plawen 1973) and from the Black Sea (Kowalevsky 1901). Correspondingly, *P. milaschewitchii* has been treated in several ecological papers (e.g. Hadl et al. 1969; Poizat 1984) and commonly considered a well-known acochlidian species (Arnaud et al. 1986). However, biological and anatomical knowledge was fragmentary, little detailed and hardly reliable. The original description by Kowalevsky (1901) mainly concentrated on external morphology and offered little data on the anatomy. Wawra (1986) supplied additional details of the reproductive system of this gonochoristic species. He described an intraepidermal vas deferens opening slightly dorsally of the mouth opening – which was the first report of a male genital opening between the oral tentacles in acochlidians – but he did not provide a complete revision of the male or female genital system. Up to now, the most detailed description of the anatomy of *P. milaschewitchii* was presented by Marcus and Marcus (1954), who examined a single male specimen from the coast of southern Brazil. On the basis of the latter description Rankin (1979) erected a new genus and species, *Gastrohedyle brasilensis*. However, Jörger et al. (2007) recently clarified the status of *G. brasilensis* as a junior synonym of *P. milaschewitchii* on a morphological basis. Molecular data will be necessary to determine whether or not Mediterranean, Black Sea and Atlantic *Pontohedyle* populations represent cryptic species.

The present study redescribes Mediterranean *Pontohedyle milaschewitchii* providing a detailed anatomical and histological revision of all major organ systems. Using computer-based three-dimensional (3D) reconstruction, we show how the anatomy of such diminutive yet complex animals can be accessed reliably and efficiently. We further discuss whether or not external SEM examination of entire specimens can provide an additional set of characters that may be useful for taxonomic and phylogenetic purposes.

Material and methods

Samples of coarse sand were taken by snorkeling in a depth range of 5–9 m at different collecting sites near Rovinj (Istria, Croatia) in June and September 2005. Specimens of *Pontohedyle milaschewitchii* were extracted from the samples following the method described by Schrödl (2006). Extracted specimens were slowly anaesthetized, using 7% isotonic $MgCl_2$ solution, to prevent them from retracting prior to and during fixation. Specimens used for semithin sectioning and SEM examination were fixed in 4% glutardialdehyde buffered in 0.2 M sodium cacodylate (0.1 M NaCl and 0.35 M sucrose, pH 7.2); specimens used for radula preparation were fixed in 75% ethanol. The glutardialdehyde-fixed specimens were rinsed in 0.2 M sodium cacodylate buffer (0.1 M NaCl and 0.35 M sucrose, pH 7.2), post-fixed in 1% OsO_4 buffered in 0.2 M cacodylate buffer (0.3 M NaCl, pH 7.2) for 1.5 h, and again rinsed in 0.2 M cacodylate buffer (0.3 M NaCl, pH 7.2). The fixed specimens were decalcified in ascorbic acid, dehydrated by a graded acetone series, and embedded in Spurr's (1969) low-viscosity epoxy resin for sectioning. The epoxy resin blocks were cut at 1.5 µm intervals with a rotation microtome (Microtom HM 360; Zeiss), using glass knives and contact cement at the lower cutting edge (Henry 1977), to receive ribboned serial sections. Four complete series were prepared and stained with methylene blue-azure II (Richardson et al. 1960). Computer-based 3D reconstruction of all major organ systems was performed with the software AMIRA 3.0 (TGS Template Graphics Software, Inc., USA). All sections have been deposited in the Zoologische Staatssammlung München (ZSM), Mollusca Section (ZSM Mol 20060522–20060525).

For SEM examination 20 glutardialdehyde-fixed specimens were dehydrated through a graded ethanol series followed by a graded acetone series. The specimens were critical-point dried in 100% acetone in a Baltec CPD 030. After mounting on SEM stubs with self-adhesive carbon stickers, the dried specimens were coated with gold in a Polaron Sputter Coater for 120 s. Seven ethanol-fixed specimens were used for SEM analysis of the radula. They were macerated up to 24 h in 10% KOH to separate the radula from the surrounding tissue. Remaining tissue was removed mechanically under a stereo microscope. Prepared radulae were rinsed in Aqua bidest and transferred to SEM stubs with self-adhesive carbon stickers. The radulae were coated with gold for 120 s (Polaron Sputter Coater). Scanning electron microscopic examinations were conducted using a LEO 1430VP SEM at 10–15 kV.

Results

External morphology and spicules

(Figs. 1, 2)

The body of *Pontohedyle milaschewitchii* is divided into a cylindrical anterior part (head–foot complex) and a posterior sac-like, elongated and broadened visceral

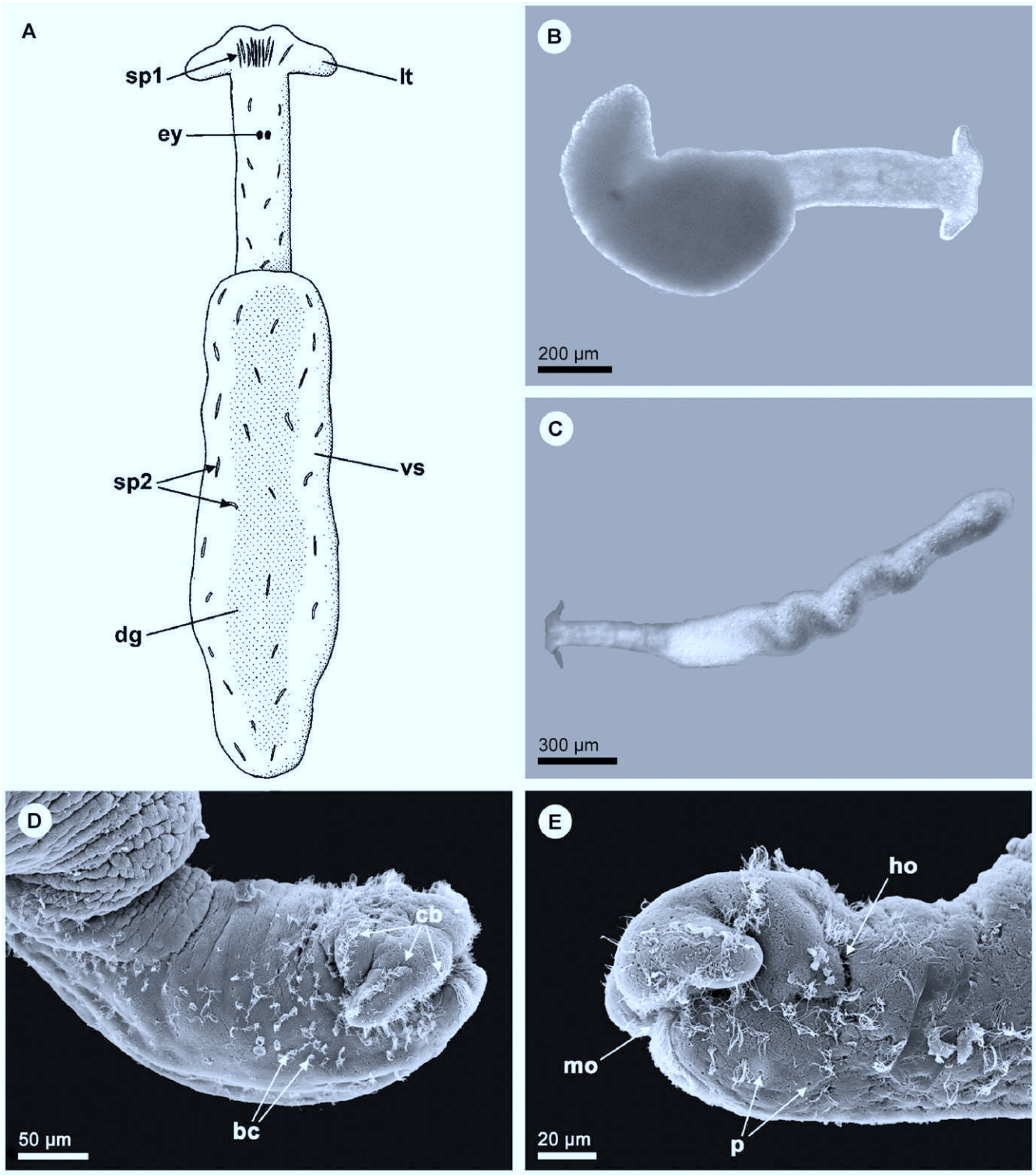

Fig. 1. External morphology of *Pontohedyle milaschewitchii*. (A) Semi-schematic drawing of an entire specimen, dorsal view. (B, C) Photographs of living specimens in dorsal view, showing range of variation in external morphology. (D, E) SEM micrographs of the head–foot complex. (D) Pattern of ciliation, dorsolateral view. (E) Hancock's organ, ventrolateral view. Abbreviations: bc = bundles of cilia, cb = ciliary band on head and tentacle, dg = digestive gland, ey = eye, ho = Hancock's organ, lt = labial tentacle, mo = mouth opening, p = pore of epidermal gland, sp1/2 = spicules of type I/II, vs = visceral sac.

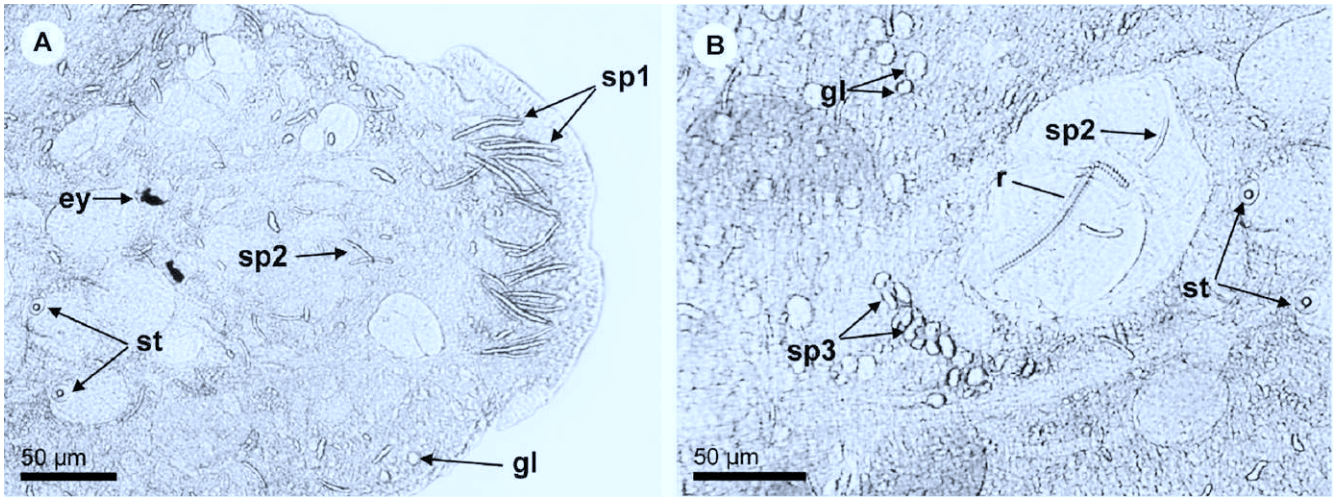

Fig. 2. Different types of spicules in *Pontohedyle milaschewitchii*. (A) Accumulation of large, needle-like spicules (type I) between oral tentacles. (B) Accumulation of small, oval spicules (type III) in posterior portion of pharynx. Abbreviations: ey = eye, gl = epidermal gland, r = radula, sp1–3 = spicules of types I–III, st = statocysts.

hump (Fig. 1A). The head–foot complex can be retracted into the visceral hump. Body length of extended mature specimens examined varied from 1.5 to 3.0 mm. Body coloration is whitish, transparent; the digestive gland is bright green to olive green. The ciliated foot is short (i.e. there is no free tail extending behind the head–foot complex); its posterior end is rounded. The head bears a pair of large, flattened oral tentacles. The shape of the oral tentacles is variable among specimens of a population, ranging from bow-shaped and curved to elongated and slightly triangular (Fig. 1B, C), and also varies depending on the stage of activity or contraction of the animal. Rhinophores are lacking completely. A pair of darkly pigmented eyes is located at approximately mid-length of the head–foot complex. An accumulation of parallel-oriented calcareous spicules occurs between the oral tentacles (Figs. 1A, 2A). The spicules are up to 40 µm long, 2 µm wide, and have a needle-like monoaxonic form (type I). Numerous monoaxonic spicules are also found irregularly distributed in the rest of the body, but smaller in size (length approximately 25 µm; type II). Spicules are embedded in the subepidermal mesenchyma. Oval to bean-shaped spicules (length about 10 µm; type III) are found in an aggregation in the posterior portion of the pharynx behind the radula (Fig. 2B).

SEM examination shows that the head–foot complex is covered laterally and in the anterior dorsal region with scattered bundles of cilia; the posterior dorsal region lacks cilia (Fig. 1D, E). On the dorsal and the anterior side of the oral tentacles run two 30 µm long and 3 µm wide ciliary bands. Another band with similar dimensions traverses the anterior dorsal region behind the oral tentacles (Fig. 1D). The visceral hump only bears a few scattered bundles in its anterior ventrolateral region; the remainder of the hump shows no ciliation. Overall, the density of cilia bundles varies among individuals, but the described pattern is always present.

Microanatomy

(Fig. 3)

The cavity of the head–foot complex contains the central nervous system (cns) and the anterior digestive organs (oral tube, pharynx, salivary glands and oesophagus). Ventral to the oral tube, around the central muscle strand, the large, bilobed anterior pedal gland extends. An unpaired duct connects the anterior pedal gland to the exterior, opening slightly ventral to the mouth opening (Fig. 3). Three strong muscle strands (one central and two lateral) extend through the head–foot complex, with the lateral strands leading far into the visceral hump. Additionally, a network of fine muscle fibres runs subepidermally in the body wall. A diaphragm separates the cavity of the head–foot complex from that of the visceral hump. The majority of the visceral hump cavity is filled with the digestive gland and the genital system (Fig. 3). Excretory and circulatory systems are located in the anterior right portion of the visceral hump. The anus opens on the right side of the visceral hump clearly behind the junction with the head–foot complex. Nephroporus and female gonopore open anterior to the anus on the right side of the head–foot complex, close to the junction with the visceral hump. The male genital system empties in the anterior-most region of the head–foot complex, just dorsal to the mouth opening.

Three different types of epidermal gland cells are present in *P. milaschewitchii*: (1) large (diameter about

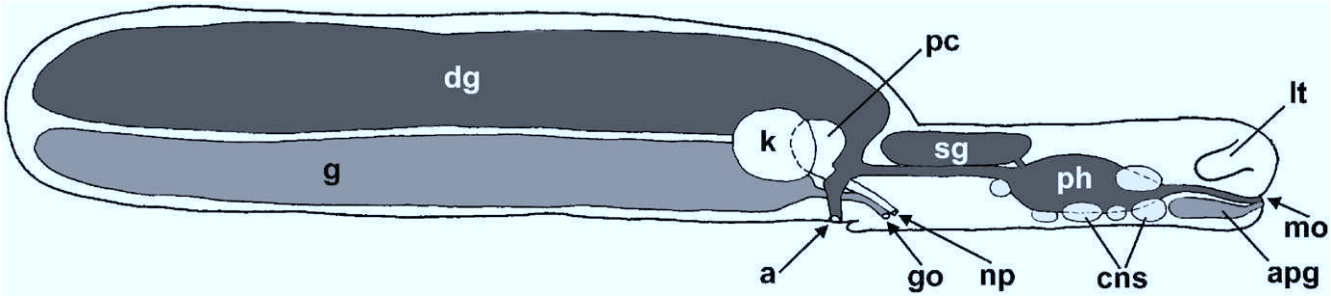

Fig. 3. Schematic overview of arrangement of internal organs in female *Pontohedyle milaschewitchii*, lateral view. White = excretory and circulatory systems, light grey = central nervous system, grey = genital system, dark grey = digestive system. Abbreviations: a = anal opening, apg = anterior pedal gland, cns = central nervous system, dg = digestive gland, g = genital system, go = genital opening, k = kidney, lt = labial tentacle, mo = mouth opening, np = nephropore, pc = pericardium, ph = pharynx, sg = salivary glands.

15 µm), spherical, whitish glandular cells (type I) forming a subepidermal sac and distributed on the head–foot complex and, in higher concentration, on the visceral hump (Fig. 4A); (2) small (5 µm), irregular-shaped ochre-colored glandular cells (type II); and (3) spherical cells (10 µm; type III) filled with dark blue-stained granules exclusively found in one row on the inner border of the visceral hump near the transition region to the head–foot complex (Fig. 4A). Dorsal to the ciliated foot sole numerous small (5–10 µm) pedal glands could be detected subepidermally, showing similar lilac staining properties as the anterior pedal gland (Fig. 4B).

Nervous system

(Figs. 5, 6)

The central nervous system (cns) of *P. milaschewitchii* consists of the paired cerebral, rhinophoral, pedal, pleural and buccal ganglia and three distinct unpaired ganglia on the short, euthyneurous visceral nerve cord (Fig. 5). Cerebral, rhinophoral and pedal ganglia are located prepharyngeally; the pleural ganglia in the anterior part of the pharynx, the ganglia of the visceral cord in its posterior part. Only the buccal ganglia are located postpharyngeally. The cns is epiathroid. The terms for the ganglia are used according to Schmekel (1985) and Haszprunar (1985), accessory ganglia are determined following Neusser et al. (2006).

Accessory ganglia

Many accessory ganglia in various sizes can be found in the anterior region of the cns of *P. milaschewitchii* (Fig. 6A). They are characterized as well-defined groups of cells showing homogenous distribution of nuclei (i.e. a lack of subdivision into cortex and medulla; see Fig. 6E), surrounded by relatively thin connective tissue. In *P. milaschewitchii* the accessory ganglia are arranged

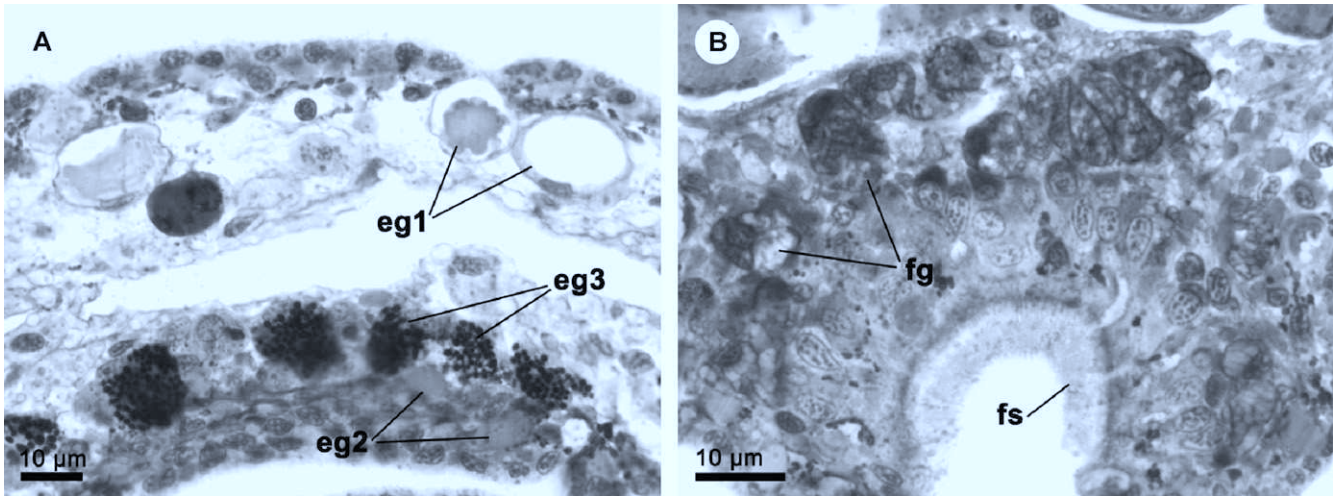

Fig. 4. Different types of glandular cells in *Pontohedyle milaschewitchii*. (A) Semithin cross-section of anterior region of visceral hump. (B) Semithin cross-section of foot. Abbreviations: eg1–3 = epidermal gland types I–III, fg = foot gland, fs = ciliated foot sole.

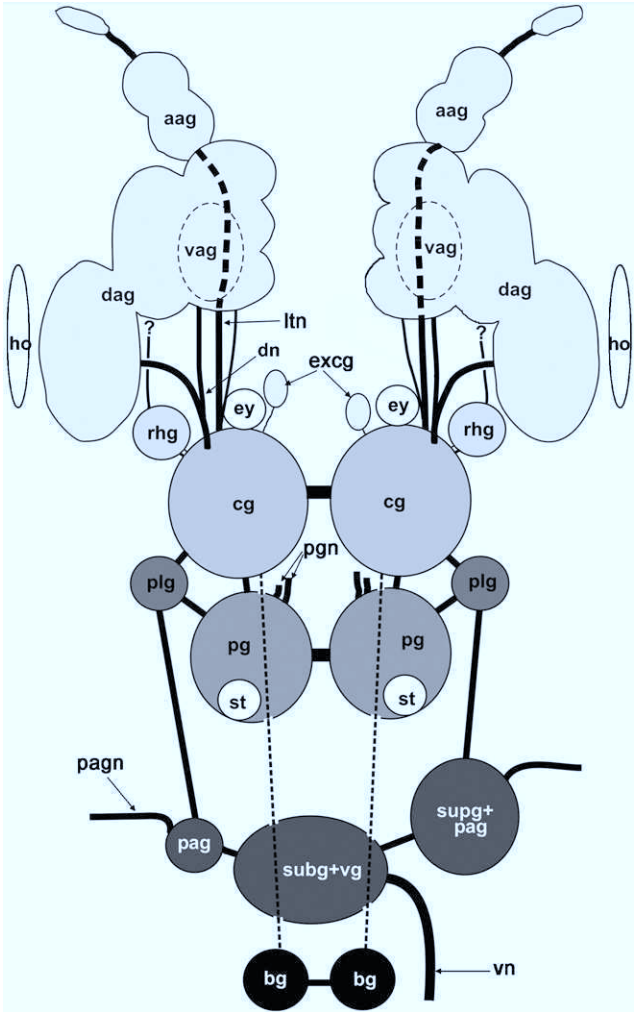

Fig. 5. Schematic of central nervous system (cns) in *Pontohedyle milaschewitchii*, dorsal view. Abbreviations: aag = anterior accessory ganglia complex, bg = buccal ganglion, cg = cerebral ganglion, dag = dorsolateral accessory ganglia complex, dn = dorsal nerve, excg = 'extra-cerebral accessory ganglion', ey = eye, ho = Hancock's organ, ltn = labiotentacular nerve, pag = parietal ganglion, pagn = nerve emerging from parietal ganglion, pg = pedal ganglion, pgn = nerve emerging from pedal ganglion, plg = pleural ganglion, rhg = rhinophoral ganglion, st = statocyst, subg = subintestinal ganglion, supg = supraintestinal ganglion, vag = ventral accessory ganglia complex, vg = visceral ganglion, vn = visceral nerve.

in three paired complexes: the anterior, the dorsolateral and the ventral accessory ganglia complex (Fig. 6C). Unfortunately, it remains unclear whether the dorsolateral accessory ganglia complex forms one continuous mass of accessory ganglia or should be subdivided into a dorsal and a lateral complex. Size and shape of the accessory ganglia complexes vary from individual to individual, and even within a single specimen between the right and left body sides.

The anterior accessory complex can be subdivided into the main complex, which is innervated by the strong labiotentacular nerve emerging ventrally from the cerebral ganglion, and a small accessory-ganglion-like swelling in the oral tentacle (Fig. 6C). A strong nerve connects the main complex with the swelling in the tentacle. At the cerebral base of the labiotentacular nerve a thinner nerve splits off and runs to the inner dorsal part of the dorsolateral accessory ganglion complex. Apart from this nerve the large dorsolateral accessory ganglia complex receives two more cerebral nerves, and most likely the nerve from the rhinophoral ganglion. The strong dorsal nerve emerges from an anterodorsal position of the cerebral ganglion. The nerve bifurcates at its cerebral base. The strong outer branch of the dorsal nerve innervates the lateral part, its thinner inner branch the dorsal part of the dorsolateral accessory ganglia complex (Figs. 5, 11A). No cerebral or other nerves innervating the ventral accessory ganglia complex could be detected. This comparatively small complex is located ventrally of the other accessory ganglia dorsolateral to the anterior pedal gland.

Additional very small (diameter 10 μm) nervous structures could be detected anterior to the cerebral ganglia. These swellings, in the following referred to as 'extra-cerebral accessory ganglia', vary in presence, number (one or two) and position from anterolateral to anterodorsal of the eyes (Fig. 6C). They are connected by a very short and thin connective to the cerebral ganglion. A thin nerve emerges from the 'extra-cerebral accessory ganglia' and runs anteriorly, probably leading to the dorsal part of the dorsolateral accessory ganglia complex.

Ganglia

A pair of large cerebral ganglia (diameter 70 μm) lies dorsally to the other ganglia and is connected by the strong and short cerebral commissure (Fig. 6B, D). Two pairs of connectives can be distinguished: Ventrally from the cerebral ganglia emerges the strong and relatively short cerebro-pedal connective. The very short cerebro-pleural connective emerges in the ventrodistal region of the cerebral ganglion. The cerebro-buccal connective could not be found.

Small rhinophoral ganglia (diameter 25 μm; for identification see Discussion below) are located anterolaterally on the cerebral ganglia. They are surrounded by a layer of connective tissue and by a second, thinner layer which they share with the cerebral ganglia (Fig. 6D). A clear division of the rhinophoral ganglia in cortex and medulla could not be detected, but on the basis of their general appearance (staining qualities, arrangement of nuclei and presence of a comparatively thick layer of connective tissue) they differ from accessory ganglia. The rhinophoral ganglia are connected to the cerebral ganglia by two extremely short

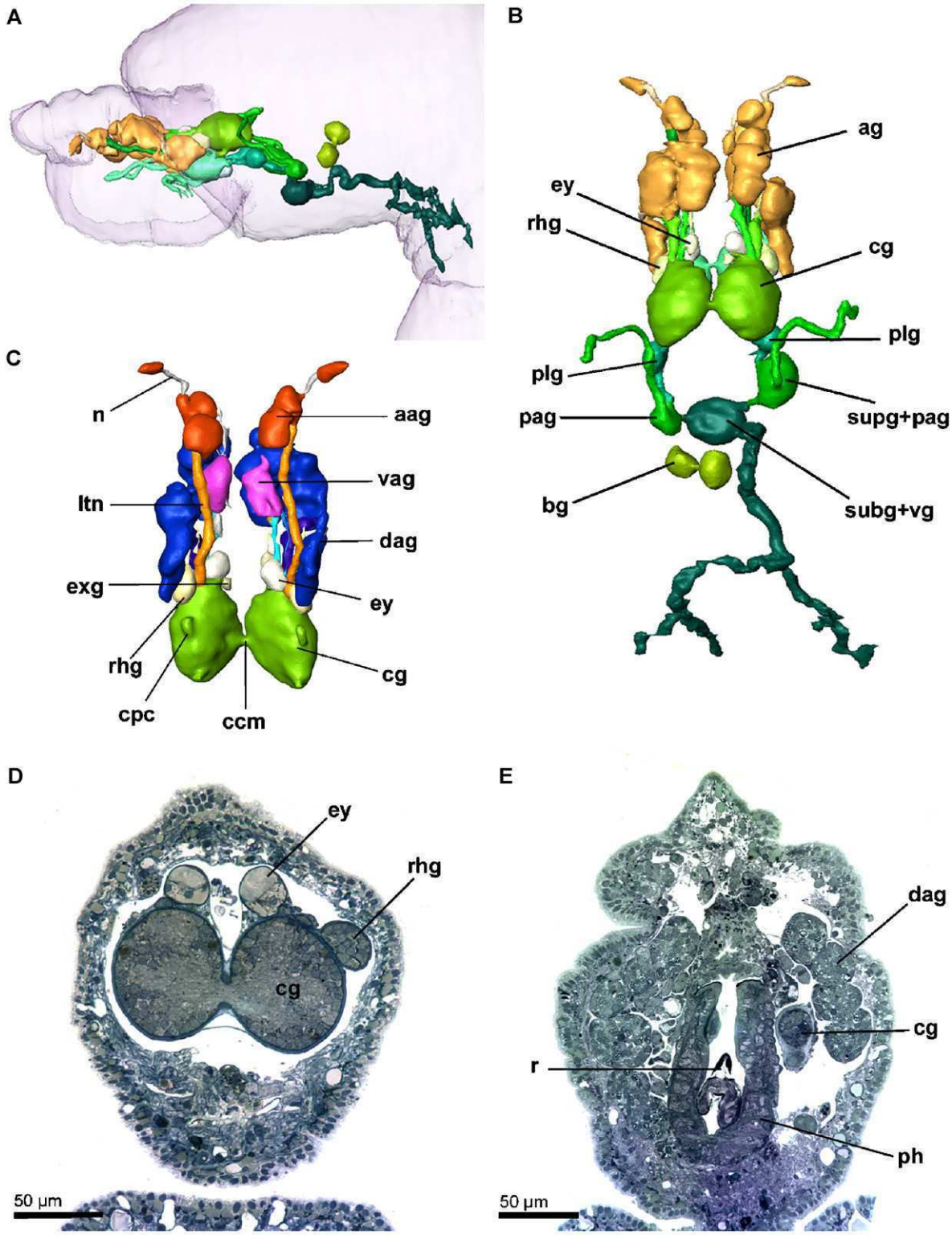

Fig. 6. Central nervous system (cns) in *Pontohedyle milaschewitchii*. (A) Overview of position of organ system in specimen, lateral view. (B) 3D reconstruction, dorsal view. (C) 3D reconstruction of innervation of accessory ganglia complexes, ventral view. (D, E) Horizontal semithin sections. (D) Cerebral ganglia with eyes. (E) Dorsolateral accessory ganglia complex. Abbreviations: aag = anterior accessory ganglia complex, ag = accessory ganglia, bg = buccal ganglion, ccm = cerebral commissure, cg = cerebral ganglion, cpc = cerebro-pedal connective, dag = dorsolateral accessory ganglia complex, exg = 'extra-cerebral accessory ganglion', ey = eye, ltn = labiotentacular nerve, n = nerve connecting parts of aag, pag = parietal ganglion, ph = pharynx, plg = pleural ganglion, r = radula, rhg = rhinophoral ganglion, subg = subintestinal ganglion, supg = supraintestinal ganglion, vag = ventral accessory ganglia complex, vg = visceral ganglion.

and thin connectives. A thin nerve leaves the rhinophoral ganglia anteriorly and runs along the dorsolateral accessory ganglia complex, most likely leading into the dorsal part of this complex.

The pedal ganglia are slightly smaller (diameter 60 μm) than the cerebral ganglia and located anteroventrally of those. They are connected by the strong and short pedal commissure. No parapedal commissure could be detected. The cerebro-pedal connective is clearly shorter than the pleuro-pedal connective. Two nerves leave each of the pedal ganglia: one runs in anterior direction and most probably innervates the anterior region of the foot, the other nerve runs to anterior as well, then twists to run backwards before turning to ventral and leading towards the posterior part of the foot.

The pleural ganglia are relatively small (diameter 25 μm) and are situated posteroventrally of the cerebral ganglia. The cerebro-pleural connectives are thin and very short; the pleuro-pedal connectives are slightly longer. Accordingly, the circumoral ring is quite narrow.

Three ganglia (for identification see Discussion) lie on the visceral cord: the right supraintestinal/parietal ganglion (40 μm), the subintestinal/visceral ganglion (55 μm), and the small left parietal ganglion (25 μm). The pleuro-supraintestinal/parietal connective is relatively short compared to the long pleuro-parietal connective on the left side of the visceral loop. A strong nerve emerges from each parietal ganglion dorsally, then passes laterally into the body wall of the visceral sac. The oval subintestinal/visceral ganglion is shifted slightly to the left side of the visceral cord. The supraintestinal/parietal-subintestinal/visceral connective is slightly longer than the parietal-subintestinal/visceral connective. The very strong, thick visceral nerve emerges laterally from the right side of the subintestinal/visceral ganglion and passes to posterior ventrally of the pharynx. Reaching the visceral sac the nerve bifurcates, with both parts running along the sides of the visceral sac.

Small buccal ganglia (diameter 25 μm) lie postpharyngeally, dorsolaterally of the pharynx-to-oesophagus transition. They are connected by a relatively long and thin commissure. One nerve leaves each of the buccal ganglia dorsolaterally, running laterally into the pharynx and passing through its epithelium in anterior direction. It is regarded as the cerebro-buccal connective. A radula nerve could not be detected. The presence of gastro-oesophagial ganglia could not be determined in the sectioned series.

Sensory organs

The subepidermal eyes nestle directly on the anterior surface of the cerebral ganglia (Fig. 6D). Each eye is approximately 20 μm long, oval, and forms a pigmented cup with a clear lens. The innervation of the eyes could not be detected using light microscopy. The eyes are surrounded by a thin layer of connective tissue which also surrounds cerebral and rhinophoral ganglia. The pair of statocysts is attached to the pedal ganglia at their posterior ends. The oval statocysts have a diameter of about 20 μm and contain one statolith each. The Hancock's organ is a pair of conspicuous folds in the epidermis just posterior to the oral tentacles (Fig. 1E). This organ is straight to bow-shaped, 60 μm long and 5 μm wide. The cells are non-glandular; some bear short cilia. A nerve innervating the Hancock's organ could not be fully ascertained, but innervation of the closely associated dorsal part of the dorsolateral accessory ganglia complex is likely.

Digestive system

(Fig. 7)

The mouth is located subterminally between the oral tentacles and leads into the oral tube. In its anterior part the thin epithelium of the oral tube is ciliated. The pharynx is bulbous and muscular; its tissue appears dark blue (in methylene blue-stained semithin sections) and folded. The entire radula lies in a radula sac in the center of the pharynx towards its posterior end (Fig. 7D). The radula is approximately 90–110 μm long, 20 μm wide, and bent to ventral in the anterior part. The dorsal part is about 2.5 times as long as the ventral part, which bears the older teeth. The number of rows in adult specimens varies between 41 and 54, 31–38 of them located on the dorsal ramus, 8–18 on the ventral one. The radula is symmetrical: each row consists of a central rhachidian tooth and one lateral plate on each side. Thus, the radula formula of *P. milaschewitchii* is 41–54 × 1-1-1. The rhachidian tooth consists of the central cusp and three lateral denticles on each side (Fig. 7C). The central cusp and the lateral denticles are triangular and slightly recurved. The lateral plates are thin, wide and slightly curved rectangular plates, each bearing one central triangular denticle (Fig. 7C). Each lateral plate has a matching notch on the anterior surface margin into which the denticle of the posterior plate fits. Jaws are absent.

The salivary glands are well developed and form a fused mass on the left side of the body (Fig. 7B). The mass fills large parts of the posterodorsal portion of the head–foot complex. Two ciliated ducts connect the salivary glands with the buccal mass laterally on the left and the right side of the transition between pharynx and oesophagus (Fig. 7D). The tube-like, ciliated oesophagus leaves the pharynx posterodorsally, connecting to the digestive gland and the intestine on the right side of the anterior part of the visceral hump. A histologically or anatomically differentiated stomach could not be detected. The digestive gland is holohepatic; it has an elongated sac-like shape with a number of bends and folds and extends over the entire length of the visceral hump (Fig. 7A). In adult specimens the digestive gland

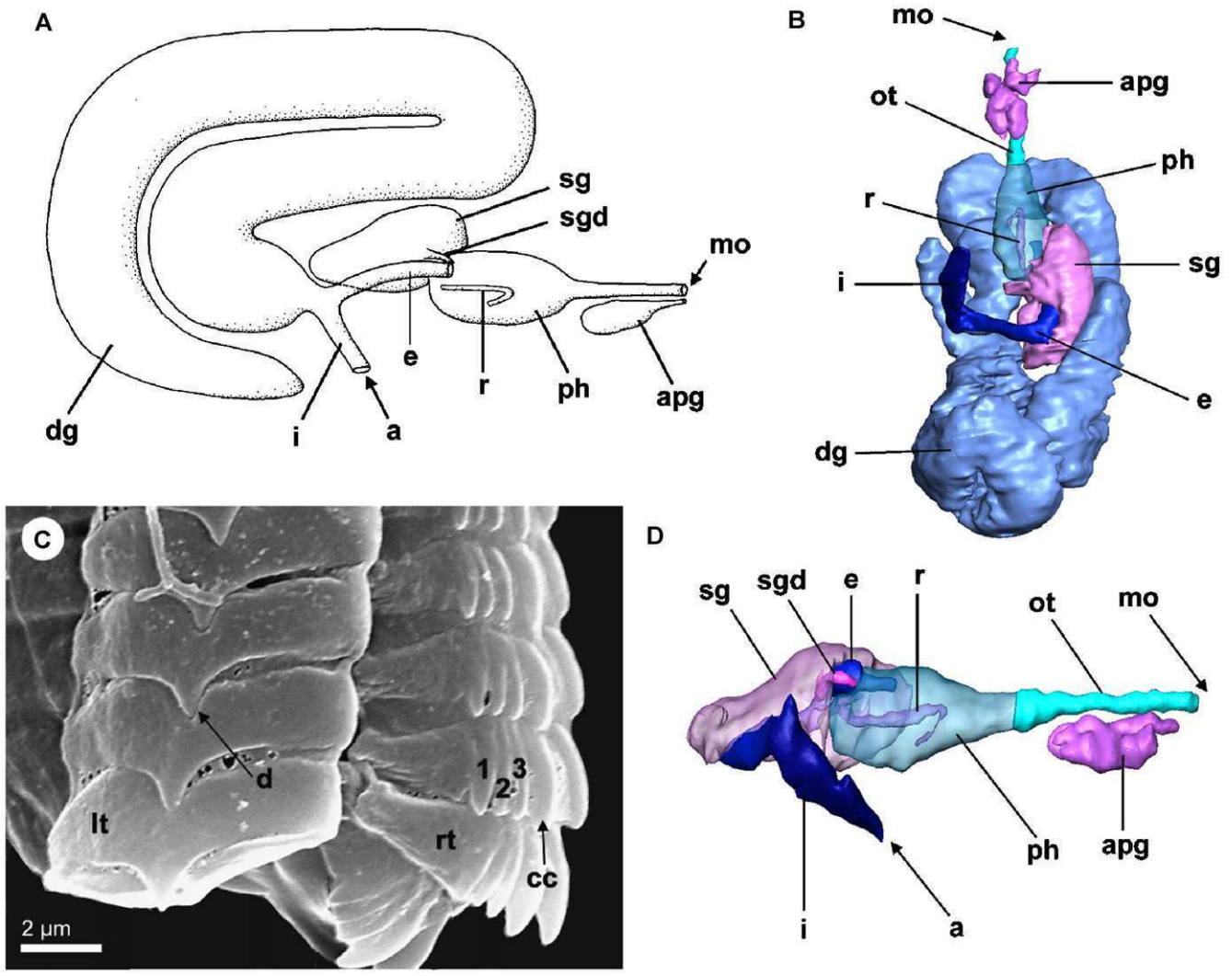

Fig. 7. Digestive system in *Pontohedyle milaschewitchii*. (A) Schematic overview, lateral view. (B) 3D reconstruction, ventral view. (C) SEM micrograph of right lateral and rhachidian tooth of radula. (D) 3D reconstruction, lateral view, digestive gland omitted. Abbreviations: a = anal opening, apg = anterior pedal gland, cc = central cusp, d = denticle, dg = digestive gland, e = oesophagus, i = intestine, lt = lateral tooth, mo = mouth opening, ot = oral tube, ph = pharynx, r = radula, rt = rhachidian tooth, sg = salivary glands, sgd = salivary gland duct, 1–3 = lateral denticles of rhachidian tooth.

is coiled around the gonad; its position seems to depend on the development of the gonads and the stage of contraction of the animal. The intestine is a relatively short, strongly ciliated tube. It leads to the anal opening, located on the right side of the visceral hump, clearly behind the transition from the head–foot complex to the visceral hump, and posterior to the female genital opening and the nephropore (Fig. 3).

Excretory and circulatory systems
(Fig. 8)

Excretory and circulatory organs are located on the anterior right side of the visceral hump (Fig. 8A); they comprise a reduced heart enclosed in a thin but relatively spacious pericardium, and a spherical kidney. The pericardium lies anteriorly to the kidney; in its posterior region it encloses the heart. The latter is a $40 \times 10 \times 10\,\mu m^3$ chamber (Fig. 8D); no subdivision into ventricle and auricle could be detected. Pericardium and kidney are connected via the very short but relatively wide renopericardial duct (Fig. 8C). No cilia could be detected in the duct's lumen. The renopericardial duct emerges laterally from the posterior end of the pericardium and enters laterally the lumen of the kidney. The slightly spherical kidney encloses the posterior third of the pericardium (Fig. 8B). The kidney (= nephridium, emunctorium) is characterized by a glandular and vacuolated epithelium. The nephroduct emerges ventrally and runs closely adjacent to the gonoduct. The nephropore opens anteriorly to the anus on the right side of the head–foot complex, close to the junction with the visceral hump. The position of

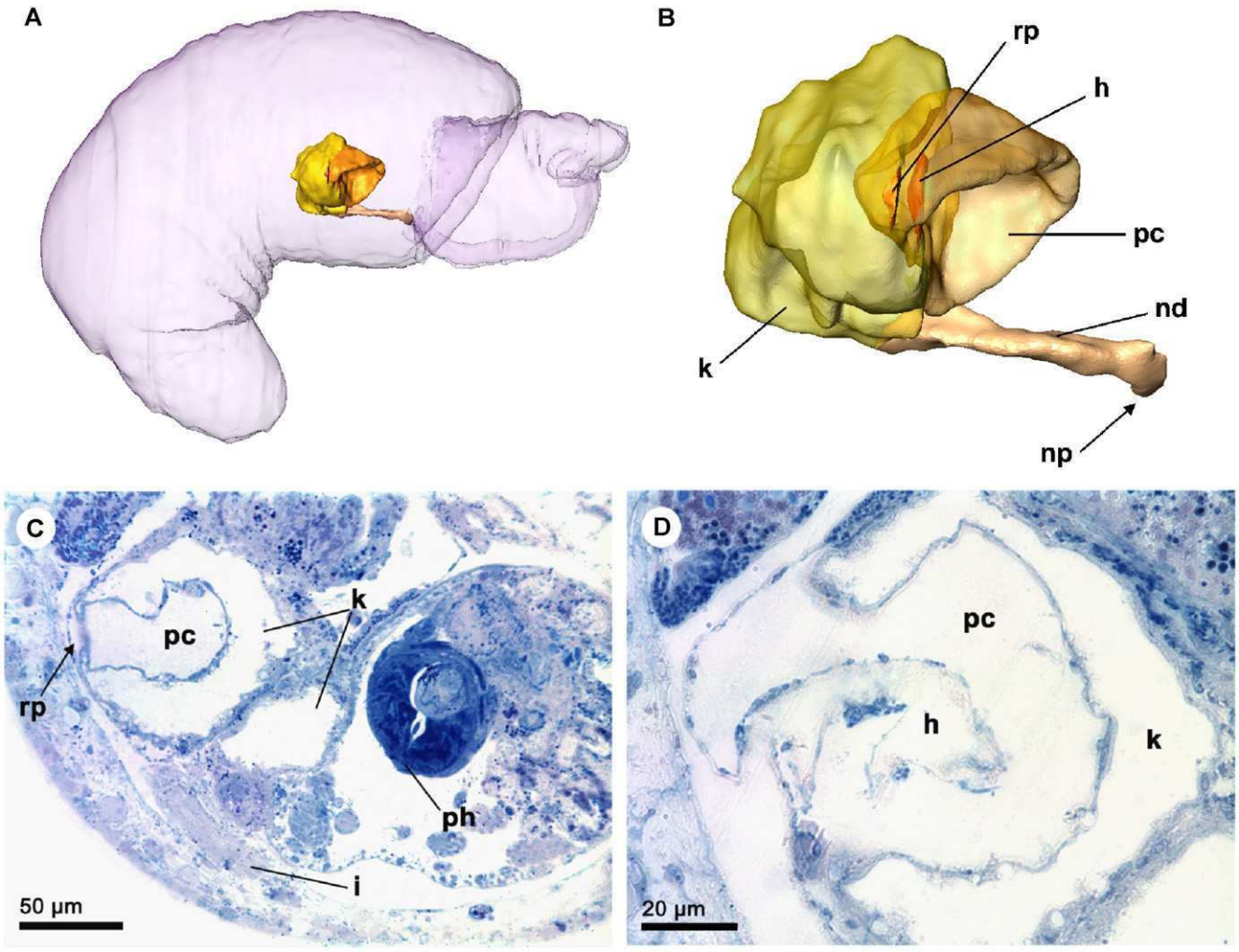

Fig. 8. Excretory and circulatory systems in *Pontohedyle milaschewitchii*. (A) Overview of positions of organ systems in specimen of 1.5 mm body length, lateral view. (B) 3D reconstruction, right-lateral view. (C, D) Semithin cross-sections. (C) Kidney and pericardium. (D) Heart. Abbreviations: h = heart, i = intestine, k = kidney, nd = nephroduct, np = nephropore, pc = pericardium, ph = pharynx, rp = renopericardial duct.

the nephropore relative to the female gonopore could not be determined, due to their close association and to poor histology in the sectioned series.

Reproductive systems

The sexes are separate in *P. milaschewitchii*. In adult specimens the reproductive system extends over the entire length of the visceral hump. The terminology used in the following description of the female and the male genital systems follows Ghiselin (1965) and Klussmann-Kolb (2001).

Female genital system

(Fig. 9)

The female reproductive system includes ovary, oviduct and nidamental glands. The sac-like ovary is closely associated with the digestive gland and extends over the entire length of the visceral sac. The ovary is loosely packed with oocytes in different stages of development: various large, vitellogenic oocytes (stage III) with a diameter of 60 µm, a series of smaller oocytes (20–30 µm; stage II), and oocytes in follicles with vitellus aggregating around them (stage I). Developing oocytes in stage II sometimes contained more than one nucleolus per nucleus (up to three nucleoli). All stage III oocytes observed had one nucleolus per nucleus only. No consistent pattern of distribution of eggs in various stages of development within the ovary could be determined. Exogenous sperm was not found in any of the sectioned females.

There are three nidamental glands connected directly to each other and apparently showing a continuous lumen throughout (Fig. 9A). No histologically or anatomically defined proximal oviduct or adhesive

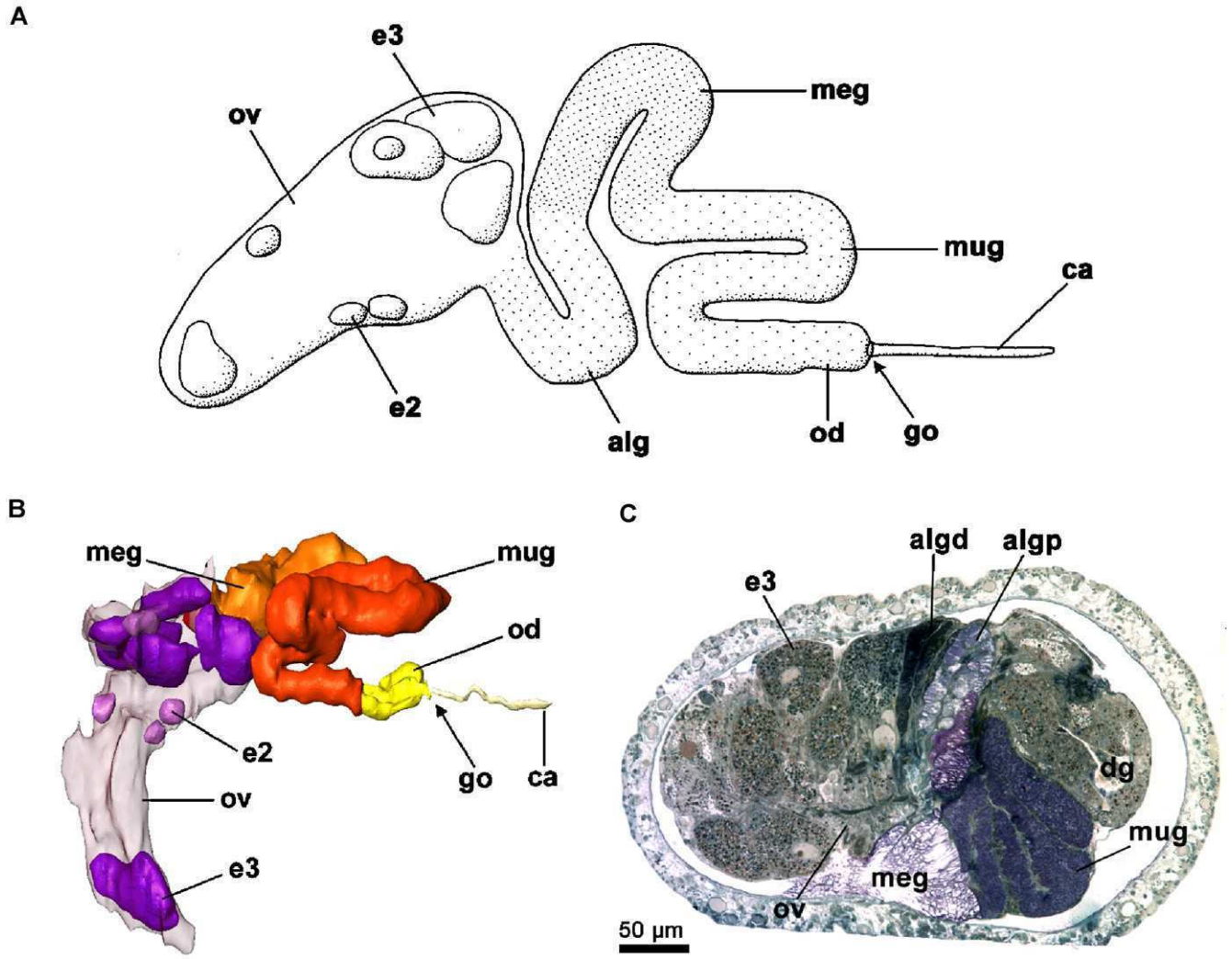

Fig. 9. Female genital system in *Pontohedyle milaschewitchii*. (A) Schematic overview, lateral view. (B) 3D reconstruction, right-lateral view. (C) Semithin cross-section of nidamental glands and ovary. Abbreviations: alg = albumen gland, algd = albumen gland distal part, algp = albumen gland proximal part, ca = ciliated area, dg = digestive gland, e2/3 = egg in stage II/III, go = genital opening, meg = membrane gland, mug = mucus gland, od = oviduct, ov = ovary.

region could be detected. The tube-like albumen gland is the smallest of the three nidamental glands. Histologically, it can be divided into a proximal and a distal part: the proximal part comprises elongated wedge-shaped secretory cells which contain a dense mass of very dark blue-stained granules; in the distal region the cells are of similar shape but do not contain granules and stain slightly purple (Fig. 9C). Over the entire gland, the epithelium bears relatively long cilia. The slightly larger membrane gland is also tube-like. Its secretory cells stain pinkish-purplish, have a glandular appearance, and contain large vacuoles. The epithelium of the membrane gland bears comparatively short cilia. The tube-like mucus gland is the largest of the nidamental glands and winds through the anterior part of the visceral hump (Fig. 9B). Its cells stain purple, contain few dark purple-stained granules, and are elongate oval in shape. The lumen partially widens to a size of $20 \times 40\,\mu m^2$; the epithelium is heavily ciliated, bearing long cilia. The distal oviduct emerges posteriorly from the mucus gland and at its beginning shows similar histology. The epithelium of the distal oviduct is ciliated. In its further course the distal oviduct loses the glandular appearance, as well as the purple staining. The duct runs ventrally of the kidney, closely adjacent to the nephroduct, and leads to the genital opening. In female *P. milaschewitchii* the genital opening is located anteriorly to the anus on the right side of the head–foot complex, close to the transition to the visceral hump. A ciliated band originates from the genital opening and runs along the right side of the head–foot complex (Fig. 9A). The band is about 15 µm wide and extends for

approximately one third of the length of the head–foot complex.

Male genital system

(Fig. 10)

The male genital system comprises the gonad and the gonoduct. The gonoduct can be divided from posterior to anterior into the ampulla, the prostatic vas deferens and the ciliated vas deferens (Fig. 10A).

The sac-like gonad is found closely associated with the digestive gland and extends over the entire length of the visceral sac (Fig. 10B). The gonad comprises various irregularly distributed groups of spermatozoids (Fig. 10E). The spermatozoids are elongated; their nuclei stain dark blue. The ampulla emerges from the anterior part of the gonad; no histologically or anatomically differentiated preampullary gonoduct could be detected. The tube-like ampulla has a diameter of about 50 µm and is bulging with sperm lying in disorder within the ampulla (Fig. 10E). A short post-ampullary gonoduct exists terminally of the ampulla. The epithelium of the post-ampullary gonoduct is thin, ciliated and bears a small lumen.

The vas deferens can be divided histologically and anatomically into a prostatic and a non-glandular section. The prostatic part has tube-like shape. Near its posterior end the diameter is about 25 µm; in its further course the prostatic part narrows to approximately 20 µm diameter. The prostatic vas deferens has elongate oval glandular cells which contain deeply pink-staining granules (Fig. 10E). Its epithelium bears long cilia. In the narrower part of the prostatic section no ciliation could be detected. The prostatic vas deferens passes to anterior on the right side of the body. In the posterior region of the head–foot complex it transforms into the non-glandular section of the vas deferens.

The non-glandular section of the vas deferens passes to anterior on the right side of the head–foot complex, slightly subepidermally. The duct has a diameter of about 10 µm; its epithelium is heavily ciliated. Before reaching the level of the oral tentacles it turns to dorsal. After passing the right oral tentacle it turns towards the midline of the head–foot complex. There it continues to the anterior tip of the head–foot complex (Fig. 10B, D). The male genital opening is located dorsally of the mouth opening (Fig. 10C).

Discussion

External morphology and spicules

All adult Acochlidia (as defined by Wawra 1987) are externally characterized by the absence of a shell and the presence of a visceral hump, which is axially elongated and clearly distinct from the head–foot complex, and into which the latter can be retracted at least partially. *Pontohedyle milaschewitchii* conforms to these general characteristics, but differs from the majority of other acochlidians in the lack of rhinophores.

We show that the shape of the oral tentacles is variable in *P. milaschewitchii*. This intraspecific variation caused some confusion in the past. Kowalevsky (1901) illustrated the oral tentacles of his Black Sea specimen with a curved bow-like shape. Marcus and Marcus (1954) described a *P. milaschewitchii* from Brazil with the oral tentacles "flat, triangular, the margins straight and angular." Challis (1970) suggested that because of the differences in the shape of the head (among others), the Brazilian specimen possibly represented a species different from Kowalevsky's. Rankin (1979) took this one step further by erecting the genus and species *Gastrohedyle brasilensis* based solely on the description by Marcus and Marcus (1954). Unfortunately, the type specimen of *Gastrohedyle brasilensis* from the Marcus collection seems to be lost, thus has not been available for re-examination (Jörger et al. 2007). The variation in the oral tentacles reported above, within our Mediterranean populations and even within single specimens, shows that this character by itself does not justify species separation. Flattened oral tentacles also occur in *Pontohedyle verrucosa* (see Challis 1970), and in the genera *Ganitus* (Marcus 1953) and *Hedylopsis* (e.g. Odhner 1937; Sommerfeldt and Schrödl 2005). Members of *Hedylopsis*, however, have significantly broader oral tentacles than those of *Pontohedyle*; in *Ganitus* the oral tentacles are not tapered towards the tip (Jörger et al. 2007). Thus, the quoted authors considered the flat, elongated to bow-shaped oral tentacles as a diagnostic feature for the genus *Pontohedyle*.

Subepidermal, calcareous spicules are a characteristic feature in many interstitial opisthobranchs, i.e. in *Rhodope, Helminthope* and most Acochlidia (Rieger and Sterrer 1975; Arnaud et al. 1986; Salvini-Plawen 1991). In *Asperspina* and *Hedylopsis* elongated and fairly long (up to 250 µm) needle-like spicules occur in high densities, forming dense covers and giving the visceral hump a stiff shape (e.g. Odhner 1937; Swedmark 1968a; Salvini-Plawen 1973; Morse 1976; Kudinskaya and Minichev 1978; Sommerfeldt and Schrödl 2005). In other acochlidians, only significantly smaller spicules (up to max. 40 µm) of various shapes (e.g. oval, plate-, star- or needle-like) occur randomly distributed in the tissue (e.g. Marcus 1953; Marcus and Marcus 1954; Challis 1968; Westheide and Wawra 1974; Neusser and Schrödl 2007), or spicules are lacking completely (Challis 1970; Neusser et al. 2006). Swedmark (1968b) assumed that densely arranged spicules as in *Hedylopsis* and *Asperspina* might serve the same protective purpose

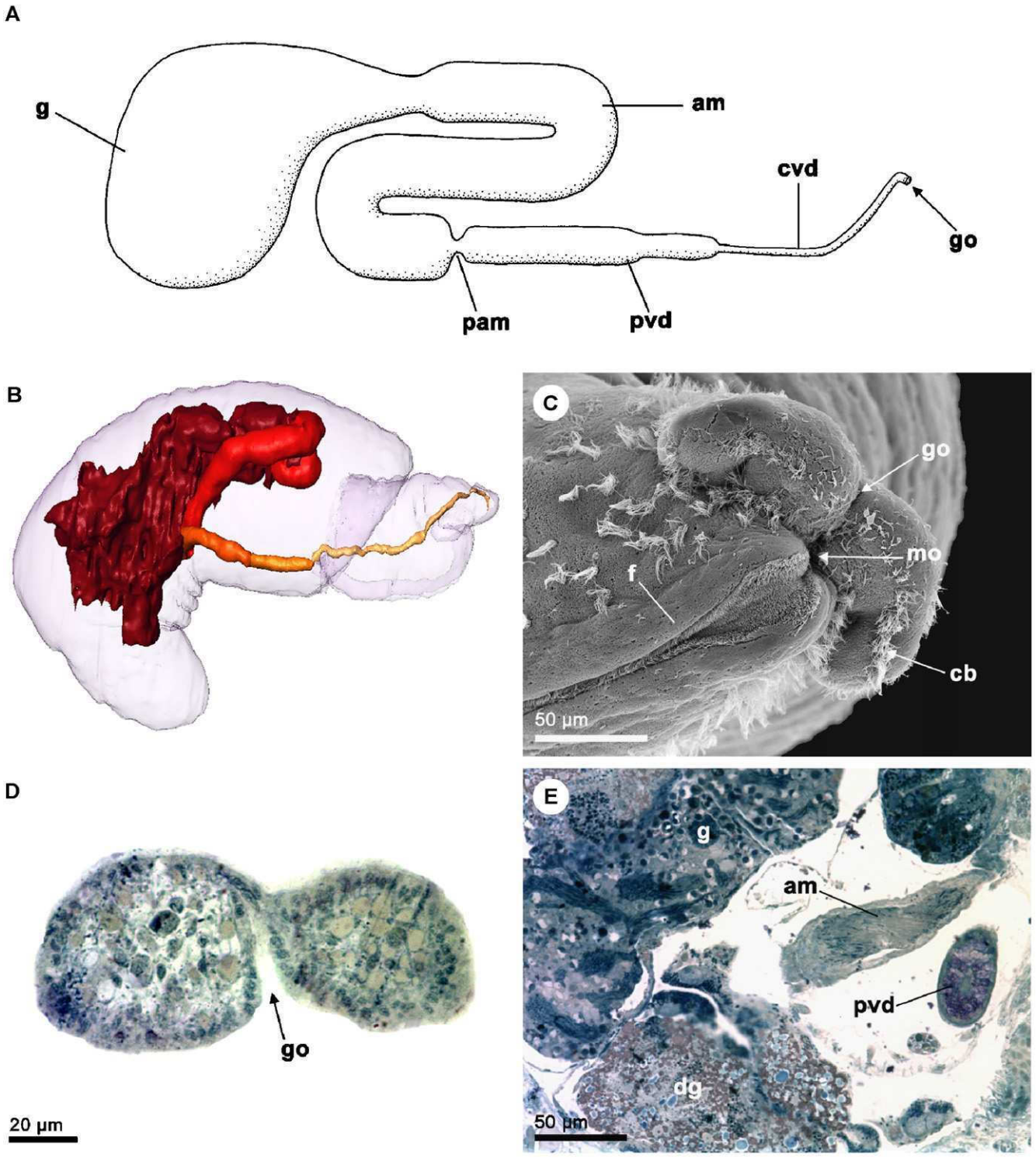

Fig. 10. Male genital system in *Pontohedyle milaschewitchii*. (A) Schematic overview, lateral view. (B) Overview of position of organ system in specimen of 1.5 mm body length, lateral view. (C) SEM micrograph of head showing male genital opening dorsally of mouth opening. (D) Semithin cross-section of male genital opening between oral tentacles. (E) Semithin cross-section of gonad and ampulla. Abbreviations: am = ampulla, cb = ciliary band on oral tentacle, cvd = ciliated vas deferens, dg = digestive gland, f = foot, g = gonad, go = genital opening, mo = mouth opening, pam = post-ampullary gonoduct, pvd = prostatic vas deferens.

as a shell. It is, however, unlikely that such a 'secondary spicule-shell' could resist direct wave action without being ground or smashed by the sand. Rigidly armored species also lose flexibility and might therefore prefer coarser sand and gravel habitats with larger interstices; in fact, most armored species are known from coarse subtidal sands. An exception is the polar *Asperspina murmanica*, which occurs in the intertidal (Kudinskaya and Minichev 1978), but this species' biological preferences and population densities in deeper sands are unknown. Spicule armor might offer some protection against potential interstitial predators such as polychaetes, considering that the head–foot complex can be retracted completely into the protected visceral hump. In contrast, loosely distributed, small spicules are unlikely to provide any special mechanical protection (Swedmark 1968b; Rieger and Sterrer 1975), but they allow higher flexibility and deformability of the body. 'Unprotected', flexible Microhedylidae, Ganitidae, and *Pseudunela* thus might be able to colonize finer sands, with higher mechanical energy, than their stiff counterparts. In addition to the poorly investigated, rigid *Asperspina murmanica*, the flexible *Parhedyle cryptophthalma*, *Ganitus evelinae*, *Paraganitus ellynae,* and *Pseudunela cornuta* (Westheide and Wawra 1974; Morse 1987; MS, pers. obs.) are the only acochlidians that occur in, prefer or even exclusively inhabit intertidal high-energy zones (i.e. sands directly exposed to wave action). The role of potential predators remains to be investigated.

What, then, are spicules good for in flexible species? Even small spicules may serve to stabilize the surrounding tissue or body region, especially when arranged in clusters (Rieger and Sterrer 1975). The aggregation of needle-like spicules between the oral tentacles of *P. milaschewitchii*, for example, might give the head additional stabilization while the animal is moving and digging between sand granules in the interstitial environment. Another conspicuous cluster of small oval or bean-shaped spicules (length around 10 µm) is found in *P. milaschewitchii* near the posterior end of the pharynx. Morse (1976) reported similar aggregations of irregular, amorphous spicules (measuring 15–38 × 9–12 µm) at the base of the buccal mass in *Asperspina riseri*. The function of these accumulations requires further investigation.

External SEM examination showed a conspicuous distribution of bundles of cilia in *P. milaschewitchii*. Although the density of the bundles varied between the specimens, a constant pattern could be detected, conforming to SEM micrographs of *P. milaschewitchii* published by Wawra (1986). Our preliminary SEM examinations of *Asperspina murmanica, Hedylopsis spiculifera* and *Paraganitus ellynnae* have revealed a unique overall ciliary pattern for each species. *Asperspina murmanica* shows constant ciliation over the entire head–foot complex and the anterior region of the visceral hump, with slightly more dense concentration of ciliary bundles on the rhinophores and oral tentacles. A similar pattern of cilia distribution was reported for *A. riseri* by Morse (1976). *H. spiculifera* shows an extremely dense ciliation over the entire head-foot complex, and randomly distributed cilia on the entire visceral hump, whereas *Paraganitus ellynnae* has only very few, scattered bundles of cilia in the anterior region of the head–foot complex.

Aside from the overall pattern, acochlidian species can be distinguished by special ciliated structures on the head appendages (two bands on the oral tentacles and one transverse band in *P. milaschewitchii*) or by ciliated areas originating from the gonopore. Differences occur also in the density and size of pores of epidermal gland cells. While the visceral hump of *P. ellynnae* is densely covered with large pores, *P. milaschewitchii* has fewer pores of various sizes, and *H. spiculifera* only some small pores.

These observations show that SEM examination can offer an additional set of external characters for taxonomic purposes that might also be of phylogenetic value. Thus, the method is recommended as the standard technique for describing acochlidian species. Suggested diagnostic characters for species are: (1) the general distribution pattern of cilia bundles on the head–foot complex and visceral hump; (2) the presence/absence, number and development of ciliary bands on the head appendages; (3) ciliated areas associated with the gonopore; and (4) the distribution, size and amount of pores of epidermal gland cells.

Microanatomy

The large anterior pedal gland in *P. milaschewitchii* accompanies the oral tube ventrally and discharges its mucus to the exterior via an opening slightly anterior of the mouth opening. Frequently in the past, similar glands in acochlidians have been termed "oral" or "vestibular" glands, e.g. by Challis (1970) in *Pontohedyle verrucosa* and *Hedylopsis cornuta* or by Doe (1974) in *Microhedyle nahantensis* (as *Unela*). However, the 'vestibular gland' of *M. nahantensis* has histochemical properties identical to those of the small pedal glands, shows no connection to the oral tube but instead an opening to the exterior ventral of the mouth opening, and thus was reinterpreted as an anterior pedal gland by Robinson and Morse (1976). Concerning the histology of the gland cells and the position of the gland, *P. milaschewitchii* closely resembles *M. nahantensis*. Robinson and Morse (1976) suggested that the mucous substances of the pedal glands in *M. nahantensis* play a role as a lubricant, aiding in locomotion and/or contributing to the ability to adhere to sand grains. A potential, perhaps additional role during feeding can

neither be suggested nor excluded in the absence of any knowledge on the food and feeding habits of acochlidians.

Nervous system

The cns of *Pontohedyle milaschewitchii* conforms to what has been shown recently for other acochlidian species (Sommerfeldt and Schrödl 2005; Neusser et al. 2006; Neusser and Schrödl 2007) concerning the high concentration, prepharyngeal position, and the euthyneurous and epiathroid condition.

Accessory ganglia

Neusser et al. (2006) defined accessory ganglia as distinct cell groups displaying a homogenous distribution of nuclei (i.e. without subdivision into cortex and medulla). Additionally, accessory ganglia can be characterized here as being surrounded by connective tissue that appears to be thinner than the one surrounding true ganglia. Several accessory ganglia on the anterior cerebral nerves are found in members of the Asperspinidae, Microhedylidae, Ganitidae, i.e. in 3 out of 6 families according to the classification of Wawra (1987). The latter author considered the lack of accessory ganglia as diagnostic for Hedylopsidae, Acochlidiidae and Tantulidae. Recently published data on *Hedylopsis ballantinei* and *H. spiculifera* (see Sommerfeldt and Schrödl 2005) agree with this assessment for the genus *Hedylopsis*. However, there are "some" accessory ganglia in the, according to Wawra, hedylopsid *Pseudunela cornuta* (see Challis 1970). Neusser and Schrödl (2007) reported aggregations of accessory ganglia in one examined specimen of *Tantulum elegans*, while there were no detectable accessory ganglia in other specimens. *Tantulum* was shown to be a truly sequential hermaphrodite, and the development or reduction of accessory ganglia may be related to preceding reorganizations at least of the reproductive organs. However, in the gonochoristic *P. milaschewitschii* accessory ganglia were present in all sections series (also in an early juvenile stage); therefore, their presence seems to be independent of the ontogenetic stage. No detailed data are available on cns features of the limnic *Strubellia*, also a sequential hermaphrodite, nor on any other Acochlidiidae.

In the present study the accessory ganglia of *P. milaschewitchii* could be grouped into three highly variable complexes. Marcus and Marcus (1954) recognized two groups of accessory ganglia in the Brazilian *P. milaschewitchii* ("tentacle ganglia" and "ganglia-like anterolateral groups of sensory neurons"), possibly referring to the anterior and the dorsolateral accessory ganglia complexes determined here. The ventral accessory ganglia complex is comparatively small and might have been overlooked by the earlier authors. The precerebral positions and cerebral innervation show obvious association of the accessory ganglia complexes to the cerebral ganglia, but the function of the accessory ganglia is still a matter of speculation. Haszprunar and Huber (1990) suspected the development of accessory ganglia in small opisthobranchs (e.g. *Platyhedyle* (Sacoglossa), *Philinoglossa* (Philinoidea)) to be a reaction to a lack of space for the neuronal tissue due to the small size of the animals, and to be a special adaptation to the interstitial environment. However, this does not explain why some similarly small acochlidians lack accessory ganglia, whereas the benthic runcinids, for example, also show precerebral nervous structures similar to accessory ganglia (Huber 1993). Immunocytochemical investigation and labeling against different neurotransmitters will be necessary to draw conclusions on the function of accessory ganglia and to determine whether certain groups of accessory ganglia are associated with certain sensory organs. Such an association can be suspected, e.g., for the anterior accessory ganglia complex in *P. milaschewitchii* with the oral tentacles and their associated ciliary bands, as well as for the accessory tentacle and rhinophoral ganglia in *Microhedyle remanei* (see Neusser et al. 2006). A possible neurosecretory function of the accessory ganglia should be investigated by TEM or ICC.

Cerebral nerves

The present examination of the cerebral nerves of *P. milaschewitchii* shows two strong bifurcating cerebral nerves (one emerging dorsally, one ventrally) and another thin nerve emerging from the rhinophoral ganglion (Fig. 11A). The static nerve could not be detected in *P. milaschewitchii*, but since statocysts are present, static nerves are assumed to be present as well. Edlinger (1980b) described three cerebral nerves for *P. milaschewitchii*: nerve 1 and 2 emerging anteriorly, nerve 3 laterally from the cerebral ganglion (Fig. 11B). A static nerve was assumed to be present, too. The most striking differences between the present study and Edlinger's (1980b) concern our findings of fully separate (rather than fused) cerebral and pleural ganglia and of a rhinophoral ganglion anterolateral of the cerebral ganglion. Instead, Edlinger (1980b) reported "nerve 3" in a posterolateral position and "with a lobe-like broadening" (see Fig. 11B). Since there is no such large, broadened nerve in this position, the "broadened nerve 3" of Edlinger (1980b) might correspond to the rhinophoral ganglion detected in the present study. The thin nerve emerging from the rhinophoral ganglion, however, does not resemble the splitting of "nerve 3" into several nerves illustrated by Edlinger. "Nerve 1" in Edlinger (1980b) clearly corresponds to the labiotentacular nerve of the present study (emerging ventrally). "Nerve 2" in Edlinger (1980b) corresponds to the strong bifurcating nerve emerging dorsally, here interpreted as

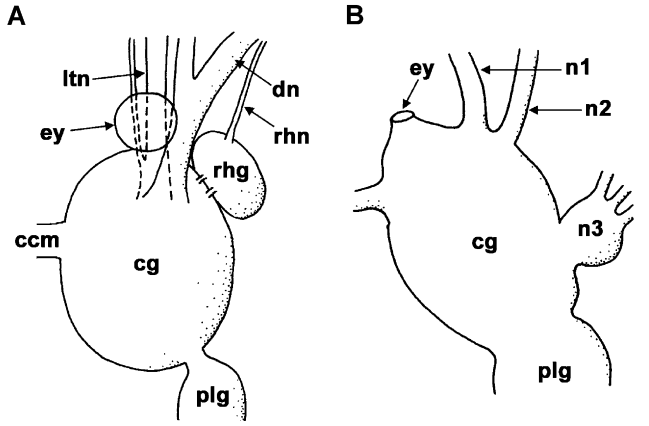

Fig. 11. Schematic drawings of cerebral nerve setting in *Pontohedyle milaschewitchii*; static nerve not shown. (A) Specimen from present study. (B) According to Edlinger (1980b, Fig. 5; as *Microhedyle milaschewitchii*). Abbreviations: ccm = cerebral commissure, cg = cerebral ganglion, dn = dorsal nerve, ey = eye, ltn = labiotentacular nerve, n1–3 = cerebral nerves 1–3, plg = pleural ganglion, rhg = rhinophoral ganglion, rhn = rhinophoral nerve.

a bifurcating oral nerve. Huber (1993) assumed a reduced number of cerebral nerves (labiotentacular, dorsal = fused rhinophoral/oral, and static nerves) as characteristic for Acochlidia, but overlooked a true rhinophoral ganglion in *Hedylopsis spiculifera* from which the dorsal nerve emerged (Sommerfeldt and Schrödl 2005). All acochlidians in which the cerebral nerves have been examined in detail share a strong ventral nerve that innervates the labial tentacles and thus is considered as the labiotentacular nerve (Sommerfeldt and Schrödl 2005; Neusser et al. 2006; Neusser and Schrödl 2007). Additionally, *Hedylopsis spiculifera*, *H. ballantinei* and *Tantulum elegans* possess true rhinophoral ganglia from which the strong rhinophoral nerve arises (Sommerfeldt and Schrödl 2005; Neusser and Schrödl, 2007). In *Hedylopsis* the rhinophoral nerve is joined with the thin optic nerve (Sommerfeldt and Schrödl, 2005), whereas in *Tantulum* the optic nerve emerges from an additional small optic ganglion and Hancock's nerve splits of from the rhinophoral nerve (Neusser and Schrödl, 2007). In *M. remanei* no true rhinophoral ganglion is present: the rhinophoral nerve emerges dorsally from the cerebral ganglion leading into an accessory ganglion (Neusser et al. 2006). An oral nerve could not be detected in any of these species. The nerve configuration in *P. milaschewitchii* is complicated by the numerous accessory ganglia, into which the cerebral nerves lead, making it difficult to determine which organs the cerebral nerves innervate. The (reduced) rhinophoral nerve in *P. milaschewitchii* probably leads into the dorsolateral accessory ganglion complex and might be involved with the innervation of Hancock's organ. However, the outer branch of the dorsal nerve also leads into this complex, and might therefore also innervate Hancock's organ. This would agree with Edlinger's (1980b) claim that "nerve 2" innervates Hancock's organ in *P. milaschewitchii*. But the author also stated that the situation in *Microhedyle glandulifera* is "similar" to *P. milaschewitchii*, with "nerve 2" also innervating the rhinophores. This seems questionable due to the presence of a true rhinophoral ganglion in *P. milaschewitchii*; reinvestigation of *M. glandulifera* is required. In general the settings and homologies of acochlidian cerebral features are far from being fully understood; comparative analyses of further acochlidians, related opisthobranchs and also pulmonates (see Neusser et al. 2007) could be facilitated by special histochemical or immunocytochemical techniques.

Ganglia

Rhinophoral ganglia in Acochlidia can be determined by their positions anterior to the cerebral ganglia, the cerebral innervation and by bearing the nerve innervating the rhinophores (Neusser et al. 2007). *Pontohedyle milaschewitchii* lacks rhinophores, but rhinophoral ganglia were recognized as such from their positions anterolateral to the cerebral ganglia and their cerebral innervation. Additionally, the rhinophoral ganglia of *P. milaschewitchii* are located in a second layer of connective tissue shared with the cerebral ganglia, as reported for the rhinophore-bearing *Tantulum elegans* (see Neusser and Schrödl 2007). In *P. milaschewitchii* the rhinophoral ganglia lack a clear division into cortex and medulla, but due to their general appearance (staining properties, arrangement of nuclei, and possession of relatively thick connective tissue) they are considered as ganglia rather than as accessory ganglia here. Accessory rhinophoral ganglia are known from the rhinophore-bearing *Microhedyle remanei* (see Neusser et al. 2006). More data are needed on different ontogenetic stages in *P. milaschewitchii*, and on related acochlidian species bearing rhinophores, in order to finally clarify the situation. A thin, double cerebro-rhinophoral connective has been detected for the first time in acochlidians. Neusser et al. (2007) found another double cerebro-rhinophoral connective in *Tantulum elegans* and pointed out that these tiny nerves can be overlooked easily or misinterpreted, thus might be present in other acochlidian species after all. Haszprunar and Huber (1990) also described a double cerebro-rhinophoral connective for *Rhodope veranii*; Huber (1993, figs. 9C and 10) showed a similar situation for, e.g. *Runcina adriatica* and *Philinoglossa praelongata*. With the double cerebro-rhinophoral connection the acochlidian rhinophoral ganglion and those of other opisthobranch groups show a condition similar to that in the pulmonate procerebrum (Van Mol 1967). Therefore, further study addressing the possibility of homology is needed.

Rankin (1979) concluded from the small, semi-schematic drawings by Kowalevsky (1901, figs. 46, 48) of an entire specimen of *P. milaschewitchii* that the pleural ganglia are fused with the cerebral ganglia. Edlinger (1980b) also illustrated these ganglia to be fused in his investigation of the cerebral nerve setting of *P. milaschewitchii* (Fig. 11B). However, the results of the present study clearly show that the pleural ganglia in *P. milaschewitchii* are fully separate from the cerebral ganglia. Cobo Gradin (1984) reported fused cerebro-pleural ganglia for *Asperspina loricata*; no pleural ganglia whatsoever had been mentioned in the original description by Swedmark (1968a). Huber (1993) considered the non-fused pleural ganglia as a characteristic feature in acochlidians and, indeed, all well-described acochlidian species show non-fused pleural ganglia (Neusser and Schrödl 2007). Accordingly, the cns of *A. loricata* requires reinvestigation.

Pontohedyle milaschewitchii has three distinct ganglia on the visceral cord. According to the pentaganglionate hypothesis of the nervous system of euthyneurans (Haszprunar 1985; Schmekel 1985), the basal condition shows five ganglia on the visceral cord: left parietal, right parietal, subintestinal, visceral, and supraintestinal ganglion. Following this hypothesis, two of the five ganglia must have either undergone fusion or been lost in *P. milaschewitchii*. While the left ganglion on the visceral cord of *P. milaschewitchii* reaches only about the size of the pleural ganglia, the median ganglion on the loop attains about double that size, and the right ganglion is only slightly smaller than the median one. Therefore, it can be assumed that the right parietal ganglion has fused with the supraintestinal ganglion, and the visceral ganglion with the subintestinal ganglion. Thus, the ganglion arrangement on the visceral cord in *P. milaschewitchii* resembles the one reported from *Microhedyle remanei* (see Neusser et al. 2006). It is also similar to those of *Hedylopsis ballantinei* and *H. spiculifera* (see Sommerfeldt and Schrödl 2005), with the only difference that the *Hedylopsis* species have an additional, 'osphradial' ganglion connected to the supraintestinal/parietal ganglion. Pattern differences exist mainly with the limnic *Tantulum elegans*, described with four separate ganglia on the visceral cord and an additional (probably penial) ganglion attached to the fused supraintestinal/parietal ganglion (Neusser and Schrödl 2007).

The cns of *P. milaschewitchii* reported here resembles the one described by Marcus and Marcus (1954) from their Brazilian specimen, except as follows: Marcus and Marcus (1954) detected only two ganglia on the visceral loop (determined as the median subintestinal/visceral ganglion and the supraintestinal ganglion on the right side), but a third one is indicated in their plate 26, fig. 13. It can be assumed that the authors overlooked the left parietal ganglion due to its small size and vicinity to the pedal ganglia, just like it probably had happened before in *Microhedyle remanei* (Marcus 1953 versus Neusser et al. 2006). Moreover, Marcus and Marcus (1954) indicated that the ganglia of the visceral cord are located close to the entrance of the pharynx rather than in the posterior region of the pharynx. However, in their plate 26, fig. 13, these ganglia seem to be located near the midline of the pharynx. Possibly, this slight shift to anterior is due to retraction or bending of the animal.

Sensory organs

Edlinger (1980a) first mentioned the presence of a paired Hancock's organ for *P. milaschewitchii* and described it as an "irregular system of folds" situated laterally at the anterior head–foot complex. In a second publication, Edlinger (1980b) referred to Hancock's organ in *P. milaschewitchii* as an "irregular system of folds, lying in a lateral furrow". No system of folds could be detected in the present study; but judging from the described position it can be assumed that Edlinger referred to a conspicuous fold in the epidermis just posterior to the oral tentacles. Edlinger (1980b) described the cerebral nerves 2 and 3 (Fig. 11B) as innervating Hancock's organ. In the specimens we examined, no nerves could be detected as leading directly to the potential Hancock organ. However, an innervation by the closely associated dorsal part of the dorsolateral accessory ganglia complex is likely.

The ciliary bands on the oral tentacles and across the head of *P. milaschewitchii* most likely also have a sensory function. No distinct nerves could be detected, but an innervation by the anterior accessory ganglia complex (which innervates the tentacles) is likely for the bands on the oral tentacles. Due to the more posterior position of the transverse ciliary band, the latter could be innervated by either the anterior or the dorsolateral accessory ganglia complex. Because of its rhinophore-like position and probable sensory function this band might be interpreted either as a (homologous) relic of the rhinophores or as a convergently developed substitute. Such a transverse ciliary band is absent in the examined rhinophore-bearing *Paraganitus ellynnae*, which only bears ciliary bands on oral tentacles and rhinophores. Additional SEM examination of other rhinophore-lacking species, such as *Pontohedyle verrucosa* and *Ganitus evelinae*, is necessary.

Digestive system

According to Marcus and Marcus (1954), the radula of their Brazilian specimen of *P. milaschewitchii* was symmetrical, with the radula formula $44 \times 2\text{-}1\text{-}2$. However, their drawings (op. cit., pl. 26, figs. 16, 17) show an almost identical radula configuration as the present

SEM examination. Thus, it can be assumed that the authors only misinterpreted the central denticle of the lateral plate as separation in the lateral plates. This has probably also been assumed by Wawra (1987), who defined the genus *Pontohedyle* with a radula formula of 1-1-1. The radula of *P. verrucosa* closely resembles the one of *P. milaschewitchii*, concerning both the radula formula (43 × 1-1-1) and the assemblage of the rhachidian tooth bearing three lateral denticles (Challis 1970). It differs, however, in the lack of a central denticle on the lateral plate (for comparison of the different *Pontohedyle* species, see Jörger et al. 2007). In sacoglossans the tooth size frequently increases with age (Jensen 1997), unlike in *P. milaschewitchii* where tooth size is uniform throughout. In *P. milaschewitchii* the entire radula lies in a radula sac in the pharynx, a condition also differing from the sacoglossan-typical ascus containing the descending limp (Jensen 1997).

A histologically and anatomically differentiated stomach could not be detected in specimens of *P. milaschewitchii* studied here. Marcus and Marcus (1954) described a spacious, spherical stomach in their Brazilian specimen, but this can be interpreted as an artefact resulting from fermenting stomach contents (Jörger et al. 2007).

The digestive gland in acochlidians is usually sac-like in shape (Rankin 1979). In some species the digestive gland reaches a length which makes internal folding within the visceral hump necessary for the digestive gland to fit into the cavity, as described for, e.g., *Microhedyle glandulifera* (see Salvini-Plawen 1973; as *M. glomerans*). A similar long, holohepatic digestive gland with internal folding has been observed for *P. milaschewitchii*. However, in some living specimens a conspicuously elongated visceral hump could be detected; in these cases the digestive gland could be observed as an unfolded sac (see Fig. 1C). Therefore, it can be assumed that folds of the digestive gland highly depend on the stage of contraction of the animals and cannot be seen as a constant character. This is supported by Marcus' (1953) observations of folded as well as unfolded digestive glands in *Ganitus evelinae* and *Microhedyle remanei*.

Excretory and circulatory systems

The reduced single-chambered heart of *P. milaschewitchii* found here was overlooked in previous studies (Kowalevsky 1901; Marcus and Marcus 1954). Similar hearts have been reported for *Hedylopsis spiculifera* (see Kowalevsky 1901), *Pseudunela cornuta* (see Challis 1970; as *Hedylopsis*) and *Tantulum elegans* (see Rankin 1979). However, the revision of *T. elegans* by Neusser and Schrödl (2007) detected a two-chamber heart, as also reported for *Microhedyle remanei* (see Neusser et al. 2006) and *Hedylopsis ballantinei* (see Fahrner and Haszprunar 2002; as *Hedylopsis* sp.). The detection and determination of the assemblage of the thin-walled hearts is difficult using light microscopy. Therefore, TEM re-examination of single-chambered hearts, and especially of acochlidians originally described as heartless, e.g. of *Ganitus evelinae* (see Marcus 1953) or *Parhedyle tyrtowii* (see Kowalevsky 1901), should be attempted. A grouping of the acochlidians based on the development of the excretory and circulatory systems, as globally stated by Rankin (1979), remains doubtful until reliable data exist.

Reproductive system

Most opisthobranchs are simultaneous or protandric hermaphrodites (Schmekel 1985). Uniquely within the opisthobranchs some gonochoristic species occur within the Acochlidia (among others *P. milaschewitchii*).

Female genital system

The female genital system in *P. milaschewitchii* basically resembles the ancestral form of the female genital system in Opisthobranchia as hypothesized by Ghiselin (1965), but differs in the lack of any sperm-storing structures (bursa copulatrix or receptaculum seminis).

Due to the similar histological, histochemical and ultrastructural characteristics, Klussmann-Kolb (2001) supposed the three nidamental glands to be homologous throughout the opisthobranchs, although the albumen gland might be modified into a capsule gland. Following this hypothesis and studies on other acochlidian species (Neusser et al. 2006; Neusser and Schrödl 2007), the nidamental glands in *P. milaschewitchii* were identified from proximal to distal as albumen, membrane and mucus gland. The albumen gland was termed as such due to its proximal position, its tube-like shape and the lack of internal folding (Klussmann-Kolb 2001). In contrast to *M. remanei* with a sac-like albumen and mucus gland (Neusser et al. 2006), the nidamental glands of *P. milaschewitchii* are all tube-like and show a continuous lumen throughout. The pattern of ciliation (albumen gland: relatively long cilia; membrane gland: short cilia; mucus gland: long cilia) also differs from *M. remanei*, with long cilia in the membrane gland but no cilia in the mucus gland (Neusser et al. 2006). However, the positions of the nidamental glands, their staining properties and histology (e.g. no internal folding in the albumen gland) are similar in the two species.

The genital pore in female *P. milaschewitchii* is located anteriorly of the anus, at the posterior end of the head–foot complex close to the transition to the visceral hump (Wawra 1986; present study). In contrast,

Kowalevsky (1901) originally described the female genital pore in *P. milaschewitchii* as located in the anterior region of the pharynx. Wawra (1986) suggested that this difference probably results from mobility of the internal organs (i.e. their positions depending on the stage of retraction). This is very unlikely, however, with regard to the relative positions of the genital (and other) openings. Wawra (1986) described a ciliated band in the female specimens of *P. milaschewitchii* originating from the genital pore, extending anteriorly to about one third of the length of the head-foot complex. This observation could be confirmed here from serial sections of female specimens. Similar ciliated areas have been reported from *Ganitus evelinae* (see Marcus 1953), *Paraganitus ellynnae* (see Challis 1968), *Hedylopsis ballantinei* (see Sommerfeldt and Schrödl 2005), and from *M. nahantensis* where such an area extends from the genital opening to the right rhinophore (Morse 1994). A transport function during egg deposition seems to be likely for the ciliated area (Wawra 1986), but observations *in vivo* are lacking.

Male genital system

The male genital system of *P. milaschewitchii* basically conforms to the hypothetic ancestral form of male portions of hermaphroditic opisthobranch genital systems according to Ghiselin (1965). Differences concern the reduction of the anterior genital organs in *P. milaschewitchii*, the absence of a copulatory apparatus, and sperm transfer taking place via spermatophores (Wawra 1992). While copulatory organs are present in many hermaphroditic acochlidians, e.g. in *Acochlidium fijiense* (see Haase and Wawra 1996), *Pseudunela cornuta* (see Challis 1970) and *Tantulum elegans* (see Neusser and Schrödl 2007), a reduction of the male anterior genital organs is common in gonochoristic species, e.g. in *Parhedyle cryptophthalma* (see Westheide and Wawra 1974), *Ganitus evelinae* (see Marcus 1953) and *Microhedyle remanei* (see Neusser et al. 2006), as well as in some hermaphrodites such as *Hedylopsis ballantinei* (see Sommerfeldt and Schrödl 2005).

In contrast to the unusual, frontal male genital pore observed in the present study, Marcus and Marcus (1954) described the genital pore in their Brazilian specimen as located on the right side of the head–foot complex close to the transition to the visceral hump. A ciliated vas deferens was not described, but the anterior part of the animal could not be sectioned because it was used for radula preparation. Thus, the authors had no possibility to detect the ciliated vas deferens. Either they observed an ontogenetic stage with a posterior genital opening, or they may have simply assumed the presence of a male genital opening in its usual posterior position in microhedylids (Jörger et al. 2007).

According to Ghiselin (1965) and Haszprunar (1985) the ancestral male reproductive system in opisthobranchs includes an open, ciliated seminal groove, which connects the pallial gonoduct with the copulatory apparatus. Sommerfeldt and Schrödl (2005) considered the open ciliary sperm groove as a plesiomorphic condition within the Acochlidia, even though the copulatory apparatus can be reduced. It may be assumed that a ciliated vas deferens evolved from the ciliated sperm groove by submerging into the epidermis and forming a closed tube (Ghiselin 1965). The ciliated part of the vas deferens has been described first in *P. milaschewitchii* by Wawra (1986), who termed it the "intraepidermal duct". However, it is a fully closed subepidermal duct attached to the epidermis and running towards the right side of the head. Similar ciliated male sperm ducts with a cephalic male genital opening have been described for the hermaphroditic *Pseudunela cornuta* by Challis (1970), and recently for *Tantulum elegans* by Neusser and Schrödl (2007). In both species the vas deferens opens on the level of the right rhinophore, and both bear a cephalic penis. Because of similar position and structure, homology between the ciliated vas deferens in the gonochoristic *P. milaschewitchii* and the hermaphroditic species is likely. The anterior part of the vas deferens in *P. milaschewitchii* entering the head cavity may be homologous to the backwards-leading part of the vas deferens in hermaphroditic species as well; like all aphallic acochlidians *P. milaschewitchii* can be assumed to have lost the associated glands. The unique anterior position of the male genital opening in *P. milaschewitchii* may be an adaptation to a more rapid and better-directed spermatophore transfer to a mate; frontal sperm transfer might be an advantage over a more lateral one, especially in an interstitial environment. The sensory oral tentacles might play a role in positioning of the spermatophore or in recognition of a potential spermatophore receiver. It can also be speculated that the mucous substances from the anterior pedal gland might be involved in attaching the spermatophore to other specimens.

Recent redescriptions of tiny acochlidian species (Neusser et al. 2006; Neusser and Schrödl 2007) have underscored the need for close and careful revision of primary data in order to gain a reliable and rich data set for phylogenetic analysis. The present study shows that even in a common and putatively well-known species, such as *Pontohedyle milaschewitchii*, reinvestigation of the anatomy with computer-based 3D reconstruction is rewarded with new and detailed results. To put these in a broader perspective the virtually unknown biology and ontogeny of this enigmatic opisthobranch group need to be revealed.

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3.3 Jörger KM, **Neusser TP** & Schrödl M 2007. **Re-description of a female *Pontohedyle brasilensis* (Rankin, 1979), a junior synonym of the Mediterranean *P. milaschewitchii* (Kowalevsky, 1901) (Acochlidia, Gastropoda).** *Bonn zoological Bulletin* **55**(3/4): 283-290.

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Re-description of a female *Pontohedyle brasilensis* (Rankin, 1979), a junior synonym of the Mediterranean *P. milaschewitchii* (Kowalevsky, 1901) (Acochlidia, Gastropoda)*

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Abstract. Currently 27 species are considered to be valid in the still enigmatic opisthobranch group of the Acochlidia. The taxonomic status of the acochlidian species, *Pontohedyle brasilensis* (Rankin, 1979), remained unclear due to a lack of primary data. The present study provides the first structural and some histological data on a female *P. brasilensis* from northern Brazil. The female genital system is reconstructed 3-dimensionally from serial semithin sections using AMIRA software. Our new results are compared with published data on a male *P. brasilensis* from southern Brazil and on *P. milaschewitchii* (Kowalevsky, 1901) from the Mediterranean and Black Sea; in absence of morphological differences we consider *P. brasilensis* as a junior synonym. The genus *Pontohedyle* thus comprises two valid species, the Atlantic/Mediterranean *P. milaschewitchii* and the tropical Indopacific *P. verrucosa* (Challis, 1970). Both possess unique bow-shaped, flattened oral tentacles, which are diagnostic for the genus and, thus, a probable autapomorphy.

Keywords. Mollusca, Opisthobranchia, taxonomy, morphology, anatomy, 3D reconstruction.

1. INTRODUCTION

Currently, 27 nominal acochlidian species are considered to be valid (SOMMERFELDT & SCHRÖDL 2005) which were conventionally classified into 12 different genera in 6 families (WAWRA 1987). All acochlidians have a characteristic shell-less body with a head-foot complex that can be at least partly retracted into a more or less elongate visceral hump. Most species belong to tiny members of worldwide coastal mesopsammic communities, while others are inhabitants of brackish waters or even limnic (see NEUSSER & SCHRÖDL 2007). Uniquely within the usually hermaphroditic opisthobranchs, microhedylid acochlidian species have separate sexes. While most acochlidians have two pairs of cephalic tentacles, a few gonochoristic species lack any rhinophores, i.e. of the genera *Ganitus* Marcus, 1953 and *Pontohedyle* Golikov & Starobogatov, 1972. There are three nominal *Pontohedyle* species: the tropical Indopacific *P. verrucosa* (Challis, 1970), the Atlantic/ Mediterranean species *P. milaschewitchii* (Kowalevsky, 1901), and the Atlantic *P. brasilensis* (Rankin, 1979) with uncertain taxonomic status.

Pontohedyle milaschewitchii was originally described from the Black Sea (KOWALEVSKY 1901) and later found throughout the Mediterranean (see HADL et al. 1969; JÖRGER et al. in press; POIZAT 1984; WAWRA 1986). Additionally, MARCUS & MARCUS (1954) described one single male specimen of *P. milaschewitchii* from Ilhabela (São Paulo State), the coast of southern Brazil. Solely based on that literature information, RANKIN (1979) established the new genus and species *Gastrohedyle brasilensis* and separated it from the Mediterranean *P. milaschewitchii* (as *Mancohedyle*); her diagnosis of *P. milaschewitchii* then was limited to the original description by KOWALEVSKY (1901). ARNAUD et al. (1986) listed *Gastrohedyle brasilensis* as *Pontohedyle brasilensis* with a question mark, and WAWRA (1987) regarded it as a probable synonym of *P. milaschewitchii*, however without giving any discussion on an entire set of putative external and internal morphological differences that were raised by RANKIN (1979).

Unfortunately, anatomical information of *P. brasilensis* is restricted to a single male specimen. This type specimen of *P. brasilensis* has not been discovered in the Marcus' collection of the Museu de Zoologia da Universidade de São Paulo (C. Magenta, São Paulo, pers. comm. 2006), and thus appears to be lost. Specimens of *Pontohedyle* from Brazil remain very rare. Even after exhaustive search at the original location, MARCUS & MARCUS (1954) were not able to rediscover further specimens. We conducted collections at Ilhabela, the type locality of *P. brasilensis*, along the coast of Santa Catarina, Paraná and São Paulo State, southern Brasil, and at many sites in Pernambuco

and Paraíba, northern Brazil. This search only resulted in two specimens, one of them usable for histological analysis.

The present study provides the first structural and histological data on a female *Pontohedyle* from northern Brazil. The taxonomy of *P. brasilensis* is revised by critically comparing our results with the published data on *P. brasilensis* from Brazil and *P. milaschewitchii* from the Mediterranean.

2. MATERIAL AND METHODS

Two *Pontohedyle* specimens were extracted from sand samples (see SCHRÖDL 2006 for method of extraction), collected by scuba diving on the northern coast of Brazil (approx. 5 km off Porto de Galinhas, at 20 m depth) in January 2004. One retracted and damaged specimen was used for molecular analysis. The posterior part of the visceral hump of the second specimen was also damaged. The specimens were slowly anaesthetised using 7 % isotonic $MgCl_2$ solution and fixed in 75 % ethanol. The preserved specimen used for histological analysis in the present study was transferred into Bouin solution for decalcification and afterwards stained with 0.5 % safranin. Then it was dehydrated by a graded acetone series and embedded in Spurr´s low viscosity epoxy resin (SPURR 1969) for sectioning. The epoxy resin block was cut at 1.5 µm with a rotation-microtome (Microtom HM 360; Zeiss), using glass knives and contact cement at the lower cutting edge (HENRY 1977) to receive ribboned serial sections. The sections were stained with methylene blue-azure II (see RICHARDSON et al. 1960). Computer based 3D reconstruction of the female genital system was performed with the software AMIRA 3.0 (TGS Template Graphics Software, Inc., USA). The section series was deposited in the Zoologische Staatssammlung München (ZSM), Mollusca Section (ZSM Mol 20041037). For morphological and anatomical comparison, serial sections and 3D reconstructions of five individuals of *P. milaschewitchii* from the Mediterranean were used (ZSM Mol 20060522-20060525).

3. RESULTS

3.1. External morphology and spicules

Our examined living Brazilian specimen used for structural analysis, showed the usual body shape of marine interstitial acochlidians, with a cylindrical anterior head-foot complex that is completely retractable into a broadened and elongated visceral hump. The crawling individual measured approximately 2 mm, but the visceral hump was damaged. The overall body coloration was whitish, with the brownish digestive gland shining through the tissue. The oral tentacles were bow-shaped and curved, rhinophores were lacking (see Fig. 3A). The ciliated foot was short, i.e. there was no free tail extending behind the head-foot complex, and its posterior edge was rounded. Monoaxone (i.e. needle shaped) spicules (about 25 µm length) were found randomly distributed over the head-foot complex and visceral hump. Additionally, an accumulation of parallel orientated monoaxone spicules was detected between the oral tentacles. Light microscopic investigation of the sectioned head region indicates cilia on the anterior border of the head and oral tentacles.

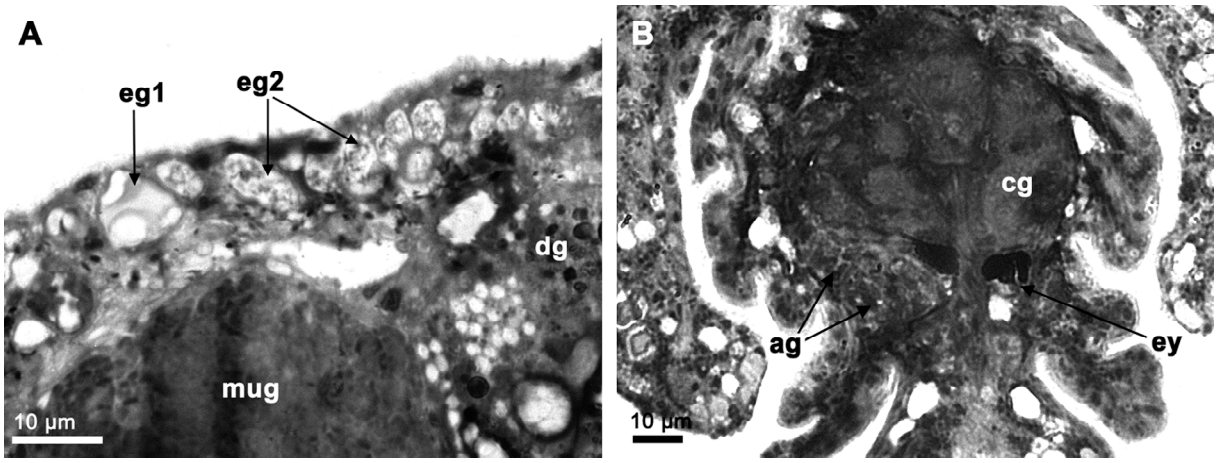

Fig. 1. Semithin sections of the female *Pontohedyle* from Brazil. (A) Cross-section of the visceral hump, showing the epidermis and the epidermal gland cells. (B) Horizontal section of the central nervous system. Abbreviations: ag accessory ganglia, cg cerebral ganglia, dg digestive gland, eg1 epidermal gland type I, eg2 epidermal gland type II, ey eyes, mug mucous gland.

3.2. Microanatomy

The bad condition of the only Brazilian specimen available for structural analysis did not allow detailed histological investigations of all major organ systems. Nevertheless, a brief treatise is given on the recognisable organs, focusing on the female genital system which could be reconstructed from serial sections.

3.2.1. Epidermal glands

The epidermis contains large spherical glandular cells (5-10 µm in diameter). They form a sub-epidermal sac, which is more or less filled with a homogenous whitish secretion (= epidermal glands type I, see Fig. 1A). Smaller vacuoles could be detected in the epidermis containing pinkish to violet stained granular material. These vacuoles occur in large numbers (= epidermal glands type II, see Fig. 1A).

3.2.2. Central nervous system

Praepharyngeal, large oval cerebral ganglia (approximately 50 µm), smaller pedal ganglia (approximately 30 µm) and groups of accessory ganglia could be detected. The cerebral ganglia are connected by a very strong and short commissure. A pair of dark pigmented eyes (diameter about 12 µm) nestles on the anterior side of the cerebral ganglia (see Fig. 1B). Groups of accessory ganglia are located anteriorly and laterally of the cerebral ganglia. Different from true ganglia true ganglia, accessory ganglia are well defined cell groups with a homogenous distribution of nuclei and without subdivision into cortex and medulla (NEUSSER et al. 2006). Here the accessory ganglia are more or less spherically shaped and grouped together like pearls on a chain (Fig. 1B).

3.2.3. Digestive system

The mouth opening is located subterminally between the oral tentacles. The thin walled oral tube is collapsed. The muscular pharynx extends in the posterodorsal part of the head-foot complex and contains the radula in its posterior region. The salivary glands form one mass on the left side of the head-foot complex, slightly extending into the visceral sac. The cells of the salivary glands contain dark blue stained granules. The tube-like oesophagus leaves the pharynx posterodorsally and connects to the digestive gland in the anterior region of the visceral hump. There is no histologically or anatomically detectable stomach. The digestive gland extends over the length of the remaining visceral hump and extrudes through the ruptured epidermis. It is sac-like in shape and its cells contain small dark blue and red stained granules. The epithelium of the digestive gland bears a series of small whitish and oval vacuoles. Neither the intestine nor the anal opening could be detected due to the bad condition of the animal.

3.2.4. Excretory and circulatory systems

Only the kidney could be detected. It is triangular in shape and squeezed in between the digestive gland and the body wall on the right side of the anterior region of the visceral hump. The epithelium of the kidney is characterized by its usual vacuolated structure.

3.2.5. Female genital system

The examined individual is a mature female, recognisable by the presence of vitellogenic oocytes in the ovary. The female genital system is composed of the ovary, the nidamental glands and the oviduct (Fig. 2A, B). The ovary extruded through the ruptured epidermis of the visceral sac and was partially falling apart. Nevertheless, seven large vitellogenic oocytes are still *in situ* (Fig. 2F). The oocytes are comprised of a nucleus containing one nucleolus and yolk (characterised by dense aggregations of blue stained granules). The oocytes reach a diameter of about 50–60 µm. The albumen gland is tube-like in shape and its secretory cells are stained dark blue to dark violet (Fig. 2E). The secretory cells are alternated by supporting cells, which bear cilia. The membrane gland is comparably large and tube-like in shape. Its secretory cells are stained pinkish with glandular appearance and containing vacuoles (Fig. 2D). The supporting cells bear cilia. The long tube-like mucous gland runs parallel to the digestive gland in the anterior region of the visceral sac. The supporting cells of the mucous gland are also ciliated and the secretory cells are stained dark violet. The three nidamental glands connect directly to each other (i.e. without any defined proximal oviduct or adhesive region, see Fig. 2A). The distal ciliated oviduct (Fig. 2C) ventrally passes the digestive gland and leads to the right anterior region of the visceral hump. The genital opening is located on the right side of the body, at the transition from the head-foot complex to the visceral hump. A short ciliated band originates at the genital opening. It has a diameter of about 10 µm and runs anteriorly along the right side of the head-foot complex.

4. DISCUSSION

According to WAWRA (1987), acochlidians belonging to the genus *Pontohedyle* share microhedylid features such as having separate sexes and lacking copulatory organs. *Pontohedyle* species were characterized by the absence of rhinophores and a radula formula of 1.1.1. The combination of these features is unique among acochlidians, but may refer to plesiomorphies. The special shape of

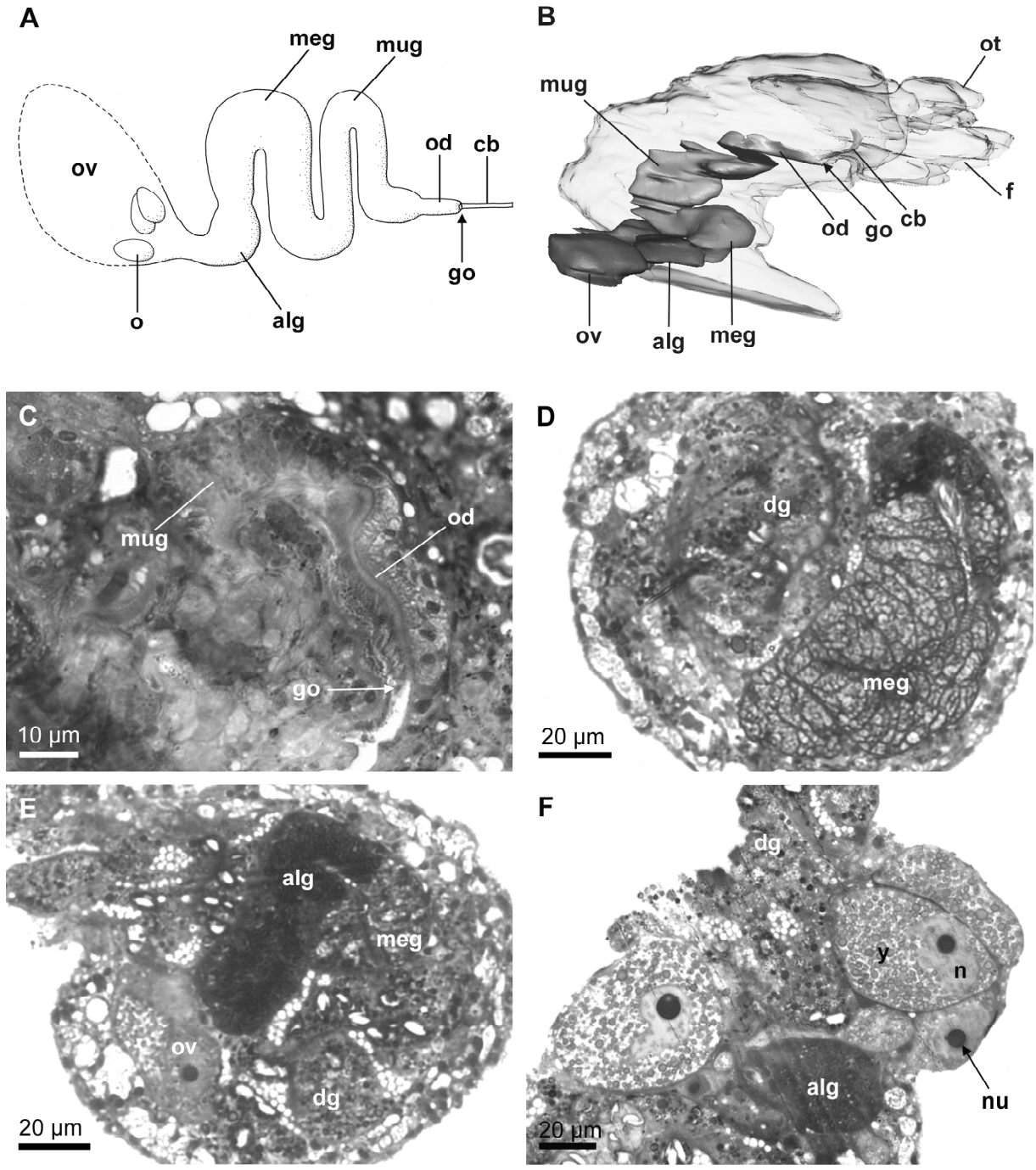

Fig. 2. Female genital system of the *Pontohedyle* from Brazil. (A) Schematic overview, lateral view. (B) 3D reconstruction, lateral right view, specimen retracted, approximately posterior half of visceral sac and gonad missing (ruptured and therefore not reconstructed). (C) Semithin cross-section of oviduct and genital opening. (D) Semithin cross-section of membrane gland. (E) Semithin cross-section of the transition from albumen gland to membrane gland. (F) Semithin cross-section of mature oocytes. Abbreviations: alg albumen gland, cb ciliary band, dg digestive gland, f foot, go genital opening, meg membrane gland, mug mucous gland, n nucleus, nu nucleolus, o oocyte, od oviduct, ot oral tentacle, ov ovary, y yolk.

acochlidian oral tentacles may provide more phylogenetic information. Apart from species of the genus *Pontohedyle,* flat oral tentacles only occur in the genera *Hedylopsis* and *Ganitus.* While the oral tentacles of the *Hedylopsis* are much broader than those of *Pontohedyle* (see Fig. 3F), the ones of *Ganitus* appear similar. *Ganitus* can be differentiated since the oral tentacles are never tapered towards the end (see Fig. 3E). In fact, the flat, elongated to bow-shaped oral tentacles of *Pontohedyle*, which are tapered towards the end (see Fig. 3A-D), are unique and diagnostic, and thus, a probable autapomorphy of *Pontohedyle*.

WAWRA (1987) regarded two *Pontohedyle* species as being valid, the tropical Indopacific *P. verrucosa* (Challis, 1970) and the temperate *P. milaschewitchii* (Kowalevsky, 1901). RANKIN (1979) however, established an additional species *P. brasilensis* on the basis of a literature description of a single male specimen from Brazil. Table 1 compares potential distinguishing features of all three nominal *Pontohedyle* species, including the results of the present study on the female Brazilian specimen and the specimens of *P. milaschewitchii* from the Mediterranean used for comparison.

4.1. External morphology and spicules

Externally, the investigated specimen from Brazil confirms with the general acochlidian characters (e.g. visceral hump in which the head-foot complex can be at least partially retracted; see WAWRA 1987) and those of the genus *Pontohedyle* (lack of rhinophores). Using external characters, RANKIN (1979) differentiated *P. brasilensis* from *P. milaschewitchii* by referring to the flat triangular versus bow-shaped oral tentacles, and the absence or presence of cilia on head and oral tentacles. However, the shape of the oral tentacles is variable within specimens of Mediterranean *P. milaschewitchii* (see JÖRGER et al. in press). They vary from bow-shaped to elongated triangular, including the flat and triangular form described by MARCUS & MARCUS (1954) for the Brazilian specimen (see Fig. 3B). Already MARCUS & MARCUS (1954) illustrated that the tentacles can have a more rounded tip (see fig. 13, 14). This character clearly varies for one individual, depending on the contraction of the animal (see Fig. 3D: *P. verrucosa* with supposedly slightly retracted tentacles). The variability of this character between individuals is underlined by the observation of our northern Brazilian specimen that had bow-shaped oral tentacles in living condition (see Fig. 3A).

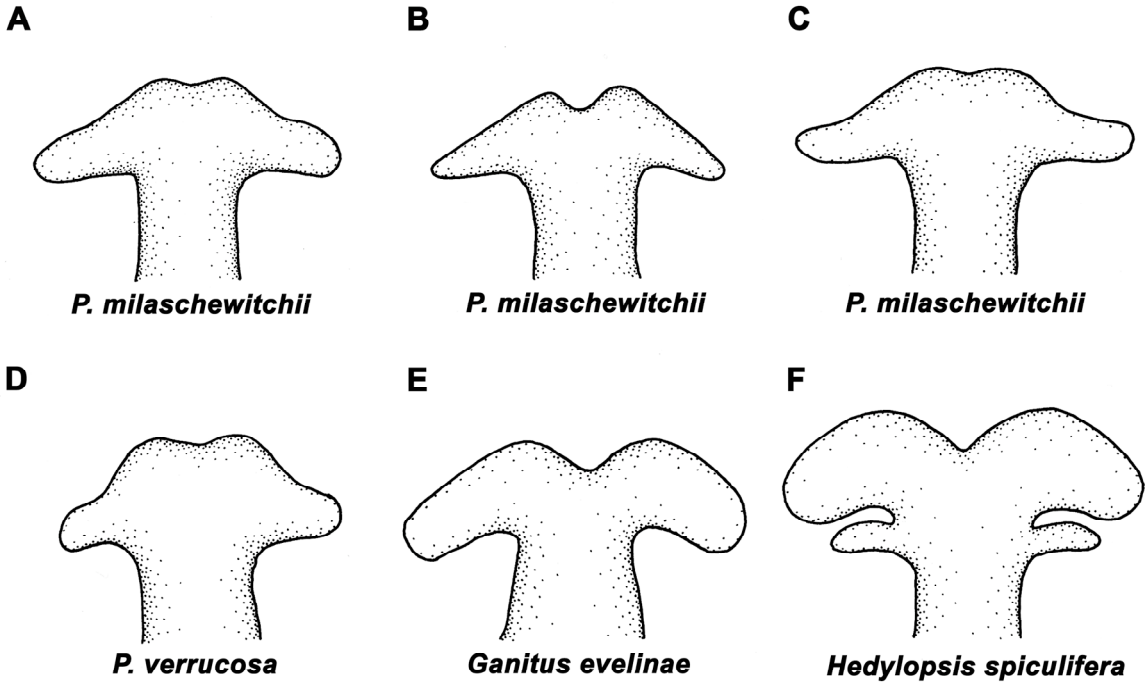

Fig. 3. Different types of flattened oral tentacles in acochlidians. (A) *Pontohedyle* from northern Brazil (present study), as *P. milaschewitchii*. (B) *P. milaschewitchii* from southern Brazil after MARCUS & MARCUS (1954; fig. 10). (C) *P. milaschewitchii* from the Mediterranean after KOWALEVSKY (1901; fig. 46). (D) *P. verrucosa* after CHALLIS (1970; fig. 5A). (E) *Ganitus evelinae* after MARCUS & MARCUS (1954; fig. 19). (F) *Hedylopsis spiculifera* (juvenile) after KOWALEVSKY (1901; fig. 49).

Table 1 . Comparison of characters previously used for species delineation in the genus *Pontohedyle*.? = no data available; * MARCUS & MARCUS (1954) originally described the radula as 44 (2.1.2) misinterpreting the central denticle of the lateral plate as incomplete cleavage (CHALLIS 1970; JÖRGER et al. in press).

	P. milaschewitchii **(Kowalevsky, 1901)**	***P. brasilensis*** **(Rankin, 1979)**	***P. verrucosa*** **(Challis, 1970)**
Data source	KOWALEVSKY (1901) WAWRA (1986) JÖRGER et al. (in press)	MARCUS & MARCUS (1954) present study	CHALLIS (1970)
Collecting site	Sebastopol (Black Sea) Princess Islands, Lesbos Island, Istria (Mediterranean Sea)	São Paulo, Porto de Galinhas, Brazil (Atlantic Ocean)	Maraunibina Island, Solomon Islands (Pacific Ocean)
Collecting habitat	coarse and fine sands, sublittoral (2–9m)	coarse and shell sands, intertidal and 20m depth	coarse, shell sand, intertidal
Foot	very short, posterior tip rounded	very short, posterior tip rounded	very short, posterior tip free and pointed
Oral tentacles	bow-shaped to triangular/elongated	bow-shaped to triangular/elongated	bow-shaped
Spicules	- aggregation of needle-shaped parallel orientated spicules between the tentacles - randomly distributed needle-shaped spicules throughout the body	- aggregation of needle-shaped parallel orientated spicules between the tentacles - randomly distributed needle-shaped spicules throughout the body	absent (?)
Eyes	present	present	absent
Radula	- 41–54 (1.1.1) - rhachidian tooth with 1 central cusp and 3 lateral denticles - lateral plate with 1 central denticle	- 44 (1.1.1)* - rhachidian tooth with 1 central cusp and 3 lateral denticles - lateral plate with 1 central denticle	- 43 (1.1.1) - rhachidian tooth with 1 central cusp and 3 lateral denticles - lateral plate without denticle
Digestive system	- no stomach detectable - salivary glands form one mass on the left side of the body, discharging into the oesophagus close to the transition of the pharynx	- “large, spherical stomach” according to MARCUS & MARCUS (1954), but no stomach detectable in the present study - salivary glands form one mass on the left side of the body	- no stomach described - salivary glands paired, discharging into the oesophagus “near its posterior end”
Male genital system	ciliated vas deferens, extending to the level anterior to the oral tentacles, genital opening dorsal to the mouth opening	genital opening on the posterior end of the head-foot complex	?
Female genital system	ciliary band extending from the genital pore to about one third of the head-food complex	short ciliary band extending from the genital pore (present study)	?

RANKIN (1979) claimed cilia to be absent from the head and oral tentacles of *P. milaschewitchii,* in contrast to *P. brasilensis*. However, a constant pattern of cilia could be detected on the oral tentacles of Mediterranean *P. milaschewitchii* (see JÖRGER et al. in press). Similar cilia were described for the Brazilian specimen by MARCUS & MARCUS (1954) and were also observed for the northern Brazilian specimen herein. Therefore, these external characters cannot be further used for separating species. In contrast, the Brazilian specimen described by MARCUS & MARCUS (1954) and the one studied herein resemble specimens of *P. milaschewitchii* (see JÖRGER et al. in press; KOWALEVSKY 1901) in all examined details, e.g. 1) body size and coloration, 2) shape of oral tentacles, 3) foot (short, posterior end rounded), 4) type (monoaxone) and position (accumulation between oral tentacles and randomly distributed all over the body) of spicules, and 4) presence of cilia on head and oral tentacles.

4.2. Microanatomy

Anatomically, RANKIN (1979) saw differences between *P. milaschewitchii* and *P. brasilensis* regarding fused versus separated cerebral and pleural ganglia, the radula formula, the presence/absence of a well developed stomach, and the development of the salivary glands.

4.2.1. Central nervous system

Probably based on small semi-schematic drawings of an entire specimen of *P. milaschewitchii* by KOWALEVSKY (1901; fig. 46, 48), RANKIN (1979) claimed the cerebral ganglia to be fused with the pleural ganglia in *P. milaschewitchii*, while they were described to be separated in *P. brasilensis.*We could not clearly detect pleural ganglia in our damaged northern Brazilian specimen. However, JÖRGER et al. (in press) showed that the cerebral and pleural ganglia in Mediterranean *P. milaschewitchii* specimens are clearly separated, as usual for Acochlidia (HUBER 1993; SOMMERFELDT & SCHRÖDL 2005; WAWRA 1987).

4.2.2. Digestive system

MARCUS & MARCUS (1954) described a radula formula of 44 x 2.1.2 for their Brazilian specimen. However, CHALLIS (1970) suggested that the denticle in the lateral tooth might have been misinterpreted as a gap that appears to separate one broad lateral tooth into two. This explanation was accepted by WAWRA (1987) and is indeed very convincing. The radula of Mediterranean *P. milaschewitchii* closely resembles the one described by MARCUS & MARCUS (1954) for the Brazilian specimen: there is a triangular rhachidian tooth with one central cusp that is bordered by three lateral denticles, and just one broad lateral plate on each side with one central denticle, thus with the formula 1.1.1 (JÖRGER et al. in press).

MARCUS & MARCUS (1954) saw an unusual large, spherical stomach in their Brazilian specimen, which was reflected in Rankin's generic name *Gastrohedyl*e. No special stomach was detected in our Brazilian specimen, but an oesophagus passing into a moderately developed digestive gland cavity which was filled with particles. This reflects the normal condition found in Mediterranean *P. milaschewitchii* (see JÖRGER et al. in press), and all other marine acochlidians. The large "stomach" described by MARCUS & MARCUS (1954) maybe easily explained as referring to a digestive gland cavity filled with particles or artificially swollen by gases due to decomposition.

RANKIN (1979) declared the salivary glands of *P. milaschewitchii* as "paired, well separated, long, thin, and tapering" in contrast to the large spherical salivary glands of *P. brasilensis* forming one mass on the left side of the body. However, the salivary glands in Mediterranean *P. milaschewitchii* are just like those described by MARCUS & MARCUS (1954) for the Brazilian specimen and also those observed herein (JÖRGER et al. in press).

4.2.3. Genital system

MARCUS & MARCUS (1954) described their male Brazilian specimen as having a genital opening located on the right side of the head-foot complex close to the transition to the visceral hump. This is the usual position for the female genital pore *P. milaschewitchii* and of other male and female genital pores in microhedylid acochlidians. However, Mediterranean male *P. milaschewitchii* show a male genital pore in an unusual cephalic position dorsal to the mouth opening (JÖRGER et al. in press; WAWRA 1986). MARCUS & MARCUS (1954) used the anterior part of the head-foot complex of their specimen for radula preparation and were therefore unable to detect a male genital opening in an anterior position. The putative posterior opening in the male Brazilian specimen maybe thus explained by generalization and misinterpretation or maybe due to different ontogenetic stages. If additional male Brazilian specimens in different ontogenetic stages did not show any ciliated duct leading anterior to a cephalic male genital opening but a posterior genital opening, this would be the first serious indication for a specific separation of *P. brasilensis* from *P. milaschewitchii*.

The female genital system of our Brazilian specimen closely resembles the one observed for *P. milaschewitchii* (JÖRGER et al. in press; WAWRA 1986) in 1) presence of a ciliary band originating from the genital opening; 2) position of the genital opening; 3) development and histo-

logy of the nidamental glands; 4) comparably small size of mature oocytes (around 60 µm). No differentiating features between the Brazilian specimen and its Mediterranean counterparts could be detected concerning the female genital system.

4.3. Taxonomy

All the differences between *P. milaschewitchii* and *P. brasilensis* claimed by RANKIN (1979) are non existent (cilia pattern, radula formula, shape of salivary glands, fusion of cerebral and pleural ganglia), variable (shape of oral tentacles) or can be easily explained by biological factors and artefacts (presence of large "stomach"). Morphological knowledge available at present (Table 1) strongly supports WAWRA (1987) in considering *P. brasilensis* as a junior synonym of *P. milaschewitchii*. However, the considerable geographical distance between the Mediterranean and the northern and southern Brazilian populations of an interstitial species and the hydrographic differences between warm temperate and tropical waters require molecular investigation as soon as abundant Brazilian populations can be found.

Pontohedyle milaschewitchii as defined above is a Mediterranean and Atlantic species, while *P. verrucosa* was described from the Solomon Islands in the tropical Indopacific (CHALLIS 1970). Main differences to *P. milaschewitchii* are the absence of spicules, eyes and lateral radula denticles (Table 1). However, at least the lack of spicules might be due to a preservation artefact; *P. verrucosa* urgently needs redescription and comparison with some other potentially undescribed *Pontohedyle* species found in the tropical Indopacific (see SCHRÖDL et al. 2003).

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3.4 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**(6): 1159-1170.

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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

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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

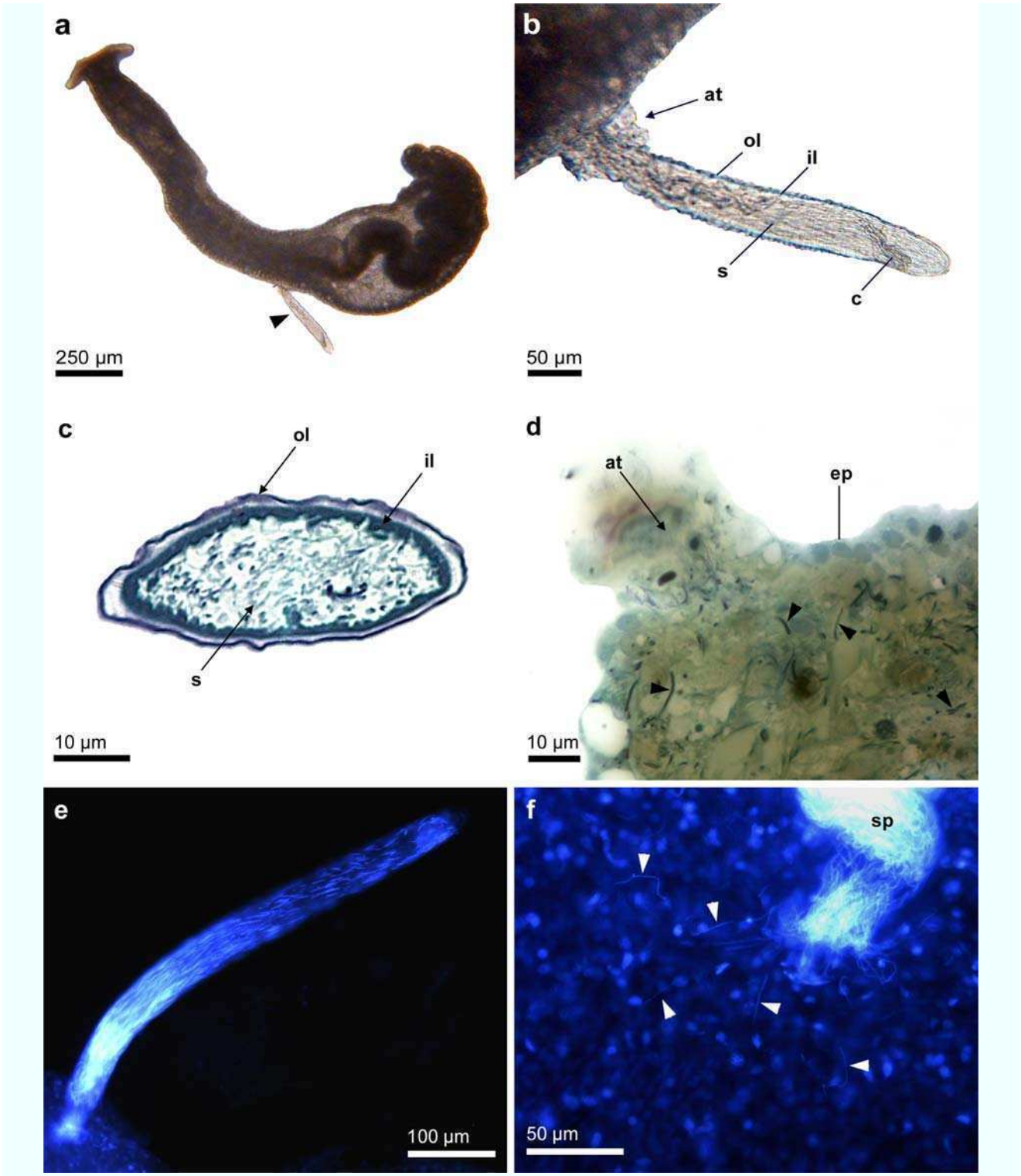

Fig. 1 Spermatophore of *Pontohedyle milaschewitchii* (light microscopy). **a** Female *P. milaschewitchii* with a spermatophore (*arrowhead*) attached to the left-anterior region of the visceral hump. **b** Close up of spermatophore filled with spermatozoa. **c** Semithin cross-section of spermatophore. **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

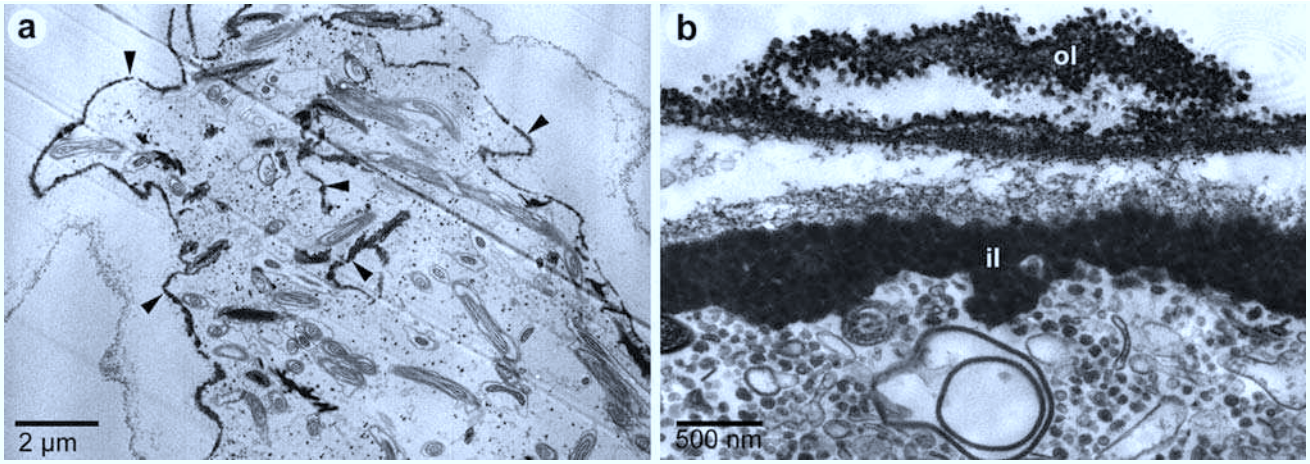

Fig. 2 TEM micrographs of a spermatophore of *P. milaschewitchii*. **a** Overview of the distal region of a spermatophore containing randomly orientated spermatozoa in a matrix with granulae and vesicles (*arrowheads* represent inner layer of spermatophore). **b** Close up of the layered wall of the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

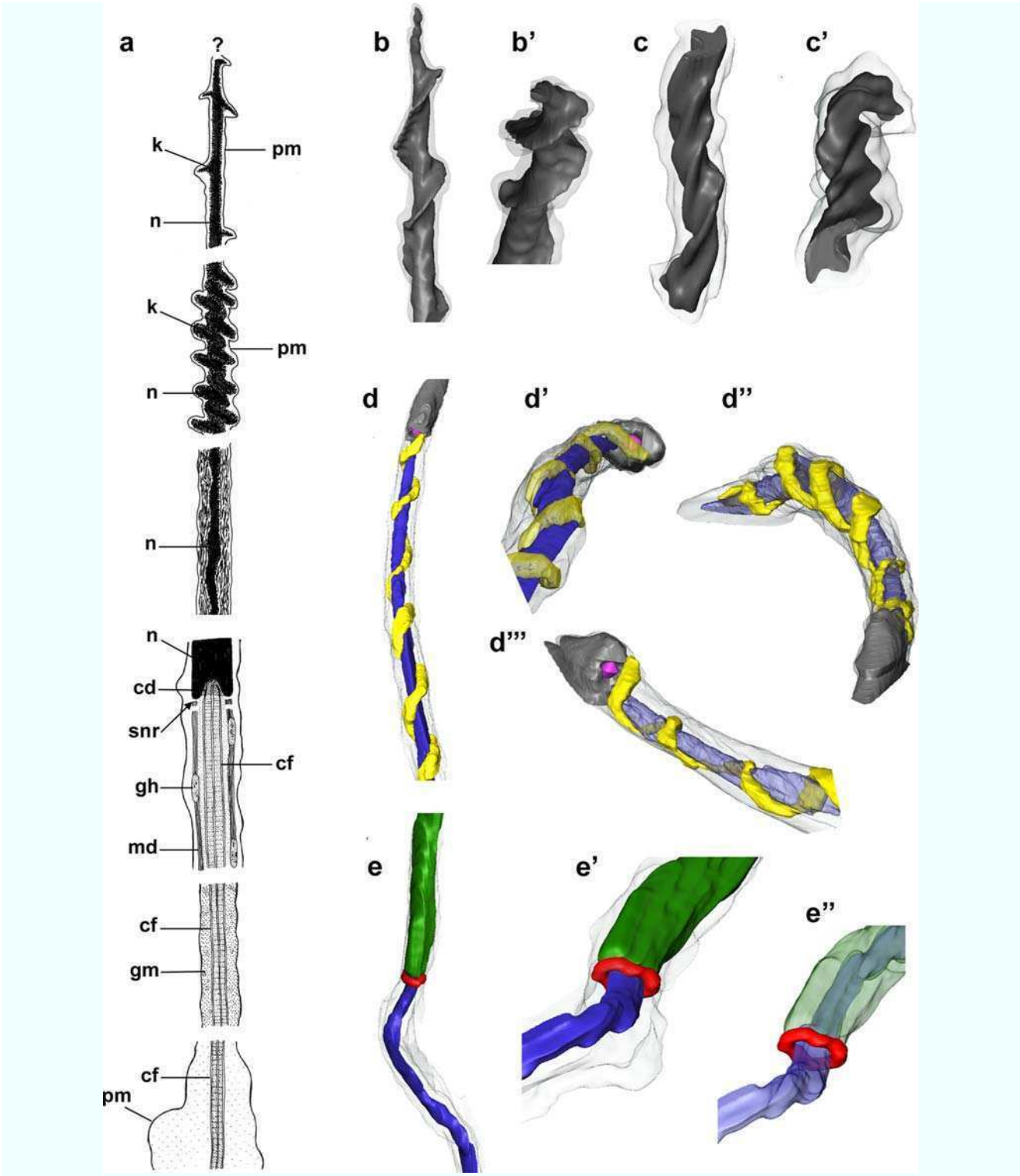

Fig. 3 Schematic overview and 3D-reconstructions of a sperm cell of *P. milaschewitchii*. **a** Schematic overview of the different structural elements. **b**–**e** 3D-reconstruction from ultrathin section series in different perspectives and transparencies. **b**, **b′** Corkscrew-shaped, one-keeled 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

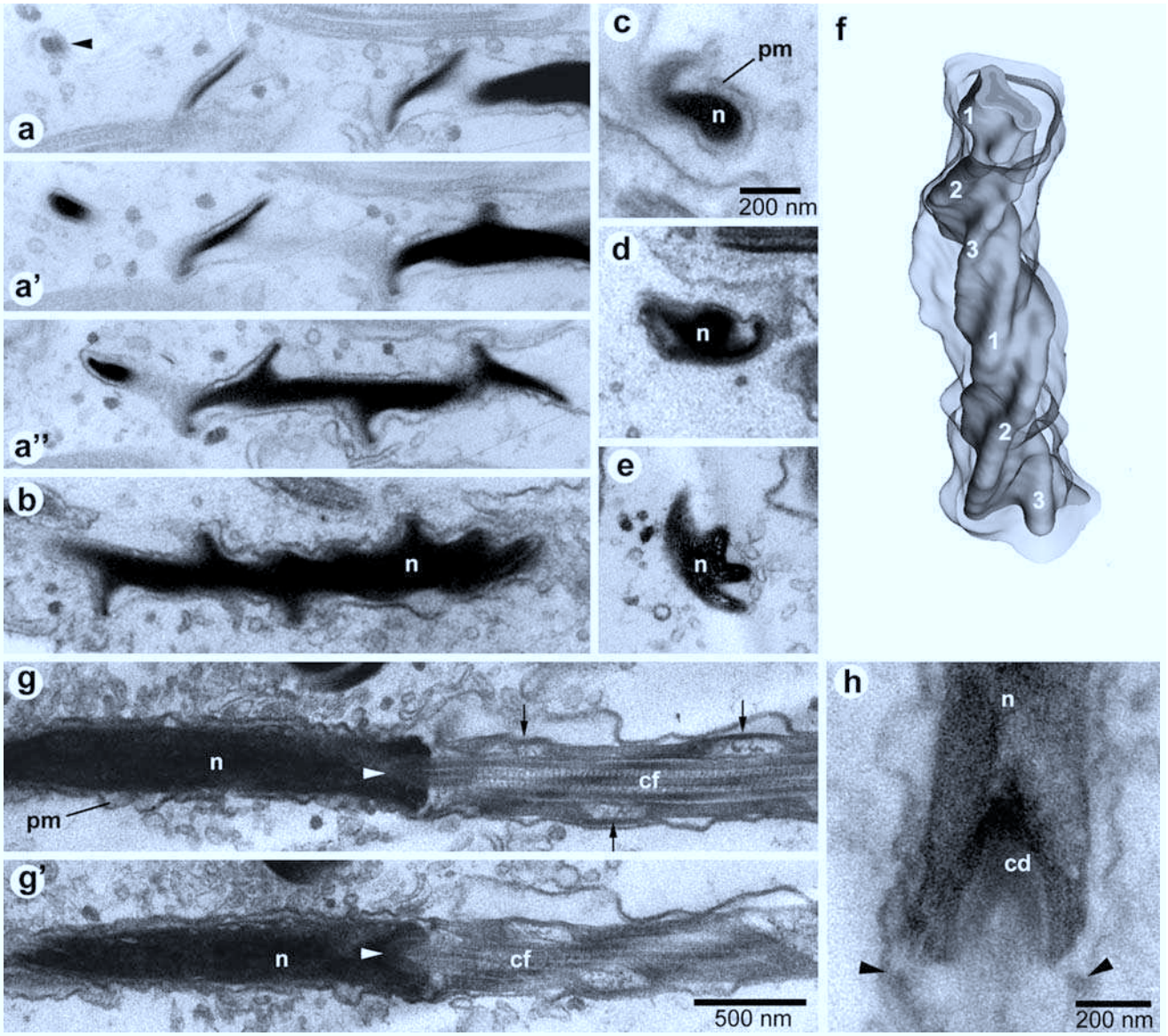

Fig. 4 TEM micrographs of sperm nucleus and mid-piece in *P. milaschewitchii*. **a**–**a**″ Longitudinal section series (*z*-spacing 80 nm) at the very tip (*arrowhead*) of a sperm nucleus. Note corkscrew-like convolution of the terminal nuclear keel (compare with Fig. 3b). **b** Longitudinal section 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

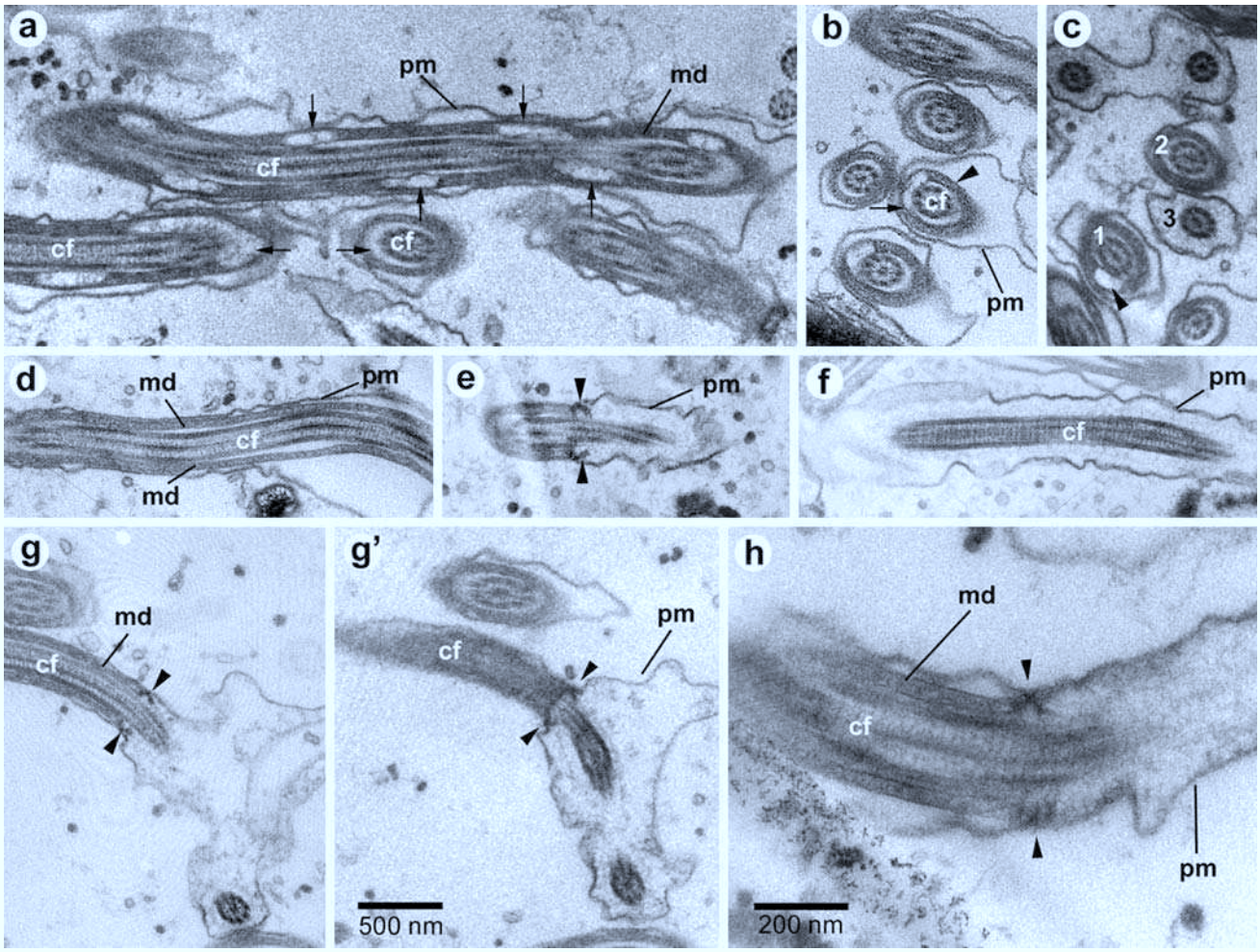

Fig. 5 TEM micrographs of sperm mid-piece and tail in *P. milaschewitchii*. **a** Oblique section through the frontal halves of sperm mid-pieces with glycogen helices (*arrows*) within the mitochondrial derivatives. **b**, **c** Cross-sections of sperm tails and mid-pieces at different positions along 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

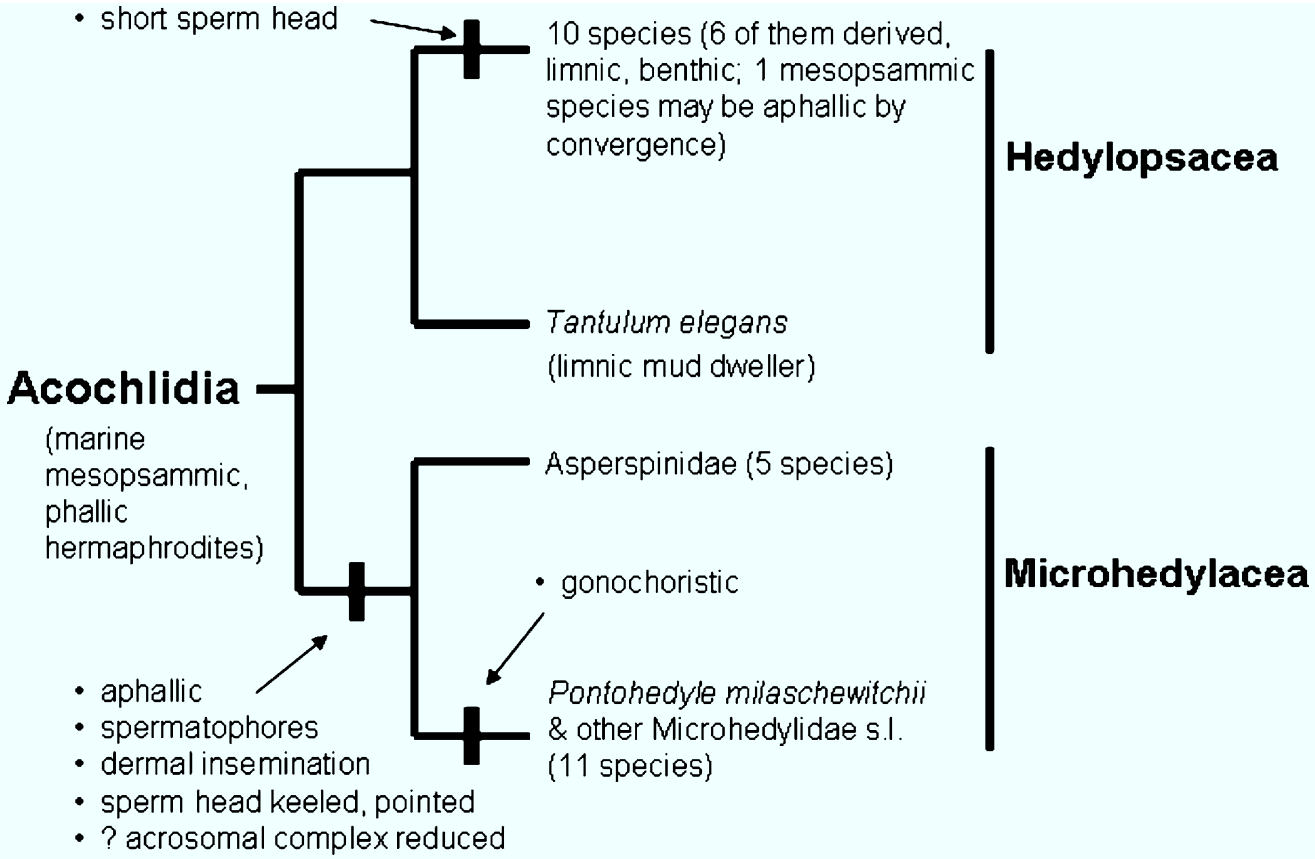

Fig. 6 Evolution of sperm structure, spermatophores and dermal insemination in the Acochlidia. Topology and apomorphies modified after Schrödl and Neusser (in press). The evolution of sperm transfer via spermatophores, dermal insemination and screw-like keeled sperm heads are regarded as key innovations 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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Tantulum elegans reloaded: a computer-based 3D-visualization of the anatomy of a Caribbean freshwater acochlidian gastropod

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Abstract. Acochlidian gastropods combine several aberrant biological and morphological features. The poorly known Caribbean *Tantulum elegans* is one of the few opisthobranch species inhabiting a freshwater system, and the only one found in muddy interstices of a Caribbean mountain spring swamp. Morphological details of this tiny species were either unknown or not fully reliable, especially with regard to the complex central nervous and reproductive systems. We critically re-examined original paratype section series and prepared semi-thin serial sections of two additional paratypes. All organ systems were three-dimensionally reconstructed using AMIRA software. Our results show several discrepancies from the original description: the pharynx is a complex system of different muscles, but similar to that of other acochlidian species; the circulatory system shows a two-chambered heart; in the nervous system there are separate optic and rhinophoral ganglia, the latter innervating a pair of small sensory pits we assume to be Hancock's organs, and large aggregations of precerebral accessory ganglia were found. Nephropore, anus, and female gonopore open dextroventrally. To our surprise, adults of *T. elegans* are sequential hermaphrodites with an unusual androdiaulic reproductive system and a well-developed cephalic penial complex. In *T. elegans,* there is a mix of character conditions found in different genera, e.g., *Pseudunela* and *Asperspina*. The phylogenetic position of *T. elegans* still remains unclear.

Additional key words: Mollusca, Opisthobranchia, three-dimensional reconstruction, phylogeny

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The Acochlidia are poorly known opisthobranch gastropods. Currently, there are 27 valid species recognized that all show a characteristic body shape with a head–foot complex separated from, but at least partially retractable into, the shell-less visceral hump (Wawra 1987). Combining several unique morphological and biological features with an array of either primitive conditions or secondary reductions, the origin and phylogeny of Acochlidia are still unclear (Sommerfeldt & Schrödl 2005; Neusser et al. 2006). Successful cladistic analysis and significant evolutionary conclusions are so far hindered by incomplete or unreliable morphological data sets on many acochlidian and other, potentially related, opisthobranch species (see Dayrat & Tillier 2002; Wägele & Klussmann-Kolb 2005).

Most acochlidian species are marine mesopsammic; their tiny body sizes ($\sim$1–5 mm), uniform vermiform body shape, the loss of shell, development of spicules, and the more or less extensive reduction of foot, body pigments, and eyes have been regarded as adaptations to extreme environmental conditions (Swedmark 1971; Arnaud et al. 1986; Westheide 1987). The reproductive system of acochlidians is monaulic and thus resembles the hypothetic basal condition in opisthobranchs (Ghiselin 1965). However, within Acochlidia, there is a wide variety of special reproductive features. These may include modification or loss of the copulatory organs and, instead of reciprocal copulation, sperm transfer by hypodermal impregnation or spermatophores (see Swedmark 1968; Wawra 1992; Morse 1994). Many, but not all, marine species have separate sexes, i.e., the Microhedylidae and Ganitidae (gonochoristic microhedylaceans according to Sommerfeldt & Schrödl 2005); this is an exclusive feature among the usually hermaphroditic opisthobranchs.

While opisthobranchs are generally marine with some species tolerating brackish waters, several acochlidian species exclusively inhabit brackish or

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freshwater systems. There is an array of large-sized ($\leqslant$25–30 mm) limnic acochlidians, i.e., *Acochlidium amboinense* STRUBELL 1892, *Strubellia paradoxa* STRUBELL 1892, *Palliohedyle weberi* BERGH 1895, *Palliohedyle sutteri* WAWRA 1979, *A. bayerfehlmanni* WAWRA 1980, and *A. fijiense* HAYNES & KENCHINGTON 1991, that are distributed over different tropical Indo-Pacific islands. They all live benthically in rivers and streams close to the sea.

In contrast, Rankin (1979) described a small (2-mm living body length) freshwater species from the Caribbean island of St. Vincent: members of *Tantulum elegans* RANKIN 1979 inhabit the muddy interstices of a single, known, mountain spring marsh, situated 411 m above sea level and obviously well isolated from the sea. Major organ systems in *T. elegans* were extensively described by Rankin (1979) from histological sections. However, sexual condition and reproductive organs remained unknown, and several original statements regarding excretory, circulatory, and nervous features of *T. elegans* have been doubted in recent studies (Fahrner & Haszprunar 2002; Sommerfeldt & Schrödl 2005). The lack of comparative data, unreliable structural information, and uncritical use of literature data contributed to Rankin's (1979) reorganization of acochlidian systematics, which was criticized severely by subsequent authors (Arnaud et al. 1986; Wawra 1987; Sommerfeldt & Schrödl 2005).

The present study thus aims to re-examine Rankin's observations on *T. elegans* and add detailed information on all major organ systems. Serial semi-thin sections of two further museum specimens were prepared and analyzed using computer-based three-dimensional (3D) organ reconstruction with AMIRA software. This method has recently been proven to be an efficient tool for obtaining accurate and reproducible anatomical information from tiny acochlidian specimens (Neusser et al. 2006). Structures are comparatively discussed, conclusions on the reproductive biology of *T. elegans* are drawn, and potential implications of the new findings on acochlidian phylogeny are outlined.

Methods

Several specimens of *Tantulum elegans* were collected in Golden Grove, St. Vincent, West Indies, in July 1972 by Dr. A.D. Harrison, described, and deposited at the Royal Ontario Museum (ROMCN M1118). According to the Royal Ontario Museum, there are no traces of the holotype, which was a whole mount according to Rankin (1979). The ROM provided us with four paratype slide-sections for re-examination; additionally, two specimens preserved in 70% ethanol were obtained for semi-thin sectioning (see Table 1). These specimens were decalcified with Bouin's solution, dehydrated in a graded series of acetone dilutions, and embedded in Spurr's low-viscosity resin (Spurr 1969). Two complete, ribboned, serial sections (1.5 μm) were prepared using ''Ralph'' glass knives and contact cement at the lower cutting edge according to Henry (1977), and stained with methylene-azure II (Richardson et al. 1960). Computer-based 3D reconstructions of the major organ systems were made with the software AMIRA 3.1 (TGS Template Graphics Software Inc., San Diego, CA, USA) as described by Neusser et al. (2006).

Table 1. Material used in present study, including original paratype sections of Rankin (1979) and two newly made serial semi-thin section series. +, present; −, absent; ?, feature cannot be detected; ZSM, serial sections made at the Zoologische Staatssammlung München.

Specimen No. used in present study	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Corresponding number of slides of Rankin's sections	40	9	9	19	ZSM	ZSM
Cutting plane	Transverse	Sagittal	Sagittal	Sagittal	Transverse	Transverse
Approximate body size (mm) of fixed specimen	?	2.8	3.0	2.7	1.8	2.4
Maturity	?	Immature	Immature	Mature	Immature	Mature
Mature male gonad	?	−	−	+	−	−
Anterior male genitalia	−	−	−	−	−	+
Female reproductive system	?	−	−	−	−	+
Female gonopore	?	?	?	?	+	+
Male gonopore	?	?	?	?	Traces	+
Accessory ganglia	−	−	−	+	−	−

Results

External morphology

In *Tantulum elegans,* body shape conforms to that characteristic for the Acochlidia. The body is vermiform, with an anterior head–foot complex and a posterior, conical, and elongate visceral sac. The foot is approximately as broad as the body, and a cephalopedal groove is developed. The tail, i.e., the posterior part of the foot, which is separated from the visceral sac, is narrower; it is shorter than the visceral sac and tapered at its end. The foot sole is densely ciliated throughout. Individuals show one pair of digitiform labial tentacles and, more posteriorly, one pair of slightly shorter digitiform rhinophores. The body length of fixed specimens is between 1.8 and 3.0 mm. Remnants of eyes were not visible through the body integument in the fixed material. Calcareous spicules (Fig. 4C,E) are found posterior to the tentacles in the region of the cerebral ganglia.

Microanatomy

The head–foot complex is filled with the central nervous system (cns), the anterior part of the digestive system (oral tube, pharynx, salivary glands, and esophagus), and the anterior male genitalia (Fig. 1). Ventral to the mouth, the bilobed anterior pedal gland opens to the outside, forming a ciliated patch. It extends to the level of the cerebral ganglia (Figs. 1, 4A, and 5A,C). Anteriorly, the anterior pedal gland is narrow and stained deep purple, like the small pedal glands that are situated along the entire length of the foot (Fig. 5C). Posteriorly, the anterior pedal gland mass is larger and stained slightly grayish-blue (Fig. 4A,B). The visceral sac contains the excretory and circulatory systems on the right side, with the reproductive system and the digestive gland filling most of the space. The anus and nephropore open close together, dextroventral to the visceral sac. The female gonopore lies dextroventrally, slightly posterior to the junction of the foot with the visceral sac. The male gonopore opens just anterior to the right rhinophore.

Nervous system and sensory organs

The cns is composed of paired rhinophoral, cerebral, optic, pedal, pleural, buccal, and gastro-esophageal ganglia, four distinct, separated ganglia on the visceral nerve cord, and a presumed genital ganglion (Fig. 2). Apart from the buccal ganglia, all ganglia are situated pre-pharyngeally. Subsequently used terms for ganglia and nerves are according to Schmekel (1985), Haszprunar (1985), and Huber (1993).

All ganglia are surrounded by a layer of connective tissue, and subdivided into an outer cortex with dark blue-stained nuclei and an inner medulla (Fig. 4C–F). The medulla, nerves, commissures, and connectives lack nuclei, and are stained slightly blue-grayish. Giant neurons are present in the cerebral, pedal, and, especially, the visceral ganglia (Fig. 4F). The cerebral ganglia are $\sim$75–100 μm in diameter and located dorsolaterally at the anterior of the pharynx (Fig. 4C,D). They are connected by a short and thick cerebral commissure (Fig. 3). Dorsal bodies could not be detected.

Large aggregations of accessory ganglia (Fig. 5C) were only detectable in one examined specimen (No. 4). They are situated anterior to the cerebral ganglia and consist of spherical cell aggregations of neuronal tissue similar to ganglia, but are lacking the characteristic separation into cortex and medulla, and any layer of surrounding connective tissue. Anteroventrally, each cerebral ganglion gives rise to a labiotentacular nerve, leading to the labial (= oral) tentacle (Figs. 2, 3, and 4C). Dorsal of the labiotentacular nerve, each cerebral ganglion bears a short connective to the small rhinophoral ganglion (Fig. 4C). From the latter, a thick nerve arises and immediately bifurcates into the rhinophoral nerve, leading to the rhinophores, and a nerve innervating a field of non-glandular cells surrounding a small, ciliated ridge just posterior to the rhinophores (Figs. 2 and 4A). This occurs on both sides of the head and is regarded to be the Hancock's organ (Figs. 3B and 4B). Just posterior to the rhinophoral ganglion, a small optic ganglion is attached to each cerebral ganglion (Fig. 2). Both rhinophoral and optic ganglia are surrounded by a layer of connective tissue shared with the cerebral ganglion. Anteriorly, the fine optic nerve emerges from the optic ganglion, running anteriorly and leading toward a single-layered, epithelial and pigment-less, hollow sphere that is assumed to be the remnant of an eye (Figs. 2, 3 and 4A). The Hancock's nerve gives off a fine nerve that joins the optic nerve (Fig. 2). Ventrally, arising near the cerebro-pedal-connective, a thin cerebral nerve runs posteriorly into the pharynx; it appears to be the cerebro-buccal connective (Fig. 2).

There is a statocyst with one statolith attached to each of the pedal ganglia (Figs. 2 and 4D). The very fine static nerve innervating the statocyst could not be detected.

The pedal ganglia are not much smaller than the cerebral ganglia (60–90 μm in diameter), but show a thinner and longer commissure (Figs. 2, 3 and 4C–F). The pedal ganglia are situated lateroventral to the pharynx and almost ventral to the cerebral ganglia.

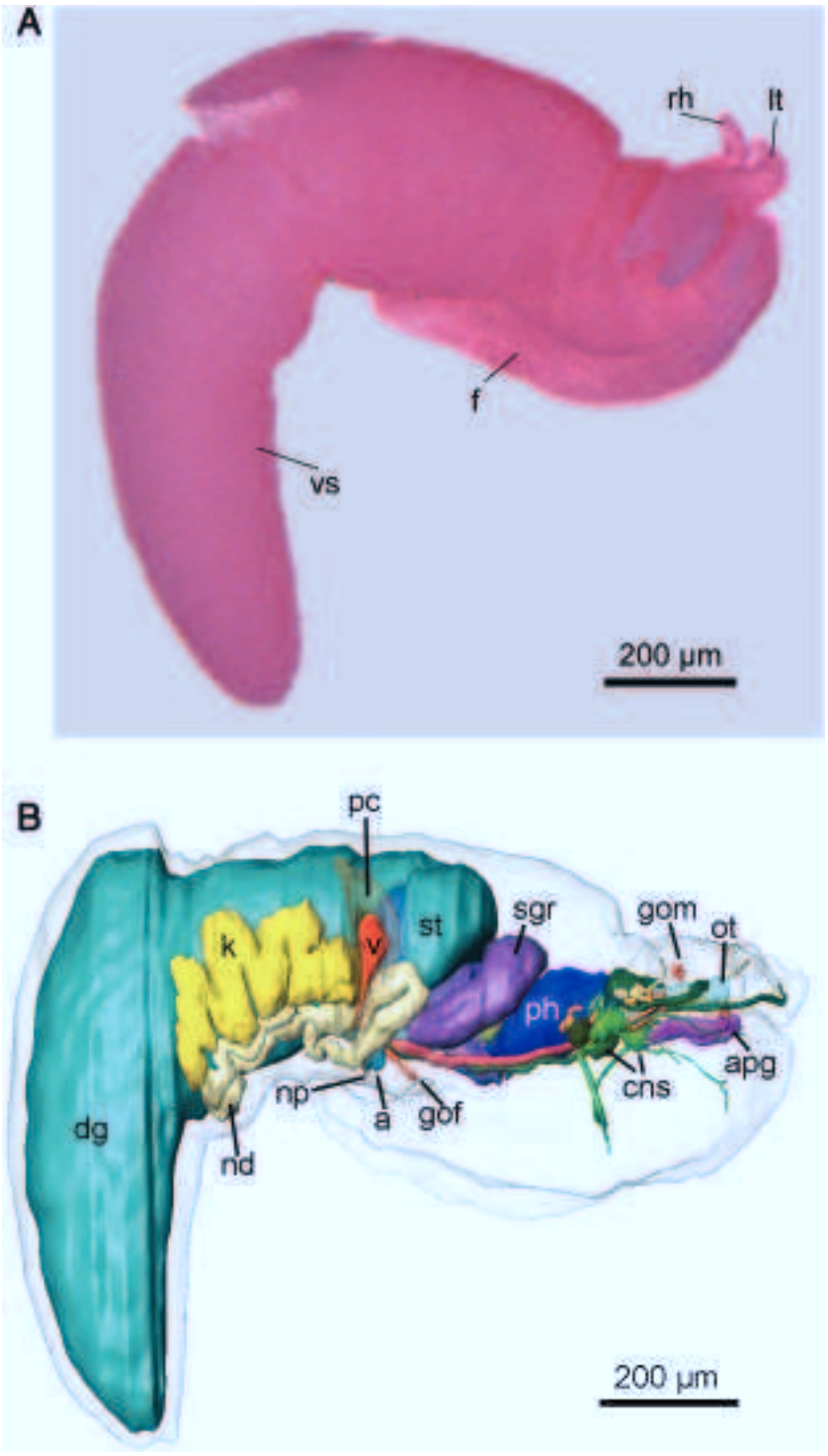

Fig. 1. External morphology and microanatomy of *Tantulum elegans* (immature specimen No. 5, right view). **A.** Photograph of preserved and stained paratype. **B.** Three-dimensional reconstruction, positions of internal organs. Green: central nervous system, blue: digestive system, yellow/orange: excretory and circulatory system, red: reproductive system. a, anus; apg, anterior pedal gland; cns, central nervous system; dg, digestive gland; f, foot; gof, female genital opening; gom, male genital opening; k, kidney; lt, labial tentacle; nd, nephroduct; np, nephropore; ot, oral tube; pc, pericardium; ph, pharynx; rh, rhinophore; sgr, right salivary gland; st, stomach; v, ventricle; vs, visceral sac.

In addition to cerebro-pedal connectives (Fig. 4C), each pedal ganglion gives off four nerves innervating the foot; the first and second arise anteriorly and ventrally, respectively, and lead to the anterior part of the foot (Fig. 3B). A lateral nerve leads to the posterior part of the foot, and a dorsal nerve seems to innervate the anterior part, but could not be followed over the whole length.

The pleural ganglia (25–30 µm in diameter) lie posterior to the cerebral ganglia (Figs. 2, 3, and 4D). Cerebro-pleural connectives are very short, as are pleuro-pedal connectives. There are four separate

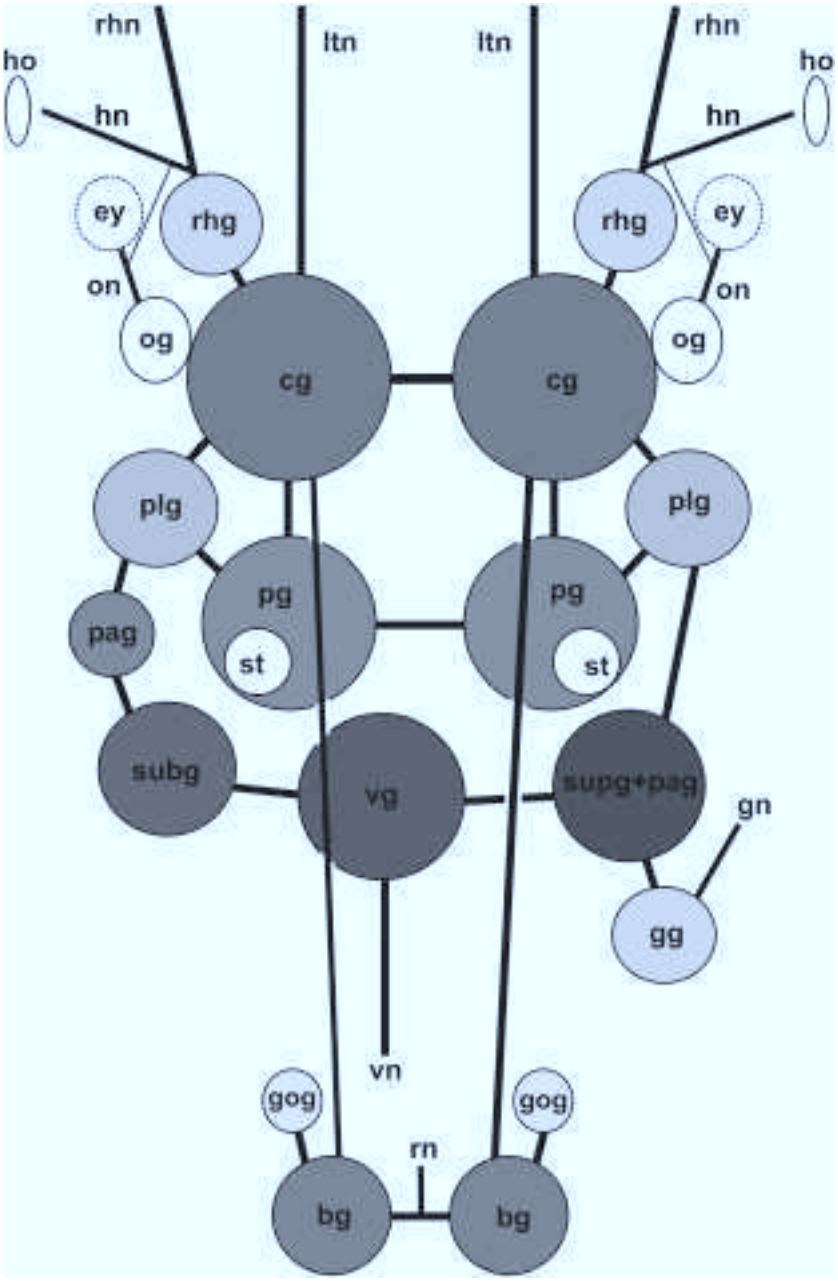

Fig. 2. Central nervous system of *Tantulum elegans* (schematic, dorsal view). bg, buccal ganglion; cg, cerebral ganglion; ey, eye remnant; gg, penial ganglion; gn, penial nerve; gog, gastro-esophageal ganglion; hn, Hancock's nerve; ho, Hancock's organ; ltn, labio-tentacular nerve; og, optic ganglion; on, optic nerve; pag, parietal ganglion; pg, pedal ganglion; plg, pleural ganglion; rhg, rhinophoral ganglion; rhn, rhino-phoral nerve; rn, radular nerve; st, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve.

ganglia on the visceral nerve cord (Fig. 2). The left parietal ganglion is ~20 μm in diameter; the pleuro-parietal connective is as short as the parietal–subintestinal connective. The subintestinal ganglion (30–35 μm in diameter) is slightly larger than the pleural ganglion (Fig. 3A). A short connective leads to the visceral ganglion, which reaches almost the size of the pedal ganglia. The visceral ganglion bears the thick visceral nerve that runs, flanking the aorta, through the visceral hump (Figs. 2, 3 and 5E). The connective that links the visceral with the smaller supraintestinal/parietal ganglion on the right side is as long as the pedal commissure; the pleuro-supraintestinal/parietal connective is short (Fig. 3). A small ganglion is attached dorsolaterally to the supraintestinal/parietal ganglion (Figs. 2, 3 and 5D); a nerve leads anteriorly toward the penial sheath and obviously innervates the anterior male genitalia.

The buccal ganglia are situated postpharyngeally and linked by a thin commissure ventral to the esophagus (Fig. 5E). They are similar in size to the pleural ganglia. The thin nerve emerging anteriorly from each ganglion is regarded to be the cerebro-buccal connective, but it could not be detected over

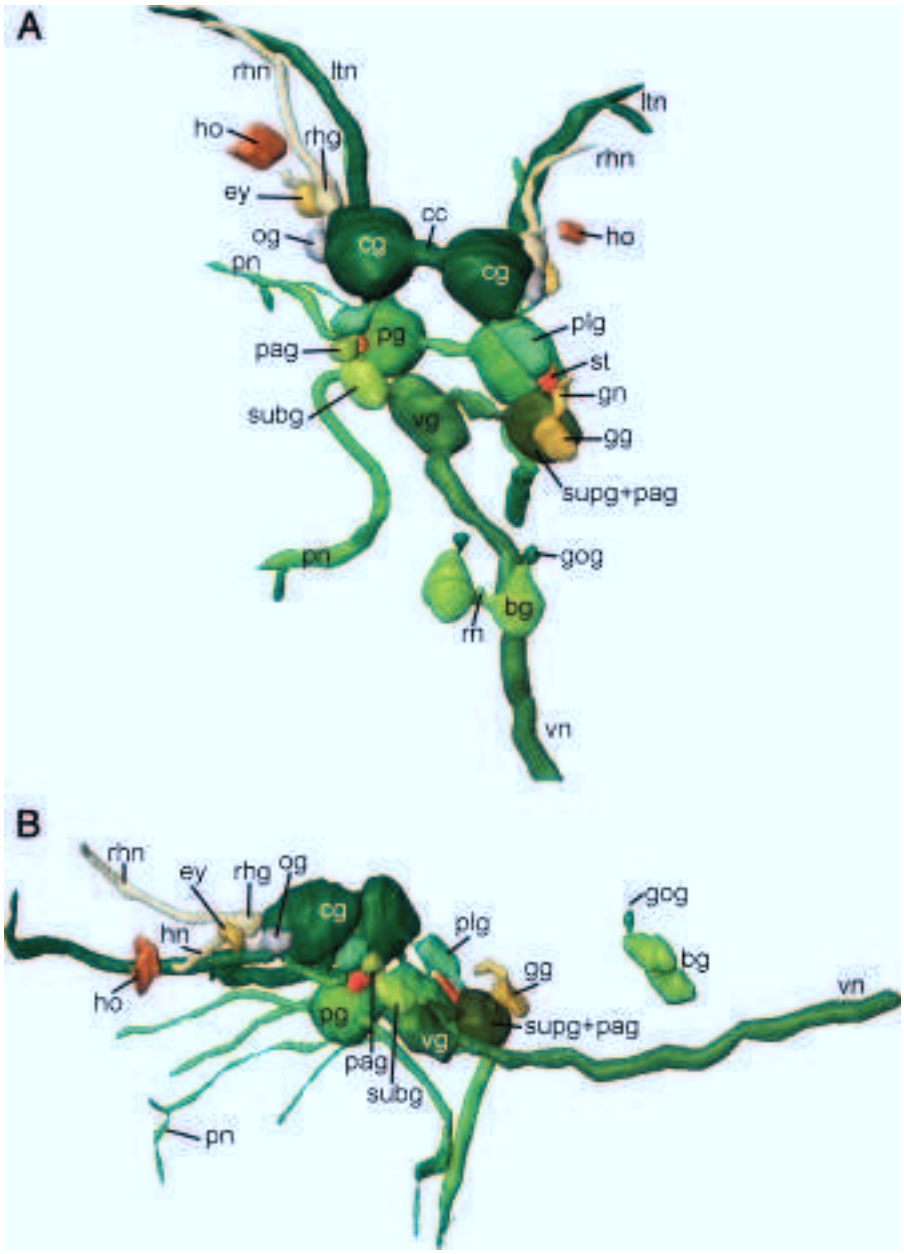

Fig. 3. Three-dimensional reconstruction of the central nervous system of *Tantulum elegans* (specimen No. 5). **A.** postero-dorsal view. **B.** left view. bg, buccal ganglion; cc, cerebral commissure; cg, cerebral ganglion; ey, eye remnant; gg, penial ganglion; gn, penial nerve; gog, gastroesophageal ganglion; hn, Hancock's nerve; ho, Hancock's organ; ltn, labiotentacular nerve; og, optic ganglion; pag, parietal ganglion; pg, pedal ganglion; plg, pleural ganglion; pn, pedal nerve; rn, radular nerve; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; st, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve.

the whole length. The radular nerve arises from the buccal commissure and leads to the radular sac at the posterior pharynx (Fig. 2 and 5E). Each buccal ganglion is connected by a thin, vertical connective with the gastro-esophageal ganglion, which is situated above the esophagus (Fig. 2, 3 and 5E).

Digestive system

The mouth lies ventrally between the oral tentacles. The single-layered oral tube is long and not ciliated. The dark blue-stained pharynx is bulbous and composed of a complex system of longitudinal muscles and a sphincter (Figs. 4F, 5D and 8B). The radula is ~275 µm long and U-shaped, with the dorsal ramus longer than the ventral one (Figs. 5B and 8B). The rachidian tooth is triangular and bears four or five denticles on each side. The lateral teeth are plate-like and elongated. Jaws are absent.

The paired salivary glands are well developed and situated posterior to the pharynx, one on each side of the esophagus (Figs. 5E and 8B). The glands are tubular with a narrow lumen. The secretory cells are filled with dark-blue-stained granules. Leaving the anterior end of the salivary gland, the lumen widens into a muscular ampulla or reservoir, here termed the salivary pump (Fig. 5B,E). At the junction of the salivary gland with the salivary pump, cells bearing cilia

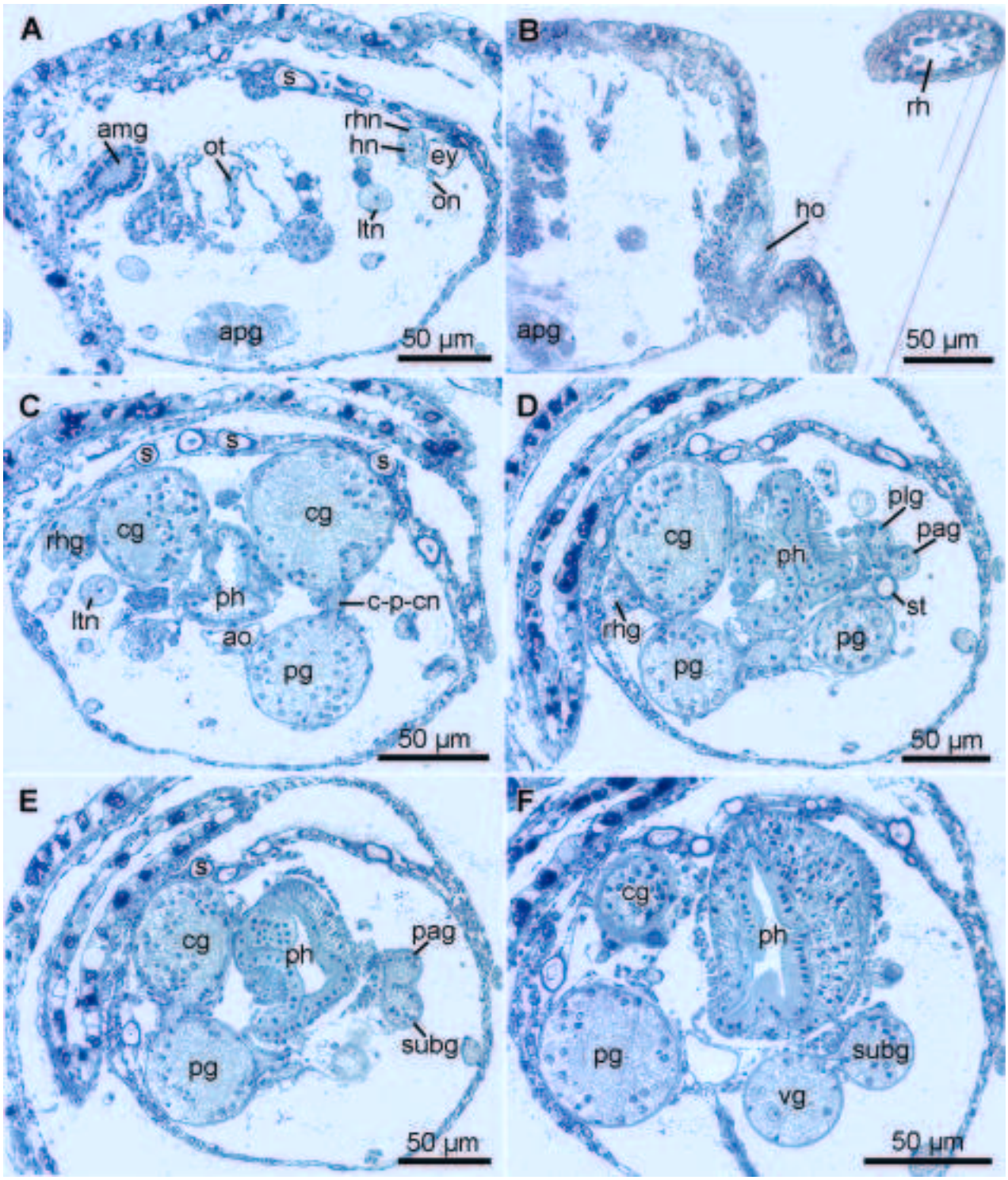

Fig. 4. Semithin transverse sections of the central nervous system of *Tantulum elegans* (specimen No. 5). **A.** Eye remnant. **B.** Hancock's organ. **C.** Cerebral and rhinophoral ganglion. **D.** Pleural and left parietal ganglion, statocysts. **E.** Subintestinal ganglion. **F.** Visceral ganglion. amg, remnant of anterior male genitalia; ao, aorta; apg, anterior pedal gland; cg, cerebral ganglion; c-p-cn, cerebro-pedal-connective; ey, eye remnant; hn, Hancock's nerve; ho, Hancock's organ; ltn, labiotentacular nerve; on, optic nerve; ot, oral tube; pag, parietal ganglion; pg, pedal ganglion; ph, pharynx; plg, pleural ganglion; rh, rhinophore; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; s, spicule; st, statocyst; subg, subintestinal ganglion; vg, visceral ganglion.

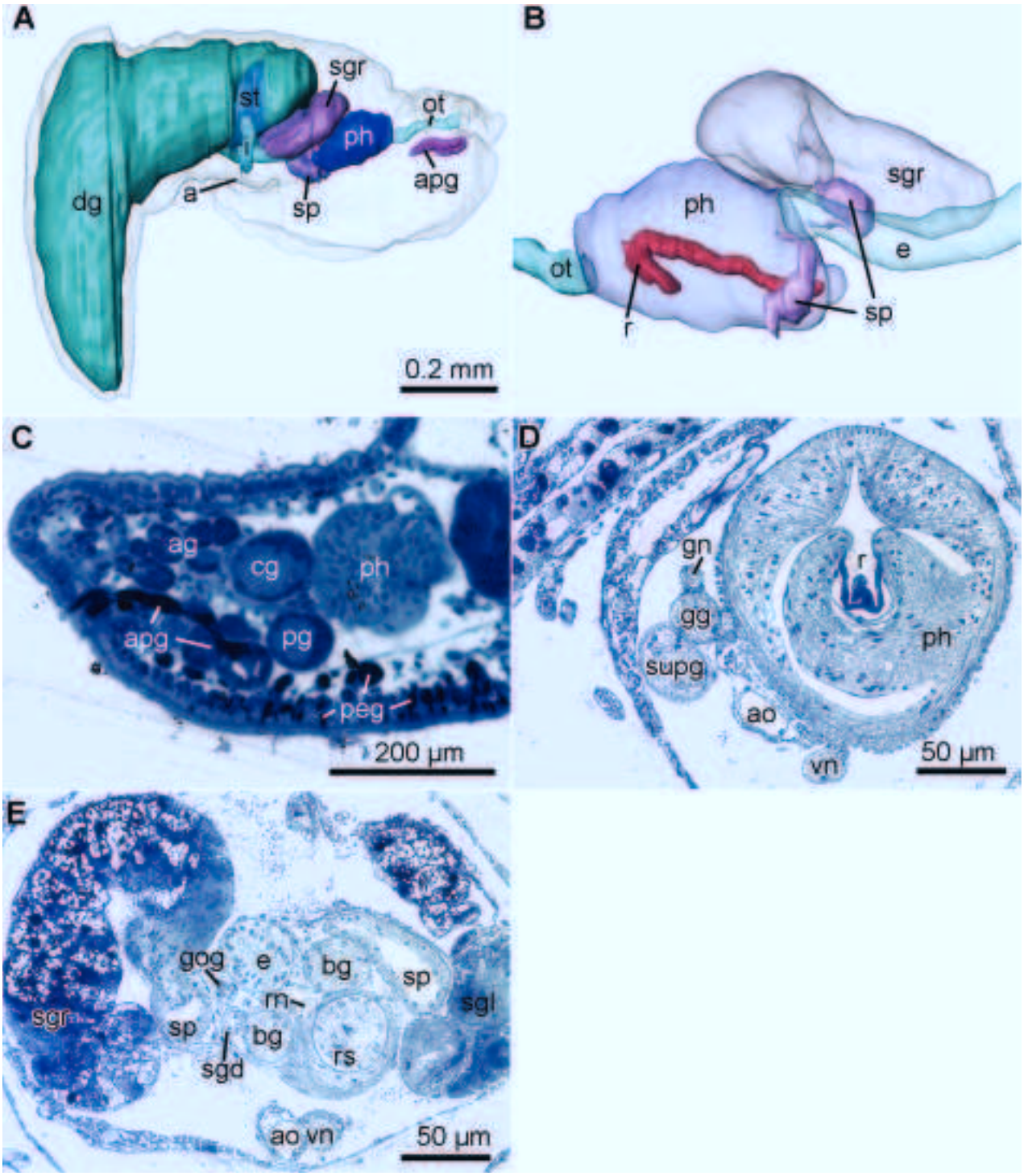

Fig. 5. Digestive system of *Tantulum elegans* (all specimen No. 5 except 5C which is specimen No. 4). **A.** Three-dimensional (3D) reconstruction, position of the organ system in the specimen (right view). **B.** 3D reconstruction, salivary pumps (left view). **C.** Semithin sagittal section, anterior pedal gland. **D.** Semithin transverse section, pharynx and radula. **E.** Semithin transverse section, salivary glands and salivary pumps. a, anus; ag, accessory ganglia; ao, aorta; apg, anterior pedal gland; bg, buccal ganglion; cg, cerebral ganglion; dg, digestive gland; e, esophagus; gg, penial ganglion; gn, penial nerve; gog, gastroesophageal ganglion; i, intestine; ot, oral tube; peg, pedal gland; pg, pedal ganglion; ph, pharynx; r, radula; rn, radular nerve; rs, radula sac; sgd, salivary gland duct; sgl, left salivary gland; sgr, right salivary gland; sp, salivary pump; st, stomach; supg, supraintestinal ganglion; vn, visceral nerve.

$\sim$10 μm in length are found. The thin salivary duct (Fig. 5E) joins the food channel at the posterior end of the pharynx. The esophagus leaves the pharynx posterodorsally; it is quite thick and accompanied by well-developed longitudinal muscular tissue (Fig. 6C,D). Epithelial cells are ciliated. At the junction of the digestive gland with the esophagus, there is an expansion serving as the stomach. It is fused with the digestive gland and separated from the latter only by a deep groove (Fig. 6C,D). The epithelia of the digestive gland and of the stomach show the same histological characteristics, but that of the digestive gland is not ciliated, whereas short cilia are found in the epithelium of the entire stomach.

The holohepatic digestive gland is very voluminous and shaped like a long sac, without forming diverticula. It nearly fills the visceral sac in immature specimens (Fig. 5A). The central lumen is unbranched and broad. In some specimens, the remains of ingested food material are found (Fig. 6C,D). The densely ciliated intestine emerges from the stomach near the entry of the esophagus. The intestine is short and vertical (Fig. 6C). The anus opens ventrally at the right side of the visceral sac, slightly anterior to, but separated from, the nephropore (Fig. 6D).

Circulatory and excretory system

The circulatory and excretory systems are placed at the right side of the body (Figs. 1 and 6A). The two-chambered heart is surrounded by the pericardium (Fig. 6B,D). The thin-walled pericardium is teardrop-shaped, with the tapered end pointing ventrally. It is situated anterior of the kidney at the beginning of the visceral sac (Fig. 6B). The pericardial complex is arranged longitudinal to the body axis.

The heart consists of a very small, thin-walled auricle and a muscular, elongate ventricle. The ventricle lies at the anteroventral end of the pericardium and ventral to the auricle (Fig. 6B,D). The thick aorta arises from the anterior end of the ventricle. It runs vertically downward and then passes forward, leading to the tentacle region of the head (Fig. 6A,B). The aorta lies closely parallel to the visceral nerve; a common layer of longitudinal muscles surrounds both (Fig. 5E). The pericardium is connected with the kidney by a renopericardial duct, which emerges at the anteroventral end of the pericardium (Fig. 6B,E). The renopericardial duct is not muscular, but composed of flagellated cells forming a ciliated funnel. It runs posteriorly and opens ventrally into the anterior part of the kidney.

The kidney lies posterior to the pericardium and extends for more than half the length of the visceral sac (Fig. 6A). The kidney is an elongated, sinuously bent sac (Figs. 1 and 6A,E), with a U-shaped duct running from the anterior to the posterior, and back to the front. In its first section, the tube shows only a small lumen surrounded by cells with small vacuoles. The second portion is characterized by a wide lumen and cells filled with large vacuoles (Fig. 6E). Anteroventrally, the kidney connects with the very long, looped nephric duct by a small, ciliated pore (Fig. 6E). First, the nephric duct runs posteriorly for approximately half of the visceral sac; then, it turns and leads back to its beginning. Finally, after a dorsal loop, the nephroduct opens through the nephropore (Fig. 6A,B). The latter is situated ventrally, just posterior to the anus.

Reproductive system

Members of *T. elegans* are protandric hermaphrodites (see Table 1). In the juvenile specimen No. 5, we could locate only traces of the genital system. The gonopore is situated dextroventrally, slightly posterior to the junction of the head/foot complex with the visceral sac. A thin gonoduct leads posteriorly, but truncates abruptly. No gonad is developed. Anteriorly, between the right tentacle and the right rhinophore, a short, ciliated invagination is found. The latter is regarded here as the early developmental stage of the anterior male genitalia (Fig. 4A). The incomplete sections of specimen No. 4 show a mature male gonad that extends over large parts of the visceral sac. It is filled with sperm cells (Fig. 8F,F′).

There is only one complete series of sections of a mature specimen available (No. 6). The following description thus refers to this single individual that recently entered the female phase. The small, sac-like ovary is situated in the ventral part of the visceral hump (Fig. 8E). It is filled with oocytes showing different stages of development. The largest egg cells contain yolk material, and reach $\sim$60 μm in diameter. Aggregations of mature sperm attach along the cell wall of the largest oocytes, with heads directed toward the egg (Fig. 8E′). Anterior to the gonad, an ampulla-like reservoir is situated, and is filled with sperm (Fig. 8E). The origin of sperm is unknown; it thus may be either autosperm or allosperm. Inside the reservoir, there are also a few egg cells. Just anterior to the reservoir, the ciliated spermoviduct arises, containing the nidamental gland mass (Fig. 7). Three short, blind diverticula can be distinguished branching off the gonoduct; they are regarded as the early developmental stages of the female glands. Terms used here for the different female glands follow Klussmann-Kolb (2001). According to their

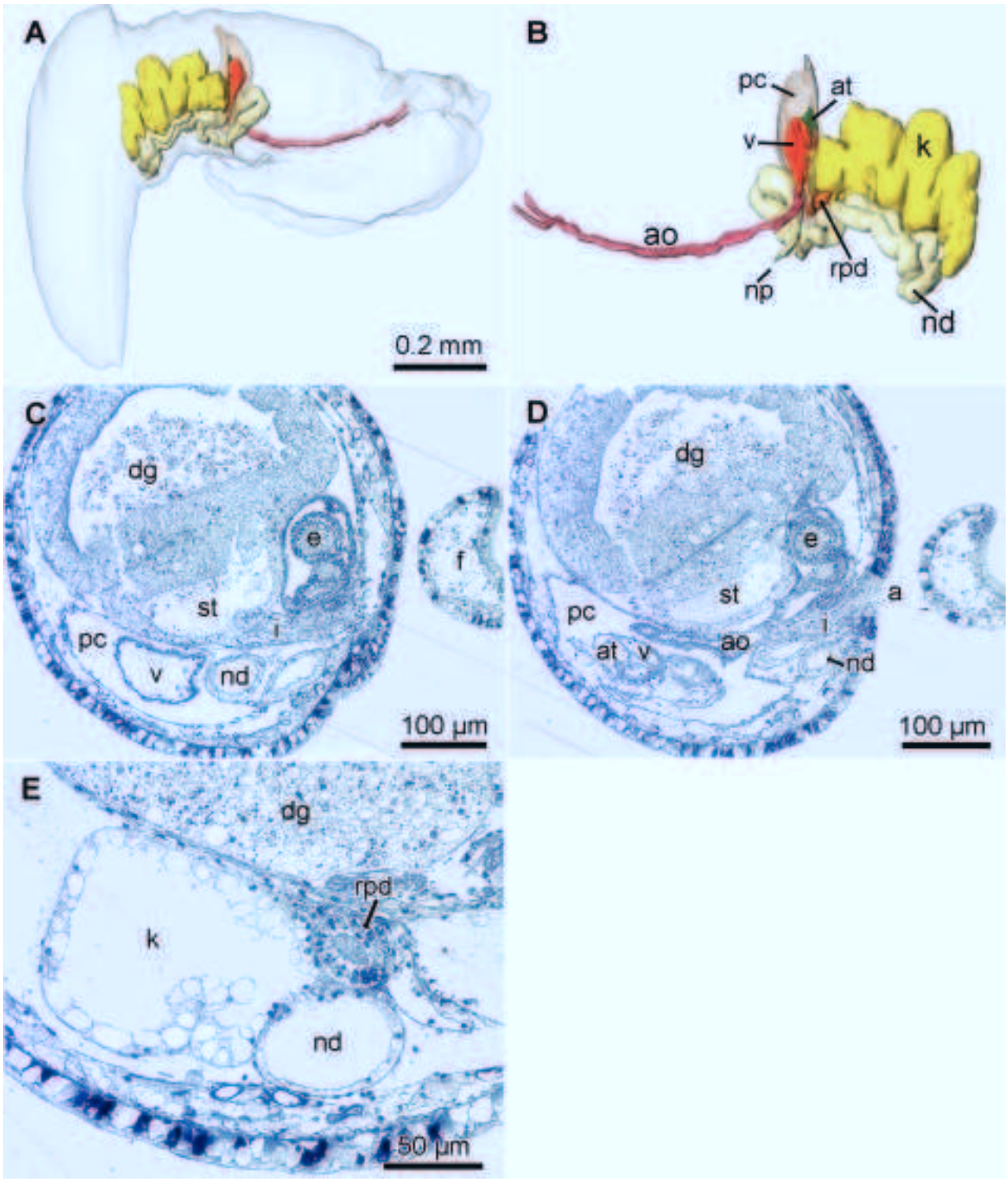

Fig. 6. Excretory and circulatory systems of *Tantulum elegans* (specimen No. 5). **A.** Three-dimensional (3D) reconstruction, position of the organ systems in the specimen (right view). **B.** 3D reconstruction (left view). **C–E.** Semithin transverse sections. **C.** Pericardium and ventricle. **D.** Atrium. **E.** Kidney and nephroduct. a, anus; ao, aorta; at, atrium; dg, digestive gland; e, esophagus; f, foot; i, intestine; k, kidney; np, nephropore; nd, nephroduct; pc, pericardium; rpd, renopericardial duct; st, stomach; v, ventricle.

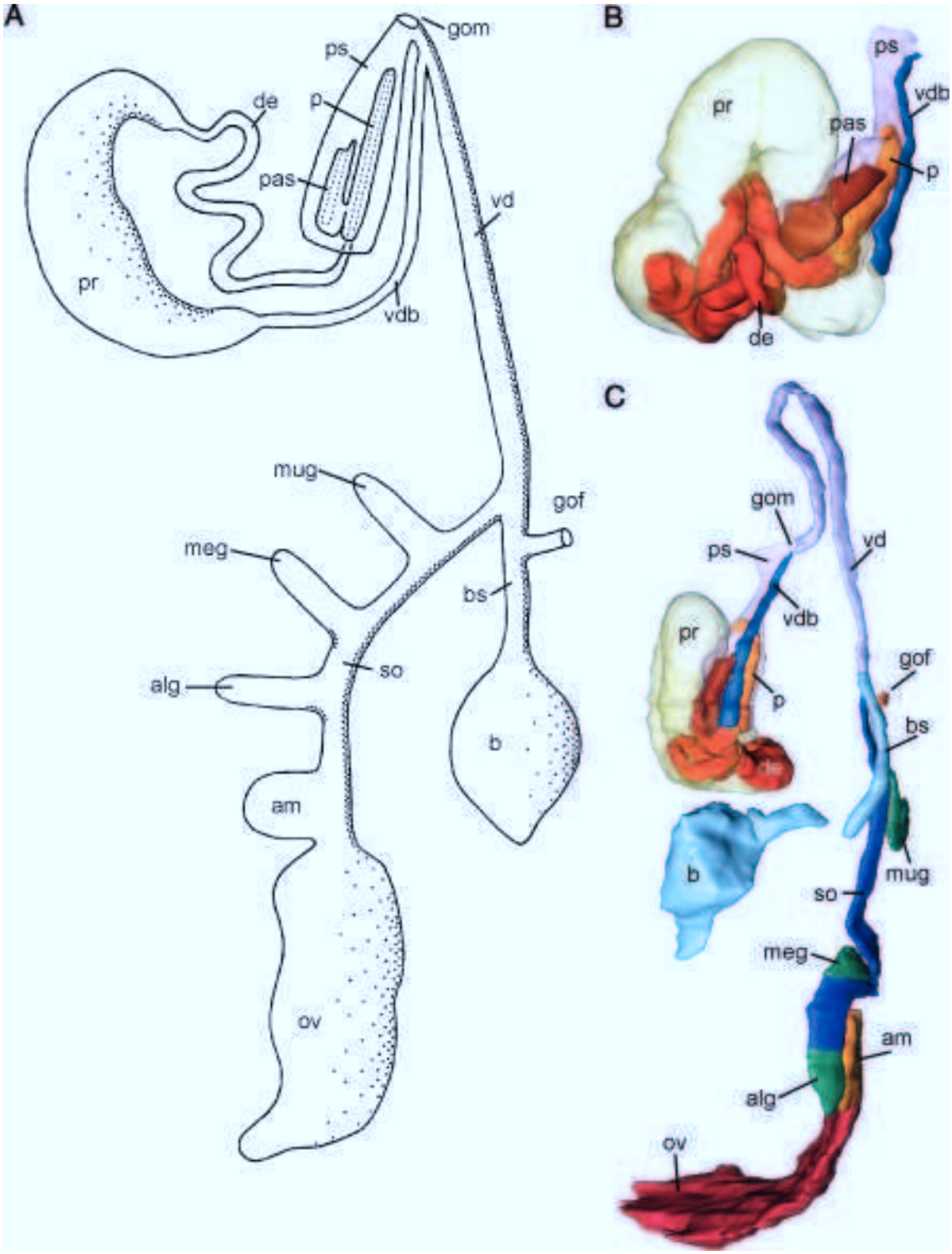

Fig. 7. Genital system of *Tantulum elegans* (specimen No. 6). **A.** Schematic drawing (dorsal view). **B.** Three-dimensional (3D) reconstruction: anterior male genitalia (dorsal view). **C.** 3D reconstruction (right view). alg, albumen gland; am, ampulla; b, bursa copulatrix; bs, stalk of bursa copulatrix; de, ductus ejaculatorius; gof, female genital opening; gom, male genital opening; meg, membrane gland; mug, mucous gland; ov, ovotestis; p, penis; pas, penis-associated structure; ps, penial sheath; pr, prostate; so, spermoviduct; vd, vas deferens; vdb, back-leading vas deferens.

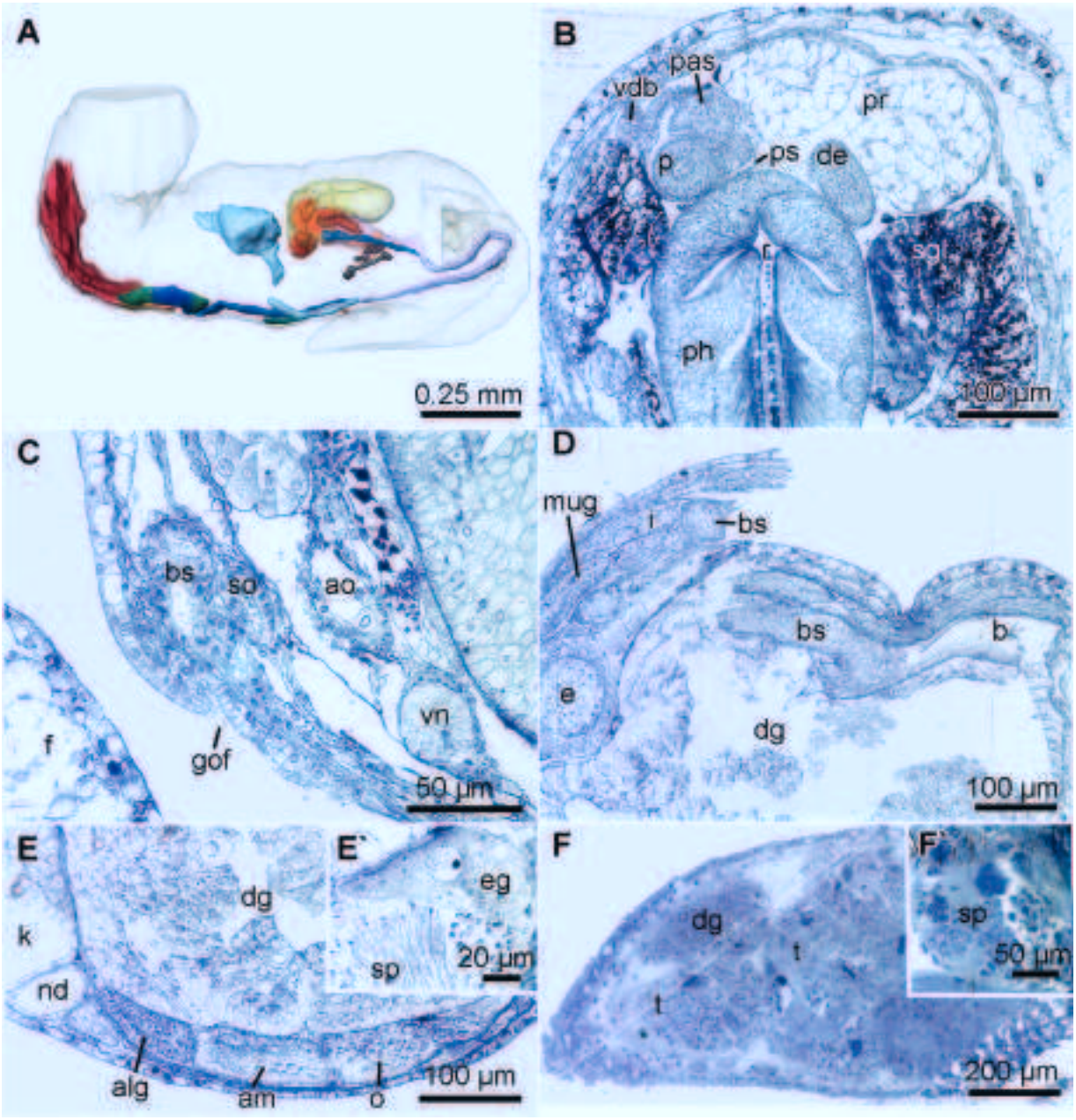

Fig. 8. Genital system of *Tantulum elegans* (specimen No. 6, Fig. 8F/F′ specimen No. 4). **A.** Three-dimensional (3D) reconstruction: position of the organ system in the specimen (right view). **B–F.** Semithin transverse sections. **B.** Penis and prostate. **C.** Female gonopore. **D.** Artificial rupture in the specimen, bursa copulatrix (right side of section is dorsal). **E.** Ampulla and ovary. **E′.** Egg and sperm cells. **F.** Male gonad. **F′.** Sperm cells. alg, albumen gland; am, ampulla; ao, aorta; b, bursa copulatrix; bs, stalk of the bursa copulatrix; de, ductus ejaculatorius; dg, digestive gland; e, esophagus; eg, egg cell; f, foot; gof, female genital opening; i, intestine; k, kidney; mug, mucous gland; nd, nephroduct; o, ovary; p, penis; pas, penis-associated structure; ph, pharynx; ps, penial sheath; pr, prostate; r, radula; sgl, left salivary gland; so, spermoviduct; sp, sperm cells; t, testis; vdb, back-leading vas deferens; vn, visceral nerve.

position in the gonoduct from proximal to distal, female glands are identified as albumen/capsule, membrane, or mucous gland (Fig. 7). Histologically, the developing glands cannot be distinguished from each other in this specimen. They all show the same glandular cells, with blue- and lilac-stained granules alternating with ciliated cells as in the spermoviduct (Fig. 8D,E). At least at this ontogenetic stage, no receptaculum seminis is developed. The spermoviduct (Fig. 8C) divides into the vas deferens and female

gonoduct near the junction of the foot with the visceral sac. An empty sac-like organ occurs distally, posterior to the female gonopore (Figs. 7 and 8D). It is interpreted here to be a bursa copulatrix. Its stalk is ciliated, stained light blue, and not glandular. Unfortunately, there is an artificial rupture in the specimen's body wall; thus, the stalk of the bursa copulatrix is interrupted and could not be reconstructed continuously (Figs. 7 and 8D).

The vas deferens is ciliated and stained light blue. It leads forward, running first ventrally then laterally just under the epidermis, and opens anterior to the right rhinophore (Fig. 7). The anterior male genitalia consist of the back-leading part of the vas deferens, the prostate, a muscular ejaculatory portion, and the penis within a sheath (Fig. 7). The ciliated back-leading vas deferens branches off at the distal end of the vas deferens and runs backwards to the prostate; this portion is laterally attached to the penial sheath (Fig. 7). The prostate is tubular and, probably due to retraction of the head, bent backward (Fig. 7). Histologically, highly glandular, unciliated tissue surrounds a narrow lumen (Fig. 8B). Distally, the connection to the ejaculatory portion of the vas deferens is densely ciliated. This ciliated and muscular duct, after several coils, enters the muscular, long penial papilla and opens terminally at its tip (Fig. 7). The penis is surrounded by a thin-walled penial sheath (Fig. 8B). Penial spines and an apical penial stylet are absent. Within the penial sheath, there is another bulbous, muscular structure associated with the penis and both are connected only basally by muscular tissue (Figs. 7A,B and 8B). This "penis-associated structure" shows a narrow cavity that opens irregularly, apically, into the penial sheath cavity. The "penis-associated structure" lumen is not connected to the lumen of the ductus ejaculatorius, nor to any other glandular structure (Fig. 7A). The penial sheath opens together with the distal vas deferens, closely anterior to the right rhinophore (Fig. 7).

Discussion

Microanatomy

Glands closely attached to the oral tube have been described with a very variable appearance for different acochlidian species. Rankin (1979) described a bilobed gland ventral to the mouth and leading to the exterior, but used different names to describe the same structure: oral organ, suprapedal gland, and oral gland. The freshwater acochlidian *Acochlidium amboinense* shows a field of glandular cells, each of them opening to the exterior (Bücking 1933, as *Hedyle amboinensis*). Challis (1968) described a paired gland with two different ducts joining the oral tube in *Paraganitus ellynnae* Challis 1968. Doe (1974) reported an unpaired "vestibular gland," with one deferent duct, joining the oral tube in *Microhedyle nahantensis* Doe 1974. However, Robinson & Morse (1979) showed the "vestibular gland" of *M. nahantensis* to be a large anterior pedal gland not connected to the oral tube, but opening to the exterior ventral to the mouth. Their histochemical investigations showed that the anterior pedal gland is very similar to the pedal glands. The oral gland in *Tantulum elegans,* described by Rankin (1979) and in the present study, seems to be anatomically and histologically identical to the anterior pedal gland investigated by Robinson & Morse (1979). Therefore, we propose the term anterior pedal gland for this structure in *T. elegans*.

Nervous system and sensory organs

Rankin's (1979) original description of central nervous features in *T. elegans* contains considerable detail. Besides correcting some discrepancies with our results and supplementing her data, we homologize and name structures according to standard works, e.g., Huber (1993) and Gosliner (1994).

The structure of the cns in *T. elegans* agrees with recent results on *Hedylopsis ballantinei* Sommerfeldt & Schrödl 2005 (Hedylopsidae) and *M. remanei* Marcus 1953 (Microhedylidae) according to its prepharyngeal location, probably epiathroid condition, and high concentration of ganglia (Neusser et al. 2006). Rankin's (1979:figs. 39, 40) assumption of fused cerebropleural ganglia in several acochlidian species is not supported by data. Pleural ganglia are separate from cerebral ganglia in *T. elegans,* which seems to be the usual condition in all acochlidians (see Wawra 1987; Huber 1993; Sommerfeldt & Schrödl 2005).

Rankin (1979) described "larger neurons … particularly around the posterior periphery of each ganglion." The presence of giant neurons in *T. elegans* is confirmed here, but giant neurons could not be detected in every ganglion. Giant neurons of different sizes have been reported in pulmonates and opisthobranchs (see Hanström 1929) and should be reinvestigated in other acochlidian species.

"Large, branching clumps" of precerebral "mixed neural and secretory tissue" termed "anterior" and "cephalic sensory organs" by Rankin (1979:p. 21) are identified here as complexes of accessory ganglia as also reported in *M. remanei* and other microhedylacean species (Neusser et al. 2006). According

Table 2. Three cerebral nerves characteristic for Acochlidia according to Huber (1993), and corresponding nerves in other studies.

Huber (1993)	Edlinger (1980b)	Rankin (1979)		Present study
Joint oral/	–	Dorsal cephalic	Superior labial nerve	–
Rhinophoral nerve	N2	nerve	Posterior tentacular nerve	Rhinophoral nerve
	N3		Nuchal nerve	Hancock's nerve
Labiotentacular nerve	N1	Ventral cephalic nerve		Labiotentacular nerve
Static nerve	–	Statocyst nerve		Static nerve

to Rankin (1979), the ''anterior'' complex is situated above and alongside the buccal tube and innervated by the ventral (labiotentacular) nerve. The large ''cephalic sensory'' complexes fill the anterior body cavity and were said to be innervated by both labiotentacular, rhinophoral and ''median labial'' nerves. These large aggregations of precerebral accessory ganglia are clearly visible in sections of specimen No. 4, but only unclearly in other specimens. Specimen No. 4 is also the only available one showing pedal glands and other cells in the foot with good staining properties. Owing to incomplete section series, the innervation of accessory ganglia in specimen No. 4 cannot be reconstructed.

As Sommerfeldt & Schrödl (2005) suspected, the present study shows that Rankin's (1979) ''small lobe'' (al) attached to the cerebral ganglion is the rhinophoral ganglion. According to Huber (1993), acochlidians develop only three cerebral nerves: the dorsal nerve corresponding to the joint oral and rhinophoral nerve, the ventral nerve corresponding to the labiotentacular nerve, and the static nerve innervating the statocysts (see Table 2). The latter is usually very fine and could not be detected in the present study. Rankin (1979) reported the static nerve originating from the cerebral ganglion in *T. elegans*. Accordingly, the labiotentacular nerve in *T. elegans* corresponds to Rankin's ventral cephalic nerve, the rhinophoral nerve to the posterior tentacular nerve. The superior labial nerve of Rankin may refer to the oral nerve according to Huber (1993), but it could not be detected in the present study. Rankin's description lacks the optic ganglion and the optic nerve leading to the eye remnant. A distinct, accessory ganglion at the base of the optic nerve has been reported for *Heliacus* (Heterobranchia), aplysiomorph opisthobranchs, and the nudibranch *Tritonia* by Huber (1993).

Rankin's ''nuchal nerve'' is identified here as Hancock's nerve leading to Hancock's organ. Rankin (1979:p. 28) described: ''just posterior to the left posterior tentacle there is a small ridge, of nonglandular epidermis in which there is a small canalicular opening, leading inward to a thin-walled saccule.'' The ''small ridge'' is not part of the ''male intromittant apparatus'' but refers to Hancock's organ. The thin-walled saccule is the remnant of the eye, lying very close to, but independent of, Hancock's organ.

Acochlidians were generally believed to have lost architectibranch and cephalaspidean Hancock's organs completely (e.g., Wawra 1987; Sommerfeldt & Schrödl 2005; Neusser et al. 2006). Huber (1993) did not find any Hancock's organs either, but he regarded the ''cephalic sensory organs'' of *T. elegans* as homologous to Hancock's organs ''because of its position, function and innervation by two different cerebral nerves'' (Huber 1993:p. 411). Here, these large aggregations of neural tissue, filling the anterior body cavity in *T. elegans,* are thought to refer to the precerebral accessory ganglia, with so far unclear function and innervation. Edlinger (1980a, b) already reported small Hancock's organs, from the marine microhedylacean *Pontohedyle milaschewitchii* Kowalevsky 1901 (as *Microhedyle*) and *M. glandulifera* Kowalevsky 1901, as a pair of regularly folded epidermal structures, lying in lateral grooves and showing abundant chemoreceptor cells. Epidermal folds were small without forming discrete organs, i.e., there are no well-developed folded plates as in some cephalaspidean species. However, the presence of a pair of ciliated, sensory epidermal folds in a posterolateral cephalic position suggests that it is a homolog of Hancock's organs (see Gosliner 1994).

Huber (1993) and Gosliner (1994) characterized cephalaspidean Hancock's organs to be divided into an anterior and posterior portion, innervated by two different cerebral nerves: the anterior portion by the posterior branch of the labiotentacular nerve, and the posterior portion by the rhinophoral nerve. The anterior part is believed to have derived into labial tentacles and the posterior one into rhinophores in nudibranchs and anaspideans, or jointly innervating the cephalic tentacles of most sacoglossans; all of these taxa lack any Hancock's organs. In *P. milaschewitchii,* which is devoid of rhinophores, Edlinger's (1980b) ventral cerebral nerve 1 (N1) innervates

the labial tentacle, and the dorsal N2 the anterior part, and N3 the posterior part of Hancock's organ. In *M. glandulifera*, N2 additionally innervates the rhinophores. In *T. elegans,* the rhinophoral nerve (= N2) divides into one branch leading to the rhinophores, and a posterior branch (= N3?) innervating (rudimentary?) Hancock's organ. While Rankin (1979) doubted that the posterior tentacles in *T. elegans* are true rhinophores, their joint innervation with Hancock's organs clearly indicates they are. Whatever the exact set and homology of sensory organs, cephalic appendages, and cerebral nerves in acochlidians, there seems to be much more variety than previously thought.

Huber's (1993) concept of cerebral systems of heterobranch gastropods thus needs some refinement. Opisthobranchs were generally considered to show either Hancock's organs (most Cephalaspidea *s.l.*) or rhinophores (most Nudipleura, Anaspidea; most Sacoglossa with joint cephalic tentacles). Some shelled sacoglossans (e.g., *Ascobulla*) are also known to possess Hancock's organs (Rudman & Willan 1998). Both Hancock's organs and rhinophores may be subject to reduction or modification in all of these taxa. However, the acochlidians *M. glandulifera* and *T. elegans,* to our knowledge, are the only opisthobranchs showing both Hancock's organs and rhinophores. Other acochlidian species should be reexamined carefully for remnants of potential Hancock's organs, and their ultrastructure and innervation should be comparatively investigated.

Some aberrant nervous features of acochlidians, such as the possession of precerebral ganglia, the reduction of cerebral nerves, the separation of cerebral and pleural ganglia, and the presence of three or four distinct ganglia on the visceral loop, may be due to small body sizes, that is, evolutionary adaptations to an interstitial mode of life. Heterochrony (progenesis) has already been discussed as an important mechanism during acochlidian evolution by Westheide (1987). However, Hancock's organs present in *T. elegans* and at least some other acochlidians can hardly be explained as progenetic adaptations to an interstitial mode of life. Instead, they are interpreted as retaining the plesiomorphic condition as also, and exclusively, expressed by architectibranchs and cephalaspideans. Ultrastructural comparisons between these groups may reveal valuable information for clarifying the still unknown origin of Acochlidia.

In contrast to other acochlidian species studied in detail, i.e., the hedylopsids *H. spiculifera* Kowalevsky 1901 and *H. ballantinei* (see Sommerfeldt & Schrödl 2005), and the microhedylid *M. remanei* (see Neusser et al. 2006), *T. elegans* shows four different, separate ganglia on the visceral nerve cord (Rankin 1979; this study). The identity of visceral loop ganglia is always problematic. According to the hypothesis of the opisthobranch nervous system by Schmekel (1985) and to the pentaganglionate hypothesis of Haszprunar (1985, 1988), basal euthyneurans show a visceral nerve cord with five separate ganglia: left and right parietal, subintestinal, supraintestinal, and visceral ganglia. Thus, one of the four ganglia on the visceral loop in *T. elegans* is apparently fused with another, or was lost. In accordance with Rankin (1979), the largest, posteriormost ganglion on the visceral loop, with a strong nerve leading posterior toward the visceral sac, is regarded to be the visceral ganglion. The first ganglion on the left side is even smaller than the pleural ganglion and thus confirmed to be the left parietal ganglion. The second one is larger and, here, called the subintestinal ganglion (termed ''buccal'' ganglion by Rankin). The first ganglion on the right side is more than twice the diameter of the left parietal ganglion and, therefore, is considered to be the fused supraintestinal/parietal ganglion (fused ''parietal-buccal-visceral'' ganglion according to Rankin).

However, in the mature specimen No. 6, we detected only three ganglia on the visceral loop. Owing to the larger body size of specimen No. 6, all ganglia are larger, too; thus, the comparison between specimen No. 6 and specimen No. 5, and the identification of the two fused ganglia, is difficult. Both pleural ganglia show the same size; therefore, fusion of the parietal ganglion with the pleural ganglia seems improbable. The first ganglion on the right side of the visceral nerve cord is only slightly larger than in specimen No. 5 and is regarded to be the fused supraintestinal/parietal ganglion. The first ganglion on the left side in specimen No. 6 is as large as the subintestinal ganglion in specimen No. 5. Thus, it is unlikely to be the fused left parietal/subintestinal ganglion, but can be regarded as the left parietal ganglion only. The second ganglion on the visceral nerve cord bears a strong nerve running posterior into the visceral sac and is most likely the visceral ganglion. It is considerably larger in specimen No. 6 than in No. 5 and, therefore, might be the fused subintestinal/visceral ganglion. According to Gosliner (1994), many cephalaspideans show a subintestinal ganglion close to or fused with the visceral ganglion.

Either fusion of the subintestinal/visceral ganglia shows intraspecific variability in *T. elegans*, or it occurs in comparably late developmental stages. Ruthensteiner (1999) described the fusion or separation of ganglia in the pulmonate *Ovatella myosotis*

DRAPARNAUD 1801 occurring usually in the larval stage of development. No data concerning other acochlidian species are available.

In acochlidians, an additional ganglion attached to the supraintestinal ganglion was reported for the hedylopsacean *Strubellia paradoxa* (see Wawra 1988), *H. spiculifera* (see Wawra 1989), and *H. ballantinei* (see Sommerfeldt & Schrödl 2005). Owing to its position, it was tentatively identified as the osphradial ganglion (Huber 1993), but an osphradium has never been found in any acochlidian. The additional ganglion attached to the supraintestinal/parietal ganglion of *T. elegans* was detected and termed "accessory visceral ganglion" by Rankin (1979). Because of a thick nerve arising dorsally and leading anterior to the penial sheath, this ganglion is assumed here to control copulatory functions. However, it is problematic to identify it as a usual heterobranch genital ganglion, as this is thought to be located either on the visceral nerve cord or connected (or fused) with the visceral ganglion (Mikkelsen 2002). The additional ganglion in *T. elegans* may thus be considered a penial ganglion and the same might be assumed for those of *S. paradoxa* and *H. spiculifera*, which also have well-developed anterior male genitalia at least in a certain ontogenetic stage (Wawra 1988, 1989; this study). In contrast, Sommerfeldt & Schrödl (2005) found no trace of any anterior copulatory organs in *H. ballantinei*; therefore, a ganglion controlling the penial complex would be useless in the latter species; reinvestigation of the hedylopsid species and *S. paradoxa* is needed.

A pair of additional buccal, that is, gastroesophageal ganglia (Rankin: suprabuccal ganglia) are present in *T. elegans* and were reported from *S. paradoxa* and *H. spiculifera* (see Wawra 1988, 1989). *Hedylopsis ballantinei* and *M. remanei* lack gastroesophageal ganglia (Sommerfeldt & Schrödl 2005; Neusser et al. 2006). No data concerning the gastroesophageal ganglia on further Acochlidia are available. Elsewhere in opisthobranchs, gastroesophageal ganglia are known for many nudibranchs, e.g., *Bathydoris*, *Jorunna*, *Armina*, *Dermatobranchus*, *Tritonia*, *Aeolidia*, and *Flabellina* (see Gosliner 1994; Wägele & Klussmann-Kolb 2005).

In *T. elegans,* there is a single, unpaired radular nerve originating from the buccal commissure, as reported from *S. paradoxa* (see Wawra 1988), while this is unknown for other acochlidians. Wägele & Willan (2000) reported an unpaired radular nerve in various opisthobranchs, e.g., Aplysiidae, *Pleurobranchus*, *Armina*, *Haminoea*, and *Tylodina*.

The cns of *T. elegans* shows a very similar arrangement to that of *H. ballantinei,* which was thought to reflect the usual and possibly basal condition in Acochlidia (Sommerfeldt & Schrödl 2005). Some discrepancies, such as the presence and innervation of Hancock's organ, the absence of gastroesophageal ganglia, and the identity of the genital ganglion, require further comparative investigations.

Digestive system

In *T. elegans,* the set and arrangement of digestive organs differ from those of other opisthobranchs and acochlidians in some ways. Rankin (1979) discussed two different types of buccal cavities in acochlidians. The first type, described in *Paraganitus ellynnae* and *Ganitus evelinae* MARCUS 1953 (both Ganitidae), represents a strongly modified pharynx with strongly developed longitudinal muscles connecting the ventral cuticular radular cushion with a pair of cuticular jaws (Marcus 1953; Challis 1968). Jaw-like cuticular structures were also reported from the microhedylid *M. glandulifera,* but need to be confirmed and studied in detail. Rankin's second type includes a series of: (1) a poorly developed pharynx with a small radular cushion, as in *Parhedyle tyrtowii* KOWALEVSKY 1900 (see Kowalevsky 1901, as *Microhedyle*); (2) a well-developed pharynx, as in *A. amboinens*e (see Bücking 1933); and (3) a very complex buccal cavity showing a highly muscular and bulbous pharynx, as in *T. elegans* (see Rankin 1979). Bücking (1933) described *A. amboinense* with a muscular pharynx being broad in the ventral part and narrower in the dorsal part. His drawings show both parts connected, whereas Rankin's schematic drawings (Rankin 1979:p. 63) do not match the original drawings of Bücking and give the impression of a deep groove between the dorsal and the ventral part. Recent results on *M. remanei* show a pharynx very similar to that of *T. elegans,* except that the posteroventral part with the radula sac extends more posteriorly (Neusser et al. 2006). While there is no doubt about the modified character of ganitid buccal masses, the pharynx of other acochlidian species appears to be quite similarly structured.

Differences in the buccal cavity structure refer to the more or less protruding radular sac, the different length of the radula limbs, the symmetry of the radula and the teeth. The radula of *T. elegans* was described in detail by Rankin (1979). The median, rhachidian tooth shows 4 or 5 denticles. The asymmetry of the radula, described by Rankin (1979), cannot be re-examined in serial semithin sections. According to Rankin (1979), the lateral, rectangular tooth plates show two denticles, one on each anterior and posterior border. Where present in acochlidian

species, there is normally one denticle on the anterior border of the lateral plate and, additionally, there is a notch on the posterior border corresponding to the denticle of the following lateral plate. Denticles and notches are difficult to distinguish in serial sections. Therefore, the radula formula and the structure of the teeth should be re-investigated by scanning electron microscopy.

Well-developed salivary glands are known for many acochlidian species and have been described by various authors (e.g., Challis 1968; Morse 1976; Sommerfeldt & Schrödl 2005; Neusser et al. 2006). In all cases, they are connected to the pharynx via a pair of salivary ducts. The latter were reported to have conspicuous swellings between the salivary glands and the salivary ducts in *T. elegans* by Rankin (1979), which is confirmed here. Their function may be to collect the secretion of the salivary glands and eject it into the salivary ducts and pharynx when needed. We would propose to term these organs "salivary pumps" instead of Rankin's term "pharyngeal pumps," as is usually used for sacoglossan or suctorian nudibranch organs, i.e., strongly muscular sucking pumps directly attached to the pharynx. A similar salivary pump is already reported from *Palliohedyle weberi* by Bergh (1895) as a spherical or spindle-shaped ampulla. According to Rankin (1979), there are also small salivary reservoirs situated close to the pharynx. These could not be detected in the present study and are not confirmed.

Only a few Indo-Pacific freshwater acochlidian species have been reported to possess a well-developed and differentiated stomach, e.g., *P. weberi* (see Bergh 1895) or *A. amboinense* (see Bücking 1933). The swollen "stomach" of *P. milaschewitchii* should be histologically reinvestigated. Other species, such as *M. remanei*, lack any separate stomach (Neusser et al. 2006) or show a stomach almost or completely fused with the digestive gland, e.g., *Pseudunela cornuta* Challis 1970, *Asperspina riseri* Morse 1976, or *T. elegans* (see Rankin 1979; this study).

The sac-like, holohepatic digestive gland of *T. elegans* conforms to the description of those in most other limnic and all marine acochlidian species (see Sommerfeldt & Schrödl 2005); it shows a large central lumen and one opening into the stomach area. So far, the freshwater *P. weberi* was reported to be "cladohepatic," i.e., a ramified digestive gland with two branches entering the stomach via separate ducts (Bergh 1895). And the lobuled digestive gland of *A. amboinense* forms $\leqslant 14$ diverticulae that fuse before entering the stomach (see Bücking 1933). The intestine is short and ciliated in all acochlidians. The position of the anus is usually situated at the junction of the head–foot complex and the visceral hump as in *M. remanei* (see Neusser et al. 2006), *Strubellia paradoxa* (see Küthe 1935), *H. ballantinei* (see Sommerfeldt & Schrödl 2005), and *A. riseri* (see Morse 1976, as *H. riseri*), or more posteriorly at the visceral sac as in *T. elegans* (see Rankin 1979; present study), *M. glandulifera* (see Kowalevsky, 1901), *P. milaschewitchii* (see Marcus & Marcus 1954), *M. nahantensis* (see Doe 1974), and *A. murmanica* Kudinskaya & Minichev 1978. Furthermore, the anus always opens dextral and usually ventrolateral. Only *A. murmanica* (see Kudinskaya & Minichev 1978) and *T. elegans* (see Rankin 1979; present study) show an almost ventral anal opening. Only sparse data are available concerning the feeding habits of acochlidians; more are crucial for a better understanding of the different features of acochlidian digestive systems.

Circulatory and excretory systems

The knowledge of the circulatory and excretory systems of Acochlidia is still limited. Rankin (1979) described *T. elegans* to have a one-chambered heart consisting only of the ventricle. The presence of a "sinu-cardiac valve" is confirmed here but this structure is interpreted here as a very small auricle. Rankin also regarded *Hedylopsis* and all microhedylids to have a one-chambered or a completely reduced heart, respectively. However, recent histological and ultrastructural studies have shown that *H. ballantinei* has a well-developed, two-chambered heart (Fahrner & Haszprunar 2002, as *Hedylopsis* sp.; Sommerfeldt & Schrödl 2005), and *M. remanei* has a small, two-chambered heart as well (Neusser et al. 2006). Large Pacific freshwater acochlidian species such as *S. paradoxa* (see Küthe 1935, as *A. paradoxum*) and *A. amboinense* (see Bücking 1933, as *H. amboinensis*) show a two-chambered heart with a still unknown ultrastructure.

Within the Acochlidia, the shape of the kidney varies. Marine species show a simple, sac-like kidney usually with a short nephroduct, e.g., *H. ballantinei* or *M. remanei* (see Sommerfeldt & Schrödl 2005; Neusser et al. 2006). But all freshwater acochlidian species have a well-developed excretory system with a long and, in some species, looped kidney. According to Bücking (1933), *A. amboinense* (as *H. amboinensis*) has "numerous ciliated nephrostomes" originating in the pericardium; the kidney is tubular and as long as the visceral sac, and the nephric duct is short. A ciliated nephrostome is also present in *T. elegans* (forming a ciliated funnel: Rankin 1979; present study), *S. paradoxa* (see Küthe 1935), and *H. ballantinei* (see Sommerfeldt & Schrödl 2005), whereas *M. remanei*

shows a narrow renopericardioduct without a ciliated funnel (Neusser et al. 2006). *Strubellia paradoxa* also resembles *T. elegans* in showing a long tubular kidney and a long, looped nephroduct (Küthe 1935). There appear to be narrow connections between the arms of the looped nephroduct in *S. paradoxa* (see Küthe 1935) that are not present in *T. elegans*. While there is a common exit of the digestive and excretory systems in *S. paradoxa*, the anus and nephropore are separated in *T. elegans* (see Küthe 1935; Rankin 1979; present study). According to Rankin (1979), the "enlarged" excretory system of *T. elegans* is a modification for the freshwater habitat and required for increased osmoregulation. Ultrastructural investigations are needed to reveal and compare specific features in the excretory system of *T. elegans* and specimens of Pacific freshwater acochlidian species.

According to Rankin (1979), the nephropore can be associated either with the anus, as in suborders Pedoneura, Proprioneura, and Pharyngoneura, or with the gonopore as in the subclass Cerebroneura. But recent examination shows the nephropore of *H. ballantinei* (according to Rankin: subclass Proprioneura) closely associated with the gonopore (Fahrner & Haszprunar 2002; Sommerfeldt & Schrödl 2005), and the nephropore of *M. remanei* (according to Rankin: subclass Cerebroneura) situated at the junction of the head/foot complex, together with the female gonopore and the anus (Neusser et al. 2006). The relative position of the nephropore, anus, and gonopore may have phylogenetic significance within acochlidians as proposed by Rankin (1979), but first, these features have to be reinvestigated in detail in all known acochlidian species before such generalities can be made.

Reproductive system

The reproductive features of *T. elegans* revealed here show several significant discrepancies from the original description. According to Rankin (1979): (1) all specimens examined presented a "reduced reproductive system," (2) with neither eggs nor sperm developed, (3) the "small ridges" just posterior to the left rhinophore were regarded as "remnants of a male intromittant apparatus," and (4) the gonoduct opens into a "genital pouch." Re-examining the original sections, we found one specimen (No. 4) showing a well-developed, mature male gonad filled with autosperm. Furthermore, in specimen No. 5, traces of the anterior male genitalia can be detected, although anterior to the level of the right rhinophore. According to our new findings, Rankin's "small ridge" is part of Hancock's organ. Finally, we cannot confirm the existence of a "genital pouch" that, according to Rankin (1979:p. 28), is "homologous with a mantle cavity"; specimens reconstructed here show a simple genital opening posteroventral to the right mantle fold. A reduced mantle cavity was reported for *A. murmanica* (see Kudinskaya and Minichev, 1978; as *Hedylopsis*) and *H. ballantinei,* and may also be present in *Pseudunela cornuta* and *Paraganitus ellynnae* (see discussion in Sommerfeldt & Schrödl 2005). Our results show that there is no trace of any rudimentary mantle cavity in *T. elegans*.

According to Schmekel (1985), most opisthobranchs are simultaneous or protandric hermaphrodites showing well-developed and complex reproductive systems. Uniquely within opisthobranchs, members of the Microhedylidae and Ganitidae have separate sexes; other acochlidian species with known reproductive conditions are hermaphroditic. Several species are clearly protandric; *S. paradoxa* and *H. spiculifera* are sequential hermaphrodites that completely reduce the male gonads and copulatory organs in their later female phase (Wawra 1988, 1989). Opisthobranchs and hermaphrodite acochlidians usually develop an ovotestis, but the acochlidian *A. riseri* shows two separate gonads (Morse 1976). The present study reveals *T. elegans* to be a protandric hermaphrodite developing an ovotestis; from the specimens available, it cannot be determined whether *T. elegans* reduces the anterior copulatory organs during the later female phase. In specimen No. 4, with the mature testis filling a great part of the visceral sac, we were unable to find anterior male organs, either due to the fragmentary original sections or because of the special ontogentic stage. However, in specimen No. 6, the male copulatory organs are (still?) present and appear to be fully functional, although the ovotestis only produces egg cells.

The female genital system in *T. elegans* agrees with the hypothetic ancestral opisthobranch genital system (see Ghiselin 1965; Mikkelsen 2002), but lacks a receptaculum seminis for allosperm storage.

A female gonad with many small oocytes is described for *H. spiculifera* (see Wawra 1989), *H. ballantinei* (see Sommerfeldt & Schrödl 2005), *A. fijiense* (see Haynes & Kenchington 1991), and *S. paradoxa* (see Wawra 1988). In contrast, *A. riseri* develops few, large "vitellogenic eggs" according to Morse (1976:p. 227), as also reported from some microhedylid and ganitid species. According to Ghiselin (1965), opisthobranch eggs are surrounded by three different layers. Klussmann-Kolb (2001) reveals the nidamental glands as a complex structure, usually consisting of three different glands from proximal to distal: albumen or a modified capsule gland,

membrane gland, and mucous gland. They are considered to be homologous within the Opisthobranchia due to their relative position, similar histology, mode of secretion, and histochemical staining properties. The three developing glands of *T. elegans* can be identified only by their relative position in the pallial gonoduct, due to the identical staining properties of the whole pallial gonoduct in this stage of development. Until now, there were few histological data available on acochlidian female glands. But recent investigations of Neusser et al. (2006) present data for *M. remanei* as having three well-developed female glands with a characteristic pattern of ciliation and staining properties similar to those described by Klussmann-Kolb (2001).

The sperm cell reservoir just anterior to the female gonad cannot be regarded as a receptaculum seminis, as sperm lie in disorder rather than being attached to the wall by their heads. This reservoir may either be an ampulla with autosperm remaining of the male phase and (accidentally?) entering the female gonad. However, there is no testicular tissue with active spermiogenesis detectable in this specimen, and there are also some oocytes inside the reservoir. If this sperm is allosperm waiting for mature egg cells, the reservoir has to be considered as some kind of storage vesicle.

Wawra (1988) reported *S. paradoxa* to possess a receptaculum seminis and a distal bursa copulatrix. *Pseudunela cornuta* presents a ''short blind sac … from the wall of the cloaca,'' interpreted as a bursa copulatrix by Challis (1970:p. 35). All other acochlidian species known in detail lack any allosperm storing sac. However, *T. elegans* shows distally a sac-like organ without sperm. With the present level of knowledge, and assuming an ovotestis in *T. elegans,* the latter is probably a bursa copulatrix. If further examinations of specimens in different stages of development reveal *T. elegans* to have two separate gonads, this sac-like structure could be an ampulla for autosperm storage.

The anterior male genitalia of *T. elegans* are similar to those described for *Pseudunela cornuta* (see Challis 1970, as *Hedylopsis*) and *A. fijiense* (see Haase & Wawra 1996), but show the following differences: (1) an unarmed penis (vs. one or two spines in *P. cornuta* and numerous penial spines in *A. fijiense*), (2) an internal ductus ejaculatorius (vs. ejaculatory finger in *A. fijiense*), and (3) the absence of a paraprostate (present in *A. fijiense* and *P. cornuta*). The identification of the penis-associated structure at the base of the penis in *T. elegans* is difficult. Owing to its position and muscular tissue, it might be a penial retractor muscle. Its elongate shape with a central cavity, however, resembles a basal finger, as was described for *A. fijiense* by Haase & Wawra (1996). In contrast to a basal finger, the penis-associated structure of *T. elegans* has no cuticular spines, and the central cavity shows no connection to any glandular structure, i.e., there is no paraprostate detectable.

However, reduction or entire loss of parts of the anterior male genital organs at the beginning of the female maturation cannot be excluded.

The reproductive system of *T. elegans* shows some modifications that, according to Ghiselin (1965), are improvements on inefficient features of the ancestral opisthobranch hermaphroditic reproductive system: (1) sequential hermaphroditism is considered to be an adaptation to alleviate interferences between egg and sperm cells, (2) an internal and closed vas deferens avoids the loss of sperm, (3) a closed ductus ejaculatorius and a prostate are regarded to accelerate the transfer of sperm, and (4) the division of the pallial gonoduct makes gamete transport easier, avoiding interferences between allosperm, autosperm, and egg cells. Ghiselin (1965) proposed a monaulic, diaulic (either andro- or oodiaulic), or triaulic reproductive system having, respectively, one, two, or three separated ducts for autosperm, allosperm, and eggs. *Tantulum elegans* shows a separate vas deferens to accommodate autosperm and an otherwise undivided pallial gonoduct for allosperm and eggs. Therefore, *T. elegans* is the only known acochlidian that appears to be androdiaulic; all others were described to have a monaulic reproductive system (or are gonochoristic). The supposedly monaulic reproductive system of *A. fijiense* (see Haase & Wawra 1996), showing an internal gonoduct and only one gonopore at the level of the right rhinophore, should be reinvestigated carefully for the existence of a posterior female gonopore that may be easily overlooked.

One crucial question remains: how does sperm transfer occur in *T. elegans*? Wawra (1992) proposed three strategies for transferring sperm in acochlidians: transfer by (reciprocal) copulation, hypodermal injection, or spermatophores (see discussion in Neusser et al. 2006). Transfer by spermatophores is known from various asperspinid, microhedylid, and ganitid species showing reduced anterior male copulatory organs or lacking them, such as in *A. brambelli* Swedmark 1968, *M. glandulifera* (see Wawra 1978), and *M. remanei* (see Kirsteuer 1973; Neusser et al. 2006). None of these species has a complex copulatory organ system as is present in *T. elegans*. Hypodermic impregnation, as is known in *H. spiculifera* and *A. fijiense* (see Haase & Wawra 1996), requires a penial spine or some similar structure for use as a hypodermic needle. No cuticular structure has been found associated with the copulatory organs of

T. elegans, nor has sperm been found outside of the gonad that might have originated from hypodermal impregnation. In acochlidian species with hypodermal impregnation, sperm obviously are injected unspecifically into the body of the mate and not necessarily into the reproductive system (Wawra 1978 for *H. spiculifera;* Haase & Wawra 1996 for *A. fijiense*). The presence of copulatory organs and the absence of penial stylets in *T. elegans* are reasons to believe that, in this species, the mode of sperm transfer is copulation. Although no specialized sperm receptacle was present in the specimens examined here, such organs may develop during later female maturation as in *S. paradoxa* (see Wawra 1988). However, if there is copulation between *T. elegans* specimens, this may differ from the reciprocal copulation as is usual in opisthobranchs because of the large distance between the cephalic penis and the posterior female opening.

Systematic implications

The biologically and structurally aberrant *T. elegans* inspired Rankin (1979) to establish a separate monotypic family Tantulidae in its own suborder Pharyngoneura. A total of 25 acochlidian species were reorganized into 13 families (10 of them new), two superfamilies (both new), and five suborders (all new). The order Acochlidioidea, a new order Platyhedyloidea, and philinoglossid cephalaspideans were united as a new gastropod subclass Ceratobranchia by Rankin (1979). This classification has been abandoned in reviews of acochlidian systematics by Arnaud et al. (1986) and Wawra (1987), but Tantulidae has been retained as a monotypic family; it was placed into the Hedylopsacea together with Hedylopsidae (the limnic *Strubellia,* the marine *Pseudunela* and *Hedylopsis*), and Indo-Pacific large-sized limnic Acochlidiidae (*Acochlidium* and *Palliohedyle*). During a revision of the genus *Hedylopsis*, Sommerfeldt & Schrödl (2005) expressed doubts about the monophyly of Hedylopsacea. Besides being limnic and having a rather well-developed foot, there was no indication of *Tantulum* being related to either Hedylopsidae or Acochlidiidae. Our results, however, show several features until now only known from (at least some) members of these groups: (1) true rhinophoral ganglia, (2) a salivary pump (as in *P. weberi;* (see Bergh 1895), (3) a complex anterior copulatory organ system with a well-developed muscular penial papilla, (4) large prostatic and muscular ejaculatory vas deferens sections, and (5) protandric hermaphroditism (as in *S. paradoxa* and *H. spiculifera;* see Wawra 1988; Sommerfeldt & Schrödl 2005). However, the supposedly microhedylacean Asperspinidae are also hermaphrodites; no information exists on possible protandry in these species. The androdiaulic condition of *T. elegans* seems unique within acochlidians, but further studies on the otherwise similar monaulic genital systems of Acochlidiidae and *Strubellia* should be performed. In contrast to all other known phallic hedylopsacean species, the penis of *T. elegans* does not show any cuticular armature or spines, but more material from different phases of sexual maturation should be examined.

Large associations of precerebral ganglia, as were considered diagnostic for microhedylacean taxa by Wawra (1987), were present in at least one specimen of *T. elegans* examined here; "a few" accessory ganglia were also mentioned in the hedylopsid *Pseudunela cornuta* by Challis (1970). An internal vas deferens with an anterior opening was also reported in *A. fijiensis* (see Haase & Wawra 1996) and the microhedylid *P. milaschewitchii* (see Wawra 1986). Sperm of *T. elegans* under light microscopy show a similarly coiled, but shorter head than those of microhedylacean species. The sperm in *H. ballantinei,* under light microscopy, show a short head as in *T. elegans,* but seems not to be coiled (T. P. Neusser, unpubl. data). However, adequately fixed specimens of *T. elegans* are required for ultrastructural examination.

In conclusion, *T. elegans* is neither a member of the limnic Acochlidiidae, which are characterized by a large body size and giant armed penial papillae, nor a member of the Microhedylidae or Ganitidae, which have separate sexes. Instead, *T. elegans* shows a mixture of character conditions as is present in Wawra's (1987) Hedylopsidae (*Strubellia, Pseudunela, Hedylopsis*) and Asperspinidae, taxa that were assumed to be at least paraphyletic by Sommerfeldt & Schrödl (2005). Cladistic analyses considering our new results are thus overdue.

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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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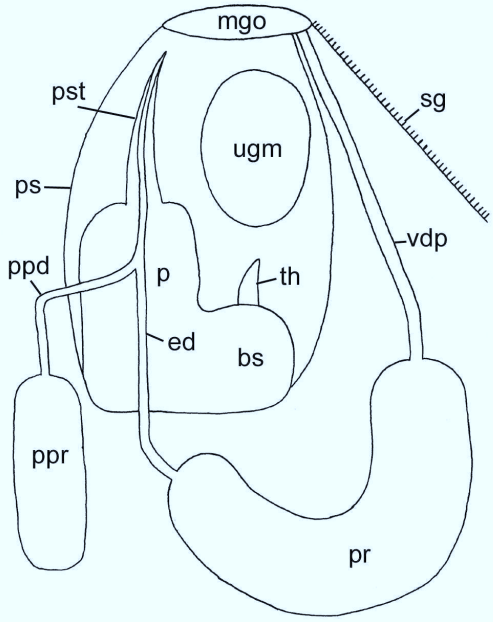

Figure 1:
Schematic overview of the male cephalic copulatory organs with associated glands of Hedylopsis ballantinei. Abbreviations: bs, basal swelling; ed, ejaculatory duct; mgo, male gonopore; p, penis; ppd, paraprostatic duct; ppr, paraprostate; pr, prostate; ps, penial sheath; pst, hollow penial 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

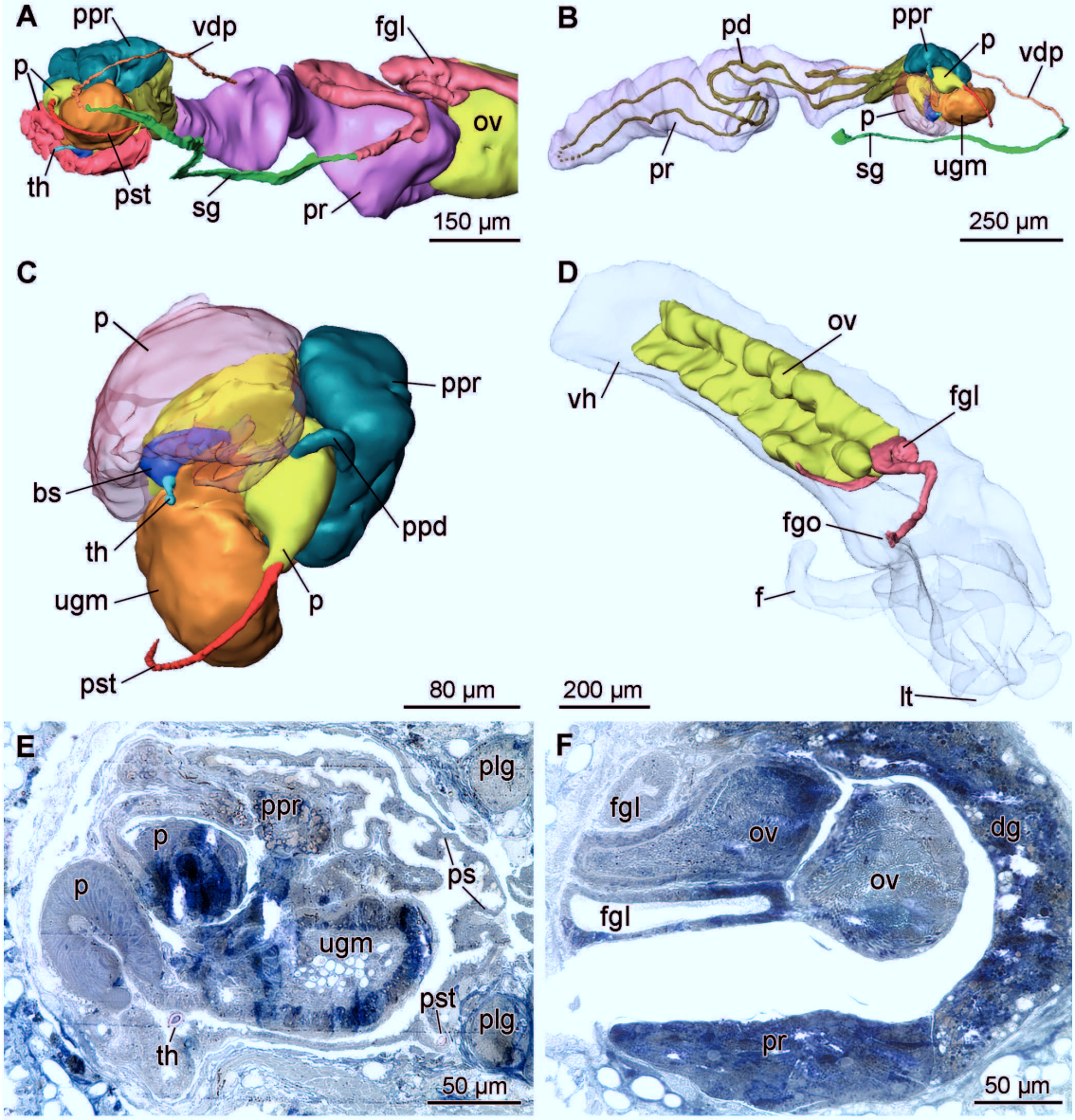

Figure 2:
3D-reconstruction and histological semithin sections of the male reproductive system of Hedylopsis ballantinei. A, Hermaphroditic reproductive system (ventral view); B, Male cephalic copulatory organs (right view); C, Penis and basal swelling with glands and armature (anterior view); D, Body 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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Tiny but complex - interactive 3D visualization of the interstitial acochlidian gastropod *Pseudunela cornuta* (Challis, 1970)

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Abstract

Background: Mesopsammic acochlidians are small, and organ complexity may be strongly reduced (regressive evolution by progenesis), especially in microhedylacean species. The marine interstitial hedylopsacean *Pseudunela cornuta* (Challis, 1970), however, was suggested as having a complex reproductive system resembling that of much larger, limnic and benthic species. The present study aims to reconstruct the detailed anatomy and true complexity of *P. cornuta* from serial, semithin histological sections by using modern computer-based 3D visualization with Amira software, and to explain it in an evolutionary context.

Results: Our results demonstrate considerable discordance with the original species description, which was based solely on paraffin sections. Here, we show that the nervous system of *P. cornuta* has paired rhinophoral, optic and gastro-oesophageal ganglia, three distinct ganglia on the visceral nerve cord, and a putative osphradial ganglion, while anterior accessory ganglia are absent. The presence of an anal genital cloaca is clearly rejected and the anus, nephropore and gonopore open separately to the exterior; the circulatory and excretory systems are well-differentiated, including a two-chambered heart and a complex kidney with a long, looped nephroduct; the special androdiaulic reproductive system shows two allosperm receptacles, three nidamental glands, a cavity with unknown function, as well as highly complex anterior copulatory organs with two separate glandular and impregnatory systems including a penial stylet that measures approximately a third of the whole length of the preserved specimen.

Conclusion: In spite of its small body size, the interstitial hermaphroditic *P. cornuta* shows high complexity regarding all major organ systems; the excretory system is as differentiated as in species of the sister clade, the limnic and much larger Acochlidiidae, and the reproductive system is by far the most elaborated one ever observed in a mesopsammic gastropod, though functionally not yet fully understood. Such organ complexity as shown herein by interactive 3D visualization is not plesiomorphically maintained from a larger, benthic ancestor, but newly evolved within small marine hedylopsacean ancestors of *P. cornuta*. The common picture of general organ regression within mesopsammic acochlidians thus is valid for microhedylacean species only.

Background

The meiofauna of marine sands includes species of nearly all taxa of invertebrates, many of which show regressive characteristics in their anatomy or specialized features in their organ systems [1]. Compared to their supposed basal opisthobranch relatives [2,3], mesopsammic acochlidian sea slugs display many of such reductions, e.g., they have a small and worm-like body, lack a shell, are unpigmented, cephalic tentacles and eyes are reduced in several lineages, many species are aphallic, and in general, the reproductive, excretory and circulatory systems have a very simple organization. Due to such reductions, which are especially pronounced in one subclade, the Microhedylacea, the Acochlidia were hypothesized to have undergone "regressive evolution" [4], as a result of progenesis [5]. However, several recent studies [6,7] show that original, macroscopic or paraffin-based histological descriptions of small acochlidian species could hardly give a reliable picture even of simple organs. In contrast, computer-based 3D-reconstruction of serial semithin histological slices is highly efficient to obtain detailed and reliable knowledge even on tiny and complex structures, such as the considerably differentiated acochlidian central nervous system [8-10].

Species of the second acochlidian subclade, the Hedylopsacea, may show fewer tendencies for reductions; in contrast to the microhedylaceans, the circulatory and excretory systems, and reproductive and copulatory organs may be highly complex and are derived especially in members of the Acochlidiidae s.l., a clade of larger-sized, benthic, limnic members [3]. According to a phylogenetic analysis [3], the genus *Pseudunela* is the sistergroup to such derived acochlidians, despite species of *Pseudunela* being small, marine, interstitial forms. Only two *Pseudunela* species are known, *P. eirene* Wawra, 1988 [11] and *P. cornuta* [12]. The description of *P. eirene* is brief and based on a single specimen with ganglia of the nervous system and stylets of copulatory organs studied on a whole-mount by light microscopy only. No histological sections were made, and the radula was studied light-microscopically after dissolving the soft parts and stylets. Information on other organ systems is absent, and no further specimens are available for study. In contrast, the original description of *P. cornuta*, the type species, is based on paraffin sections, and quite detailed data about the central nervous and the digestive systems is included. However, information about the excretory system is fragmentary and improper, and data about the reproductive system is confusing. Well-preserved specimens of *P. cornuta* were made available for detailed 3D-reconstruction. The present study thus explores the complex anatomy and potential role of a member of the stemgroup of a radiation that accounted for major evolutionary changes, i.e. a habitat switch to freshwater systems and an evolution towards highly complex copulatory systems that culminated in a giant, trap-like "rapto-penis".

Methods

Sampling and specimen preparation

During an expedition to Guadalcanal, Solomon Islands in October 2007, two specimens of *Pseudunela cornuta* were collected at the beach of Komimbo Bay near Tambea Village (09°15.843'S, 159°40.097'E). They were extracted from sand samples (fine sand of the lower intertidal) according to Schrödl [13] and relaxed using 7% $MgCl_2$ solution. Both specimens were preserved in 75% ethanol.

Later in the laboratory, the visceral sac of one specimen was removed for further molecular analysis. The remaining anterior body and the other entire specimen were decalcified with Bouin's solution overnight. For better visibility of the translucent specimens and an appropriate orientation during the embedding procedure, the material was stained with Safranin (0.5% Safranin in 80% ethanol) for a few minutes and rinsed with 80% ethanol. Finally, the two specimens (in one case only anterior part) were dehydrated in a graded series of acetone in distilled water (80, 90 and 100%) and embedded in Spurr's low viscosity resin [14]. Two 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 at the lower cutting edge [15], and finally stained with methylene blue-azure II according to Richardson *et al.* [16]. The sections were deposited at the Zoologische Staatssammlung München, Mollusca Section (entire specimen: ZSM N° 20071911 and anterior body: ZSM N° 20071809).

3D reconstruction

Digital photographs of every slice (420 images in total) 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 1120 × 840 pixels (resulting pixel size: 0.8 μm) 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 all major organ systems was conducted with the software AMIRA 4.1 and 5.2 (TGS Europe, Mercury Computer Systems, Merignac Cedex, France) following basically the procedure explained by Ruthensteiner [15]. The interactive 3D model for the electronic 3D PDF version were prepared using the 3D tools of Adobe Acrobat Professional Extended 9.0 (Adobe Systems Incorporated) according to Ruthensteiner & Heß [17]. The 3D model (accessible by clicking onto Fig. 1 in the 3D PDF version of this article; see also additional files 1 and 2) permits standard operations as zoom and rotation, the selection of the recon-

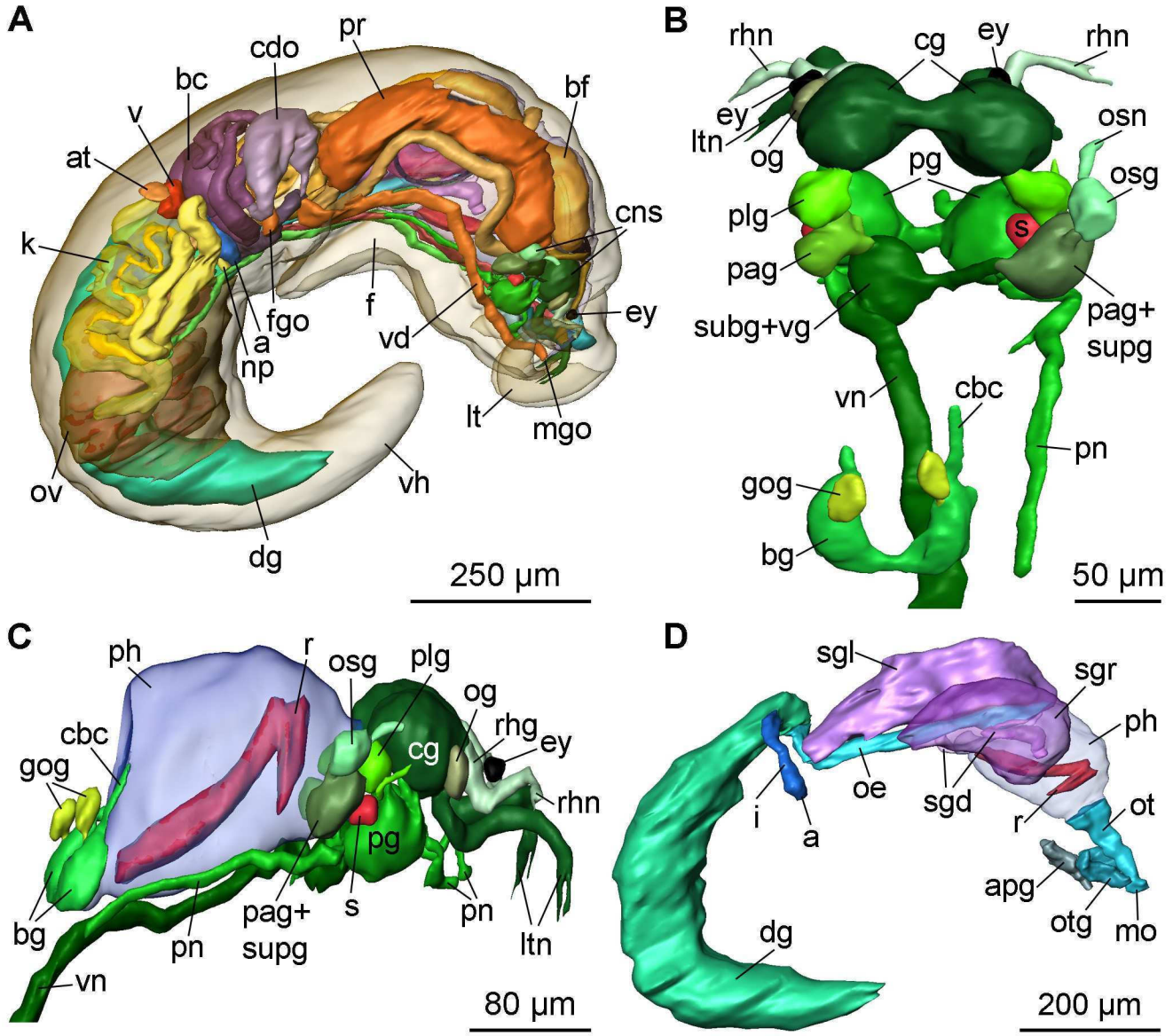

Figure 1

3D reconstruction of the general anatomy, the CNS and the digestive system of *P. cornuta*. A: general anatomy, right view. B: CNS, dorsal view. C: CNS with pharynx, right view. D: digestive system, right view. Abbreviations: **a**, anus; **apg**, anterior pedal gland; **at**, atrium; **bc**, bursa copulatrix; **bf**, basal finger; **bg**, buccal ganglion; **cbc**, cerebro-buccal connective; **cdo**, cavity of distal oviduct; **cg**, cerebral ganglion; **cns**, central nervous system; **dg**, digestive gland; **ey**, eye; **f**, foot; **fgo**, female gonopore; **gog**, gastro-oesophageal ganglion; **i**, intestine; **k**, kidney; **lt**, labial tentacle; **ltn**, labial tentacle nerve; **mgo**, male gonopore; **mo**, mouth opening; **np**, nephropore; **oe**, oesophagus; **og**, optic ganglion; **osg**, osphradial ganglion; **osn**, osphradial nerve; **ot**, oral tube; **otg**, oral tube gland; **ov**, ovotestis; **pag**, parietal ganglion; **pg**, pedal ganglion; **ph**, pharynx; **plg**, pleural ganglion; **pn**, pedal nerve; **pr**, prostate; **r**, radula; **rhg**, rhinophoral ganglion; **rhn**, rhinophoral nerve; **s**, statocyst; **sgd**, salivary gland duct; **sgl**, left salivary gland; **sgr**, right salivary gland; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **v**, ventricle; **vd**, vas deferens; **vg**, visceral ganglion; **vn**, visceral nerve; **vh**, visceral sac. **The interactive 3D-model** of *P. cornuta* can be accessed by clicking onto Fig. 1 in the 3D PDF version of this article; see also additional files 1 and 2 (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.).

structed structures and switching between prefabricated views.

Original material and neotype

According to Challis [12], the holotype of *Pseudunela cornuta*, 20 paratypes and a slide with the radula of a further paratype were deposited in The Natural History Museum, London; furthermore, 10 paratypes and a slide with another radula were deposited in the Museum of New Zealand Te Papa Tongarewa, Wellington; the remaining paratypes and the sectioned material were stored in the private collection. We contacted both museums above mentioned - there is no trace of the material or any evidence that it ever arrived there. Obviously, no type material of *P. cornuta* was ever deposited in any public institution.

We consider our recently collected specimens as the species *Pseudunela cornuta* described by Challis [12] due to 1) the same collecting site as part of the material that was used for the original description, 2) the undoubted placement into the genus *Pseudunela* and 3) the same external morphology as described by Challis [12]. The section series ZSM N° 20071911 is designed herein as neotype due to the apparent non-existence of the original type material, and to avoid taxonomic confusion with congeners and a number of similar but still unnamed species found by the authors and mentioned in the literature [18-20].

Results

The following description is based on the entire specimen, which shows mature reproductive organs.

External morphology

Pseudunela cornuta shows an anterior head-foot complex and a posterior elongated visceral hump (vh) (Figs. 1A; 2) in which the animal can partly retract when disturbed. The paired labial tentacles (lt) (Figs. 1A; 2) are broad at the base, tapering to the end and usually held at 45°-90° to the longitudinal axis of the specimen. The paired rhinophores (rh) (Fig. 2) are tapered and usually point forward like horns in crawling animals. Eyes (ey) are present (Fig. 1A-C), but not visible externally. The densely ciliated foot (f) is as broad as the anterior head-foot complex and extends about one third of the visceral hump in the crawling animal. The free end of the foot is pointed (Fig. 2).

The body size of living specimens is about 3 mm and the body colour is whitish translucent. In the anterior part of the visceral hump the heart bulb (hb) (Fig. 2) is visible externally on the right body side. A few elongate, subepidermal spicules of up to 40 μm in length can be found in the posterior part of the visceral hump.

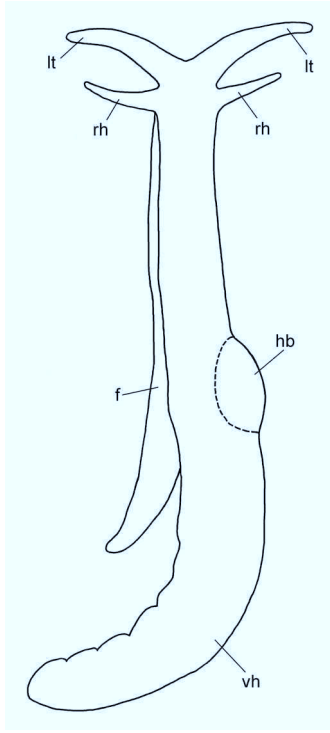

Figure 2
External morphology of *P. cornuta* (schematic drawing, dorsal view). Abbreviations: **f**, foot; **hb**, heart bulb; **lt**, labial tentacle; **rh**, rhinophore; **vh**, visceral hump.

Microanatomy

Central nervous system (CNS)

The CNS of *Pseudunela cornuta* is euthyneurous and composed of the paired cerebral (cg), rhinophoral (rhg), optic (og), pedal (pg), pleural (plg), buccal (bg) and gastro-oesophageal ganglia (gog) as well as three distinct ganglia on the visceral nerve cord, plus a presumed osphradial ganglion (osg) (Figs. 1B, C; 3). All ganglia excluding the buccal and gastro-oesophageal ganglia are situated prepharyngeally (Fig. 1C). The CNS is epiathroid; the pleural ganglion is located closer to the cerebral ganglion than to the pedal one. All ganglia consist of an outer cortex containing the nuclei and an inner medulla (Fig. 4A-C). The large cerebral ganglia are linked by a robust commissure (Figs. 1B; 3) and lie dorsal to the pedal ganglia (Fig. 1C). Anteroventrally, the robust labiotentacular nerve (ltn) (Figs. 1C; 3; 4B) emerges innervating the labial tentacle. A rhinophoral ganglion (Figs. 1C; 3; 4A) is situated anterodorsally to each cerebral ganglion connected by a short, single cerebro-rhinophoral connective. The rhinophoral nerve (rhn) (Figs. 1B, C; 3) arises from the rhinophoral ganglion extending to the rhinophore. A small, unpigmented eye (Figs. 1A, C; 4A) is connected by the thin optic nerve (on) (Fig. 3) to the rhinophoral nerve, slightly anterior to the rhinophoral ganglion. An optic ganglion (Figs.

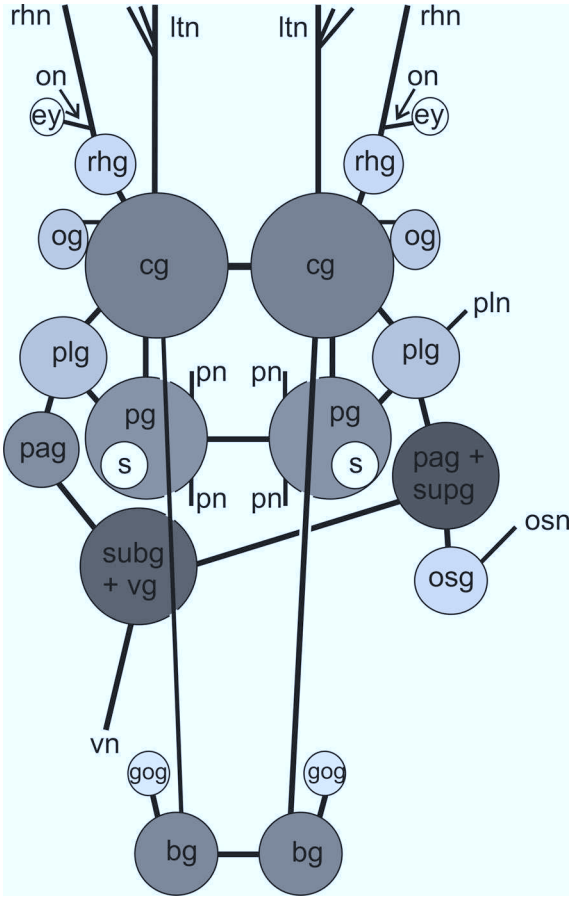

Figure 3 *cornuta* (schematic overview, dorsal view)
CNS of *P. cornuta* (schematic overview, dorsal view). Abbreviations: **bg**, buccal ganglion; **cg**, cerebral ganglion; **ey**, eye; **gog**, gastro-oesophageal ganglion; **ltn**, labial tentacle nerve; **og**, optic ganglion; **on**, optic nerve; **osg**, osphradial ganglion; **osn**, osphradial nerve; **pag**, parietal ganglion; **pg**, pedal ganglion; **plg**, pleural ganglion; **pln**, pleural nerve; **pn**, pedal nerve; **rhg**, rhinophoral ganglion; **rhn**, rhinophoral nerve; **s**, statocyst; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **vg**, visceral ganglion; **vn**, visceral nerve. Not to scale.

1C; 3; 4B) is attached laterally to each cerebral ganglion and connected to the latter by a thin nerve (Fig. 3). The optic ganglion is surrounded by an additional layer of connective tissue shared with the cerebral ganglion. Precerebral anterior accessory ganglia, as described for microhedylacean acochlidians and *Tantulum elegans* Rankin, 1979 [6-8,10], are absent. A Hancock's organ could not be detected.

The paired pedal ganglia (Figs. 1B, C; 3) lie posteroventrally to the cerebral ganglia, and are connected by a commissure which is slightly longer than the cerebral commissure (Figs. 1B; 3). A statocyst (s) with a single otolith (Figs. 1B, C; 3; 4C) is attached dorsally to each pedal ganglion. The static nerve could not be detected. Two pedal nerves (pn) (Figs. 1C; 3) emerge from each pedal ganglion, one in the anterior and another in the posterior part, both innervating the foot. The pleural ganglion is located posterior to the cerebral ganglion (Figs. 1B, C; 3; 4C) and connected to the latter and the pedal ganglion by short connectives forming the pre-pharyngeal nerve ring. The pleural ganglia are connected by very short connectives to the visceral nerve cord, so that the latter is located at the very beginning of the pharynx (Fig. 1C). There are three distinct ganglia on the short visceral nerve cord: the left parietal ganglion (pag) (Figs. 1B; 3; 4C), the fused subintestinal/visceral ganglion (subg+vg) (Figs. 1B; 3; 4C) and the fused right parietal/supraintestinal ganglion (pag+supg) (Figs. 1B, C; 3). While the left pleuro-parietal, the parietal-subintestinal/visceral and the right pleuro-parietal/supraintestinal connectives are very short, the subintestinal/visceral-parietal/supraintestinal connective is long (Fig. 3). An additional presumed osphradial ganglion (Figs. 1B, C; 3) is linked to the fused parietal/supraintestinal ganglion. Anteriorly, a nerve emerges (Figs. 1B; 3; 4C) and innervates the right body wall; no histologically differentiated osphradium could be detected. The buccal ganglia are positioned posterior to the pharynx (Fig. 1C) and are linked to each other by a short buccal commissure ventral to the oesophagus (Fig. 4E). The thin cerebro-buccal connective (Figs. 1B; 3) emerges anteriorly from each buccal ganglion and was not traceable along the entire length. A smaller gastro-oesophageal ganglion (Figs. 1B, C; 3; 4E) lies dorsally to each buccal ganglion and is connected to the latter by a short connective.

Digestive system

The mouth opening (mo) (Fig. 1D) lies ventrally between the labial tentacles. The paired anterior pedal glands (apg) (Figs. 1D; 4A) discharge ventral to the mouth opening to the exterior. The oral tube (ot) (Figs. 1D; 4A, B) is long and not ciliated. Paired oral tube glands (otg) (Figs. 1D; 4A, B) are flanking the oral tube and discharge in its anterior part. The muscular pharynx (ph) (Figs. 1C; 4C, D) is bulbous and narrows to the posterior; it contains the hook-shaped radula (r) (Figs. 1C, D; 4D). The upper ramus is longer than the lower one (Fig. 1C). The radula formula could not be examined. Jaws are absent. The long, ciliated oesophagus (oe) (Figs. 1D; 4E, F) emerges posterodorsally from the pharynx and is flanked by longitudinal muscles. One pair of large salivary glands (sgl, sgr) (Figs. 1D; 4E) discharges into the oesophagus via narrow salivary gland ducts (sgd) (Figs. 1D; 4E) directly behind the pharynx.

The large, sac-like digestive gland (dg) (Fig. 1D) is placed at the left side of the visceral hump flanking the ovotestis

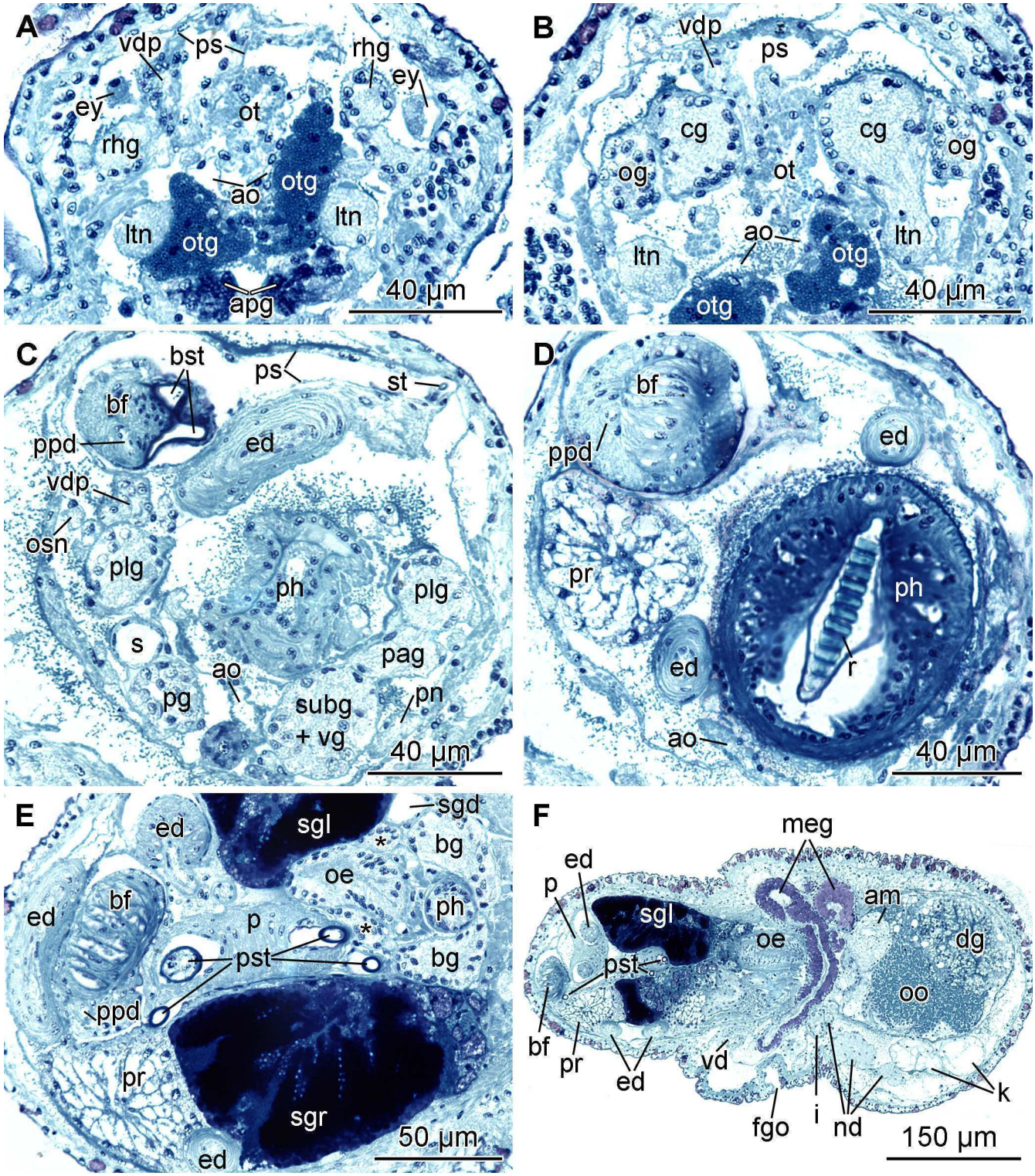

Figure 4 *(see legend on next page)*

Figure 4 *(see previous page)*
Histological cross-sections of *P. cornuta*. A: eye and rhinophoral ganglion. B: cerebral and optic ganglia. C: pleural, parietal and fused subintestinal/visceral ganglion. D: pharynx and basal finger. E: buccal ganglion and penial stylet. F: female gonopore and membrane gland. Abbreviations: **am**, ampulla; **ao**, aorta; **apg**, anterior pedal gland; **bf**, basal finger; **bg**, buccal ganglion; **bst**, stylet of basal finger (base); **cg**, cerebral ganglion; **dg**, digestive gland; **ed**, ejaculatory duct; **ey**, eye; **fgo**, female gonopore; **i**, intestine; **k**, kidney; **ltn**, labial tentacle nerve; **meg**, membrane gland; **nd**, nephroduct; **oe**, oesophagus; **og**, optic ganglion; **oo**, oocyte; **osn**, osphradial nerve; **ot**, oral tube; **otg**, oral tube gland; **p**, penis; **pag**, parietal ganglion; **pg**, pedal ganglion; **ph**, pharynx; **plg**, pleural ganglion; **pn**, pedal nerve; **ppd**, paraprostatic duct; **pr**, prostate; **ps**, penial sheath; **pst**, penial stylet; **r**, radula; **rhg**, rhinophoral ganglion; **s**, statocyst; **sgd**, salivary gland duct; **sgl**, left salivary gland; **sgr**, right salivary gland; **st**, stylet of basal finger (tip); **subg**, subintestinal ganglion; **vd**, vas deferens; **vdp**, posterior-leading vas deferens; **vg**, visceral ganglion; *****, gastro-oesophageal ganglion.

(Figs. 1A; 5E) and extends almost up to the end of the visceral hump (Fig. 1A). The intestine (i) is densely ciliated and short (Figs. 1D; 5A, B). The anus (a) (Fig. 1A, D) opens slightly anterior, but separate to the nephropore and ventrolaterally on the right side of the visceral hump.

Excretory and circulatory systems

The excretory and circulatory systems are located at the right side of the body (Fig. 1A) just at the beginning of the visceral hump.

The circulatory system shows a large two-chambered heart consisting of an anterior ventricle (v) (Figs. 1A; 5F; 6; 7A, B) and a smaller, posterior atrium (at) (Figs. 5F; 6; 7A, B). The thin-walled pericardium (pc) (Fig. 6) surrounding the heart could not be detected due to the very compressed tissue. The aorta (ao) (Figs. 5A; 6; 7A, B) arises anteriorly from the ventricle and leads to the head, where the aorta bifurcates (Figs. 4A, B; 6) approximately at the level of the eyes ending in blood sinuses. The renopericardioduct (rpd) (Figs. 6; 7B) is a well-developed and heavily ciliated funnel (Figs. 5B; 6B). The kidney (k) is a sinuously bent sac and extends over almost the half of the visceral hump (Fig. 1A). Internally it is divided into a narrow lumen (kn) (Figs. 5D; 6A; 7A, B) bordered by tissue with small vacuoles, and a wide lumen (kw) (Figs. 5C, D; 6; 7A, B) limited by highly vacuolated tissue. Both lumina join in the posterior part of the kidney (Fig. 6). The renopericardial duct is connected to the narrow lumen in the anterior part of the kidney (Figs. 6B; 7B). The connection between the kidney and the nephroduct is narrow and ciliated. The nephroduct is long and looped with a dorsal branch (ndd) extending backward and a ventral branch (ndv) forward (Figs. 6; 7A, B). The ventral branch is looped dorsally in its distal part (Figs. 6; 7A, B). The nephropore (np) (Fig. 1A) opens just posterior, but separate to the anus and ventrolaterally on the right side of the visceral hump.

Reproductive system

Terms used below are based on Ghiselin [21]. The nidamental glands are identified according to Klussmann-Kolb [22] and the anterior male copulatory organs are named following the terminology of Haase & Wawra [23].

The reproductive system of *Pseudunela cornuta* is (simultaneous) hermaphroditic (Fig. 8). The anterior genitalia show a special androdiaulic condition: the vas deferens does not branch off in a proximal position as usual in androdiaulic nudibranch or acteonoidean species [2,24,25], but more distally, i.e. autosperm must pass through the nidamental glands. Nevertheless this reproductive system is not strictly monaulic, because the internal vas deferens (for autosperm) is separated from the distal portion of the oviduct.

The sac-like ovotestis (ov) extends over the half of the right side of the visceral hump (Fig. 1A) and is not separated into follicles; oocytes are located more in the exterior part of the gonad and the spermatocytes are positioned more in the centre. Sperm heads are short (Fig. 5E). Approximately 10 yolky oocytes (oo) were noted in the examined specimen (Figs. 5A, E; 7C). Anterior to the ovotestis there is a small receptaculum seminis (rs) (Figs. 5A, B; 7C; 8) containing sperm cells orientated with their heads to the wall, as well as a sac-like ampulla (am) (Figs. 7C, D; 8) filled with unorientated autosperm (Figs. 4F; 5A). Three nidamental glands can be distinguished: the albumen (alg), membrane (meg) and mucus gland (mug) from proximal to distal, respectively (Figs. 7C, D; 8). The tube-like albumen gland is characterized by cells containing dark blue stained vesicles and long cilia (Fig. 5A-D). The membrane gland is tube-like with long cilia as well. In the proximal part, vesicles are stained purple, in the distal part, lilac (Fig. 5A, D). The mucus gland is sac-like with short cilia. It shows the same histological staining properties as the distal membrane gland (Fig. 5B, D). The distal part of the mucus gland extends to the right side of the body wall where the hermaphroditic duct divides into the vas deferens (vd) and the oviduct (Fig. 8). The oviduct widens to a cavity (cdo) (Figs. 5B-D, F; 7C, D; 8). At the distal end of the cavity a long, narrowly coiled bursa stalk (bs) (Figs. 5B-D, F; 7C, D; 8) branches off leading to the large bursa copulatrix (bc) (Figs. 5D, F; 7C, D; 8). No sper-

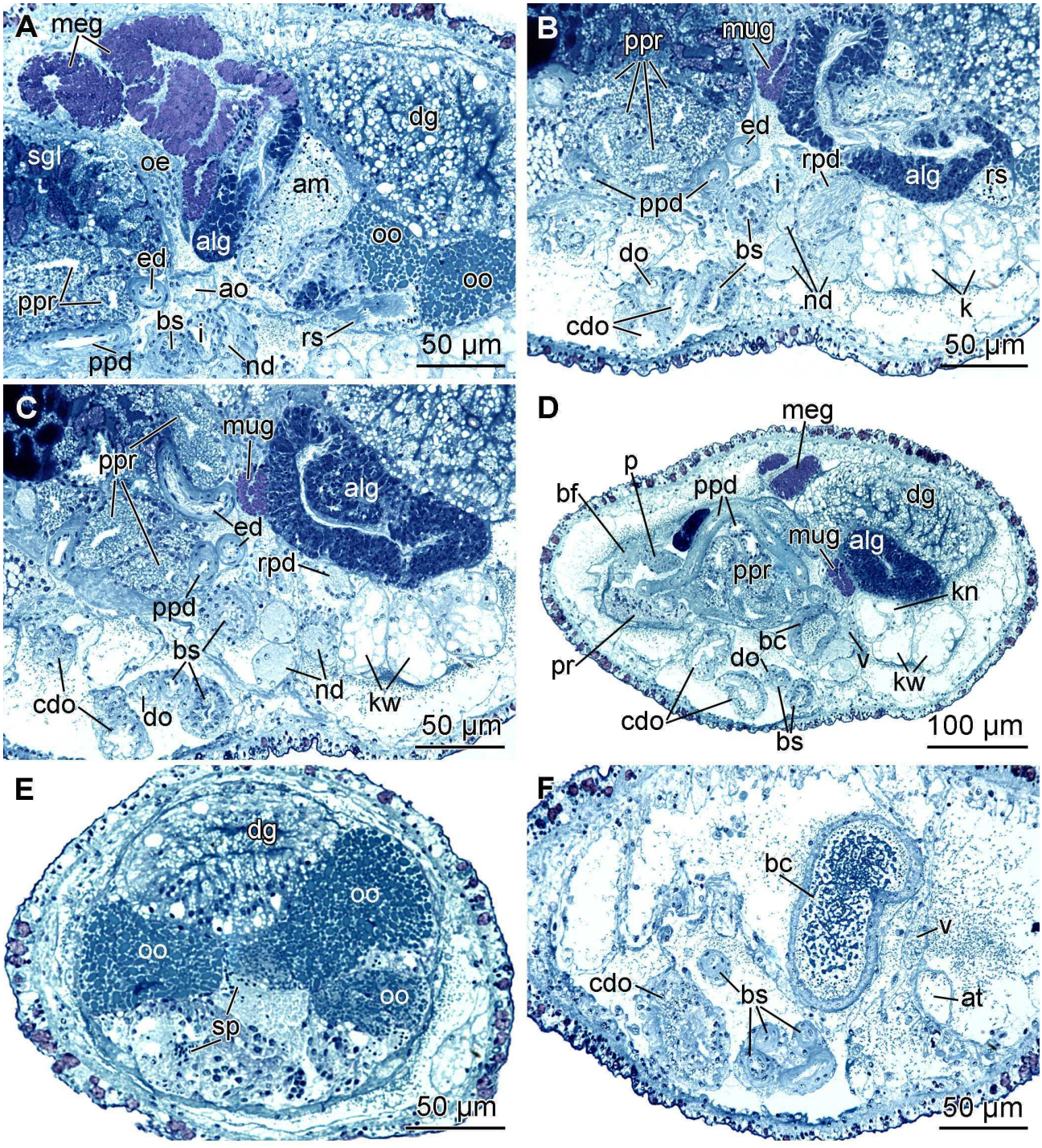

Figure 5

Histological cross-sections of *P. cornuta*. A: ampulla and receptaculum seminis. B: renopericardioduct. C: albumen gland. D: paraprostate. E: ovotestis with oocytes and spermatocytes. F: bursa copulatrix and atrium. Abbreviations: **alg**, albumen gland; **am**, ampulla; **ao**, aorta; **at**, atrium; **bc**, bursa copulatrix; **bf**, basal finger; **bs**, bursa stalk; **cdo**, cavity of distal oviduct; **dg**, digestive gland; **do**, distal oviduct; **ed**, ejaculatory duct; **i**, intestine; **k**, kidney; **kn**, narrow lumen of kidney; **kw**, wide lumen of kidney; **meg**, membrane gland; **mug**, mucus gland; **nd**, nephroduct; **oe**, oesophagus; **oo**, oocytes; **p**, penis; **ppd**, paraprostatic duct; **ppr**, paraprostate; **pr**, prostate; **rpd**, renopericardioduct; **rs**, receptaculum seminis; **sgl**, left salivary gland; **sp**, spermatocytes; **v**, ventricle.

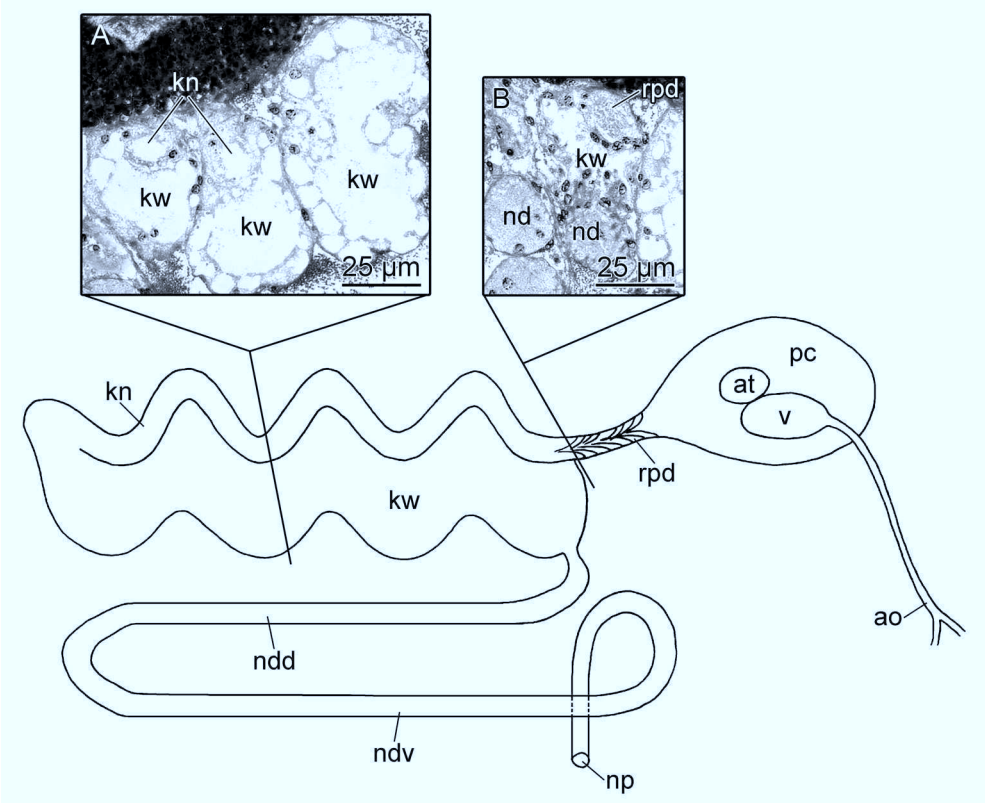

Figure 6
Circulatory and excretory systems of *P. cornuta* (schematic drawing, right view and histological cross-sections). A: narrow and wide lumen of kidney. B: transition of renopericardioduct and kidney. Abbreviations: **ao**, aorta; **at**, atrium; **kn**, narrow lumen of kidney; **kw**, wide lumen of kidney; **ndd**, dorsal branch of nephroduct; **ndv**, ventral branch of nephroduct; **np**, nephropore; **pc**, pericardium; **rpd**, renopericardioduct; **v**, ventricle. Not to scale.

matocytes can be detected inside the bursa, but an indeterminable mucous mass that might contain degenerated sperm. The distal oviduct (do) extends to the female gonopore (fgo) (Figs. 4F; 7C, D; 8) opening ventrolaterally on the right side of the visceral hump to the exterior. The female gonopore is situated considerably anterior to the anus and the nephropore (Fig. 1A).

The internal, subepidermal vas deferens extends along the right body side (Figs. 4; 8) to the right rhinophore connecting to the anterior male copulatory organs (Figs. 7E; 8). The short posterior-leading vas deferens (vdp) (Figs. 4B, C; 7C; 8) joins the large, tubular prostate gland (pr) (Figs. 4D, E; 7C, E; 8). Anteriorly, the long and highly coiled, muscular ejaculatory duct (ed) arises from the prostate (Figs. 4C-F; 5A-C; 7C, E; 8). The ejaculatory duct enters the muscular penis (p) (Figs. 4E, F; 7E, F; 8) at its base and discharges at the top of the penis through a long hollow stylet. The penial stylet (pst) is about 600 µm long and corkscrew-like coiled with one and a half spirals (Figs. 4E, F; 7F; 8). This stylet can be partly retracted into the penial muscle (Figs. 4E; 7F) that is able to evert to a certain extent. The blind ending glandular paraprostate (ppr) (Figs. 5A-D; 7E; 8) is longer and thinner than the prostate, and in contrast to the latter, highly coiled. It is connected by the paraprostatic duct (ppd) (Figs. 5A-D; 7E; 8) to the muscular basal finger (bf) (Figs. 4C-F; 7C, E, F; 8), which is united to the penial muscle mass at its base. The paraprostatic duct enters the basal finger approximately in the middle of the muscle (Fig. 7E) and opens terminally via a hollow curved stylet (bst, st) (Figs. 4C; 7E, F; 8) of about 110 µm length. The penis, the basal finger and parts of the ejaculatory and paraprostatic ducts are surrounded by a thin-walled penial sheath (ps) (Figs. 4C; 7E, F; 8). The latter, together with the copulatory organs, probably can be protruded through the male gonopore (mgo) (Fig. 1A) just at the base of the right rhinophore during the sperm transfer. However, sperm transfer has never been observed in living specimens.

Discussion

External morphology

The body of *Pseudunela cornuta* is divided into an anterior head-foot complex and the elongated visceral hump, as

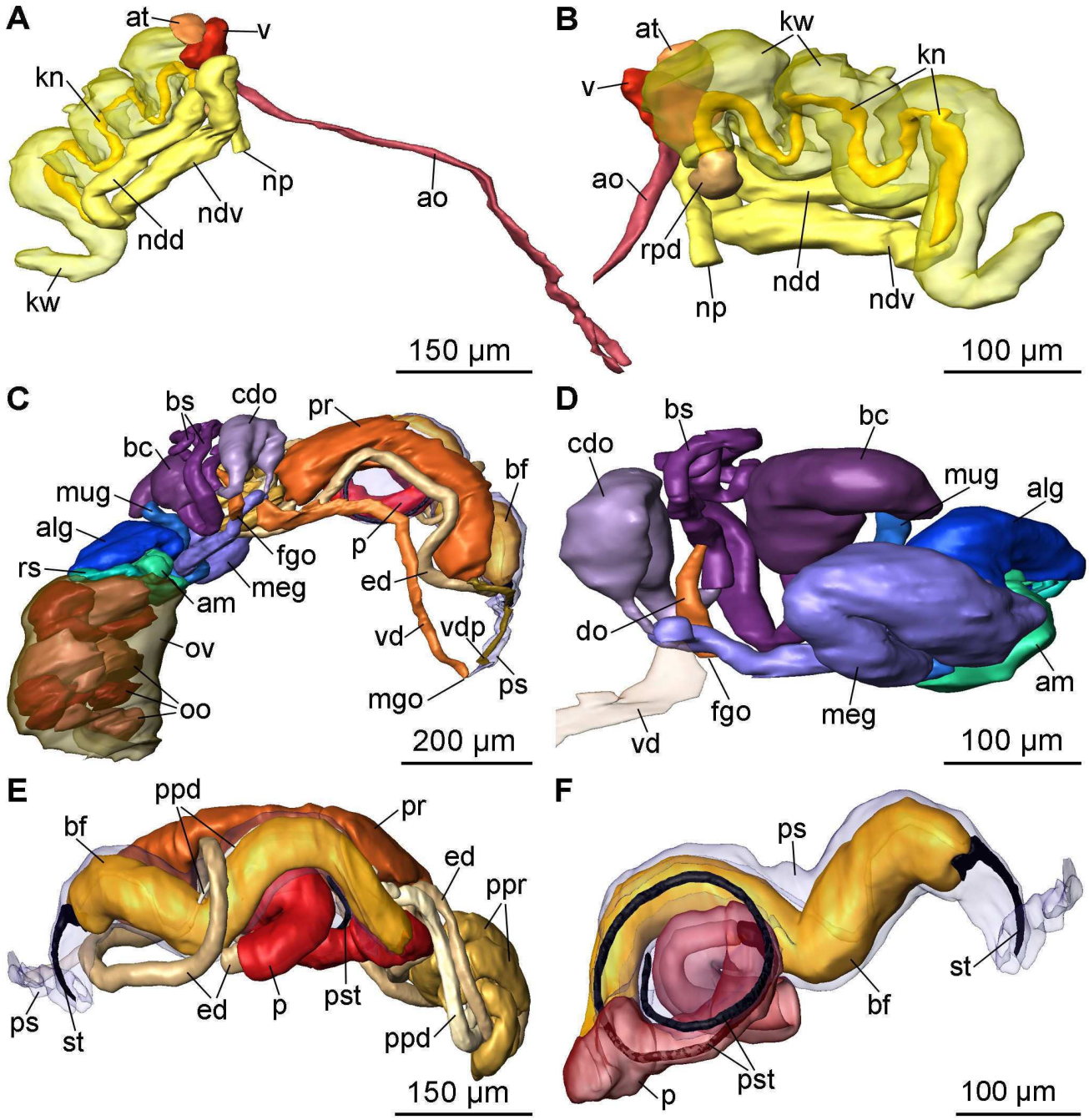

Figure 7

3D reconstruction of the excretory and circulatory systems and the reproductive system of *P. cornuta*. A: circulatory and excretory systems, right view. B: circulatory and excretory systems, left view. C: complete reproductive system, right view. D: nidamental glands and sperm storing receptacles, left view. E: anterior male copulatory organs, left view. F: penis and basal finger, anterolaterally right view. Abbreviations: **alg**, albumen gland; **am**, ampulla; **ao**, aorta; **at**, atrium; **bc**, bursa copulatrix; **bf**, basal finger; **bs**, bursa stalk; **cdo**, cavity of distal oviduct; **do**, distal oviduct; **ed**, ejaculatory duct; **fgo**, female gonopore; **kn**, narrow lumen of kidney; **kw**, wide lumen of kidney; **meg**, membrane gland; **mgo**, male gonopore; **mug**, mucus gland; **ndd**, dorsal branch of nephroduct; **ndv**, ventral branch of nephroduct; **np**, nephropore; **oo**, oocyte; **ov**, ovotestis; **p**, penis; **ppd**, paraprostatic duct; **ppr**, paraprostate; **pr**, prostate; **ps**, penial sheath; **pst**, penial stylet; **rpd**, renopericardioduct; **rs**, receptaculum seminis; **st**, stylet of basal finger; **v**, ventricle; **vd**, vas deferens; **vdp**, posterior-leading vas deferens.

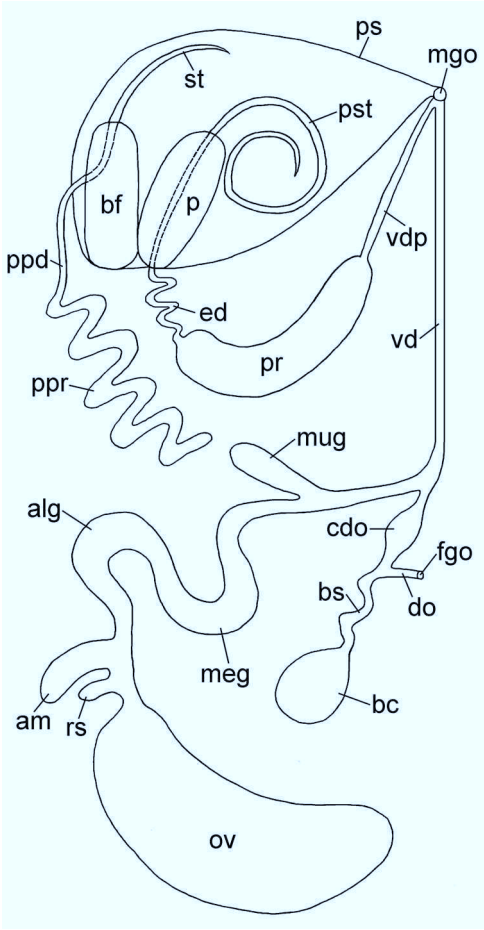

Figure 8
Reproductive system of *P. cornuta* (schematic drawing, dorsal view). Abbreviations: **alg**, albumen gland; **am**, ampulla; **bc**, bursa copulatrix; **bf**, basal finger; **bs**, bursa stalk; **cdo**, cavity of distal oviduct; **do**, distal oviduct; **ed**, ejaculatory duct; **fgo**, female gonopore; **meg**, membrane gland; **mgo**, male gonopore; **mug**, mucus gland; **ov**, ovotestis; **p**, penis; **ppd**, paraprostatic duct; **ppr**, paraprostate; **pr**, prostate; **ps**, penial sheath; **pst**, penial stylet; **rs**, receptaculum seminis; **st**, stylet of basal finger; **vd**, vas deferens; **vdp**, posterior-leading vas deferens. Not to scale.

characteristic for Acochlidia [3]. The digitiform shape and the position of the cephalic tentacles identify this species as belonging to the genus *Pseudunela*, according to Salvini-Plawen [26], Rankin [27] and Wawra [28]. Our results of the external morphology match with the original description of Challis [12], except for the presence of subepidermal spicules in living specimens. Most probably Challis overlooked the sparsely arranged spicules in the visceral hump of *P. cornuta* or they were already dissolved in preserved specimens.

Microanatomy

Central nervous system

Challis' original description of the CNS in *Pseudunela cornuta* contains some substantial details [12]. In the present study we supplement and correct the original data, and, in addition, homologize and name ganglia according to standard works [29]. The ganglia on the visceral nerve cord were interpreted according to the pentaganglionate hypothesis proposed by Haszprunar and recent studies on other acochlidians [6,30,31].

The CNS of *P. cornuta* follows the usual arrangement of ganglia in other hedylopsacean acochlidian species such as *Hedylopsis ballantinei* Sommerfeldt & Schrödl, 2005 and *Tantulum elegans* [6,31]. In contrast to *T. elegans*, precerebral ganglia are lacking in *P. cornuta*. Challis [12] described precerebral anterior accessory ganglia for *P. cornuta* as "anterior nerves in the form of two chains of ganglia". According to the drawing in Challis [12], the highly undulated and curled nerves might have been misinterpreted as anterior accessory ganglia. Anterior accessory ganglia are absent in a recently discovered congener from Vanuatu [32], but have been reported for *P. eirene* by Wawra [11] and, thus, should be re-examined carefully in this species.

Although Challis [12] described some very tiny nerves, such as the static nerve and the cerebro-buccal connectives, he overlooked or misinterpreted quite larger structures, such as the paired rhinophoral, optic and gastro-oesophageal ganglia. Our results show the eye is innervated by the optic nerve which emerges from the rhinophoral nerve; this condition is very unusual for opisthobranch species and, to our knowledge, only known for the closely related acochlidians *Hedylopsis ballantinei* and *H. spiculifera* (Kowalevsky, 1901) [31,33]. In contrast, the eye in the more basal *Tantulum elegans* is innervated by the optic nerve arising from the optic ganglion; additionally, the optic nerve is connected to the Hancock's nerve [6]. Challis [12] described only two ganglia on the visceral nerve cord, namely the sub- and the supraintestinal ganglia, which are identified in the present work as the fused subintestinal/visceral and the fused right parietal/supraintestinal ganglion, respectively. The small left parietal ganglion has been overlooked, probably due to its very close position to the pleural ganglion. The additional ganglion attached to the fused parietal/supraintestinal ganglion, which has been described originally as visceral ganglion [12], is interpreted herein as the osphradial ganglion, according to Huber [29].

Digestive system

The digestive system of *Pseudunela cornuta* was well-described by Challis [12] and conforms to the general ground-pattern of the digestive system in acochlidian spe-

cies. The stomach reported in the original description, however, could not be detected in the present study. While a stomach fused with the anterior cavity of the digestive gland is present in some acochlidian species, such as *T. elegans* and *Asperspina murmanica* (Kudinskaya & Minichev, 1978) [6,7], a histologically and anatomically distinct organ is absent in all Acochlidia studied in detail.

Acochlidians generally have reduced or lost the mantle cavity. While in *Hedylopsis ballantinei* a small remainder could be detected by histological and ultrastructural investigations [34], a well-developed "mantle-cavity" originally described from *A. murmanica* was shown to be completely absent [7,35]; the genital system, intestine and nephroduct open separately at the right lateral body surface [7]. The presence of common exit ducts, such as cloacae, could indicate that there are remnants of mantle cavities in some acochlidians. Challis described an anal-genital cloaca into which the intestine is discharging from *P. cornuta*; however, this assumption is clearly rejected by our results. In *P. cornuta* the genital opening, anus and nephropore open separately to the exterior (from anterior to posterior, respectively). Additionally, the anus is associated with the nephropore; the female gonopore opens more anteriorly. The same arrangement of the orifices of the body can be found in *T. elegans* [6], whereas the nephropore is situated anterior to the anus in the microhedylacean *Microhedyle remanei* (Marcus, 1953), *A. murmanica* and *Pontohedyle milaschewitchii* (Kowalevsky, 1901) [7,8,10]. Another acochlidian species, *Asperspina rhopalotecta* (Salvini-Plawen, 1973), which was reported to show a true cloaca [28], should be re-examined carefully.

Excretory and circulatory systems

The excretory and circulatory systems of *P. cornuta* were rudimentarily described by Challis who identified a pericardium, a heart without evident division into ventricle and atrium, and a short aorta "discharging almost immediately into the haemocoele" [12]. In contrast, our results show a two-chambered heart and an aorta extending up to the head. Well-developed two-chambered hearts have been reported for *Hedylopsis ballantinei*, *Microhedyle remanei* and *Tantulum elegans* [6,8,34]. In contrast, only a one-chambered heart could be detected recently in *Asperspina murmanica* and *Pontohedyle milaschewitchii* in spite of detailed re-examinations [7,10]. Jörger *et al*. [10] suggest a thorough examination by TEM for all acochlidian species reported with a one-chambered heart or described as being even heart-less, such as *Ganitus evelinae* Marcus, 1953 and *Parhedyle tyrtowii* (Kowalevsky, 1901) [36,37].

The kidney of *P. cornuta* has been depicted as a "large unfolded sac" [12] without any internal and histological data given. Surprisingly, our present data reveal that the kidney is a large, complex organ showing histologically distinguishable sections with supposedly different, but yet unknown function. In contrast, all marine acochlidian species studied in detail (*M. remanei*, *P. milaschewitchii* and *A. murmanica*) have a small, simple, sac-like kidney [7,8,10]. The marine *Hedylopsis ballantinei* was reported to show a long, sac-like kidney extending almost over the entire visceral sac [31,34]; however, our re-examination revealed a complex kidney with a narrow duct extending posteriorly and a wide one leading anteriorly (own unpubl. data), just as in *P. cornuta*. The kidney of *P. cornuta* also resembles those described for limnic hedylopsaceans such as *T. elegans* [6]. The original description of *P. cornuta* does not provide any information about the length and the shape of the nephroduct, nor the position of the nephropore. Whereas marine acochlidian species usually have a short, straight nephroduct (such as *M. remanei*, *P. milaschewitchii*, *A. murmanica*), the present study reveals *P. cornuta* to have a long, looped nephroduct as present in limnic Acochlidiidae (own unpubl. data) [38].

Unfortunately, Wawra [11] did not mention any excretory or circulatory features in the description of *Pseudunela eirene*, thus no comparison to other *Pseudunela* species can be drawn.

Reproductive system

The original description of the genital organs [12] shows major discrepancies relative to our results. Besides revising the differences, we add new data and name structures according to Haase & Wawra [23].

The reproductive system of the opisthobranch common ancestor likely was monaulic and the pallial gonoduct undivided [21]. Most acochlidian species may have a monaulic reproductive system as well (or are gonochoristic). In contrast, a special type of an androdiaulic reproductive system with the distal portion of the female gonoduct separated from the vas deferens exists in *Pseudunela cornuta* and *Tantulum elegans* [6]. Challis [12] noticed the presence of a distal bursa copulatrix as a short blind sac emerging from the "cloaca", but, in contrast to our observations, there is no report of a proximally situated receptaculum seminis. In the past, only the limnic acochlidian *Strubellia paradoxa* (Strubell, 1892) from Solomon Islands was known to possess both allosperm receptacles [39]. While in the original description no ampulla was described, we could find a well-developed, sac-like ampulla in *P. cornuta*. A sac-like ampulla is reported from *Asperspina murmanica* and *Tantulum elegans* [6,7], whereas the ampulla is a tubular swelling of the gonoduct in *Microhedyle remanei* and *Pontohedyle milaschewitchii* [8,10]. Opisthobranch eggs are surrounded by three layers of nutritive and protective materials that are

secreted by three different glands [21]. Challis described two nidamental glands, the proximal albumen and the distal mucous gland, but gave no data about their shapes or histological appearances. Following Klussmann-Kolb [22], the nidamental glands in this study were interpreted based on their position in the reproductive system. These are the albumen, membrane and mucus gland, from proximal to distal, respectively. The albumen and membrane glands are tubular in all acochlidian species studied in detail. The mucus gland shows more structural variety and may be tubular as in *A. murmanica* and *P. milaschewitchii* [7,10], but is a blind sac in *P. cornuta* and *M. remanei* [8]. The cavity of the distal oviduct in *P. cornuta* that is situated near to the female gonopore was not described by Challis [12] and has never been observed in any other acochlidian species up to now. The function of this structure is yet unknown. A function as fertilization chamber is not likely due to its very distal position in the reproductive system. However, a role during sperm transfer is imaginable (see below).

The posterior part of the reproductive system is connected to the anterior male reproductive system by the completely internal vas deferens. According to Ghiselin [21] the latter is a mechanism to hasten the transfer of sperm and, therefore, is an improvement compared with the external sperm groove of the hypothetic ancestor of the opisthobranchs.

The original description of the complex, anterior copulatory organs includes a drawing by Challis [12]; unfortunately, the interpretation of the different ducts, glands and stylets remains confusing. Wawra [11] interpreted the penial spine in Challis' drawing as the penial stylet. In contrast, we consider herein the penial spine of 100 μm in fact being the stylet of the basal finger (which measures approx. 110 μm in our specimen), so that the following conclusions can be drawn: 1) the stylet-bearing muscle at the base of the penis in Challis' drawing is the basal finger; 2) the penial gland was misinterpreted and is in fact the paraprostate; 3) the duct connecting Challis' penial gland with the penial spine is considered as the paraprostatic duct; 4) the prostate gland is the prostate; 5) the spermatic duct running from the rhinophore to the prostate gland is the cephalic, posterior-leading vas deferens; 6) the efferent male duct probably is the penial sheath through which the anterior male copulatory organs can be protruded. Furthermore, the ejaculatory duct connecting the prostate to the penis was overlooked, as well as the large hollow stylet that we found at the tip of the penis. May be the stylet was totally retracted into the penial muscle in the specimen examined by Challis, or perhaps it was broken away during the last sperm transfer. Wawra [40] suggested this possibility for *Hedylopsis spiculifera*, as he found a detached stylet in the visceral sac of one specimen. The extremely complex copulatory system found in *P. cornuta* is similar to that of species of the much larger, limnic Acochlidiidae, and particularly the genus *Strubellia* (own unpubl. data).

Reproductive functions

While the generally marine microhedylacean species are aphallic, the basal, limnic hedylopsacean, *Tantulum elegans*, possesses a muscular copulatory organ [6]. Similar, but more complex anterior copulatory organs can be found in the marine hedylopsaceans *Hedylopsis spiculifera*, *Pseudunela cornuta* and *P. eirene* [11,40], as well as in other, limnic hedylopsacean species. The hollow penial stylet of all these latter species indicates that sperm transfer occurs by injection [3,41]. Hypodermal injection in the sequential hermaphrodite *H. spiculifera*, which lacks any allosperm receptacles, may be an imprecise one, as indicated by the finding of lost penial stylets in the body cavity [40]. In *P. cornuta*, we found an extremely long, tubular penial stylet and two allosperm storing receptacles. Due to the presence of the latter, we suggest a more precise sperm injection in *P. cornuta* into the genital system of the mate. In the present species, the cavity of the distal oviduct may serve as the site of sperm injection, or any other place within the genital system. Injected sperm then would move to the receptaculum seminis for long term storage and/or to the bursa copulatrix for short term storage and digestion. Passing through the nidamental glands without being trapped is obviously possible, presumably during periods without active glandular secretion. Challis proposed either the bursa stalk or the cloaca as region of fertilization in *P. cornuta*. This is unlikely due to the absence of the cloaca and the position of the bursa stalk distal to the nidamental glands. Fertilization of oocytes certainly occurs proximally, close to the receptaculum seminis, where allosperm is stored and nourished as indicated by the heads that are embedded into the organ walls.

Peculiar and noteworthy is the very long and curled, hollow penial stylet in *P. cornuta*. While other *Pseudunela* species have a penial stylet not exceeding 200 μm, the penial stylet of *P. cornuta* is approx. 600 μm long, which represents nearly one third of the body length in the fixed specimen. The functionality of such a curled stylet, however, is not understood. The curl may be a fixation artefact or more likely, due to the immense length of the stylet and the little space available in the head, the curled position signifies a "space saving storage". During sperm transfer the stylet may be uncoiled due to the pressure of emergent fluids and be operative for "long distance" hypodermal impregnation; in this case, the specimen can inject autosperm without approximating too closely the mate and thus, without the risk of being "hit" by the mate. Since the stylet in its extended condition measures over 2 times the

complete body width of a potential mate, we cannot imagine of any basic functional needs for developing such an organ, such as injection of sperm into a certain organ or body region of the mate. Instead, we may be observing the product of an evolutionary race of arms within *P. cornuta*. Similarly obscure is the exact function of an additional, paraprostatic impregnatory system that was described from *Acochlidium fijiense* Haynes & Kenchington, 1991 [23]. Schrödl & Neusser [3] discussed a probable role in the production of anaesthetics as known in cephalaspidean species with complex penial structures [42] or of fluids stimulating sperm transfer, as known from the sacoglossan *Elysia timida* (Risso, 1818) [43]. In *P. cornuta*, however, the penial stylet is extremely long, and it is difficult to imagine how the much shorter stylet of the basal finger may hit and affect the mate.

Regression or innovation? Evolution of acochlidian organ systems

Based on our recent results on acochlidian phylogeny [3], the evolution of organs and whole organ systems can be reconstructed at least for the major clades. In contrast to earlier generalizations [4,5], the various lineages show different trends; an overview of reductions and increasing complexity of the organ systems in Acochlidia is given in Fig. 9. The topology of the phylogenetic tree (parsimony analysis for all nominal 27 acochlidian species and 11 outgroup taxa based on 107 morphological characters) is simplified according to Schrödl & Neusser [3].

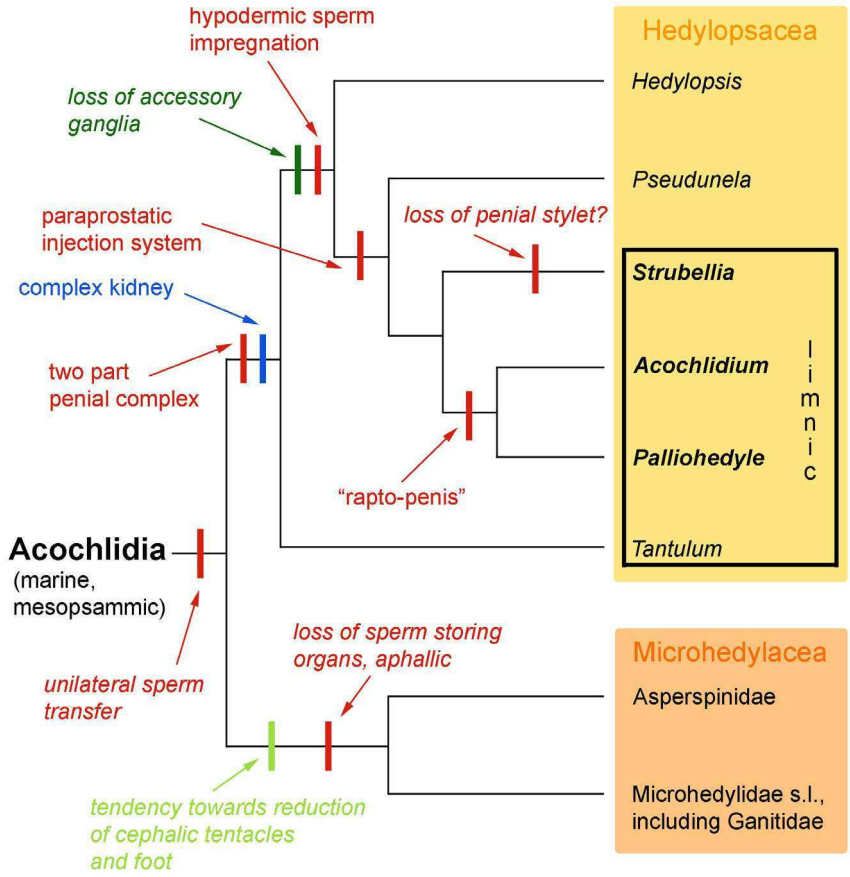

Figure 9 of organ complexity in acochlidian lineages

Evolution of organ complexity in acochlidian lineages. A selection of major organ reductions or innovations of several systems is mapped on a phylogenetic tree (strict consensus tree from Schrödl & Neusser [3], simplified. The parsimony analysis was based on 107 morphological characters with all 27 valid acochlidian species and 11 outgroup taxa included). Within the basally marine mesopsammic Hedylopsacea, the reproductive and excretory systems evolved towards higher complexity. With current state of knowledge the special hedylopsacean kidney appears ancestral and can be interpreted as a preadaptation and key feature to successful invasions of freshwater habitats. In contrast, the microhedylacean lineage shows regressive tendencies, especially with regard to external and reproductive features. Light green: external morphology. Dark green: central nervous system. Blue: excretory system. Red: reproductive system. Features in *italic* are reductions/losses, taxa in **bold** refer to large, benthic members of the Acochlidiidae according to Schrödl & Neusser [3].

The external morphology with the anterior head-foot complex retractile into an elongated visceral hump is similar in all acochlidian species and certainly ancestral. Only in the microhedylacean species is there a tendency towards reduction of the cephalic tentacles, the foot length and the foot width (Fig. 9), whereas *P. cornuta* shows, together with all other hedylopsacean species, well-developed tentacles and foot. The digestive system of *P. cornuta* is quite simple and conforms to the usual ground-pattern in acochlidian species. The CNS is plesiomorphically complex and the arrangement of ganglia is more or less similar in all acochlidian species. Differences concern precerebral accessory ganglia which, after splitting off *Tantulum*, were lost in the hedylopsacean lineage (Fig. 9), still by marine ancestors. In contrast, aggregations of accessory ganglia are present in microhedylacean species. The acochlidian excretory system varies considerably between marine and limnic species. All microhedylacean species known in detail show a small, simple and sac-like kidney and a short nephroduct [7,8,10]. While members of *Hedylopsis* were reported to have a simple, but long kidney [31,34,44], our re-examination of *Hedylopsis ballantinei* showed this species having a complex, bent kidney, as well (own unpubl. data). Since this special type of kidney seems present in all Hedylopsacea (Fig. 9), but neither in microhedylacean acochlidians nor in potential outgroup taxa, we propose that it has evolved in the mesopsammic ancestor of hedylopsaceans. This organ thus is of marine origin, still occurs in marine species and is equally structured in limnic species such as the basal, small Caribbean *Tantulum elegans* and members of the more derived, large Acochlidiidae that inhabit rivers of tropical Pacific islands. The hedylopsacean kidney thus is assumed to be a preadaptation and key feature to both, independent invasions of a limnic habitat known from opisthobranchs. The evolution of excretory systems and the invasion of freshwater systems in acochlidians clearly merit further study.

The most variable organ system within the Acochlidia is the reproductive system. Lacking any sperm storage or copulatory organs, the latter is considerably reduced from a usual basal opisthobranch condition in all microhedylacean species [3,45]. In contrast, the special androdiaulic genital system of *P. cornuta* with highly elaborated cephalic copulatory organs is clearly more complex than that assumed for the basal opisthobranch acochlidian ancestors. In fact, the hedylopsacean topology as revealed by Schrödl & Neusser [3] points towards the successively increasing complexity of the copulatory system of hypodermal injectors in the hedylopsacean stem line. This is confirmed herein (Fig. 9). The basal *T. elegans* lacks any stylet on the penial muscle and sperm transfer occurs probably by copulation [6]. *Hedylopsis spiculifera* shows a single penial stylet for sperm transfer [40]. While *H. ballantinei* was described to potentially being aphallic [31], we could detect two copulatory stylets or thorns in this species (own unpubl. data); details must be explored in a future study. In contrast, *P. cornuta* has an additional paraprostatic glandular system connected to another stylet (Fig. 9). This is similar to the condition in *Strubellia* (own unpubl. data), the most basal known member of Acochlidiidae. Schrödl & Neusser [3] assume that the function of this accessory impregnation system might be the production of special fluids to enforce unilateral insemination or stimulate sperm transfer. Thus, it might be to the best advantage for each individual being the first in injecting its own sperm and other fluids. Finally, the evolution of complex copulatory organs peaks in the so-called giant "rapto-penis" [3] of *Acochlidium* and *Palliohedyle* (Fig. 9). A schematic overview of the different penial structures is given in Schrödl & Neusser [3].

An increasing complexity of excretory and reproductive organs that evolved in the hedylopsacean stemline already in the mesopsammon (Fig. 9) clearly contradicts Swedmark's [4] hypothesis of a general evolutionary regression in marine mesopsammic acochlidians.

But what are the reasons for the remarkable reduction of the reproductive system in microhedylacean species on the one hand and an otherwise increasing complexity in hedylopsacean species on the other hand? Recently, Jörger *et al*. [45] pointed out that the spatially limited interstitial environment may favour unidirectional sperm transfer while quickly passing by. In basally still hermaphroditic microhedylaceans this occurs by means of spermatophores, dermal insemination (spermatophores are placed somewhere on the body surface) and dermal fertilization (allosperm penetrate the body wall and migrate to the gonad for fertilization). Unidirectional sperm transfer, together with the reduction of the copulatory system might have been prerequisites for the evolution of gonochorism in the ancestor of Microhedylidae s.l., and they all may have been key features for the successful radiation of microhedylacean species [3]. Both the hypothetical acochlidian ancestor and the most basal known hedylopsacean offshoot, *Tantulum elegans*, still use copulation for sperm transfer. Since the latter species is a sequential hermaphrodite, sperm transfer is unilateral; this is, thus, the ancestral condition for acochlidians (Fig. 9). According to our data, unidirectional hypodermal impregnation within the Acochlidia was established in the still mesopsammic hedylopsacean lineage (Fig. 9); first in its most simple form as expressed by *Hedylopsis spiculifera*. Comparisons with other, non-mesopsammic opisthobranchs (e.g. Sacoglossa, Nudibranchia) using hypodermal impregnation [43], will show whether or not an already unilateral mode of sperm transfer may be a precondition for evolving hypodermal impregnation systems. Once established,

this more or less quick and violent mode of sperm transfer grants for a selective advantage for injectors. Consequently, along the hedylopsacean stem lineage, more and more sophisticated sperm and auxiliary injection systems, such as very long penial and accessory paraprostatic stylets in *P. cornuta*, have evolved already in marine mesopsammic environments (Fig. 9). These are similarly retained by the benthic limnic *Strubellia*, but were elaborated into the even more complex and potentially harmful copulatory systems with a giant, armed "rapto-penis" [3] in the ancestor of an array of large-sized benthic, limnic *Acochlidium* and *Palliohedyle* species (Fig. 9), which are no more such spatially limited in their habitat.

Conclusion

Although miniaturization and reductions of organs are characteristic for many interstitial acochlidian species [4], *P. cornuta* shows a complex and complete set of organ systems in spite of the small body size. Remarkable is the high complexity of reproductive organs that resembles that of species of the much larger, limnic Acochlidiidae, and especially the genus *Strubellia*. Unexpectedly, the elaborated excretory system of the marine *P. cornuta* also resembles that of limnic hedylopsacean acochlidians, such as *Tantulum* and Acochlidiidae; the looped kidney and nephroduct are interpreted as evolutionary preadaptations that contributed to successful invasions of limnic systems within the otherwise generally marine Opisthobranchia. Structurally, *Pseudunela cornuta* thus links basal marine with basal and derived limnic clades, reflecting its recently proposed position on the acochlidian tree [3]. Importantly, organ complexity as seen in *P. cornuta* (regarding excretory and reproductive features, at least) is not plesiomorphically retained from a larger, benthic ancestor, but represents innovations that evolved in small, mesopsammic marine acochlidians. Earlier general statements on regressive, progenetic evolution in acochlidians may be relevant for explaining the origin of Acochlidia or that of microhedylacean lineages; *P. cornuta*, however, definitely is an example for evolution of a wealth of sophisticated structures within hedylopsaceans, the exact function of some of which, such as the extremely long spiral penial stylet, still cannot be explained.

Challis' achievement of a quite detailed description has to be acknowledged, since it was almost impossible to describe the complexity of the reproductive system of *P. cornuta* in detail without modern methods. This study once again shows that semithin-histology combined with computer-based 3D reconstruction is highly recommendable for studying the anatomy of micromolluscs, especially for obtaining reliable results that can be used for phylogenetic analyses. An interactive way of publishing 3D models even more impressively demonstrates the complexity of organs in tiny specimens - in the accurate dimensions, positions and relations.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TPN carried out the morphological analyses and drafted a manuscript version that was discussed and improved jointly. MH and TPN prepared the interactive 3D model. MS planned and supervised the study. All authors read and approved the final manuscript.

Additional material

Additional file 1

***Interactive 3D-model of* Pseudunela cornuta**. *The file provided includes an interactive 3D-model of the anatomy of* Pseudunela cornuta. *The interactive 3D-model of* P. cornuta *can be accessed by clicking into Fig. 1. 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.*
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Additional file 2

Pdf file of this article with interactive figure*1 - for details see Additional file 1.*
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Between Vanuatu tides: 3D anatomical reconstruction of a new brackish water acochlidian gastropod from Espiritu Santo

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ABSTRACT
The majority of known acochlidian sea slug species are marine mesopsammic, while some others are limnic. The structural, functional and evolutionary background of the invasion of freshwater systems was hardly explored. During the expedition SANTO 2006 to Espiritu Santo, Vanuatu, we discovered a unique new acochlidian species in a brackish water habitat. *Pseudunela espiritusanta* n. sp. inhabits the underside of intertidal rocks deeply embedded into coarse sand, the interstices of which are filled with a mixture of fresh subsoil and seawater. *Pseudunela espiritusanta* n. sp. is herein described in full external and anatomical detail using computer-based 3-dimensional reconstruction techniques from serial histological sections. This new species possesses a typical acochlidian central nervous and digestive system; it is a simultaneous hermaphrodite with a special androdiaulic reproductive system and complex, stylet-bearing copulatory organs with associated glands. Such penial features may indicate a relationship with marine mesopsammic *Pseudunela* (Pseudunelidae) species, while e.g., the larger body size, the broad foot, and the presence of a special ventricular cell layer may be potential synapomorphies with limnic, benthic Acochlidiidae (*Strubellia* and Acochlidiidae *s.s.*). *Pseudunela espiritusanta* n. sp. shares its special shape of head tentacles with both *Pseudunela* and *Strubellia*, while other characters are potentially synapomorphic with either one or the other taxon. Regardless of its unresolved exact systematic position, *Pseudunela espiritusanta* n. sp. evidently links marine and limnic taxa by its intermediate ecological and morphological features. Its considerable body size and well-developed heart and kidney can be considered as preadaptations to overcome osmotic challenges when colonising rivers from brackish coastal sands.

KEY WORDS
Mollusca,
Opisthobranchia,
marine,
freshwater,
limnic,
morphology,
histology,
penial stylet,
Vanuatu,
new species.

RÉSUMÉ

Dans la zone intertidale du Vanuatu : reconstruction anatomique 3D d'un nouveau gastéropode acochlidiacé d'eau saumâtre d'Espiritu Santo.

La majorité des acochlidiacés sont des espèces marines mésopsammiques, bien que quelques espèces vivent dans les eaux douces. Le contexte structural, fonctionnel et évolutif de la conquête des eaux douces a rarement été exploré. Au cours de l'expédition SANTO 2006, menée dans l'île d'Espiritu Santo au Vanuatu, nous avons découvert une nouvelle espèce remarquable d'acochlidiacé vivant dans un milieu saumâtre. *Pseudunela espiritusanta* n. sp. vit sous les blocs de la zone intertidale profondément enfoncé dans un sable grossier, dont les espaces sont remplis d'un mélange d'eau de mer et d'eau douce résurgente. La morphologie et l'anatomie de *Pseudunela espiritusanta* n. sp. sont décrites ici de manière détaillée à partir de sections histologiques sériées et d'un logiciel de reconstruction 3D utilisant des séries de coupes histologiques. Le système nerveux central et le système digestif sont typiques des acochlidiacés ; l'espèce est hermaphrodite simultané, avec un appareil reproducteur androdiaulique particulier et des organes copulateurs comportant un stylet et des glandes associées. Ces caractéristiques du pénis pourraient traduire une parenté avec des espèces mésopsammiques de *Pseudunela* (Pseudunelidae), alors que d'autres caractères comme par exemple la grande taille du corps, un pied large, et la présence d'une couche particulière de cellules ventriculaires pourraient représenter des synapomorphies potentielles avec des Acochlidiidae limniques benthiques (*Strubellia* et Acochlidiidae *s.s.*). *Pseudunela espiritusanta* n. sp. présente la même forme particulière des tentacules de la tête que *Pseudunela* et *Strubellia*, mais d'autres caractères sont communs avec l'un ou l'autre de ces taxons. Indépendamment de sa position taxonomique incertaine, *Pseudunela espiritusanta* n. sp. relie de manière évidente taxons marins et taxons d'eaux douces, tant par son écologie que par ses caractères morphologiques intermédiaires. La très grande taille du corps et le développement du cœur et du rein pourraient représenter des préadaptations pour faire face aux changements osmotiques rencontrés lors de la colonisation des rivières depuis les sables littoraux saumâtres.

MOT CLÉS
Mollusca,
Opisthobranchia,
marin,
eau douce,
limnique,
morphologie,
histologie,
stylet pénien,
Vanuatu,
espèce nouvelle.

INTRODUCTION

Among the otherwise marine Opisthobranchia, the Acochlidia comprise the only opisthobranch group that successfully invaded freshwater systems (Neusser & Schrödl 2007; Strong *et al.* 2008). First phylogenetic studies based on morphological characters indicate that colonisation of limnic habitats occurred twice independently within acochlidians; once by the ancestor of an array of large-sized, benthic species distributed over different Indo-Pacific Islands (*Strubellia* Odhner, 1937, *Acochlidium* Strubell, 1892, *Palliohedyle* Rankin, 1979), and second in the small, interstitial Caribbean *Tantulum elegans* Rankin, 1979 from St. Vincent Island (Schrödl & Neusser in press). However, up to now, we have no exact information about 1) how the osmotic challenges presented by the colonisation of limnic environments were overcome, and 2) what are the morphological adaptations in limnic acochlidian species. Studying species living in marine habitats that are temporarily or permanently influenced by freshwater input may provide a clue.

Recently, a new acochlidian species was discovered in a brackish water environment, inhabiting the intertidal off Luganville, Espiritu Santo Island, Vanuatu. The goal of this study is to describe this species externally and internally by computer-aided

3D reconstruction of serial histological sections and to compare it with externally similar marine and limnic representatives of the genera *Pseudunela* Salvini-Plawen, 1973 and *Strubellia*.

MATERIAL AND METHODS

The material (six specimens) was collected during the SANTO 2006 Expedition to the island of Santo, Vanuatu, in October 2006. For a narrative of the expedition, see Bouchet *et al.* (2008), and for a review of the geography and natural history of Santo, we refer to Bouchet *et al.* (in press). The specimens were collected by brushing the embedded surfaces of intertidal boulders and relaxed by a solution of isotonic $MgCl_2$. One specimen was fixed in 80% EtOH. Three specimens were fixed in 95% and 99% EtOH for molecular studies. The pharynx of one of the latter specimens was removed for analysis of the radula by SEM and macerated in 10% KOH. Remaining tissue was separated using an ultrasonic bath (Sonorex TK52, Bandelin, Berlin, Germany) for 20 minutes. The radula was mounted on a SEM stub, sputter-coated with gold for 120 s (SEM-Coating-System, Polaron) and analyzed using a LEO 1430 VP SEM (15 kV).

Two specimens were fixed in 4% glutardialdehyde in 0.2 M sodium cacodylate buffer (0.1 M NaCl, 0.35 M sucrose, pH 7.2), followed by post-fixation in 1% OsO_4 buffered in 0.2 M sodium cacodylate (0.3 M NaCl, pH 7.2) for 2 h in the dark. Subsequently the specimens were decalcified in 1% ascorbic acid overnight and dehydrated in an acetone series (30, 50, 70, 90, 100%), embedded in Spurr's low viscosity resin (Spurr 1969) and serially sectioned (thickness: 1.5 µm) using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) and contact cement on the lower cutting edge to form ribbons (Ruthensteiner 2008). Sections were stained with methylene-azure II (Richardson *et al.* 1960). Every second section (for cns: every section) of series no. 20080791 was photographed with a CCD microscope camera (Spot Insight, Diagnostic instruments, Sterling Heights, USA) on a Leica DMB-RBE (Leica Microsystems, Wetzlar, Germany) microscope. Computer-aided 3D reconstruction of all major organ systems was performed with the software AMIRA 4.1 (TGS Europe, Mercury Computer Systems, Mérignac, France). Sections were deposited at the ZSM, Mollusca Section (no. 20070968 and 20080791).

ABBREVIATIONS

cns	central nervous system;
MNHN	Muséum national d'Histoire naturelle, Paris;
SEM	Scanning electron microscopy;
ZSM	Zoologische Staatssammlung, München.

SYSTEMATICS

Family PSEUDUNELIDAE Rankin, 1979
Genus *Pseudunela* Salvini-Plawen, 1973

Pseudunela espiritusanta n. sp.

TYPE MATERIAL. — Holotype: ZSM 20080115, 3.5 mm preserved body length, stored in 75% EtOH. Paratypes: ZSM 20070968 and 20080791 (two serially sectioned specimens), ZSM 20080116 (head removed for radula analysis, stored in 96% EtOH), ZSM 20080117 and 20071118 (used for molecular studies).

TYPE LOCALITY. — Vanuatu, Espiritu Santo Island, leaving Luganville to Palikulo Bay. Expedition SANTO 2006, stn VM 53, 15°30'58"S, 167°11'52"E.

ETYMOLOGY. — *Pseudunela espiritusanta* n. sp. is named according to the type locality on the island of Espiritu Santo.

DISTRIBUTION. — Known only from the type locality.

DESCRIPTION

Habitat

Intertidal zone of type locality characterized by rocks, dead coral pieces and coarse sand (Fig. 1A). During high tide, seawater reaches the bank beside the road. *Pseudunela espiritusanta* n. sp. inhabits lower intertidal in brackish environment. It lives underside of rocks deeply embedded into coarse sand (Fig. 1B); interstices filled with mixture of sea and emerging fresh subsoil water. Habitat keeps wet during low tide. Despite further sampling, the new species could not be found in neighbouring sand and gravel patches. Other molluscan species associated with *P. espiritusanta* n. sp. in the same

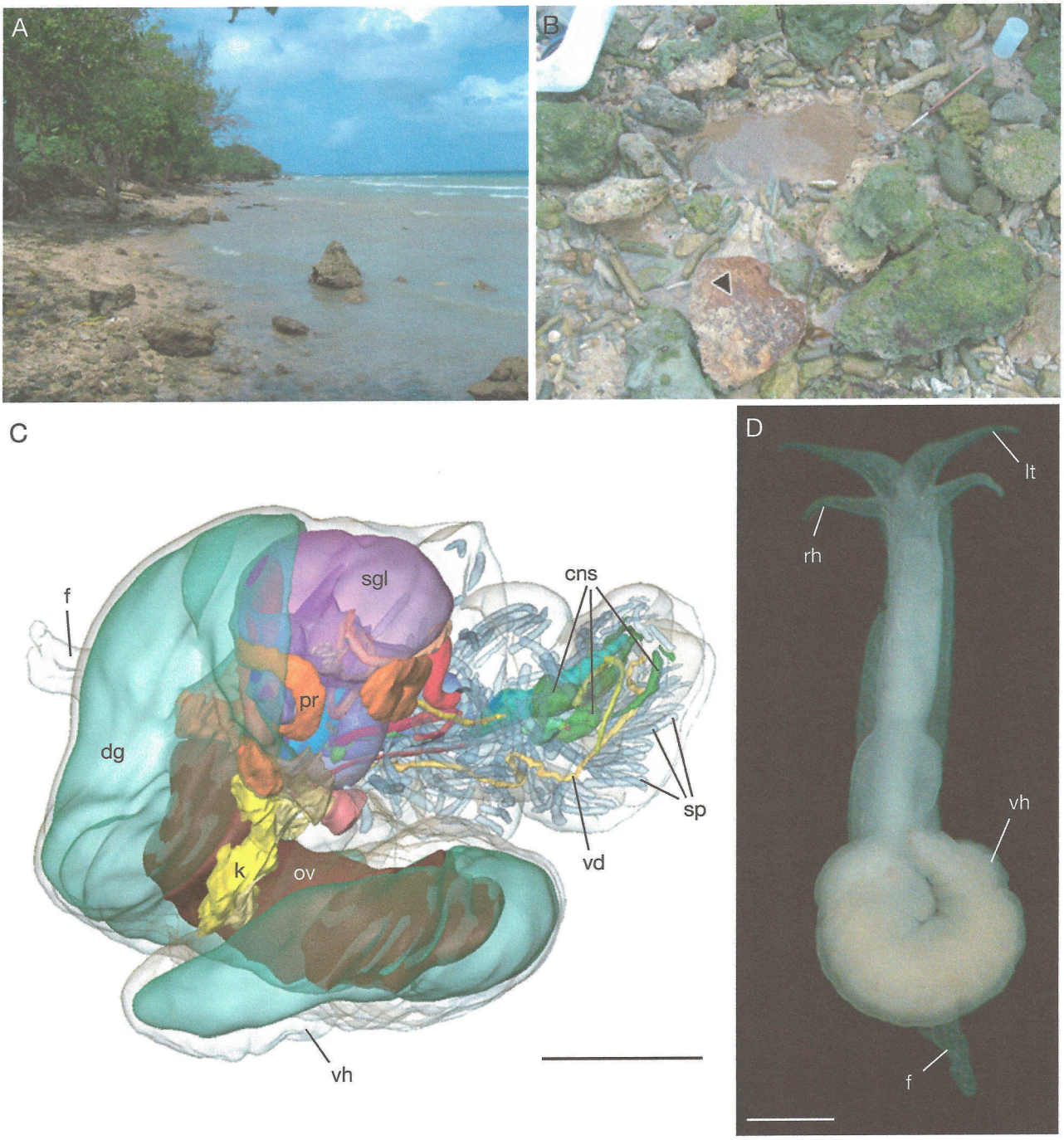

FIG. 1. — Habitat, external morphology and general anatomy of *Pseudunela espiritusanta* n. sp.: **A**, type locality when tide is just coming in; **B**, habitat of *P. espiritusanta* n. sp.: underside of rocks embedded in coarse sand (arrowhead points to exact place where specimens were found); **C**, 3D reconstruction, position of internal organs: green, central nervous system; blue/lilac, digestive system; yellow, circulatory and excretory systems; red/brownish, reproductive system; **D**, photograph of living specimen. Abbreviations: **cns**, central nervous system; **dg**, digestive gland; **f**, foot; **k**, kidney; **lt**, labial tentacle; **ov**, ovotestis; **pr**, prostate; **rh**, rhinophore; **sgl**, salivary gland; **sp**, spicule; **vd**, vas deferens; **vh**, visceral hump. Scale bars: C, 500 µm; D, 1 mm.

habitat were at least *Neritilia littoralis* Kano, Kase & Kubo, 2003 and two undescribed *Neritilia* spp. (Kano pers. comm.).

External morphology

Body divided into anterior head-foot complex and posterior elongated visceral hump (vh) (Fig. 1C, D).

Head-foot complex partially retractable into temporary cavity of visceral hump. Length of crawling specimen, 9 mm. Body colour of living specimens translucent-whitish, digestive gland yellowish. Labial tentacles (lt) broad at base (Fig. 1D), tapering to distal end, usually held at 45° to mid line. Rhinophores (rh) slightly shorter and narrower, tapering (Fig. 1D). Pigmented eyes, only clearly visible laterally. Densely ciliated foot (f) broader than anterior head-foot complex (Fig. 1D), tail extending about two-thirds of visceral hump; tip pointed. Visceral hump in living specimens usually curved (Fig. 1D). Heart visible within prominent bulb at anterior right side of visceral hump. Subepidermal spicules (sp) bean-shaped (Figs 1C; 8A), small (70-135 µm) in tentacles, larger (200-300 µm) in foot and around cns and pharynx (Fig. 3D).

Microanatomy

Central nervous system. cns euthyneurous. Paired rhinophoral (rhg), cerebral (cg), pedal (pg), pleural (plg), optic (og), buccal (bg) and gastro-oesophageal (gog) ganglia; three distinct separated ganglia on visceral nerve cord, plus presumed osphradial ganglion (osg) (Fig. 2). All ganglia excluding buccal and gastro-oesophageal ganglia pre-pharyngeal (Fig. 3C, D). Large cerebral ganglia (*c.* 130 µm in diameter) with short commissure (Figs 2; 3A, B). Labiotentacular nerve (ltn) emerging anteroventrally from cerebral ganglion, ramifying into (at least) 6 branches within labial tentacles (Figs 2; 3A). Rhinophoral ganglion (*c.* 40 µm in diameter) anterodorsal to each cerebral ganglion (Figs 2; 3A); short, single cerebro-rhinophoral connective. Rhinophoral nerve (rhn) emerging from rhinophoral ganglion (Figs 2; 3A). Thin nerve arising at base of rhinophoral nerve (Fig. 2), extending towards ciliated ridge posterior to rhinophore. Optic ganglion (Figs 2; 3A, B) (*c.* 35 µm in diameter) situated posterior to rhinophoral ganglion, attached to cerebral ganglion. Thin optic nerve (on) (Figs 2; 3B) innervating pigmented eye (ey) (Figs 2; 3A, B) (*c.* 45 µm in diameter). Anterior accessory ganglia absent.

Pedal ganglia (*c.* 100 µm in diameter) connected by thin, long commissure (Figs 2; 3B). Statocyst (st) (Figs 2; 3B) posterior to each pedal ganglion.

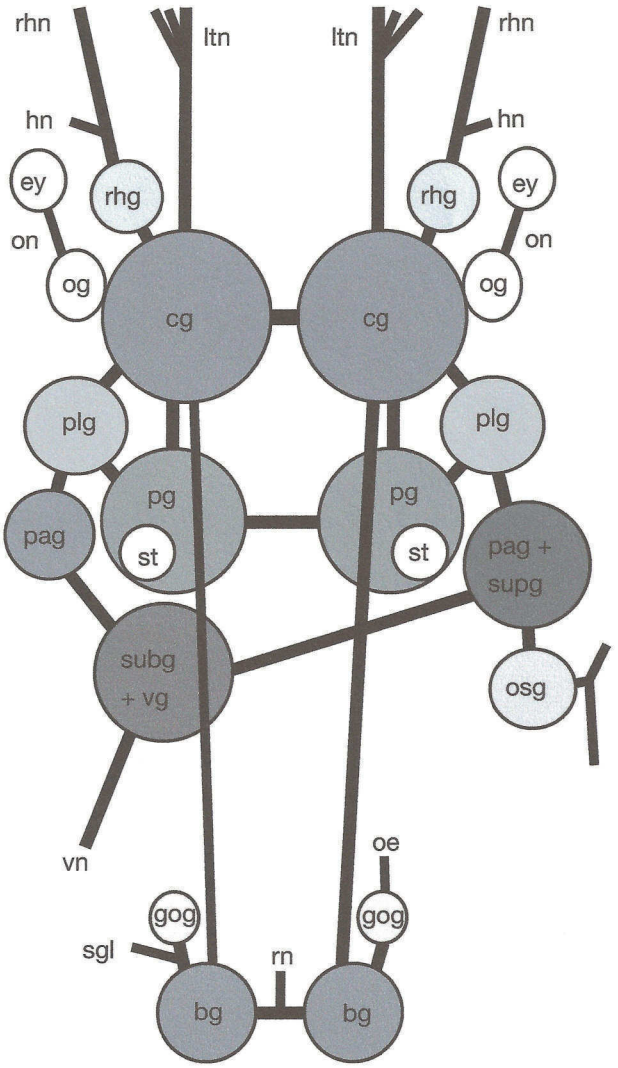

FIG. 2. — Central nervous system of *Pseudunela espiritusanta* n. sp. (schematic, dorsal view). Abbreviations: **bg**, buccal ganglion; **cg**, cerebral ganglion; **ey**, eye; **gog**, gastro-oesophageal ganglion; **hn**, Hancock's nerve; **ltn**, labial tentacle nerve; **oe**, nerve innervating oesophagus; **og**, optic ganglion; **on**, optic nerve; **osg**, osphradial ganglion; **pag**, parietal ganglion; **pg**, pedal ganglion; **plg**, pleural ganglion; **rhg**, rhinophoral ganglion; **rhn**, rhinophoral nerve; **rn**, radula nerve; **sgl**, nerve innervating salivary gland; **st**, statocyst; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **vg**, visceral ganglion; **vn**, visceral nerve. Not to scale.

Four pedal nerves (pn) per ganglion (Fig. 3A), extending anteriorly, posteriorly and ventrally. Pleural ganglion (Figs 2; 3A, B) (*c.* 55 µm in diameter) posterior to and equidistant from cerebral and pedal ganglion, connected to both by short connectives forming pre-pharyngeal nerve ring. Visceral nerve cord short with three distinct ganglia: left parietal (pag) (Figs 2; 3B) (*c.* 55 µm in

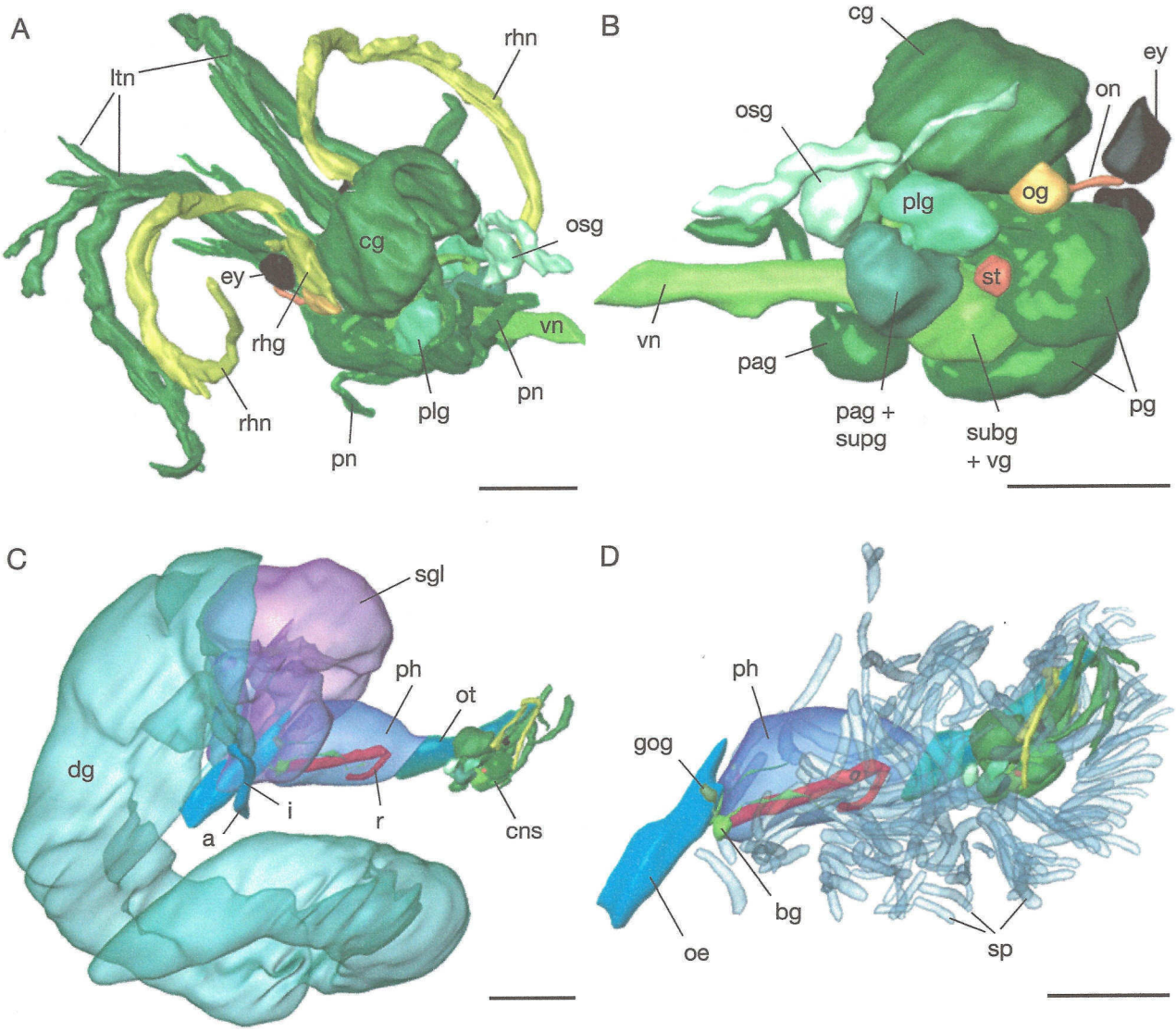

FIG. 3. — 3D reconstruction of the central nervous system and digestive system of *Pseudunela espiritusanta* n. sp.: **A**, cns, dorsolateral left view; **B**, cns without cerebral nerves, right view; **C**, cns and digestive system, right view; **D**, spicules surrounding cns and buccal mass, right view. Abbreviations: **a**, anus; **bg**, buccal ganglion; **cg**, cerebral ganglion; **cns**, central nervous system; **dg**, digestive gland; **ey**, eye; **gog**, gastro-oesophageal ganglion; **i**, intestine; **ltn**, labial tentacle nerve; **oe**, oesophagus; **og**, optic ganglion; **on**, optic nerve; **osg**, osphradial ganglion; **ot**, oral tube; **pag**, parietal ganglion; **pg**, pedal ganglion; **ph**, pharynx; **plg**, pleural ganglion; **pn**, pedal nerve; **r**, radula; **rhg**, rhinophoral ganglion; **rhn**, rhinophoral nerve; **sgl**, salivary gland; **sp**, spicule; **st**, statocyst; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **vg**, visceral ganglion; **vn**, visceral nerve. Scale bars: A, B, 100 µm; C, D, 300 µm.

diameter), fused subintestinal/visceral (subg+vg) (Figs 2; 3B) (*c.* 75 µm in diameter) and fused right parietal/supraintestinal (pag+supg) ganglia (Figs 2; 3B) (*c.* 70 µm in diameter). Left pleuro-parietal, parietal-subintestinal/visceral and right pleuro-parietal/supraintestinal connectives short, subintestinal/visceral-parietal/supraintestinal connective longer. Left parietal ganglion producing one nerve. Robust nerve emerging from subintestinal/visceral ganglion extending to visceral hump (Fig. 3A, B). Tentative osphradial ganglion (Figs 2; 3A, B) (*c.* 45 µm in diameter) with one bifurcating nerve linked to parietal/supraintestinal ganglion. Buccal ganglion (*c.* 65 µm in diameter) posterior to pharynx (ph) (Figs 2; 3D; 7B), thin buccal commissure situated ventral to oesophagus. Radular nerve (rn) thin, branching from buccal commissure (Fig. 2). Thin cerebro-buccal connective emerging anteriorly

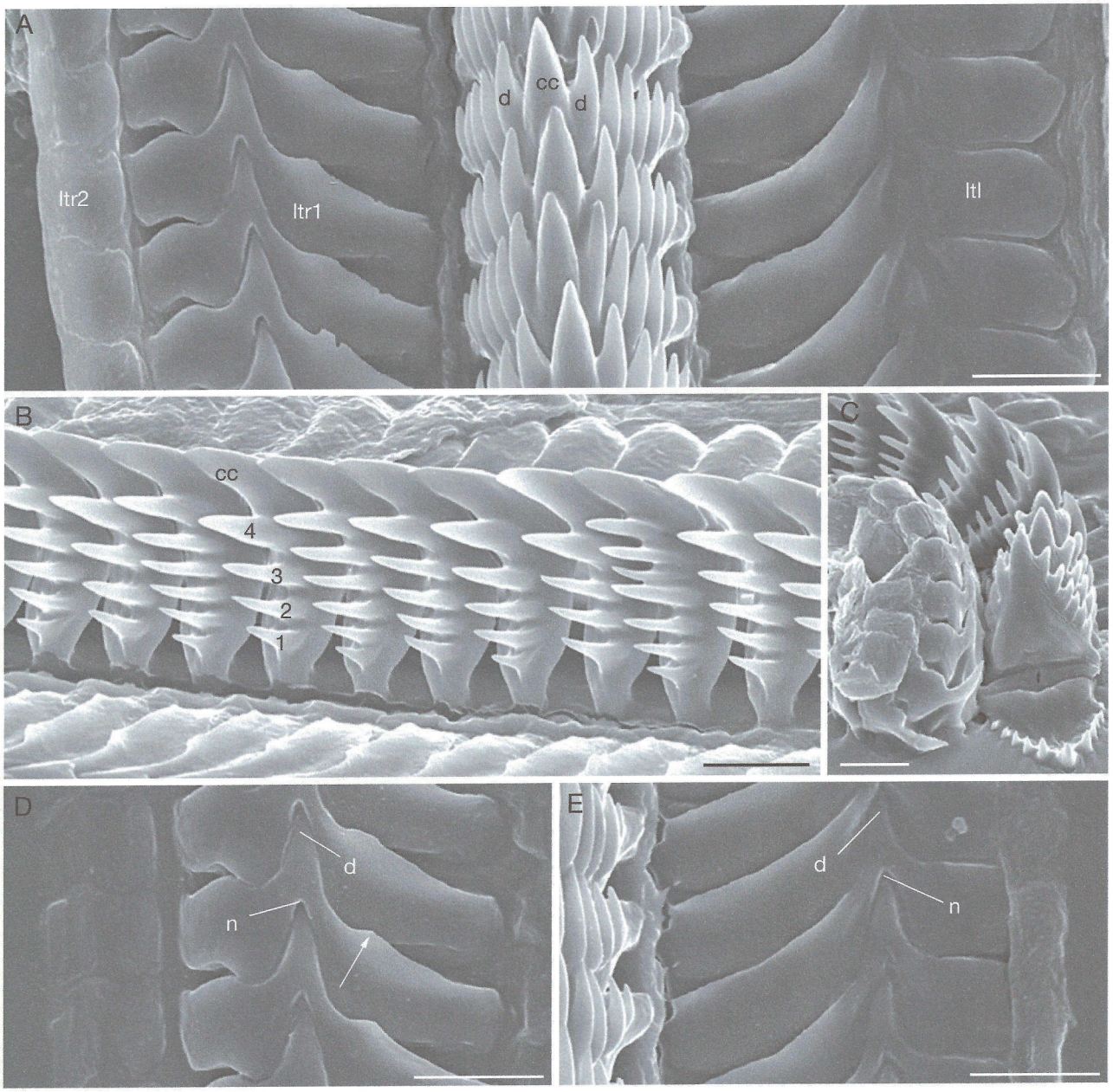

FIG. 4. — Radula of *Pseudunela espiritusanta* n. sp., SEM-micrographs: **A**, row of radular teeth; **B**, rhachidian teeth, right view; **C**, rhachidian tooth, anterior view; **D**, right lateral teeth, arrow points to blunt protrusion; **E**, left lateral teeth. Abbreviations: **cc**, central cusp; **d**, denticle; **ltl**, left lateral tooth; **ltr1**, first right lateral tooth; **ltr2**, second right lateral tooth; **n**, notch; **1-4**, lateral denticle on rhachidian tooth. Scale bars: 10 µm.

from each buccal ganglion, not traceable along entire length. Small gastro-oesophageal ganglion (Figs 2; 3D; 7B) (*c.* 30 µm in diameter) slightly dorsally to each buccal ganglion, innervating oesophagus (oe). Thin nerve innervating the salivary gland (sgl), branching from buccal-gastro-oesophageal connective (Fig. 2).

Digestive system. Mouth opening ventrally between labial tentacles. Anterior pedal gland (apg) (Fig. 8A) opening ventral to mouth. Oral tube (ot) long, unciliated (Fig. 3C). Paired oral glands (otg) flanking oral tube (Fig. 8A). Pharynx (ph) bulbous and muscular (Figs 3C, D; 7A). Jaws absent. Radula (r) *c.* 575 µm long, hook-shaped

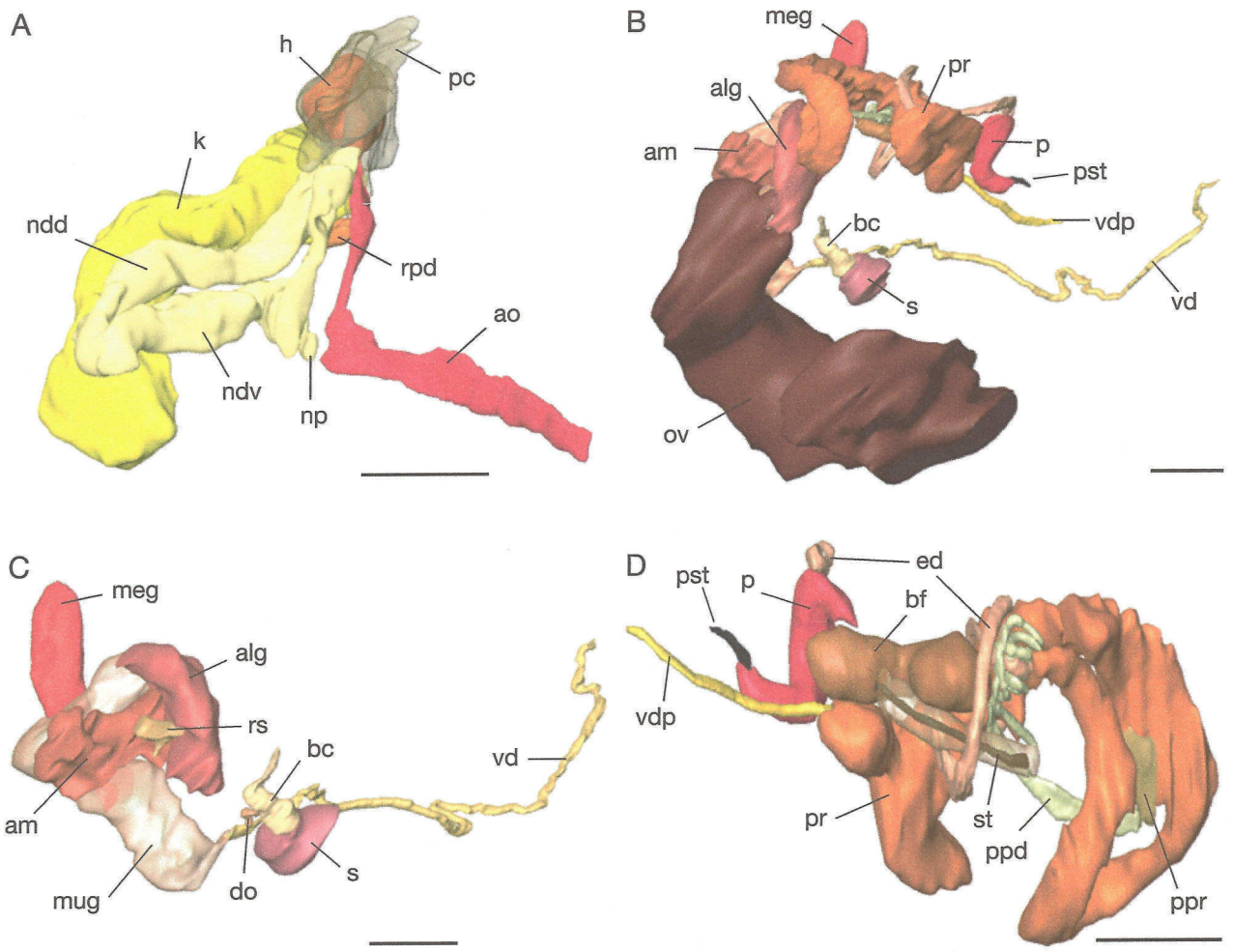

FIG. 5. — 3D reconstruction of the circulatory, excretory and reproductive systems of *Pseudunela espiritusanta* n. sp.: **A**, circulatory and excretory systems, right view; **B**, complete reproductive system, dorsolateral view from right; **C**, nidamental glands, sperm storing receptacles and sphincter, right view; **D**, anterior male copulatory organs, left view. Abbreviations: **alg**, albumen gland; **am**, ampulla; **ao**, aorta; **bc**, bursa copulatrix; **bf**, basal finger; **do**, oviduct; **ed**, ejaculatory duct; **h**, heart; **k**, kidney; **meg**, membrane gland; **mug**, mucus gland; **ndd**, nephroduct dorsal branch; **ndv**, nephroduct ventral branch; **np**, nephropore; **ov**, ovotestis; **p**, penis; **pc**, pericardium; **ppd**, paraprostatic duct; **ppr**, paraprostate; **pr**, prostate; **pst**, penial stylet; **rpd**, renopericardioduct; **rs**, receptaculum seminis; **s**, sphincter; **st**, stylet of basal finger; **vd**, vas deferens; **vdp**, posterior-leading vas deferens. Scale bars: 200 µm.

(Fig. 3C, D) and asymmetric with formula 67 × 1.1.2; upper ramus with 48 rows (*c.* 410 µm long), lower ramus with 19 rows (*c.* 165 µm long). Rhachidian tooth triangular (21 µm high, 18 µm wide) with prominent central cusp (cc) and 4-7 thinner denticles (d) per side (Fig. 4A-C). Lateral teeth plate-like (Fig. 4A). Left lateral tooth (ltl) (5-7 µm high, 29 µm wide) with prominent, cuspid denticle on anterior margin 6 µm in length; deep triangular notch (n) on posterior margin of tooth (Fig. 4E) receiving denticle of subsequent tooth. First/inner right lateral tooth (ltr1) similar to left lateral but slightly smaller (5-6 µm high, 25 µm wide), with small blunt protrusion at inner side of denticle (Fig. 4D). Outer margin of left lateral tooth rounded; outer margin of first right lateral tooth straight (Fig. 4A). Second/outer right lateral tooth (ltr2) small and quadratic plate (8 × 8 µm) (Fig. 4D).

Ciliated oesophagus (oe) long, emerging posterodorsally from pharynx (Figs 3C, D; 7B), flanked by longitudinal muscles. Paired, large salivary glands (sgl) (Figs 3C; 7B) discharging into posterior pharynx via salivary gland ducts (sgd) (Fig. 7B).

Digestive gland (dg) sac-like (Fig. 3C), inner surface heavily folded, lumen empty, situated dorsally of ovotestis (ov), extending to end of visceral hump (Fig. 1C). Stomach continuous with broad, anterior, lobed part of digestive gland. Intestine (i) short (Fig. 3C), densely ciliated (Fig. 8C, D). Anus (a) (Fig. 3C) ventrolaterally on right side of visceral hump, slightly anterior to nephropore.

Circulatory and excretory systems. Circulatory and excretory systems situated at right of body (Fig. 1C), at anterior end of visceral hump. Heart (h) (*c.* 160 µm) with one well-developed muscular ventricle (Figs 5A; 8D), thin valve at posterior end (Fig. 8D). Atrium not apparent. Aggregations of discrete cells in lumen of ventricle (v) (Fig. 8D). Aorta (ao) (Figs 5A; 8C) emerging anteriorly from ventricle, extending to anterior head region. Heart surrounded by thin-walled pericardium (pc) (Fig. 5A); thick layer of blue stained epicardial cells of unknown function embracing ventricle (Fig. 8D). Kidney (k) elongated (Fig. 5A), internally divided into narrow lumen (kn) defined by tissue with small vacuoles, and wide lumen (kw) bordered by highly vacuolated tissue (Fig. 8C, D). Both lumina joining in posterior part of kidney. Renopericardial duct (rpd) well developed and densely ciliated (Figs 5A; 8C). Connection between kidney and nephroduct narrow and ciliated (Fig. 8B). Nephroduct long; dorsal branch (ndd) extending posteriorly, continuing anteriorly via ventral branch (ndv) (Fig. 5A); distally looped. Nephropore (np) ventrolateral at right side of visceral hump, slightly posterior to anus.

Reproductive system. Simultaneously hermaphroditic, androdiaulic. Ovotestis (ov) sac-like (Figs 1C; 5B; 6), extending over almost entire visceral hump, ventral to digestive gland; not separated into follicles, but spermatocytes situated more anteriorly and ventrally, oocytes dorsally and posteriorly. Details of individual oocytes obscured by large amount of yolk. Anterior to ovotestis sac-like ampulla (am) filled with unorientated autosperm in disorder (Figs 5B, C; 6; 7D). Small receptaculum seminis (rs) with orientated allosperm (Figs 5C; 6; 7D). Sperm heads short. Three female nidamental glands: tubular albumen (alg), sac-like membrane (meg)

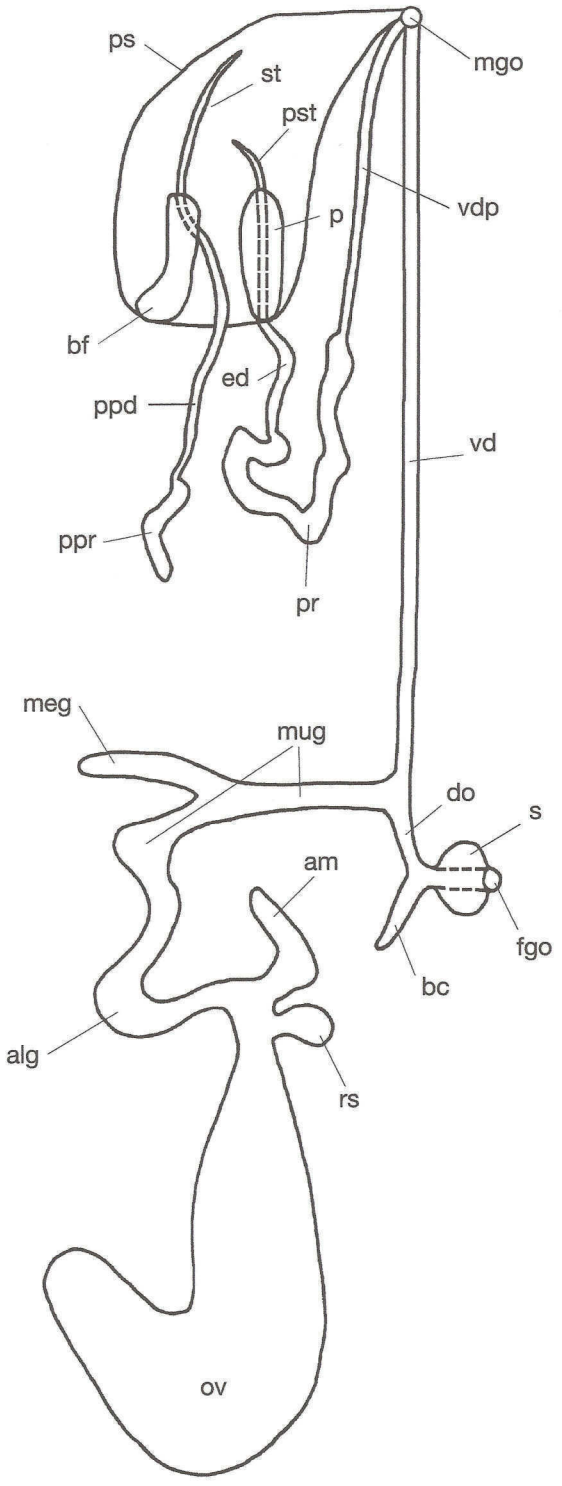

FIG. 6. — Reproductive system of *Pseudunela espiritusanta* n. sp. (schematic drawing). Abbreviations: **alg**, albumen gland; **am**, ampulla; **bc**, bursa copulatrix; **bf**, basal finger; **do**, oviduct; **ed**, ejaculatory duct; **fgo**, female gonopore; **meg**, membrane gland; **mgo**, male gonopore; **mug**, mucus gland; **ov**, ovotestis; **p**, penis; **ppd**, paraprostatic duct; **ppr**, paraprostate; **pr**, prostate; **ps**, penial sheath; **pst**, penial stylet; **rs**, receptaculum seminis; **s**, sphincter; **st**, stylet of basal finger; **vd**, vas deferens; **vdp**, posterior-leading vas deferens. Not to scale.

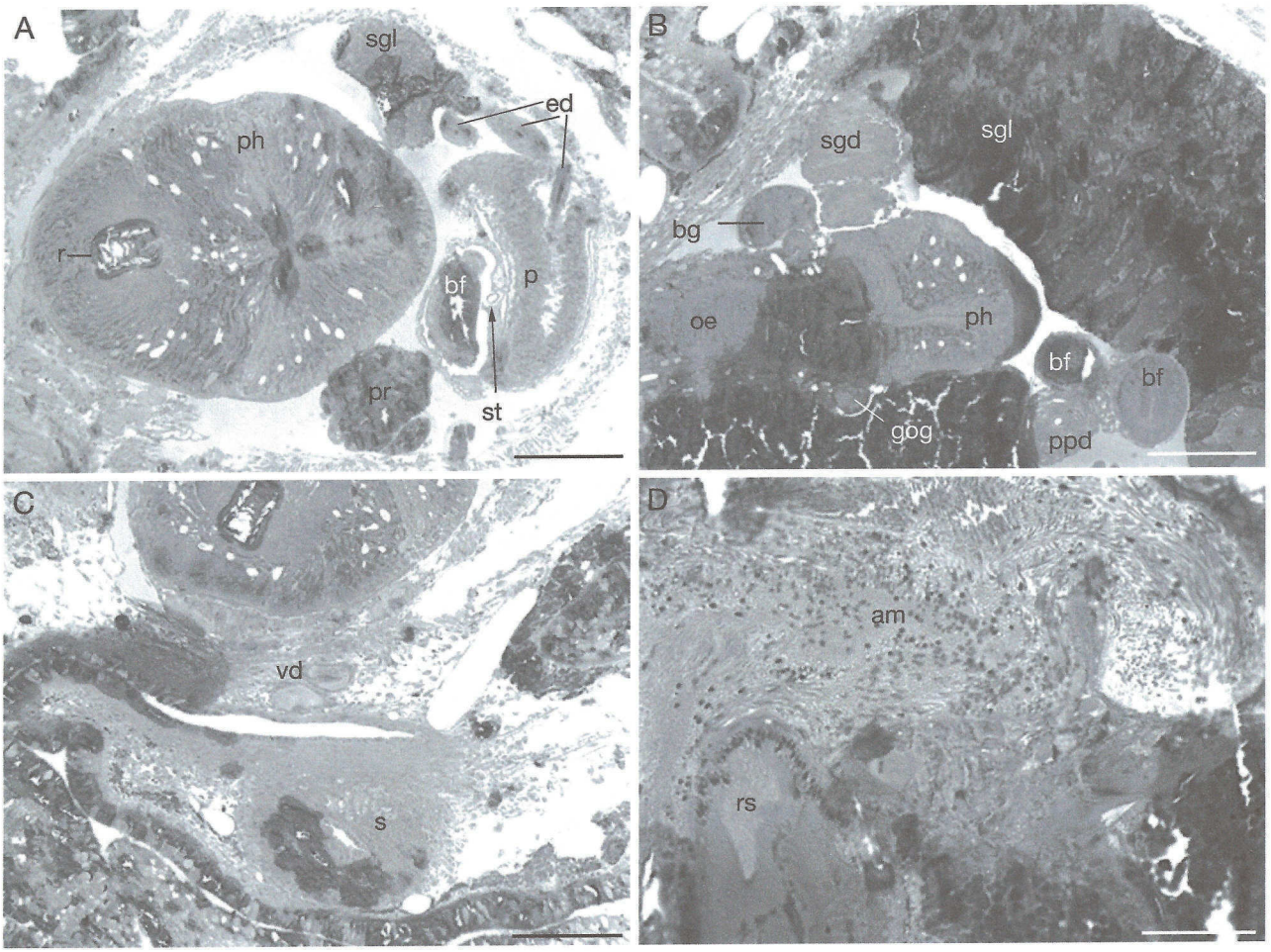

FIG. 7. — Transverse histological sections of *Pseudunela espiritusanta* n. sp.: **A**, pharynx with radula, penis, basal finger; **B**, salivary gland, buccal ganglion; **C**, sphincter; **D**, sperm storing receptacles. Abbreviations: **am**, ampulla; **bf**, basal finger; **bg**, buccal ganglion; **ed**, ejaculatory duct; **gog**, gastro-oesophageal ganglion; **oe**, oesophagus; **p**, penis; **ph**, pharynx; **ppd**, paraprostatic duct; **pr**, prostate; **r**, radula; **rs**, receptaculum seminis; **s**, sphincter; **sgd**, salivary gland duct; **sgl**, salivary gland; **st**, stylet of basal finger; **vd**, vas deferens. Scale bars: A-C, 100 µm; D, 25 µm.

and tubular mucus gland (mug) (Figs 5B, C; 6). Membrane gland branching off in middle of mucus gland. Albumen gland characterized by cells containing dark blue-stained granules; cells of mucus and membrane glands with violet-staining contents. All nidamental glands densely ciliated. Distal part of mucus gland extending to right side of body wall where hermaphroditic duct separates into vas deferens (vd) and oviduct (do) (Figs 5C; 6). Oviduct short, narrow (Fig. 5C; 6), dividing into long slender, blind duct (bc) (bursa copulatrix or bursa stalk) (Fig. 5B, C; 6) and distal gonoduct extending to female gonopore (fgo). Large muscular sphincter (s) surrounding female gonopore (Figs 5B, C; 6; 7C). Female gonopore ventrolateral at right side of visceral hump, anterior to anus and nephropore.

Subepidermal, ciliated vas deferens (Figs 5C; 6; 7C) extending along right body side to opening at base of right rhinophore. Anterior male copulatory organs cephalic. Posterior-leading vas deferens (vdp) connecting to large tubular prostate (pr) (Figs 5D; 6; 7A). Ejaculatory duct (ed) connecting to elongated, muscular penial papilla (p) (Figs 5D; 6; 7A). Curved hollow penial stylet (pst) (*c.* 80 µm long) partially retractable into muscular penial bulb (Figs 5D; 6). Blind glandular paraprostate (ppr) (Figs 5D; 6) considerably smaller than prostate, connected by paraprostatic duct (ppd) to muscular

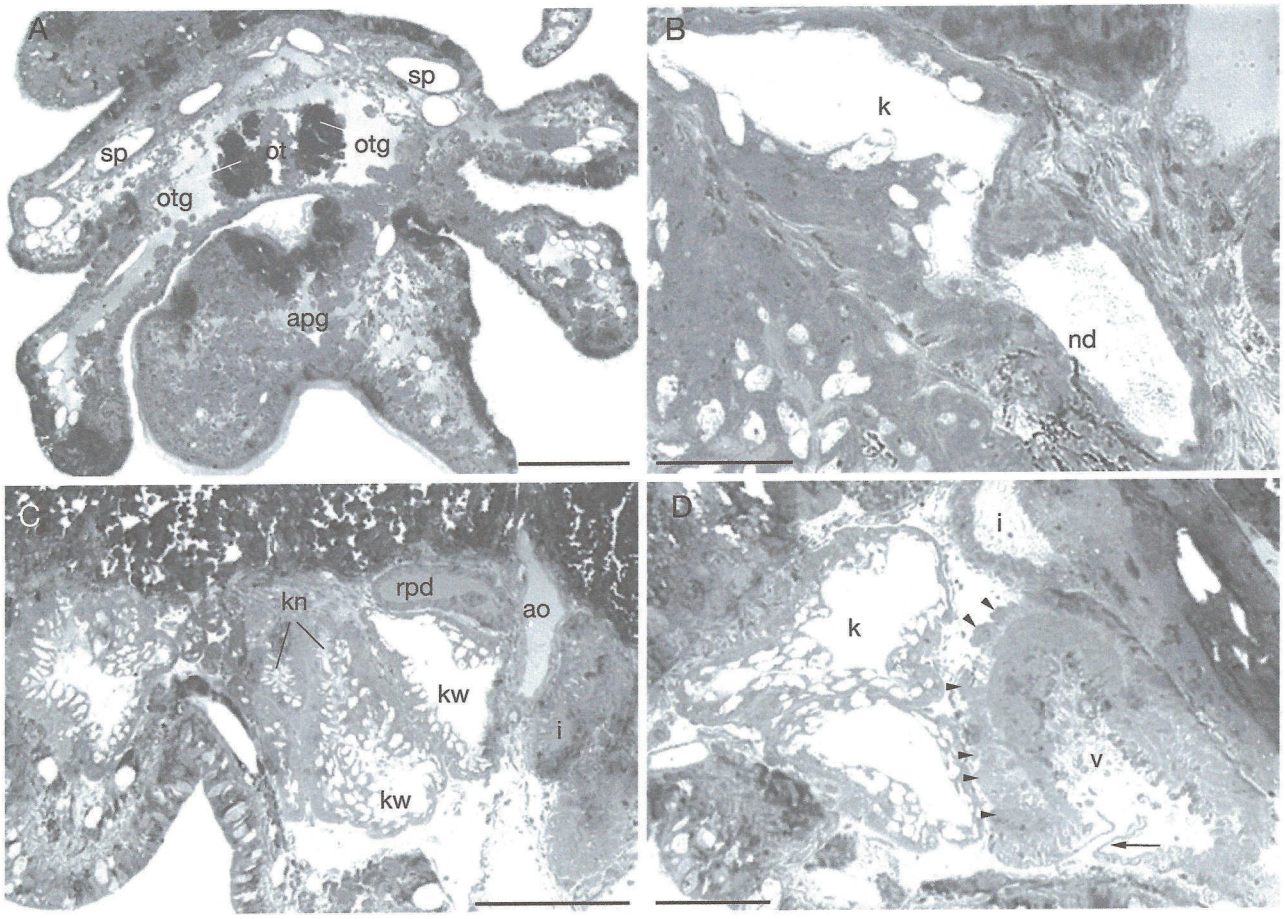

FIG. 8. — Transverse histological sections of *Pseudunela espiritusanta* n. sp.: **A**, oral tube gland, anterior pedal gland; **B**, transition kidney-nephroduct; **C**, renopericardial duct; **D**, heart, arrow points to valve; arrowheads point to epicardial cells of unknown function. Abbreviations: **ao**, aorta; **apg**, anterior pedal gland; **i**, intestine; **k**, kidney; **kn**, narrow lumen of kidney; **kw**, wide lumen of kidney; **nd**, nephroduct; **ot**, oral tube; **otg**, oral tube gland; **rpd**, renopericardioduct; **sp**, spicule cavity; **v**, ventricle. Scale bars: A, C, 100 µm; B, 25 µm; D, 50 µm.

basal finger (bf) (Figs 5D; 6; 7A, B). Paraprostatic duct entering basal finger subapically and opening apically via a long hollow, slightly curved stylet (st) (*c.* 340 µm long). Penis and basal finger not connected, surrounded by common penial sheath (ps) (Fig. 6).

DISCUSSION

HABITAT

Most of the Acochlidia are marine mesopsammic including *Pseudunela cornuta* (Challis, 1970) and *P. eirene* Wawra, 1988; only six of 27 valid species are limnic. *Palliohedyle weberi* (Bergh, 1895) is reported to live in a "brackish" habitat "in a river mouth on [...] Flores" (Bergh 1895). However, despite a detailed sampling at the type locality, we could not find any acochlidian species near the sea (own unpubl. data). While all marine species are mesopsammic, the limnic species live benthically in coastal rivers of Indo-Pacific Islands or, i.e. the only Caribbean limnic species *Tantulum elegans*, in the interstices of a muddy mountain spring swamp on St. Vincent Island. *Pseudunela espiritusanta* n. sp. is thus unique regarding its habitat, not only by dwelling in a true brackish-water environment but also by living on the underside of large intertidal rocks. Kano *et al.* (2003) already described a neritiliid gastropod from this special, rarely explored habitat. Feeding habits of *P. espiritusanta* n. sp. are as yet unknown. Potential prey organisms living on the underside

TABLE 1. — Comparison of the external morphology within the genera *Pseudunela* Salvini-Plawen, 1973 and *Strubellia* Odhner, 1937. Abbreviations: **cns**, central nervous system; **vh**, visceral hump; **?**, no data available.

	Pseudunela cornuta	***Pseudunela eirene***	***Pseudunela espiritusanta* n. sp.**	***Strubellia paradoxa***	***Strubellia paradoxa***
Data source	Challis 1970; **Neusser *et al.* 2009b**	Wawra 1988a	present study	Küthe 1935	Wawra 1988b
Body size (mm)	3; **3**	4 (fixed)	9	20 (fixed)	20-30
Colour of body	translucent-whitish; **translucent-whitish**	?	translucent-whitish	light brown	
Colour of digestive gland	?; **orange-brownish**	?	yellowish	red-brownish	
Eyes visible externally	no; **no**	?	small, visible lateral	large, visible lateral	
Foot width	as broad as head; **as broad as body**	as broad as body	broader than body	broader than body	broader than body
Foot length	slightly longer than head; **1/2 of vh**	?	2/3 of vh	2/3 of vh	2/3 of vh
Visceral hump	elongated; **elongated**	?	recurved	elongated	elongated
Heart bulb visible	?; **yes**	?	yes	yes	yes
Subepidermal calcareous spicules	absent; **few in vh**	?	bean-shaped; in cephalic tentacles, foot, vh, around cns	present	absent (after fixation)

of stones may be limited. *Pseudunela espiritusanta* n. sp. thus may prey upon mesopsammic organisms, although it was not yet found in the adjacent sandy interstices. Reporting a limpet-like brachiopod species attached to rubble deeply embedded in coarse sand in the mid intertidal, Kato (1996) emphasized potential advantages of this habitat, which provides protection from ultraviolet rays, desiccation, physical turbulence caused by wave action and benthic predators. Subsoil freshwater influence additionally may deter marine predators, but also requires mechanisms to scope with osmotic stress for all species including potential prey.

EXTERNAL MORPHOLOGY

The external appearance of *Pseudunela espiritusanta* n. sp. resembles that of the marine *Pseudunela cornuta* and *P. eirene* and the limnic *Strubellia paradoxa* (Strubell, 1892). While the shape of body and tentacles coincide in all four species (Küthe 1935; Challis 1970; Wawra 1974, 1988a), there are some differences regarding body sizes and colour, foot width and length, shape of the visceral hump and the absence or presence of subepidermal calcareous spicules (Table 1).

MICROANATOMY

The anatomy of the cns of *P. espiritusanta* n. sp. broadly agrees with the typical acochlidian nervous system and resembles in different features the cns of *P. cornuta*, *S. paradoxa* (Challis 1970; Wawra 1988b) and *Tantulum elegans* (Neusser & Schrödl 2007). While anterior accessory ganglia were described as present in *T. elegans* and possibly *P. eirene*, they are absent in *P. cornuta*, *S. paradoxa* and our new species (Table 2) (Wawra 1988a, b; Neusser & Schrödl 2007; Neusser *et al.* 2009b). The optic ganglion innervating the eye is reported only from *T. elegans* and *P. espiritusanta* n. sp. (Neusser & Schrödl 2007), while the optic nerve emerges from the rhinophoral ganglion in *P. cornuta*, although an optic ganglion is present in this species as well (Neusser *et al.* 2009b). The thin nerve emerging at the base of the rhinophoral nerve and leading

TABLE 2. — Comparison of different anatomical characteristics within the genera *Pseudunela* Salvini-Plawen, 1973 and *Strubellia* Odhner, 1937. **?**, no data available.

	Pseudunela cornuta	***Pseudunela eirene***	***Pseudunela espiritusanta* n. sp.**	***Strubellia paradoxa***	***Strubellia paradoxa***
Data source	Challis 1970; **Neusser *et al.* 2009b**	Wawra 1988a	present study	Küthe 1935	Wawra 1988b
Accessory ganglia	present; **absent**	?	absent	?	absent
Optic ganglion	absent; **present**	?	present	absent	absent
Osphradial ganglion	absent; **present**	present	present	?	present
Gastro-oesophageal ganglia	absent; **present**	absent	present	absent	present
Radula formula	50 × 1.1.1; **?**	52 × 1.1.2	67 × 1.1.2	48-56 × 2.1.2	48-51 × 2.1.2
Rhachidian cusp	projecting; **?**	?	projecting	very elongate	very elongate
Rhachidian tooth denticles	3 or 4; **?**	3 or 4	4-7	numerous, tiny	numerous, tiny
Anal-genital cloaca	present; **absent**	?	absent	present	?
Kidney	large, unfolded sac; **long, internally divided**	?	long, internally divided	long, internally divided	?
Long nephroduct with 2 branches	?; **present**	?	present	present, branches interconnected	?
Hollow curved penial stylet (µm)	100; **600**	200	80	500	1000
Solid basal thorn (µm)	absent; **absent**	30	absent	present	present
Hollow curved stylet on basal finger (µm)	?; **110**	?	340	?	?
Glands associated with copulatory organs	prostate, penial gland; **prostate, paraprostate**	?	prostate, paraprostate	2 glands	?
Receptaculum seminis	?; **present**	?	present	present	present
Bursa copulatrix	present; **present**	?	present	?	present

to a ciliated ridge just posterior to the rhinophore might be interpreted as the nerve leading to the Hancock's organ, just as reported for *T. elegans* (Neusser & Schrödl 2007). The ganglion connected to the supraintestinal ganglion in *P. cornuta*, *P. eirene*, *P. espiritusanta* n. sp. and *S. paradoxa* is interpreted to be the osphradial ganglion according to Huber (1993), even though an osphradium has never been identified in any acochlidian species so far. Gastro-oesophageal ganglia were reported by Wawra (1988b, 1989) for *Strubellia paradoxa* and *Hedylopsis spiculifera* (Kowalevsky, 1901), as well as for *P. cornuta*, *Tantulum elegans* and *Asperspina murmanica* (Kudinskaya & Minichev, 1978) (Neusser & Schrödl 2007; Neusser *et al.* 2009a, b).

The digestive system of *P. espiritusanta* n. sp. conforms with that of other acochlidian species studied in detail (Sommerfeldt & Schrödl 2005; Neusser *et al.* 2006, 2009a; Jörger *et al.* 2008). The radula resembles that of *Pseudunela cornuta* (Challis 1970) and *Asperspina murmanica* (Neusser *et al.* 2009a), but is considerably larger reflecting the overall larger body size. Both latter species show a rhachidian tooth with large, well-defined denticles and a large denticle on the first lateral tooth which has a pronounced notch at least in *A. murmanica*. While *P. cornuta* was described to have a symmetric radula with one lateral tooth (Challis 1970), *P. eirene* shows an asymmetric radula (Wawra 1988a) like the new species. The radula of *P. cornuta* should be re-examined in detail, as well as the radula of *Strubellia paradoxa* which was reported as symmetric with two lateral teeth on each side (Küthe 1935).

Only sparse data on the circulatory and excretory systems of Acochlidia are available. *Pseudunela cornuta*, *Microhedyle remanei* (Marcus, 1953) and *Pontohedyle milaschewitchii* (Kowalevsky, 1901) were found to possess well-developed, two-chambered hearts (Neusser *et al.* 2006, 2009b; Jörger *et al.* 2008). The heart of *P. espiritusanta* n. sp. is large, and the ventricle is strongly muscular. The valve detected at the posterior, thin-walled margin of the ventricle might be the connection to an equally thin-walled atrium, which possibly is collapsed or compressed and, thus, could not be detected. Highly conspicuous is the layer of irregular bulbous (epicardial?) cells covering the ventricle. Küthe (1935) has previously reported a layer of bulbous cells covering the heart of *S. paradoxa*. Their weak similarity to so-called pericardial glands of doridoidean nudibranchs (see e.g., Schrödl & Wägele 2001), which were shown to be podocytes by Fahrner & Haszprunar (2002a), might indicate that they are involved in ultrafiltration. The unique ventricular podocyte-like cells are thus shared by *Pseudunela espiritusanta* n. sp. and at least one of the limnic Acochlidiidae. If they are indeed podocytes, they likely enhance production of primary urine in species exposed to brackish or limnic conditions. Furthermore, *P. espiritusanta* n. sp. shows a complex excretory system as was reported from limnic acochlidian species, e.g., *Acochlidium amboinense* (Strubell, 1892), *S. paradoxa* and *T. elegans* (Bücking 1933; Küthe 1935; Rankin 1979; Neusser & Schrödl 2007). While marine acochlidians usually show a simple sac-like kidney with a short nephroduct (Neusser *et al.* 2006, 2009a; Jörger *et al.* 2008), all the freshwater species have a well-developed, complex kidney. The kidney of *P. espiritusanta* n. sp. is divided into two inter-connected compartments, and also has a long looped nephroduct which increases the surface area for resorption. *Strubellia paradoxa* was reported to have an internally divided kidney just as our new species (Küthe 1935); whether or not the branches of the nephroduct really show anastomose (Table 2) requires re-examination. Challis (1970) described the kidney of *P. cornuta* as a "large unfolded sac"; unfortunately without mentioning the (shape and length of the) nephroduct. Re-examination of *P. cornuta* from the type locality shows this species has in fact a large, internally divided kidney and a long looped nephroduct (Neusser *et al.* 2009b). The marine *Hedylopsis ballantinei* was reported to possess a long, sac-like kidney extending almost over the entire visceral sac (Fahrner & Haszprunar 2002b; Sommerfeldt & Schrödl 2005); however, our re-examination revealed a complex kidney with a narrow duct extending posteriorly and a wide one leading anteriorly (own unpubl. data), just as in *P. cornuta*. Functionally, the well-developed excretory system of *P. espiritusanta* n. sp. clearly enhances excretion and resorption and can be regarded as an adaptation to their brackish-water habitat. Species adapted to subsoil freshwater influence such as *P. espiritusanta* n. sp. or intertidal mesopsammic species like *P. cornuta* which at least temporarily tolerate freshwater input (e.g., during heavy rainfalls) may have evolved preadaptations necessary for colonising limnic systems. Consequently, these habitats may have served as stepping stones for the colonisation of freshwater. Neusser *et al.* (2009b) proposed that the complex kidney has evolved in the mesopsammic ancestor of the marine Hedylopsacea and is assumed to be a preadaptation and key feature to both, independent invasions of a limnic habitat known from opisthobranchs.

The posterior reproductive system of *P. espiritusanta* n. sp. is interesting in possessing a proximal receptaculum seminis and a bursa-like duct in the typical distal position. In acochlidians, a full set of allosperm receptacles is only known from *Strubellia paradoxa* from Solomon Islands (Wawra 1988b), but is also present in *Pseudunela cornuta* (Neusser *et al.* 2009b). A sac-like ampulla is only reported from *Asperspina murmanica*, *Tantulum elegans* and *P. cornuta* (Neusser & Schrödl 2007; Neusser *et al.* 2009a, b), while the ampulla is a tubular swelling in *Pontohedyle milaschewitchii* and *Microhedyle remanei* (Neusser *et al.* 2006; Jörger *et al.* 2008). The nidamental glands were identified based on their position in the reproductive system from proximal to distal, i.e. the albumen, membrane and mucus gland, respectively. The mucus gland may be tubular as in *A. murmanica* and *P. milaschewitchii* but is a blind sac in *M. remanei* and *P. cornuta* (Neusser *et al.* 2006, 2009a, b; Jörger *et al.* 2008). In contrast, the membrane gland is tubular in all acochlidian species

examined except *P. espiritusanta* n. sp. A sphincter at the female gonoduct has never been observed in acochlidians, the function is unknown. A similar structure is present in the nudibranch *Goniodoris castanea* Alder & Hancock, 1845 (Wägele & Cervera 2001). The complex anterior male genitalia of *P. espiritusanta* n. sp. presents some similarities to marine species of the genus *Pseudunela* (Table 2). While *P. eirene* and the limnic *Strubellia paradoxa* seem to possess one larger, hollow penial stylet and a second smaller solid thorn (Küthe 1935; Wawra 1998a, b), the re-examination of *P. cornuta* revealed a hollow penial stylet connected to the prostate and an additionally stylet on the basal finger connected to the glandular paraprostate (Neusser *et al.* 2009b). Küthe (1935) observed two glands associated with the penial muscle in *S. paradoxa*, but did not identify them. There are no data concerning male glands in *P. eirene* available. A detailed (re-)examination of the basal marine genera *Hedylopsis* and *Pseudunela* and of the limnic *Strubellia paradoxa* is essential to homologise structures of the complex copulatory organs and associated glands.

Taxonomic remarks

External features such as the special tentacle shape leave no doubt that *P. espiritusanta* n. sp. is associated with the acochlidian genera *Pseudunela* and *Strubellia*, according to Schrödl & Neusser (in press) belonging to the hedylopsacean families Pseudunelidae Rankin, 1979 and Acochlidiidae *sensu* Wawra (1987), respectively. Comparing external and anatomical features clearly shows that *P. espiritusanta* n. sp. differs in many significant ways from other known *Pseudunela* and *Strubellia* species (Tables 1; 2).

A sketch of an acochlidian specimen found in marine sands of Lizard Island was provided by Burn (1998: fig. 16.42B as *Microhedyle cornuta*). Its tentacle shape is characteristic for both marine *Pseudunela* and limnic *Strubellia* species. While its broad foot contradicts identification as *P. cornuta* or *P. eirene*, the marine environment and still smaller body size (10 mm) argues against a placement into *Strubellia*. Burn's specimen resembles our new species by its size and body shape, and especially by also having a bent visceral hump. However, the foot appears considerably shorter, and no spicules were recognized by Burn. In contrast to our species, Burn's species was found in marine sand samples (R. Burn pers. comm.) and most likely represents an undescribed *Pseudunela* species.

Although the specimens from Espiritu Santo clearly represent a new species, the generic placement of this species is problematic. From a taxonomic point of view, *P. espiritusanta* n. sp. does not correspond to any of the current generic diagnoses by Wawra (1987). According to the acochlidian phylogeny based on morphological data (Schrödl & Neusser in press), *P. espiritusanta* n. sp. possesses a mixture of features that are apomorphic for acochlidians (e.g., the asymmetric radula with relatively small rhachidian teeth), hedylopsaceans (short sperm head), and a clade composed of *Pseudunela* (marine interstitial), *Strubellia*, and Acochlidiidae (both limnic, benthic). Unique synapomorphies of *P. espiritusanta* n. sp., *Pseudunela* and Acochlidiidae *sensu* Arnaud *et al.* (1986), i.e. *Strubellia* plus Acochlidiidae *sensu* Wawra (1987), are the well-developed and externally visible heart bulb, and the fusion of the anterior portion of the visceral hump with the headfoot without showing a discernable mantle border (Schrödl & Neusser in press). With present, limited knowledge the only potential synapomorphies for *Pseudunela* and *P. espiritusanta* n. sp. refer to the very similar and special structure and arrangement of copulatory organs and associated glands in *P. espiritusanta* n. sp. and *P. cornuta* (Neusser *et al.* 2009b). On the other hand, the presence of bean-shaped spicules, a broad foot, and the layer of bulbous cells around the ventricle may be synapomorphic for *P. espiritusanta* n. sp. and *Strubellia*. For several features, *P. espiritusanta* n. sp. displays intermediate conditions, e.g., it is still marine but under freshwater influence, it is presumably neither mesopsammic nor lives benthically on stones, it is larger than marine interstitial species but still smaller than limnic ones. We conclude that the generic placement of our new species is uncertain; it could be either a basal offshoot of *Pseudunela* or of the *Strubellia*-Acochlidiidae clade (see Schrödl & Neusser in press). Its exact position will depend on a detailed re-examination of the inadequately known *Pseudunela* (Pseudunelidae) and *Strubellia* (Acochlidiidae *s.l.*)

species and a subsequent phylogenetic analysis. At the moment, we do not wish to introduce new generic or familiar names; we thus place our new and very special species into *Pseudunela*, with the caveats expressed above.

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Redescription and three-dimensional reconstruction of the limnic acochlidian gastropod *Strubellia paradoxa* (Strubell, 1892) (Gastropoda: Euthyneura) from Ambon, Indonesia

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This study is the first to examine the entire microanatomy of a representative of the limnic family Acochlidiidae s.l., belonging to the otherwise marine mesopsammic Acochlidia. A paratype of *Strubellia paradoxa* (Strubell, 1892) was reconstructed three-dimensionally from serial semi-thin sections using the software AMIRA; additional material recently collected close to the type locality on Ambon Island, Indonesia was examined by scanning electron microscopy. Results are critically compared with the original description by Küthe of 1935. The genital system of *S. paradoxa* is monaulic, with two allosperm receptacles in the male phase, suggesting that the species is a sequential, protandric hermaphrodite rather than gonochoric; an open seminal groove connects to a complex cephalic copulatory apparatus. The two-chambered heart with conspicuous epicardium, the elongated kidney and the looped nephroduct are interpreted as possible adaptations to the freshwater habitat. Differences from *S.* "*paradoxa*" *sensu* Wawra 1974 and 1988 from the Solomon Islands are discussed.

Keywords: Mollusca; Heterobranchia; Opisthobranchia; histology; microanatomy

Introduction

The Mollusca have been a rich source of information about animal evolution and biodiversity, historically starting from a morphological and palaeontological perspective to one that is increasingly focused on molecular phylogenetics (e.g. Dayrat and Tillier 2002; Grande et al. 2004a; Klussmann-Kolb et al. 2008; Ponder and Lindberg 2008). A combination of both morphological and molecular techniques has recently been employed to obtain knowledge on molluscan evolution per se and also the generation of biodiversity over time (e.g. Glaubrecht 2009).

The Opisthobranchia are a traditional and exceptionally diverse group of gastropods and comprise about 6000 aquatic species that have commonly modified or lost their shell (e.g. Wägele and Klussmann-Kolb 2005). Molecular systematic studies of the affiliation of the major opisthobranch and pulmonate taxa contradict the classic idea of a sister relationship between these taxa (e.g. Grande et al. 2004b; Vonnemann et al. 2005; Wägele et al. 2007). Relationships appear more complex than expected; in particular, the supposedly opisthobranch group Acochlidia showed a tendency to

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cluster among pulmonate clades (Klussmann-Kolb et al. 2008; Jörger et al. 2010). In contrast, morphological datasets – especially those lacking hard to obtain or small species – still support the traditional view (e.g. Wägele and Klussmann-Kolb 2005; Schrödl and Neusser 2010).

The enigmatic Acochlidia is a small group of close to 30 valid species of ecologically divergent opisthobranch-like slugs that have successfully colonized the marine interstitial worldwide and, uniquely among shell-less Gastropoda, limnic habitats (see Sommerfeldt and Schrödl 2005; Schrödl and Neusser 2010). A number of minute mesopsammic species has been the focus of exemplary studies using modern three-dimensional (3D) reconstruction techniques that were able to reveal a wealth of details on microanatomy needed to establish a foundation for phylogenetic hypotheses (Neusser et al. 2006, 2007, 2009; Neusser and Schrödl 2007; Jörger et al. 2009). However, none of the large limnic Indo-Pacific species have yet been examined. The Indo-Pacific species were the first acochlidians to be discovered, namely *Acochlidium amboinense* Strubell, 1892 and *Strubellia paradoxa* (Strubell, 1892) from Ambon, Indonesia (Bergh 1895; Bücking 1933; Küthe 1935). The six described species known today from streams of tropical islands in Southeast Asia and the southwestern Pacific are classified as forming the family Acochlidiidae *sensu* Arnaud et al. (1986), i.e. including the genera *Acochlidium*, *Palliohedyle* and *Strubellia* (see Schrödl and Neusser 2010). According to Schrödl and Neusser (2010), Acochlidiidae is the sister group to the small marine mesopsammic Pseudunelidae (as part of hedylopsacean Acochlidia), with *Strubellia* being most basal. The acochlidiids differ from their mesopsammic counterparts by their comparatively enormous size and a complex copulatory apparatus (e.g. Wawra 1979, 1980; Haynes and Kenchington 1991; Haase and Wawra 1996). Their circulatory and excretory systems appear to exhibit morphological changes related to the limnic habitat, but are nevertheless similar to the condition already found in the coastal mesopsammic and brackish-water *Pseudunela* species recently examined (Neusser et al. 2009; Neusser and Schrödl 2009). The recent studies using 3D reconstruction have considerably expanded knowledge on acochlidian evolution and biology and because many original studies were unreliable or lacking in important detail, a redescription of selected taxa is needed and is expected to reveal details on anatomical structures not examined so far. This study is the first to use 3D reconstruction to analyse the entire microanatomy of a limnic acochlidiid, and briefly highlights differences from congeners from the South Pacific Solomon Islands and Vanuatu (own unpublished data).

Materials and methods

The 5 mm paratype specimen used for serial sectioning was originally collected by A. Strubell in Batu Gatja stream running through Ambon city, Amboina (Ambon) Island, Moluccas archipelago, Indonesia (Strubell 1892; Küthe 1935) and had been stored in 75% ethanol (Berlin Museum für Naturkunde: ZMB Moll. 90761).

Two further specimens were examined, collected by M.G. in October 2008 close to the type locality on Ambon, Maluku Utara, Indonesia [ZMB Moll. 193.943, from Kemeri (east of Kemeri, stream at road Passo to Liliboi, western part of Leitihu) and ZMB Moll. 193.944, from Watatiri road Passo to Natsepa, eastern part of Leitihu]. The former 4 mm specimen was used for serial sectioning, the latter for scanning electron microscope (SEM) examination of the radula.

Specimens used for serial sectioning were decalcified by an overnight immersion in 10% Bouin's solution and dehydrated in a graded acetone series from 30% to 100%. The specimens were infiltrated first overnight with a mixture of equal parts of epoxy resin and 100% acetone [paratype: Spurr's low viscosity epoxy resin (Spurr 1969); second specimen: Epon], then left to polymerize in pure resin at 70°C for 1 day.

Serial sections of 1.5 μm thickness were made using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) with a rotation microtome (Microm HM 360, Zeiss); contact cement was applied to the lower cutting edge to obtain ribbons of serial sections (Henry 1977; Ruthensteiner 2008). Ribbons collected on microscopy slides were stained with Richardson's stain (methylene-blue/azure II; Richardson et al. 1960) and sealed with Araldite resin (Romeis 1989) and cover slips.

Of the paratype, every second section was photographed through a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany) with a mounted CCD camera using SPOT 3.1 software (Spot Insight, Diagnostic Instruments, Inc., Sterling Heights, MI, USA) and unfiltered bright-field illumination or (slight) phase contrast. For 3D reconstruction, data were downsized to 8-bit grey-scale and 50% resolution, every second photograph was used for the 3D reconstruction with AMIRA 4.1 software (TGS Europe, Mercury Computer Systems, Mérignac, France) resulting in a stack of 532 photographs.

For SEM examination, the radula of the third specimen was removed and macerated in 10% KOH. The cleaned radula was mounted on an SEM stub, sputter coated in gold for 120 seconds (Polaron sputter coater) and viewed in an LEO 1430 VP scanning electron microscope at 15 kV.

Results

General morphology (Figures 1A,B, 2A,B)

Both the examined paratype and the recently collected specimens of *Strubellia paradoxa* display the typical acochlidian bauplan with the projecting posterior visceral sac separated from the anterior head–foot complex, the latter being divided by a lateral cephalopedal groove. In the present fixed specimens the body is contracted in a defensive posture with the head retracted partially into the arched and somewhat flattened visceral sac (Figure 1A). The four pointed head appendages are tucked in between skin folds of the retracted head; the labial tentacles are flattened at their base and connect broadly to form the upper lip, while the more posterodorsal rhinophores appear to be round in cross-section. The contracted broad foot is contained by the convex side of the visceral sac and has several lateral folds.

In living specimens, the anterior foot margin has flaring edges (Figure 2A,B); the tail end is long and free. The rhinophores (with the eyes visible just posterior to their base) are held erect while labial tentacles extend parallel to the substratum. The visceral sac is more or less circular in cross-section with its tip curling slightly towards the right.

Internally, the cavity of the head–foot is separated from that of the visceral sac by a transversal muscular diaphragm (visible in Figure 3A) that is penetrated by the aorta, the oesophagus and the visceral nerve.

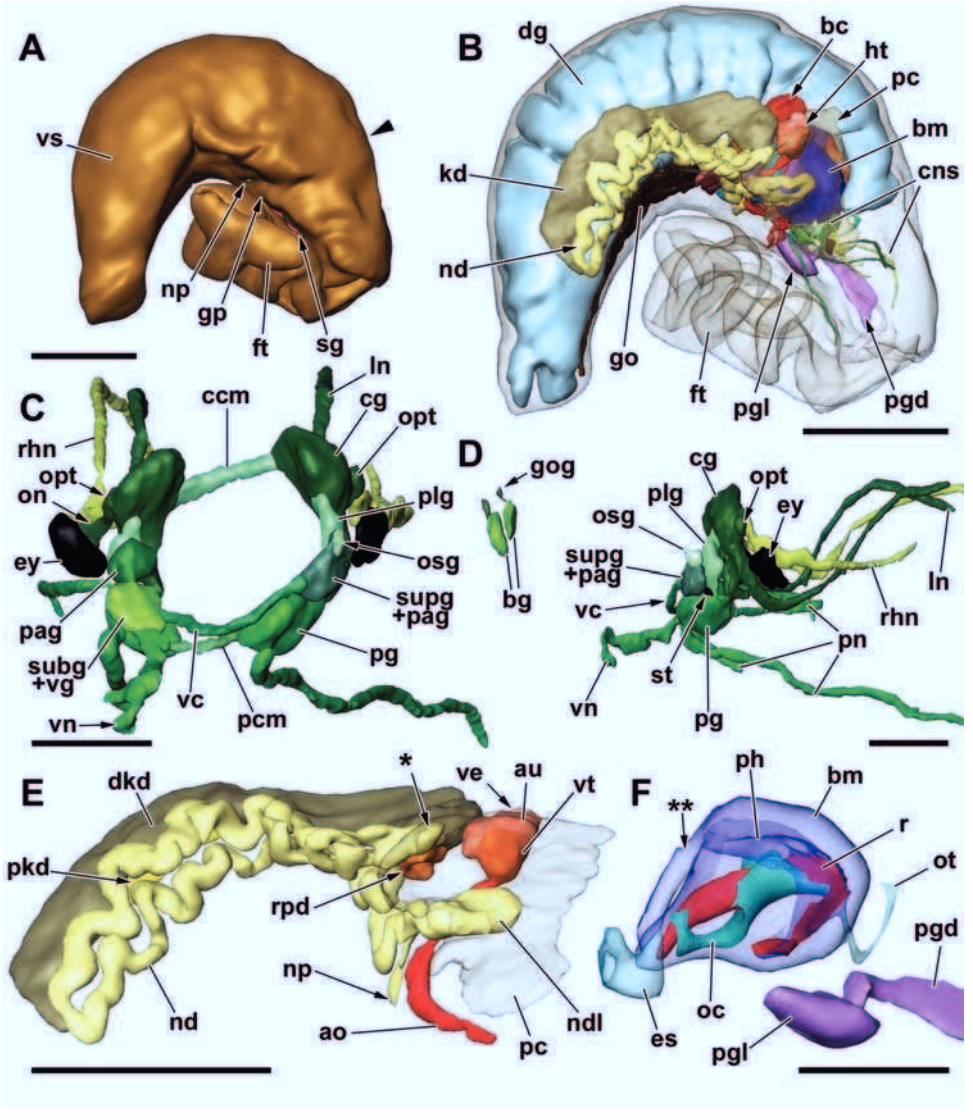

Figure 1. Three-dimensional reconstruction of general anatomy, central nervous (CNS), circulatory, excretory, and anterior digestive systems of *Strubellia paradoxa*, paratype. (A) External morphology (contracted specimen, right view), arrowhead indicates position of diaphragm between head–foot and visceral sac; (B) overview of microanatomy showing internal organ systems; (C) CNS (posterior view, buccal ganglia omitted); (D) CNS, including buccal ganglia at left (right view); (E) excretory and circulatory systems (ventrolateral right view), asterisk indicates intersection between kidney and nephroduct; (F) buccal apparatus and anterior pedal gland, right view, asterisks indicate ciliated part of oesophagus. Abbreviations: ao, aorta; au, auricle; bc, bursa copulatrix; bg, buccal ganglion; bm, buccal mass; ccm, cerebral commissure; cg, cerebral ganglion; cns, central nervous system; dg, digestive gland; dkd, distal lumen of kidney; es, oesophagus; ey, eye; ft, foot; go, gonad; gog, gastro-oesophageal ganglion; gp, genital pore; ht, heart; kd, kidney; ln, labial tentacle nerve; nd, nephroduct; ndl, distal loop of nephroduct; np, position of nephroporus; oc, odontophore cavity; on, optic nerve; opt, optical

Epidermis (Figure 3A,B)

The smooth epidermis consists largely of monocellular glands filled with either pink-staining granules (very common, rounded cells with apical pore) or, more rarely, a smooth or slightly granular blue-staining substance (slender cells, no apical pore detected). The epidermis is thickest on the anterior part of the visceral sac and conspicuously thin and non-glandular along the cephalopedal groove. Apical ciliation is obvious only on the foot sole, which also has interspersed glands of the pink-staining type.

Glandular structures

Numerous clusters of apparent glandular cells are embedded in the connective tissue of the foot (Fig. 3B); those closer to the margin of the foot stain darker and less grainy than those in the middle. Thin ducts connect the clusters to the outside on the ciliated pedal epidermis.

The large anterior pedal gland (Figures 1B, F, 3F) is located anteroventrally to the pharynx and appears to open into a strongly ciliated, invaginated groove of epidermis on the mediodorsal margin of the foot, ventral of the mouth opening. The distal part of the gland is two-lobed and is indistinctly divided into corticular cells (staining grainily dark blue) and more central greyish cells containing tiny blue granules. Anteriorly, both lobes merge.

Musculature

Body musculature consists of fibres that either independently span the body cavities or are closely associated with organs. A thin and more or less regular sheath of radial and longitudinal muscle fibres is located just below the epidermal basal lamina; a similar sheath envelops the entire genital system except for the gonad. Oral tube and oesophagus are lined with longitudinal muscle fibres.

A thin transverse septum of muscles separates the cavities of the visceral sac from the anterior body (Figures 3A, 5C); this diaphragm is punctured medially by the aorta, oesophagus and visceral nerve.

Several distinct muscles are discernable: dorsoventral muscles span the head–foot from the cephalopedal groove to the dorsal body wall; numerous thin fibres span the foot in a dorsoventral fashion, sometimes crossing (Figure 3B). A thick longitudinal muscle (about 90 μm high and 45 μm wide) stretches from close to the mouth opening to the end of the tail. At least three pairs of strong muscles extend from the oral tube to the sides, the first pair close to the mouth, the last pair just in front of the pharynx.

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ganglion; osg, osphradial ganglion; ot, oral tube; pag, parietal ganglion; pc, pericardium; pcm, pedal commissure; pg, pedal ganglion; pgd, pedal gland duct; pgl, anterior pedal gland; ph, pharyngeal cavity; pkd, proximal lumen of kidney; plg, pleural ganglion; pn, pedal nerve; r, radula; rhn, rhinophoral nerve; rpd, renopericardioduct; sg, external seminal groove; st, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vc, long visceral connective between subg+vg and supg+pag; ve, short venous vessel; vn, visceral nerve; vs, visceral sac; vt, ventricle. Scale bars: A, B, F, 1 mm; C, D, 200 μm; E, 1 mm; F, 500 μm.

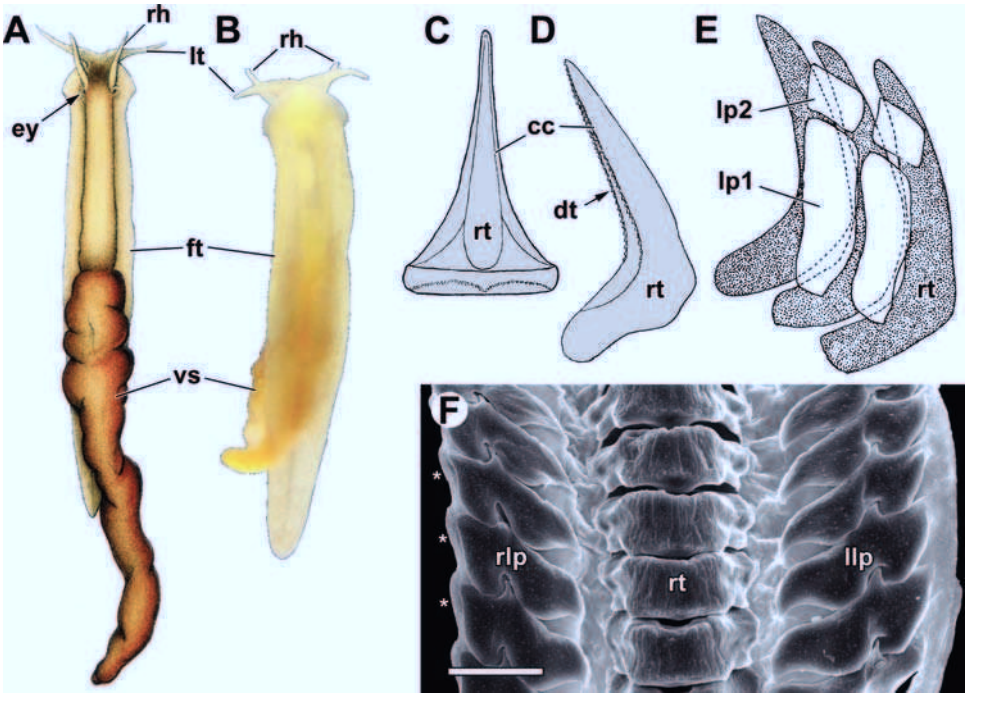

Figure 2. External anatomy and aspects of radula. (A) Dorsal view of live specimen from Batu Gatja River, Ambon; original drawing by A. Strubell, 1892 (modified from Küthe 1935, used with permission); (B) ventral view of live juvenile specimen from Watatiri, Ambon; (C–E) aspects of radula (modified from Küthe 1935, used with permission): (C) anterior view of rhachidian tooth, (D) right view of rhachidian tooth showing denticulate margins; (E) portion of folded radula with lateral plates, note lack of denticle in lp1; (F) scanning electron micrograph of radula of Watatiri specimen, note rhachidian teeth worn down to their bases and strong denticle on each lateral plate, asterisk indicates presumed position of second lateral plate folded down in the sample. Abbreviations: cc, central cusp of rhachidian tooth; dt, denticles; ey, eye; ft, foot; llp, left lateral plate; lp1 and 2, first and second lateral plates; lt, labial tentacles; rh, rhinophore; rlp, (first) right lateral plate; rt, rhachidian tooth. Scale bar: 20 μm.

Connective tissue and spicules (Figure 3B, C, E)

Connective tissue consists of irregularly shaped large cells that stain very light blue. The cells are tightly packed in the foot but form loose aggregates in the rest of the body, notably the flanks of the head–foot complex and the anterolateral visceral sac. The cells are always located between the epidermis and a thin sheet of connective tissue that separates the main cavities of the head–foot and the visceral sac from the peripheral haemocoel underneath the epidermis (Figure 5A, C, D). All major organ systems are located inside this membrane, except for the circulatory and excretory systems on the right side of the visceral sac and the patches of characteristic large-celled connective tissue mentioned above.

Cylindrical spicules are embedded in the entire connective tissue. The bulk of the calcareous spicules' bodies is dissolved, but there is commonly a concentrically layered, supposedly organic residue left in the empty cavities that are surrounded by a thin membrane. This membrane appears to have closely surrounded the spicules in

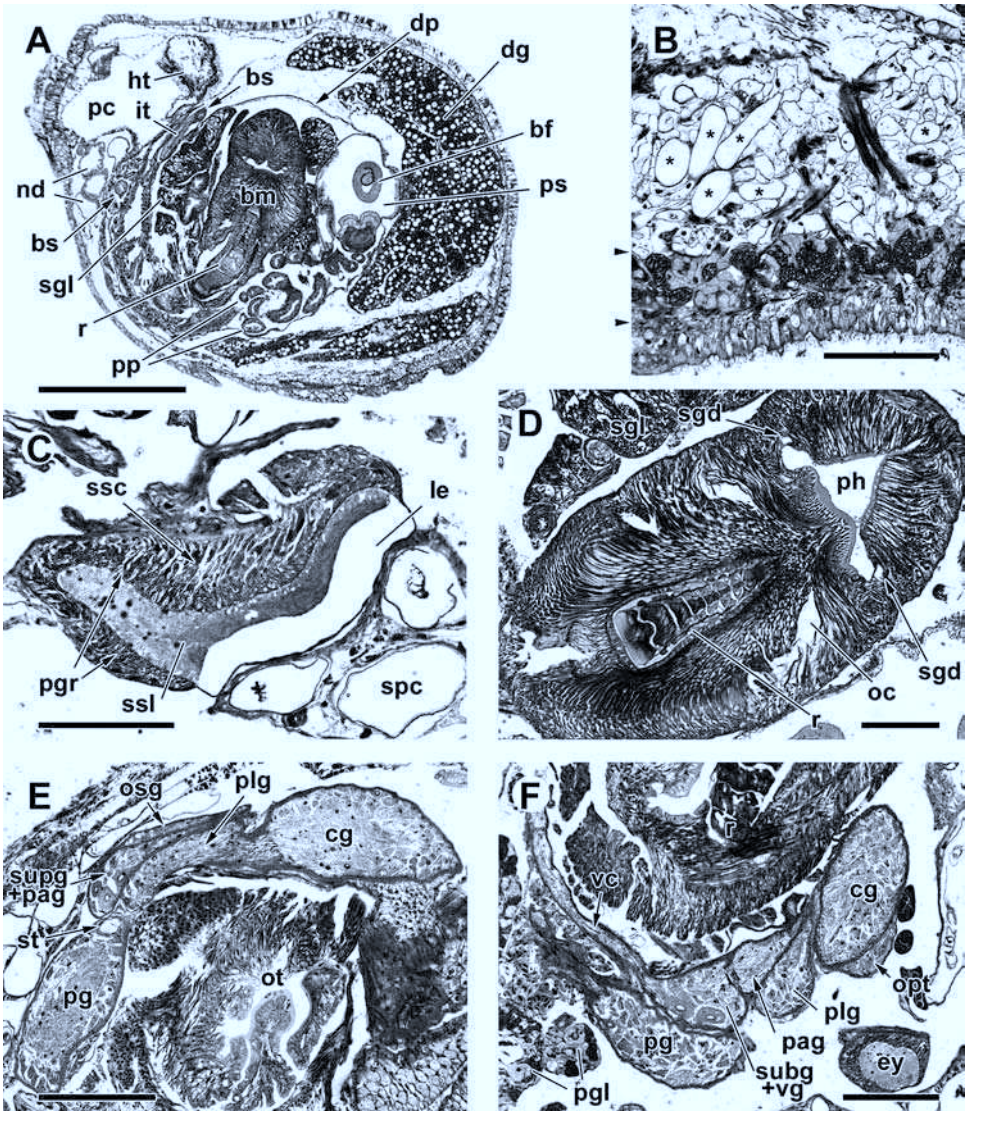

Figure 3. Semi-thin cross-sections of anterior head–foot. (A) Cross-section through body at level of buccal mass (retracted); (B) foot with ciliated sole, subepidermal foot glands (arrowheads indicate upper and lower margins of glandular layer), connective tissue with spicules (asterisks), note crossing muscle fibres; (C) left eye; (D) cross-section through buccal mass with cuticularized pharynx and folded dorsal branch of radula (young branch still separated from pharyngeal cavity); (E) ganglia on the right side of CNS; (F) ganglia on the left side (note giant nerve cells in subg+vg). Abbreviations: bf, basal finger; bs, bursa stalk; bm, buccal mass; cg, cerebral ganglion; dg, digestive gland; dp, diaphragm separating cavities of head–foot and visceral sac; ey, eye; ht, heart; it, intestine; kd, kidney; le, eyelens; nd, nephroduct; oc, odontophore cavity; opt, optic ganglion; osg, tentative osphradial ganglion; ot, oral tube; pag, left parietal ganglion; pc, lumen of pericardium; pg, pedal ganglion; pgl, anterior pedal gland; pgr, granular pigment layer; ph, pharynx; plg, pleural ganglion; pp, loops of paraprostate; ps, lumen of penial sheath; r, radula; sgd, salivary gland duct; sgl, salivary gland; spc, spicules; ssc, sensory cell of eye; ssl, sensory layer; st, statocyst; subg+vg, combined subintestinal and visceral ganglion; supg+pag, combined supraintestinal and right parietal ganglion; vc, long connective between subg+vg and supg+pag. Scale bars: A, 500 μm; B, C, 50 μm; D–F, 100 μm.

the living animal. The resulting cavities are straight or slightly curved and round in cross-section with smoothly rounded tips. Spicule size can be estimated by measuring the empty cavities, which are maximally between 20 and 150 μm long and 5 to 40 μm wide. The spicules are most numerous in the foot (50–90 μm long, 20–25 μm wide), where they are distributed irregularly. Thin spicules (90–135 μm long, 25 μm wide) are present inside each head appendage, sorted longitudinally. In the visceral sac, spicules of normal size are found only in the connective tissue of the anterior flanks, whereas only few and very small spicules (20 × 5 μm) appear in the rest of the visceral sac. The largest spicules (150 μm long, 40 μm thick) are located dorsolateral to the central nervous system and buccal mass (Figure 3C, E); they appear to be inside the longitudinal connective tissue membrane, as opposed to all other spicules.

Digestive system (Figures 1F, 2F, 3A, D)

The digestive system of *S. paradoxa* resembles closely the pattern found in other acochlidian species. The laterally flattened oral tube, flanked by radiating bundles of muscle fibres and clusters of small glands resembling those found in the epidermis, leads into the cuticle-lined pharynx; jaws are absent. The large, bulbous pharynx consists of an egg-shaped (approximately 850 μm long, 630 μm wide) and compact mass of buccal muscles (Figures 1F, 3D). The muscle fibres stain strongly blue; they expand radially from the pharyngeal cavity; ventrally, the fibres are oriented in longitudinal and oblique bundles.

The hook-shaped radula lies in the posteroventral part of the buccal mass (Figure 1F). It consists of a column of rhachidian teeth with a rectangular base (60 μm wide and about 18 μm long) and a very slender and pointed central cusp. Flat lateral plates fold onto the rhachidian teeth along the proximal branch of the radular ribbon where vacuolate cells embed the base and teeth. The ribbon's distal branch (about one-third of its total length) is bent down and backwards (Figure 1F) with the lateral plates spread open and the median teeth projecting freely into the pharyngeal cavity.

The radula examined via SEM has 38 rows with three teeth each (see Discussion); each tooth is equipped with denticles facing the next younger teeth in the ribbon. The rhachidian teeth consist of a very pointed and triangular central cusp with approximately 26–30 small denticles on each margin in the younger teeth (as in Figure 2D), whereas in the recurving older branch they are worn down to the rectangular base with no denticles left (Figure 2F). The lateral plates are largely rectangular with rounded corners, each plate has one strong and slightly curved denticle fitting into a notch in the border of the neighbouring younger tooth. Although the left lateral plates have a rounded and convex outer margin, it is comparatively straight and slightly concave in the right lateral plates.

The pharyngeal cavity anteriorly and dorsally to the radula is divided into three longitudinal folds. In cross-section, the pharyngeal cavity is shaped like a three-pointed star (Figure 3D); the salivary ducts open into the left and right fold, and the upper fold houses the projecting rhachidian teeth in its anterior part. The pharyngeal cavity is lined with a cuticle up to 25 μm thick that stains homogeneously light blue. It extends from the radular membrane underneath the base of the median teeth and is thickest dorsally to the pointed rhachidian teeth. Ventrally and laterally, the radula is

supported by an independent fluid-filled sinus within the buccal muscle (odontophore cavity in Figures 1F, 3D).

The large mass of paired salivary glands surrounds the posterior third of the buccal muscle and reaches posteriorly to the digestive gland. The glandular mass stains dark blue with large, elongate cells projecting radially from a (collapsed) central lumen. The salivary glands open laterally into the posterodorsal buccal cavity via paired thin ducts (Figure 3D).

The oesophagus projects from the posterodorsal part of the buccal mass. It is thicker than the oral tube with the wall being more muscular. It is ciliated only in the most anterior part following the pharyngeal cavity (double asterisk in Figure 1F). Posteriorly, the oesophagus widens and connects to the digestive gland at the anteroventral left of the visceral sac. The distal part of the oesophagus gradually turns more glandular and is lined with colourless cells similar to the cells of the digestive gland but lacking blue-stained vesicles. There is no anatomically distinct stomach.

The digestive gland is by far the largest organ of the examined specimens and is shaped like an elongate sac (Figure 1B). It fills most of the visceral sac except for the anterior right part (the position of the pericardium and kidney) and a small ventral part that is occupied by the gonad. The gland's surface has irregular and transverse folds, with the outer glandular cortex forming most of gland's volume, and a mostly collapsed central lumen. The cortex consists of large glandular cells situated in lobes that create an irregular inner surface. The loosely packed cortical cells stain pale but are filled with innumerate small, dark blue-staining spherical vesicles (1.5–7 μm diameter) and, more conspicuously, numerous unstained spherical vesicles (10–20 μm diameter) evenly distributed inside the glandular lobes (Figure 3A). Inside the lumen of the digestive gland there are large patches of non-cellular, dark-blue staining material that contains a few clumps of round, blue-stained cells, apparently remnants of food.

On the anterior right of the visceral sac, the digestive gland connects to the short but thick intestine which runs anteroventrally. The muscular intestine consists of a longitudinally folded wall of high (30 μm) epithelial lining with strong apical ciliation (Figure 5E). Inside, there is blue-staining material similar to the contents of the digestive gland. The intestine appears to terminate close to the distal nephroduct, whereas the anus itself was not detectable, probably as a result of contraction of the specimens (see Figure 5C, E).

Central nervous system and sense organs (Figures 1C, D, 3E, F, 4)

The central nervous system of *S. paradoxa* is euthyneurous and slightly epiathroid, i.e. the cerebropleural connective is slightly shorter than the pleuropedal connective. There are 18 ganglia: the paired cerebral, pedal, optic, pleural, rhinophoral, buccal and gastro-oesophageal ganglia and three unpaired ganglia on the visceral nerve cord plus an additional unpaired one (Figure 4); nomenclature of the ganglia herein follows Haszprunar (1985) and Sommerfeldt and Schrödl (2005). Except for the buccal and gastro-oesophageal ganglia, all ganglia are located more or less prepharyngeally, and the visceral cord runs ventrally to the anterior buccal mass. All ganglia are enclosed by a darker blue stained sheath of connective tissue (Figure 3E, F). The ganglia themselves are separated into an outer cortex of mostly somewhat shrunken cell bodies (with the nucleus sometimes well visible as a lighter, unstained sphere and a dark blue

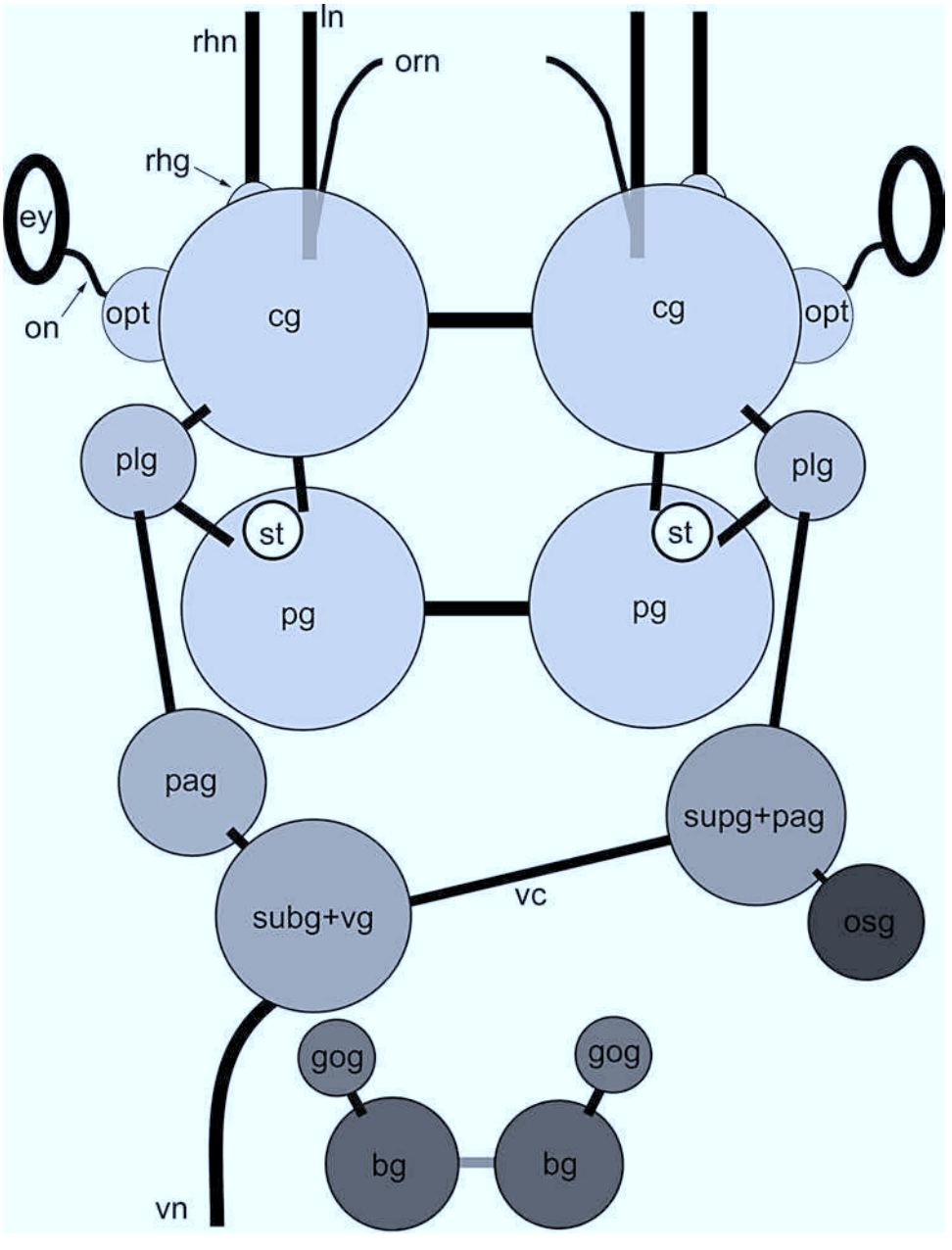

Figure 4. Schematic overview of the central nervous system, dorsal view. Pedal nerves omitted and length of pleuroparietal connectives exaggerated for reasons of visualization. Buccal commissure and cerebrobuccal connective not found. Abbreviations: bg, buccal ganglion; cg, cerebral ganglion; ey, eye; gog, gastro-oesophageal ganglion; ln, labial tentacle nerve; on, optic nerve; orn, oral nerve; opt, optical ganglion; osg, tentative osphradial ganglion; pag, parietal ganglion; pg, pedal ganglion; plg, pleural ganglion; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; st, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vc, long visceral connective between subg+vg and supg+pag; vg, visceral ganglion; vn, visceral nerve.

nucleolus), and the interior medulla that does not contain cell bodies and stains more or less homogeneously light blue to grey. The nerves, connectives and commissures lack cell bodies and so resemble the medulla in histology.

All ganglia except the cerebral ganglia have several conspicuous giant nerve cells; in the paratype these are best visible in the subintestinal/visceral ganglion (with two or three with 30 × 18 μm diameter and large nucleus, Figure 3F), and less distinct in the pedal ganglia.

The prepharyngeal nerve ring is formed by three pairs of ganglia: the cerebral, pedal ganglia and smaller pleural ganglia. The large and oval cerebral ganglia (180 μm high, 90 μm wide) are the most anterodorsal ganglia. They are connected by a stretched cerebral commissure of about 200 μm length and 30 μm diameter (Figure 1C). The thick (30 μm) rhinophoral nerve emerges from the ventrally attached elongate rhinophoral ganglion (this is regarded as true ganglion here although it appears to lack a clear separation into cortex and medulla). The equally thick labial nerve emerges from each cerebral ganglion ventrally and bifurcates shortly after; the lateral branch runs into the labial tentacles. A small cap-like optic ganglion (60 × 30 μm) sits laterally on each cerebral ganglion; it is surrounded by a separate sheath of connective tissue (Figure 3F) and connects to the eyes via a thin, rather long (55 μm) optic nerve. A nervous connection between the optical and the cerebral ganglia could not be detected.

The pigment-cup eyes are located anterolaterally to the cerebral ganglia (Figure 1D). They are elongate and bean-shaped and about 130 μm long and 90 μm thick. The cup consists of two distinct layers; the outer layer consists of light blue-staining cells, the inner layer is filled with an exterior grainy dark pigment and a layer of blue-stained material (Figure 3C, F). The inside of the cup is filled with a clear, unstained lens that is covered with a thin layer of flattened cells over the anterodistal and anterolateral opening of the cup.

The large pedal ganglion (170 × 100 μm) is connected to the cerebral ganglion posteroventrally by a cerebropedal connective about 120 μm long. The pedal commissure is about 130 μm long (Figure 1C), running ventrally of the oral tube. Four large, paired nerves emerge from the pedal ganglion laterally and posteroventrally and extend towards the flanks and the foot. A spherical statocyst (diameter 24 μm) is embedded mediodorsally in each pedal ganglion close to the cerebropedal connective (Figure 1D); the statocysts consist of a single layer of flat cells surrounding the central spherical and optically empty lumen (Figure 3E). A static nerve could not be detected. The small pleural ganglion (70–90 by 30–40 μm) is located posteriorly beneath the cerebral ganglion and is connected to the latter by a very short (25 μm) cerebropleural connective. Posteriorly, each pleural ganglion connects to the visceral loop via a short connective. There are three medium-sized to large ganglia on the visceral loop, plus an additional small one connecting only to the very right one (Figure 1C, D). Beginning on the left side, there is the medium-sized left parietal ganglion (70 × 80 μm). Via a very short connective, this ganglion connects to the large fused subintestinal/visceral ganglion (100 × 90 μm) located to the left of the midline. The very thick visceral nerve (approximately 40 μm) emerges from the subintestinal/visceral ganglion and extends posteriorly inside a muscular sheath; this nerve can be tracked to the left side of the gonad. The subintestinal/visceral ganglion joins to the large fused right parietal/supraintestinal ganglion situated on the right via a long, thin transverse connective (approximately 250 μm long, 16 μm thick)

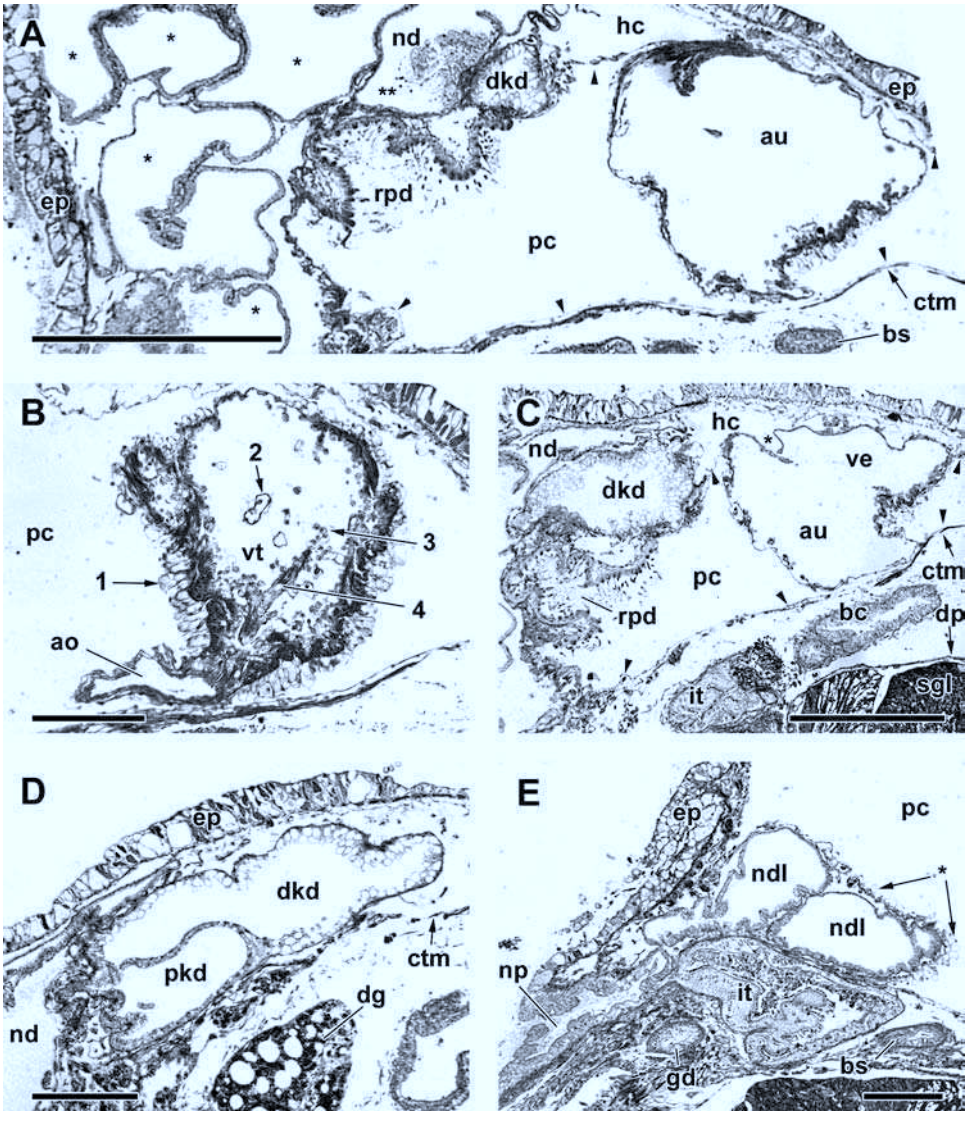

Figure 5. Semithin sections of anterior right visceral sac with the pericardium, heart and kidney; dorsal side to the upper right. (A) Overview of anterior right visceral sac with pericardial complex (arrowheads indicate membrane of pericardium, asterisks indicate loops of nephroduct, ** indicates ciliary tuft at intersection between distal kidney and nephroduct); (B) section more anterior to A showing medioventral tip of heart with protruding aorta, (1) vacuolate epicardium, (2) tentative "rhogocyte", (3) loose cells and (4) muscular bridge inside the ventricular lumen; (C) section posterior to A with kidney and heart at base of renopericardioduct, asterisk marks thickened margins of venous vessel opening to the haemocoel; (D) kidney posterior toheart showing proximal and vacuolate distal lumina; (E) most anterior portion of excretory system with nephroduct loop proximal to nephroporus, vacuolate cells inside pericardium (asterisk) and distalmost intestine. Abbreviations: ao, aorta; au, lumen of auricle; bc, bursa copulatrix; bs, bursa stalk; ctm, connective tissue membrane; dg, digestive gland; dkd, distal lumen of kidney; dp, diaphragm separating body cavities; ep, epidermis; gd, gonoduct; hc, haemocoel

(Figures 1C, 3F). The right parietal/supraintestinal ganglion connects to two further ganglia: anteriorly to the right pleural ganglion via a short (pleuroparietal) connective (closing the visceral loop) and posterodorsally to the small, cap-like "osphradial" ganglion (following Huber 1993) (Figures 1C, D, 3E). There are no detectable nerves leaving the latter two. Giant nerve cells are detectable in all ganglia of the visceral cord.

Posteriorly to the buccal mass, there are the paired and medium-sized buccal ganglia (90 × 60 μm) (Figure 1D); a buccal commissure could not be detected. A small gastro-oesophageal ganglion (50 × 25 μm) is connected dorsally to each buccal ganglion.

Circulatory system (Figures 1E; 5A–C)

The bulbous heart lies in the dorsal part of the thin-walled, spacious pericardium (Figure 5A), which itself fills much of the anterior right of the visceral sac (Figure 1B). The heart consists of a thin-walled auricle and a muscular ventricle; both are oriented on an axis running from dorsal right to ventral left.

The visceral haemocoel between epidermis and diaphragm opens into the single venous vessel on the posterodorsal side of the heart (Figure 1E). The vessel consists only of a short (about 100 μm) and broad projection of the auricular wall, with the margins slightly thickened in comparison to its otherwise thin wall (Figure 5C). The spacious auricle is characterized by a thin muscular wall that continues smoothly into the ventricular walls (Figure 5A,B); the ventricle is located more to the medioventral left. It is a conspicuous thick-walled muscular bulb and about 160 μm long and 230 μm wide. No valve could be detected separating auricle and ventricle, but there are a few muscular bridges spanning the lumen of the ventricle (Figure 5B). Conspicuous elongate cells (15 × 6 μm) with a strongly refracting, dark-bordered vacuole somewhat resembling a spicule are found separate and free inside the lumen of the heart, most numerously in the ventricle and also embedded in its wall. Between this vacuole and the cell wall there is a grainy material staining brown to yellow; some of these cells are also found in the connective tissue surrounding the kidney. Furthermore, there are small spherical blue cells in the lumen, mainly between the muscular bridges inside the ventricle. The outer surface of the ventricle is covered with a dense layer of conspicuously vacuolated high cells. These cells contain a large vacuole that stains light blue; the nucleus is mostly flattened and located laterally or apically. The anteromedian tip of the ventricle leads into the aorta, a vessel with a large lumen and a muscular wall. The aorta exits the pericardium in an anteroventral direction (Figure 1E) and penetrates the diaphragm to the cavity of the head–foot. There it runs anteriorly towards the ventral side of the buccal mass, where it is no longer detectable.

The spacious pericardium fills a large portion of the anterior right visceral sac located dorsally to the anterior end of the kidney and to the right of the intestine. The pericardium envelops all of the heart except for the small dorsal part with

at right body side; it, intestine; nd, nephroduct; ndl, distal nephroduct loop; np, nephroporus; pc, lumen of pericardium; pkd, proximal lumen of kidney; rpd, funnel of renopericardioduct; sgl, salivary gland; ve, venous vessel; vt, lumen of ventricle. Scale bars: A, C, 200 μm; B, D, E, 100 μm.

the venous opening; medioventrally, the thin-walled pericardium is punctured by the aorta. The renopericardioduct exits from the pericardium posteroventrally to the heart (Figure 1E, 5A, C). Anteroventrally, the pericardium is in close contact with a distal loop of the renal tube (Figure 1E); there, the inside of the pericardium is lined with a layer of vacuolated cells resembling those on the outside of the ventricle but that are shaped more irregularly (asterisk in Figure 5E). There are no further cells found in the lumen of the pericardium.

Excretory system (Figures 1E, 5)

The excretory system is located posteroventrally to the pericardium. It begins posteroventrally to the heart, with the short renopericardioduct connecting the pericardial lumen to the kidney (Figure 1E). The renopericardioduct consists of an about 170 μm wide and longitudinally folded funnel that is lined with cuboidal cells from which bundles of conspicuous and large cilia emerge (Figure 5A, C). The cilia-bearing cells with a diameter of about 10 μm are rounded basally; the bundles of cilia originate close to the central large nucleus. Some sections have cilia reaching far into the pericardium; however, the general orientation of the very long (30 μm) cilia is down the renopericardial funnel towards the kidney.

The kidney extends along three-quarters of the visceral sac (Figure 1E); internally, the kidney is divided into two separate lumina by a longitudinal epithelial wall (Figure 5D). The lumina connect only in the posterior part of the kidney, forming an anterior–posterior loop. The proximal and more ventral part of this loop is lined with simple blue-staining cells, whereas the distal and dorsal lumen is more voluminous and has a conspicuously vacuolated cellular lining (25 μm high, 5–6 μm wide) with a basal nucleus and a bulbous, clear apical vacuole (Figure 5C, D). The vacuolated cells appear to lie above each other in a layer up to 40 μm thick, giving the wall a "spongy" appearance. This second part accounts for much of the kidney's volume. The distalmost and anterior end of the kidney leads into the nephroduct via a short, curved passage of about 60 μm diameter. On the inside of its bend, this connecting part is lined with a small patch of densely ciliated cells (double asterisk in Figure 5A).

The nephroduct is located ventral to the kidney and consists of an undulated tube that extends back and forth in parallel (Figures 1E, 5A). The nephroduct wall is lined with cuboidal, light blue-staining cells with darker nuclei and is between 3 and 9 μm thick. There are only sparsely distributed apical vacuoles projecting into the otherwise smooth renal tube lumen. Apical ciliation is not detectable.

The distal part of the nephroduct forms an anterodorsal loop embedded in a fold of the pericardium that is internally lined with vacuolate cells only in this place (see circulatory system; Figures 1E, 5E). The last part of the nephroduct is separated from the nephropore by a slight constriction. This distalmost portion is strongly ciliated and displays a few small pink glands resembling those commonly found in the epidermis.

Genital system (Figures 6–8)

The paratype specimen used for 3D reconstruction appears functionally male (except for a lack of mature sperm), with two apparent allosperm receptacles present in the posterior part of the genital system; nomenclature follows Ghiselin (1966),

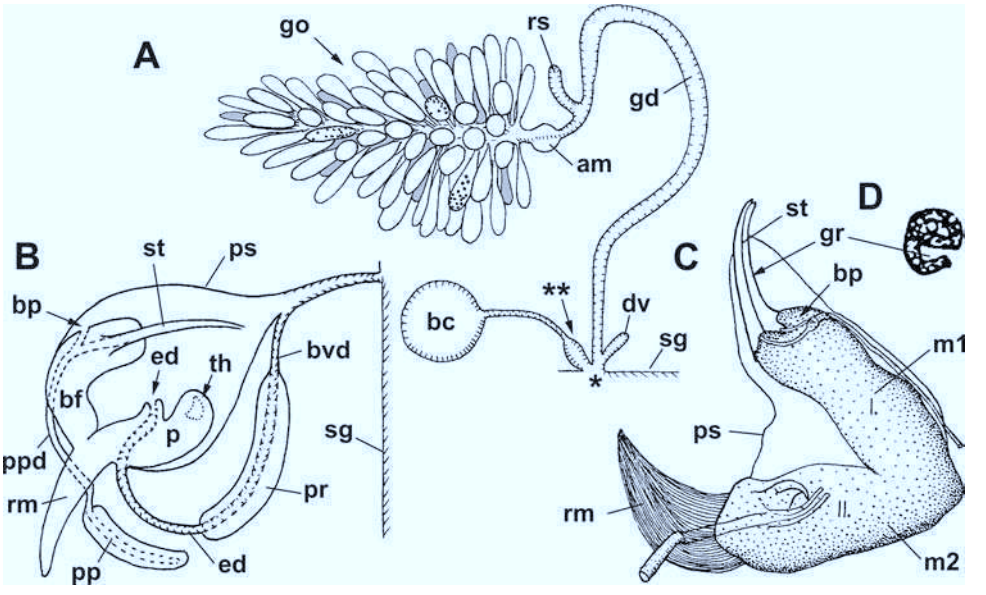

Figure 6. Schematic overview of the genital system, male phase. (A) Overview of posterior genital system of paratype, * indicates genital pore, ** indicates widening in distal bursa stalk, dorsal view; (B) overview of cephalic copulatory apparatus of paratype, dorsal view; (C, D) adapted from Küthe (1935), used with permission: (C) copulatory apparatus with anterior muscle (showing external duct with distal bifurcation and bypass leading into lumen and groove of the stylet) and posterior muscle (with duct passing through the retractor muscle and exiting through a papilla close to a hooked thorn); (D) cross-section of stylet showing lumen and groove. Abbreviations: am, ampulla; bc, bursa copulatrix; bf, basal finger; bp, bypass of paraprostatic duct; bvd, "posterior-leading" vas deferens; dv, diverticle; ed, ejaculatory duct on raised papilla; gd, gonoduct; go, gonad; gr, groove of cuticular stylet; m1, "anterior" muscle, confused with the penis by Küthe; m2, "horseshoe-shaped posterior" muscle; p, penis; pp, paraprostate; ppd, paraprostatic duct; pr, prostate; ps, penial sheath; rm, retractor muscle of copulatory apparatus; rs, receptaculum seminis; sg, external sperm groove on right body side; st, hollow stylet; th, thorn.

Klussmann-Kolb (2001) and Neusser and Schrödl (2007). The more recently collected specimens appear to be juvenile.

Posterior genital system (Figures 6A, 7A–D)

The posterior genital system consists of the acinar gonad, from which the gonoduct extends to the genital opening in a wide loop. The genital opening is located on the right anteroventral side of the visceral sac, slightly anterior to the renal and anal opening.

The gonad, located beneath the digestive gland and extending along two-thirds of the visceral sac, is formed by numerous oval acini extending mostly laterally from a central lumen (Figure 6A, 7B,D: acini not labelled singly in 3D reconstruction). The epithelial walls and the lumina of the acini are built up by numerous small, round cells (2–6 μm) stained strongly blue; the central lumen is ciliated along its ventral wall

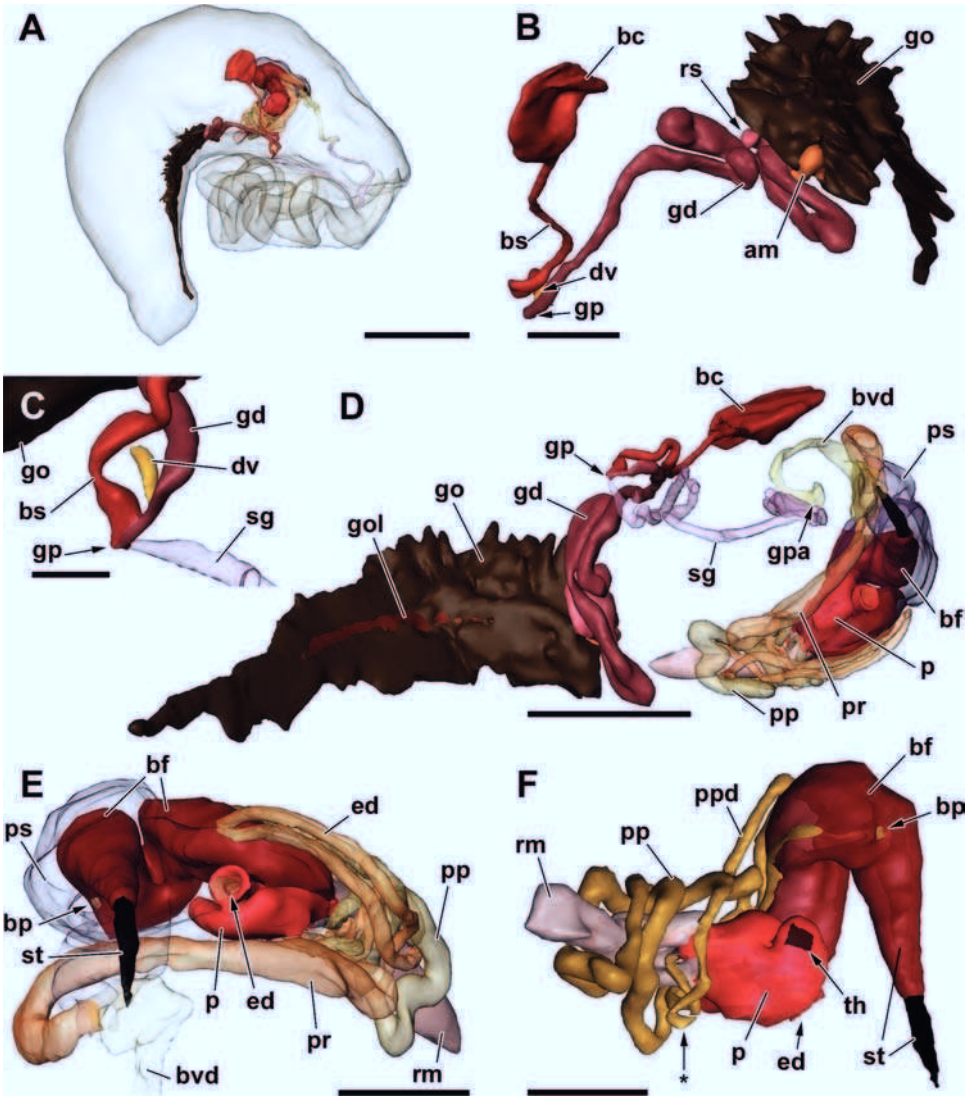

Figure 7. Three-dimensional reconstruction of the genital system, male phase. (A) Genital system (right view); (B) posterior genital system (oblique anterior view); (C) detail of distal gonoduct and genital opening (right view); (D) complete genital system (ventral view); (E) copulatory apparatus (anteroventral view); (F) copulatory apparatus (right view, * indicates blind ending of paraprostate; prostate and penial sheath omitted). Abbreviations: am, ampulla; bc, bursa copulatrix; bf, basal finger; bp, bypass of paraprostatic duct; bs, bursa stalk; bvd, "posterior-leading" vas deferens; dv, diverticle; ed, ejaculatory duct; gd, gonoduct; go, gonad; gol, central lumen of gonad; gp, genital pore; gpa, anterior genital opening; p, penis; pp, paraprostate; ppd, paraprostatic duct, pr, prostate; ps, penial sheath; rm, retractor muscle; rs, receptaculum seminis; sg, external sperm groove; st, stylet; th, thorn. Scale bars: A, 1 mm; B, D–F, 250 μm; C, 100 μm.

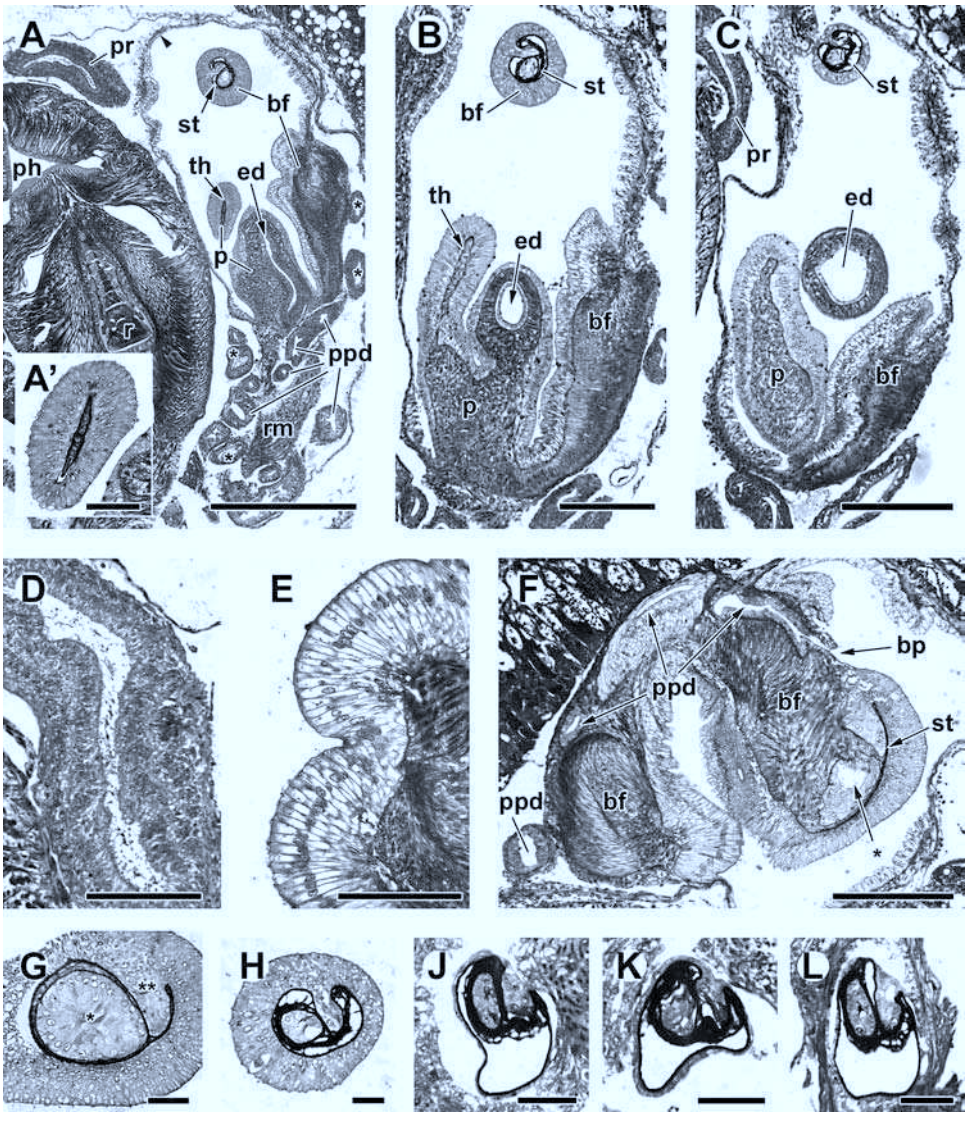

Figure 8. Semi-thin sections showing aspects of the cephalic copulatory apparatus, male phase. (A) Buccal mass and copulatory apparatus, arrowhead, opening direction of penial sheath, asterisks, ciliated loops of prostatic duct, note paraprostatic duct passing through retractor muscle (anterior view, dorsal side is up); (A') tip of penis with chitinous thorn; (B, C) more anterior sections showing relation of penis and basal finger, note widening of ejaculatory duct; (D) prostate; (E) regular epithelium covering the basal finger; (F) curved base of basal finger showing course of distal paraprostatic duct, note bypass to lumen of penial sheath, open base of stylet, asterisk indicates lumen inside stylet that will connect to paraprostatic duct; (G) stylet showing lumen filled with epithelial cells (*) and outer groove (**); (H–L) series of sections through stylet, L closest to tip. Abbreviations: bf, basal finger; bp, bypass of paraprostatic duct into lumen of penial sheath; ed, ejaculatory duct; p, penis; ph, pharynx; pr, prostate; ppd, paraprostatic duct; r, radula; rm, retractor muscle of copulatory apparatus; st, stylet of basal finger; th, thorn of penis tip. Scale bars: A, 250 μm; B, C, F, 100 μm; D, E, 50 μm; A', G, H, J–L, 25 μm.

and folded transversally. Tetrads of cells obviously in a meiotic stage are observable in places; however, no mature sperm cells could be detected.

In the gonoduct following the most distal acini, there is a sparsely ciliated bulbous widening (diameter 80 μm) continuous with the ciliation along the ventral gonad lumen (Figures 6A, 7B: am); judging from position and form, this thin-walled bulb appears to be an ampulla.

The ampulla is followed by the long gonoduct, which is characterized by a thicker epithelium of slightly glandular appearance and a surrounding muscular sheath. Closely following the ampulla, a blind, curved tube of about 220 μm length emerges from the side of the gonoduct (Figures 6A, 7B). It appears to be a receptaculum seminis, but is not histologically separable from the gonoduct: the lumen is ciliated internally; externally it is surrounded by a thin layer of muscle.

Distally, the gonoduct runs to the left before forming a horizontal loop and arching widely to the ventral genital opening on the right side. The proximal part of the gonoduct is slightly thicker than the distal part leading to the genital opening. The genital opening is surrounded by a mass of muscle fibres. Externally, it is raised only slightly above the normal level of the epidermis. Besides the distal gonoduct, there are two additional tubes connected to the genital opening, both are lined with a ciliated epithelium of 5 to 9 μm thickness. First, a short and blind tube (90 μm long, 20 μm thick) emerges from the part of the gonoduct close to the genital opening. This "genital diverticulum" does not extend out of the mesh of muscle fibres surrounding the genital opening (Figures 6A, 7B, C). Posterior to the diverticulum, another ciliated tube runs posterodorsally. This tube is about 600 μm long, begins with a slight widening (Figure 6A) and ends in a large and ciliated flat bulb (Figures 4C, 7B) of about 380 μm length and 350 μm height, located rather dorsally between the heart and digestive gland (Figures 3A, 4C). Judging from its position, this bulb is a flattened bursa copulatrix, although it is empty in the examined specimen.

The sperm groove, which is a 25 μm deep ciliated furrow overhung by a longitudinal rim of raised epidermis, extends from the genital opening to the right rhinophore. The groove is positioned inside the cephalopedal groove and connects the genital opening to the base of the right rhinophore, from where a thin duct leads into the penial sheath, which contains the cephalic copulatory apparatus.

Cephalic copulatory apparatus (Figures 6B, C, 7E, F, 8)

The cephalic copulatory apparatus is a complex organ consisting of two large, curved muscles (penis and basal finger) that are connected basally. The apparatus is retracted into the thin-walled penial sheath, which is located left of the buccal mass and posterior to the central nervous system in its retracted state. A strong, cylindrical retractor muscle – continuous with the penis – runs from the base of the penial sheath to the ventral midline of the body; the open anterior end of the penial sheath connects to the base of the right rhinophore and the anterior end of the sperm groove via a single duct that runs obliquely over the cerebral commissure. From the distal end of this duct, the ciliated "posterior-leading" vas deferens splits off and leads towards the base of the penis, continuing as the ciliated and slightly glandular prostate (approximately 700 μm long and 100 μm thick) (Figures 7E, 8A). Following the prostate, the ejaculatory duct enters the curved penial muscle and exits through a trumpet-shaped papilla on the convex side (Figures 6B, 7E, 8B, C). The tip of the penis is equipped with a flat

and apparently chitinous thorn about 50 μm wide (Figures 7E, 8A') which does not protrude from the muscle in the retracted state.

Splitting from the base of the penis is the even larger and strongly curved basal finger, a separate muscle that is equipped with a 600 μm long and slightly curved chitinous stylet that projects apically (Figure 7E). The stylet is hollow and opens at the tip; a cuticular groove runs along the side of the stylet giving it a shape resembling the letter "e" in cross-section (Figure 8G, H). The base of the stylet is approximately 90 μm wide, the tip only about 30 μm. The lumen of the stylet, filled with loose epithelial cells (Figure 8G), is connected to the paraprostate, another glandular tube (approximately 30 μm thick) located basal of the penial sheath. In contrast to the prostate, the paraprostate is not ciliated interiorly and ends blindly (see asterisk in Figure 7F). Proximally, the paraprostate (and also the ejaculatory duct) loops around the retractor muscle of the penial sheath; the paraprostatic duct passes through the muscle before entering the basal finger (Figures 6B, 7E, F; 8F). Just before leading into the hollow stylet, the distal paraprostatic duct divides; a short (about 30 μm), thin bypass leads directly into the lumen of the penial sheath on the side facing the groove of the stylet (Figures 6B, 7E, 8F).

The penial sheath itself is formed by an invagination of epithelium surrounded by a layer of muscle. The wrinkly inner lining is formed by bright blue, small (6–20 μm high) and closely stacked cells without apical ciliation (see Figure 8B, C). Both penis and basal finger muscles are covered with the same epithelial layer, although the lining of penis and basal finger differs (from the lining of the wall) in being a lot thicker (up to 40 μm high) and in having a very smooth surface. The cells are also stacked regularly and stain light blue, with darker nuclei sorted along an equatorial plane (Figure 8E). A thinner layer of irregularly shaped epithelial cells covers much of the stylet's length, also appearing to fill the lumen of the stylet (Figure 8G–L). The penial thorn is completely embedded in the epithelium, beneath its basal laminae.

Remarks on taxonomy

Order **ACOCHLIDIA**: **Hedylopsacea** *sensu* Wawra, 1987
Family **ACOCHLIDIIDAE** *sensu* Arnaud et al., 1986; Schrödl and Neusser, 2010
Genus ***Strubellia*** Odhner, 1937
***Strubellia paradoxa* (Strubell, 1892)**

Acochlidium paradoxum Strubell, 1892: Verhandl. naturh. Verein preuss. Rheinlande, 48. Jahrg., Sitzung d. niederrhein. Ges. 13. Juni 1892: 62
Acochlidium paradoxum Küthe 1935: Zool. Jahrb. Syst. 66: 513–540
Strubellia paradoxa Odhner 1937: Zool. Anz. 120: 237–238

The bipartite copulatory apparatus and complex kidney identify *S. paradoxa* as a member of the hedylopsacean Acochlidia, (Wawra 1987) whereas the limnic habitat separates it from the smaller but otherwise rather similar *Pseudunela* species. *Strubellia paradoxa* differs from the likewise limnic *Acochlidium* species in the following characters: a uniformly coloured body with an elongate visceral sac more or less round in cross-section, very slender median cusps of the rhachidian teeth, a copulatory apparatus with only a single penial spine and a stylet-bearing basal finger that is larger

than the penis, and in being a sequential hermaphrodite (see Wawra 1988; Neusser and Schrödl 2009; Schrödl and Neusser 2010 for discussion).

Strubellia paradoxa differs from *Strubellia* "*paradoxa*" *sensu* Wawra (1974, 1988) from the Solomon Islands and, apparently, Vanuatu (mentioned in Haynes 2000; own unpublished data) in the length and form of the basal finger's stylet: in *S. paradoxa*, the stylet is approximately 0.5–0.6 mm long [measured from Küthe (1935) and 3D reconstruction; see Figures 6C, 7E], has a continuous curve and is rather stout compared with that of *Strubellia* from the Solomon islands. There, it is approximately 1 mm long, slender and has a hooked tip (Wawra 1974: fig. 4).

We are currently processing specimens for DNA analysis to determine whether there are genetic differences between the Ambon, Solomon Islands and Vanuatu populations.

Discussion

The results of our 3D reconstruction of the type material of *Strubellia paradoxa* supplement and correct the original description by Küthe (1935). Important details of the nervous, genital, circulatory and excretory systems are comparatively discussed, and several novel features for Acochlidia are recognized and their potential functions are inferred.

Central nervous system

Küthe (1935) described the cerebral nerve ring as comprising only four paired ganglia: the paired cerebral, pedal and pleural ganglia and additional paired "visceral ganglia" posterior to the pharynx. Küthe further found a connective between the "visceral ganglia" ventrally of the oesophagus, but he did not find a connection to the anterior ganglia. These "visceral ganglia" presumably refer to the buccal ganglia [as already discussed by Wawra (1988)] which Küthe explicitly stated to be missing, while the visceral cord itself remained undetected.

Our results show that the general organization of the central nervous system of *S. paradoxa* broadly resembles that of most other Acochlidia and include the following: no "accessory" precerebral ganglia (as defined in Neusser et al. 2006) but a pair of optic and rhinophoral ganglia attached to the cerebral ganglia, three large to medium-sized ganglia on the visceral cord with an additional osphradial ganglion on the right side, and two pairs of small ganglia posterior to the buccal apparatus (buccal and gastro-oesophageal ganglia). This general condition resembles closely that described for the hedylopsacean *Hedylopsis ballantinei* Sommerfeldt and Schrödl, 2005, *Pseudunela espiritusanta* Neusser and Schrödl, 2009 and *Pseudunela cornuta* (Challis, 1970) (see Neusser et al. 2009; Neusser and Schrödl 2009) and also that of the microhedylacean *Microhedyle remanei* (Marcus, 1953) and *Pontohedyle milaschewitchii* (Kowalevsky, 1901) (see Neusser et al. 2006; Jörger et al. 2008). The eye is innervated by an optic nerve that emerges from the optic ganglion, as is the case in *Tantulum elegans* Rankin, 1979 and *Pseudunela espiritusanta* but not *Pseudunela cornuta,* where the optic nerve splits from the rhinophoral nerve (an optic ganglion is nevertheless present) (Neusser et al. 2007, 2009). Rhinophoral ganglia have been reported for a number of acochlidian species (see Neusser et al. 2007), even in *Pontohedyle milaschewitchii* which does not have rhinophores; the ganglia often integrate input from paired sensory folds on the sides of the head, the Hancock's organs

(found recently in *Tantulum elegans*, see Neusser and Schrödl 2007). These organs could not be detected in the present material of *Strubellia* from Ambon but are present in congeneric specimens from the Solomon Islands; in these *Strubellia*, there is a small osphradium on the right side of the body that is innervated by the osphradial ganglion (own unpublished data).

Digestive system

The digestive system of *S. paradoxa* was well described by Küthe (1935) and conforms with the general acochlidian organization with a strong buccal muscle followed by paired salivary glands and a large and undivided digestive gland filling most of the visceral sac (see Schrödl and Neusser 2010).

Most of the recently examined species of the limnic Acochlidiidae and the closely related Pseudunelidae Rankin, 1979 have been shown to possess a characteristic asymmetric radula with a formula of $n \times 1.1.2$ (lacking a second lateral plate on the left side) and with more or less strongly serrated edges of the pointed rhachidian tooth (e.g. Wawra 1979; Haynes and Kenchington 1991; Neusser and Schrödl 2009). Küthe correctly showed the rhachidian tooth of *S. paradoxa* to have an elongate central cusp with finely serrated edges but mentioned a second lateral plate on both sides of the radula resulting in a formula of $48–56 \times 2.1.2$, which would be a unique feature for Acochlidia. Moreover, *Strubellia* from the Solomon Islands were shown to possess an asymmetric radula (Wawra 1989; own unpublished data). A SEM examination of the radula of the *Strubellia* specimens collected from Ambon clearly showed the lack of a second lateral plate on the left side, in contrast to Küthe's observation. The existence of the second plate on the right side (as in all closely related species) remains to be confirmed confidently, so the radula formula of *S. paradoxa* is $38 \times 1.1.2$ or, possibly, $38 \times 1.1.1$.

The rhachidian teeth of *Strubellia* are more pointed than in any other hedylopsacean species and have finely serrated saw-like margins. While the number of denticles was not mentioned by Küthe (1935) for his material, his fig. 3 shows 35 per side of the rhachidian tooth (i.e. 70 per tooth, reproduced in Figure 2C–E). In the material re-examined herein, there are approximately 26 to 30 denticles per margin.

Circulatory and excretory systems

The original description of *Strubellia* mentions a strongly muscular two-chambered heart (with the auricle at the left, separated from the ventricle by a valve), a superficial layer of "vacuolated cells", muscular strings spanning the lumen of the ventricle and at least two types of cells floating freely in the haemocoel of the heart (Küthe 1935).

The separation into auricle (rather ample, thin-walled) and ventricle (oval and muscular) can be clearly seen if the heart is present in its diastolic phase (as in the paratype), otherwise the auricle is barely detectable. There appears to be no proper valve, but the conspicuous muscular bridges spanning the ventricular lumen could easily be interpreted as such, likely to improve the performance of the comparably large heart.

The vacuolated layer on the ventricle is a striking feature; the large cells form a closed but irregular layer almost as thick as the muscular wall itself. This feature is so far only described from the brackish-water *Pseudunela espiritusanta*, and may be a

novel site of ultrafiltration involved in production of primary urine if the cells function as podocytes similar to the "pericardial glands" found in doridoidean nudibranchs (see Fahrner and Haszprunar 2002) and many bivalves (e.g. Meyhöfer et al. 1985; Andrews and Jennings 1993). This possible new site of ultrafiltration on the ventricle shall be investigated by future studies of ultrastructure as well as the identity of the two types of free-floating cells inside the heart that Küthe theorized to be "blood cells"; so far only *Pseudunela espiritusanta* was also mentioned as having similar cells inside the heart.

Whereas most members of the mesopsammic Microhedylacea possess a simple sac-like kidney followed by a short nephroduct, the excretory system of Hedylopsacea is generally described as more elaborate: the tubular lumen of the elongate kidney is separated into histologically different proximal and distal parts, and the nephroduct is elongate and forms a loop parallel to the kidney, more so in the brackish water *Pseudunela espiritusanta* than in the mesopsammic *Pseudunela cornuta* (see Neusser et al. 2009; Neusser and Schrödl 2009). In the limnic *Strubellia,* kidney and nephroduct appear to be even more pronounced than in the aforementioned species, similar to what is described for the limnic *Acochlidium amboinense* Strubell, 1892 (see Bücking 1933). There are at least four histologically distinct epithelia found along the excretory system: the renopericardioduct (conspicuously ciliated), the proximal lumen of the kidney (slightly vacuolated with cuboidal epithelium and small lumen), its distal lumen (densely vacuolated, large lumen) and the nephroduct (rather flat cuboidal epithelium) that forms an additional distal loop not mentioned by Küthe. Interconnections of the proximal and distal parts of the nephroduct loop as described by Küthe could not be found in the material examined herein and are likely to be observational errors.

Ultrastructural studies are expected to yield more information on the role of the strongly enlarged and histologically specialized tissues found in the excretory system which is adapted to life in fresh water.

Genital system

Küthe (1935) considered *S. paradoxa* to be a gonochoric species because all mature specimens examined by him were without doubt either male or female, with no simultaneous presence of oocytes and spermatocytes in the gonad. However, he also considered the possibility of protogyny, because the only female specimen (the smallest mature one in the collection) had a small, blind tube in the position of the male copulatory apparatus. Both interpretations are at odds with current knowledge on other hedylopsaceans which all are hermaphroditic and some protandric (Schrödl and Neusser 2010).

While the specimens examined herein were male or juvenile, the presence of three seminal receptacula *sensu lato* together with an elaborate copulatory organ in the male suggests sequential hermaphroditism, specifically protandry. This conclusion is also supported by comparative studies of *Strubellia* specimens from the Solomon Islands (Wawra 1974, 1988; own unpublished data).

Posterior genital system

The posterior genital system of acochlidians most commonly consists of a sac-like gonad filling much of the ventral part of the visceral sac, followed by a long and ciliated, undivided gonoduct leading to the genital opening on the anteroventral right

side of the visceral sac (Schrödl and Neusser, 2010). This is also the case in *Strubellia*, where the posterior part of the genital system closely matches the condition inferred to be ancestral in hermaphroditic opisthobranchs (Ghiselin 1966; p. 332c). There, the gonoduct is usually associated with seminal receptacles and/or female glands that are either expansions of the duct or sac-like extensions from the main gonoduct lumen. *Strubellia* "males" possess three receptacles of which two can be regarded as allosperm receptacles following more recent works on opisthobranch genital systems [e.g. Klussmann-Kolb (2001) and the account on Solomon island *Strubellia* by Wawra (1988)]: the proximal ampulla, a bulbous widening of the gonoduct following the gonad, was found to contain sperm in loose packaging, presumably autosperm (Wawra 1988), it is followed closely by the receptaculum seminis, a short blind tube emerging from the side of the gonoduct. The receptaculum seminis was detected both in males (this study) and females where it was shown to contain sorted spermatozoa with their heads lodged onto the wall (see Wawra 1988; own unpublished data). Finally, there is the stalked and voluminous distal bursa copulatrix (Wawra 1988: filled with coagulated sperm) which was the only receptacle s.l. found by Küthe and so was misinterpreted as an organ for autosperm storage ("vesicula seminalis") before copulation.

Next to the three receptacles, the paratype has a very small blind pouch originating from the distal base of the gonoduct, termed genital diverticle here. This ciliated tube was also found in Solomon Island *Strubellia* "males" by Wawra (1988) but did not contain sperm. Similar structures have been mentioned, for example for the cephalaspidean *Philinopsis* Pease, 1860 (see Klussmann-Kolb 2001); in *Strubellia*, one might infer a function during copulation.

As in *Hedylopsis spiculifera* (Kowalevsky, 1901), *S. paradoxa* has an open seminal groove leading to a separate genital opening on the right side of the head (Wawra 1989). This is in contrast to several hedylopsaceans already examined in detail, such as *Tantulum elegans*, *Pseudunela cornuta*, *Pseudunela espiritusanta* and *Acochlidium fijiense*, which all have a closed distal vas deferens (Haase and Wawra 1996; Neusser and Schrödl 2007, 2009; Neusser et al. 2009).

Female genital system

Küthe described the "oviduct" in "females" as a strongly glandular tube, containing two separable parts that were assumed to be a "protein" and a "mucus" gland ("*Eiweiß*- und *Schalendrüse*"). These parts were shown to differ in their glandular epithelium (larger nuclei sorted peripherally, granular secretion more homogeneous in second part) and the type of ciliary cells surrounding the glandular lumen (spindle-shaped nuclei only in second part). After several glandular loops, the oviduct is described as opening "in the same place as the vas deferens in the male animal" without any "accessory glands". While the paratype yields no information on this, the description fits with the findings in *Strubellia* "females" from the Solomon Islands (Wawra 1988; own unpublished data). The nidamental glands of acochlidiids have been reported to contain only two different glandular parts where they were examined (Wawra 1988; Haase and Wawra 1996). This is in contrast to studies on opisthobranch nidamental glands in general (Klussmann-Kolb 2001) or more specifically on Acochlidia where three histologically different nidamental glands were detected (e.g. *Pontohedyle milaschewitchii*, *Asperspina riseri* (Morse, 1976) and *Tantulum elegans*; see Morse 1976; Neusser and Schrödl 2007; Jörger et al. 2008).

Copulatory apparatus

Our results indicate that Küthe's structural and functional interpretation of the copulatory apparatus has to be corrected. He obviously confused the basal finger to be the sperm-transferring "penis", whereas the latter was not considered as such, and he missed the ciliated "posterior-leading" vas deferens connecting to the base of the prostate. In Küthe's interpretation, sperm would have entered the penial sheath through a duct connecting to the seminal groove. Once inside, sperm would have to move along the outer side of the basal finger (although the latter was correctly stated to be covered with a smooth monolayered epithelium, there is no ciliation that could transport sperm); from there the sperm would be transmitted by the stylet during copulation, together with the secretions of the paraprostate. However, our results show that *Strubellia* possesses the bipartite copulatory apparatus typical for Hedylopsacea, with a paraprostatic system consisting of the basal finger with stylet, and the sperm pathway formed by the posterior-leading vas deferens, the prostate and the penis, the latter not bearing a hollow stylet (as in *Hedylopsis spiculifera*; Wawra 1989) but a basal thorn.

Küthe's description of the copulatory apparatus and stylet are remarkably detailed; he even detected the thin bypass of the paraprostatic duct and found it to connect to the base of the groove of the basal finger stylet. This is not evident from the paratype because of partial retraction of the stylet into the muscle, the bypass and base of the stylet groove are separate by at least 300 μm. The function of the thinner branch of the paraprostatic duct (leading into the stylet groove, as suggested by Küthe, or bypassing into the lumen only as a drain off) and consequently the groove itself (transporting paraprostatic excretion, and perhaps having a stabilizing function of the stylet) remains unclear. A stabilizing function however appears at least somewhat unlikely because of the groove being rather deep but not supported by an exceptionally thick rim. Gascoigne (1974) discussed two types of cuticular elements found in the copulatory apparatus of sacoglossans, showing hollow stylets to have a function in injecting sperm, whereas curved structures were shown to be coupling devices. Following this argument for *Strubellia*, the curved penial thorn works as a coupling device, holding the penis in place during the transmission of sperm. The basal finger would work as a hypodermic injecting device for the secretion of the paraprostate, possibly before copulation. Potential functions include facilitating copulation, avoiding reciprocal copulation, and sperm competition effects, among others.

Strubellia paradoxa is therefore a phallic hermaphrodite like other hedylopsaceans, having a complex copulatory system that resembles that of *Pseudunela* (Neusser et al. 2009). However, the absence of a hollow penial stylet and the possession of allosperm receptacles suggest that sperm transfer in *Strubellia* is via copulation rather than by hypodermic impregnation. This is similar to conditions in the basal limnic but interstitial *Tantulum*, but a unique trait within higher hedylopsaceans that may be explained by a secondarily benthic lifestyle offering sufficient space for adequate positioning of specimens. In members of *Acochlidium*/*Palliohedyle*, the evenly limnic and benthic sister clade to *Strubellia*, the penis complex is apically enlarged, and bears a crown of multiple spines (see e.g. Bücking 1933; Bayer and Fehlmann 1960; Wawra 1979, 1980; Haynes and Kenchington 1991) that was suspected to function as a catch (Schrödl and Neusser 2010). There, the penis supposedly transfers sperm via hypodermic injection

because sperm have been found free in the body cavity, and there is no bursa copulatrix in *Acochlidium fijiense*, which is the only species studied in detail (Haase and Wawra 1996).

As many old descriptions and following interpretations vary considerably from modern investigations, further members of the Acochlidiidae s.l. should be critically reinvestigated using modern 3D morphological methods to further elucidate the potential morphological and functional adaptations to their limnic habitat and benthic lifestyle, giving clues about the biology of these unique freshwater slugs.

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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.

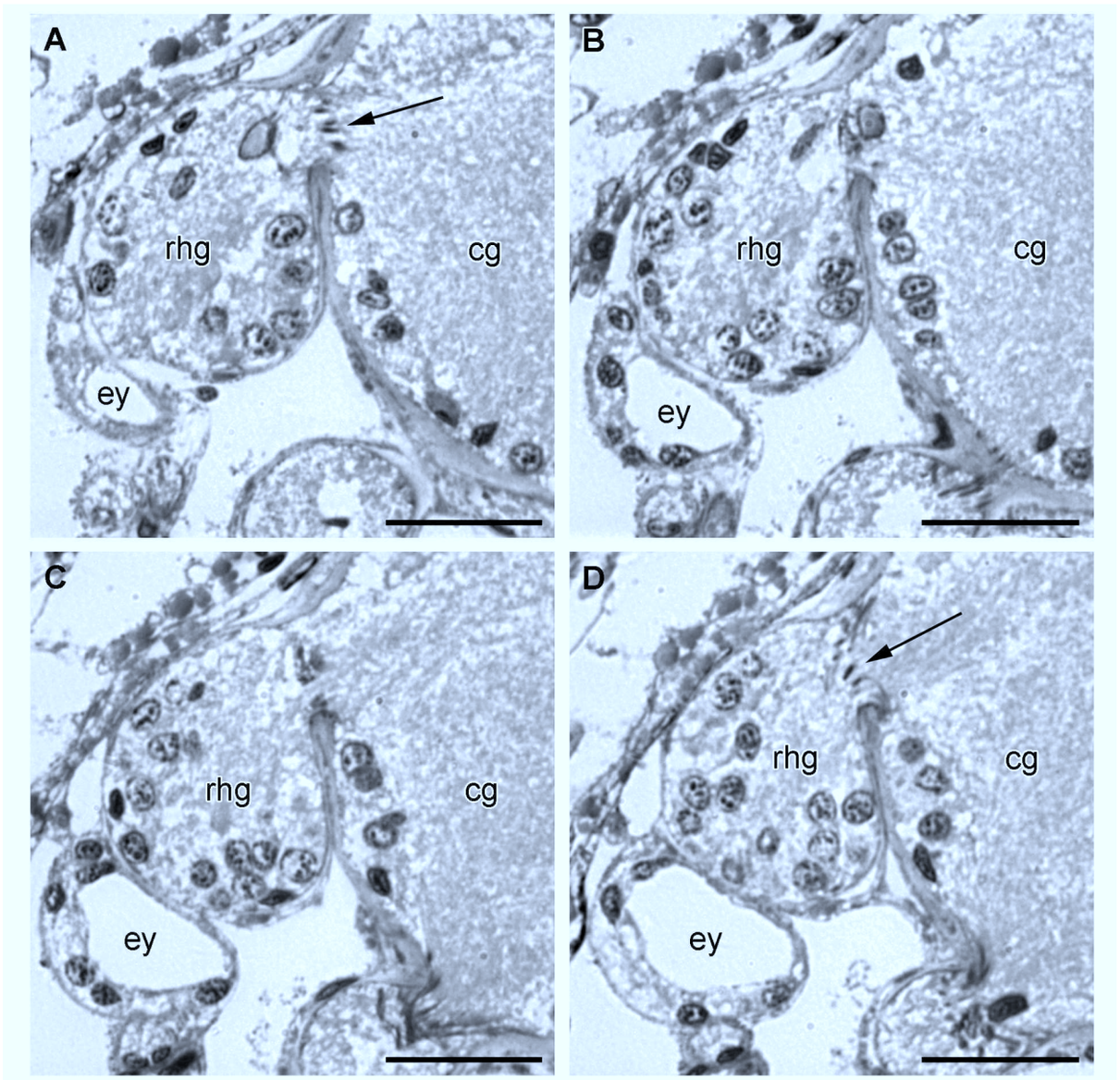

Fig. 1. Double cerebro-rhinophoral connective in *Tantulum elegans*. Four consecutive cross sections of series ROM N° 8E1, 3.slide, 6. ribbon, section N° 17–20. A: section N° 17, first cerebro-rhinophoral connective. B and C: section N° 18 and 19, respectively, without connective. D: section N° 20, second 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

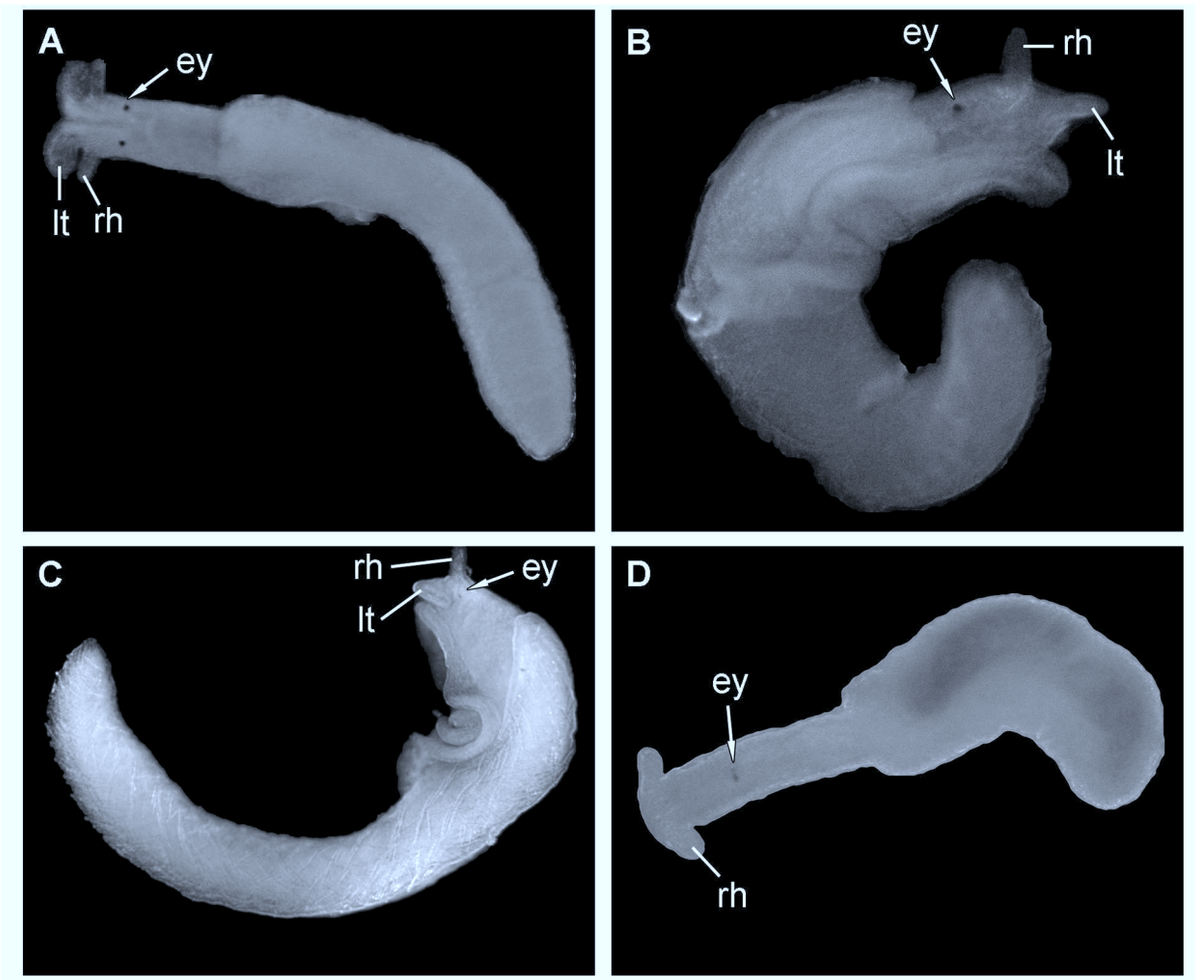

Fig. 2. Position of eyes in different acochlidian species, external view. A: *Hedylopsis spiculifera*, dorsal view, length 3.5 mm. B: *Hedylopsis spiculifera*, lateral view, length 3.5 mm. C: *Hedylopsis ballantinei*, lateral view, length 5 mm. D: *Pontohedyle milaschewitchii*, dorsal view, length 2.5 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-

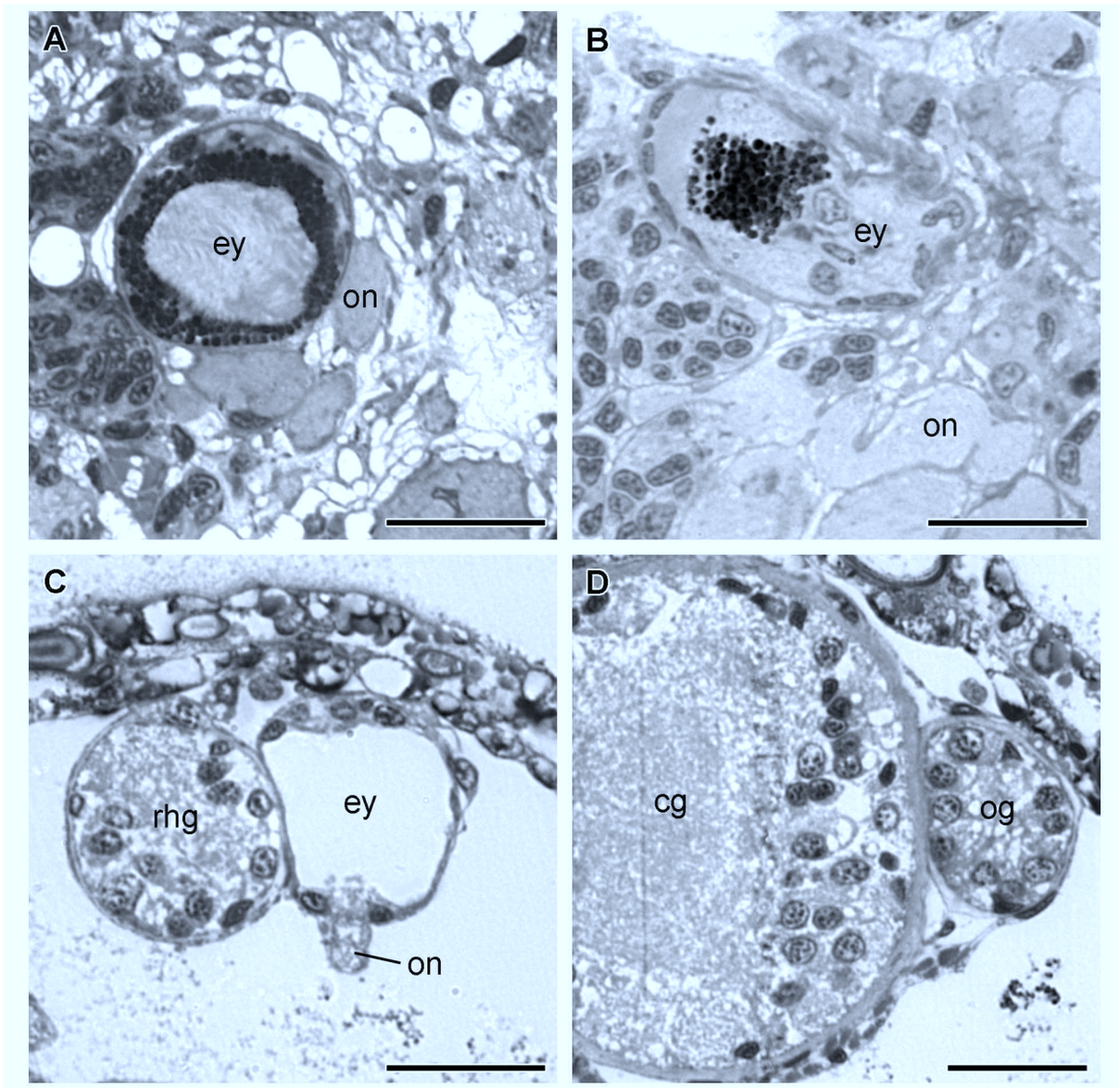

Fig. 3. Eyes and optic ganglion (cross sections). A: Pigmented eye in *Hedylopsis spiculifera* ZSM N° 20070391. B: Pigmented eye in *Hedylopsis ballantinei* ZSM N° 20004766/1. C: Unpigmented eye in *Tantulum elegans* ROM N° 8E1. D: Optic ganglion attached to the cerebral ganglion in *Tantulum elegans* 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

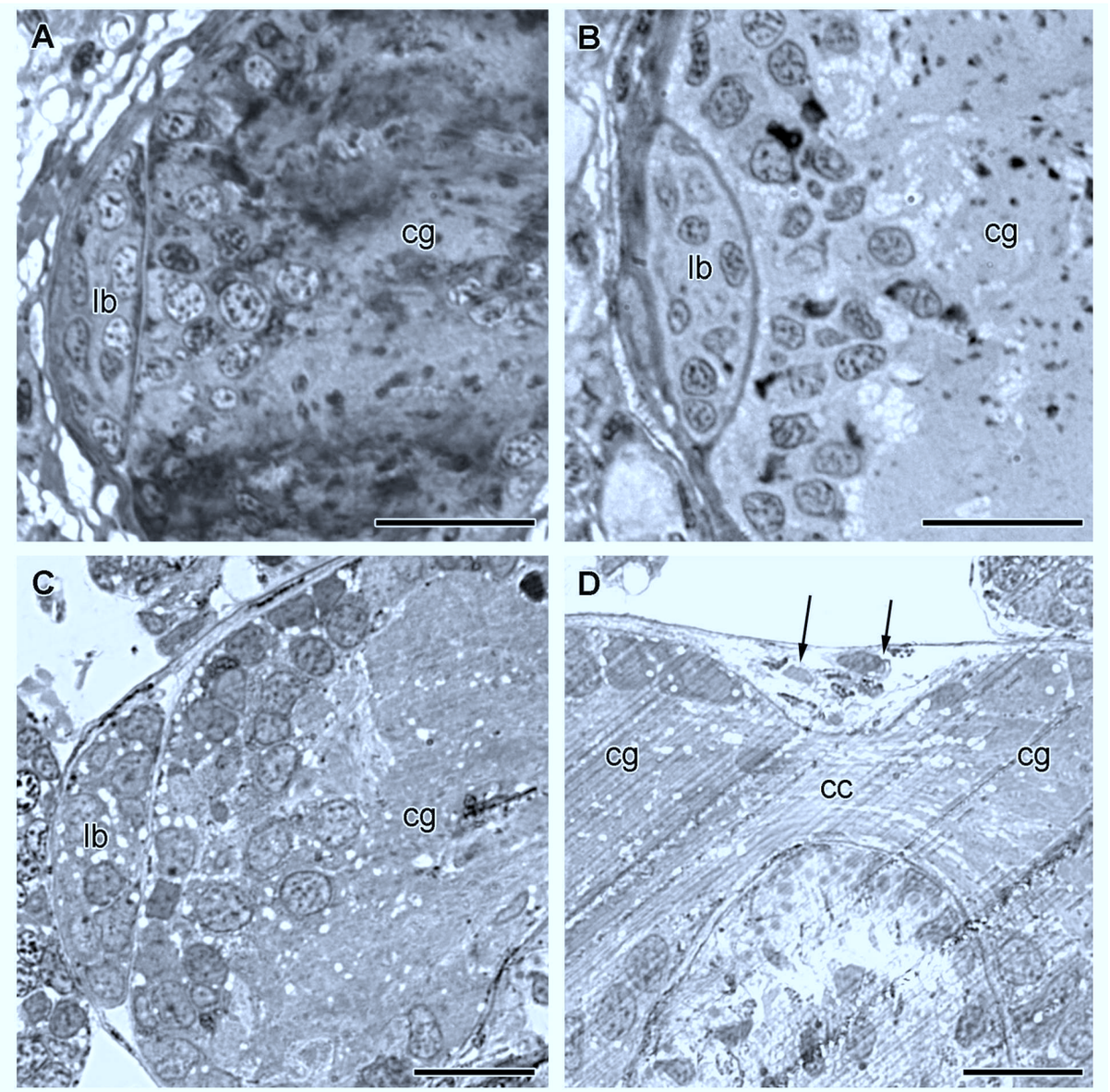

Fig. 4. Aggregates attached to the cerebral ganglia (cross sections). A: "Lateral body" in *Hedylopsis spiculifera* ZSM N° 20070391. B: "Lateral body" in *Hedylopsis ballantinei* ZSM N° 20004766/1. C: "Lateral body" in *Asperspina murmanica* ZSM N° 20062163. D: Cells above cerebral commissure in *Asperspina 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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Towards a phylogeny and evolution of Acochlidia (Mollusca: Gastropoda: Opisthobranchia)

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The Acochlidia are unique among opisthobranch gastropods in combining extremely high morphological and ecological diversity with modest species diversity. The phylogeny of acochlidians has never been addressed by cladistic means, as their evolution has remained unknown. This study gives a first overview on more than 150 biological and morphological characters that are potentially useful for phylogenetic analysis. Based on 107 characters, a parsimony analysis (PAUP) was performed for all 27 valid acochlidian species together with 11 (plus two) outgroup taxa. The resulting strict consensus tree shows a moderate overall resolution, with at least some bootstrap support for most resolved nodes. The Acochlidia are clearly monophyletic, and originate from an unresolved basal opisthobranch level. The Acochlidia split into the Hedylopsacea (*Tantulum* (*Hedylopsis* (*Pseudunela* (*Strubellia* ('*Acochlidium*', '*Palliohedyle*'))))) and Microhedylacea (*Asperspina* (*Pontohedyle*, '*Parhedyle*', '*Microhedyle*', (*Ganitus*, *Paraganitus*))). The formerly enigmatic Ganitidae, resembling sacoglossan opisthobranchs by having dagger-like rachidian radular teeth, are likely to be highly derived microhedylids. The paraphyly of some of the traditionally recognized family level taxa induced a preliminary reclassification. From the phylogenetic hypothesis obtained, we conclude that the acochlidian ancestor was marine mesopsammic. The colonization of limnic systems occurred twice, independently: first in the Caribbean (with the development of the small interstitial *Tantulum elegans*), and second in the Indo-Pacific, with a radiation of large-sized benthic acochlidian species. The evolution of extraordinary reproductive features, such as hypodermic impregnation by a complex copulative aparatus in hedylopsaceans, cutaneous insemination via spermatophores in microhedylaceans, and gonochorism in Microhedylidae *s.l.* (including Ganitidae), is discussed.

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ADDITIONAL KEYWORDS: cladistic analysis – gonochorism – hermaphroditism – hypodermal impregnation – interstitial – limnic – mesopsammic – morphology – spermatophores – taxonomy.

INTRODUCTION

Considerable advances have been made in reconstructing the phylogeny within major traditional opisthobranch groups ('orders'), such as the Cephalaspidea (Mikkelsen, 1996, 2002), Anaspidea (Klussmann-Kolb, 2004), Sacoglossa (Jensen, 1996a; Mikkelsen, 1998), and Nudibranchia (Wägele & Willan, 2000), by the cladistic analyses of morphological data sets. The Thecosomata and Gymnosomata were recently analysed based on molecular markers (Klussmann-Kolb & Dinapoli, 2006). In contrast, the phylogeny of the Acochlidia is completely unclear, and has never been addressed by cladistic means. Up to now, Acochlidia have been considered 'fascinating' (Dayrat & Tillier, 2003), i.e. enigmatic, poorly known, and morphologically and biologically extremely aberrant. Most of the 27 species currently regarded to be valid (Wawra, 1987; Sommerfeldt & Schrödl, 2005) inhabit interstitial spaces of coastal marine sands worldwide. Special morphological adaptations include tiny body sizes, elongate body shapes, the loss of the shell, the development of subdermal spicules, the absence of body pigments, and the reduction of eyes (see Swedmark, 1971; Arnaud, Poizat & Salvini-Plawen, 1986; Westheide, 1987). All acochlidians have a narrow radula (with one or two lateral teeth on each side of a central tooth), which is asymmetrical in several species (in having two lateral teeth on the

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right side with just one on the left); in a few species, the radula is monostichoglossate, i.e. reduced to 12–15 dagger-like rhachidian teeth. Acochlidian species have a variety of aberrant reproductive features (Swedmark, 1968; Wawra, 1992; Morse, 1994), such as sperm transfer by hypodermic injection, via a hollow penial stylet in *Hedylopsis spiculifera* (Kowalevsky, 1901) (see Wawra, 1989), and the use of spermatophores, at least in Asperspinidae and Microhedylidae (see Sommerfeldt & Schrödl, 2005; Neusser *et al.*, 2006, 2007a, Neusser, Martynov & Schrödl, 2008). Although euthyneuran gastropods generally possess male copulatory organs (Dayrat & Tillier, 2003), some acochlidian species become aphallic during ontogeny (Wawra, 1988a, 1989), and others lack any penial apparatus. Of the latter species, at least several are gonochoristic, another condition that is unique amongst the otherwise hermaphroditic opisthobranchs or euthyneurans in general. Perhaps the most surprising extravagance within the generally marine opisthobranchs refers to some acochlidian species that have succeeded in inhabiting a variety of freshwater systems, including mountain spring swamps on the Caribbean island of St. Vincent (Rankin, 1979) and coastal rivers in the tropical Indo-Pacific (Bergh, 1895; Bücking, 1933; Küthe, 1935; Wawra, 1979, 1980; Haynes & Kenchington, 1991). Thus, Acochlidia are especially interesting not only for anatomical and functional reasons, but also for phylogenetic and evolutionary investigations.

The traditionally assumed monophyly of Acochlidia was confirmed by cladistic studies on euthyneuran and opisthobranch phylogeny in which acochlidian species were included, by both using morphological characters (Dayrat & Tillier, 2002; Wägele & Klussmann-Kolb, 2005) and molecular markers, such as combined 18S and 28S rRNA gene sequences (Vonnemann *et al.*, 2005) and multiple markers (Klussmann-Kolb *et al.*, 2008). Most recent studies have shown the Acochlidia to be a basal opisthobranch offshoot, as previously proposed by Odhner (1937) and Marcus (1953); whereas in Klussmann-Kolb *et al.*'s (2008) main analysis, Acochlidia form part of a clade composed of Sacoglossa, pulmonates, and Pyramidelloidea. The cladistic morphological and histological analysis of opisthobranchs by Wägele & Klussmann-Kolb (2005), however, shows acochlidians nested within a clade composed of tiny, partly mesopsammic, and enigmatic taxa, such as Rhodopidae, Runcinidae, and Philinoglossidae. Such an assemblage might easily result from convergent reductions; their tree topology is poorly supported: performing a bootstrap analysis of the original data set (1000 replications, 50% majority rule; our own re-analysis), the acochlidian clade received a high bootstrap value (99), whereas all of the more basal nodes collapsed. The Acochlidia, Rhodopidae, and Philinoglossidae form independent offshoots of a basal polytomy comprising 37 different clades. The morphological cladistic analysis of Salvini-Plawen & Steiner (1996) has already suggested a sister-group relationship between Acochlidia and the equally enigmatic, small-sized, and, in part, interstitial Rhodopemorpha. However, as in the case with tiny runcinids and mesopsammic philinoglossids, this result may have also been driven by convergent organ reductions and adaptations to extreme environments, such as interstitial spaces in the phytal or mesopsammon zones.

Older hypotheses considered the Acochlidia to be related to the sacoglossan *Platyhedyle* (see Salvini-Plawen, 1973; Rankin, 1979), but were mainly based on misinterpretations of central nervous and reproductive features of *Platyhedyle* (see Wawra, 1987, 1988b, 1991). Jensen (1996a) convincingly showed that *Platyhedyle* is the sister group of *Gascoignella aprica* Jensen, 1985, a benthic elysioid sacoglossan that feeds on intertidal algae. Close morphological similarities between *Platyhedyle* and *Gascoignella* were confirmed by Rückert, Haszprunar & Schrödl (2006): a unique muscular septum dividing the digestive gland medially into two rami was considered as a synapomorphy of *Platyhedyle* and *Gascoignella* by Rückert, Altnöder & Schrödl (2008).

Gosliner (1994) suggested that at least parts of the Acochlidia, i.e. the Ganitidae, were derived from sacoglossan opisthobranchs, implying that Acochlidia could also be diphyletic. All of these studies suffered either from considering only a few (i.e. available) acochlidian species, or from using a generalized bauplan that does not necessarily reflect the basal conditions within the heterogenous Acochlidia. The phylogeny within Acochlidia was completely unclear, resulting in two controversial classifications, i.e. that of Rankin (1979) versus that of Wawra (1987), and a modified version that was implemented in Arnaud *et al.* (1986).

Recently, *Hedylopsis ballantinei* Sommerfeldt & Schrödl, 2005 was described as a model organism for acochlidian microanatomy and ultrastructure. Using the detailed structural information obtained, Sommerfeldt & Schrödl (2005) re-evaluated former results on other species, and tried to reconstruct the phylogeny of Acochlidia using apomorphy-based systematics. Sommerfeldt & Schrödl (2005) concluded that: (1) the Acochlidia is a monophyletic group, (2) the Acochlidia originates from a basal opisthobranch level, (3) several taxa defined by Wawra (1987), such as Hedylopsacea and Hedylopsidae, are paraphyletic at best, and (4) all gonochoristic acochlidian species have one common ancestor. However, successful reclassification was once again hindered by the poor anatomical

knowledge of many species, for which the bulk of descriptions were not always reliable, and were at best derived from paraffin-based histology and hand-based graphical reconstruction (see Neusser *et al.*, 2006), and by difficulties to interprete potential organ reductions and the array of mosaic-like distributed aberrant features. Computer-based 3D visualization techniques from serial semithin histological sections allowed full anatomical (re)examination of members of several acochlidian groups: the results showed an unexpectedly high degree of errors within original descriptions (e.g. Neusser & Schrödl, 2007; Neusser *et al.*, 2008), and provided a wealth of structural and histological detail (e.g. Jörger, Neusser & Schrödl, 2007a; Jörger *et al.*, 2008; Neusser, Jörger & Schrödl, 2007b) that can now be compared and used for phylogenetic analyses.

The present study creates a comprehensive list of over 150 discernable biological and structural acochlidian characters and sets, many of which are suitable for cladistic analysis, for the first time. The main concerns are to test the monophyly of acochlidians, and, especially, to present the first parsimony-based hypothesis on inner acochlidian phylogeny that includes all valid species, which enables us to address some of the most interesting aspects of acochlidian evolution, such as the invasion of interstitial and especially limnic systems, and the derivation of aberrant radula and reproductive features.

PHYLOGENETIC ANALYSIS

Taxa

Previous cladistic studies based on morphology (Dayrat & Tillier, 2002) and on molecular markers (Vonnemann *et al.*, 2005; Klussmann-Kolb *et al.*, 2008) indicated the Acochlidia to be a basal euthyneuran or opisthobranch offshoot with still uncertain relationships. Thus, a variety of 11 pyramidellid, pulmonate, actenoidean, and other basal opisthobranch outgroup taxa has been selected (Table 1). *Toledonia*, tiny runcinids, and mesopsammic Philinidae and Philinoglossidae (all Cephalaspidea) were included, as these taxa had been assumed to be potentially closely related to acochlidians (e.g. Odhner, 1937; Sommerfeldt & Schrödl, 2005; Wägele & Klussmann-Kolb, 2005). For an additional analysis, the mesopsammic and worm-like *Platyhedyle* (Sacoglossa) and *Rhodope* (Opisthobranchia *incertae sedis*) were also considered.

Our ingroup comprises all 27 nominal acochlidian species considered to be valid at present (Wawra, 1987; Sommerfeldt & Schrödl, 2005; Jörger *et al.*, 2007a), regardless of the heterogenous state of knowledge of these species.

Characters

Characters have been selected according to the following criteria. Outgroup-specific characters are only included to an extent that guarantees a reasonable framework for rooting the Acochlidia. In contrast, for the ingroup, all characters that are discernable, available, and relevant to acochlidians, except for molecular markers, have been collected from the literature and have been defined (see lists below). Definitions were made so as to minimize a priori assumptions on homology of problematic structures (e.g. the identity of the visceral loop ganglia, and the homology of lateral radula teeth portions). Where sufficient information on at least some acochlidian species was availabe, characters have been preliminarily coded and polarized by outgroup comparison. A priori, uninformative or problematic characters, i.e. autapomorphies of single terminal taxa, or characters showing too much ambiguity or lack of information within the ingroup, were excluded. Characters not considered for this analysis are listed and briefly discussed separately.

The morphological information on outgroups was obtained from several recent reviews and phylogenetic studies (Challis, 1969; Hubendick, 1978; Brown, 1979; Haszprunar & Huber, 1990; Gosliner, 1991, 1994; Salvini-Plawen, 1991; Huber, 1993; Jensen, 1996a, b; Poizat, 1978; Mikkelsen, 1996, 2002; Collin & Wise, 1997; Ponder & Lindberg, 1997; Ruthensteiner, 1999; Wägele & Willan, 2000; Dayrat & Tillier, 2002; Wägele & Klussmann-Kolb, 2005; Golding, Ponder & Byrne, 2007). Information on *Toledonia* was derived from Odhner (1926), Hoffmann (1939), and from our own unpublished information on living *Toledonia* spp. from Antarctica (see Sirenko & Schrödl, 2001). Data on *Platyhedyle* were derived from Salvini-Plawen (1973), Wawra (1988b, 1991), Huber (1993), and Rückert *et al.* (2006, 2008). Data on *Pluscula* were derived from Marcus (1953) and from our own unpublished external examinations of living specimens from the type locality.

For acochlidian species, all of the available original or secondary literature was considered (e.g. Bergh, 1895; Kowalevsky, 1901; Bücking, 1933; Küthe, 1935; Odhner, 1937, 1938, 1952; Marcus, 1953; Marcus & Marcus, 1954, 1955; Bayer & Fehlmann, 1960; Challis, 1968, 1970; Swedmark, 1968, 1971; Kirsteuer, 1973; Doe, 1974; Wawra, 1974, 1978, 1979, 1980, 1986, 1987, 1988a, c, 1989, 1992; Westheide & Wawra, 1974; Morse, 1976, 1994; Kudinskaya & Minichev, 1978; Haynes & Kenchington, 1991; Huber, 1993; Haase & Wawra, 1996; Fahrner & Haszprunar, 2002; Sommerfeldt & Schrödl, 2005; Neusser *et al.*, 2006, 2007a, b, 2008; Neusser & Schrödl, 2007; Jörger *et al.*, 2007a, b, 2008). Rankin's (1979) literature

Table 1. Data matrix

	0										1										2										3										4									
Character number	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	6	7	8	9
Odostomia	0	0	1	0	0	0	0	0	–	0	0	–	0	0	0	0	0	0	–	0	0	0	0	1	0	0	0	–	0	–	–	0	–	–	–	–	–	0	–	0	0	0	0	0	0	0	0	0	–
Amphibola crenata	3	0	0	0	0	0	0	0	–	0	0	–	0	0	0	0	0	0	–	0	0	0	1	1	0	0	0	–	0	–	–	0	–	–	–	–	–	0	–	0	?	0	0	0	0	?	0	0	–
Myosotella myosota	3	0	0	0	0	0	1	0	–	0	0	–	0	0	0	0	0	0	–	0	0	0	1	1	0	0	0	–	0	–	–	0	–	–	–	–	–	0	–	0	?	0	0	0	1	1	1	0	–
Chilina	2	0	0	0	0	0	1	0	–	0	0	–	0	0	0	0	0	0	–	0	0	0	1	1	0	0	0	–	0	–	–	0	–	–	–	–	–	0	–	0	?	0	0	0	1	1	1	0	–
Acteon	0	0	0	0	0	0	0	1	1	0	0	–	0	0	0	0	0	0	–	0	0	0	0	0	0	0	0	–	1	1	7	0	–	–	–	–	–	0	–	0	0	0	0	0	0	0	0	0	–
Cylindrobulla	0	{0,1}	0	0	0	0	1	1	1	0	0	–	0	0	0	0	0	0	–	0	0	0	0	0	0	0	0	–	1	0	–	0	–	–	–	–	–	0	–	0	?	0	0	0	0	0	0	0	–
*Platyhedyle denudata**	0	1	1	1	1	–	1	0	–	0	0	–	0	1	1	0	0	1	2	0	0	0	3	1	1	0	0	–	1	0	–	1	0	0	–	0	0	0	0	0	1	0	0	1	0	0	0	0	–
Toledonia	0	0	1	0	0	0	1	0	–	0	0	–	0	0	0	0	0	0	–	0	0	0	0	0	0	0	0	–	1	0	–	0	–	–	–	–	–	0	–	?	?	0	0	1	0	?	?	?	–
Colpodaspis	0	0	1	0	0	0	1	0	–	1	0	–	0	0	0	0	0	0	–	0	0	0	0	0	0	0	0	–	1	1	7	0	–	–	–	–	–	0	–	?	?	?	0	1	0	?	?	?	–
Metaruncina	0	0	1	1	0	1	1	1	0	1	0	–	1	–	1	–	0	–	3	0	0	0	0	0	2	0	0	–	1	0	–	0	–	–	–	–	–	0	–	?	?	?	0	1	1	0	0	0	–
Philine exigua	0	1	1	1	0	1	1	1	0	1	0	–	1	–	1	–	0	–	3	1	1	0	0	1	2	0	0	–	1	0	–	0	–	–	–	–	–	0	–	0	1	1	0	1	0	0	0	0	–
Pluscula	0	1	1	1	0	1	1	1	0	1	0	–	1	–	1	–	0	–	3	1	1	0	0	1	2	0	0	–	1	0	–	0	–	–	–	–	–	0	–	?	?	1	2	?	0	0	?	0	–
*Rhodope**	0	{0,1}	1	1	1	–	1	0	–	0	1	1	1	–	1	1	0	–	3	3	0	0	3	1	5	0	0	–	1	0	–	1	0	0	–	0	0	–	0	0	–	1	0	1	0	0	1	0	0	–
Hedylopsis spiculifera	0	1	1	1	1	–	1	0	–	0	1	0	0	1	1	0	0	0	0	0	0	0	3	1	1	0	1	0	1	1	3	1	0	1	1	0	1	0	0	1	0	0	0	?	0	0	0	1	0
Hedylopsis ballantinei	0	1	1	1	1	–	1	0	–	0	1	0	0	1	1	0	0	0	0	0	0	0	2	1	1	0	1	0	1	1	3	1	0	1	1	0	1	0	0	1	0	0	0	?	0	0	0	1	0
Pseudunela cornuta	0	1	1	1	1	–	1	0	–	0	1	0	0	1	1	1	0	0	0	0	0	0	3	1	1	?	1	0	1	1	0	1	1	0	0	–	0	0	0	1	0	0	1	?	0	0	0	0	?
Pseudunela eirene	0	1	1	1	1	–	1	0	–	0	1	0	0	?	1	?	0	0	0	0	0	0	?	1	?	?	1	0	?	1	?	?	?	0	0	–	?	?	0	?	?	?	{1,2}	?	0	?	?	?	?
Strubellia paradoxa	2	0	0	1	1	–	1	0	–	0	1	0	0	1	1	1	0	0	0	1	0	1	3	1	4	0	1	0	1	1	0	1	1	0	0	–	0	0	0	1	0	0	0	1	0	0	0	0	0
Acochlidium amboinense	2	0	0	1	1	–	1	0	–	0	1	1	0	1	1	1	1	2	0	1	0	1	3	1	1	0	1	0	?	1	0	1	1	0	0	–	0	0	0	?	?	0	0	?	0	0	0	?	0
Acochlidium bayerfehlmanni	2	0	0	1	1	–	1	0	–	0	1	1	0	1	1	1	1	2	0	1	0	1	3	1	?	?	1	0	?	1	0	1	1	0	0	–	0	0	0	?	?	?	0	?	0	?	?	?	?
Acochlidium fijiense	2	0	0	1	1	–	1	0	–	0	1	1	0	1	1	1	1	2	0	1	0	1	3	1	1	?	1	0	?	1	0	1	1	0	0	–	0	0	0	?	?	0	0	?	0	?	?	?	0
Palliohedyle sutteri	2	0	0	1	1	–	1	0	–	0	1	1	0	1	1	1	1	2	0	1	0	0	3	1	?	?	1	0	?	1	0	?	?	0	0	–	?	?	0	?	?	0	0	?	0	?	?	?	?
Palliohedyle weberi	1	0	0	1	1	–	1	0	–	0	1	1	0	1	1	1	1	2	0	?	0	1	3	1	1	?	1	0	?	1	1	1	1	0	0	–	0	0	0	1	?	0	0	?	0	0	0	?	0
Tantulum elegans	2	1	1	1	1	–	1	0	–	0	1	0	0	0	1	0	0	0	0	0	0	0	3	1	4	0	1	0	1	1	0	1	0	0	0	–	1	0	0	1	0	1	1	1	0	0	0	0	1
Asperspina brambelli	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	0	0	0	1	0	0	0	3	1	1	?	1	1	?	1	?	1	0	0	1	1	1	0	?	?	?	1	2	?	0	0	0	?	–
Asperspina loricata	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	0	0	0	1	2	0	0	3	1	1	0	1	1	?	1	2	1	0	0	1	1	1	0	1	?	?	?	2	?	0	0	0	?	–
Asperspina murmanica	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	0	0	0	1	0	0	0	3	1	4	0	1	1	1	1	2	1	0	0	1	1	1	0	1	1	0	1	2	?	0	0	0	1	–
Asperspina riseri	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	0	0	0	1	2	0	0	3	1	1	0	1	1	?	1	2	1	0	0	1	1	1	1	1	?	?	1	2	?	0	0	0	?	–
Asperspina rhopalotecta	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	0	0	0	1	0	0	0	{2,3}	1	1	0	1	1	1	1	2	1	0	0	1	1	1	0	1	1	?	1	{1,2}	?	0	0	0	?	1
Microhedyle glandulifera	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	1	2	0	0	3	1	3	1	1	0	1	1	5	1	{0,2,3}	0	0	–	0	1	0	1	1	1	0	1	0	0	0	?	1
Microhedyle nahantensis	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	1	2	0	0	3	1	3	1	1	0	?	1	5	1	2	0	0	–	0	1	0	?	1	1	1	?	0	0	0	?	1
Microhedyle odhneri	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	1	2	0	0	3	1	1	0	1	0	?	1	5	?	?	0	0	–	?	?	0	?	1	1	1	?	0	?	?	?	?
Microhedyle remanei	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	1	2	0	0	3	1	1	0	1	0	1	1	5	0	–	–	–	–	–	1	0	1	0	1	2	?	0	0	0	0	–
Pontohedyle milaschewitchii	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	2	2	0	0	3	1	3	0	0	–	1	1	4	1	0	0	0	–	1	0	0	1	0	1	0	1	0	0	0	0	1
Pontohedyle verrucosa	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	{1,2}	2	0	0	3	1	?	0	0	–	?	1	4	1	0	0	0	–	1	0	0	?	?	1	{1,2}	?	0	0	0	?	?
Parhedyle cryptophthalma	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	1	2	0	0	3	1	?	?	1	0	?	1	5	1	2	0	0	–	0	1	0	?	?	1	1	?	0	?	?	?	1
Parhedyle gerlachi	0	1	1	1	1	–	1	0	–	0	1	0	0	?	1	1	0	0	?	2	0	0	?	1	?	?	1	?	?	1	?	?	?	0	0	–	?	?	0	?	?	?	0	?	?	?	?	?	?
Parhedyle tyrtowii	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	1	2	0	0	3	1	3	?	1	0	?	1	5	1	2	0	0	–	0	1	0	?	?	1	0	?	0	0	0	?	1
Ganitus evelinae	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	2	2	0	0	3	1	1	0	0	–	1	1	6	1	2	0	0	–	0	1	0	?	?	1	2	?	0	0	0	?	–
Paraganitus ellynnae	0	1	1	1	1	–	1	0	–	0	1	0	0	0	1	1	0	0	1	2	0	0	3	1	?	?	1	0	?	1	5	0	–	–	–	–	0	1	0	?	?	1	2	?	0	0	0	?	–

Table 1. *Continued*

	5	6		7	8 9	10
Character number	0 1	2 3 4 5 6 7 8 9 0 1 2	3	4 5 6 7 8 9 0	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8	9 0 1 2 3 4 5 6 7
Odostomia	0 0	1 0 1 1 1 2 3 2 2 – –	–	– – – – – – –	– 0 0 – ? – ? ? ? ? 0 0 0 0 – 0 0 0 0 1 0 0 0 1 0 0 0 0	– 0 0 0 0 – 1 0 0
Amphibola crenata	0 0	0 0 1 0 1 0 0 0 0 0 –	0	0 2 3 0 0 0 –	2 0 0 – 0 – 2 0 0 0 0 0 0 0 – 0 0 1 0 0 – 0 0 1 1 1 2 0	– 0 0 0 0 – 1 0 ?
Myosotella myosota	0 0	0 0 1 0 1 0 0 0 0 0 –	0	0 1 – – 0 0 –	2 0 0 – 0 – 2 ? 0 0 0 0 0 0 – 0 0 0 0 0 – 0 0 1 1 1 0 0	– 0 0 0 0 – 0 1 ?
Chilina	0 0	0 0 2 0 0 0 0 0 0 0 –	0	0 0 1 0 0 0 –	2 0 0 – 0 – 2 ? 0 0 0 0 0 1 1 0 2 0 0 1 0 0 0 1 1 1 0 0	– 0 0 0 0 – 0 0 ?
Acteon	0 0	0 1 0 0 0 0 0 0 0 0 –	0	1 – – – 0 0 –	2 0 1 0 0 – 0 ? 0 0 0 0 0 1 1 0 2 1 0 0 – 0 0 0 0 1 0 0	– 0 0 0 0 – 0 0 0
Cylindrobulla	0 1	0 1 1 0 1 0 1 0 0 1 1	0	0 2 0 1 2 – –	– 1 0 – 0 – ? ? 0 0 0 0 0 1 1 0 0 0 0 0 – 0 0 1 0 1 0 0	– 0 0 0 0 – 0 0 ?
Platyhedyle denudata	0 0	1 1 1 1 1 1 1 0 0 1 1	1	0 3 – 1 2 – –	– ? 0 – 0 – 0 0 2 – 0 0 0 1 1 0 2 1 0 0 – 0 0 1 0 1 0 1	0 0 0 0 0 – 0 1 ?
Toledonia	0 0	0 0 1 0 1 0 1 0 0 0 –	0	0 2 0 0 1 1 0	3 1 ? ? 1 0 ? 0 0 0 0 0 0 0 – 0 0 0 0 0 – 0 0 1 ? 1 0 0	– 0 0 0 0 – 0 1 ?
Colpodaspis	0 0	1 0 1 0 1 0 1 0 0 0 –	0	1 – – – 1 0 –	2 ? 0 – 1 0 ? ? 0 0 0 0 0 0 – 0 0 0 0 0 – 0 0 1 0 1 0 0	– 0 0 0 0 – 0 0 ?
Metaruncina	1 ?	1 1 ? 0 1 0 0 0 0 0 –	{0,1}	? ? ? ? 1 0 –	3 1 1 1 1 0 1 ? 1 0 0 ? 0 0 – 0 0 0 0 1 0 0 0 1 0 1 0 0	– 0 0 0 0 – 1 0 ?
Philine exigua	0 0	1 0 1 0 1 0 1 0 0 0 0	1	1 – – – 1 0 –	3 0 1 1 2 – 1 0 1 0 0 0 0 0 – 0 0 ? 0 0 – 0 0 1 0 1 0 0	– 0 0 0 0 – 0 1 ?
Pluscula	– –	0 0 1 0 1 ? 1 0 0 1 0	1	1 – – – 1 0 –	3 0 0 – 1 0 1 0 1 0 0 0 0 ? ? 0 0 ? 0 0 – 0 0 1 0 1 0 0	– 0 0 0 0 – 0 1 ?
Rhodope	1 0	1 1 3 1 1 0 1 3 2 – –	–	– – – – – – –	– ? ? ? 1 0 0 0 2 – 0 0 ? 0 – 0 2 1 0 ? ? 0 ? ? 1 1 0 {0,1}	0 0 0 0 0 – 0 1 ?
Hedylopsis spiculifera	0 {0,1}	1 0 1 1 1 1 1 0 1 1 {0,1}	0	0 2 1 0 1 1 {0,2}	1 0 0 – 1 0 0 1 1 0 0 1 1 0 – 0 0 0 0 0 – 0 0 1 0 1 0 1	0 1 0 1 0 – 1 1 1
Hedylopsis ballantinei	0 0	1 0 1 1 1 1 1 0 1 1 1	0	0 2 1 0 1 1 {0,2}	1 0 0 – 1 0 0 1 1 0 0 0 1 0 – ? 0 ? 0 ? ? ? 1 – – – – –	– – – – – – 1 1 1
Pseudunela cornuta	0 0	1 0 1 1 1 1 1 0 ? 1 0	0	0 2 1 0 1 1 0	? 0 0 – 1 0 0 2 1 1 0 0 2 1 0 0 2 1 0 0 – 0 0 1 0 1 0 1	1 0 1 0 0 – 0 0 1
Pseudunela eirene	? ?	? 0 ? ? ? 1 ? 0 1 1 0	0	0 2 ? 0 1 1 0	0 0 ? ? 1 0 0 ? ? ? 0 0 ? ? ? ? ? ? ? ? 0 – 0 0 1 0 1 ? 1	1 ? ? ? 0 – ? ? 1
Strubellia paradoxa	0 1	1 0 1 1 1 1 1 0 1 1 0	0	0 2 2 2 1 1 0	0 0 0 – 1 0 0 2 1 1 0 1 0 0 – 0 0 0 0 0 – 0 0 1 0 1 0 0	– 0 1 1 0 – 0 0 1
Acochlidium amboinense	? 1	? 0 ? ? ? 1 1 0 ? 1 1	0	0 2 2 3 1 1 1	0 0 0 – 3 – 0 ? 1 1 0 0 ? ? ? ? 1 ? ? 0 – 0 0 1 ? ? 1 1	? ? ? ? 1 0 ? ? ?
Acochlidium bayerfehlmanni	? 1	? ? ? ? ? ? 1 0 1 1 1	0	0 2 2 3 1 1 0	0 ? ? ? 3 – 0 ? 1 1 0 0 ? 1 0 ? 2 ? 0 0 – 0 0 1 0 1 1 ?	? 0 ? ? 1 0 ? ? ?
Acochlidium fijiense	? 1	? ? ? ? ? ? 1 0 1 1 ?	0	0 2 2 2 1 1 0	0 ? ? ? 3 – 0 ? 1 1 0 0 3 0 – 0 2 1 0 0 – 0 0 1 0 1 1 1	1 0 1 1 1 1 1 1 1
Palliohedyle sutteri	? 1	? ? ? ? ? ? 1 0 1 1 ?	0	0 2 2 2 1 1 0	0 0 ? ? ? ? ? ? ? ? ? 0 0 ? ? ? ? 2 1 0 0 – 0 0 1 0 1 1 1	1 0 1 ? 1 0 ? ? ?
Palliohedyle weberi	? 1	? 0 ? ? ? 1 1 0 1 1 0	0	0 2 2 1 1 1 0	0 0 0 – 3 – 0 ? 1 ? 0 0 0 ? ? ? ? ? ? ? 0 – 0 0 1 ? ? 1 ?	? ? ? ? 1 1 ? ? ?
Tantulum elegans	0 ?	1 0 1 1 1 1 1 0 1 1 0	0	0 2 0 0 1 1 0	0 0 0 – 1 0 0 2 1 0 0 1 2 1 0 0 2 1 0 0 – 0 0 1 0 1 0 0	– 1 0 0 0 – 0 1 0
Asperspina brambelli	– –	1 0 ? 1 1 1 1 0 0 1 {0,1}	0	0 2 0 1 1 1 0	0 0 ? ? 1 0 0 ? ? 0 0 0 ? 0 – 0 0 0 0 1 1 1 1 – – – – –	– – – – – – 1 1 0
Asperspina loricata	– –	1 ? ? 1 1 1 1 0 0 1 0	0	0 2 0 0 1 1 0	3 0 ? ? 1 0 0 0 ? 0 0 0 ? 0 – 0 0 0 0 ? ? ? 1 – – – – –	– – – – – – ? ? ?
Asperspina murmanica	– –	1 0 1 1 1 1 1 0 1 1 0	0	0 2 0 0 1 1 0	0 0 0 – 1 0 0 0 1 0 0 0 2 0 – 0 0 0 0 1 1 ? 1 – – – – –	– – – – – – 1 1 0
Asperspina riseri	– –	1 0 ? 1 1 1 1 0 0 1 0	0	0 2 0 0 1 1 0	3 0 0 – 1 0 0 0 ? 0 0 0 0 0 – 0 0 0 0 1 1 1 1 – – – – –	– – – – – – 1 1 0
Asperspina rhopalotecta	? ?	1 0 1 1 1 1 1 0 1 1 ?	0	0 2 0 0 1 1 0	0 0 0 – 1 0 0 ? ? 0 0 0 ? 0 – ? 0 ? ? ? ? ? 1 – – – – –	– – – – – – ? ? ?
Microhedyle glandulifera	1 0	1 0 1 1 1 1 ? 0 0 1 0	0	0 2 0 0 1 1 1	3 0 0 – 1 1 0 0 ? 0 1 – ? – – 1 0 1 1 1 1 ? 1 – – – – –	– – – – – – 1 1 0
Microhedyle nahantensis	1 0	1 0 ? 1 1 1 1 0 0 1 0	0	0 2 1 0 1 1 2	3 0 0 – 1 1 0 0 ? 0 1 – ? – – 1 0 1 1 1 1 1 1 – – – – –	– – – – – – 1 1 0
Microhedyle odhneri	1 0	? ? ? ? ? ? 1 0 0 1 ?	0	0 2 0 0 1 1 2	3 0 ? – 1 ? 0 ? ? 0 1 – ? – – ? ? 1 ? ? ? ? 1 – – – – –	– – – – – – 1 1 ?
Microhedyle remanei	– –	1 0 1 1 1 1 1 0 0 1 0	0	0 2 0 0 1 1 1	3 0 0 – 1 1 0 0 1 0 1 – 0 – – 1 0 1 1 1 1 1 1 – – – – –	– – – – – – 1 1 0
Pontohedyle milaschewitchii	1 0	1 0 1 1 1 1 1 0 0 1 0	0	0 2 0 0 1 1 0	3 0 0 – 1 1 0 0 1 0 1 – 0 – – 1 3 1 0 1 1 1 1 – – – – –	– – – – – – 1 1 0
Pontohedyle verrucosa	? ?	? ? ? 1 1 1 1 0 0 1 0	0	0 2 0 0 1 1 2	3 0 ? ? 1 1 0 0 ? 0 1 – ? – – ? 0 ? ? ? ? ? 1 – – – – –	– – – – – – 1 1 ?
Parhedyle cryptophthalma	1 0	? 0 1 ? ? 1 1 0 0 1 0	0	0 2 0 0 1 1 2	0 0 0 – 1 1 0 0 1 0 1 – ? – – ? 0 1 0 1 1 1 1 – – – – –	– – – – – – ? ? ?
Parhedyle gerlachi	? ?	? ? ? ? ? 1 ? ? 0 1 0	0	0 2 0 0 1 1 0	3 ? ? ? ? ? ? ? 1 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?	? ? ? ? ? ? ? ? ?
Parhedyle tyrtowii	1 0	1 ? ? 1 1 1 1 0 ? 1 ?	0	0 2 1 0 1 1 0	0 0 0 – 1 1 0 0 ? 0 ? ? 0 ? ? ? 0 1 0 ? ? ? 1 – – – – –	– – – – – – 1 1 0
Ganitus evelinae	– –	1 0 ? 1 1 1 2 1 0 1 1	1	0 3 – 3 2 – –	– 0 0 – 1 1 0 0 1 0 1 – 0 – – 1 0 1 0 1 1 1 1 – – – – –	– – – – – – 1 1 0
Paraganitus ellynnae	– –	1 0 ? 1 1 1 2 1 0 1 1	1	0 3 – 3 2 – –	– 0 0 – 1 1 0 0 ? 0 1 – 0 – – 1 0 1 1 1 1 ? 1 – – – – –	– – – – – – 1 1 0

Outgroup and acochlidian ingroup taxa listed with the following character states (**Platyhedyle denudata* and *Rhodope* were considered in an additional analysis only): 0, plesiomorphic condition; 1–7, apomorphic conditions; ?, missing data; –, inapplicable (also coded as missing).

review on acochlidian morphology was shown to be seriously flawed and full of misinterpretations (e.g. Wawra, 1987; Neusser & Schrödl, 2007), especially with regard to inner organ systems: most of her comparative statements and schematic drawings were not considered herein.

In addition to literature data, we consider our own unpublished external information from living specimens of *Acochlidium fijiense* Haynes & Kenchington, 1991, *Acochlidium bayerfehlmanni* Wawra, 1980, *Asperspina rhopalotecta* Salvini-Plawen, 1973, *Ganitus evelinae* Marcus, 1953, *Microhedyle glandulifera* (Kowalevsky, 1901), *Parhedyle cryptophthalma* (Westheide & Wawra, 1974), *Paraganitus ellynnae* Challis, 1968, *Pontohedyle verrucosa* (Challis, 1970), and *Pseudunela cornuta* (Challis, 1970), most of which were collected at the type localities (M. Schrödl, T. P. Neusser & K. Jörger, unpubl. data). Anatomical information on *P. cryptophthalma*, *P. cornuta*, and *Strubellia paradoxa* (Strubell, 1892) was obtained from the 3D reconstruction of serial histological sections (M. Schrödl, T. P. Neusser, K. Jörger & B. Brenzinger, unpubl. data).

The presence or condition of structures is coded only when mentioned in the literature description or shown in illustrations. In case of discrepancies, the more recent, detailed, and reliable data source was preferred, or coding was set to unknown. In contrast, the absence of conspicuous structures was also concluded from not mentioning their presence in an otherwise detailed study, if this was not contradicted by any other means.

The following 107 characters (Table 1) were used for parsimony analysis (PAUP 4.0b10; Swofford, 2001), and the character discussion concentrates on acochlidians.

Ecology

1. All opisthobranchs but a few acochlidian species are marine-based (0), *Palliohedyle weberi* (Bergh, 1895) inhabits brackish waters (1), whereas *Acochlidium amboinense* (Strubell, 1892), *A. bayerfehlmanni*, *A. fijiense*, *S. paradoxa*, *Palliohedyle sutteri* (Wawra, 1979), and *Tantulum elegans* Rankin, 1979 are freshwater inhabitants (2). *Amphibola* and *Myosotella* live amphibiously in the intertidal zone (3).
2. Most euthyneurans, including some Acochlidiidae, live (epi-)benthically or on the substrate (0). Other acochlidians, *Platyhedyle*, *Pluscula*, and some *Rhodope* spp. inhabit the interstitial spaces of marine sands (i.e. they are mesopsammic) (1).

External organization

3. Body size. The vast majority of opisthobranchs including Acochlidiidae grow larger than 5-mm long (0). *Toledonia*, *Tantulum elegans*, and mesopsammic acochlidians are smaller than 5-mm long (1).
4. Body symmetry. Basal Heterobranchia and most shelled euthyneurans show at least a certain degree of body asymmetry, e.g. they have a coiled visceral sac (0). All acochlidians, *Platyhedyle*, *Metaruncina*, *Rhodope*, *Philine exigua* Challis, 1969, and *Pluscula* are externally symmetric (1), but the visceral sac may be irregularly formed or bent in some species.
5. Shell. Most heterobranchs retain a shell (0). All acochlidians, *Platyhedyle*, and *Rhodope* lack any adult shell (1).
6. Shell location. Although the shell is usually external (0), it is internalized in *Pluscula*, *Philine exigua*, and *Metaruncina* (1).
7. Operculum. Although basal heterobranchs, Amphibolidae, all but one Acteonidae species, Ringiculidae, and some *Retusa* species still possess an operculum in adults (0), the vast majority of euthyneurans including acochlidians do not (1).
8. Head shield. The head may be free in most euthyneurans (0), or is covered with a (mantle) shield in many cephalaspideans, supposedly used for digging (1).
9. Head shield division. Where present, a head shield may be entire (0) or medially divided (1).
10. Posterior shield. Although usually absent (0), a pallial shield or lobe covers the posterior body portions in several cephalaspideans (1).
11. Retraction of head–foot complex into a temporal visceral cavity. Although absent in other gastropods (0), at least some *Rhodope* species and all acochlidians are able to retract (parts of) their anterior body temporarily into a cavity built by a partial inversion of the visceral sac (1).
12. Degree of retractibility. Although marine acochlidians, *Tantulum*, and *Strubellia* retract their head–foot completely (Fig. 1G) (0), *Rhodope*, *A. fijiense*, and probably all other large limnic acochlidians only partially retract the head–foot (1).
13. Visceral sac. The visceral sac is largely separated from the rest of the body in most shelled gastropods, *Platyhedyle*, and in Acochlidia (0), whereas it is an integrative part of the body in limpets and most externally shell-less gastropods (1).
14. Free visceral sac connection. The head–foot has a narrow connection to the free visceral sac in most shelled species and several acochlidians (Fig. 1H) (0), whereas a broader area of fusion is present in *P. cornuta* (Fig. 1C), *Hedylopsis* (Fig. 1J), *Strubellia* (Fig. 1A), *Palliohedyle*, and *Acochlidium* (1).
15. Mantle. The visceral sac is covered by a thin integument in shelled species (0), whereas it is

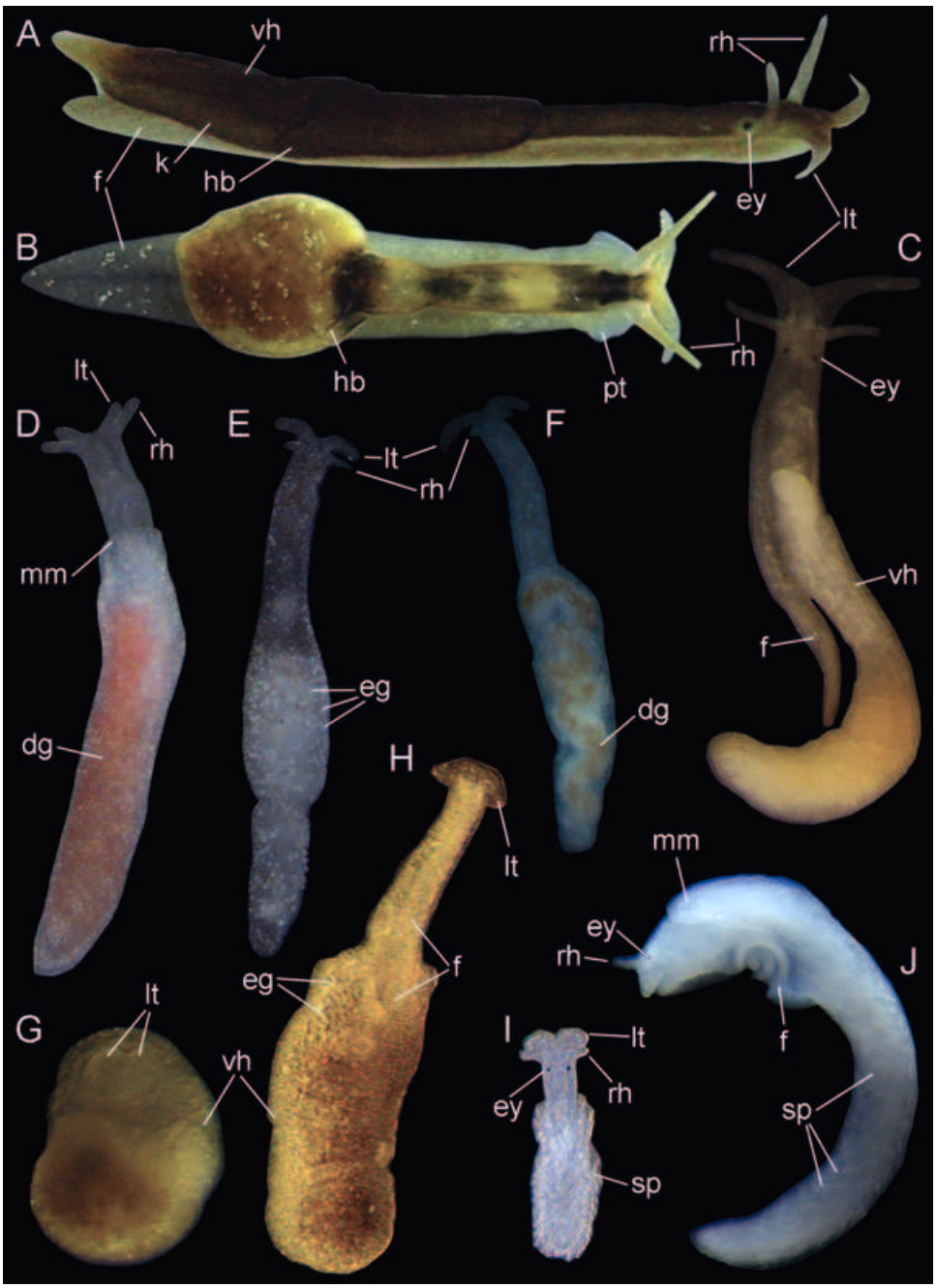

Figure 1. External morphology of living specimens of limnic (A and B) and marine (C–J) Acochlidia. A, *Strubellia* sp. from Vanuatu (subadult, 2-cm long), with large lateral eyes and a broad foot; B, *Acochlidium fijiense* from Fiji (2-cm long), note the propodial tentacles and the heart bulb; C, *Pseudunela* sp. from Vanuatu (3.5-mm long), with long, free posterior foot; D, *Asperspina rhopalotecta* from Italy (2-mm long), note anterior mantle margin forming a permanent rim; E, *Microhedyle glandulifera* from Italy (2-mm long), with epidermal glands; F, *Paraganitus* sp. from Vanuatu (1.5-mm long), with convoluted digestive gland; G, *Pontohedyle milaschewitchii* from Croatia (3-mm long; ventral view), with head–foot completely retracted into visceral hump; H, *P. milaschewitchii* from Italy (3-mm long), with short and blunt free posterior foot; I, *Hedylopsis spiculifera* from Italy, (juvenile, 1-mm long); J, *Hedylopsis ballantinei* from Egypt (5.5-mm long), note the net-like arrangement of spicules. Abbreviations: dg, digestive gland (shining through the integument); eg, epidermal gland; ey, eye; f, foot; hb, heart bulb; k, kidney (shining through the integument); lt, labial tentacle; mm, anterior mantle margin; pt, propodial tentacle; rh, rhinophore; sp, spicule; vh, visceral hump. A, right view; B–F, I, dorsal view; G, H, ventral view; J, left view.

covered with a robust mantle in externally shell-less species (1).

16. Anterior mantle margin. The anterior mantle margin forms a clearly distinct and permanent rim in most externally shelled heterobranchs, *Platyhedyle*, and several acochlidians, such as *Hedylopsis* (Fig. 1I, J) and *Asperspina* (Fig. 1D) (0), whereas it does not form a permanent rim in *Rhodope*, *P. cornuta*, *Strubellia*, *Palliohedyle*, *Acochlidium*, Microhedylidae, and Ganitidae (1).
17. Width of visceral sac. The visceral sac is usually narrower, to about as wide as the body (Fig. 1C) (0), whereas it is considerably broader than the head–foot in *Acochlidium* (Fig. 1B) and *Palliohedyle* (1).
18. Visceral hump shape. A visceral hump, i.e. a free visceral sac covered by a robust mantle, is present in *Platyhedyle* and Acochlidia only. The visceral hump is conical in living and preserved specimens of microhedylacean (Fig. 1E, H) and several hedylopsacean species (Fig. 1A, I) (0), is rather conical but medially depressed in *Platyhedyle* (1), whereas it is more or less depressed and oval in living specimens, changing to leaf-like and flattened in preserved specimens of *Acochlidium* (Fig. 1B) and *Palliohedyle* (2).
19. Tail length. The tail (free posterior foot) is relatively long in most gastropods (Fig. 1A–C) (0), is short and pointed in asperspinid and microhedylid acochlidians, and in *Paraganitus* (1), and is very short and blunt in *Platyhedyle*, *Pontohedyle milaschewitchii* (Kowalevsky, 1901) (Fig. 1H), and *Ganitus* (2), whereas the foot is fused with the body, i.e. with the head and the visceral sac, (almost) along its entire length in *Metaruncina*, *Philine*, *Pluscula*, and *Rhodope* (3).
20. Foot width. The foot is approximately as broad as the body, showing a cephalopedal groove in many gastropods, *Tantulum*, *Pseudunela* (Fig. 1C), *Hedylopsis* (Fig. 1J), and at least some Asperspinidae (0). The foot is broader than the body, e.g. in *Acochlidium* (Fig. 1B), *P. sutteri*, and *Strubellia* (Fig. 1A) (1), is narrow without showing a cephalopedal groove in *Asperspina loricata* (Swedmark, 1968), *Asperspina riseri* (Morse, 1976), Microhedylidae (Fig. 1H), and Ganitidae (2), whereas *Rhodope* has no discernable foot (3).
21. Parapodia. The whole foot sole may be used to crawl (0), or the foot edges may be bent upwards to form (short) parapodia in *Pluscula* and *P. exigua* (1).
22. Propodial tentacles. The anterior foot edge may be rounded (0) or laterally elongated into propodial tentacles in *Strubellia*, *Acochlidium* (Fig. 1B), and *P. weberi* (1).
23. Mantle cavity. A permanent mantle cavity is usually present in externally shelled marine gastropods, *Metaruncina*, *P. exigua*, and *Pluscula* (0). Pulmonates (except for *Smeagol*) have (at least parts of) the cavity modified into a lung (1). The cavity is largely reduced in *H. ballantinei* (2). Such cavities are absent in other acochlidians, *Rhodope*, and *Platyhedyle* (3). In contrast to their original descriptions, *Asperspina murmanica* (Kudinskaya & Minichev, 1978) and *P. cornuta* do not possess any mantle cavity or cloaca, respectively (Neusser & Schrödl, 2008; unpubl. data), and are coded as (3). *Asperspina rhopalotecta* was mentioned as forming a true cloaca by Wawra (1987); however, without mentioning the presence of a cavity, it is thus coded as (2, 3).
24. Gill. Although plicate gills are present in some basal heterobranchs and shelled opisthobranchs (0), plicate gills are entirely absent in *Odostomia*, pulmonates, *Platyhedyle*, *Rhodope*, *P. exigua*, *Pluscula*, and Acochlidia (1).
25. Anus position. In shelled heterobranchs, the anus usually opens in a more or less dorsal anterior position at the junction of the head–foot complex and mantle (0). In *Platyhedyle* (see Rückert *et al.*, 2008) and most Acochlidia, the anal opening is dextral, and is usually ventrolateral at the junction (1). Runcinids, *Pluscula*, and *P. exigua* have the anus in a terminal posterior position (2). In some acochlidians the anus opens on the visceral hump, either laterally in some microhedylids such as *P. milaschewitchii* (see Jörger *et al.*, 2008) (3), or ventrally in *Asperspina murmanica* (Kudinskaya & Minichev, 1978), *Strubellia*, and *Tantulum* (see Neusser & Schrödl, 2007) (4). *Rhodope* has the anal opening in a dorsolateral posterior position (5).
26. Juxtaposition between anus and nephropore. Opening into the mantle cavity, the anus and nephropore are more or less closely associated in most gastropods, *Rhodope*, and in most acochlidians that were studied sufficiently (0). In some microhedylid species, the anus and nephropore are apart from each other (1).
27. Rhinophores. According to Huber (1993), rhinophoral nerves are present in pyramidellids and opisthobranchs. Rhinophores, i.e. chemosensoric head appendages innervated by the rhinophoral nerves, are only present in nudipleurans, many sacoglossan taxa, and most acococchlidians (1). Rhinophores are absent in other euthyneurans, and also absent in *Pontohedyle* (Fig. 1H), *Ganitus*, *Platyhedyle*, and *Rhodope* (0).
28. Rhinophore length and shape. Acochlidian rhinophores are solid and smooth. They are more or less elongate digitiform and pointed in most

species (Fig. 1A–C) (0), but are relatively short, hardly mobile, and blunt in *Asperspina* (Fig. 1D) (1).

29. Oral tentacle nerves. According to Huber (1993), head appendages innervated by the nervus labiotentacularis are absent in most gastropods (0), but are present in opisthobranchs, including the acochlidians examined (1).
30. Oral (= labial) tentacles. Distinct organs are absent in e.g. *Platyhedyle*, *Rhodope*, *Metaruncina*, and Philinoglossidae (0), whereas they are present in all valid acochlidian species (1). Taxa without nervus labiotentacularis are coded as inapplicable.
31. Oral tentacle shape. Where present as distinct organs, oral tentacles are digitiform, with a broad base, and tapering in *Tantulum*, *Strubellia* (Fig. 1A), *P. cornuta* (Fig. 1C), *Acochlidium* (Fig. 1B), and *P. sutteri* (0), were illustrated as short and tapering in *P. weberi* by Bergh (1895: pl. 1, fig. 4) (1), are short and stout, hardly mobile, and somewhat flattened in *Asperspina* (Fig. 1D) (2), are flattened rounded lobes in *Hedylopsis* (Fig. 1I) (3), are flattened and hammerhead shaped in *Pontohedyle* (Fig. 1H) (4), are long and slightly recurved in microhedylids (Fig. 1E) and *Paraganitus* (Fig. 1F) (5), are short recurved lobes in *Ganitus* (6), and are more or less triangular lobes in *Acteon* and *Colpodaspis* (7).

Integument

32. Calcareous spicules. The vast majority of gastropods and some microhedylid acochlidians do not possess calcareous spicules (0), whereas other acochlidians (e.g. Fig. 1I, J), *Platyhedyle* (see Wawra, 1979), and *Rhodope* do have spicules (1).
33. Spicule shape. Where present, spicules may be needle-like, as in *Hedylopsis* or *A. murmanica* (see Neusser *et al.*, 2008) (0), chunky cylindrical rods, as in Acochlidiidae (1), irregular rounded rods, as in *P. cryptophthalma* (see Westheide & Wawra, 1974: fig. 3) (2), or stellate, as in *M. glandulifera* (see Kowalevsky, 1901: fig. 54) (3).
34. Spicules stiffening edge of visceral hump. Although absent in other acochlidians (0), both *Hedylopsis* species have a band of longitudinal spicules along the lateral visceral hump (1).
35. Spicule 'shell'. Where present in the visceral hump, spicules may be scattered irregularly (0), or may form a roof-like 'secondary shell' in *Hedylopsis* (see Sommerfeldt & Schrödl, 2005) and *Asperspina* (1).
36. Arrangement of roof spicules. Spicules are irregularly net-like in *Hedylopsis* (Fig. 1J) (0), and are transversally arranged in parallel in *Asperspina* (1).
37. Aggregation of spicules in head. Although usually absent (0), *Pontohedyle*, *Hedylopsis*, *A. rhopalotecta*, *Asperspina brambelli* (Swedmark, 1968), *A. loricata*, *A. murmanica*, most *A. riseri*, and *Tantulum* show aggregations of (short fusiform) spicules on the anterior head (1).
38. Integumental concrements. Although absent in other gastropods and most acochlidians (0), some microhedylids, ganitids, and *A. riseri* possess rounded or ring-shaped concrements that may be arranged in pearl strings (see Westheide & Wawra, 1974: figs 3, 4) (1).
39. Dorsomedian keel on visceral hump. Such a keel is absent in *Platyhedyle* and most acochlidians (0), whereas it is present (1) in all *Asperspina* species excepting *A. brambelli*, which is coded as unknown.

Central nervous system (CNS) and sensory organs

40. Tentacle nerves. According to Huber (1993), the nervus clypei capitis is present in caenogastropods and heterobranchs studied in sufficient detail (0), except for *Rhodope*, and all nudibranchs and acochlidians studied so far, where it is absent (1). *Metaruncina*, *Colpodaspis*, *Toledonia*, and *Pluscula* are coded as unknown.
41. Rhinophoral ganglia. According to Huber (1993), rhinophoral ganglia bearing a rhinophoral nerve (or the homologous nerve leading to the posterior part of the Hancock's organ) are present in several opisthobranchs, including some acochlidians (0), whereas they are described as being absent in pulmonates, some microhedylid acochlidians, and others (1). Because of its potential homology with opisthobranch rhinophoral ganglia (Neusser *et al.*, 2007b), we code the pulmonate procerebrum as unknown.
42. Accessory ganglia. Aggregations of precerebral 'accessory ganglia' are absent in most gastropods (0), whereas they are present in *Platyhedyle*, *Rhodope*, *Pluscula*, *P. exigua*, and most acochlidians such as *Tantulum*, Asperspinidae, Microhedylidae, and Ganitidae (1). In contrast to its original description, we could not detect any accessory ganglia in *P. cornuta*; *Pseudunela eirene* Wawra, 1988 is coded as unknown.
43. Cephalic eyes. Although usually present in most gastropods and acochlidians (0), eyes may be reduced or pigmentless as in *Tantulum* (1), or are completely lost (2) in some acochlidian species such as *Microhedyle remanei* (Marcus, 1953). *Pluscula* also lacks eyes.
44. Hancock's organ. Although absent in basal heterobranchs, *Acteon*, and pulmonates (0), such cerebrally innervated lateral sensory organs are present in most shelled opisthobranchs. Han-

cock's organs were also discovered in *Tantulum*, *P. milaschewitchii*, and *M. glandulifera* (see Neusser *et al.*, 2007b), and in *Strubellia* (B. Brenzinger, T.P. Neusser & M. Schrödl, unpubl. data) (1). Further acochlidian species are coded as unknown, and must be examined in sufficient histological detail.

45. Position of CNS. All acochlidian species known in detail show a prepharyngeal CNS (0), as do lower heterobranchs, *Amphibola*, *Acteon*, and many cephalaspideans including *P. exigua* and Philinoglossidae. In contrast, *Chilina*, *Myosotella*, Runcinidae, and sacoglossans including *Platyhedyle*, and *Rhodope*, show a postpharyngeal CNS (1).
46. Dorsal bodies. Although generally absent (0), dorsal bodies (see Saleuddin, 1999) associated with the dorsal surface of cerebral ganglia or the cerebral commissure are present in *Chilina* and *Myosotella* (1).
47. Cerebral glands. Although generally absent (0), the so-called cerebral glands are associated with the cerebral ganglia in pulmonates (1).
48. Lateral bodies. Although generally absent (0), the so-called lateral bodies (Neusser *et al.*, 2007b) attached to the lateral surface of cerebral ganglia are present in some acochlidian species (1).
49. Eye position in relation to rhinophores. Of the taxa with rhinophores, the eyes are situated closely posterior to the basis of the rhinophores in *Hedylopsis* (Fig. 1I, J) and several limnic acochlidians (Fig. 1A) (0), or are clearly posterior to the rhinophores and usually close to the cerebral ganglia (1). Although *P. milaschewitchii* lacks rhinophores, the eyes are located clearly posterior to the usual rhinophore position (1).
50. Length of optic nerves. Optic nerves are relatively long, e.g. in pulmonates, *P. exigua* and *Hedylopsis* (0), whereas they are short in *Rhodope* and several microhedylids (1).
51. Lateral eyes. Eyes are dorsally situated in most heterobranchs (0), whereas they are laterally situated (and laterally visible) in *Cylindrobulla*, *Strubellia* (Fig. 1A), *Acochlidium*, and *Palliohedyle* (1).
52. Cerebral commissure. According to Mikkelsen (1996), the cerebral commissure is long in acteonoids (except for *Hydatina*) and (in at least most) cephalaspideans. Although also long in *Amphibola*, *Myosotella*, and *Chilina* (0), the cerebral commissure is short in e.g. pyramidellids, runcinids, and all acochlidians studied in sufficient detail (1). The CNS of *Acochlidium* and *Palliohedyle* species was either not described or too poorly described to rely on.
53. Cerebropleural ganglia. Cerebral and pleural ganglia are separate in e.g. *Amphibola*, many basal pulmonates and cephalaspideans, and all acochlidians studied in sufficient detail (0), whereas they are fused in e.g. *Acteon*, *Rhodope*, and *Platyhedyle* (1).
54. Visceral loop ganglia. A pentaganglionate state is present in *Acteon* (Hoffmann, 1933) (0), whereas pyramidellids, most basal pulmonates, most cephalaspideans, *Platyhedyle*, and all acochlidians studied in detail by the authors have three (or sometimes four in *Tantulum*) separate ganglia (1). *Chilina* was considered to show a hexaganglionate condition: there are five separate ganglia including a fused right parietal and supraoesophageal ganglion, and an additional ganglion between the left parietal and suboesophageal ganglia (Haszprunar, 1985) (2). *Rhodope* has just one visceral loop ganglion (3). Some acochlidian species such as *Acochlidium amboinense, Asperspina riseri*, and *G. evelinae* were described to have only two separate ganglia on the visceral loop, but this is not yet considered reliable.
55. Visceral loop length. The visceral nerve loop is long in most of the outgroup taxa included (0), but is short in *Odostomia*, *Platyhedyle*, *Rhodope*, and Acochlidia species known in sufficient detail (1).
56. Euthyneury. The visceral loop is streptoneurous in *Chilina* and *Acteon* (0), whereas it is euthyneurous in other outgroup taxa and Acochlidia (1).

Digestive system

57. Oral tube. It is usually short (0), but is long in *Platyhedyle* and acochlidians (1), and forms a very long proboscis in pyramidellids (2).
58. Jaws. Jaws composed of cuticular elements are present in the pulmonates included and in *Acteon* (0). Jaws are lacking in Diaphanidae, sacoglossans and most acochlidians (1). A pair of thickened massive cuticular structures ('jaws' with unclear homology) seems to be present in Ganitidae (2). *Microhedyle glandulifera* may have jaw-like cuticular structures (Wawra, 1978), and is coded as unknown. Pyramidellids have a stylet (3).
59. Pharynx. The pharynx is usually bulbous and composed of a complex system of various muscles (0), whereas the pharynx is considerably modified in Ganitidae (see Rankin, 1979), showing well-developed longitudinal muscles that connect the jaws with a ventral cuticular radular cushion (1). Pyramidellids have a highly modified buccal sac with elongate buccal pump (2). The pharynx in *Rhodope* is poorly differentiated, wide, and sac-like (3).
60. Radula. Gastropod radulae are usually bilaterally symmetric with the same number of lateral teeth on each side (0); this is also true for some

acochlidian species such as *P. milaschewitchii*, with a radula formula of 41–54 × 1.1.1. (Jörger *et al.*, 2008). However, radulae are asymmetric in several other acochlidians (e.g. *H. ballantinei*; see Sommerfeldt & Schrödl, 2005), in having an additional tooth on the right side (1). Indonesian material of *S. paradoxa* was described as having a symmetric radula (Küthe, 1935), as were specimens from the Solomon Islands (Wawra, 1974); later, the latter were corrected to be asymmetric by Wawra (1979). Obviously, Bergh (1895) was not aware of asymmetric radulae in *H. weberi* either: whereas Bergh's text mentions one lateral plus one marginal teeth, his figures (plate 1, fig. 13a, b) suggest a marginal tooth on the right side only. Bücking (1933) illustrated marginal teeth on both radula sides of *A. amboinense*; however, this needs reconfirmation, and is coded here as unknown. In contrast to its original description, the radula of *A. murmanica* is asymmetric (Neusser *et al.*, 2008). *Odostomia* and *Rhodope* lack a radula (2).

61. Descending limb. Although usually absent (0), a descending radula limb (Mikkelsen, 1996: figs 28–29) is present in sacoglossans and acochlidians (1). According to Marcus (1953), *Pluscula* also has a 'lower limb'.
62. Radular limb proportions. In Acochlidia, the upper ('ascending') ramus with unused, younger teeth is usually considerably longer than the ramus with teeth either in use or used (0); whereas rami are roughly equally long in several acochlidian species and in many sacoglossans, such as *Platyhedyle* and *Cylindrobulla* (1).
63. Radula row number. Although usually (many) more than 20 rows are present (0), *P. exigua*, *Pluscula*, *Platyhedyle*, and especially ganitid species have a greatly reduced number of rows, i.e. less than 20 (1).
64. Rachidian teeth. Although usually present (0), *Acteon*, *Colpodaspis*, and *P. exigua* lack rachidian teeth (1).
65. Rachidian tooth shape. Where present, there is an enormous variety of different shapes of heterobranch rachidians. The pulmonate *Chilina* shows an asymmetric, tricuspid central tooth (0), whereas it is elongate and unicuspid in *Myosotella myosotis* (Draparnaud, 1801) (1). Within basal opisthobranchs, *Cylindrobulla* and *Toledonia* have well-developed, triangular rachidians, with broad bases, which are similarly present in most acochlidians and *Amphibola* (2). Rachidians are dagger-shaped in *Platyhedyle* and Ganitidae (3).
66. Rachidian cusp. Central cusps may be small (0), projecting (1), very elongate in Acochlidiidae and *Strubellia* (2), or are large and flat in *Amphibola* (3).
67. Rachidian tooth denticles. The triangular central teeth have well-developed denticles (between two and five denticles on each side of a prominent central cusp) in *Toledonia* and in most acochlidians (0), have more than six well-developed denticles in *Cylindrobulla*, *A. brambelli*, and *P. weberi* (1), and have numerous tiny denticles in some limnic Acochlidiidae and *Strubellia* (2); denticles are absent (3) in *A. amboinense*, *A. bayerfehlmanni*, and Ganitidae.
68. Lateral teeth. Although most euthyneurans have at least several lateral teeth (0), *Toledonia* and most acochlidians only possess one or two lateral teeth (1). *Cylindrobulla*, *Platyhedyle*, and ganitid acochlidians lack any lateral teeth (2).
69. Rectangular first lateral tooth. Although usually absent (0), *Toledonia* and acochlidian species (where present) have delicate rectangular plates (1).
70. Denticles on rectangular first lateral tooth. *Toledonia* and several acochlidian species such as *A. murmanica* or *P. milaschewitchii* (see Jörger *et al.*, 2008: fig. 7c) have rectangular first lateral teeth with one spiny denticle (0), with a blunt projection, e.g. *M. remanei* (see Neusser *et al.*, 2006) (1), or lacking any such structure (2).
71. Second lateral tooth. The second lateral tooth may be a quadrangular plate, as in *A. murmanica* (0), a slender spine, as in *Hedylopsis* (1), more or less hook-like (2), or is absent (3).
72. Oesophageal caecum. Although usually absent (0), a pouch or diverticle is present in *Toledonia* and *Cylindrobulla* (1). The potential homologue in runcinids is also coded as present.
73. Posterior oesophagus cuticle. Although generally absent (0), a cuticular lining in the posterior oesophagus is present in *Acteon* and many cephalaspideans (1).
74. Gizzard plates. The cuticular lining in the posterior oesophagus may be smooth (0) or form plates (1).
75. Digestive gland. The digestive gland forms two major lobes in *Acteon*, *Platyhedyle*, and many basal pulmonates (0), is lobe-like (i.e. at least externally compact) in *Pluscula*, most basal opisthobranchs, and most acochlidians, including the limnic *Tantulum* and *Strubellia* (1), bears several tubes (Challis, 1969) in *P. exigua* (2), but is ramified (cladohepatic) in at least *A. amboinense*, *A. bayerfehlmanni*, *A. fijiense*, and *P. weberi* (3).
76. Digestive gland shape. The single-lobed digestive gland may be a large elongate sac that fills out the visceral hump (Fig. 1D) (0), or a long,

slender, actively contractile and mobile, sometimes looped, tube (Fig. 1F) (1).

77. Intestine. The intestine is short in *Platyhedyle* and all acochlidians (0), is a prolonged tube in *P. exigua*, philinoglossids, and runcinids (1), and is longer and looped in others (2).

Excretory and circulatory systems

78. Kidney. The kidney is small, simple, and sac-like in *Toledonia* and most marine acochlidians (0); it is a considerably elongated sac in *Hedylopsis* (1), and is a long, longitudinally divided tube in *T. elegans*, *Strubellia*, and *Pseudunela cornuta* (2). The kidney of *A. amboinense* was inadequately described by Bücking (1933), and is coded as unknown here.
79. Position of pericardium and heart. The pericardium and heart are situated on the left side of the body cavity in many basal euthyneurans, either transversely or with the atrium anterior of the ventricle (0). The pericardium/heart is on the right side in *P. exigua* and *Pluscula*, and with the atrium posterior of the ventricle in acochlidian species (1). *Rhodope* and *Platyhedyle* lack a heart (2).
80. Heart bulb. Although absent in all other species analysed herein (0), the heart is situated within a bulbous expansion of the (right body) wall in *P. cornuta*, *Strubellia* (Fig. 1A), and at least some Acochlidiidae (Fig. 1B), in a strict sense (1).

Reproductive system

81. Sexes. Pyramidellids and euthyneurans are generally hermaphrodites (0), except for the gonochoric acochlidian Microhedylidae and Ganitidae (1). *Parhedyle gerlachi* (Marcus & Marcus, 1959) and *Parhedyle tyrtowii* (Kowalevsky, 1900) need (re-)examination.
82. Differential maturity. Mikkelsen (2002) reports that all opisthobranchs are simultaneous hermaphrodites, although the gonads of many species appear to be functionally protandric prior to becoming simultaneous hermaphrodites later, as is observed in many pulmonates (0). A few acochlidian species such as *Tantulum*, *S. paradoxa*, and *H. spiculifera* are known to be real sequential hermaphrodites that completely reduce male parts of their reproductive system during female maturation (1).
83. Ampulla. Although supposed to be present in most opisthobranchs (Gosliner, 1994), an ampulla is undescribed for many taxa. An ampulla may be a simple tubular swelling, e.g. in *P. milaschewitchii* and *M. remanei* (0), a large, blind-ending sac with separate entrances of the spermoviduct in *Hedylopsis* (1), a large sac with one opening in *A. murmanica* and, probably, *Tantulum* (2), or, according to Haase & Wawra (1996), a system of 'communicating chambers' in *A. fijiense* (3).
84. Reproductive system. Pyramidellids and many lower heterobranchs and acochlidians have a monaulic system with female and male gonoducts sharing a common opening (0), whereas *Acteon*, *Tantulum*, and *P. cornuta* have an androdiaulic system (1). Gonochoric species are coded as inapplicable. Several species such as *Pluscula* and *A. amboinense* need to be re-examined, and are coded here as unknown.
85. Gonoduct separation. In diaulic species, the vas deferens separates from the vaginal duct in a very distal position in *Tantulum* and *P. cornuta* (0), whereas it separates in a more proximal position in *Acteon*, *Chilina*, and the sacoglossan *Cylindrobulla* and *Platyhedyle* (1).
86. Female ciliary band. Although generally absent (0), a more or less broad dextrolateral ciliary band is present in females of at least several microhedylid and all ganitid species (1). A potential function could be the transport of eggs. The ciliary band-like structure of the hermaphroditic *H. ballantinei* (see Sommerfeldt & Schrödl, 2005: fig. 2a) is coded as unknown because of the unclear homology.
87. Position of male genital opening. As in most basal heterobranchs the spermoviduct of *Hedylopsis* and *Strubellia*, and the sperm duct of most microhedylacean species (males), is short, and opens dextrolaterally at the level of the mantle fold (0). The male genital opening of *A. amboinense* is positioned more anteriorly, at the level of the posterior end of the pharynx (1); that of *P. cornuta* and most Acochlidiidae is positioned below the right rhinophore (2). *Pontohedyle milaschewitchii* has a frontal male genital opening (i.e. above the mouth; Jörger *et al.* (2008) (3).
88. Sperm groove. Although usually present in monaulic species as well as in the diaulic *Chilina* and *Cylindrobulla* (0), a sperm groove is absent in other androdiaulic species and *Amphibola* (1). *Philine exigua*, *Pluscula*, *A. amboinense*, and *A. bayerfehlmanni* are coded as unknown.
89. Vas deferens appendix. Although usually absent (0), a blind ending duct with unknown function is connected to the distal vas deferens in some microhedylids (1).
90. Spermatophores. Most heterobranchs do not have spermatophores (0), whereas pyramidellids, runcinids, and most aphallic microhedylacean species are already known to use spermatophores for sperm transfer (1).

91. Spermatophore placement. Although spermatophores are usually transferred into (or placed at) the vagina of the mate (0), asperspinid and microhedylid acochlidians place their spermatophores anywhere on the body of the mate (1).
92. Cutaneous insemination. Although generally absent (0), allosperm directly penetrates the body tissue in some acochlidians, such as *P. milaschewitchii* (1).
93. Copulatory organ. Although usually present in heterobranchs (0), acochlidian Asperspinidae, Microhedylidae, Ganitidae, and *H. ballantinei* appear to lack any copulatory organ (1).
94. Protrusible penis. The copulatory organ is not protrusible in *Acteon* (0), whereas it is retractile in most other euthyneurans (1).
95. Cephalic prostate. The cephalic (backwards leading) vas deferens may connect to the penis via a tubular prostate, as in *T. elegans* (see Neusser & Schrödl, 2007: fig. 7) (0), or the tubular prostate may be absent (1).
96. Ejaculatory duct. The penis bears an external sperm furrow in pyramidellids (0), whereas most euthyneurans have an ejaculatory duct (1).
97. Penis shape. Where present, the penis is a muscular papilla, e.g. in *Tantulum* (Fig. 2A) (0), but is a giant and blunt organ in all other limnic acochlidian species (1). *Amphibola* has a complex spermovipositor (2).
98. Penial stylet. Although usually absent, e.g. in *Tantulum* and *Strubellia* (Fig. 2A, D) (0), the penis bears an apical hollow cuticular stylet (obviously used for hypodermal injection) in *Platyhedyle*, in at least some *Rhodope*, in *Pseudunela* (Fig. 2C), and in *Hedylopsis spiculifera* (Fig. 2B), and also in other limnic acochlidians (1). *Acochlidium bayerfehlmanni* and *P. weberi* are not known in sufficient detail.
99. Ejaculatory finger (see Haase & Wawra, 1996). Although usually absent (0), the penis with stylet is elongated into a slender muscular ejaculatory finger in at least *Pseudunela* (Fig. 2C), *A. fijiense*, and probably *Palliohedyle sutteri* (1).
100. Basal swelling. Although usually absent (0), a muscular basal penial swelling that is neither directly associated with the ejaculatory duct, nor with a paraprostate (see below), is present in *Tantulum* and *H. spiculifera* (Fig. 2A, B) (1).
101. Basal finger (see Haase & Wawra, 1996). Although usually absent (0), an accessory paraprostate connected to a so-called basal finger with apical hollow stylet is present in *P. cornuta* (Fig. 2C), *Strubellia* (Fig. 2D), *A. fijiense*, and probably *P. sutteri* (1).
102. Basal penial thorn. Although usually absent (0), the penial complex shows a basal curved thorn in *H. spiculifera* (Fig. 2B), *Strubellia* (Fig. 2D), and *A. fijiense* (1).
103. Rows of cuticular spines. Although absent in other phallic species (0), members of *Acochlidium* and *Palliohedyle* show semicircles or ascending spirals of cuticular penial spines (1). As demonstrated by Haase & Wawra (1996), the arrangement of penial spine rows (semicircles vs. ascending spiral) in *A. fijiense* depends on the degree of penis contraction.
104. Number of penial spines. A low number of 14–18 spines was mentioned for *A. sutteri*, *A. bayerfehlmanni*, and *A. amboinense* (0), whereas *A. fijiense* and *P. weberi* have more than 30 spines (1).
105. Bursa copulatrix. A distal bursa copulatrix or gametolytic gland is present in many heterobranchs, in *Tantulum*, *P. cornuta*, and in the female phase of *S. paradoxa* (0), whereas it is absent in other acochlidians with genital systems studied in enough detail (1). *Acteon* has an allosperm receptacle with sperm storage and lytic function.
106. Receptaculum seminis. An allosperm-nourishing receptacle is present in many heterobranchs, as well as in *P. cornuta* and *S. paradoxa* (0), whereas it is absent in other acochlidians (1).
107. Sperm heads. Although elongate in *Odostomia*, *Acteon*, *Tantulum*, and all sufficiently studied asperspinid, ganitid and microhedylid species (0), sperm heads are short in *Hedylopsis*, *Pseudunela*, *Strubellia*, and *A. fijiense* (1).

The following characters or character sets were not considered for this analysis. They are likely to be useful for future cladistic analyses as soon as more information on the homology of characters, outgroup conditions and distribution of character states within the acochlidian species are available.

Central nervous system (CNS)

108. Rhinophoral ganglia are separated into medulla and cortex in *Hedylopsis*, *A. murmanica*, and *Tantulum* (see e.g. Neusser & Schrödl, 2007: fig. 4c). Rhinophoral ganglia are homogenous in *M. remanei*, *P. milaschewitchii* (see e.g. Jörger *et al.*, 2008: fig. 6d), and possibly in some other acochlidians with accessory ganglia. No reliable data exist on other acochlidians and outgroup taxa.
109. A double connective between cerebral and rhinophoral ganglia was found in *P. milaschewitchii* and *T. elegans* (see Neusser *et al.*, 2007b; Jörger *et al.*, 2008). A similar situation occurs in *Rhodope*, as well as in pulmonates

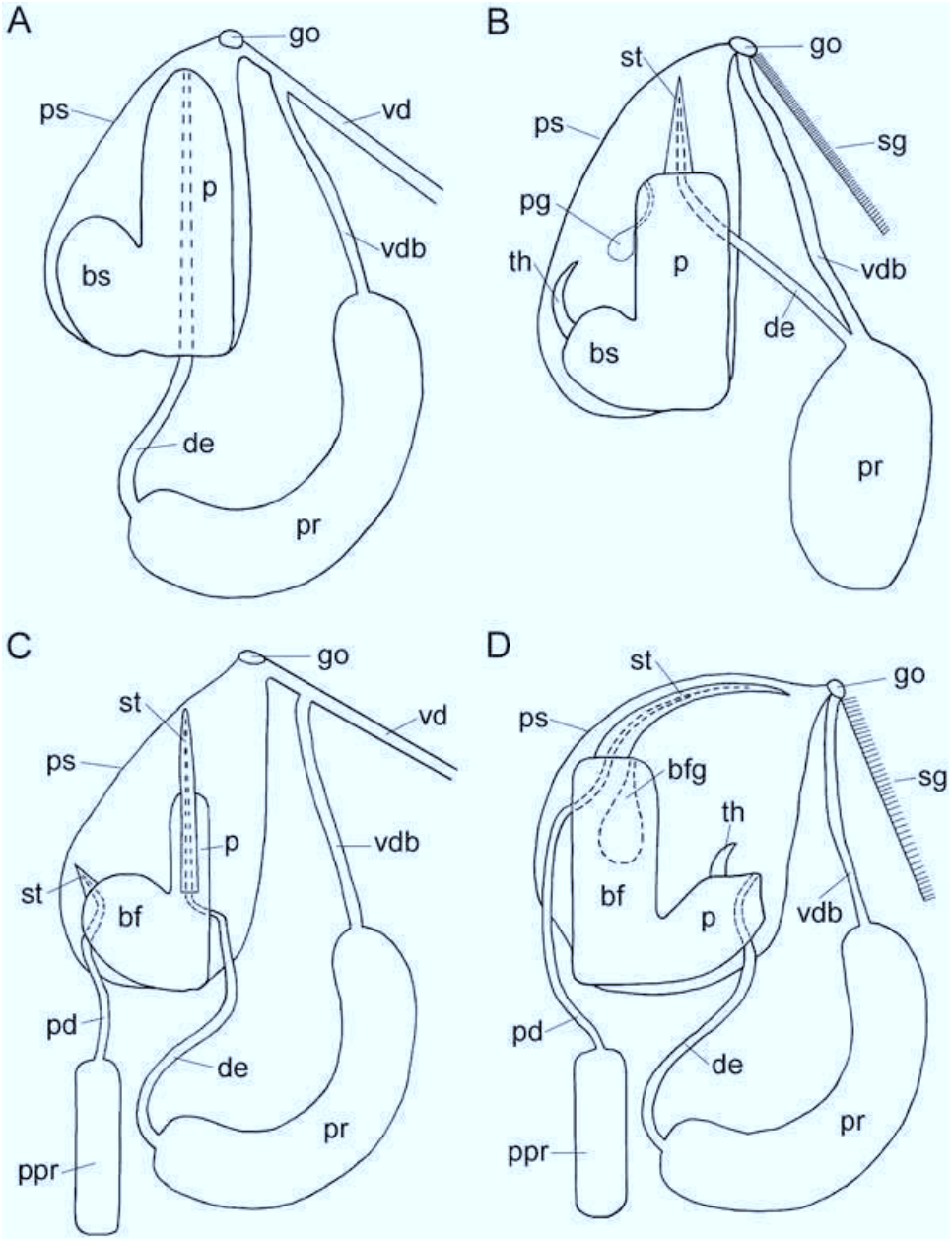

Figure 2. Schematic overview of the cephalic copulatory organs of different acochlidian species. A, *Tantulum elegans*; B, *Hedylopsis spiculifera*; C, *Pseudunela* spp.; D, *Strubellia* spp. Abbreviations: bf, basal finger; bfg, gland inside basal finger; bs, basal swelling; de, ejaculatory duct; go, copulatory/male genital opening; p, penis; pd, paraprostatic duct; pg, penial gland; ppr, paraprostate; pr, prostate; ps, penial sheath; sg, external sperm groove; st, hollow stylet; th, solid thorn; vd, vas deferens; vdb, back leading vas deferens. Not drawn to scale.

where the procerebrum has double connectives to the cerebral ganglia.

110. Eye sizes and diameters of optic nerves differ considerably between acochlidian species, as dicussed by Neusser *et al.* (2007b).
111. Distinct optic ganglia are present in *T. elegans* (see Neusser & Schrödl, 2007), as well as in *Strubellia* and *Pseudunela* species (B. Brenzinger, T.P. Neusser & M. Schrödl unpubl. data), whereas they are either absent or fused with other ganglia in other species (Neusser *et al.*, 2007b).
112. Optic nerve arrangement. Optic and rhinophoral nerves rise jointly from a rhinophoral ganglion in *H. ballantinei* and *H. spiculifera* (see Sommerfeldt & Schrödl, 2005; T.P. Neusser & M. Schrödl unpubl. data), whereas there is a separate optic

nerve rising from the cerebral (or intermediate optic) ganglion in other acochlidians, e.g. in *P. milaschewitchii* and *Tantulum* (Neusser & Schrödl, 2007; Jörger *et al.*, 2008).

113. Hypo/epiathroid CNS. This feature is difficult to assess in small acochlidian species that have a highly concentrated CNS. At least in *H. ballantinei*, pleurals are closer to the cerebral ganglia (Sommerfeldt & Schrödl, 2005).
114. The arrangement and branching of labial tentacular, oral, and rhinophoral nerves may also bear some variation within Acochlidia. For example the rhinophoral nerve inserts the cerebral rather than the rhinophoral ganglia in an unidentified *Asperspina* species from Florida (Hochberg, 2007), and *A. murmanica* (see Neusser *et al.*, 2008). Hochberg (2007) showed that applying immunocytochemical staining and confocal laser scanning techniques can greatly advance our understanding of nerve and ganglia arrangement, homology, and function in tiny acochlidians.
115. Otokonia vs. otolith. Several acochlidian species, e.g. *P. milaschewitchii* and *A. murmanica*, were found to have statocysts with one otolith (Jörger *et al.*, 2008; Neusser *et al.*, 2008), whereas many other opisthobranchs apparently possess several otoconia per statocyst (Wägele & Willan, 2000).
116. Gastrooesophageal ganglia were detected in *Strubellia* by Wawra (1988a), in *Tantulum* by Neusser & Schrödl (2007), and in *A. murmanica* by Neusser *et al.* (2008), whereas such ganglia were not found in *H. ballantinei*, *M. remanei*, and *P. milaschewitchii* (see Sommerfeldt & Schrödl, 2005; Neusser *et al.*, 2006; Jörger *et al.*, 2008). Elsewhere, gastro-oesophageal ganglia were reported from a variety of nudibranchs (see Wägele & Klussmann-Kolb, 2005) and at least some pleurobranchoideans (e.g. Martynov & Schrödl, 2008).
117. The radular nerve is single and unpaired in several acochlidian species, such as *Strubellia*, as described by Wawra (1988a), and *T. elegans* (see Neusser & Schrödl, 2007: fig. 2), whereas details are unknown from other acochlidians.
118. An osphradial ganglion is connected or attached to the supraintestinal ganglion in many prosobranchs and lower heterobranchs. An additional, osphradial ganglion connected with the supraintestinal ganglion is also present in several acochlidian species, such as *A. murmanica*, *H. ballantinei*, *Strubellia*, and *Tantulum* (e.g. Neusser & Schrödl, 2007: fig. 2) (0). An osphradial ganglion is absent in some other species studied in sufficient detail, e.g. *M. remanei* and *P. milaschewitchii* (1).
119. Pedal commissure. The pedal commissure is long in most basal opisthobranchs [but not in *Diaphana glacialis* (Odhner, 1907)], *Akera*, and *Aplysia* (0), whereas it is relatively short (1) in the Acochlidia studied in enough detail so far.
120. Lengths of the left pleuro-parietal and the right pleuro-supraintestinal/parietal connectives. They are short in *A. murmanica*, *H. ballantinei*, and *T. elegans* (see Sommerfeldt & Schrödl, 2005; Neusser & Schrödl, 2007; Neusser *et al.*, 2008); accordingly, the visceral nerve cord is short and the ganglia are located in the anterior part of the pharynx. In contrast, these connectives are longer in the microhedylid species, e.g. *M. remanei* and *P. milaschewitchii* (see Neusser *et al.*, 2006; Jörger *et al.*, 2008), and thus the visceral nerve cord is longer and the position of the ganglia is more posterior.
121. Genital ganglion. Although absent in all acochlidians studied in sufficient histological detail, the visceral nerve bears a separate genital ganglion in a posterior position in several cephalaspideans (Mikkelsen, 1996) and *Platyhedyle* (see Rückert *et al.*, 2008). The original report of a genital ganglion in *A. murmanica* by Kudinskaya & Minichev (1978) was shown to be erroneous by Neusser *et al.* (2008).

Digestive system

122. Salivary pumps and reservoirs. Pumps are stable organs situated proximally at the salivary duct; they are easier to detect than reservoirs, which attach to the pharynx and may collapse. Pumps and reservoirs are present in *Tantulum* (see Neusser & Schrödl, 2007: fig. 5b, e). A pump-like organ was reported for *P. weberi* by Bergh (1895: pl. 1, figs 5, 6), whereas a reservoir was detected in *A. murmanica* by Neusser *et al.* (2008: fig. 6c). There is inadequate information on most other acochlidians so far.
123. Stomach. A distinct stomach is present in most gastropods, including many opisthobranchs, and was described for some Acochlidiidae, such as *P. weberi* by Bergh (1895) and *A. amboinense* by Bücking (1933) (0). The stomach is considerably or completely fused with the digestive gland in other hedylopsacean, and, as far as is known, all microhedylacean acochlidians (1). The 'stomachs' originally described for *A. murmanica* and *P. milaschewitchii* are also fused with a distal cavity of the digestive gland, as shown by histological investigations (Jörger *et al.*, 2007a, 2008; Neusser *et al.*, 2008); a re-examination is also required for Acochlidiidae.

Reproductive system

124. Prostate-like glands. Glandular, possibly prostatic tissue covers the male deferent duct(s) in at least several gonochoric acochlidian species, such as *M. remanei* (see Neusser *et al.*, 2006). The character distribution and homology are as yet unclear.
125. Female glands. This is a complex organ system that shows some variation regarding the presence, arrangement, and structure of glands involved between the few acochlidian species studied in sufficient detail so far. For example, the mucus gland may be tubular as in *A. murmanica* and *P. milaschewitchii* (Jörger *et al.*, 2008; Neusser *et al.*, 2008), but is a blind sac in *M. remanei* (see Neusser *et al.*, 2006).
126. Gonad. In most lower heterobranch and opisthobranch species (see Wägele & Klussmann-Kolb, 2005) there may be an ovotestis with sperm and oocytes developing within the same follicles; according to Haase & Wawra (1996) and Sommerfeldt & Schrödl (2005), this is also the case in *A. fijiense* and *Hedylopsis* (0). Other opisthobranch species such as *Rhodope* may have separate male and female follicles (1), or the ovary and testis may be completely separated as described for *A. riseri* by Morse (1976) (2).
127. Female genital opening. In most heterobranchs and acochlidians, the distal oviduct (or spermoviduct) is short, and opens dorsolaterally or laterally on the right side at the level of the anterior mantle fold (0). Haase & Wawra (1996) described *A. fijiense* as having a very long internal spermoviduct extending anteriorly towards the female genital opening that is near to (but separate from) the cephalic penis (1). *Amphibola* has an anterior female genital opening at a common spermovipositor (2). The situation in other acochlidiids is unclear.
128. Reciprocal copulation. Most opisthobranchs with a penial papilla copulate reciprocally. This is possibly true also for *Strubellia* (see Wawra, 1992) and *Tantulum* (see Neusser & Schrödl, 2007) (0). The lack of allosperm receptacles and/or the possession of penial stylets or an aphallic condition indicates other acochlidians do not show reciprocal copulation (1); no observations on living specimens are available so far.
129. Hypodermic impregnation. Although generally absent, some acochlidian (e.g. *H. spiculifera* and *A. fijiense*), rhodopemorph, cephalaspidean, sacoglossan, and a few nudibranch species may show hypodermal injection via hollow penial stylets. Sperm may be injected specifically into the vaginal duct, the genital system, or elsewhere into the body. The details of many hedylopsacean species are still unknown.
130. Apical penial gland. At the basis of the penial stylet *H. spiculifera* has an accessory sac (Fig. 2B) joining the ejaculatory duct, whereas this organ is absent in any other phallic acochlidians studied in sufficient detail so far (T.P. Neusser & M. Schrödl unpubl. data).
131. Connection between genital system and digestive gland. Although not known from any other opisthobranch, such connections (one regular duct between the digestive gland and the distal gonoduct, and a transient connection between the ampulla and the digestive gland) were reported for *A. fijiense* by Haase & Wawra (1996). An examination of further specimens of this and other species is necessary.
132. Size and shape of spermatophores. Swedmark (1968) reported the spermatophores of *M. glandulifera* [described therein as *Microhedyle lactea* (Hertling, 1930)] to be thin sacs that were as long as the visceral sac, whereas they were half as long in *A. brambelli* (as *Hedylopsis*), and were very small in *P. milaschewitchii* (as *Microhedyle*) (see also Westheide & Wawra, 1974). Kirsteuer (1973) described spermatophores of *M. remanei* to be spindle-shaped, thin-walled, of about 200 μm in length and 25 μm in width. Thus, there seems to be considerable variation amongst asperspinid and microhedylid species, which should be studied in detail.
133. Sperm ultrastructure. The sperm of *H. ballantinei* is very different from sperm of *M. remanei*, *A. murmanica*, and *P. milaschewitchii* on the ultrastructural level regarding head and midpiece structure (Sommerfeldt & Schrödl, 2005; Neusser *et al.*, 2007a, 2008; Jörger *et al.*, in press). The latter three microhedylacean species have especially elongate spiral heads. Asperspinid and microhedylid nuclei show considerable variation with regard to absolute lengths and arrangement of nuclear keels. So far, no acrosomal vesicles could be detected, i.e. they are either very small or are absent. Also, the number and arrangement of glycogen helices differ considerably among acochlidian species. Sperm features are thus a very promising character set.

Ontogeny

134. Shape and structure of egg mass. Not enough information is available for comparison.
135. Egg number. *Acochlidium fijiense*, *M. glandulifera*, and *H. spiculifera* are able to produce ~20–50 mature eggs (0), whereas only one or a few large, yolky eggs mature at the same time in

some microhedylid species, such as *M. remanei* (see Neusser *et al.*, 2006) and *Microhedyle nahantensis* (Doe, 1974) (see Morse, 1994) (1). Comparative data is lacking.

136. Egg size and structure. Westheide & Wawra (1974) reported eggs of *P. cryptophthalma* to reach 450 µm in length. Eggs of other acochlidian species appear to be considerably smaller. Not enough comparative data is available.
137. Larval development. A free swimming planktonic veliger stage is usual for lower heterobranchs, *Chilina*, and most opisthobranchs (0). A shortened interstitial veliger stage was suspected for *M. glandulifera* and *H. spiculifera* by Swedmark (1968) (1). The development of *A. riseri* is completely intracapsular, releasing a crawling juvenile state (see Morse, 1994) (2). *Acochlidium fijiense* has a free-swimming veliger with unknown habitat preferences and timespan until metamorphosis. No data is available on other acochlidian species.

Further organs / characters

138. Food. Bergh (1895) reported *A. amboinense* as having animal remains in the stomach. Hadl *et al.* (1969) showed the mesopsammic *P. milaschewitchii* to prefer substrates with microbial mats, which could be a potential food source. There is no further data available on acochlidian food or feeding habits.
139. Visceral hump flexing. Being stressed by careful frontal touching, *A. fijiense* reacts by suddenly flexing its visceral hump upwards (M. Schrödl pers. observ.). Neither living *Strubellia* from Vanuatu nor any marine species observed by us shows such behaviour.
140. Body colour. Mesopsammic species are usually whitish, with a green to brownish digestive gland in *Pontohedyle* species, a brownish gland in *Paraganitus*, and an orange gland in *A. rhopalotecta*, *A. murmanica*, and *A. riseri*. Living limnic *A. amboinense* and *P. sutteri* are green, the visceral hump of *Strubellia* is brown in specimens from Vanuatu and brownish orange in specimens from the Solomon Islands, *A. fijiense* is cream-coloured with dark dorsal stripes; the living coloration of *P. weberi* is still unknown.
141. Special epidermal glands. The possession of large spherical epidermal glands giving the living animals a dotted appearance was mentioned to be characteristic for several microhedylaceans, such as *M. glandulifera* and *M. remanei* (see Kowalevsky, 1901; Marcus, 1953), but apparently not for *P. tyrtowii* and *P. cryptophthalma*; large glands may be also present in *A. riseri* and *A. brambelli*, and in some further species. Fluids of these glands may be responsible for the extreme adhesion of specimens to any kind of substrate and particle. The homology and distribution of such glands needs to be reinvestigated.
142. Mantle margin glands. Spherical epidermal gland cells (10 µm in diameter; type III according to Jörger *et al.*, 2008: fig. 4a) filled with dark-blue stained granules were found exclusively in one row on the anterior mantle margin in *P. milaschewitchii*.
143. Body ciliation. The head–foot complex and the visceral sac of *H. spiculifera* and *A. fijiense* are densely covered by cilia; just a few bundles of cilia are found on the head of *Paraganitus ellynnae* and *Parhedyle cryptophthalma* (see Jörger *et al.*, 2007b). Aggregations of long cilia, scattered especially on the head–foot complex, were described for at least *A. riseri*, *A. rhopalotecta*, *A. murmanica*, *M. glandulifera*, and *P. milaschewitchii* by scanning electron microscopy (Morse, 1976; Wawra, 1987; Jörger *et al.*, 2007b). Other acochlidians and outgroup conditions are unknown.
144. Cephalic ciliary bands. Although absent in several acochlidian species examined by Jörger *et al.* (2007b), two bands of cilia run along the oral tentacles of *P. milaschewitchii*, and one transversal band is in a rhinophore-like position (Jörger *et al.*, 2008). There is not enough comparative information yet, e.g. on the congener *P. verrucosa*.
145. Osphradium. Such chemosensoric organs are usually associated with the mantle cavity aperture of shelled gastropods. Several hedylopsacean species such as *Hedylopsis* and *Tantulum* have a putative osphradial ganglion (e.g. Sommerfeldt & Schrödl, 2005; Neusser & Schrödl, 2007), but appear to lack any histologically detectable osphradia. Ultrastructural research is needed.
146. Postpharyngeal spicule collar. Some acochlidians including *T. elegans* and *P. milaschewitchii* may show a special collar-like aggregation of spicules posterior to the pharynx (Neusser & Schrödl, 2007; Jörger *et al.*, 2008).
147. Anterior pedal gland. A distinct pedal gland that opens anteriorly between the mouth and the foot is present in *A. riseri*, *A. murmanica*, *Tantulum* elegans, and *P. milaschewitchii* (e.g. Morse, 1976; Robinson & Morse, 1976; Jörger *et al.*, 2008). Its distribution within Acochlidia, and homology with similar structures in some heterobranchs and pulmonates, is unclear.

148. Adductor/retractor muscles. One or two pairs of longitudinal muscles were mentioned in several acochlidian species: they are probably used for retraction of the head–foot complex. A comparison of their structure and position (i.e. homology with shell retractors) amongst acochlidian taxa is necessary before coding is possible.
149. Renopericardioduct. Although short in *Philine exigua*, *Pontohedyle milaschewitchii*, *M. remanei*, and *A. murmanica* (0), it is longer in *Pluscula*, *Hedylopsis*, *Tantulum*, *A. amboinense*, and *Strubellia* (1).
150. Ciliated funnel. Similar to the syrinx of nudibranchs, *H. ballantinei*, *Tantulum* and *Strubellia* show a funnel-shaped nephrostome with a strong ciliary tuft at the beginning of the renopericardioduct (e.g. Neusser & Schrödl, 2007: fig. 6e). A ciliated proximal region is also present in *Paraganitus* and *Ganitus*, but seems absent at least in *P. milaschewitchii*, *M. remanei*, and *A. murmanica*. *Acochlidium amboinense* was said to have numerous ciliated nephrostomes originating in the pericardium (Bücking, 1933).
151. Nephroduct. The nephroduct is short and/or undifferentiated in most opisthobranch and marine acochlidian species (0), whereas it is long and looped in *T. elegans* (see Neusser & Schrödl, 2007), *S. paradoxa*, and, judging from Bücking's (1933) scetchy drawings, also in *A. amboinense* (1).
152. Heart. *Hedylopsis ballantinei*, *M. remanei*, and *T. elegans* were shown to possess a two-chambered heart (Fahrner & Haszprunar, 2002; Sommerfeldt & Schrödl, 2005; Neusser *et al.*, 2006; Neusser & Schrödl, 2007), as is usual in opisthobranchs (0). This is in contrast to Rankin's (1979) claim that all microhedylids have reduced hearts. Some variation of heart sizes and structures may, however, exist amongst acochlidian species, and this must be investigated with an adequate methodology. For example, *P. milaschewitchii* and *A. murmanica* have a reduced, probably one-chambered heart (Jörger *et al.*, 2008; Neusser *et al.*, 2008) (1).
153. Excretion and locality of ultrafiltration. *Hedylopsis ballantinei* was shown to possess an auricular filtration and excretion system that is plesiomorphic for opisthobranchs (Fahrner & Haszprunar, 2002). In particular, limnic or heartless (if any) acochlidians may show considerable modifications, e.g. Küthe (1935) reported on special cell layers (with still unknown function) on the ventricle of *S. paradoxa*.
154. Diaphragma. A diaphragma separating the head–foot from the visceral cavity is present in many basal opisthobranchs, and in all acochlidians studied in sufficient detail.

RESULTS

A parsimony analysis was performed on 38 taxa (11 outgroup and 27 ingroup taxa) using 107 characters based on ecology (2) and morphology (105). All characters were unordered, and all were given equal weight. Five characters are parsimony-uninformative (numbers 54, 56, 72, 94, and 96). Accelerated transformation (ACCTRAN) was used for character state optimization. Trees were unrooted. The heuristic search produced 600 equally parsimonious trees, with a length of 262 steps. The consistency index (CI) is 0.5725. The homoplasy index (HI) is 0.4275. The CI excluding uninformative characters is 0.5625, and the HI excluding uninformative characters is 0.4375. The retention index (RI) is 0.8140, and the rescaled consistency index (RC) = 0.4660. Of the 102 parsimony-informative characters, 57 show homoplasies in the strict consensus tree, i.e. character states that either evolved more than once or showed at least one reversal within the ingroup.

In the strict consensus tree (Fig. 3) the pyramidellid *Odostomia*, pulmonates, a clade of *Acteon* (Architectibranchia) and *Cylindrobulla* (Sacoglossa), and a clade composed of cephalaspidean opisthobranchs, and Acochlidia form a basal polytomy. After *Colpodaspis* and *Toledonia* (both Diaphanidae) branch off from the stem line, the Acochlidia originate as a sister group to a clade composed of *Metaruncina* (Runcinidae) and a clade with *Philine* (Philinidae) and *Pluscula* (Philinoglossidae). The Acochlidia is clearly monophyletic, giving a bootstrap value (BT) of 100 and a Bremer support value (BS) of 9. There are six nonhomoplastic synapomorphies in the main analysis (i.e. loss of shell, head–foot being retractile into temporary cavity, loss of mantle cavity, loss of the tentacle nerve, long oral tube, and imprecise placement of spermatophores). Seven further acochlidian synapomorphies are homoplastic, i.e. have character states that also evolved elsewhere or show reversals within acochlidian subgroups, e.g. acochlidian rhinophores were lost in *Pontohedyle* and *Ganitus*, calcareous spicules were described to be absent in *P. ellynnae* and *M. remanei*, and a descending radula limb also occurs in sacoglossans. The basal acochlidian dichotomy bears one clade (BT 68, BS 3) comprising all hedylopsacean taxa *sensu* Wawra (1987). The limnic Caribbean *T. elegans* is the first offshoot. The remaining hedylopsaceans (BT 59, BS 3) are composed of marine interstitial *Hedylopsis* (BT 99, BS 6) plus a clade (BT 58, BS 1) of the likewise marine *Pseudunela* (BT 53, BS 1), and a

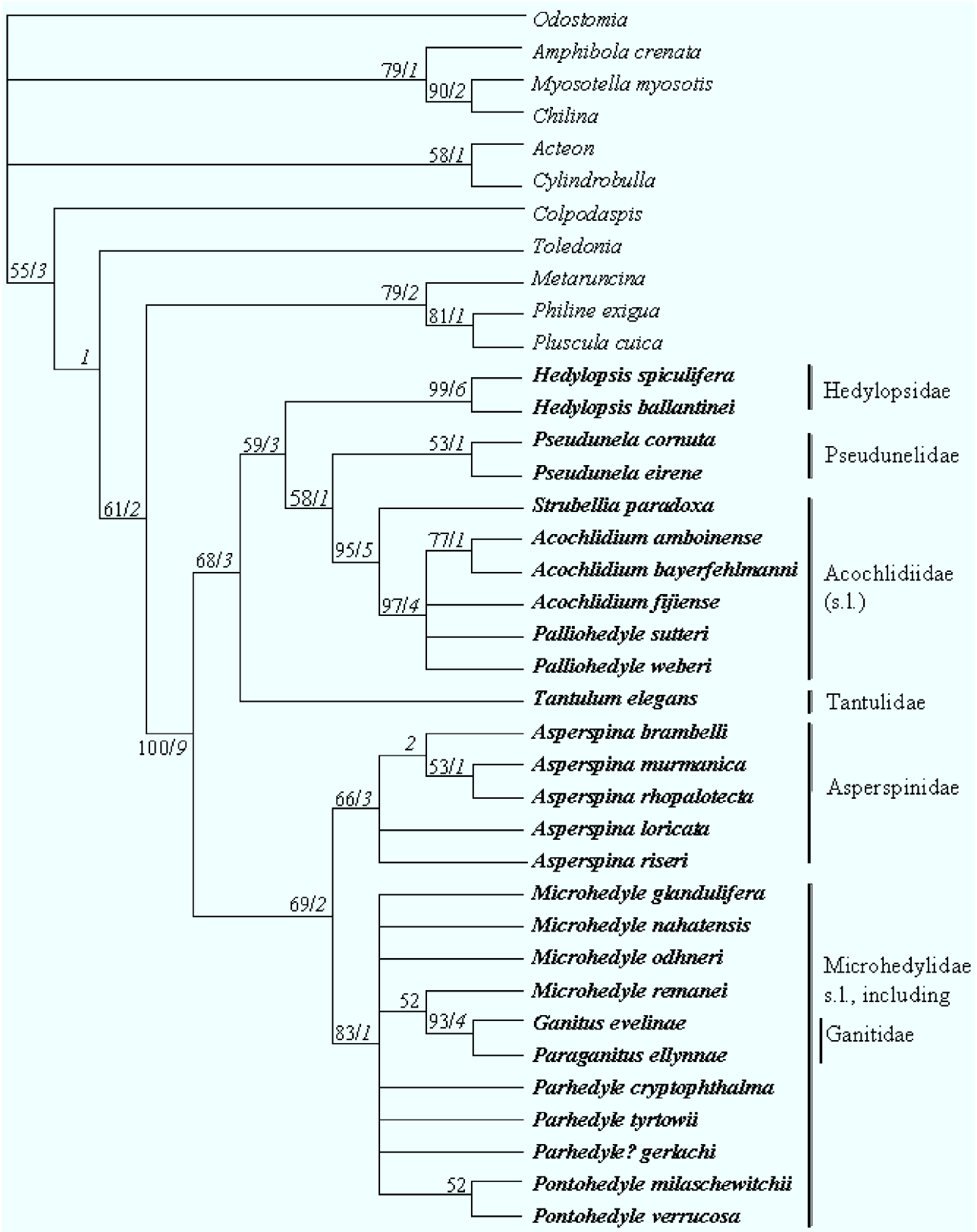
Odostomia
Amphibola crenata
79/1
Myosotella myosotis
90/2
Chilina
Acteon
58/1
Cylindrobulla
Colpodaspis
Toledonia
55/3
Metaruncina
79/2
Philine exigua
81/1
Pluscula cuica
1
Hedylopsis spiculifera
99/6
Hedylopsis ballantinei
Hedylopsidae
59/3
53/1
Pseudunela cornuta
Pseudunela eirene
Pseudunelidae
58/1
61/2
Strubellia paradoxa
Acochlidium amboinense
77/1
95/5
68/3
Acochlidium hayerfehlmanni
Acochlidium fijiense
97/4
Palliohedyle sutteri
Palliohedyle weberi
Acochlidiidae (s.l.)
Tantulum elegans
Tantulidae
Asperspina brambelli
2
100/9
53/1
Asperspina murmanica
Asperspina rhopalotecta
66/3
Asperspina loricata
Asperspina riseri
Asperspinidae
Microhedyle glandulifera
69/2
Microhedyle nahatensis
Microhedyle odhneri
Microhedyle remanei
52
93/4
Ganitus evelinae
83/1
Paraganitus ellynnae
Ganitidae
Parhedyle cryptophthalma
Parhedyle tyrtowii
Parhedyle? gerlachi
52
Pontohedyle milaschewitchii
Pontohedyle verrucosa
Microhedylidae s.l., including

Figure 3. Phylogeny of the Acochlidia. Strict consensus tree of 600 equally parsimonious trees, obtained by cladistic analysis (PAUP) of the data matrix given in Table 1 (excluding *Platyhedyle* and *Rhodope*). All characters were treated as unweighted and unordered. The tree was unrooted. Numbers above the branches refer to bootstrap values (< 50 not indicated), and were obtained by a separate analysis (1000 replications, PAUP) with the same settings. Numbers in italics are Bremer decay values (> 0) calculated with PRAP 2.0 (see Müller, 2004). Acochlidian species are set in bold face. Vertical bold lines with family names indicate the modified acochlidian classification proposed herein.

well-supported clade (BT 95, BS 5) of all known large, limnic or brackish-water, tropical Indo-Pacific species. *Strubellia paradoxa* is the sister group of the Acochlidiidae *sensu* Wawra (BT 97, BS 4), comprising the genera *Acochlidium* and *Palliohedyle*, which, however, may not be monophyletic. *Acochlidium amboinense* and *A. bayerfehlmanni* result as a clade (BT 77, BS 1). The other basal acochlidian clade comprises the marine mesopsammic Microhedylacea (BT 69, BS 2). Within the Asperspinidae (BT 66, BS 3), *A. murmanica* and *A. rhopalotecta* form a poorly supported clade (BT 53, BS 1) as a sister group to *A. brambelli* (BS 3). Asperspinids are the sister group of a clade (BT 83, BS 2) composed of largely unresolved Microhedylidae *s.l.*, including the Ganitidae (BT 93, BS 4) as a poorly supported sister group (BT 52, BS 1) to *M. remanei* (Fig. 3). The genus *Pontohedyle* has a low BT of 52. The 50% majority rule bootstrap tree is identical to the strict consensus tree, except it does not recover the sister-group relationship of *A. brambelli* with *A. rhopalotecta* and *A. murmanica*. Omitting the two ecological characters from the analysis had no influence on the ingroup topology of the strict consensus tree.

When including the mesopsammic sacoglossan *Platyhedyle denudata* Salvini-Plawen, 1973 as an additional taxon (tree not shown), it results as the sister group of Acochlidia. The latter is still monophyletic, with a BT of 69. The topology of the strict consensus tree within acochlidians does not change. The addition of the enigmatic *Rhodope* to the main analysis results in its placement as a sister group of the still monophyletic Acochlidia: their internal topology remains unchanged. Adding both *Platyhedyle* and *Rhodope*, they form the sister clade to Acochlidia: the BT for monophyletic Acochlidia decreases to 61, but again, the topology of the strict consensus tree for acochlidians is not affected.

DISCUSSION

Origin of Acochlidia

This first cladistic analysis of Acochlidia was based on the available bibliographic data on morphology and biology of all valid acochlidian species. A broad set of euthyneuran outgroup taxa was used to appropriately root the Acochlidia for reconstructing inner relationships, rather than to clarify the origin of Acochlidia. However, some tendencies are evident from the present analysis (Fig. 3). The Acochlidia do not form part of a clade composed of Sacoglossa, pulmonates, and Pyramidelloidea, as resulted from the analysis of multiple molecular markers by Klussmann-Kolb *et al.* (2008). Neither is there support for earlier assumptions of a direct relationship of Acochlidia with diaphanid cephalaspideans such as *Toledonia* (see Sommerfeldt & Schrödl, 2005), which shows a similar radular morphology with acochlidians. Instead, the main analysis indicates a sister-group relationship of acochlidians with small runcinid and mesopsammic philinid and philinoglossid cephalaspideans, as was already suggested by Wägele & Klussmann-Kolb (2005). However, such a topology contradicts the available molecular studies (Vonnemann *et al.*, 2005; Klussmann-Kolb *et al.*, 2008), and is not robust against including further small and interstitial opisthobranch species into the morphological analysis: when the sacoglossan *P. denudata*, the still enigmatic *Rhodope*, or both taxa are added, they always appear as direct sister taxa of Acochlidia (trees not shown). The concerted convergent evolution of small, worm-like bodies, and reductions expressed by mesopsammic members of different clades, appears to outnumber and mask the true phylogenetic signal in morphological analyses. From the morphological and molecular analyses available at present, we conclude that the Acochlidia thus may be best regarded as a basal opisthobranch or early cephalaspidean offshoot, as previously proposed by Odhner (1937) and Marcus (1953). A much broader taxon sampling and combined morphological and molecular approach is needed.

The fossil record dates the first opisthobranchs back to approximately 200 Myr, and most cephalaspidean families were present before some 150 Mya (see compilation of data in Wägele *et al.*, 2008). According to the phylogenetic hypothesis herein, this would be the (Jurassic) time frame expected for the origin of Acochlidia, which, because of their shell-less nature, lack any fossil record.

Monophyly of Acochlidia

Herein, the Acochlidia is clearly monophyletic, with a BT of 100 in the main analysis (Fig. 3). This is in accordance with traditional taxonomic observations

(e.g. Odhner, 1937; Marcus, 1953; Arnaud *et al.*, 1986; Wawra, 1987; Sommerfeldt & Schrödl, 2005), as well as with molecular results using combined sequence data from complete 18S and partial 28S rRNA genes (Vonnemann *et al.*, 2005), and multiple marker analysis (Klussmann-Kolb *et al.*, 2008). The only hypothesis thus far questioning the monophyly of Acochlidia, i.e. Gosliner (1994), who suspected the Ganitidae to be derived from *Platyhedyle*-like sacoglossan ancestors, is clearly dismissed by the present study. Even considering *Platyhedyle* as an acochlidian sister group, ganitids are always nested within Microhedylidae, and are thus confirmed as highly derived acochlidians. The presence of dagger-like teeth in both groups is undoubtedly the result of convergence. Such teeth are used for piercing algal cells in sacoglossans, and in ganitids may have a similar function; the food of ganitids (and of any of the other marine acochlidians) is, however, still unknown.

Adding the enigmatic euthyneuran taxon *Rhodope* to the main analysis, it appears as the direct sister group of Acochlidia rather than appearing within any acochlidian taxa; again, without affecting the inner acochlidian topology. On a morphological cladistic basis, the Rhodopemorpha were already regarded as a sister taxon to Acochlidia by Salvini-Plawen & Steiner (1996). Potential synapomorphies may be the presence of calcareous spicules, and an, although slight, retractibility of the anterior body portion of *Rhodope* (but not *Helminthope*) into a temporary cavity. However, unlike acochlidians, rhodopids have a truly worm-like body without showing a discernable foot or a visceral sac, making the homology of such partial body retraction questionable at best. Extreme morphological reductions, a supposedly high degree of convergence resulting from similar environmental selection pressures, similar functional constraints in tiny bodies, and a large number of unknown or inapplicable character states are serious impediments for reconstructing natural relationships. Therefore, phylogenetic analyses based on morphology alone may never become fully conclusive for rhodopids. Published molecular studies on Rhodopemorpha are not yet available. Preliminary sequence analyses on partial 28S rRNA genes of *Rhodope* and Acochlidia do not indicate any closer relationship (N. Wilson & M. Schrödl, unpubl. data).

Acochlidian apomorphies

Thirteen apomorphies support the acochlidian monophyly in the strict consensus tree; six of them represent nonhomoplastic features in the corresponding main analysis. However, none of those derived states is unique within opisthobranchs. Using a more complex character concept than that used herein, Sommerfeldt & Schrödl (2005) argued that the head–foot is retractable into a temporary cavity within the visceral hump in acochlidians only. In fact, elsewhere in opisthobranchs, only the sacoglossan *P. denudata* possesses a visceral hump that is distinctly offset from the head–foot complex and completely surrounded by a well-developed mantle. But, rather than being capable of any retraction, *Platyhedyle* curls up its body into a spiral when disturbed (e.g. Salvini-Plawen, 1973; Rückert *et al.*, 2008). Most other potential apomorphies of Acochlidia listed by Sommerfeldt & Schrödl (2005) are also confirmed herein, i.e. the presence of spicules, reduction of the mantle cavity, and the development of solid rhinophores that are innervated by the rhinophoral nerve only (Fig. 4). All of these features were, however, subject to modifications or reversals within certain acochlidian subgroups; e.g. the large limnic acochlidiids *Acochlidium* and *Palliohedyle* are no more fully retractable into their visceral humps, and *Pontohedyle* species have lost the rhinophores. According to the main analysis herein, the shell and the tentacle nerve were lost, the oral tentacles had evolved, the oral tube was elongated, and the anal opening had already shifted to the dextrolateral side in the acochlidian ancestor. However, the overall level of homoplasy is high (56% of parsimony-informative characters), and reconstructing autapomorphies of Acochlidia depends on the outgroup selection.

In contrast to assumptions made by Sommerfeldt & Schrödl (2005), a posteriori character state tracking (PAUP) in the present main analysis indicates that the absence of a receptaculum seminis is plesiomorphic for acochlidians, with the reinstatement by *P. cornuta* and *S. paradoxa*. A bursa copulatrix was apparently still present in the acochlidian ancestor, but was lost at least three times independently. Besides the probable absence of any mantle cavity or dorsal bodies (see Neusser *et al.*, 2007b, for acochlidian 'lateral bodies') in the acochlidian stem line, and the potential plesiomorphic presence of Hancock's organs, the acochlidian ground plan proposed by Sommerfeldt & Schrödl (2005) is confirmed by the present analysis as far as features are concerned.

Phylogeny and classification

The strict consensus tree obtained in the main analysis (Fig. 3) is well-resolved at the basal acochlidian and microhedylacean level, as well as throughout most of the hedylopsacean clade. This topology receives at least some statistical support for all but one node by the bootstrap analysis, and is surprisingly robust to modifications of outgroup and ingroup taxon sampling. However, some inner acochlidiid, asperspinid, and, in particular, microhedylid relation-

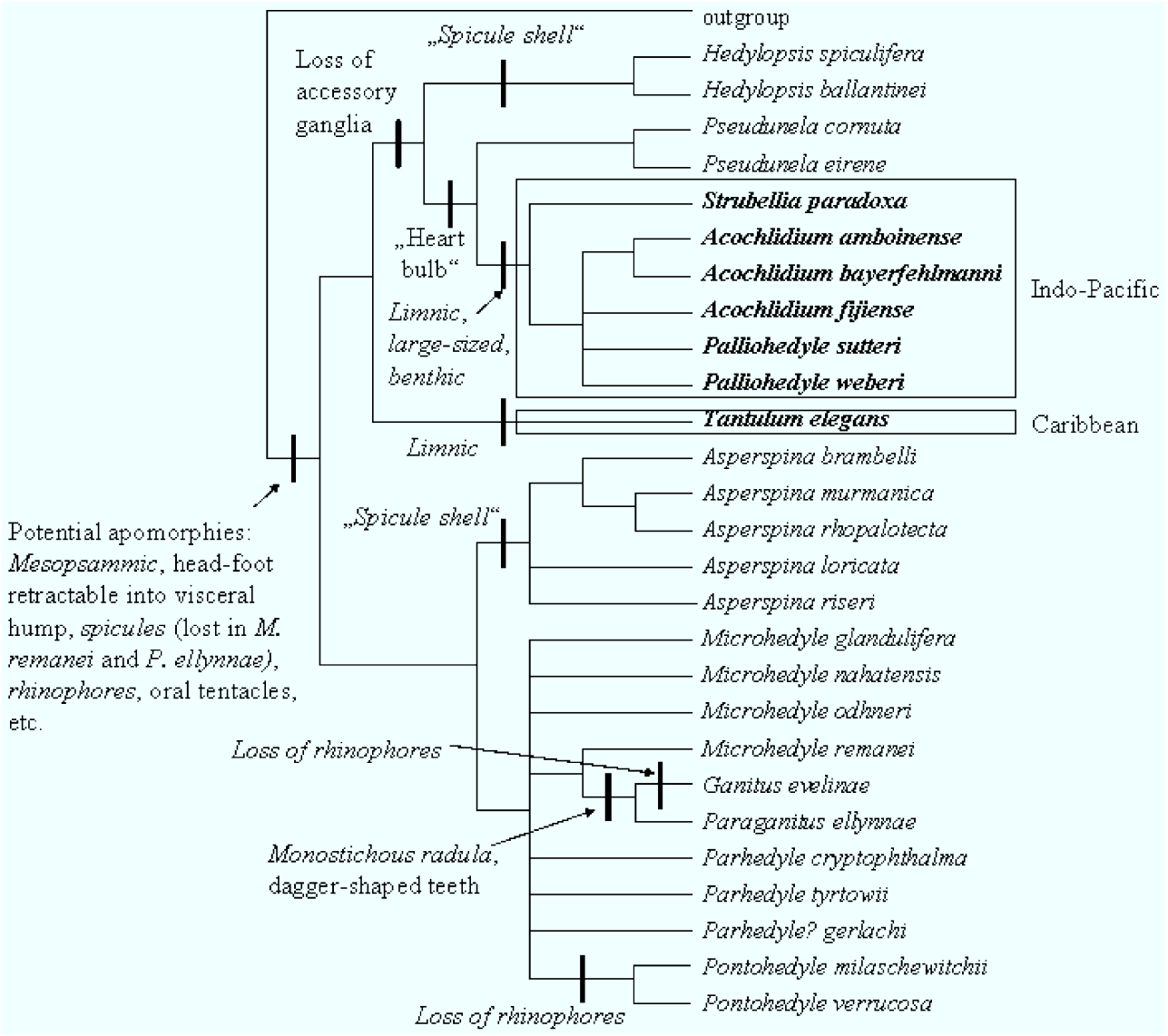

Figure 4. Evolution of the Acochlidia. Strict consensus tree (see Fig. 3, but with outgroups condensed), showing some selected apomorphies of the major groups (indicated by vertical blotches), e.g. the dagger-like radula teeth of ganitids. Homoplasies, such as the independent evolution of secondary spicule shells, are marked in italics. Limnic species (in boxes) evolved twice, independently; note the differences between the single, small-sized *Tantulum elegans* from the Caribbean and the array of large, benthic Indo-Pacific Acochlidiidae species that obviously had greater evolutionary success.

ships remain unresolved. Besides the well-known, extraordinarily high degree of parallelism within opisthobranchs (e.g. Gosliner, 1994; Wägele & Klussmann-Kolb, 2005), there are several further reasons for the high level of homoplasy within Acochlidia with just moderate branch support.

1. The taxon sampling is still limited, i.e. we know of only a fraction of the existing species and morphological variety, with only some parts of the world's coastal waters having been explored (see Schrödl, Eheberg & Burghardt, 2003).
2. The information on many species, such as *P. eirene*, *Acochlidium*, *Palliohedyle*, and *Parhedyle* species, is still insufficient or unreliable.
3. Our coding was conservative, i.e. 'unknown' was used whenever character states were undescribed for a certain species, rather than extrapolating 'normal conditions' from higher taxa, thereby weakening the tree statistics.

4. Entire character sets (such as sperm ultrastructure) are inapplicable or not considered for analysis because of the lack of data available for comparison.
5. The exact origin of Acochlidia is still unknown.

Future analyses will have to overcome these obstacles by sampling in so-far uncovered regions, by the re-examination of poorly known species, by refining characters and including further features listed above, and by applying a set of powerful techniques for comparative structure analyses. Computer-based 3D reconstruction of histological and ultrastructural serial sections greatly faciliate achieving a detailed and accurate view of tiny and complex organs (e.g. Neusser *et al.*, 2006; DaCosta *et al.*, 2007; Neusser & Schrödl, 2007); scanning and transmission electron microscopy can reveal a number of informative characters, e.g. those derived from body ciliation patterns and sperm ultrastructure (Neusser *et al.*, 2007a; Jörger, Kristof, Klussmann-Kolb & Schrödl, 2007b, 2008, in press). According to Hochberg (2007) and own observations (Sommerfeldt & Schrödl, 2005; Jörger *et al.*, unpubl. data), immunecytochemical staining and confocal laser scanning techniques are especially useful to provide information on tiny nervous structures, which may supplement and confirm histological results. Examining and considering additional outgroup taxa such as basal opisthobranchs, pulmonates, and other interstitial taxa by structural and molecular means is badly needed. All this is far beyond the scope of the present study. Although the phylogenetic hypothesis presented is not considered to be definitive, several consequences for classification emerge.

The present analysis (Fig. 3) renders the system of Rankin (1979), who had proposed four suborders, with 13 families and 19 genera for only 25 nominal acochlidian species, obsolete. The need of major modifications was already emphasized by Wawra (1987), Sommerfeldt & Schrödl (2005), and Neusser *et al.* (2006). The classification of Starobogatov (1983), creating an own genus *Minicheviella* and a monotypic family Minicheviellidae for the arctic *Hedylopsis murmanica*, Kudinskaya & Minichev (1978), can also be rejected. Wawra (1987) already transferred *H. murmanica* to the genus *Asperspina*. As assumed by Neusser *et al.* (2008), it appears to be the sister species to the Mediterranean *A. rhopalotecta*, and there is no need for own categories.

The strict consensus tree obtained herein (Fig. 3) supports a basal split of Acochlidia into Hedylopsacea and Microhedylacea, as proposed by Wawra (1987). Of the six families defined by Wawra, the Acochlidiidae (*Palliohedyle* and *Acochlidium*), Asperspinidae (*Asperspina*), Ganitidae (*Ganitus* and *Paraganitus*), and the monotypic Tantulidae (*T. elegans*) are monophyletic; however, only the Acochlidiidae *sensu* Wawra and the Ganitidae show convincing statistical support (BTs of 97 and 93, respectively). The Hedylopsidae *sensu* Wawra (*Hedylopsis*, *Pseudunela*, and *Strubellia*) became paraphyletic, with *Pseudunela* being the sister group of *Strubellia* plus Acochlidiidae *sensu* Wawra, as had already been assumed by Arnaud *et al.* (1986), and the latter two taxa form a clearly monophyletic clade (BT 95), which is the Acochlidiidae *sensu* Arnaud *et al.* (1986). Within the Microhedylacea, the Asperspinidae is the sister group to a clade containing Microhedylidae and Ganitidae. As suspected in earlier studies (Sommerfeldt & Schrödl, 2005; Neusser *et al.*, 2006), the gonochoristic Microhedylidae and Ganitidae species form a monophyletic clade; however, according to the strict consensus tree (Fig. 3), the Ganitidae nestle among species of the genus *Microhedyle*, and thus render the Microhedylidae paraphyletic. If future studies prove this position, the family rank of Ganitidae will need to be reconsidered. The genera as defined by Wawra (1987) are monophyletic, with the exception of *Acochlidium*, *Palliohedyle*, *Microhedyle*, and *Parhedyle*, which may be paraphyletic.

Our proposals for classification are as follows, until this analysis has been re-run on a broader and more detailed data basis: (1) Rankin's system and names should be abandoned; (2) Wawra's higher classification and genera can continue to be used; but (3) some families should be redefined (see Fig. 3). The Acochlidiidae *sensu* Wawra (*Acochlidium* and *Palliohedyle*) should additionally include *Strubellia*, as proposed by Arnaud *et al.* (1986). The two *Pseudunela* species constitute the sister group of Acochlidiidae in the wider sense, and may thus be termed Pseudunelidae, as already introduced by Rankin (1979) for *P. cornuta*. A synapomorphy of the Pseudunelidae (which must be confirmed for *P. eirene*) and Acochlidiidae may be the well-developed and externally visible heart bulb. Another synapomorphic and diagnostic feature is the fusion of the visceral sac and head–foot, without a discernable mantle border. The family Hedylopsidae can be restricted to the clearly monophyletic genus *Hedylopsis* for now. A substantial synapomophy for the clade of Hedylopsidae, Pseudunelidae, and Acochlidiidae is their short sperm head (Fig. 5). The sister to this unnamed clade is *T. elegans* (Tantulidae); a synapomorphy of the Hedylopsacea *sensu* Wawra may be the two-part penis forming a basal swelling adjacent to the penial papilla (see discussion of reproductive features). The Microhedylacea are characterized by the loss of the copulatory organ and by using spermatophores for sperm transfer (Fig. 5). The Asperspinidae (with Minicheviellidae as a junior synonym) *sensu* Wawra may persist. The gonochoristic Microhedylidae (*s.l.*) may informally include the clearly monophyletic Ganitidae, until the origin of the

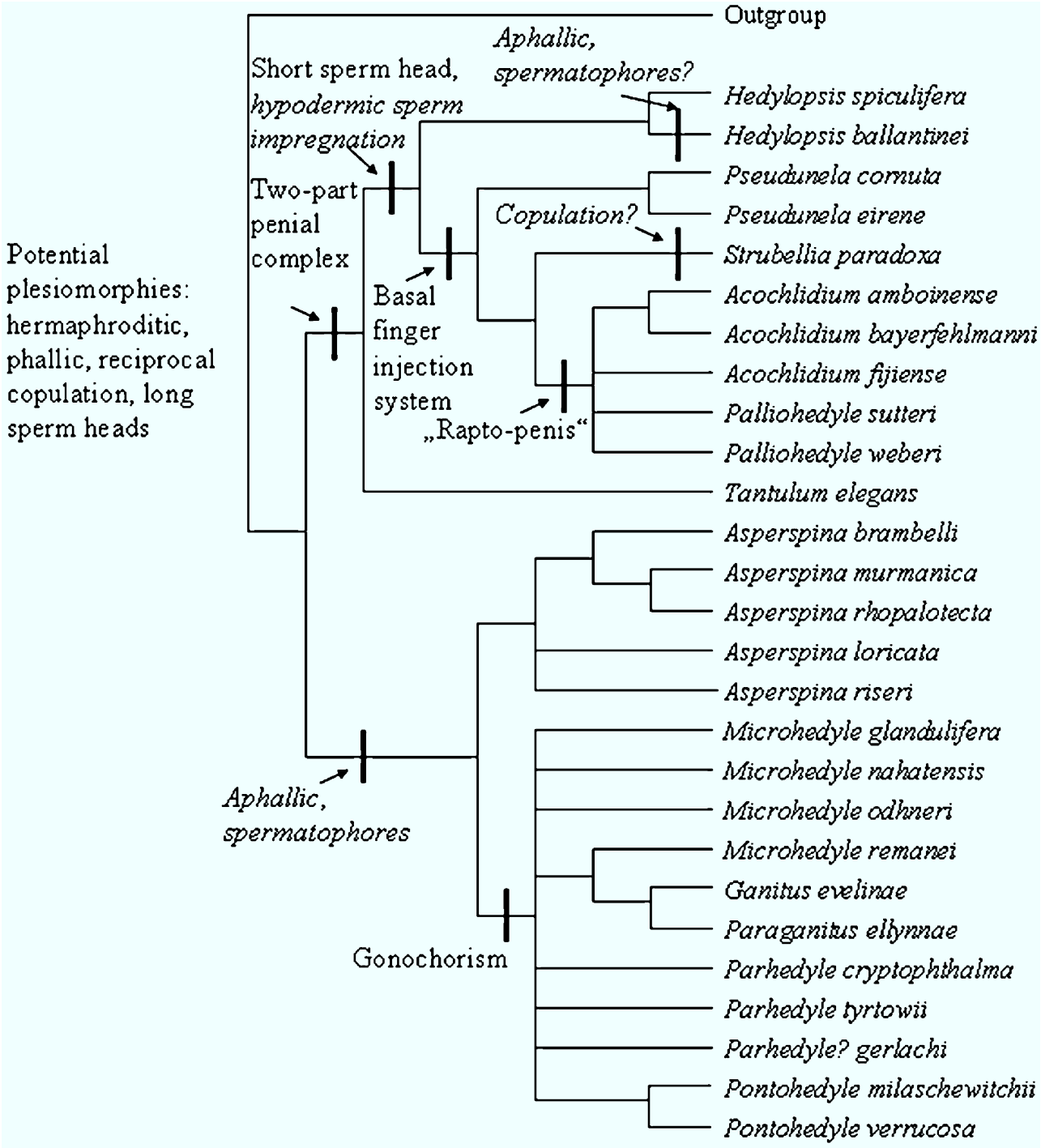

Figure 5. Evolution of special reproductive features in Acochlidia. Strict consensus tree (see Fig. 3, but with outgroups condensed). The evolution of hypodermic impregnation in hedylopsaceans (excluding *Tantulum*, with plesiomorphic copulation) was concomitant with the production of short-headed sperm. The common ancestor of both *Pseudunela* and Acochlidiidae evolved an additional, paraprostatic injection system. A potential evolutionary key feature for the radiation of *Acochlidium* and *Palliohedyle* is the giant, armed 'rapto-penis'. Within the microhedylacean clade, gonochorism may have been the key to the radiation of marine Microhedylidae *s.l.* species. An aphallic condition and sperm transfer via spermatophores, correlated at least in *Asperspina* and Microhedylidae (but still unknown for *Hedylopsis ballantinei*), may have been necessary evolutionary prerequisites.

two monotypic ganitid genera *Ganitus* and *Paraganitus* from a microhedylid stem is confirmed or rejected with higher statistical support. Once the origin of Acochlidia is clarified, intra-acochlidian categories may be adapted.

EVOLUTION

Acochlidia are unique among opisthobranchs in combing an extremely high morphological and ecological diversity with relatively low species diversity. This will ultimately allow for a comprehensive 'all species' evolutionary approach to be applied to a group of apparently Mesozoic origin. Once more reliable data are available, acochlidians may become a suitable model group for reconstructing and understanding opisthobranch evolution and the processes involved.

Invasion of marine interstitial spaces

According to the present analysis, the ancestor of acochlidians was marine in origin, and was possibly already small in size. Accepting an origin from benthic, basal opisthobranch ancestors, the Acochlidia show many autapomorphic reductions, such as the loss of shell and mantle cavity organs. In the acochlidian stem line, the shell-less visceral sac was covered by a more or less resistant integument, and calcareous spicules were also present. There was a complete external, and far-reaching internal, detorsion that resulted in a more or less longitudinally arranged heart complex, with the atrium already positioned posterior to the ventricle in the acochlidian ancestor. Many of these features can be interpreted as evolutionary adaptations to the primary invasion of the marine mesopsammic habitat, favouring small, worm-like, flexible, and symmetric body constructions (Swedmark, 1968). As discussed above, within opisthobranchs, such invasions probably occurred convergently in *Platyhedyle* (Sacoglossa), *Rhodope*, and *Helminthope* (both *incertae sedis*), as well as at least once in Philinidae and Philinoglossidae (Cephalaspidea), with similar adaptations.

Several members of these groups also show aggregations of precerebral ganglia, so far with unknown function. In acochlidians, precerebral ganglia were ancestrally present, but were then reduced in the hedylopsacean lineage after splitting from *Tantulum*. If these are aggregations of neurosensoric tissue (Marcus, 1953), i.e. olfactory tissue, the development of 'accessory ganglia' may have been a prerequisite to the reduction of the eyes in many subgroups (but not in all species). At least several acochlian species such as *T. elegans*, *S. paradoxa*, *P. milaschewitchii*, and *M. glandulifera* have either retained or reinstated an at least Hancock's-like, cerebrally innervated organ, with certainly sensoric function (see Neusser & Schrödl, 2007; Neusser *et al.*, 2007b; this study). Furthermore, the probably newly developed sensoric organs are special ciliary tufts and bands, especially in the head region of many mesopsammic acochlidian species (Jörger *et al.*, 2007b): these may also be adaptations to an interstitial environment. Whereas other interstitial opithobranchs do not possess elaborate cephalic tentacles, two pairs of solid tentacles evolved in the acochlidian ancestor. Ancestrally, oral tentacles were digitiform, but flattened, shovel-like oral tentacles evolved within *Hedylopsis*, *Pontohedyle*, and *Ganitus*. Digitiform rhinophores were reduced in size or completely lost several times independently within lineages of marine mesopsammic species.

As a 'fast' evolutionary mechanism favouring miniaturization and (adult) organ reductions, Westheide (1987) proposed that progenesis played a major role, especially in the evolution of interstitial organisms. Our analysis, however, suggests that: (1) the acochlidian ancestor may have already been small before colonizing the mesopsammon; (2) it was well equipped, with tentacular sensory organs similar to those that are also present in benthic opisthobranchs such as nudibranchs (in some acochlidian subgroups sensory organs have then been reduced in parts, and may have been substituted by other organs); and (3), apparently no adult-specific organs have been reduced. In particular, the ancestral acochlidian showed the hermaphroditic, phallic, and monaulic reproductive condition, as is usual for basal opisthobranchs. Modifications such as the loss of copulatory organs evolved within different acochlidian subclades, and are discussed below. Thus, there is neither any indication for a fast event of progenesis in the acochlidian stem line, nor any indication of larval opisthobranch features persisting in adult acochlidians. Therefore, we assume that steady selection under strong environmental pressure led to a mosaic of reductive and novel features, as displayed by extant acochlidian species. Once ontogenetic stages of basal opisthobranchs and acochlidians are available for comparison, evolutionary processes involved, such as heterochrony, may be addressed more conclusively.

Invasion into freshwater systems

According to the present phylogenetic hypothesis, limnic habitats were successfully colonized twice by opisthobranchs, i.e. acochlidians (Fig. 4): first, by the ancestor of the small interstitial Caribbean *T. elegans*, and second, by the common ancestor of all large, benthic Indo-Pacific species, the Acochlidiidae, as defined herein. A posteriori character tracking indicates that *P. weberi* had limnic ancestors and, if they truly inhabited brackish waters, they colonized such a

habitat secondarily. The timescales and evolutionary processes involved are still unknown. Remarkably, selection under limnic conditions resulted in the evolution of large body sizes (i.e. a secondary 'gigantism' resulted, because an increased volume/surface ratio may reduce osmolarity problems, although this is not true in juveniles) only in the Indo-Pacific clade, where a considerable radiation took place. In contrast, *T. elegans* is equally as small as members of related marine groups, such as species of *Hedylopsis*, *Asperspina*, and microhedylids; a radiation of limnic Caribbean species has yet to be discovered. Future histological and ultrastructural studies will enable instructive comparisons of analogous excretory structures, i.e. may show the different means by which originally small and marine opisthobranchs have overcome osmotic stress.

Evolution of asymmetric radulae

Very unusual, asymmetric radulae, all with an additional, although variably shaped, tooth on the right side, were discovered to be present in all sufficiently studied marine and limnic hedylopsaceans, and within a few marine *Asperspina* and *Parhedyle* species. Earlier classifications, e.g. by Wawra (1987) or Arnaud *et al.* (1986), implied multiple developments and reductions of radula asymmetry within several acochlidian taxa. A posteriori character tracking in the strict consensus tree favours a scenario in which asymmetric radulae evolved at the base of Hedylopsacea, and at least two times independently within microhedylacean taxa. However, we consider an alternative scenario, with such an asymmetric radula as described above representing a unique synapomorphy for Acochlidia, as being more plausible. A hypothetical evolutionary reduction row proposed by Salvini-Plawen (1973) needs to be confirmed once a better resolved microhedylacean tree is available; a trend to reduction and loss of lateral teeth within microhedylaceans culminated in the monostichous radula of ganitids.

Evolution of 'secondary shells'

Whereas normal shells were lost by the acochlidian ancestor, large needle-like subepidermal spicules are arranged to form a unique roof or net-like structure, stiffening the visceral hump, in several acochlidian species. According to our analysis (Fig. 4), such internal 'spicule shells' evolved convergently in *Hedylopsis* and in the microhedylacean *Asperspina*. Rigid visceral humps may serve a protective function, as assumed by Swedmark (1968), but possibly against predators rather than against mechanical forces caused by currents or waves (Jörger *et al.*, 2008).

Evolution of aberrant reproductive features (Fig. 5)

In a mesopsammic environment, as inferred to be the ancestral state for acochlidians, a normal opisthobranch head-to-foot copulation of two hermaphrodites, with synchronization of sexual activities and reciprocal penetration, may simply be mechanically difficult. Reciprocal copulation was neither observed nor concluded for any mesopsammic opisthobranchs, except for the aeolid nudibranch *Pseudovermis* (see Swedmark, 1968), which should be re-examined. Acochlidians, including mesopsammic and secondarily benthic lineages, are very special with regard to their reproductive biology (e.g. Swedmark, 1968), and genital structures were recognized to be of considerable value for clarifying acochlidian phylogeny (Wawra, 1987). However, the wealth of apparently different aberrant features in almost every acochlidian species faced the virtual absence of observations of living animals, and mosaic-like distributed anatomical information on just certain organs on certain ontogenetic stages of just a few members of certain clades. In the absence of a sound phylogenetic hypothesis, the evolutionary scenarios presented by Wawra (1987, 1992) and Haase & Wawra (1996) are conflictive. The following evolutionary interpretation of acochlidian reproductive features is based on the topology given in Figure 3, and on a posteriori character states analysis. This scenario (Fig. 5) is still preliminary, but may be tested and refined in future studies.

Allosperm receptacles

Hermaphroditic opisthobranchs usually use reciprocal copulation for sperm transfer and store, and/or digest sperm in at least one allosperm receptacle. In contrast, the situation in Acochlidia is more complicated. Microhedylacean species, *Hedylopsis*, *Palliohedyle*, and *Acochlidium* apparently lack any allosperm receptacle, whereas a bursa copulatrix was found in *T. elegans* by Neusser & Schrödl (2007). *Strubellia paradoxa* and *P. cornuta* possess both a bursa and a receptaculum seminis (Wawra, 1988a; this study). The phylogenetic hypothesis herein (Fig. 3) suggests that the receptaculum seminis was already absent in the acochlidian ancestor, but was reinvented by the common ancestor of *Pseudunela* and *Strubellia*, and then lost again in the ancestor of *Acochlidium* and *Palliohedyle*. In contrast, a bursa copulatrix may have been retained from an opisthobranch ancestor, but implies multiple losses in the ancestor of Microhedylacea, in *Hedylopsis*, and, apparently, in *A. fijiense*. However, *Palliohedyle* and *Acochlidium* species should be re-examined.

From reciprocal copulation to hypodermic impregnation

The possession of two allosperm receptacles in *Strubellia* led Wawra (1992) to assume normal copulation behaviour for all limnic Acochlidiidae. This has, however, never been directly observed for any acochlidian, and is questionable at least for those species apparently lacking any organs specialized for sperm storage. The basal hedylopsacean *T. elegans* has a well-developed, unarmed penial papilla (Fig. 2A) and a bursa copulatrix, and thus copulation is likely; the evolutionary fate of the muscular 'basal penial swelling' is discussed below. In *Strubellia*, the penial papilla does not possess a stylet but bears a subapical cuticular thorn (Fig. 2D): this may help to fix the penis in a copulatory position. In contrast to earlier statements (Rankin, 1979; Wawra, 1987), the vas deferens connects with a hollow apical penial stylet in members of all other hedylopsacean clades (Fig. 2B, C), strongly indicating that sperm is transferred via injection (Fig. 5). In the case of *H. spiculifera*, which lacks any allosperm storage organ, sperm may be more or less precisely injected into the gonad or reproductive system, as known from some nudibranch *Palio* species (see Haase & Wawra, 1996). The apomorphically short-headed sperm may be adapted to migrate within the genital system to the place where eggs are fertilized. However, in *H. spiculifera* and *A. fijiense*, penial stylets or sperm were found to be injected somewhere within the body of the mate (see Wawra, 1989; Sommerfeldt & Schrödl, 2005). It is unknown whether or not such an imprecise hypodermal impregnation is the normal method of sperm transfer in these species, which would imply that short-headed hedylopsacean sperm are able to migrate through tissues and epithelia, as reported for some sacoglossan species (see Haase & Wawra, 1996). Regardless of the precise or imprecise mode of hypodermal impregnation, a certain degree of injury is caused by the penis stylets perforating the body integument and underlying tissue; this was obviously overcompensated by some evolutionary advantage for injectors.

Towards a giant 'rapto-penis'

Once a more rapid, uni- or bidirectional impregnation had evolved in the hedylopsacean (excluding *Tantulum*) mesopsammic lineage (Fig. 5), there was a tendency towards more complex copulatory systems. A more or less conical penial papilla with apical stylet, as is still present in *Hedylopsis* and *Pseudunela*, evolved into the giant penial papillae present in *Palliohedyle* and *Acochlidium*. These unique organs (e.g. Haase & Wawra, 1996: figs 6–12), besides the so-called ejaculatory finger, form trap-like armed bulbs with apical rows of cuticular spines, the arrangement of which depends on the state of eversion of the whole papilla: they thus appear suitable to grasp and fix the mate. Sperm is injected via a slender subapical ejaculatory finger in at least some species (see Haase & Wawra, 1996). Even though impregnation has never been observed directly, such copulatory organs will definitely harm the mate. Selection towards efficiently transferring sperm by this kind of 'rapto-penis' requires a strategy to avoid being hit and injured by mobile, benthic mates with similar weapons. Simply grasping a mate and holding it at some distance may allow the application of sperm and prostatic liquids, but may also allow the application of other, special fluids.

Paraprostatic glands and impregnation systems

The basal penial swelling of *Tantulum*, the most basal hedylopsacean offshoot, is unarmed, whereas that of *H. spiculifera* bears a cuticular thorn (Fig. 2A, B). In *Pseudunela*, the basal swelling is already penetrated by a paraprostatic duct that opens through an apical hollow stylet (Fig. 2C); in *Strubellia*, there is an additional gland opening at the external base of the paraprostatic stylet (Fig. 2D). We assume that these accessory paraprostatic impregnation systems (Fig. 5) are homologous with the so-called basal finger, which is also a part of the complex 'rapto-penis' of *Acochlidium* and *Palliohedyle* (see Haase & Wawra, 1996). The exact funtion of such accessory glands is unknown. However, they may produce special substances, e.g. anaesthetics, as in the cephalaspidean *Siphopteron quadrispinosum* Gosliner, 1989 (see Anthes & Michiels, 2007), to enforce unilateral insemination. Auxiliary glandular fluids may also stimulate or adjust sperm transfer, as in the sacoglossan *Elysia timida* (Risso, 1818) (see Schmitt, Anthes & Michiels, 2007), or may even play a role in sperm competition, as in helicid land snails (Chase & Blanchard, 2006). The arrangement, function, and evolution of the complex hedylopsacean copulative apparatus are definitely worthy of investigation in detail.

Loss of copulatory organs and use of spermatophores

The marine hermaphroditic *H. spiculifera*, as well as the limnic *Strubellia* and *Tantulum*, were described to reduce male copulatory organs and testes during ontogeny. Thus, protandry leads to functional gonochorism at least once within Hedylopsacea. An ontogenetic loss of copulatory organs in such sequential hermaphrodites might have been a precursor of the completely aphallic condition in the still hermaphroditic *H. ballantinei* and *Asperspina*, and of the aphallic and gonochoristic condition in Microhedylidae (*s.l.*).

How is sperm transferred in a mesopsammic environment, lacking any copulatory or sperm-storing organs? Swedmark's (1971) assumption of cutaneous

insemination via spermatophores in all acochlidians is clearly limited to aphallic species. Although there is still no direct information available on the absence or presence of spermatophores in *H. ballantinei*, the loss of copulatory organs is obviously correlated with transferring sperm via spermatophores in Microhedylacea (Fig. 5). As far as is known, acochlidian spermatophores are formed somewhere in the posterior genital system, are released through the (usually) dextrolateral genital opening, and are sticked unspecifically at any potential mates (Swedmark, 1968; Morse, 1994); *P. milaschewitchii* is special in having its male genital opening situated above the mouth (see Jörger *et al.*, 2008). Sperm, having very elongated spiral heads in apparently all spermatophore-possessing microhedylaceans, may then directly penetrate the integument. This truly cutaneous insemination would be a unique condition within opisthobranchs (see Karlsson & Haase, 2002), correcting an earlier observation of apparent dermal insemination in the aeolidoidean nudibranch *Aeolidiella glauca* (Alder & Hancock, 1845) by Haase & Karlsson (2000). After that, sperm has to migrate towards the gonad, and thus penetrate connective tissue and epithelia, similarly to sperm that is imprecisely impregnated subdermally. In fact, the cutaneous insemination of microhedylaceans may be considered an imprecise, aphallic 'soft injection' executed by screw-like, mobile sperm. As in the case of hypodermal injection, such a potentially unidirectional and still comparatively fast mode of sperm transfer via spermatophores was evolutionary successful in a mesopsammic environment.

Gonochorism

Opisthobranchs in general, the acochlidian ancestor, and all basal acochlidian taxa are hermaphrodites. Extreme sequential hermaphroditism, i.e. functional gonochorism, is a potential preadaptation for evolving separate sexes in Acochlidia: true gonochorism evolved only once (Fig. 5), i.e. in the aphallic and spermatophore-using ancestor of Microhedylidae (including Ganitidae). The Microhedylidae as defined herein are the most successful acochlidian group with regard to species diversity (11 valid species). Collection in northern Sulawesi revealed several additional, obviously undescribed *Pontohedyle* and microhedylid species (see Schrödl *et al.*, 2003; Burghardt *et al.*, 2006), thus tropical regions appear to be inhabited by a much higher number of species than expected. The sister group of Microhedylidae, the genus *Asperspina*, at present only comprises five valid species, and at least four of them are hermaphrodites (*A. rhopalotecta* needs to be re-examined); some further species were reported from the north-eastern and tropical Pacific (Morse, 1994), and the Caribbean (Hochberg, 2007). This relative evolutionary success of Microhedylidae may be linked to gonochorism. However, other factors such as the much more flexible body construction in comparison to the asperspinids, which have evolved a secondary 'spicule shell', might also play a role. As discussed, an aphallic condition in the ancestor of Microhedylidae and sperm transfer via spermatophores may have been necessary preadaptations for gonochorism, which is unique amongst opisthobranchs. It is, however, unclear why gonochorism is exclusively present, and is even an advantage for opisthobranchs inhabiting interstitial spaces: there, mobility is limited, and detection of mates in a dense and 3D-structured environment may be especially difficult. Any possibility of selfing is excluded, and it would therefore be twice as difficult to find a mate of the opposite sex.

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3.12 **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(8): 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°	Pre-paration 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''.

In addition, this published work and the nomenclatural acts it contains have been registered in ZooBank, the proposed online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix ''http://zoobank.org/''. The LSID for this publication is: urn:lsid:zoobank.org:pub:08C58B19-13BC-45CE-AEF5-BD1D508A1C10.

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

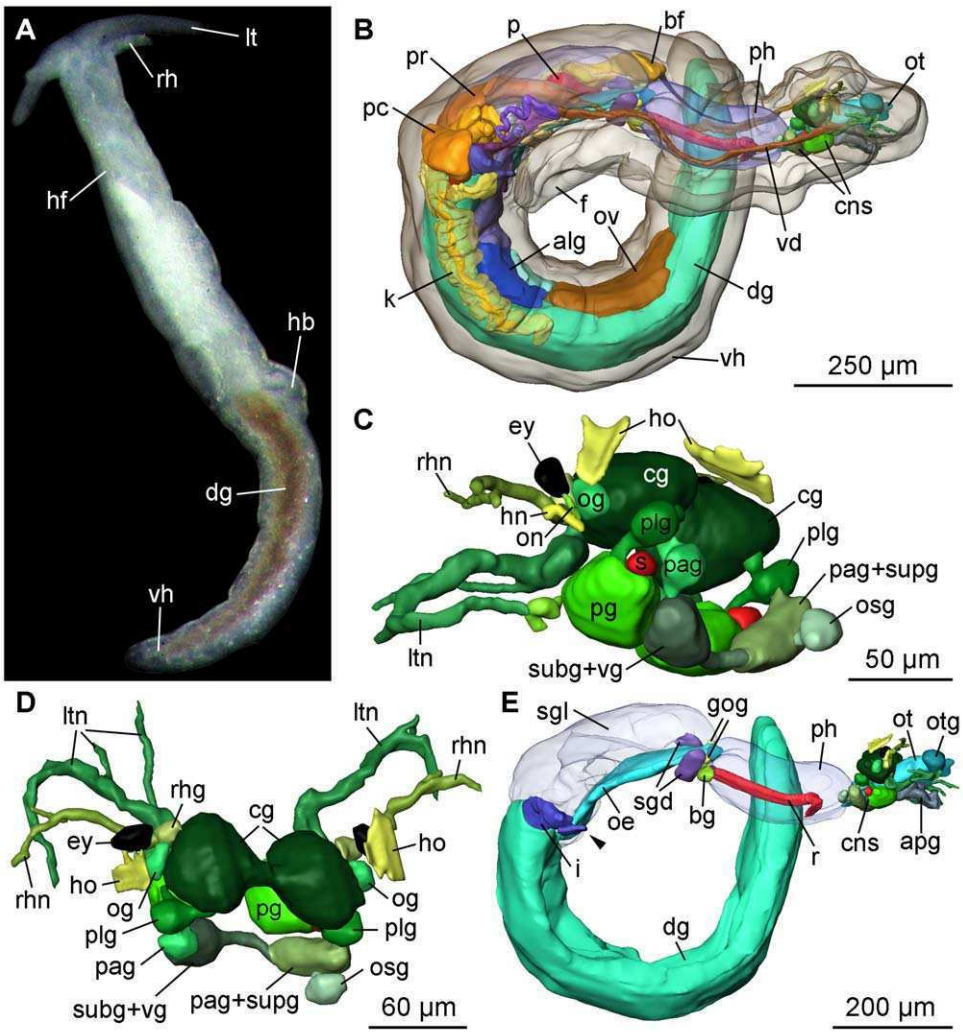

Figure 1. Photograph of a living specimen and 3D reconstruction of *P. viatoris* sp. nov. from Fiji. A: external morphology of a living specimen (body size 3 mm), dorsal view. B: general anatomy, right view. C: CNS, left view. D: CNS, dorsal view. E: digestive system with CNS, right view. Abbreviations: **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

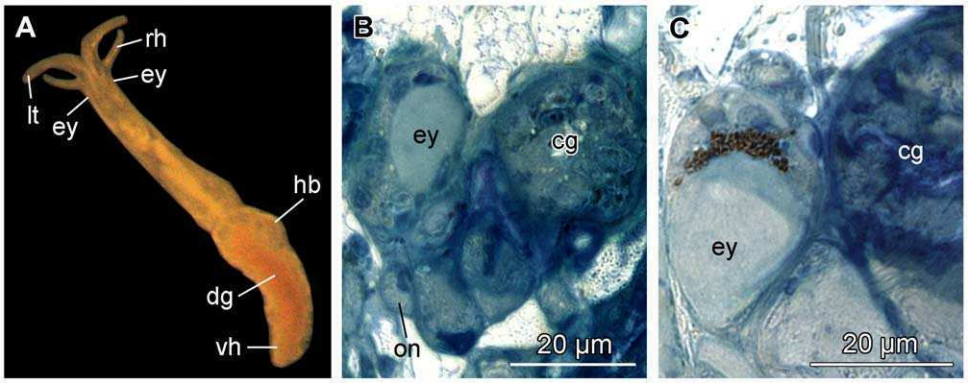

Figure 2. Photograph of a living specimen and histological cross-sections of *P. viatoris* sp. nov. from Indonesia. A: external morphology of a living specimen (body size 3 mm). B: unpigmented eye. C: pigmented eye. Abbreviations: **cg**, cerebral ganglion; **dg**, digestive gland; **ey**, eye; **hb**,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

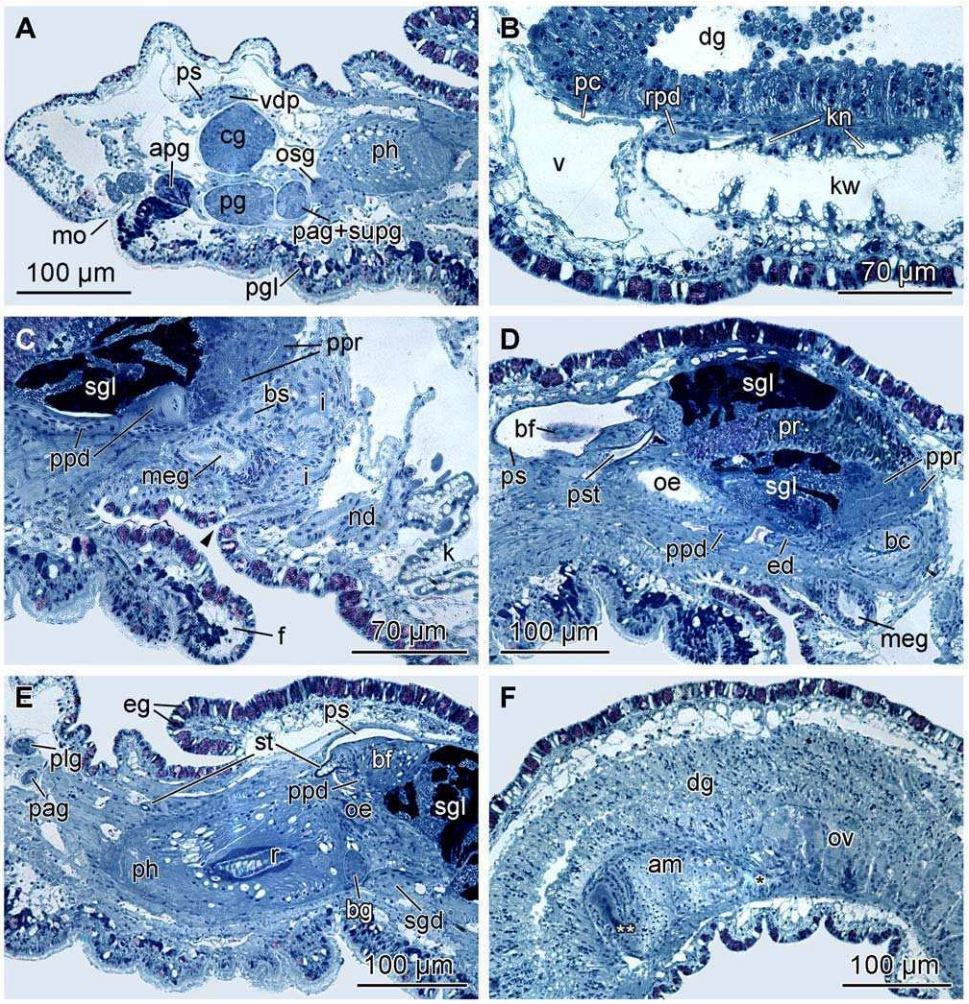

Figure 3. Histological cross-sections of *P. viatoris* sp. nov. from Fiji. A: anterior pedal gland and ganglia. B: circulatory and excretory systems. C: common opening of digestive and excretory systems. D: penial stylet and prostate. E: basal finger and pharynx. F: ampulla and ovotestis. Abbreviations: **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;

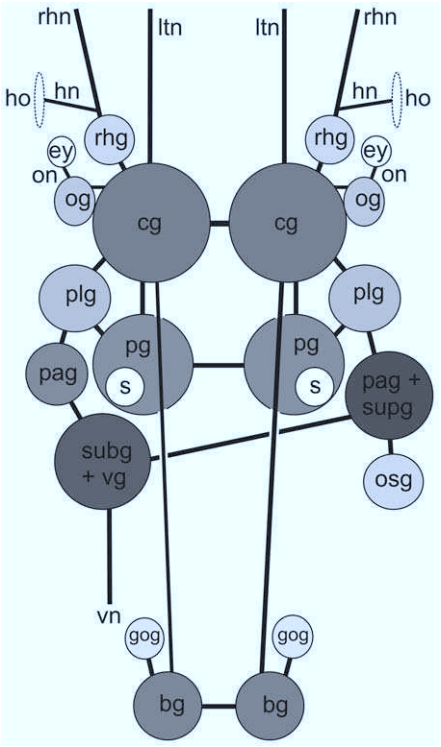

Figure 4. CNS of *P. viatoris* sp. nov. from Fiji (schematic overview, dorsal view). Abbreviations: **bg**, buccal ganglion; **cg**, cerebral ganglion; **ey**, eye; **gog**, gastro-oesophageal ganglion; **hn**, Hancock's nerve; **ho**, Hancock's organ; **ltn**, labial tentacle nerve; **og**, optic ganglion; **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

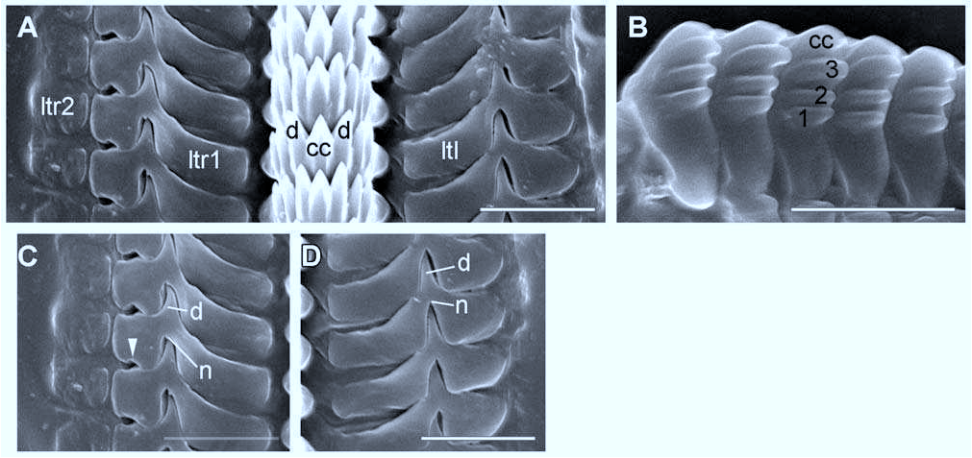

Figure 5. SEM micrographs of the radula of *P. viatoris* sp. nov. from Fiji. A: row of radular teeth. B: rhachidian tooth. C: right lateral teeth. D: left lateral tooth. Abbreviations: **cc**, central cusp; **d**, denticle; **ltl**, left lateral tooth; **ltr1**, first right lateral tooth; **ltr2**, second 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

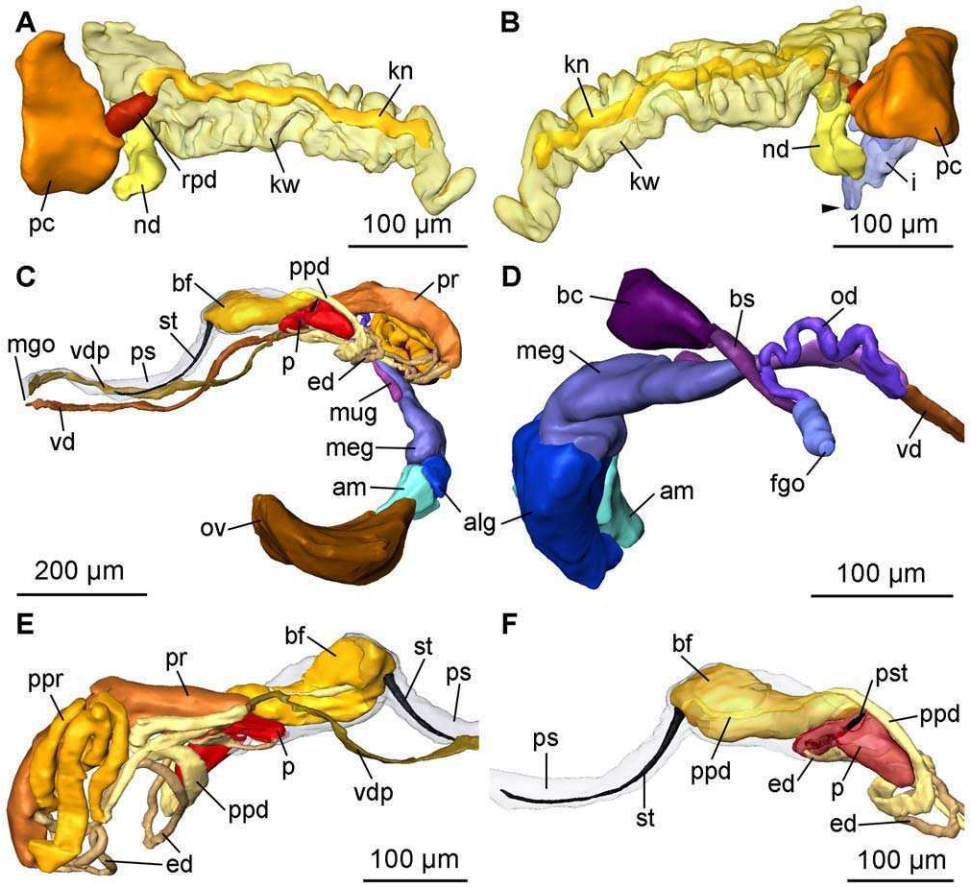

Figure 6. 3D reconstruction of the excretory and reproductive systems of *P. viatoris* sp. nov. from Fiji. A: circulatory and excretory systems, left view. B: circulatory and excretory systems, right view. C: complete reproductive system, left view. D: nidamental glands and sperm storing receptacles, 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

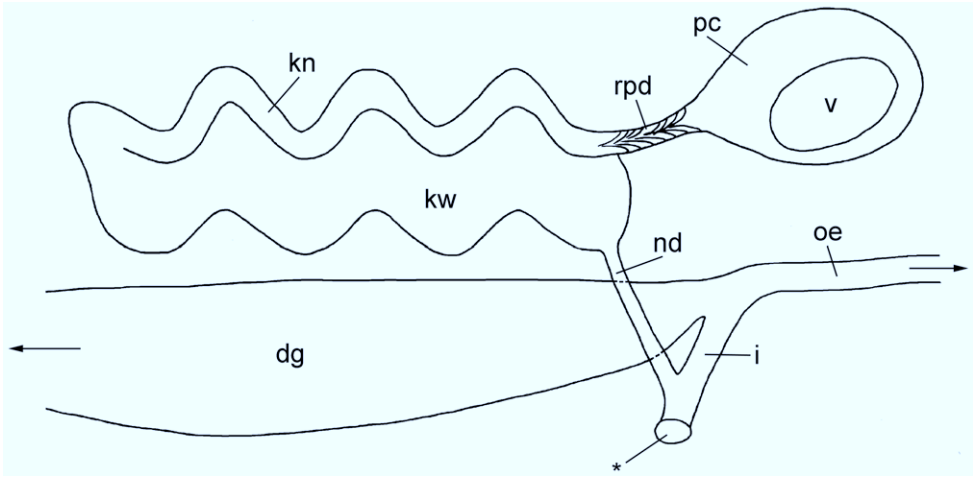

Figure 7. Circulatory and excretory systems of *P. viatoris* sp. nov. from Fiji (schematic drawing, right view). Abbreviations: **dg**, digestive gland; **i**, intestine; **kn**, narrow lumen of kidney; **kw**, wide lumen of kidney; **nd**, nephroduct; **oe**, oesophagus; **pc**, pericardium; **rpd**, 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.

urn:lsid:zoobank.org:act:77053243-8F24-4ED9-89DC-D5665814E750

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.

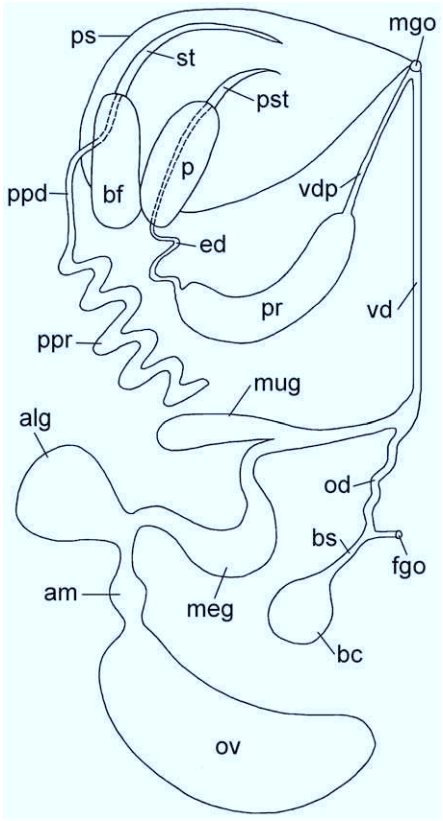

Figure 8. Reproductive system of *P. viatoris* sp. nov. from Fiji (schematic drawing, dorsal view). Abbreviations: **alg**, albumen gland; **am**, ampulla; **bc**, bursa copulatrix; **bf**, basal finger; **bs**, bursa stalk; **ed**, ejaculatory duct; **fgo**, female gonopore; **meg**, membrane gland; **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).

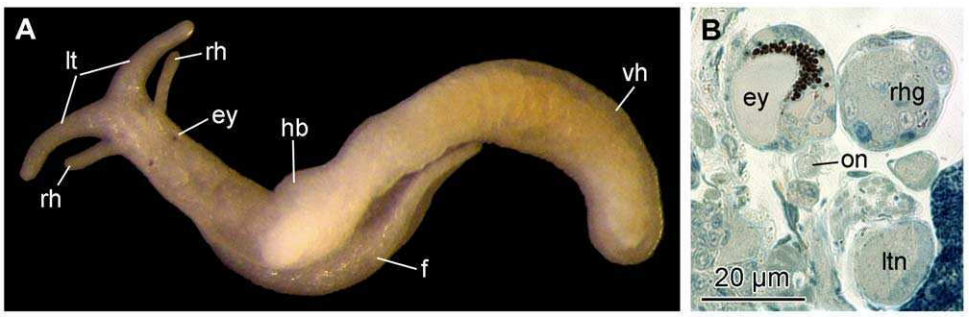

Figure 9. Photograph of a living specimen and histological cross-section of *P. marteli* sp. nov. (Solomon Islands). A: external morphology of a living specimen (body size 3 mm). B: pigmented eye. Abbreviations: **ey**, eye; **f**, foot; **hb**, heart bulb; **lt**, labial tentacle; **ltn**, labial tentacle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

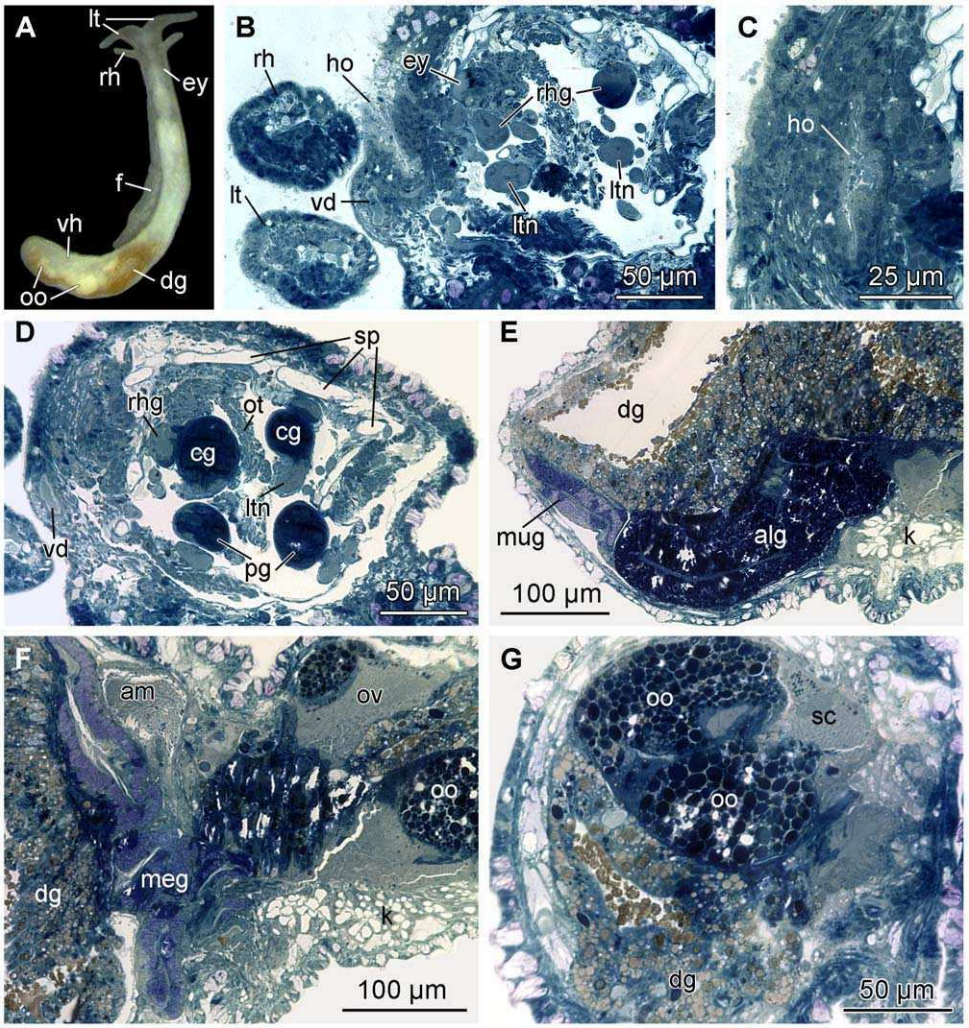

Figure 10. Histological cross-sections of *P. marteli* sp. nov. from Vanuatu. A: external morphology of a living specimen (body size 3 mm). B: Hancock's organ and eye. C: Hancock's organ. D: spicule cavities. E: albumen and mucus glands. F: ampulla and membrane gland. G: oocytes and spermatocytes. Abbreviations: **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

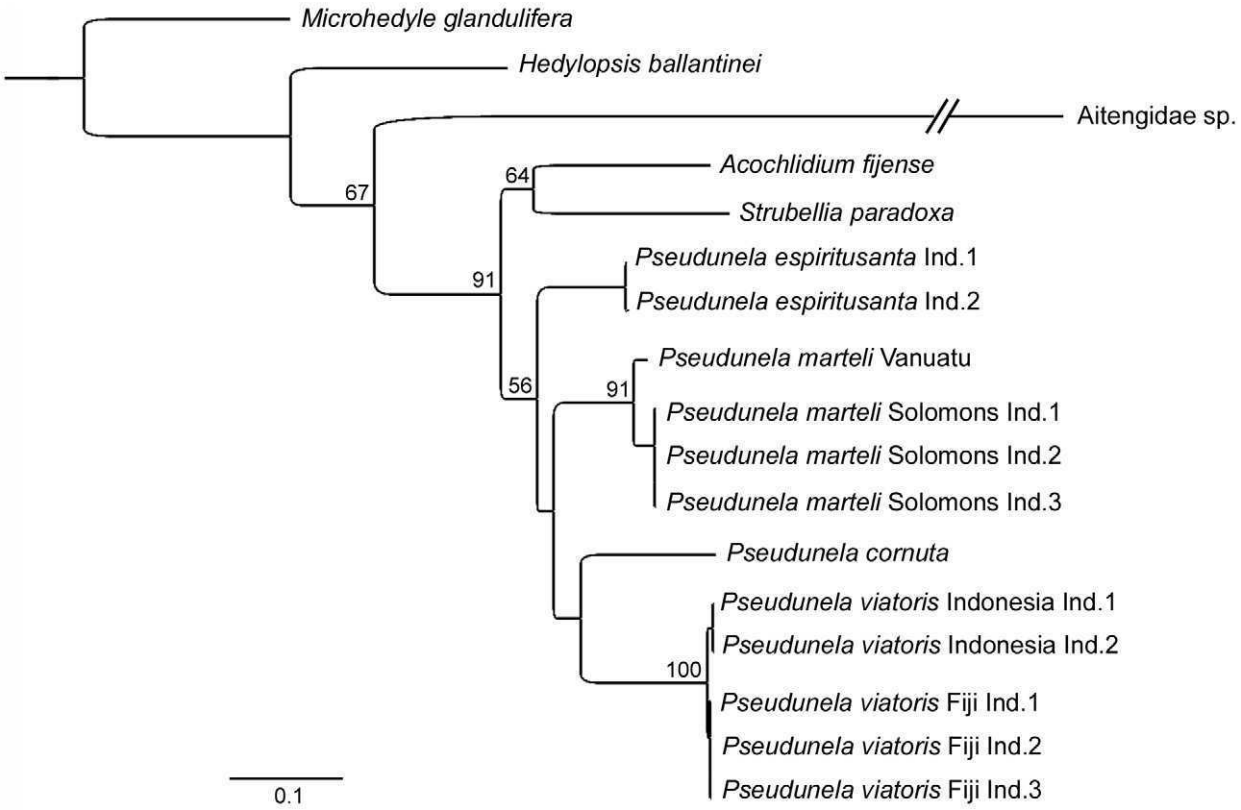

Figure 11. Molecular phylogeny of the genus *Pseudunela*. RAxML analysis of concatenated sequences of partial 18S rRNA, 16S rRNA and COI markers, analysed in four partitions. Bootstrap values (>50%) given at nodes. Sister group relationship between Pseudunelidae and limnic Acochlidiidae receives strong 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.
doi:10.1371/journal.pone.0023313.t003

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

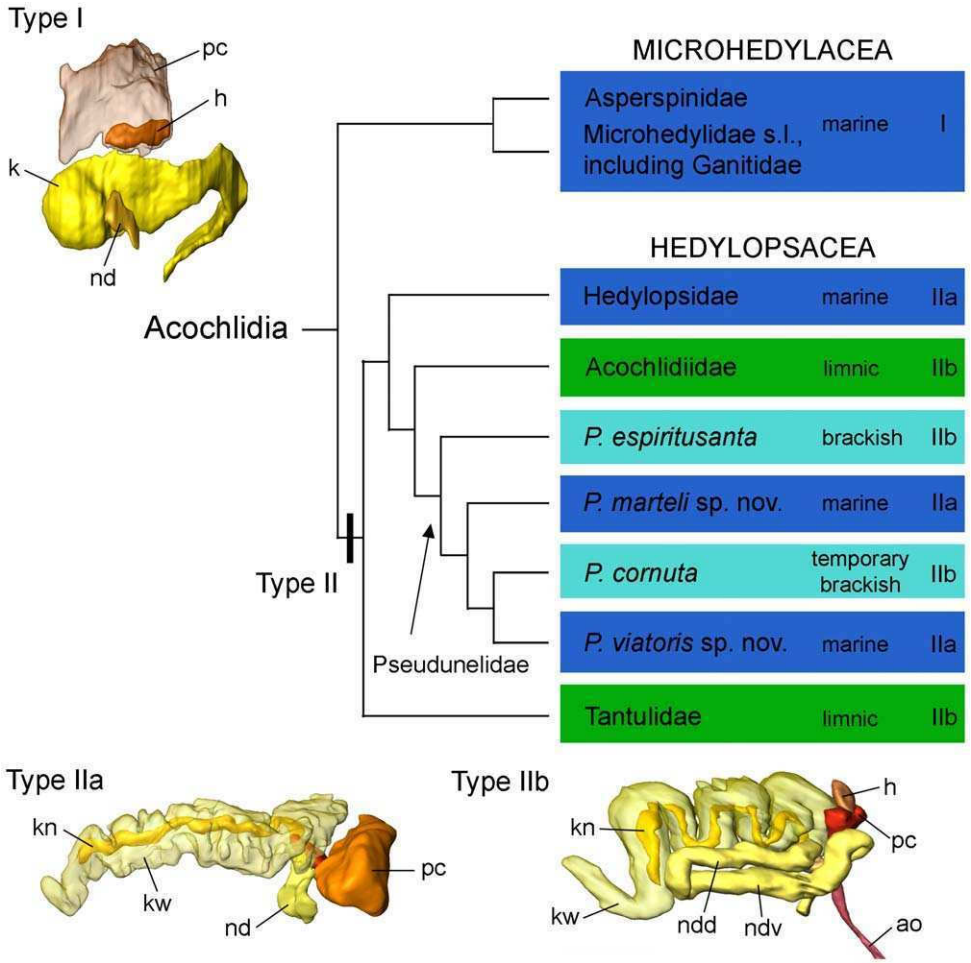

Figure 12. Evolution of excretory systems and habitat in acochlidian lineages. The habitat of the different acochlidian lineages and their types of excretory systems are plotted on a consensus tree (topology combined from Schrödl & Neusser [18] and molecular results herein; the enigmatic Aitengidae are 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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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).

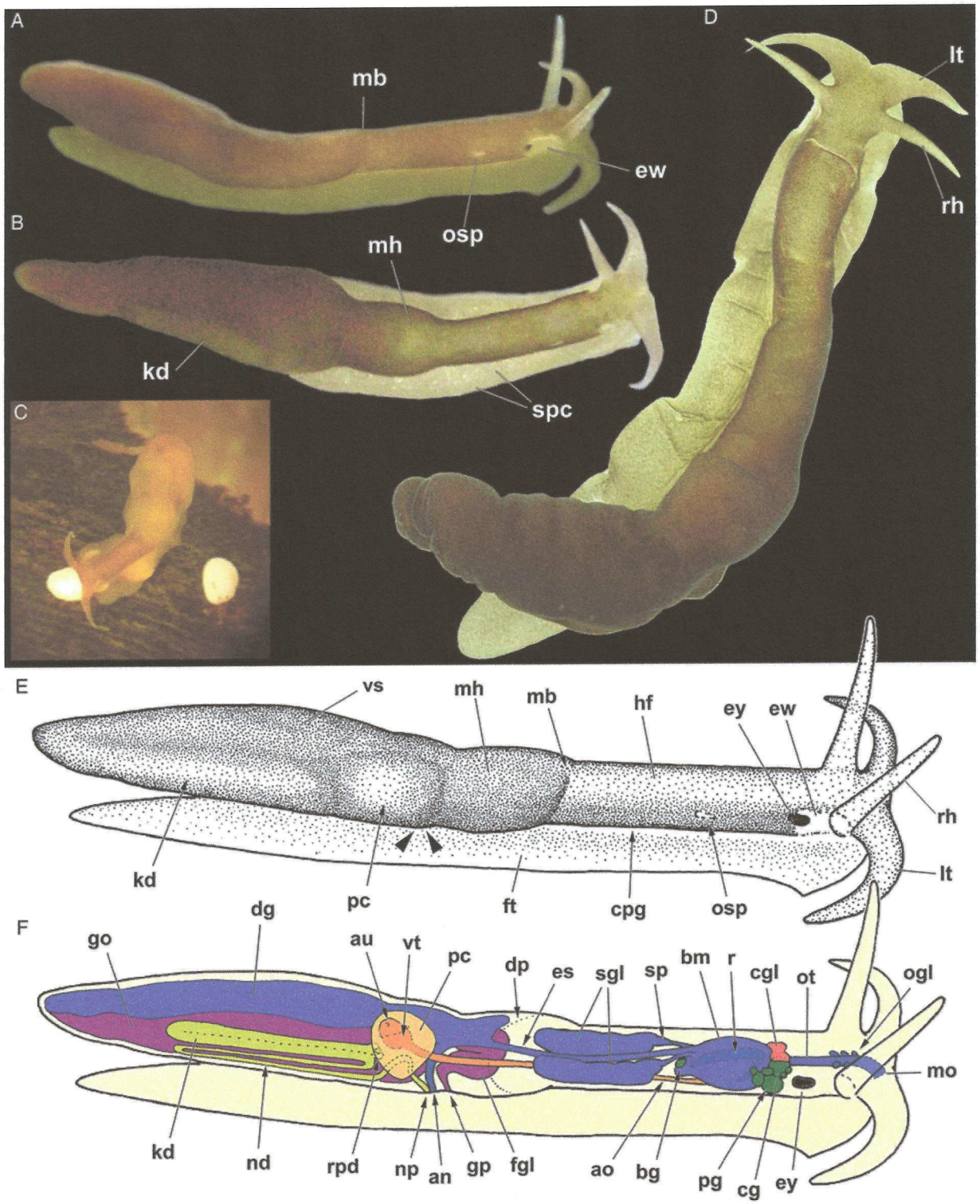

Figure 1. Live specimens and general schematic overview of the anatomy of *Strubellia wawrai* n. sp. **A–D.** External morphology of living specimens from Kohove River, Guadalcanal, Solomon Islands (**A–C**) and Tapuntari Cascades, Wounaouss River, Espiritu Santo, Vanuatu (**D**). **A.** Young specimen, *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).

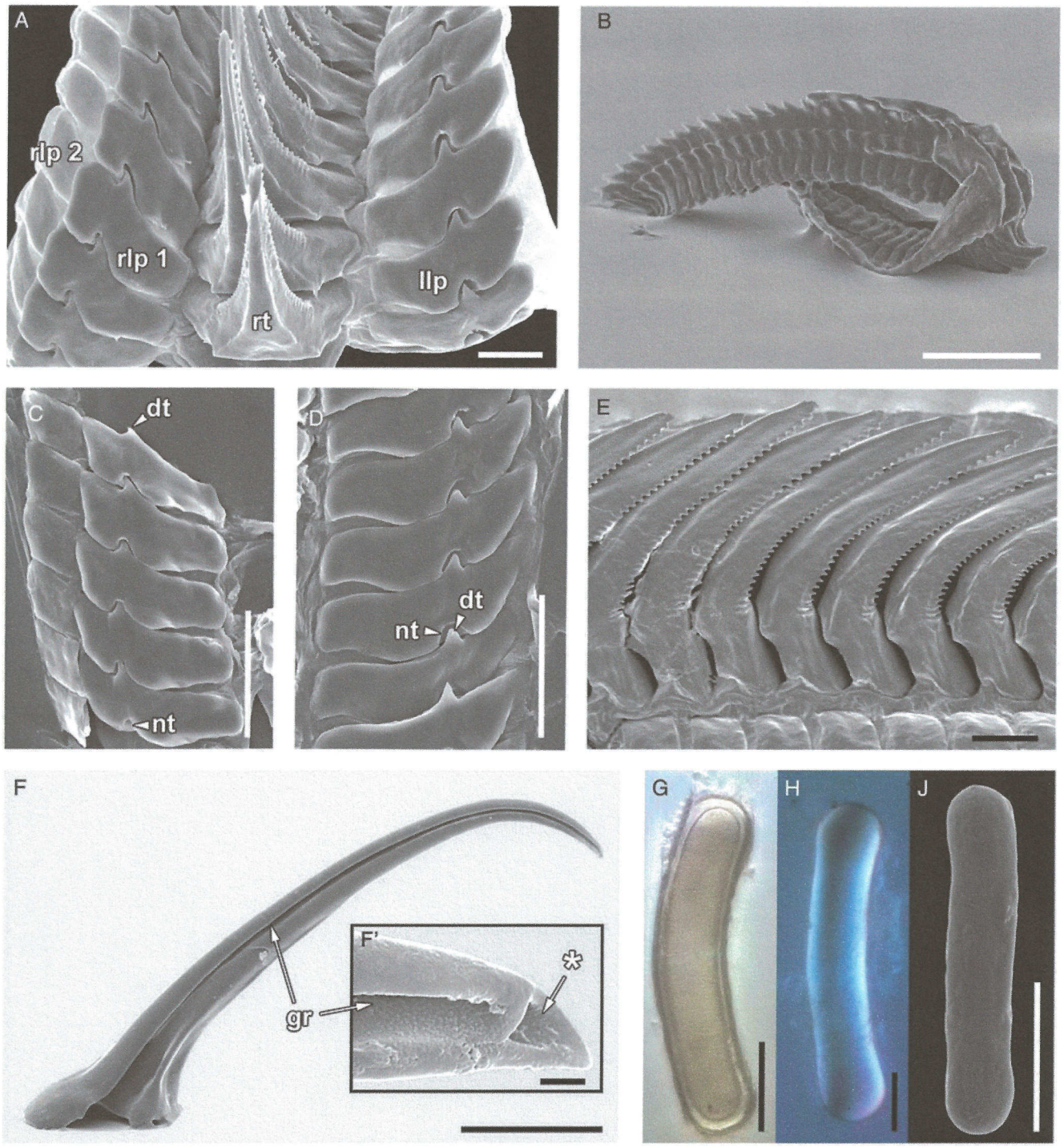

Figure 2. Microscopic views of radula (SEM), stylet of basal finger (SEM) and spicules surrounding the buccal mass (SEM, light microscopy) of *Strubellia wawrai* n. sp. **A, F, F′.** Vanuatu specimen; **others**: Solomon Islands. **A.** Functional part of radula. **B.** Complete hook-shaped radula. **C.** 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, 72°C 60 s (Phire polymerase, New England Biolabs)
	16S-R	CCG GTC TGA ACT CAG ATC ACG T	Simon *et al.* (1994)	
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) × 35–40, 72°C 3 min (Taq polymerase, Sigma)
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al.* (1994)	
	COI long r	TAA AGA AAG AAC ATA ATG AAA ATG	Stothard & Rollinson (1997)	

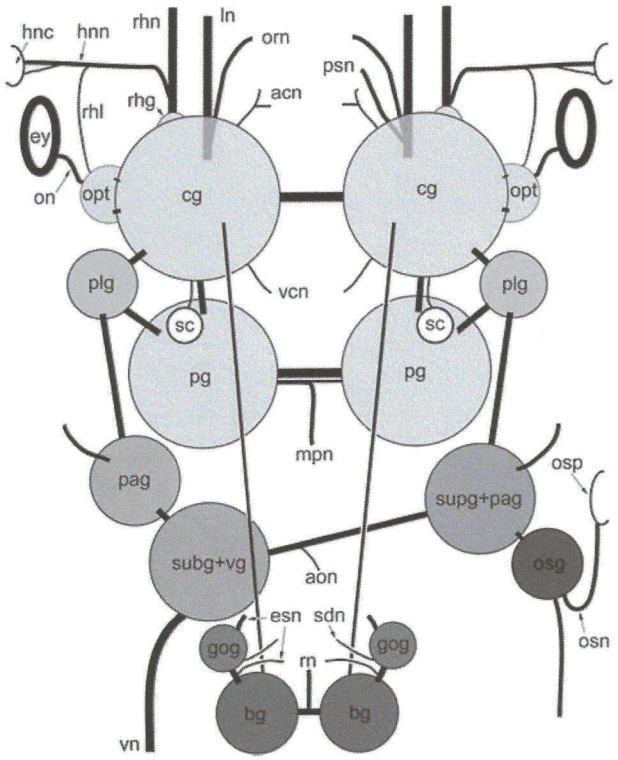

Figure 3. Schematic overview of the CNS (pedal nerves omitted for clarity) of *Strubellia wawrai* n. sp., dorsal view. Abbreviations: acn, anterior cerebral nerve; aon, aortic nerve; bg, buccal ganglion; cg, cerebral ganglion; esn, esophageal nerves; ey, eye; gog, gastroesophageal ganglion; hnc, Hancock's 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.

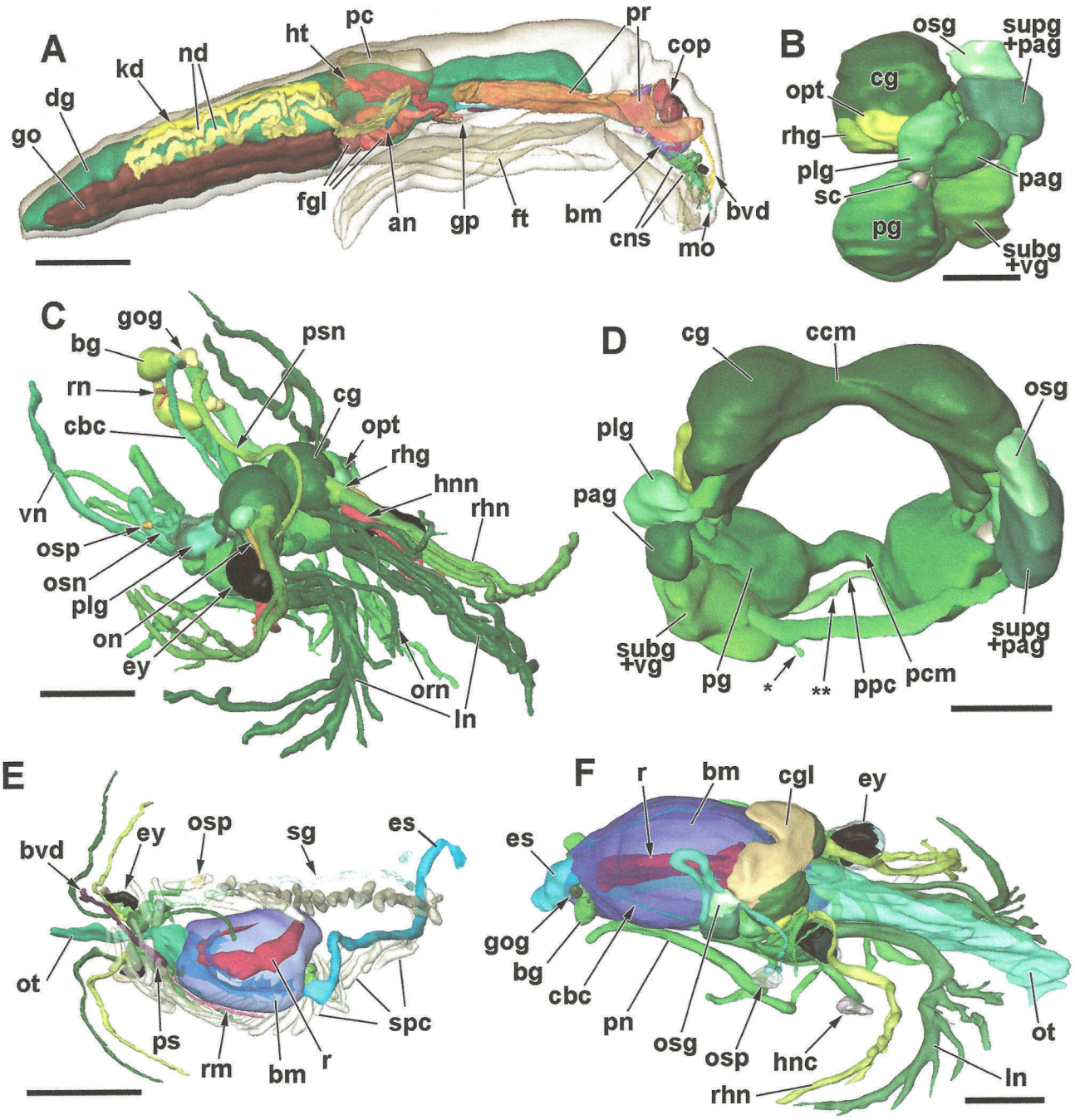

Figure 4. Three-dimensional reconstruction of general anatomy and CNS of *Strubellia wawrai* n. sp. from Vanuatu (**A, C**) and Solomon Islands (**B, D–F**). **A.** General anatomy, right view. **B.** Main ganglia, left view. **C.** CNS, anterior right view. **D.** Main ganglia, posterodorsal view. **E.** 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.

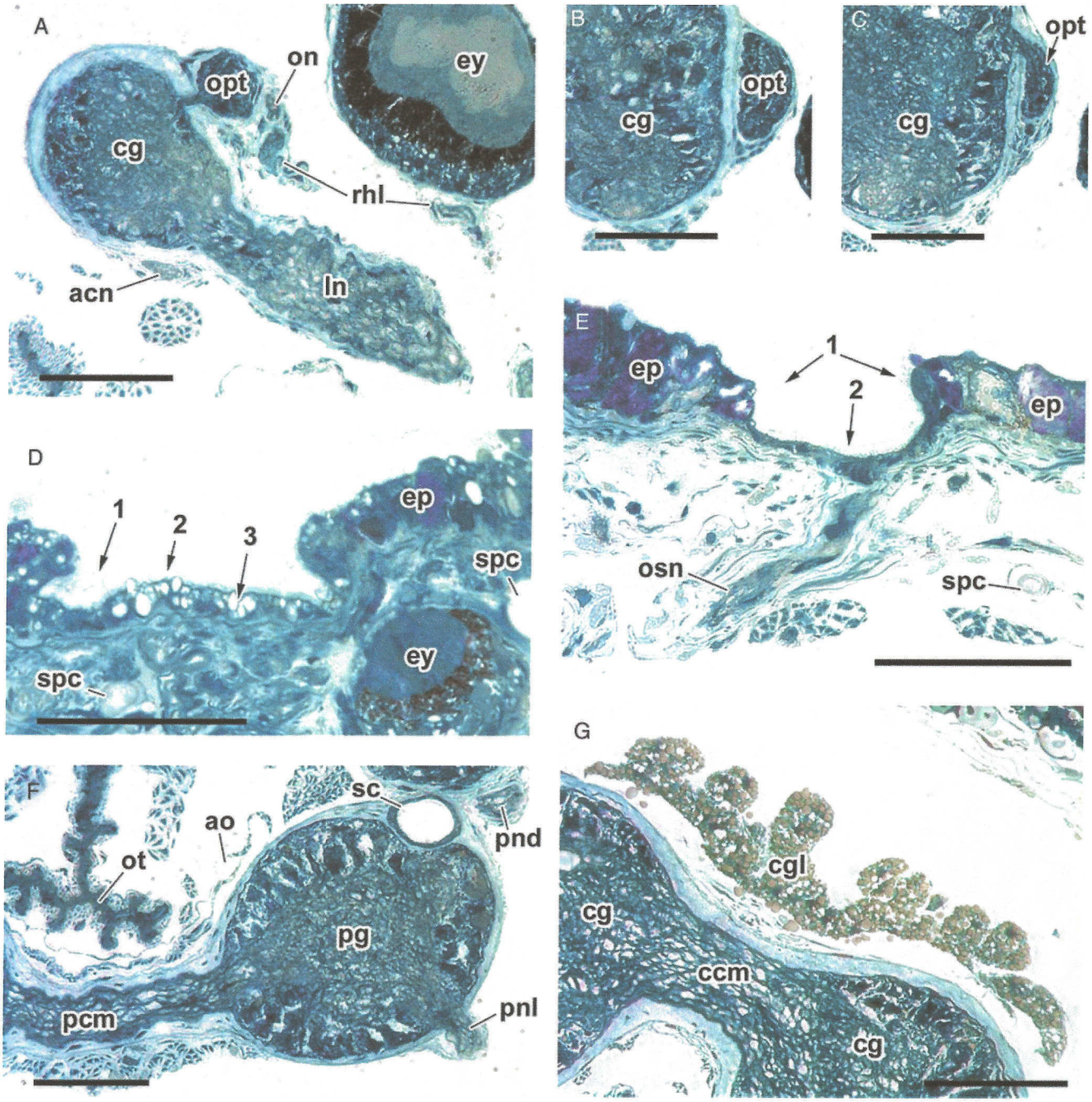

Figure 5. Semithin sections of the CNS and sensory organs (Solomon Islands specimens) of *Strubellia wawrai* n. sp. **A–C.** Cerebral ganglion and double cerebro-optic connectives. **D.** Hancock's organ. **E.** Osphradium. **F.** Pedal ganglion and statocyst. **G.** Cephalic gland dorsally to cerebral 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.

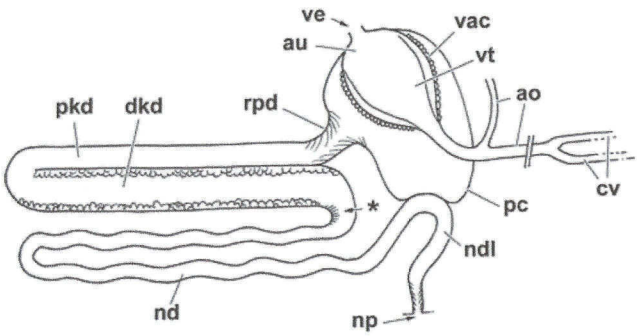

Figure 6. Schematic overview of the circulatory and excretory systems of *Strubellia wawrai* n. sp., right view. Abbreviations: ao, aorta; au, auricle; cv, paired cephalic vessels; dkd, distal kidney lumen; nd, nephroduct; ndl, nephroduct loop; np, nephropore; pc, pericardium; pkd, proximal kidney lumen; 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;

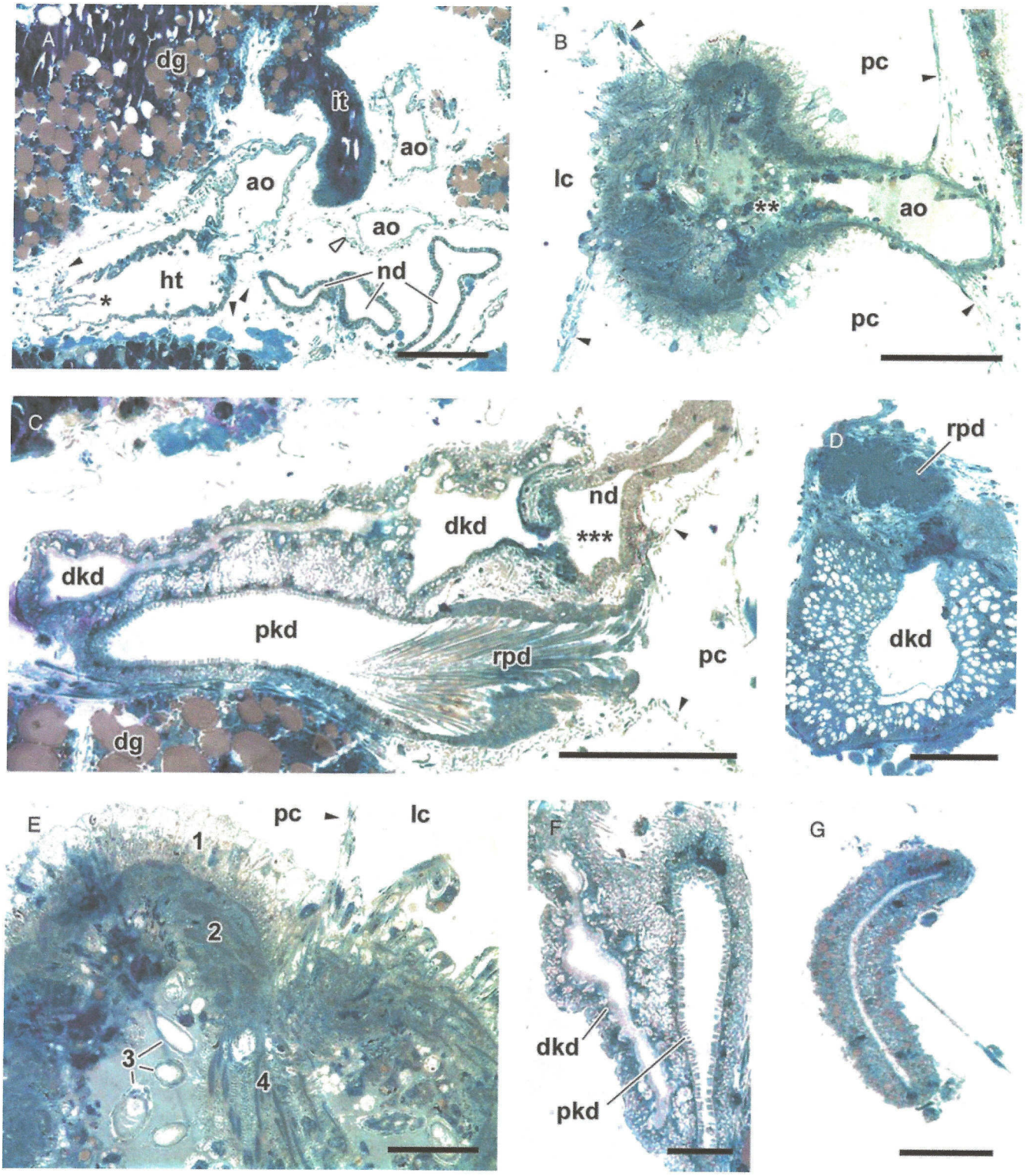

Figure 7. Semithin sections of the circulatory and excretory systems of *Strubellia wawrai* n. sp. (Solomon Islands specimens). **A.** Heart, longitudinal section. **B.** Pericardium and heart, cross-section. **C.** Anterior portion of excretory system, longitudinal section. **D.** Anterior portion of 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

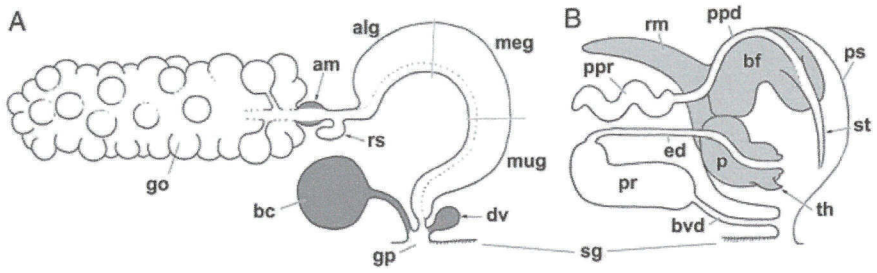

Figure 8. Schematic overview of the genital system and copulatory apparatus of *Strubellia wawrai* n. sp. **A.** Genital system, dark grey areas indicate organs that become reduced in the female phase. **B.** Copulatory apparatus. Abbreviations: alg, albumen gland; am, ampulla; bc, bursa copulatrix; 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

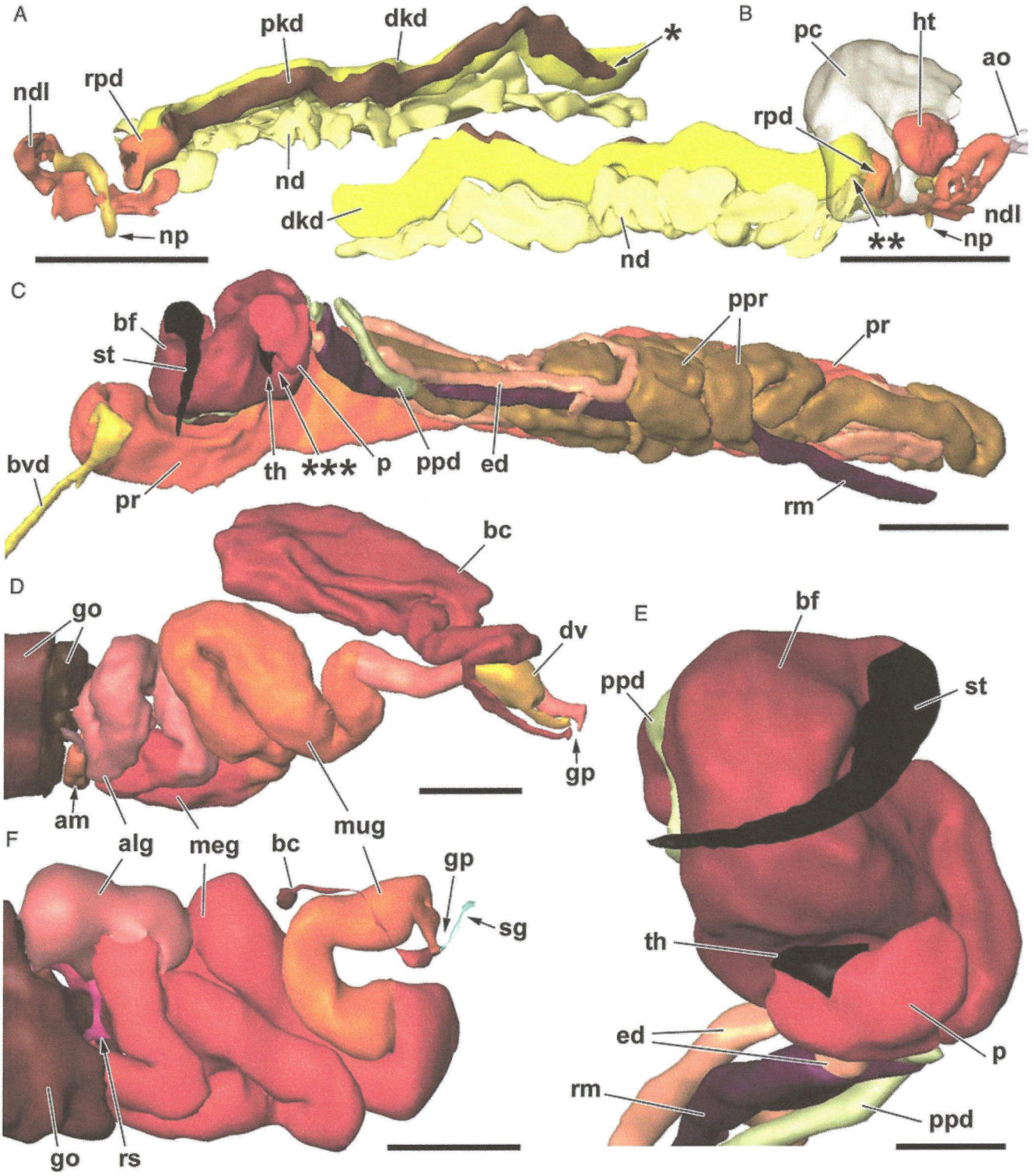

Figure 9. Three-dimensional reconstructions of the excretory, circulatory and reproductive systems of *Strubellia wawrai* n. sp. from Solomon Islands (**A, B, F**) and Vanuatu (**C–E**). **A.** Excretory system, left view. **B.** Excretory and circulatory systems, right view. **C.** Anterior male copulatory 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

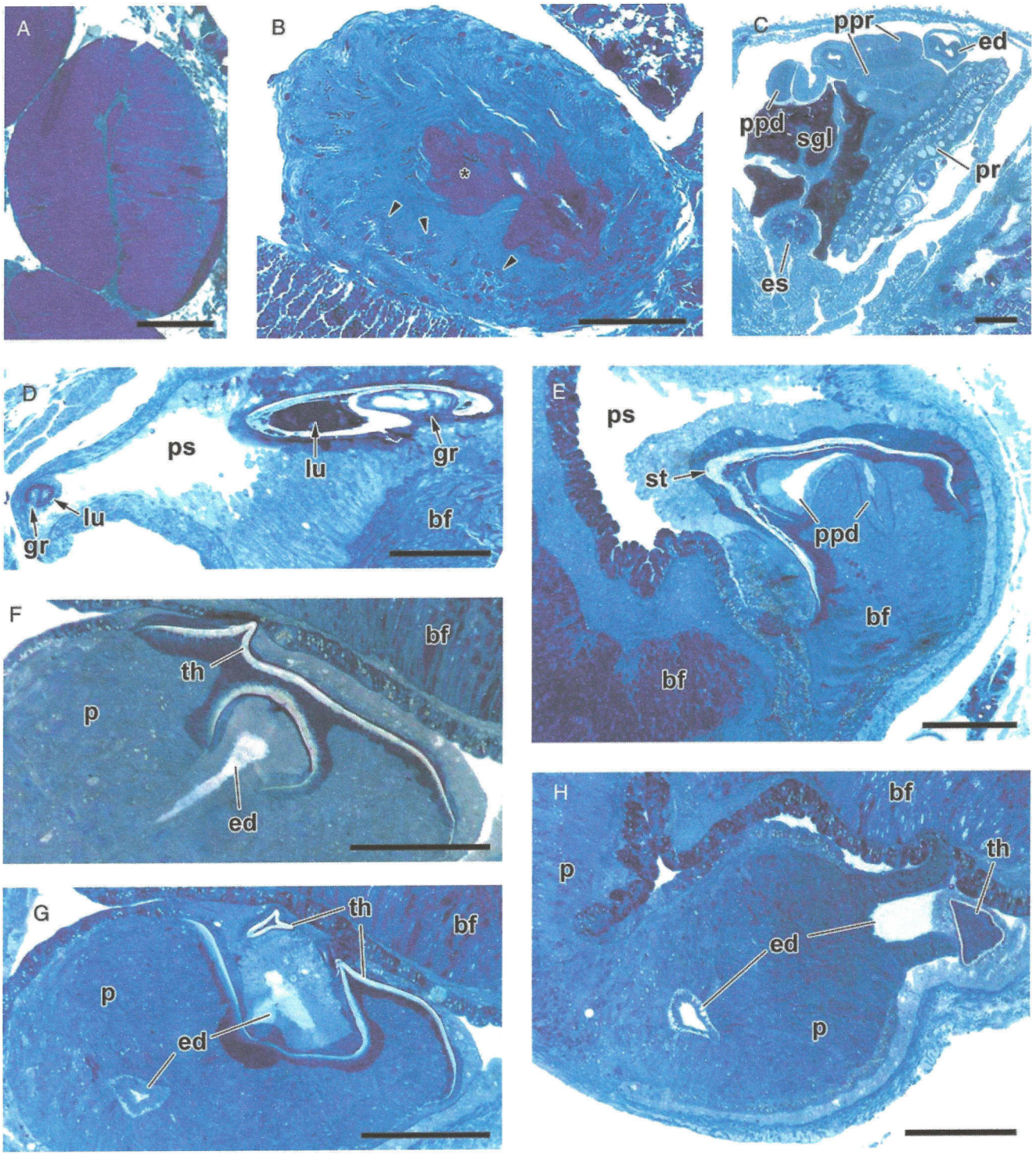

Figure 10. Semithin sections of the genital system of *Strubellia wawrai* n. sp. from Solomon Islands (**A, B**; posterior genital system in female phase) and Vanuatu (**C–H**; parts of copulatory apparatus). **A.** Membrane gland showing acentral lumen. **B.** Receptaculum seminis filled with spermatozoa, 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

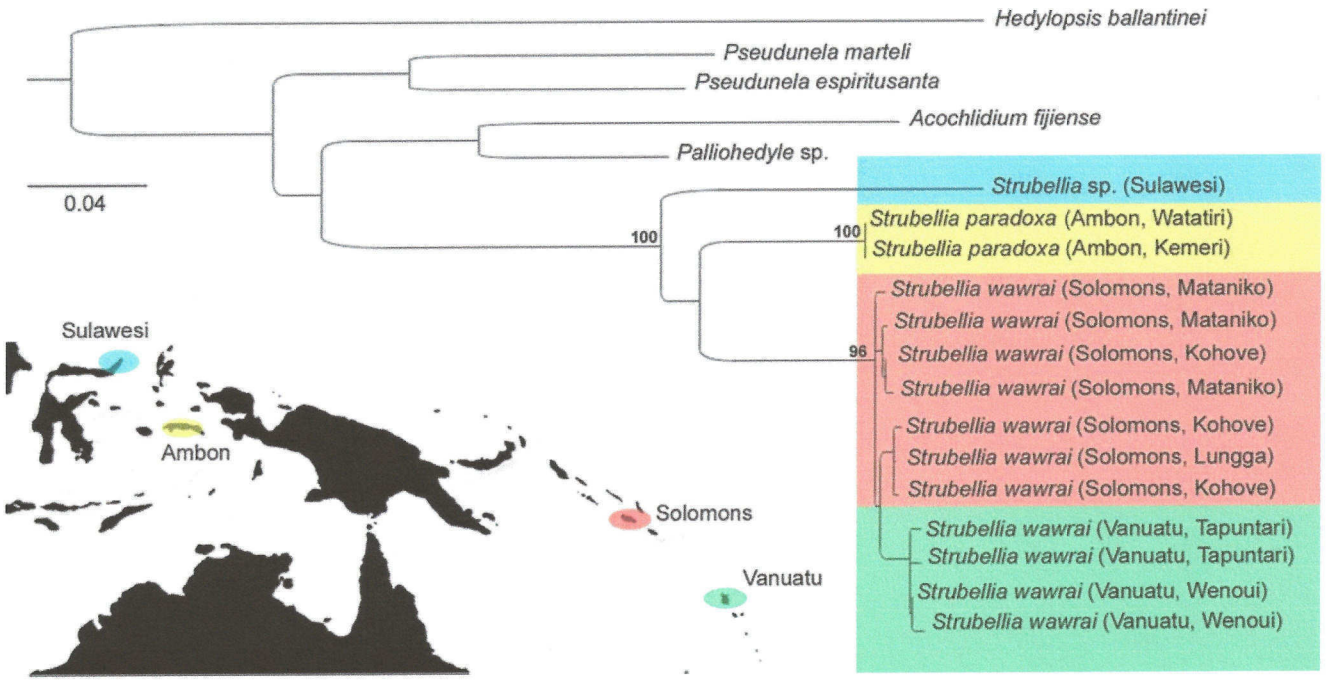

Figure 11. RAxML tree of the genus *Strubellia*, based on a concatenated dataset of mitochondrial COI and 16S rRNA (1113 bp) and colour coded distribution map of the different *Strubellia* species. Bootstrap values given above nodes.

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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3.14 **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.

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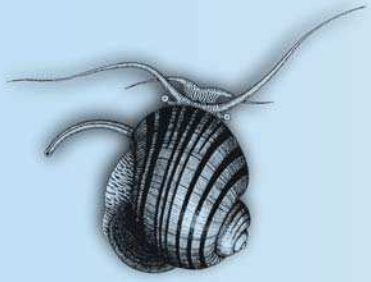

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SACOGLOSSA OR ACOCHLIDIA? 3D RECONSTRUCTION, MOLECULAR PHYLOGENY AND EVOLUTION OF AITENGIDAE (GASTROPODA: HETEROBRANCHIA)

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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.

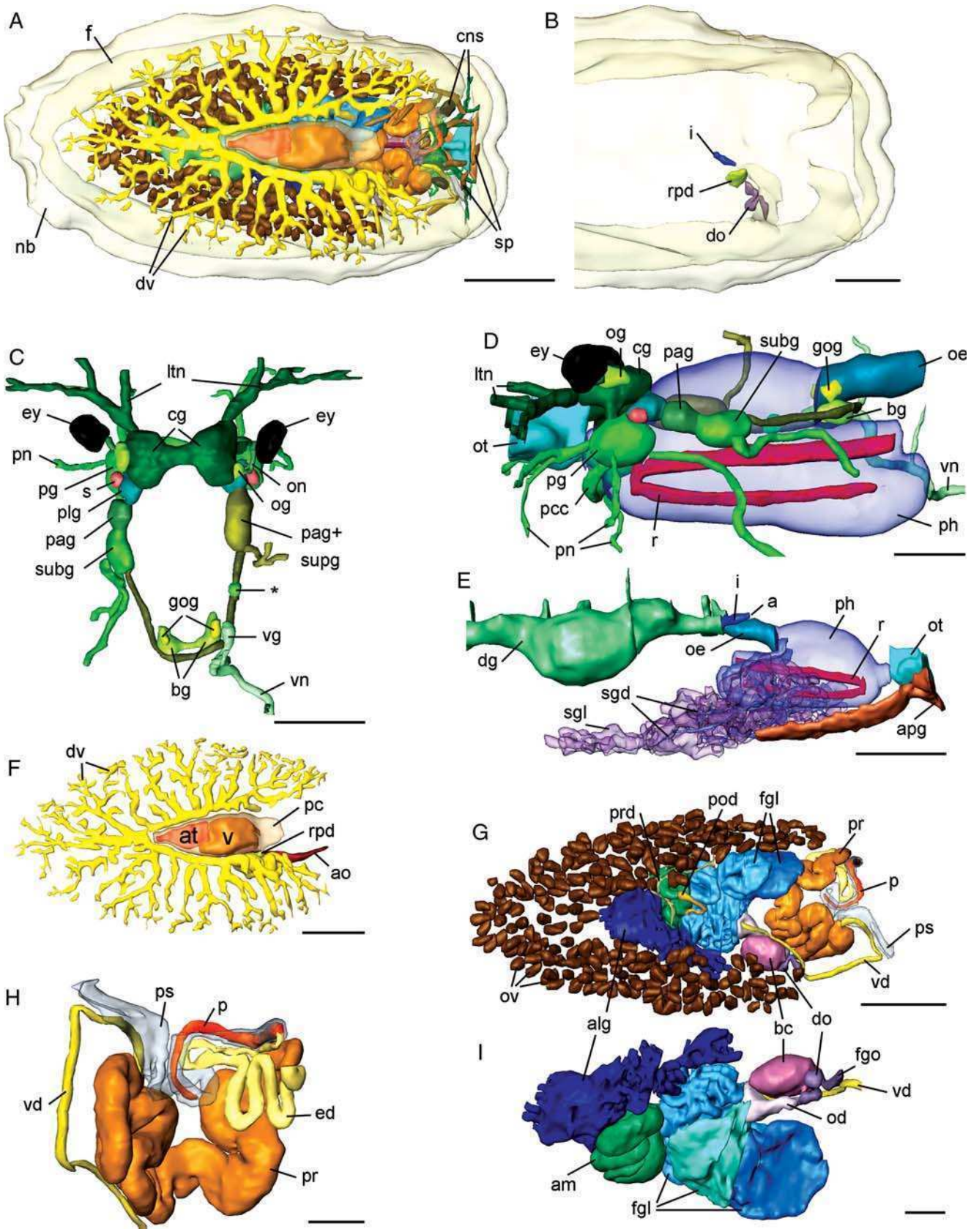
Table 4. Taxon sampling and GenBank accession numbers for the gene sequences used in the present study.

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.



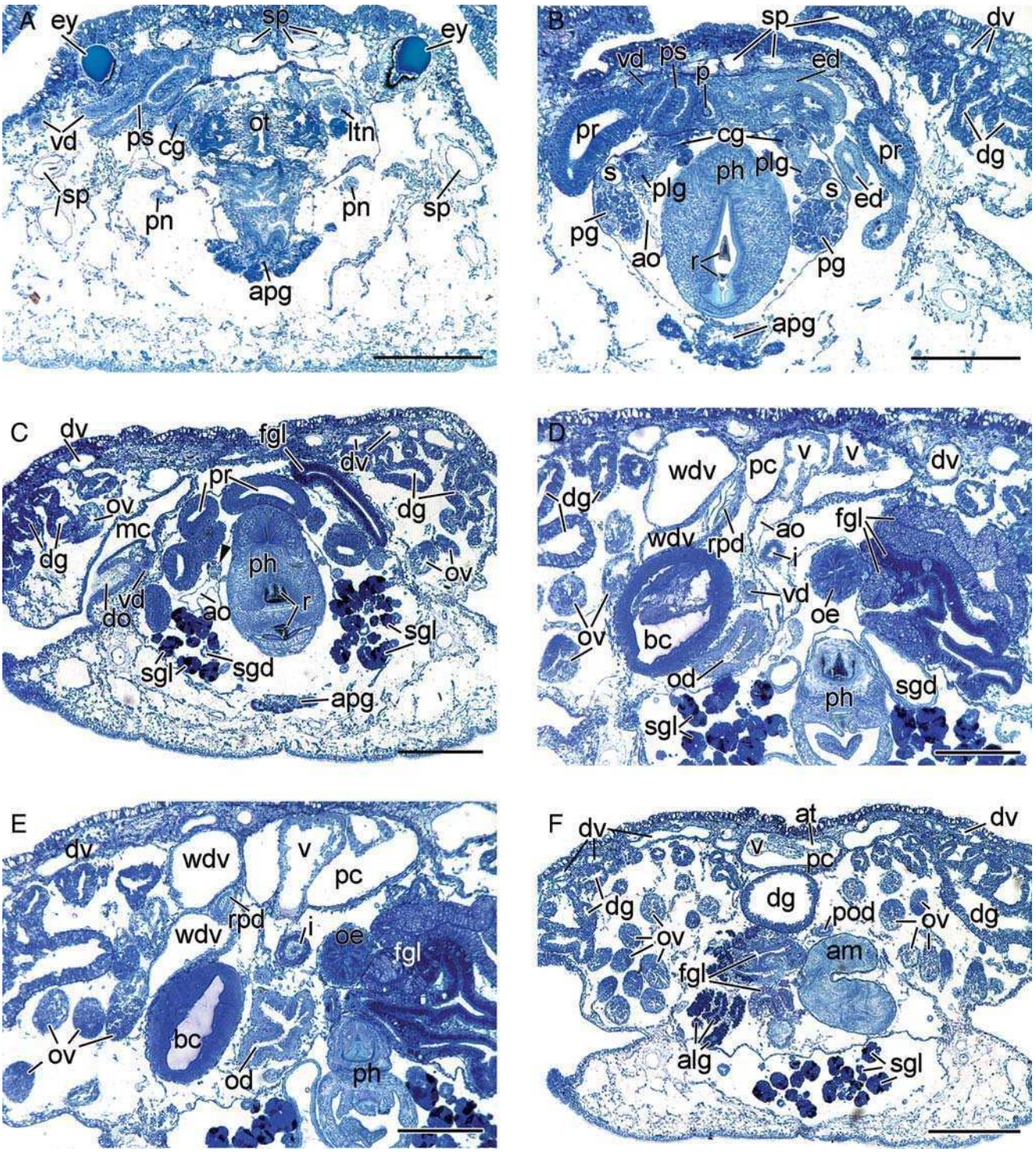


Figure 2. Histological cross-sections of *Aiteng ater*. **A.** Eyes, vas deferens and penial sheath. **B.** Ganglia, prostate. **C.** Mantle cavity. **D.** Dorsal vessels, renopericardioduct. **E.** Bursa copulatrix, ovotestis. **F.** Ampulla. Abbreviations: alg, albumen gland; am, ampulla; ao, aorta; 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

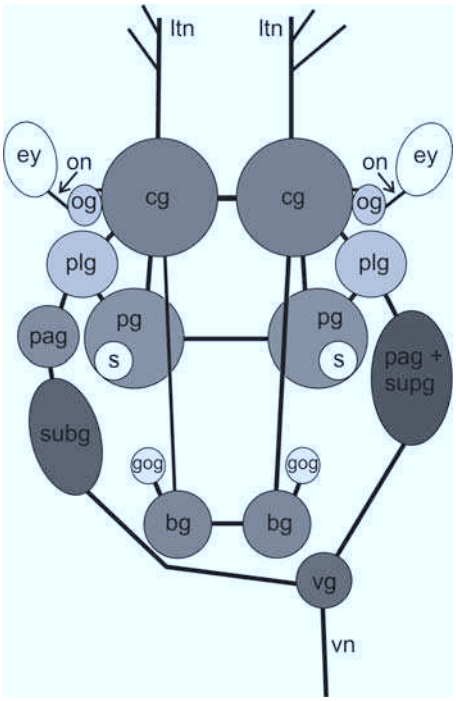


Figure 3. Schematic overview of the central nervous system of *Aiteng ater* (dorsal view). Abbreviations: bg, buccal ganglion; cg, cerebral ganglion; ey, eye; gog, gastro-oesophageal ganglion; ltn, labial tentacle nerve; og, optic ganglion; on, optic nerve; pag, parietal ganglion; pg, pedal ganglion; 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

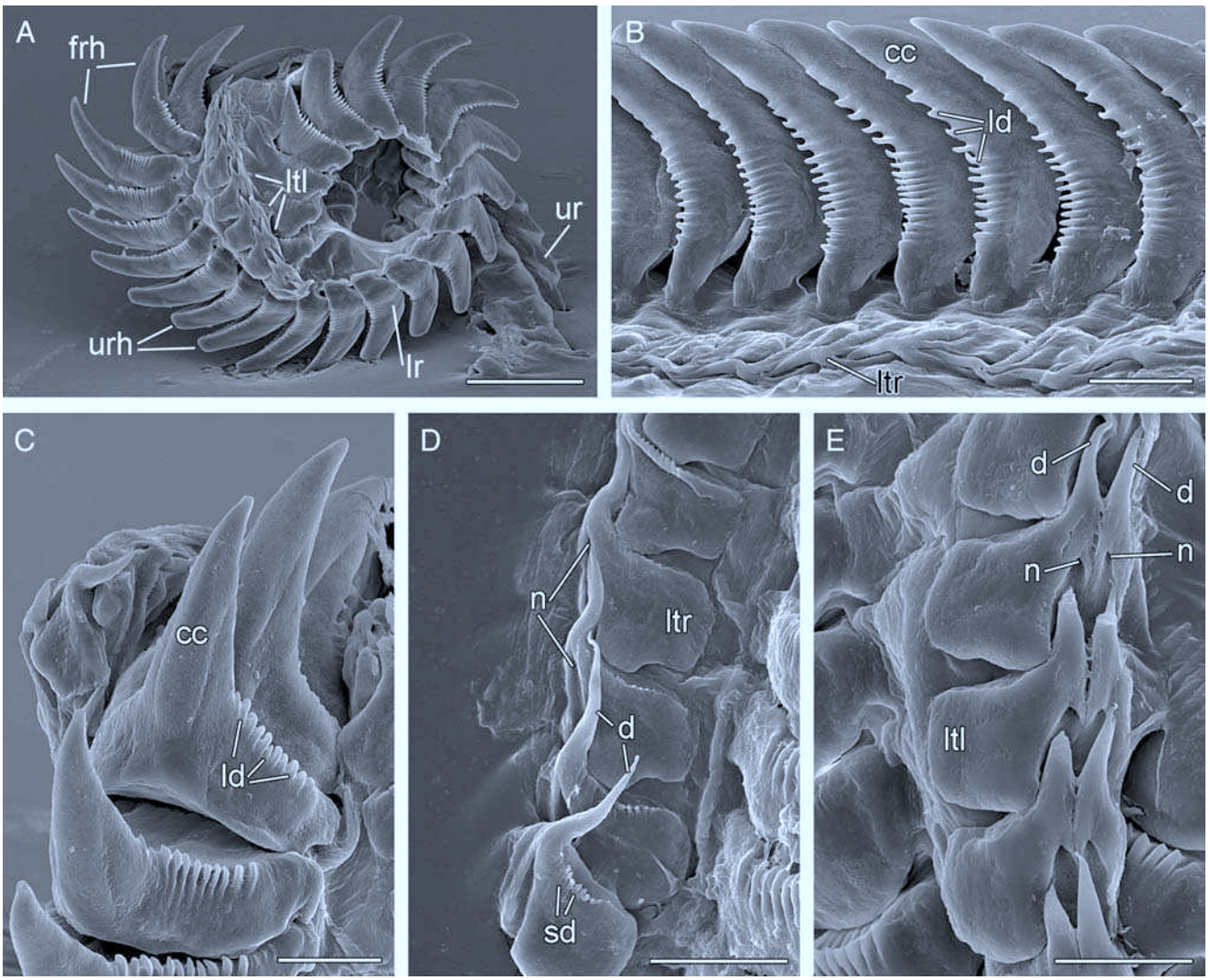


Figure 4. SEM micrographs of the radula of *Aiteng ater*. **A.** Radula, left view. **B.** Rhachidian teeth, right view. **C.** Rhachidian teeth, anterior view. **D.** Right lateral teeth. **E.** Left lateral teeth. Abbreviations: cc, central cusp; d, denticle; frh, functional rhachidian tooth; ld, lateral 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.

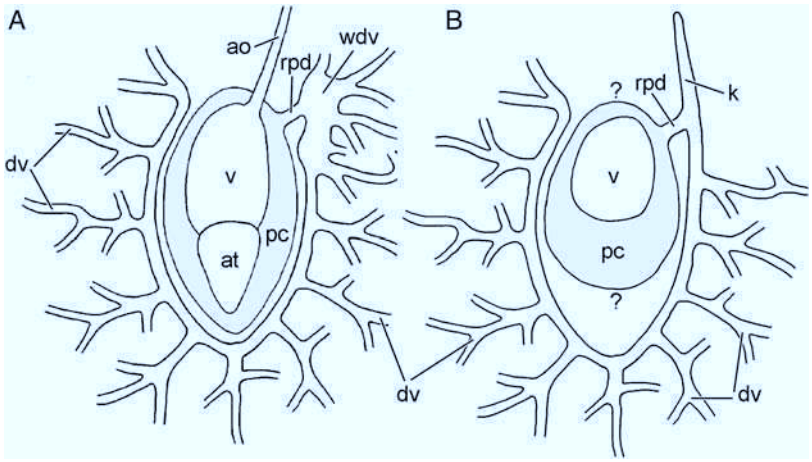


Figure 5. Schematic overview of the circulatory and excretory systems (dorsal view). **A.** *Aiteng ater*. **B.** *Aiteng mysticus* n. sp. Abbreviations: ao, aorta; at, atrium; dv, dorsal vessel; k, kidney; pc, pericardium; rpd, renopericardioduct; v, ventricle; wdv, wide lumen of dorsal vessel; ?, no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

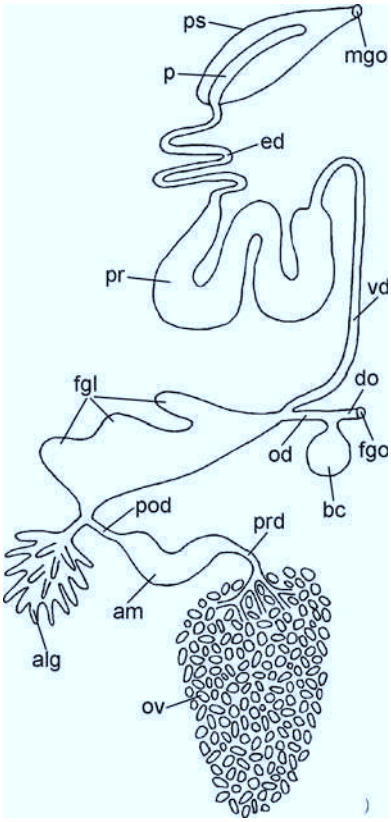


Figure 6. Schematic overview of the reproductive system of *Aiteng ater* (dorsal view). Abbreviations: alg, albumen gland; am, ampulla; bc, bursa copulatrix; do, distal oviduct; ed, ejaculatory duct; fgl, female gland; fgo, female gonopore; mgo, male gonopore; od, oviduct; ov, ovotestis; p, penis; pod, 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.

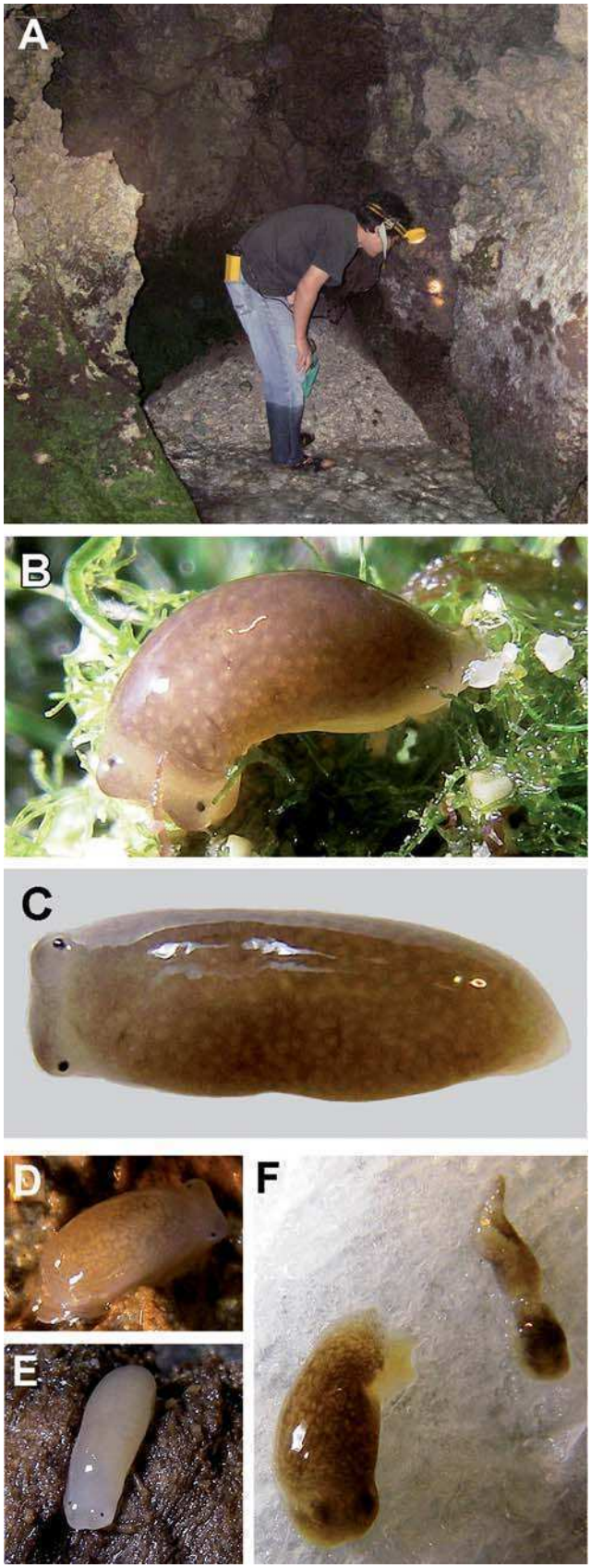


Figure 7. Habitat and external morphology of *Aiteng mysticus* n. sp. **A.** Coastal cavern on Miyako Island, Okinawa, Japan. **B–D, F.** Living specimens of *c.* 5 mm on Miyako Island. **B.** On algae. **C.** Brownish coloration. **D.** Pale coloration. **E.** Pale coloration (Yonaguni Island). **F.**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

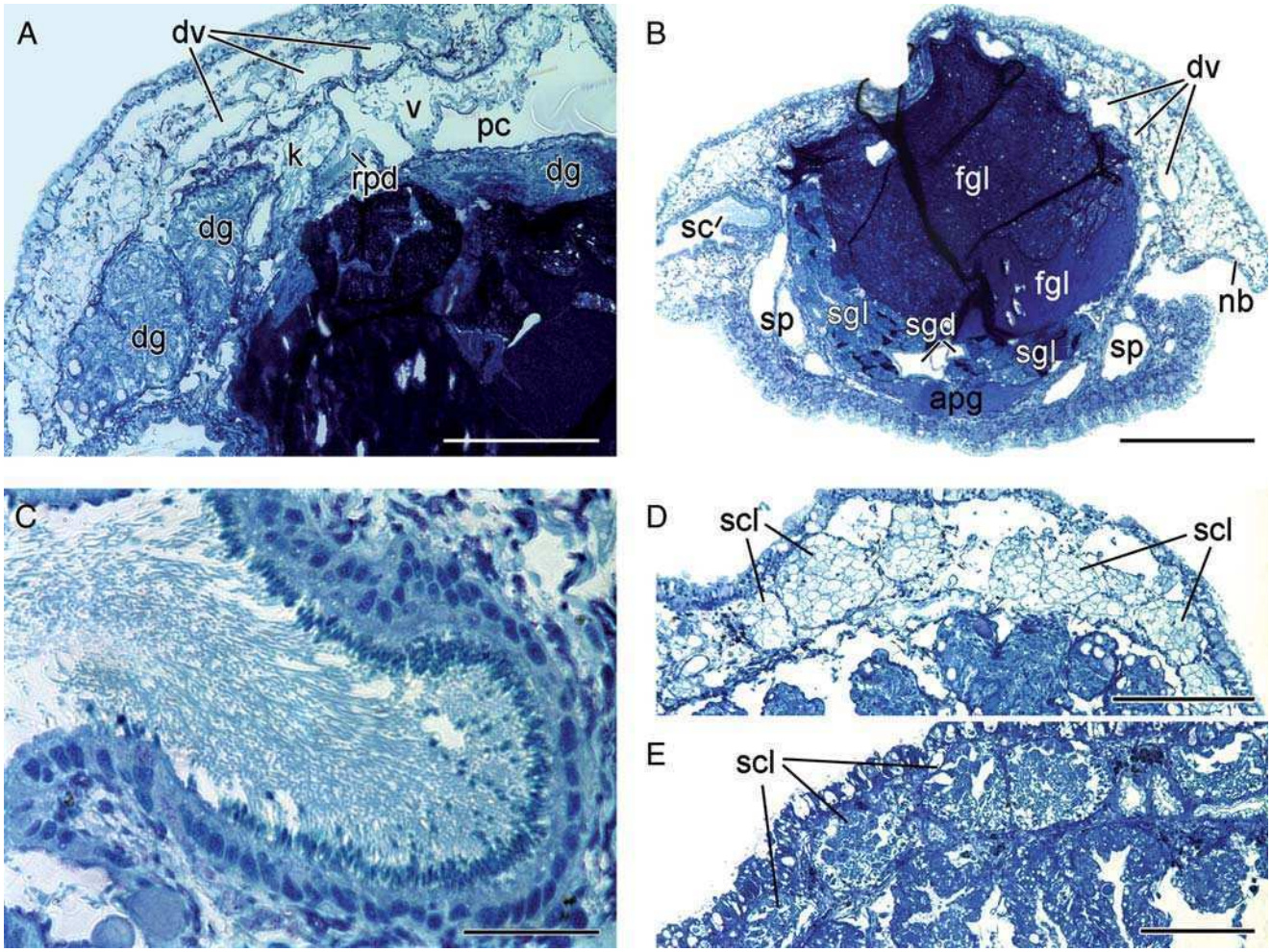


Figure 8. A–**D.** Histological cross-sections of *Aiteng mysticus* n. sp. **A.** Kidney, pericardium. **B.** Female glands, spermatocytes under notum border. **C.** Spermatocytes. **D.** Supporting cells. **E.** Supporting cells in *Aiteng ater*. Abbreviations: apg, anterior pedal gland; dg, digestive 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.

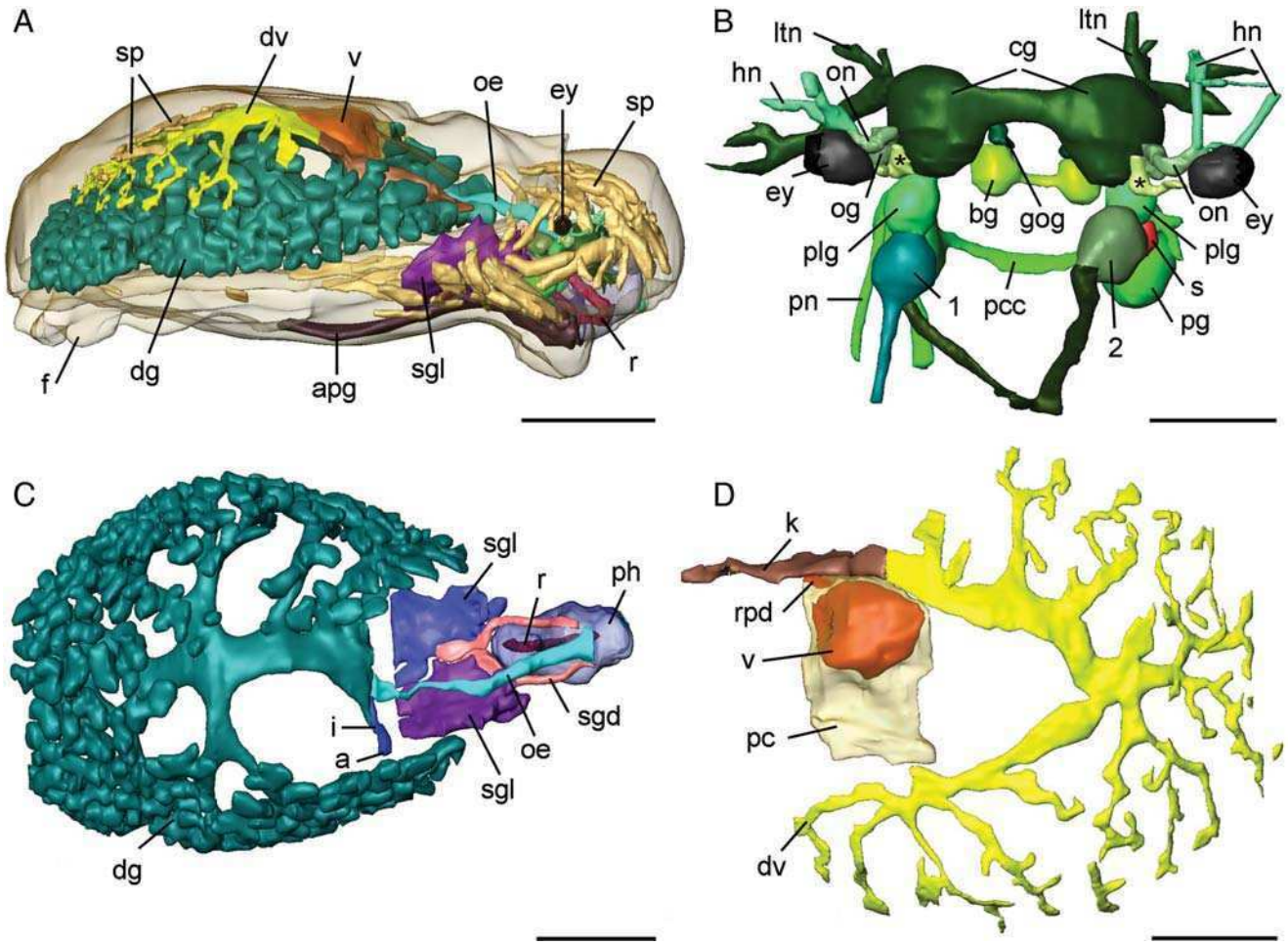


Figure 9. 3D reconstruction of *Aiteng mysticus* n. sp. **A.** General microanatomy, right view. **B.** Central nervous system, dorsal view. **C.** Digestive system, dorsal view. **D.** Circulatory and excretory systems, dorsal view. Abbreviations: a, anus; apg, anterior pedal gland; bg, buccal ganglion; 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;

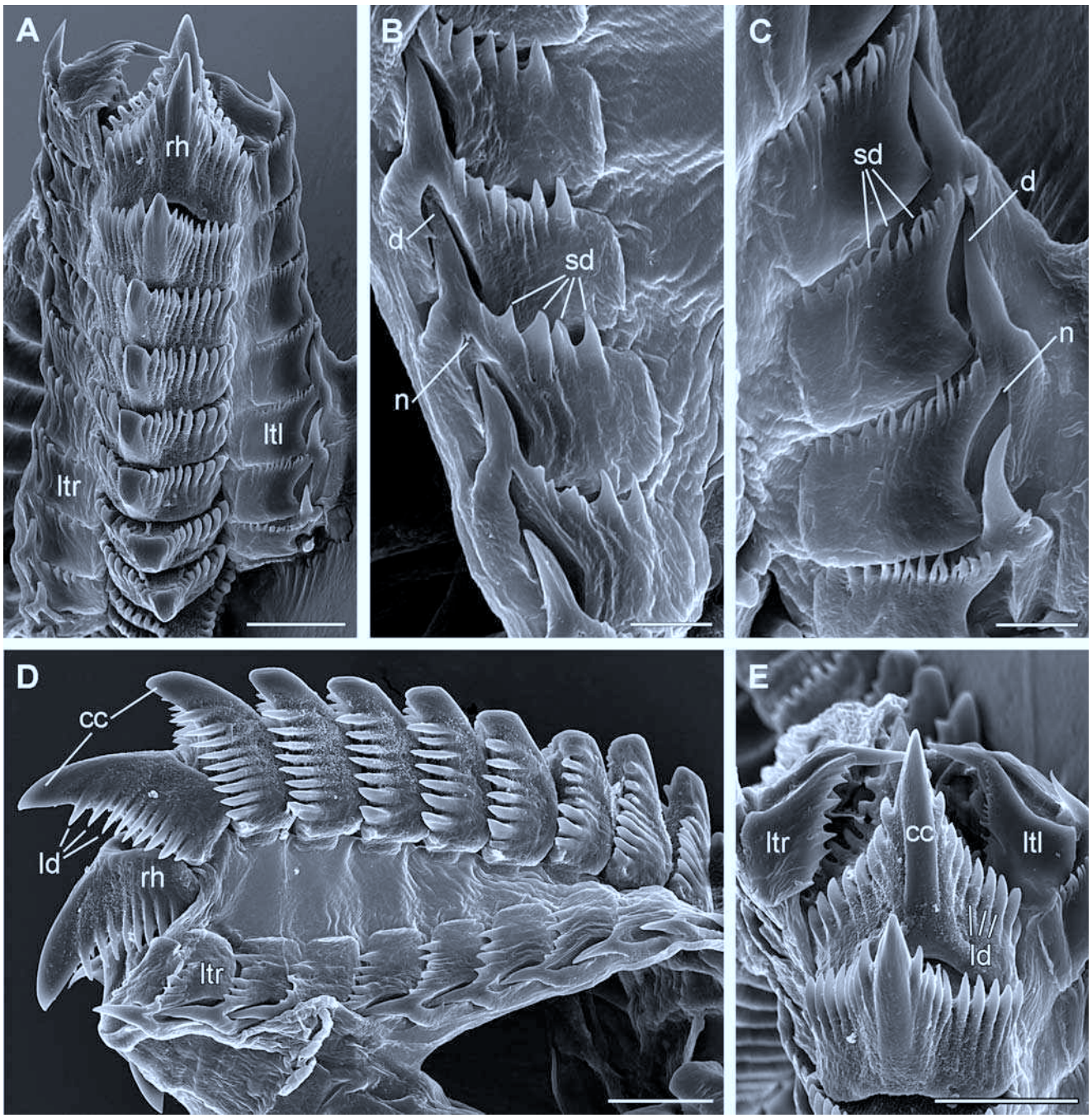


Figure 10. SEM micrographs of the radula of *Aiteng mysticus* n. sp. **A.** Rows of radular teeth (anterior view). **B.** Right lateral teeth. **C.** Left lateral teeth. **D.** Rhachidian teeth, right view; **E.** Rhachidian teeth, anterior view. Abbreviations: cc, central cusp; d, denticle; ld, lateral 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

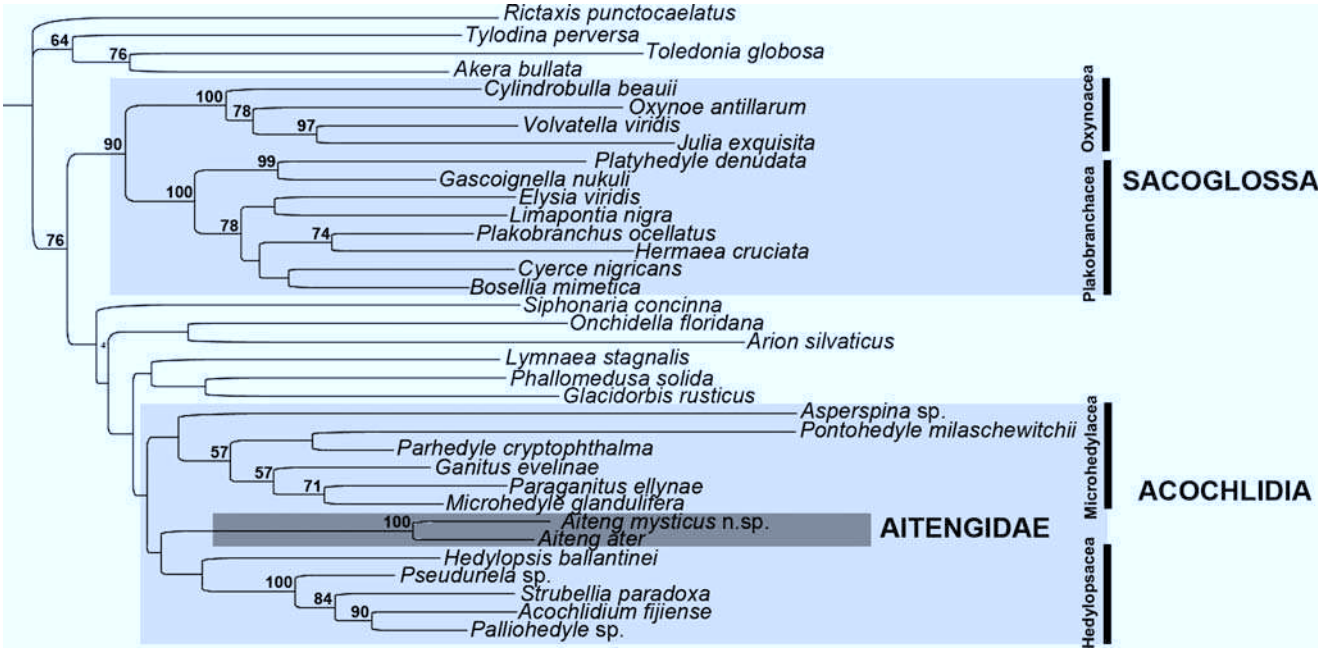


Figure 11. Maximum-likelihood tree generated with RAxML based on the concatenated 28S + COI + 16S dataset, clustering monophyletic Aitengidae basal within Hedylopsacea (bootstrap values >50% given above nodes) *Pseudunela* sp. = *P. marteli* Neusser *et al*., 2011.

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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"Nothing in biology makes sense except in the light of evolution"

DOBZHANSKY (1973)

4 DISCUSSION

Representing one key aspect of research in the work group of Michael Schrödl during the last years, we intensely explored the Acochlidia by a multimodal approach including computer-aided 3D reconstructions with Amira® based on serial semi- or ultrathin sections, analyses by TEM and SEM, molecular studies, phylogenetic analyses and observation of living specimens. In the following, I give a synopsis of the topics with focus on my results.

4.1 A powerful tool for microanatomy: 3D reconstruction with Amira® and interactive 3D modeling

In a case study for Mollusca, and for the first time for heterobranch gastropods, I explored systematically the microanatomy of Acochlidia applying computer-aided 3D reconstructions based on synthetic resin serial semi-thin sections to representatives of seven out of eight acochlidian families. The software Amira® greatly facilitated achieving a detailed, accurate and testable view of minute structures and complex organs in Acochlidia. Among these rarely reported or novel features are numerous tiny (e.g. optic, radular, Hancock`s) nerves, double cerebro-rhinophoral connectives, a Hancock`s organ, a comparted, complex kidney and the complex anterior male copulatory organs. However, an osphradium could be detected only in the large limnic *Strubellia* from the Indo-Pacific (BRENZINGER *et al.* 2011a, b), although an osphradial ganglion was described in several small marine species as well. Even the 3D ultrastructure of small spermatozoa was reconstructed successfully (JÖRGER *et al.* 2009) and offers a great insight in acochlidian morphological diversity. The level of details in the anatomical data is unreached by macropreparatory approaches such as dissectings, which were considered to provide sufficient reliable results in larger opisthobranch specimens (DACOSTA *et al.* 2007). Even conventional methods applying paraffin based histology are inadequate due to larger section thickness and lower section quality (see

RUTHENSTEINER 2008). Recently, SOMMERFELDT & SCHRÖDL (2005) provided a considerably detailed description of the small, marine acochlidian *Hedylopsis ballantinei* Sommerfeldt & Schrödl, 2005 based on serial semithin sections of 2 μm; nevertheless the graphical, handmade reconstructions included are frequently complicated in production due to complex organ systems and tax the scientist's patience. The computer-based 3D reconstruction with Amira® for comparison of anatomy is a great alternative to traditional graphical methods; the advantages are diverse and were partly discussed by DACOSTA *et al*. (2007): (1) organ structures in general and particularly looped ducts can be easily followed through the image stack on the screen, (2) the natural silhouettes and proportions are reconstructed as close to their natural condition as specimen preparation allows, (3) the orientations and the relative spatial positions of the reconstructed organ systems are precise, (4) single or combined organ systems can be easily analysed and presented from different angles of view and (5) the results are reproducible and thus, can be reliably checked in future research. In the last years, different studies appreciating the advantages of anatomical surface reconstructions using Amira® were published (e.g. BRENZINGER *et al.* 2011c; HEß *et al.* 2008; KUNZE *et al.* 2008; MARTIN *et al.* 2009, 2010; RUTHENSTEINER *et al.* 2007; RUTHENSTEINER & STOCKER 2009; SCHULZ-MIRBACH *et al.* 2011). Furthermore, Amira® provides (6) excellent 3D images which subsequently can be used for creating an interactive 3D model for electronic publication, as shown in the case of *Pseudunela* (see NEUSSER *et al.* 2009a, 2011b) and *Strubellia wawrai* Brenzinger, Neusser, Jörger & Schrödl, 2011 (see BRENZINGER *et al.* 2011b). This technical innovation allows any interested reader to explore and understand the anatomy of the reconstructed organ systems in the above mentioned species in detail and from different views – the reader participates and grasps the message in the truest sense of the word. Three-dimensionally reconstructed structures mapped on a two-dimensional layer, i.e. a sheet of paper or an electronic page of the pdf, usually are restricted in terms of the perspective; due to limited space typically only small portions of a detailed object can be represented. Thus, the propagation of 3D data was regarded to be severly hindered by the 2D medium of print publication (DE BOER *et al.* 2011). MURIENNE *et al*. (2008) proclaimed the insertion of interactive 3D models into a pdf as "a 3D revolution in communicating science" and highlighted that this novel method will increase the information content of scientific papers. Several procedures were published yet describing the incorporation of 3D models in scientific publications (BARNES & FLUKE 2008 for astrophysics; DE BOER *et al.* 2011 for medical applications; KUMAR *et al.* 2008 for protein structures; RUTHENSTEINER

& HEß 2008 for biology). Since then, several publications were released including an interactive 3D model embedded into the pdf (e.g. BÄUMLER *et al.* 2008; BRENZINGER *et al.* 2011b; HARTMANN *et al.* 2011; RUTHENSTEINER *et al.* 2010a, b; ZIEGLER *et al.* 2008) or published as online supplementary material (e.g. HASZPRUNAR *et al.* 2011; NEUSSER *et al.* 2011b).

However, 3D reconstructions based on serial histological sections show some side aspects: (1) the application of histological sections implies the permanent transformation of a whole mount to a section series, which is sometimes frowned upon for holotypes or type material consisting of only few specimens. (2) Even if the sectioning is carried out by a skilled person using diamond knifes allowing for ribboned sections (see RUTHENSTEINER 2008), the production of serial section series requires time, particularly for the larger limnic acochlidian species with a body size predestined traditionally for dissection. Additionally, several microhedylacean species are gonochoristic and therefore at least two mature specimens had to be sectioned to examine both sexes; the same applies to some hedylopsacean species that are protandrous or even sequential hermaphrodites, and examining different ontogenetic stages is of interest per se. On the other hand, sectioning only few specimens in order to save time may miss discovering relevant intraspecific variation. (3) Even if the 3D reconstructions with Amira® are computer-assisted and several procedures are semi-automated, such as the alignment of slices, this method still is a time consuming process (see NEUSSER *et al.* 2006; RUTHENSTEINER 2008).

Within the last decade, modern imaging techniques such as CLSM, μCT or μMRI, gained in importance to reveal animal anatomy in morphological studies (ZIEGLER *et al.* 2010a). These methods represent an enormous resource and raised hope for faster and less invasive 3D visualisations than the conventional histological methods (LAURIDSEN *et al.* 2011).

SOMMERFELDT & SCHRÖDL (2005) were the first to apply immunocytochemical staining techniques and CLSM in the study of the acochlidian CNS. The arrangement of major ganglia obtained by histological techniques in *H. ballantinei* was confirmed by CLSM, and some tiny cerebral nerves could be detected. More recently, HOCHBERG (2007) located for the first time a serotonergic network in the CNS of an acochlidian species of the genus *Asperspina* using CLSM and epifluorescence microscopy. JÖRGER *et al*. (2010b) complemented the 3D reconstructions in their study of the CNS and sensory organs of *Parhedyle cryptophthalma* (Westheide & Wawra, 1974), applying immunocytochemistry (staining of FMRFamide and Tyrosine Hydroxylase) in conjunction with CLSM. While

these studies contributed valuable data on the CNS of acochlidian species, they also indicated that the laser scanner is not able to penetrate the whole specimens (JÖRGER *et al.* 2010b). This is in accordance with WANNINGER (2007), who indeed advocated CLSM as an alternative solution for the traditional time consuming reconstructions based on physical histological sections, but admitted that CLSM applications in whole mount preparations are limited to approx. 100 µm. I conclude that CLSM cannot substitute 3D reconstructions based on histological sections for studying the comparative morphology in (non-larval or early juvenile) Acochlidia. Nevertheless, it provides useful additional data on the e.g. CNS, musculature or ciliation patterns originating from immunohistochemistry (e.g. WANNINGER 2009; WORSAAE & ROUSE 2009, 2010). A promising way to achieve 3D reconstructions of 300 to 500 µm thickness by means of laser scanning microscopy is the use of 2-photon microscopy, as demonstrated recently e.g. by KOCH *et al*. (2010).

MicroCT is an established and broadly applied, non-invasive technique for imaging x-ray dense, and hence high contrast-producing materials such as diverse mineralised animal tissues (e.g. bony skeletons of vertebrates or the hard exoskeletons of invertebrates) (e.g. DINLEY *et al.* 2010; RUTHENSTEINER *et al.* 2010a). In contrast, soft-bodied and especially aquatic invertebrates as the Acochlidia are considered as the most difficult biological specimens to scan as their internal tissue densities are minimally different and the tissues themselves very closely approximated (DINLEY *et al.* 2010). Although µCT was applied successfully to the study of e.g. odontophoral cartilages of Caenogastropoda (GOLDING *et al.* 2009), muscles associated with the pharynx and anterior gut in nephtyid worms (DINLEY *et al.* 2010) or to reconstruct spicule patterns in the nudibranch *Polycera quadrilineata* (Müller, 1776) (see ALBA-TERCEDOR & SÁNCHEZ-TOCINO 2012), the widespread application of µCT imaging in comparative morphology has been limited by the low intrinsic x-ray contrast of non-mineralised tissues (METSCHER 2009). Recent studies show that soft tissue borders can be enhanced using contrast media (DINLEY *et al.* 2010). The application of special high atomic weight stains, e.g. osmium tetroxide or iodine, allowed high-contrast 3D imaging of different non-mineralised animal tissues, among them of the caudofoveate mollusc *Falcidens* sp. (METSCHER 2009). However, METSCHER (2009) emphasised that each new type of sample must be tested with diverse fixations and stains to discover the best treatment for the imaging required; this fact impedes the application if only few specimens are available. Besides, the use of contrast media signifies an alteration of the material and should be considered as invasive too. The combination of µCT of resin-embedded specimens and

subsequent serial sectioning and 3D microanatomical modeling seems promising for enhancing analytical power and accuracy.

ZIEGLER *et al.* (2008; 2010b) compared the anatomy of sea urchins using MRI and considered this method as particularly suited for soft tissue studies. However, HOLLAND & GHISELIN (2009) proved that MRI failed to distinguish between smaller gut regions and larger haemal sinuses in the study of ZIEGLER *et al*. (2008). Recently, LAURIDSEN *et al*. (2011) presented promising results on bones, inner organs and blood vessels using μCT and MRI. However, their material examined included a variety of "large-sized" vertebrates and spiders, and hence these results probably cannot be projected to micromolluscs without prior validation against histology-based 3D models.

Summing up, in spite of the great advantages of "non-invasive" modern imaging techniques in different scientific areas, they cannot replace the, yet elaborate and irreversible, application of histological techniques for anatomical studies in tiny meiofaunal gastropods at the moment. Yet I agree with Ziegler & Bartolomaeus (in HOLLAND & GHISELIN 2009) that future histological studies with 3D reconstructions can benefit from employing different combinations of modern imaging techniques (e.g. LAFORSCH *et al.* 2012; SCHWAHA *et al.* 2010). The consequent examination of acochlidian representatives by means of 3D reconstruction of histological serial sections with Amira® reaches a new level of unprecedented detail and accuracy in acochlidian research. These high-quality anatomical data now available are benchmarks for future comparative morphological, taxonomic and evolutionary studies in micromolluscs and other small invertebrates.

4.2 Beyond traditional taxonomy - modern Acochlidian microanatomy

Within the scope of my dissertation I investigated the question to what extent modern microanatomy can supplement or even correct data derived from traditional taxonomy, since older species descriptions were often limited to the external morphology, the structure of spicules and the examination of the radula by light microscopy. Even original descriptions based on (partly incomplete series of) semithin histological sections of 3 μm (RANKIN 1979) turned out to often lack complete information of organ systems and required critical re-examination as well.

In the following I give a "before-and-after" comparison of selected acochlidian organs.

4.2.1 Digestive system

Oral gland versus anterior pedal gland

In the past, different glands situated in the anterior body and associated with the mouth opening or the oral tubes were subject to inconsistent naming and misidentification in different acochlidian species. Glands discharging into the oral tube were named "oral gland" in *Paraganitus ellynnae* (see CHALLIS 1968), *Pontohedyle verrucosa* and *Pseudunela cornuta* (see CHALLIS 1970) or vestibular gland in *Microhedyle nahantensis* (Doe, 1974) and *Ganitus evelinae* Marcus, 1953 (see DOE 1974 and MARCUS 1953, respectively). Glands opening to the exterior, ventral to the mouth opening, were named in the same way "oral" or "suprapedal gland" in *Tantulum elegans* (see RANKIN 1979). However, histochemical investigations by ROBINSON & MORSE (1976) showed the "vestibular gland" of *M. nahantensis* to be a large anterior pedal gland not connected to the oral tube, but opening to the exterior ventral to the mouth. Our 3D reconstructions reveal both types of glands being present in Acochlidia: the anterior pedal gland opening to the exterior and oral (tube) glands discharging into the oral tube in almost all acochlidian species examined in detail (e.g. BRENZINGER *et al.* 2011b; EDER 2011; JÖRGER *et al.* 2008; NEUSSER *et al.* 2009a, b; NEUSSER & SCHRÖDL 2007, 2009). Even the salivary glands were misinterpreted: while all acochlidian species have voluminous paired salivary glands, the 'salivary' gland of *Hedylopsis spiculifera* indicated by ODHNER (1937) was considered to be in fact the prostate by WAWRA (1989). SOMMERFELDT & SCHRÖDL (2005) reconstructed a specimen of *H. spiculifera* (det. Odhner as *H. suecica*) and confirmed a well developed prostate anterior to the large, paired salivary glands.

Pharynx

RANKIN (1979) discussed two different types of buccal cavities in acochlidians. The first type, described in Ganitidae, represents a much modified pharynx with strongly developed longitudinal muscles connecting the ventral cuticular radular cushion with a pair of cuticular jaws (CHALLIS 1968; MARCUS 1953). The second type included a series of 1) a poorly developed pharynx with a small radular cushion, as in *Parhedyle tyrtowii* (see KOWALEVSKY 1901), 2) a well-developed pharynx, as in *Acochlidium amboinense* (Strubell, 1892) (see BÜCKING 1933), and 3) a very complex buccal cavity showing a highly muscular and bulbous pharynx, as in *Tantulum elegans* (see RANKIN 1979). BÜCKING (1933) described *A. amboinense* with a muscular pharynx being broad in the ventral part and narrower in the dorsal part. His drawings show both parts connected, whereas Rankin's schematic drawings (RANKIN 1979) do not match the original drawings of

Bücking and give the impression of a deep groove between the dorsal and the ventral part. While the modified character of ganitid buccal masses was recently confirmed by EDER (2011), the pharynx of all other acochlidian species examined herein is similarly structured as in *T. elegans*; non-ganitid acochlidians cannot be differentiated merely by pharyngeal gross morphology.

Radulae

Most of the acochlidian species were described to possess bilaterally symmetric radulae with one lateral tooth on each side, such as *Pontohedyle milaschewitchii*, *Asperspina riseri* (Morse, 1976) or *A. loricata* (see JÖRGER *et al.* 2008; MORSE 1976; SWEDMARK 1968b). But several acochlidian species were originally reported to have a radula formula of n x 2.1.2, including two lateral teeth on each side of the rhachidian tooth, e.g. the marine *A. brambelli* and *A. murmanica* (see KUDINSKAYA & MINICHEV 1978; SWEDMARK 1968b) or the limnic *Acochlidium amboinense* and *Strubellia paradoxa* (Strubell, 1892) (see BÜCKING 1933; KÜTHE 1935). However, radulae with the formula of n x 2.1.2 were proven to be non-existent and were shown to be asymmetric with the formula n x 1.1.2, including an additional tooth of the right side only, e.g. in *A. murmanica* or in *S. paradoxa* (see BRENZINGER *et al.* 2011a; NEUSSER *et al.* 2009b). Only occasionally has light microscopy led to detection of asymmetrical radulae of acochlidians, such as in *A. rhopalotecta* (Savini-Plawen, 1973) (VON SALVINI-PLAWEN 1973). Obviously, BERGH (1895) was not aware of asymmetric radulae yet: whereas Bergh's text in the original description of *Palliohedyle weberi* mentions one lateral plus one marginal tooth, his figures suggest a marginal tooth on the right side only. Most of the recently examined species of the Hedylopsacea have been shown to possess a characteristic asymmetric radula with a formula of n × 1.1.2 (e.g. BRENZINGER *et al.* 2011b; NEUSSER *et al.* 2011b; NEUSSER & SCHRÖDL 2009; SOMMERFELDT & SCHRÖDL 2005).

Summing up, the traditional examination of the radula by light microscopy turned out to be methodologically inadequate. Although the asymmetry of the radula could be proven even light microscopically in some species before (e.g. RANKIN 1979; VON SALVINI-PLAWEN 1973; WAWRA 1989; WESTHEIDE & WAWRA 1974), the examination by SEM was indispensable for the detailed description of tiny structures, such as the shape and position of the lateral teeth denticles or rounded vs. straight borders of the lateral teeth (BRENZINGER *et al.* 2011a, b; NEUSSER *et al.* 2011b; NEUSSER & SCHRÖDL 2009). Furthermore, in contrast to the original description (SWENNEN & BUATIP 2009) we detected lateral teeth in *Aiteng ater* Swennen & Buatip, 2009 (NEUSSER *et al.* 2011a) and,

for the first time a (to our knowledge) unknown radular feature: in aitengid species lateral teeth are absent in the oldest part of the lower ramus and only few of the youngest teeth of the lower ramus bear lateral teeth, i.e. teeth rows with either three or one teeth are present in the same radula.

Stomach

A distinct 'stomach' was originally described for e.g. *Asperspina murmanica*, *Pontohedyle milaschewitchii*, *Pseudunela cornuta* and some Acochlidiidae, such as *Palliohedyle weberi* by BERGH (1895) and *Acochlidium amboinense* by BÜCKING (1933). While a stomach fused with the anterior cavity of the digestive gland is present in some acochlidian species, such as *Tantulum elegans*, *P. milaschewitchii*, and *A. murmanica* (see JÖRGER *et al.* 2008; NEUSSER *et al.* 2009b; NEUSSER & SCHRÖDL 2007), a histologically and anatomically distinct organ is absent in all Acochlidia studied in detail.

4.2.2 Reproductive system

The acochlidian reproductive system shows a large variety of peculiar features. The quite simple, reduced reproductive system of the aphallic Microhedylidae was often correctly described (e.g. KOWALEVSKY 1901; KUDINSKAYA & MINICHEV 1978) and some details could be supplemented recently (JÖRGER *et al.* 2008; NEUSSER *et al.* 2009b). In contrast, the hedylopsacean reproductive system was either completely unknown, e.g. despite its huge size and considerable complexity it has been overlooked in *Tantulum elegans* by RANKIN (1979), described only by the penial armature, e.g. in *Pseudunela eirene* Wawra, 1988 (see WAWRA 1988a), or remained a mystery. For instance, *Hedylopsis ballantinei* was assumed to be the only aphallic hedylopsacean species without any copulatory organs (SOMMERFELDT & SCHRÖDL 2005); the original description of the anterior copulatory organs of *Pseudunela cornuta* was incomplete (CHALLIS 1970); even the reproductive system of the majority of the large limnic species, such as *Acochlidium weberi*, *A. sutteri* (Wawra, 1979), *A. bayerfehlmanni* Wawra, 1980, *A. amboinense* and *Strubellia paradoxa* were only partly examined by dissection with special focus only of the penis and its armature (see BAYER & FEHLMANN 1960; BERGH 1895; BÜCKING 1933; KÜTHE 1935; WAWRA 1979a, 1980). Unfortunately, the interpretation of the different ducts, glands and stylets remained confusing in all the latter species. The only exception was the detailed description of the reproductive system of *Acochlidium fijiense* Haynes & Kenchington, 1991, which, however, still showed some strange features such as a connection between digestive and reproductive systems (HAASE & WAWRA 1996).

Applying modern 3D microanatomy to four re-examined (BRENZINGER *et al.* 2011a; KOHNERT *et al.* 2011; NEUSSER *et al.* 2009a; NEUSSER & SCHRÖDL 2007) and four new described (BRENZINGER *et al.* 2011b; NEUSSER *et al.* 2011b; NEUSSER & SCHRÖDL 2009) hedylopsacean species, we revealed highly complex anterior male copulatory organs within the Hedylopsacea including a bipartite penis in all members, a more or less elaborate impregnatory system with thorns and stylets in most species, and a second impregnatory system with associated glands in several hedylopsaceans (e.g. BRENZINGER *et al.* 2011a, b; NEUSSER *et al.* 2009a, 2011b; NEUSSER & SCHRÖDL 2007, 2009). We showed the "aphallic" *Hedylopsis ballantinei* is a sequential hermaphroditic species with complex and voluminous anterior copulatory organs being completely reduced in later, female stages (KOHNERT *et al.* 2011). Now, *H. ballantinei* fits well, with evolutionary traits observed, within other hedylopsacean acochlidians known in detail. Additionally we described a special type of androdiaulic reproductive system for *Tantulum elegans* and *Pseudunela* implying the transport of autosperm through the female glands, a fact which was already indicated in *Acochlidium fijiense* (see HAASE & WAWRA 1996).
A comparative compilation of the acochlidian reproductive systems by RANKIN (1979) suffered from the uncritical and in some species erroneous use of literature data without re-examination of type or newly collected material; therefore her sketchy and simple drawings of different acochlidian reproductive systems are misleading and must be considered as useless. A more realistic survey at least of copulatory organs is given in SCHRÖDL & NEUSSER (2010, fig. 2) plus additions e.g. in KOHNERT *et al.* (2011), NEUSSER *et al.* (2011b) and NEUSSER & SCHRÖDL (2009).

4.2.3 Excretory system

In the past little attention was paid to the acochlidian excretory system. Several original descriptions provided only few data on the excretory system or even lack any (e.g. BAYER & FEHLMANN 1960; BERGH 1895; BÜCKING 1933; HAYNES & KENCHINGTON 1991; SWEDMARK 1968b; VON SALVINI-PLAWEN 1973; WAWRA 1979a, 1980). For marine Acochlidia a small, sac-like kidney with a short nephroduct was generally assumed (CHALLIS 1968, 1970; DOE 1974; KUDINSKAYA & MINICHEV 1978; MARCUS & MARCUS 1954; MORSE 1976); only *Hedylopsis* was reported to show a long, sac-like kidney extending almost over the entire visceral sac (FAHRNER & HASZPRUNAR 2002; ODHNER 1937; SOMMERFELDT & SCHRÖDL 2005). In contrast, the limnic *Tantulum elegans* and *Strubellia paradoxa* were described with a complex kidney including different compartments and a long looped nephroduct (KÜTHE 1935; RANKIN 1979). By means of

the 3D reconstructions we revealed that a quite simple excretory system including a small, sac-like kidney and a short nephroduct only applies for all marine microhedylacean species known in detail (EDER 2011; JÖRGER *et al.* 2008; NEUSSER *et al.* 2009b). In contrast, all members of the Hedylopsacea possess a complex excretory system comprising an internally divided kidney with a narrow and a wide lumen. All fully marine hedylopsacean species (*Hedylopsis spiculifera*, *H. ballantinei*, *Pseudunela marteli* Neusser, Jörger & Schrödl, 2011 and *P. viatoris* Neusser, Jörger & Schrödl, 2011) additionally have a short nephroduct (NEUSSER *et al.* 2011b). However, the temporary brackish *Pseudunela cornuta* (see NEUSSER *et al.* 2009a) and the fully brackish *P. espiritusanta* Neusser & Schrödl, 2009 (see NEUSSER & SCHRÖDL 2009) possess a long, looped nephroduct with two branches as the limnic species *T. elegans* (see NEUSSER & SCHRÖDL 2007) and the Acochlidiidae (BRENZINGER *et al.* 2011a, b). For the first time in Acochlidia we described a dense layer of vacuolated cells covering the outer surface of the ventricle in *P. espiritusanta* and *Strubellia paradoxa* (see BRENZINGER *et al.* 2011a; NEUSSER & SCHRÖDL 2009). These cells were discussed as potential podocytes and hence as a novel site of ultrafiltration involved in the production of primary urine similar to the "pericardial glands" found in doridoidean nudibranchs (FAHRNER & HASZPRUNAR 2002) and many bivalves (e.g. ANDREWS & JENNINGS 1993; MEYHÖFER *et al.* 1985).

4.2.4 Central nervous system

Mosaic-like features versus a general pattern

A mosaic-like distribution of features of the acochlidian CNS was reported in the past. Several of the species (re)descriptions in Acochlidia did not include any information on the CNS (e.g. BAYER & FEHLMANN 1960; HAYNES & KENCHINGTON 1991; HUGHES 1991; KIRSTEUER 1973; MARCUS & MARCUS 1955; VON SALVINI-PLAWEN 1973; WAWRA 1979a, 1980, 1988b). Other authors limited their descriptions of the CNS to the main ganglia on the prepharyngeal nerve ring and the visceral nerve cord (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 1968b; WAWRA 1989; WESTHEIDE & WAWRA 1974). Furthermore, very few studies gave 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. SOMMERFELDT & SCHRÖDL (2005) confirmed these nerves plus an optic nerve for *Hedylopsis*. Data about sensory organs were sparse, often consisting only in the

affirmation of presence or absence of easily identified structures, such as eyes (CHALLIS 1970; MARCUS & MARCUS 1954, 1955; WESTHEIDE & WAWRA 1974). Hancock's organs like structures were reported from *Microhedyle glandulifera* and *Pontohedyle milaschewitchii* by EDLINGER (1980a, b).

In the course of my dissertation, our knowledge on the acochlidian CNS completely changed. Our studies (BRENZINGER *et al.* 2011a, b; JÖRGER *et al.* 2008, 2010b; NEUSSER *et al.* 2006, 2009a, b, 2011a, b; NEUSSER & SCHRÖDL 2009) showed that the arrangement of ganglia in the acochlidian CNS is more or less similar in all acochlidian species. It consists of prepharyngeal paired cerebral, pedal, pleural ganglia, plus paired buccal ganglia and usually three ganglia on the visceral nerve cord. Unfortunately, the identification of the small and not always well-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 (NEUSSER & SCHRÖDL 2007). Additionally, aggregations of accessory ganglia, paired rhinophoral, paired optic, paired gastro-oesophageal ganglia and/or an osphradial ganglion may be present but were undetected by conventional examination (e.g. NEUSSER *et al.* 2009a).

Hancock's organs were considered to be present in most shelled opisthobranch gastropods (GÖBBELER & KLUSSMANN-KOLB 2007), but were previously assumed to be missing in Acochlidia (SOMMERFELDT & SCHRÖDL 2005; WAWRA 1987). However, paired epidermal folds on the side of the head were reported for the microhedylacean *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 (MIKKELSEN 1996). Recently, sensory spots innervated by a branch of the rhinophoral nerve, i.e. putative Hancock's organs, were confirmed for the microhedylacean *P. milaschewitchii* and *M. glandulifera* (see EDER *et al.* 2011; JÖRGER *et al.* 2008) and were newly described for the hedylopsacean species *Pseudunela espiritusanta*, *P. viatoris*, *P. marteli*, *Tantulum elegans*, *Strubellia wawrai* and *Aiteng mysticus* Neusser, Fukuda, Jörger, Kano & Schrödl, 2011 (see BRENZINGER *et al.* 2011b; NEUSSER *et al.* 2011a, b; NEUSSER & SCHRÖDL 2007). These tiny organs can easily be overlooked and thus, their presence or absence should be critically examined in species in which Hancock's organs could not be detected. In contrast to HUBER (1993), our (re)descriptions clearly show e.g. *Strubellia wawrai* (see BRENZINGER *et al.* 2011b) having (at least) six cerebral nerves, i.e. the oral, labial tentacle, rhinophoral, Hancock's, static and optic nerves.

Novel features

Several features concerning the CNS were described for the first time in acochlidian species.

A double cerebro-rhinophoral connective was detected in *Pontohedyle milaschewitchii*, *Microhedyle glandulifera* and *Tantulum elegans* (see EDER *et al.* 2011). *Strubellia wawrai* is the only known species with a double cerebro-optic connective. 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". HASZPRUNAR & HUBER (1990) described a double cerebro-rhinophoral connective for the enigmatic opisthobranchs *Rhodope veranii* Kölliker, 1847 and *Rhodope transtrosa* Salvini-Plawen, 1991, as well as a double connective attaching the cerebral ganglion with the procerebrum in the pulmonate *Smeagol manneringi* Climo, 1980. HUBER (1993) showed a similar situation for e.g. *Runcina adriatica* Thompson, 1980 and *Philinoglossa praelongata* Salvini-Plawen, 1973. In fact, the double cerebro-rhinophoral connective of the acochlidian CNS resembles the general pulmonate condition (VAN MOL 1967). The homology of opisthobranch rhinophoral or optic ganglia and the pulmonate procerebrum (with double connectives to the cerebral ganglion) has been suggested previously (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 in *S. wawrai* and *T. elegans* (see BRENZINGER *et al.* 2011b; NEUSSER & SCHRÖDL 2007), the variable origin of the optic nerve (usually from the optic ganglion, but in *P. cornuta* it splits off from the rhinophoral nerve (NEUSSER *et al.* 2009a)), and finally the presence of double connectives in one ganglion or the other.

"Lateral bodies" were described for *Hedylopsis spiculifera*, *H. ballantinei* and *Asperspina murmanica* (see NEUSSER *et al.* 2007, 2009b; SOMMERFELDT & SCHRÖDL 2005). They consist of a more or less hemispherical cluster of neuronal 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. 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, 1799 and *Siphonaria* Sowerby, 1823 (SALEUDDIN 1999; SALEUDDIN *et al.* 1997; TAKEDA & OHTAKE 1994). The function of the "lateral bodies" in *Hedylopsis* 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). Similar positions, structures and functions, as well as the molecular data suggesting that acochlidians are part of the (pan)pulmonate diversification, support homology of dorsal and lateral bodies. Furthermore a putative endocrine gland, called the juxtaganglionar organ, has been described in several opisthobranch species (SWITZER-DUNLAP 1987). However, the homology of these structures is still unclear. Future studies by means of TEM 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* and *Microhedyle remanei* (Marcus, 1953)) (NEUSSER *et al.* 2007) may represent characters with a phylogenetic signal and may be used in future phylogenetic analyses.

A "cephalic gland" consisting of a loose aggregation of cells covering the cerebral ganglia was detected uniquely in *Strubellia wawrai*. A similarity to the "lateral bodies" of other acochlidian species, the basommatophoran dorsal bodies or the "blood gland" of some nudibranchs is discussed by BRENZINGER *et al.* (2011b), but highlighted as a novel feature, which might represent an apomorphy for either *Strubellia* or Acochlidiidae.

All hedylopsacean species known in detail, as well as the minute microhedylacean *Parhedyle cryptophthalma* (see JÖRGER *et al.* 2010b; SCHRÖDL & NEUSSER 2010; WESTHEIDE & WAWRA 1974) possess a ganglion attached to the visceral nerve cord, i.e. the supraesophageal ganglion. Concluding from its position and innervation, the ganglion was assumed to be homologous with the osphradial ganglion of other euthyneurans (HUBER 1993; SOMMERFELDT & SCHRÖDL 2005; WAWRA 1989) even in absence of any osphradium reported from acochlidians. This interpretation could be confirmed quite recently with the detection of a pit-shaped osphradium in living *Strubellia wawrai* (see

BRENZINGER *et al.* 2011b). BRENZINGER *et al*. (2011b) pointet out that the anterior position of the osphradium on the head—far anterior to what can be considered the mantle border—appears strange, since the chemosensory organ is usually part of the mantle cavity organs including the gill, anus, genital opening and nephropore (THOMPSON 1976). Apparently the osphradium has moved to a more anterior position after the loss of the mantle cavity in acochlidians. It appears possible that the osphradium as a discrete organ is expressed only in the large-bodied species. However, it is also likely to have simply been overlooked so far in the minute interstitial species. Judging from light-microscopical observations, the osphradium of *S. wawrai* resembles the corresponding organ of the cephalaspidean *Philine* (see EDLINGER 1980a) and can accordingly be divided into two zones: a microvillous inner zone and a ciliated border forming the rim, similar to the condition described for the cephalaspidean *Scaphander lignarius* (Linnaeus, 1758) by HASZPRUNAR (1985a). Since ultrastructural research on the osphradial sensory epithelia has been used to test phylogenetic hypotheses (e.g. HASZPRUNAR 1988; PONDER & LINDBERG 1997), TEM examination of the organ in *Strubellia* might reveal structural features shared with other closer panpulmonate relatives (i.e. Hygrophila and Eupulmonata), which possess an osphradium. Most members of the Hygrophila have an osphradium, while it is absent in *Lymnaea* and *Acroloxus* (see DAYRAT & TILLIER 2002). In contrast, adult stylommatophorans lack an osphradium, but some species may possess one during ontogeny (RUTHENSTEINER 1997; RUTHENSTEINER 1998). This is similar in many representatives of the Ellobioidea in which the osphradium is only present in the embryonic stage (HASZPRUNAR 1985a), e.g. in *Ovatella myosotis* (Draparnaud, 1801) in which the reduction of the osphradium is concomitant with the formation of the osphradial ganglion (RUTHENSTEINER 1998). A similar process cannot be excluded yet for small-sized acochlidians having an osphradial ganglion but no detectable osphradium.

In summary, the CNS represents the most challenging data set within acochlidian organ systems due to lacking or contradictory literature data. Rather than being simple (HUBER 1993), the acochlidian CNS offers numerous minute features, the identification of which depends critically upon preservation and staining conditions, as well as on the cutting plane. Furthermore, interspecific variation may be smaller than intraspecific variation, e.g. there are 4 visceral loop ganglia in subadult *Tantulum elegans* while just 3 in mature specimens, complicating a final evaluation of the characteristics. In general the settings and homologies of acochlidian cerebral features are far from being fully

understood; comparative analyses of further acochlidians and related panpulmonates (JÖRGER *et al.* 2010a) could shed new light on this topic. In spite of the wealth of new anatomical data obtained, an extensive comparative analysis of every different organ system was unfortunately not possible. Usually only few specimens of one species were available for sectioning and 3D reconstruction, e.g. in *Pseudunela cornuta* only a single specimen (NEUSSER *et al.* 2009a). Even in case of abundant material accessible, due to time constraints only few specimens could be sectioned. Our current understanding on acochlidian species is often based on a small number of specimens and hence, knowledge on intraspecific and ontogenetic variation for comparison is lacking in many species.

My dissertation demonstrates that traditional taxonomy including paraffin-based histology cannot provide sufficient detailed data on the acochlidian morphology and anatomy. Older comparative studies on the acochlidian organ systems (HUBER 1993; RANKIN 1979) comprised erroneous data and do not reflect the complexity of the Acochlidia at all. In contrast, modern microanatomy allowed us to investigate the morphological structures with much higher detail and accuracy. The morphological data available in the literature were re-examined and re-evaluated to a large extent and could be supplemented and improved considerably. The high-quality data obtained by modern microanatomy contributed to a new knowledge on acochlidian organ systems and, in combination with the new insights into the acochlidian taxonomy and diversity, were indispensable for the following analyses of the inner-acochlidian phylogeny and their evolution.

4.3 Towards the phylogeny of Acochlidia: optimising a morphological data set

Before my dissertation, the Acochlidia were an enigmatic, neglected taxon with detailed morphological knowledge of almost all acochlidian species lacking. In consequence, many unknown character states existed and highly ambiguous homology assumptions were made. The poor coverage of existing species in combination with unreliable information based on dubious species descriptions obviously hampered the performance of morphology-based cladistic analyses with acochlidians included (Dayrat & Tillier 2002; von Salvini-Plawen 1990; von Salvini-Plawen & Steiner 1996; Wägele & Klussmann-Kolb 2005). Cladistic analyses targeting inner acochlidian relationships were not available. Own preanalyses with literature data were extremely sensitive to changes in taxon and/or character sets and tended to result in highly implausible topologies. Our approach for reconstructing the inner-acochlidian

phylogeny thus had to break new ground regarding both quantity and quality of information used. In order to minimise selectivity and subjectivity, we intended a taxon and character sampling as complete as possible for the ingroup. On the taxon side, we considered all valid acochlidian species; a dense taxon sampling was desired also to minimise potential long branch artefacts, which may negatively affect cladistic parsimony analyses. Regarding characters, we considered any distinctive features showing variation between outgroup and ingroup or within the ingroup as potentially usefull. Character definitions were made so as to minimise topology-dependent assumptions on homology of problematic structures; a priori homology was assessed according to structural and positional similarity of complex structures that were then divided into discernable substructures and coded. Only *a priori* uninformative or problematic characters, i.e. autapomorphies of single terminal taxa, or characters showing too much ambiguity or lack of information within the ingroup, were excluded from analyses, but listed and discussed separately to provide full transparency (SCHRÖDL & NEUSSER 2010). On the primary data side, we recollected most of the valid acochlidian species at their type locality and re-examined at least one representative of every family (except the Ganitidae) by means of modern microanatomy.

The comprehensive 3D reconstructions revealed a wealth of characters potentially useful for phylogenetic studies, i.e. 154 characters of which we considered 107 as sufficiently explored to be used in our analyses. The number of characters can be probably augmented in the future when all acochlidian species are revised in detail. A lot of erroneous data within the original descriptions was identified, e.g. 8 - 15 % of the applicable characters were described incorrectly in *Asperspina murmanica*, *Pseudunela cornuta* and *Tantulum elegans*. Furthermore, up to approx. 49 % of the characters considered as relevant in our phylogenetic analyses formerly were not detected and not described in the above mentioned species.

Small and tiny structures were anyway overlooked or misinterpreted in original descriptions, such as the presence of Hancock`s organs, an osphradium, rhinophoral, optic or gastro-oesophageal ganglia or the number of cerebral nerves (see e.g. BRENZINGER *et al.* 2011b; NEUSSER *et al.* 2009a, b; NEUSSER & SCHRÖDL 2007). But even comparatively large structures were described inaccurately, e.g. a posterior genital ganglion was described erroneously in *A. murmanica* (see NEUSSER *et al.* 2009b); the precerebral accessory ganglia were overlooked in *T. elegans* (see NEUSSER & SCHRÖDL 2007), whereas undulated cerebral nerves were believed to be accessory ganglia in *Pseudunela cornuta* (see NEUSSER *et al.* 2009a). Also, complex structures were not

correctly reconstructed and illustrated, such as the excretory system with a complex kidney in *P. cornuta* (see NEUSSER *et al.* 2009a). Finally, the presence of a mantle cavity, which was considered as phylogenetically informative and important by e.g. PONDER & LINDBERG (1997) and DAYRAT & TILLIER (2002), was reported erroneously in the original description of *A. murmanica*. We demonstrated the complete absence of any ("well-developed longitudinal") mantle cavity in the latter species and reevaluated its formerly assumed basal position among acochlidians (NEUSSER *et al.* 2009b); *Asperspina murmanica* now fits well into the pattern of other asperspinid species.

The results of our cladistic analyses (SCHRÖDL & NEUSSER 2010) based on morphological data clearly show: (1) quality-proofing and supplementing the available primary data is essential, and viable using a 3D microanatomical approach; (2) a high-quantity data and 'all-species' approach aimed to minimise subjective selection and topology-dependent homology assumptions (see MARTYNOV & SCHRÖDL 2011) also works in acochlidians, in spite of a high level of convergence and still considerable missing data; (3) a (partly) well-resolved, robust and plausible topology was obtained that may allow reconstructing some aspects of evolution; (4) our morphology-based topology may reflect evolutionary history, since it was recently supported by molecular results (JÖRGER *et al.* 2010a).

Especially the last aspect is not a trivial result: there are, to my knowledge, few studies dealing with the phylogeny of heterobranchs in which "morphology matches molecules". In the past, even proposed molecular phylogenies for heterobranchs were contradictory, poorly resolved and did not match between each others (see e.g. VONNEMANN *et al.* 2005; WÄGELE *et al.* 2008). Difficulties to resolve heterobranch phylogeny based on morphological data were formerly explained by extensive parallelism and homoplastic similarity, particularly within the opisthobranch members (e.g. DAYRAT & TILLIER 2002; LAFORGE & PAGE 2007; MARTYNOV & SCHRÖDL 2011). Moreover, comprehensive anatomical data are lacking for many heterobranch subtaxa (VONNEMANN *et al.* 2005) or are limited to few morphological characteristics, such as the shell in cephalaspideans (MALAQUIAS & REID 2008), leading to morphological mini-data sets and resulting in the fragmentary knowledge about heterobranch phylogeny. Recently, MARTYNOV & SCHRÖDL (2011) generated a list of 70 characters and proposed a stable and reasonable phylogeny of corambid nudibranchs. The authors showed that morphological structures, if investigated in depth, bear the potential for an efficient phylogenetic analysis even in extremely problematic groups such as the corambids and emphasised that it is beneficial to optimise both the coverage of in-group taxa and of

structural characters. However, the success of this approach cannot be generalised and must be proofed in future studies on other heterobranch taxa. For example, members of the anatomically well-studied heterobranch Bullidae turned out to possess notably "few morphological diagnostic characters" (MALAQUIAS & REID 2008); this scarcity remains to be tested using potentially powerful analytical approaches such as histology-based 3D microanatomy. The systematic revision of *Bulla* based on morphological and molecular cytochrome *c* oxidase subunit I (COI) gene data (MALAQUIAS & REID 2008) are largely supported by the phylogenetic hypothesis based on concatenated sequences from the COI, 16S rRNA and 28S rRNA genes (MALAQUIAS & REID 2009), yet, with unresolved relationships between two species. The phylogenetic hypothesis based on molecular markers of the caenogastropod genus *Littoraria* supported largely the assumptions on the species composition recognised in a morphological approach (REID *et al.* 2010), though cryptic species remained hidden in the morphological study. Reconstructing the phylogeny of caenogastropod Calyptraeidae including shell morphology, anatomical features and molecular data, COLLIN (2003) assessed the efficacy of morphological characters in gastropod phylogenetics. The results are conflictive between the molecular and the morphological data, but inclusion of morphological data improved the resolution and the support of nodes in the topology of a combined dataset.

Thus, I do not share SCOTLAND *et al.*`s (2003) opinion that morphology cannot resolve phylogeny at any taxonomic level. My dissertation and MARTYNOV & SCHRÖDL`S (2011) results on corambid phylogeny clearly show that stable and meaningful topologies can be obtained, even within heterobranch groups which were considered enigmatic before. However, in both studies it was fundamental to optimise the taxon sampling and the quality of the morphological data. Ignoring such quality data for phylogenetic purposes because of its class rather than its signal appears spurious considering the elusive nature of many invertebrates and the yet problematic performance of molecular data in many groups. Finally, limiting the role of morphology merely to mapping certain morphological features onto molecular phylogenetic trees as proposed by SCOTLAND *et al.* (2003) requires a stable molecular phylogenetic hypothesis, which is not available in many marine invertebrate taxa.

4.4 Proposed phylogeny of Acochlidia

The phylogeny within Acochlidia was completely unclear and has never been addressed to by cladistic means. SCHRÖDL & NEUSSER (2010) presented the first parsimony-based hypothesis on the inner-acochlidian phylogeny that included all 27 valid acochlidian species and which was (partly) based on the large amount of high-quality morphological data obtained in the context of my dissertation. The Acochlidia result monophyletic and split into the Hedylopsacea (*Tantulum* (*Hedylopsis* (*Pseudunela* (*Strubellia* ('*Acochlidium*', '*Palliohedyle*'))))) and Microhedylacea (*Asperspina* (*Pontohedyle*, '*Parhedyle*', '*Microhedyle*', (*Ganitus*, *Paraganitus*))). This topology (see Fig. 2) is surprisingly robust to modifications of outgroup and ingroup taxon sampling. Within the Microhedylacea asperspinid and, in particular, microhedylid relationships still are unresolved in SCHRÖDL & NEUSSER (2010), resulting in a polytomy of the genera *Microhedyle*, *Parhedyle* and *Pontohedyle*. Reasons may include an incomplete taxon sampling (with existing species not detected yet or implying a high level of extinct species) and/or few distinguishing characters available within the reduced Microhedylacea in combination with still poor morphological knowledge on microhedylid genera. Recently, several microhedylid species were re-examined in detail. The poorly known *Parhedyle cryptophthalma* was studied using 3D reconstructions and immunocytochemistry (JÖRGER *et al.* 2010b). The results confirmed the presence of a unique spicule pattern and a special asymmetric radula and contributed new data especially on the CNS, which all indicated monophyletic *Parhedyle*. Based on recent molecular data *Pontohedyle* is the first basal offshoot of the Microhedylidae s.l. (EDER *et al.* 2011; JÖRGER *et al.* 2010a; NEUSSER *et al.* 2011a). In contrast, *Microhedyle glandulifera* was discussed in a derived position by EDER *et al*. (2011); however, the authors highlighted that an improved taxon sampling is required to shed light on the microhedylid relationships. The formerly enigmatic Ganitidae, resembling sacoglossan opisthobranchs by having dagger-like rhachidian radular teeth, are likely to be highly derived microhedylids. The systematic position of Ganitidae presented by SCHRÖDL & NEUSSER (2010) was confirmed in recent molecular studies (EDER *et al.* 2011; JÖRGER *et al.* 2010a; NEUSSER *et al.* 2011a). These recent results on some microhedylid species suggest that the relationships within Microhedylidae can be resolved in future analyses, when all valid microhedylid species are revised in detail.

In contrast, the Hedylopsacea are quite well resolved. The small limnic Caribbean *T. elegans* is the first basal offshoot. The remaining hedylopsaceans are composed of marine interstitial H*edylopsis* plus a clade of *Pseudunela* and a clade of large, limnic

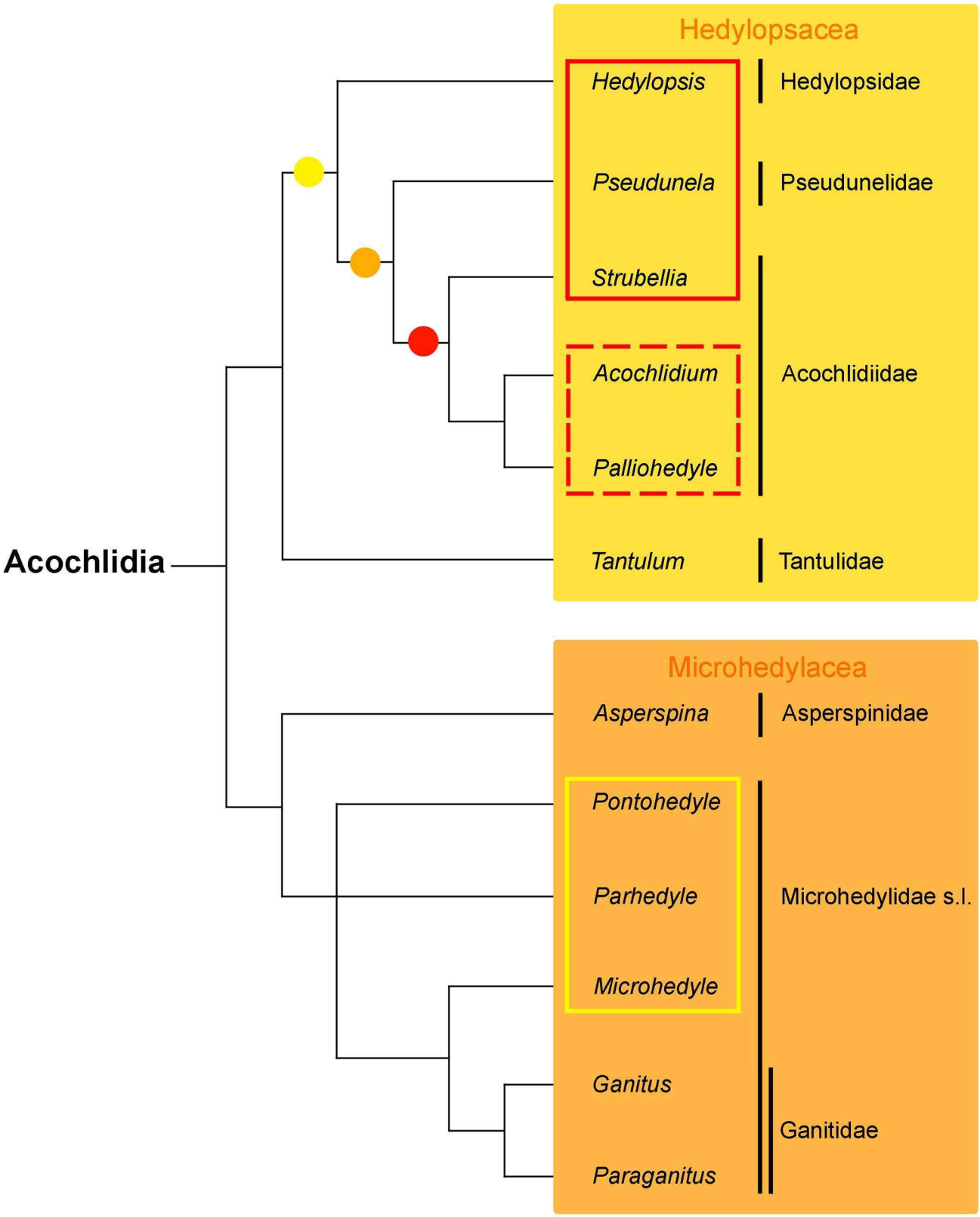

Figure 2 - Phylogeny of the Acochlidia according to SCHRÖDL & NEUSSER (2010) (topology simplified) with potential systematic positions of Aitengidae and showing differences to earlier phylogenetic considerations by WAWRA (1987). Yellow and orange points: possible origins of Aitengidae inferred from molecular markers in NEUSSER *et al.* (2011a; 2011b). Red point: systematic position of Aitengidae according to morphological results in NEUSSER *et al.* (2011a). Taxa in red box: Hedylopsidae sensu WAWRA (1987). Taxa in red dashed box: Acochlidiidae sensu WAWRA (1987). Taxa in yellow box: Microhedylidae sensu WAWRA (1987).

tropical Indo-Pacific species. Recent molecular analyses (NEUSSER *et al*. 2011b) revealed the brackish *Pseudunela espiritusanta* as the sister clade to marine or temporary brackish *Pseudunela* species. *Strubellia paradoxa* is the sister group of the Acochlidiidae, comprising the genera *Acochlidium* and *Palliohedyle*. While *Acochlidium amboinense* and *A. bayerfehlmanni* result as a clade, some inner acochlidiid relationships still remain unclear. The recently discovered, enigmatic and biologically and morphologically aberrant amphibious slug family Aitengidae was associated with the Sacoglossa (SWENNEN & BUATIP 2009). Rather surprisingly, we have shown aitengids are specialised members of the Acochlidia (JÖRGER *et al.* 2010a; NEUSSER *et al.* 2011a). According to my morphological results (NEUSSER *et al.* 2011a), the Aitengidae might be the sister group to the limnic Acochlidiidae (Fig. 2, red point); especially the large, laterally situated eyes as well as the prominent rhachidian tooth of members of Aitengidae closely resemble the anatomy in members of the large, limnic acochlidian family Acochlidiidae, e.g. in *Strubellia paradoxa* (see BRENZINGER *et al.* 2011a). In a recent multilocus molecular analysis the family Aitengidae also clusters within hedylopsacean Acochlidia (JÖRGER *et al.* 2010a); however, only a single aitengid species and single representatives of acochlidian families were included. Additional molecular analyses including a more focused taxon sampling for Acochlidia and Sacoglossa, supported Aitengidae clustering within Acochlidia as a more or less basal offshoot of the Hedylopsacea (Fig. 2, yellow point) (NEUSSER *et al.* 2011a) or as the sister group to Pseudunelidae plus Acochlidiidae (Fig. 2, orange point) (NEUSSER *et al.* 2011b). First molecular results on the acochlidian phylogeny confirmed our morphological analyses (JÖRGER *et al.* 2010a); however a molecular analyses including a more comprehensive taxon sampling is in preparation (JÖRGER *et al*., in prep.) and must be awaited for a final evaluation.

Besides the well-known, extremely high level of evolutionary parallelism within opisthobranchs hindering conventional phylogenetic reconstructions (e.g. GOSLINER 1994; WÄGELE & KLUSSMANN-KOLB 2005), there are several further reasons for the high degree of homoplasy within Acochlidia and just moderate branch support in our analyses (SCHRÖDL & NEUSSER 2010): (1) the taxon sampling included in the analysis was still limited, i.e. with only a fraction of existing species and morphological variety, with only some parts of the world`s coastal waters explored (SCHRÖDL *et al.* 2003); (2) information on many species such as *Pseudunela eirene*, *Acochlidium*, *Palliohedyle* and *Parhedyle* species was still insufficient or unreliable; (3) our coding was conservative, i.e. "unknown" was used whenever character states were undescribed for a certain species;

(4) entire character sets (such as sperm ultrastructure) were inapplicable or not considered for analysis due to lack of data for comparison; (5) the exact origin of Acochlidia was still unknown and, even worse, (6) high quality data of potential outgroups for comparison were lacking.

Recently, JÖRGER *et al.* (2010a) suggested a radical reclassification of Euthyneura showing the Acochlidia to be part of an early (pan)pulmonate radiation as sister to Eupulmonata. This result was surprising and led to a radical reclassification of euthyneurous gastropods (JÖRGER *et al.* 2010a; SCHRÖDL *et al.* 2011a, b) that is supported by broad EST-based analyses (KOCOT *et al.* 2011; SMITH *et al.* 2011). Future cladistic studies will have to seize the chance to include a wealth of new and particularly detailed morphological data and adjust the outgroup selection according to the recent hypothesis proposed by JÖRGER *et al*. (2010a) in order to try to achieve higher resolution in the cladistic analyses.

4.5 New preliminary classification of Acochlidia

Classifications are considered important to disseminate phylogenetic results to the broader public (JOHNSON & GOSLINER 2012). The acochlidian classification was controversial in history due to the unresolved acochlidian relationships and therefore, several classificatory systems were used simultaneously. The classification by TAYLOR & SOHL (1962) relied principally on external features and the radula structures and was mainly based on the original literature of ODHNER (1938; 1939; 1952) and MARCUS (1953). It comprised only three families, i. e. the Acochlidiidae, the Hedylopsidae and the Microhedylidae and did not seem to reflect natural relationships. RANKIN (1979) used her species description of *Tantulum elegans* as an occasion to reclassify the Acochlidia based on morphological similarities and differences taken mainly from the literature. Her uncritical and in several species erroneous adoption of literature data was criticised rigorously (FAHRNER & HASZPRUNAR 2002; WAWRA 1987), as well as the inflation of acochlidian taxa resulting in five new suborders, with 13 families and 19 genera for only 25 nominal acochlidian species (RANKIN 1979). Some years later, STAROBOGATOV (1983) reduced the suborders to the Hedylopsoidei and the Strubellioidei and adopted several of Rankin`s newly erected families. Additionally, he created an own genus *Minicheviella* and a monotypic family Minicheviellidae for the arctic *Hedylopsis murmanica*. However, WAWRA (1987) transferred *H. murmanica* to the genus *Asperspina*. As already assumed by NEUSSER *et al*. (2009b), our analyses (SCHRÖDL & NEUSSER 2010) reveal it to be the sister species to the Mediterranean *A. rhopalotecta*, and there is no need for own higher

categories. WAWRA (1987) was the first one who discussed potential apomorphies of the taxa included and classified the Acochlidia based on his own critical observations and species (re)examinations. A modified version of WAWRA (1987) was implemented in ARNAUD *et al*. (1986). Surprisingly, his phylogeny based on few potential synapomorphies already quite resembled our present results. Differences between Wawra`s phylogenetic hypotheses and the phylogeny presented in my dissertation are illustrated in Fig. 2. He proposed the superfamilies Hedylopsacea and Microhedylacea. While the Hedylopsidae *sensu* Wawra comprised the genera *Hedylopsis*, *Pseudunela* and *Strubellia* (Fig. 2, red box), they resulted paraphyletic in our analyses. This was already assumed by SOMMERFELDT & SCHRÖDL (2005) who tried to reconstruct the phylogeny of Acochlidia using apomorphy-based systematics, but successful reclassification was once again hindered by the poor anatomical knowledge of many species. The family Hedylopsidae thus is restricted to the genus *Hedylopsis*. Accordingly, the Acochlidiidae *sensu* Wawra only comprised *Acochlidium* and *Palliohedyle* (Fig. 2, red dotted box), while in our analyses *Strubellia* belongs to the Acochlidiidae. The Microhedylacea *sensu* Wawra consisted of the families Asperspinidae (with *Asperspina*), Microhedylidae (with *Pontohedyle*, *Microhedyle* and *Parhedyle*, see Fig. 2, yellow box) and Ganitidae (with *Ganitus* and *Paraganitus*). In contrast, in our analyses the Ganitidae are nested within the Microhedylidae rendering the latter paraphyletic. In consequence, the Microhedylacea herein comprise the Asperspinidae and paraphyletic Microhedylidae.

The latest classification of the Mollusca by BOUCHET & ROCROI (2005) recognised the controversial classification of RANKIN (1979) and STAROBOGATOV (1983) and tentatively followed the latter. The authors consulted BERGH (1895), KÜTHE (1935), ODHNER (1937, 1952), RANKIN (1979) and STAROBOGATOV (1983) rather than adopting the more recent classification proposed by ARNAUD *et al.* (1986) and WAWRA (1987). The classification of the Acochlidia proposed in the database WoRMS (World Register of Marine Species) (APPELTANS *et al.* 2011) was based on the classification according to BOUCHET & ROCROI (2005). Recently, the data were revised and a new version that is mainly based on our current results was provided (GOFAS 2011).

Although my phylogenetic hypothesis presented in Fig. 2 is not considered definitive, the paraphyly of some of the traditionally recognised family level taxa induced a preliminary reclassification of the Acochlidia. The results presented in SCHRÖDL & NEUSSER (2010) render the classification of RANKIN (1979), STAROBOGATOV (1983) and BOUCHET & ROCROI (2005) obsolete. The need of major modifications was already emphasised by WAWRA (1987), SOMMERFELDT & SCHRÖDL (2005), and NEUSSER *et al*.

(2006). Our proposals for a preliminary classification are as follows: until this analysis has been rerun on a broader and more detailed data basis, (1) RANKIN'S (1979), STAROBOGATOV'S (1983) and BOUCHET & ROCROI'S (2005) systems and names should be abandoned (exceptions are already adopted by WAWRA (1987)), (2) WAWRA'S (1987) higher classification and genera can be further used, but (3) some families should be redefined and (4) the Aitengidae should be included as a new hedylopsacean family.

The family Hedylopsidae can be restricted to the clearly monophyletic genus *Hedylopsis* for now. The Pseudunelidae comprise the two traditional *Pseudunela* species (*P. cornuta* and *P. eirene*) plus the newly described *P. viatoris*, *P. marteli* and *P. espiritusanta* and constitute the sister group of Acochlidiidae in the wider sense and may thus be termed Pseudunelidae as already introduced by RANKIN (1979) for *P. cornuta*. The Acochlidiidae *sensu* Wawra (*Acochlidium, Palliohedyle*) should additionally include the genus *Strubellia*. A synapomorphy of the Pseudunelidae (which has to be confirmed for *P. eirene*) and Acochlidiidae may be the well-developed and externally visible heart bulb. Another synapomorphic and diagnostic feature is the fusion of the visceral sac and head-foot complex without a discernable mantle border. A substantial synapomorphy for the clade of Hedylopsidae, Pseudunelidae and Acochlidiidae is their short sperm head. The sister to this unnamed clade is the monotypic Tantulidae. The Aitengidae represent a new acochlidian family including two species, i.e. *Aiteng ater* and *A. mysticus*. However, the sister relationship to other hedylopsacean taxa is not definitive yet. The Microhedylacea are characterised by the loss of the copulatory organ and the use of spermatophores for sperm transfer. The Asperspinidae (with Minicheviellidae as junior synonym) *sensu* Wawra may persist. The gonochoristic Microhedylidae (s.l.) informally may include the morphologically clearly monophyletic Ganitidae (EDER *et al.* 2011), until the origin of the two monotypic ganitid genera *Ganitus* and *Paraganitus* from a microhedylid stem is confirmed or rejected with higher statistical support.

Summing up, WAWRA`S (1987) classification was quite precise, probably due to the selected characters on which his phylogenetic assumptions were based; these were special reproductive features with, as we can confirm now, relatively low level of homoplasy. Here I present an updated and modified classification (Fig. 2) that will be further refined (NEUSSER *et al.*, in prep.) considering upcoming molecular results.

4.6 Evolutionary history of Acochlidia

The phylogenetic hypothesis presented in SCHRÖDL & NEUSSER (2010) is already based partly on our thorough, high-quality redescriptions, however, (re)examinations particularly of members of the Acochlidiidae and the Microhedylidae s.l. are still missing. We assume that it likely reflects natural relationships as its topology is robust to modifications of ingroup and outgroup taxon sampling and was recently largely confirmed by an independent genetic multi-locus study (JÖRGER *et al.* 2010a). Thus, *a posteriori* tracing character state changes on the tree (SCHRÖDL & NEUSSER 2010) already uncovered many details of the inner-acochlidian evolutionary history. More recent species discoveries (BRENZINGER *et al.* 2011b; NEUSSER *et al.* 2011a, b; NEUSSER & SCHRÖDL 2009), novel structures and habits and biological observations (e.g. BRENZINGER *et al.* 2011b) allow a more comprehensive view; I will concentrate on few selected features only.

4.6.1 Invasion into freshwater systems

Limnic habitats were successfully colonised twice, independently, by acochlidian species: first, by the ancestor of the small interstitial Caribbean *Tantulum elegans*, and second, by the common ancestor of all large, benthic Acochlidiidae from the Indo-Pacific. Interestingly, only in the Indo-Pacific species the selection under limnic conditions resulted in the evolution of large body sizes, i.e. a secondary 'gigantism' evolved, because an increased volume/surface ratio may reduce osmolarity problems. However, this is not true for juveniles of Acochlidiidae and the limnic, interstitial *Tantulum elegans*, which is equally small as marine mesopsammic acochlidian species. Up to now, a radiation of limnic Caribbean species remains to be discovered, while within Acochlidiidae a considerable radiation took place. Therefore we conclude that the large-sized Indo-Pacific species obviously had greater evolutionary success: increasing body size alone may be not strictly necessary for acochlidians invading freshwater systems, but advantageous. The reasons why Acochlidia colonised freshwater systems are unclear. However, we observed in a petri dish that several groups of marine predators, such as nudibranch *Pseudovermis* and philinoglossid sea slugs, and polychaetes, feed on marine acochlidians - at least under laboratory conditions. Therefore, limnic Acochlidia may have escaped from marine-adapted predators; no limnic members of the mentioned predatory sea slug lineages are known, and polychaetes usually inhabit marine environments and only rarely limnic ones (FAUCHALD 1977). Another reason may be that acochlidian prey is abundant in the

rivers. *Strubellia* species were known to co-occur with neritid gastropods (HAYNES 2000; STARMÜHLNER 1976), but quite recently BRENZINGER *et al*. (2011b) observed for the first time *Strubellia wawrai* feeding on egg capsules of neritid species. However, whether the presence of prey represents the cause or the consequence of acochlidian invasion remains unclear. Aitengids, potential relatives of Acochlidiidae, were observed to feed on pupae and insect larvae (SWENNEN & BUATIP 2009). Thus a carnivorous and potentially oophagous state may be ancestral for these and perhaps other hedylopsacean lineages such as *Pseudunela espiritusanta* which is large sized and lives in brackish waters under rocks together with *Neritilia littoralis* Kano, Kase & Kubo, 2003 and two undescribed *Neritilia* spp. (pers. comm. Kano). Food of interstitial hedylopsaceans and (entirely mesopsammic) microhedylaceans is unknown; in case of *Pontohedyle* feeding of microfilms was inferred by HADL *et al.* (1969). Finally, there remains much to discover regarding "simple" biology!

HAYNES & KENCHINGTON (1991) observed that recently hatched veliger larvae of *Acochlidium fijiense* apparently were not able to survive in freshwater and died after a few days. This lead us to the assumption that (at least Indo-Pacific) limnic acochlidian species have an amphidromous life style (BRENZINGER *et al*. 2011b), such as the numerous freshwater nerites occurring in rivers of different Indo-Pacific Islands (HAYNES 1988; KANO *et al.* 2002; MYERS *et al.* 2000). This implies that the larvae after hatching in freshwater are swept downstream by the current into the sea where they undergo a marine phase and grow to juveniles. After metamorphosis the juveniles migrate upstream (sometimes "hitchhiking" upstream on the shell of larger individuals (KANO 2009)) and (re)colonise the freshwater systems. This hypothesis is supported by an amazing observation made in our laboratory: in seawater, veligers of an *Acochlidium* species quickly metamorphosise into 'adhesive'-type larvae which remain alive for at least 2 months glueing themselves e.g. to the wall of the petri dish they are kept in. This suggests that limnic *Acochlidium* species, and possibly already the common ancestor with *Strubellia*, have evolved a specialised larval type that might be able to disperse between islands of archipelagos leading to the colonisation of rivers, potential epibiosis on their adult prey producers, involving a neritid-like amphidromic lifestyle (BRENZINGER *et al.* 2011b). However, it is questionable if this lifestyle can be assumed for the limnic *Tantulum elegans*, since this species was reported to live interstitially in a mountain spring marsh approx. 400 meters above the sea level on St. Vincent Island.

We found the acochlidian excretory system comprises different types which, however, are not strictly correlated with the habitat. All marine microhedylacean species possess

a simple, sac-like kidney with a short nephroduct (JÖRGER *et al.* 2008; NEUSSER *et al.* 2006, 2009b), as it is characteristic for almost all marine euthyneurans, including marine Panpulmonata, such as Siphonarioidea (HUBENDICK 1978), the sacoglossan *Platyhedyle denudata* (see RÜCKERT *et al.* 2008), Amphiboloidea (GOLDING *et al.* 2007), and marine eupulmonates. In contrast, all hedylopsacean species including marine, brackish and limnic species, have a complex excretory system comprising a complex, internally divided kidney with a narrow and a wide lumen. All fully marine hedylopsacean species (*Hedylopsis spiculifera, H. ballantinei*, *Pseudunela viatoris* and *P. marteli*) have a short nephroduct (NEUSSER *et al.* 2011b). In contrast, the temporary brackish *Pseudunela cornuta*, the brackish *P. espiritusanta* and all limnic hedylopsacean species (*Tantulum elegans* and Acochlidiidae) have additionally a long looped nephroduct with two branches (BRENZINGER *et al.* 2011a; NEUSSER *et al.* 2009a; NEUSSER & SCHRÖDL 2007, 2009). We therefore conclude that the ancestral hedylopsacean species were already adapted to a freshwater-influenced environment and had a complex kidney (NEUSSER *et al.* 2011b), 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 more likely, evolved as an adaptation facilitating invading such habitats. Thus, considering evidence from excretory systems, we favour a scenario with (1) hedylopsaceans originating in a freshwater, or at least freshwater influenced, habitat; (2) a repeated invasion into brackish or limnic habitats and (3) an apparently secondary invasion back into the fully marine mesopsammon within *Pseudunela*. Recently, the amphibious Aitengidae were shown to be a more or less basal offshoot of Hedylopsacea (JÖRGER *et al.* 2010a; NEUSSER *et al.* 2011b). This result implies a habitat switch from aquatic to amphibious lifestyle (see also 4.7.2) and further extends the ecological tolerance and evolutionary plasticity observed within the hedylopsacean lineage.

4.6.2 Sex and violence in Acochlidia

Within the Acochlidia a wide range of different reproductive features can be recognised. Lacking any sperm storage or copulatory organs, the reproductive system is considerably reduced in the vast majority of the known mesopsammic acochlidian species, i.e. all aphallic microhedylacean species known in detail (EDER 2011; JÖRGER *et al.* 2009; NEUSSER *et al.* 2006, 2009b; SCHRÖDL & NEUSSER 2010). In members of the Microhedylacea sperm is transferred probably by spermatophores and dermal insemination as shown for *P. milaschewitchii* by JÖRGER *et al.* (2009). This is in contrast to most opisthobranchs in which the usual mode of sperm transfer is reciprocal copulation

(SCHMEKEL 1985) and sperm transfer via spermatophores is rare (MANN 1984). The reason for the use of spermatophores may be correlated to the interstitial habitat: in a mesopsammic milieu, as inferred to be the ancestral state for acochlidians (SCHRÖDL & NEUSSER 2010), a normal opisthobranch head-to-foot copulation of two hermaphrodites which have to synchronise their sexual activities, may (simply) be mechanically complicated due to the limited space available. JÖRGER *et al*. (2009) discussed the disadvantages of the dermal sperm transfer including 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 apparently compensated by the benefits of transferring sperm rapidly to any available body portions of a potential mate while "passing by" in the mesopsammon.

In contrast, the hedylopsacean topology as revealed by SCHRÖDL & NEUSSER (2010) points towards an evolutionary trait from a simple, unarmed copulatory system (in *Tantulum* and *Aiteng*) towards complex hypodermal injection systems (in *Hedylopsis*, *Pseudunela* and *Strubellia*) culminating in the large, trap-like spiny "rapto-penis" of several limnic Acochlidiidae. An unarmed penial papilla and a bursa copulatrix are present in the basal hedylopsacean mud-dweller *T. elegans* and the amphibious *Aiteng* (see NEUSSER *et al.* 2011a; NEUSSER & SCHRÖDL 2007; SWENNEN & BUATIP 2009); although copulation (and any other mating behaviour) has never been observed in living hedylopsacean species, reciprocal copulation is likely and might be facilitated by sufficient space available in their habitat. Differing from microhedylacean species, the marine mesopsammic hedylopsaceans *Hedylopsis* and *Pseudunela* possess complex anterior copulatory organs. *Hedylopsis spiculifera* lacks any allosperm receptacles and shows a single penial stylet for sperm transfer (WAWRA 1989). Sperm transfer by hypodermal injection was therefore suggested by WAWRA (1992), which, however, may be an imprecise one in the sequential hermaphrodite *H. spiculifera*, as indicated by the finding of lost penial stylets in the body cavity (WAWRA 1989). While *H. ballantinei* was described as potentially aphallic (SOMMERFELDT & SCHRÖDL 2005), we could detect a penial stylet and a solid thorn in this species (KOHNERT *et al.* 2011). The special androdiaulic genital system of the marine hedylopsacean *P. cornuta* with highly elaborated cephalic copulatory organs including an extremely long, coiled penial stylet, an additional paraprostatic injection system and two allosperm storing receptacles (NEUSSER *et al.* 2009a) is clearly more complex than that of other marine hedylopsacean species. Inspite of the presence of allosperm receptacles, the hollow penial stylet of *P.*

cornuta indicates that sperm transfer occurs by injection (SCHRÖDL & NEUSSER 2010; WAWRA 1992), either through the genital opening or through the tissue. In members of the limnic *Acochlidium* and *Palliohedyle* the anterior copulatory organs are enlarged bearing rows of spines (BAYER & FEHLMANN 1960; BÜCKING 1933; HAYNES & KENCHINGTON 1991; WAWRA 1979a, 1980) and were assumed to function as a violent catch and therefore called "rapto-penis" by SCHRÖDL & NEUSSER (2010). The absence of any sperm storing organs and the presence of penial injection systems, suggest hypodermal injection in both genera. In the benthic *Strubellia*, the penial papilla lacks a penial stylet, but bears a subapical cuticular thorn. Additionally, a paraprostatic injection system is present including a hollow, curved stylet. While reciprocal copulation was assumed for *Strubellia* by WAWRA (1992), unilateral copulation was discussed by BRENZINGER *et al*. (2011a). GASCOIGNE (1974) reported of two types of cuticular elements of the copulatory organs of sacoglossans, i.e. hollow stylets for injecting sperm and curved structures functioning as coupling devices. Thus, BRENZINGER *et al*. (2011a) concluded, the curved penial thorn in *Strubellia* might work as a coupling device, holding the penis in place during the sperm transfer. The basal finger would function as a hypodermic injecting device for paraprostatic fluids, possibly before copulation. Potential functions were discussed including facilitating copulation, avoiding reciprocal copulation, and sperm competition effects.

In conclusion, within Acochlidia the mode of sperm transfers covers a wide spectrum and ranges, besides the use of spermatophores, from reciprocal to unilateral copulation to hypodermic injection. The latter is regarded as an antagonistic mechanism (ANTHES & MICHIELS 2007a) resulting from a sexual conflict, i.e. differences in objectives between males and females (see PARKER 2006). Physically injurious and violent mating behaviours, such as hypodermic injections were assumed to be more common among hermaphrodites (MICHIELS & KOENE 2006; MICHIELS & NEWMAN 1998) than in species with separate sexes. This is in accordance with our results in acochlidian species, in which the morphological features suggest an arm race concerning reproductive organs and behaviour within the hermaphroditic hedylopsaceans in contrast to aphallic Microhedylacea. Violent mating behaviours, such as hypodermic injection were also shown e.g. in the marine flatworm *Pseudoceros bifurcus* Prudhoe, 1989 (MICHIELS & NEWMAN 1998), in seed beetles (HOTZY & ARNQVIST 2009) and in the sacoglossan *Siphopteron quadrispinosum* Gosliner, 1989 (ANTHES & MICHIELS 2007b). Experimental research and particularly observations on living acochlidian species are overdue and

may allow new insights into the fascinating and miscellaneous biology and evolution of the Acochlidia.

4.7 Integrative approaches

Parallel to my anatomy-based work, Katharina Jörger investigates the acochlidian phylogeny and evolution by molecular systematic techniques. Therefore, we were able to combine morphological and molecular data in integrative approaches.

4.7.1 "Pseudo-"Cryptic diversity in *Pseudunela*

Towards realistic estimations of the diversity of marine animals, tiny meiofaunal species usually are underrepresented. Since the biological species concept is hardly verifiable 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 modeling from histological sections. However, acochlidian species traditionally were delineated applying a morphospecies concept by means of traditional taxonomy of external and radular features and this concept on tiny meiofaunal gastropods has never been tested by molecular analyses. In a first case study on meiofaunal *Pseudunela* species from different Indo-Pacific islands we tested diversity estimations from traditional taxonomy against results from modern microanatomical methodology and molecular systematics (NEUSSER *et al.* 2011b). Our study 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 modeling of micro-morphology is more suitable to delineate species and may reveal diagnosable differences among pseudocryptic species after delineating them by molecular analyses; (3) only the combined evidence of microanatomical and molecular data enabled us to uncover and describe the full range of (pseudo)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; (5) the acochlidian diversity is higher and the evolution even more complex than previously thought. Similarly, molecular studies on other marine taxa revealed formerly hidden cryptic and pseudocryptic species (e.g. KRABBE *et al.* 2010; MAHON *et al.*

2008; MEDLIN & ZINGONE 2007; ORNELAS-GATDULA *et al.* 2012). In addition, our exploration of the genus *Pseudunela* in NEUSSER & SCHRÖDL (2009) and NEUSSER *et al*. (2009a; 2011b) showed considerable ecological and structural diversity, i.e. of fully marine species, and those steadily or temporarily exposed to freshwater, having complex excretory systems. Only after disentangling the cryptic species diversity in *Pseudunela* we were able to reconstruct the complex evolution of these features.

4.7.2 Aberrant morphology of Aitengidae - induced by habitat shift

The taxon Aitengidae was established as a monotypic sacoglossan family with possible affinities to Acochlidia (SWENNEN & BUATIP 2009). Its sole species, the mysterious "bug-eating slug" *Aiteng ater* was included into the list of Top 10 bizarre new animal 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 and compact lacking any cephalic tentacles or body processes. Anatomically it shows an unusual mix of acochlidian and sacoglossan features. *Aiteng ater* was first placed within Sacoglossa, but the authors expressed their doubts and the systematic affinities remained open. We aimed to clarify the systematic relationships and evolutionary history of the Aitengidae combining evidences from detailed micromorphological descriptions and sequence marker analyses. We revisited *A. ater* within an integrative molecular and microanatomical approach including 3D reconstructions (NEUSSER *et al.* 2011a). Our results supplemented and refined the original description in several substantial features and finally, few characters were left indicating a closer relationship of *Aiteng ater* to Sacoglossa, i.e. the *Gascoignella*-like body shape lacking cephalic tentacles, the presence of a potentially elysiid-like system of dorsal vessels, and an albumen gland consisting of follicles. We compared *A. ater* to the equally small and vermiform newly described aitengid species from Japan. *Aiteng mysticus* is externally similar to *A. ater*, but different concerning the habitat, the body size and colour, the CNS and the presence of a kidney. Both aitengid species resemble acochlidians by 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 the Aitengidae, which is used to pierce insects and pupae in *A. ater* according to SWENNEN & BUATIP (2009), and the large, laterally situated eyes closely resemble the anatomy in members of the limnic acochlidian family Acochlidiidae. The

acochlidian nature of *Aiteng* is strongly indicated by our molecular analysis (NEUSSER *et al.* 2011a), forming a basal hedylopsacean offshoot or the sister clade to limnic Acochlidiidae and brackish or marine Pseudunelidae within Hedylopsacea implying a switch from aquatic to amphibious lifestyle. Such a topology would, however, mean that the Aitengidae have lost the most characteristic acochlidian apomorphy, the subdivision of the body into a head-foot complex and a free, elongated visceral hump. Considerable external dissimilarities and even aberrant anatomical structures might probably be aitengid autapomorphies that evolved during that habitat switch. The compact body shape, a short stout head and the lack of cephalic tentacles give the Aitengidae an appearance that is very different to other, strictly aquatic Acochlidia and might be interpreted as an adaptation to an amphibious lifestyle. The visceral hump connected to the foot on all its length guarantees better stability and minimises the body surface. A unique layer of large, vacuolated supporting notal cells almost certainly contributes to a more stable and robust body shape in Aitengidae and probably also provides some mechanical protection as well as protection from desiccation. The ramified system of dorsal vessels, which is a modified portion of the kidney, is assumed to enhance respiratory, secretory and excretory processes in a secondarily amphibious lineage.

Aitengidae are small but highly specialised amphibious slugs. Surveying tropical slug diversity in different, not only aquatic habitats may reveal further and perhaps even more specialised and aberrant creatures. Integrating biological observations such as "bug-eating" with microanatomical and genetic data allows us reconstructing a first evolutionary scenario that turnes a "mysterious slug" to an instructive and amazing example of animal evolution. The combination of detailed microanatomical and molecular phylogenetic studies will shed further light on the origin of acochlidians, their unexpected frequent habitat shifts during hedylopsacean evolution and their evolutionary adaptations to an extraordinarily wide range of completely different habitats. With increasing taxon sampling and details studied, the integrative approaches reveal the evolution of acochlidian panpulmonates is even more complex than expected.

In summary, the integrative approaches within my dissertation contributed considerably to our knowledge on Acochlidia: (1) the discovery of (pseudo)cryptic species within *Pseudunela* enabled us a new understanding of the actual species diversity which was boosted by the present results; (2) the verification of the

acochlidian nature of the Aitengidae by molecular data induced new morphological analyses which revealed a much higher morphological diversity than previously expected, e.g. the presence of a dorsal vessel system in some members of the Acochlidiidae; (3) new phylogenetic and evolutionary hypotheses were considered, e.g. a hedylopsacean origin influenced by freshwater inflow and the previously most mysterious aitengid slugs are now explainable within an evolutionary scenario adaptive to an amphibious habitat.

Despite the fact that traditional taxonomy based on morphological studies will remain useful in many cases, our results support recent studies postulating a change from traditional to a more integrative taxonomy including different sources of data sets (e.g. COOK *et al.* 2010; DAYRAT 2005; PFENNINGER *et al.* 2006; VALDECASAS *et al.* 2008; VARGAS *et al.* 2010). However, PADIAL & DE LA RIVA (2010) criticised recent integrative approaches to not being really integrative and promoted applying the evolutionary species concept (DE QUEIROZ 2007) in which taxonomy should be open to all disciplines offering data about the origin and evolution of species. An integrative framework including different lines of evidence will better prepare taxonomists to face the realities of inventorying the actual underestimated Earth's biodiversity (PADIAL & DE LA RIVA 2010; PADIAL *et al.* 2010). Such integrative methods are recommended for all future taxonomic approaches and biodiversity surveys on soft-bodied and small-sized invertebrates. Finally, I agree with JENNER (2004) that the greatest power of science lies in its "multifaceted nature" and any artificial limitation of approaches will cause the "impoverishment of science".

"What we know is a drop, what we don't know is an ocean."
Isaac Newton

5 CONCLUSIONS AND OUTLOOK

The extensive study of acochlidian representatives of almost all families was rewarding. My dissertation convincingly shows that traditional taxonomy is insufficient for the purpose of a detailed morphological description of not only the tiny mesopsammic acochlidian species, but also the larger limnic ones. Old literature data comprised erroneous data and did not reflect the complexity of the Acochlidia at all. At the moment, scanning electron microscopy and resin-based histological investigations are the by far best options for detailed and accurate descriptions. Three-dimensional microanatomical reconstructions with the software Amira® turned out to be a powerful tool for an in-depth description of minute structures and complex organs. This method enabled to raise the standard of anatomical descriptions resulting in an outstanding morphological data set of a previously enigmatic taxon and should be applied by default to all micromolluscs in the future. Novel imaging techniques, such as CLSM provide additional informative data sets and facilitate further insights into acochlidian anatomy. Future anatomical studies on shelled and shell-less heterobranch micromolluscs may gain new insights into molluscan diversity combining the yet established histology-based 3D reconstructions with investigations by e.g. μCT.
Our study design combining reliable and detailed high-quality data with a dense taxon sampling minimised the subjective selections and maximised not only the quality of homology assumptions but also the number of phylogenetically relevant characters. Our cladistic analysis is already based partly on thoroughly re-examined morphological characters. The resulting inner-acochlidian topology is robust to modifications of ingroup and outgroup taxon sampling and is largely confirmed by independent molecular data. As vermiform mesopsammic taxa show highly adaptive convergences to the extreme ecological environment, this topological congruence is not trivial - and quite unique among the Heterobranchia. Careful morphology-based cladistics as applied successfully in the present approach may be promising for establishing solid and plausible phylogenetic hypotheses on other enigmatic Molluscan taxa.

Having both vast morphological and biological data and our robust and crossvalidated backbone topology we are fortunate to reconstruct character evolution. We showed that a regressive evolution as suggested for all mesopsammic Acochlidia (SWEDMARK 1971) is only applicable in the Microhedylacea; in contrast, within the Hedylopsacea complex excretory and reproductive systems evolved combined with the invasion of freshwater-influenced habitats. The habitat shift in Aitengidae from an ancestrally aquatic to an amphibious lifestyle provoked adaptations in the external morphology and mainly in the excretory system. While copulation might be favored by a benthic lifestyle, reproductive features such as impregnatory stylet systems are not obviously adaptive specialisations to the habitat among the Hedylopsacea. Different aspects of potential sex conflicts and arms races should be explored in detail in future studies.

Diversity referring to species structures and traits uncovered by integrative approaches combining modern microanatomy and molecular analyses were applied successfully for detecting (pseudo)crypsis within acochlidian species. This approach seems to be promising to be applied in future studies on other mesopsammic Acochlidia. Furthermore, my dissertation revealed a much higher diversity within Acochlidia as previously thought. This includes both species diversity, e.g. the formerly enigmatic sacoglossan Aitengidae are now included in the Acochlidia, and habitat diversity, e.g. a new brackish water habitat in *Pseudunela espiritusanta*, and a habitat shift from an aquatic to an amphibious life style in Aitengidae. Further studies in yet insufficiently sampled habitats may discover many more micromolluscs with special adaptations.

The origin of the Acochlidia could not be resolved based on morphological characters due to the large amount of convergences of other mesopsammic outgroup taxa. Unexpectedly, the recent molecular approach supports the acochlidian clade emerging from a (pan)pulmonate rather than an "opisthobranch" level (JÖRGER *et al.* 2010a). The hypothesis of the new relationships proposed requires a careful reevaluation of acochlidian outgroups and morphological characters. Ultimately, this may reveal "pulmonate"-structures such as special cerebral features in "opisthobranch" acochlidian or sacoglossan species, but also some typically "pulmonate" features such as special head tentacles or certain mantle cavity features might be shown to be inherited from an "opisthobranch" ancestral grade. In spite of the urgency for speed facing the biodiversity crisis, we must push for accurate and complete species descriptions combined with biologcal observations in order to appreciate the full range of morphological, evolutionary and species biodiversity on Earth.

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8 DECLARATION OF OWN CONTRIBUTION AS CO-AUTHOR

Article I (Chapter 3.1)

Neusser TP, Martynov AV & Schrödl M 2008. **Heart-less and primitive? 3D-reconstruction of the polar acochlidian gastropod *Asperspina murmanica***. *Acta Zoologica* **90**: 228-245.

Neusser: realised SEM, histological work and morphological analyses; prepared figures; drafted and wrote the manuscript.

Article II (Chapter 3.2)

Jörger KM, **Neusser TP**, Haszprunar G & Schrödl M 2008. **Undersized and underestimated: 3D-visualization of the Mediterranean interstitial acochlidian gastropod *Pontohedyle milaschewitchii* (Kowalevsky, 1901)**. *Organisms Diversity & Evolution* **8**: 194-214.

Neusser: instructed and assisted in histological work and 3D reconstruction; discussed and improved the manuscript.

Article III (Chapter 3.3)

Jörger KM, **Neusser TP** & Schrödl M 2007. **Re-description of a female** *Pontohedyle brasilensis* **(Rankin, 1979), a junior synonym of the Mediterranean** *P. milaschewitchii* **(Kowalevsky, 1901).** *Bonner Zoologische Beiträge* **55**: 283-290.

Neusser: instructed and assisted in histological work and 3D reconstruction; discussed and improved the manuscript.

Article IV (Chapter 3.4)

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.

Neusser: instructed and assisted in 3D reconstruction; discussed and improved the manuscript.

Article V (Chapter 3.5)

Neusser TP & Schrödl M 2007. ***Tantulum elegans* reloaded: a computer-based 3D-visualization of the anatomy of a Caribbean freshwater acochlidian gastropod.** *Invertebrate Biology* **126**: 18-39.

Neusser: searched for and obtained type material; realised histological work and morphological analyses; prepared figures; drafted and wrote the manuscript.

Article VI (Chapter 3.6)

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: realised sectioning; instructed and assisted in preparing figures; drafted and wrote the manuscript.

Article VII (Chapter 3.7)

Neusser TP, Heß M & Schrödl M 2009. **Tiny but complex - interactive 3D visualization of the interstitial acochlidian gastropod *Pseudunela cornuta* (Challis, 1970).** *Frontiers in Zoology* **6**:20.

Neusser: realised histological work and morphological analyses; prepared figures and interactive 3D model; drafted and wrote the manuscript.

Article VIII (Chapter 3.8)

Neusser TP & Schrödl M 2009. **Between Vanuatu tides: 3D anatomical reconstruction of a new brackish water acochlidian gastropod from Espiritu Santo**. *Zoosystema* **31**: 453-469.

Neusser: collected material; realised SEM, histological work and morphological analyses; prepared figures; drafted and wrote the manuscript.

Article IX (Chapter 3.9)

Brenzinger B, **Neusser TP**, Glaubrecht M, Hazsprunar G & Schrödl M 2011. **Redescription and three-dimensional reconstruction of the limnic acochlidian gastropod *Strubellia paradoxa* (Strubell, 1892) (Gastropoda: Euthyneura) from Ambon, Indonesia.** *Journal of Natural History* **45**: 183-209.

Neusser: instructed and assisted in histological work and 3D reconstruction.

Article X (Chapter 3.10)

Neusser TP, Jörger K & Schrödl M 2007. **Exploring Cerebral Features in Acochlidia (Gastropoda: Opisthobranchia).** *Bonner zoologische Beiträge* **55**: 301-310.

Neusser: collected material; realised histological work; prepared figures; drafted and wrote the manuscript.

Article XI (Chapter 3.11)

Schrödl M & **Neusser TP** 2010. **Towards a phylogeny and evolution of Acochlidia (Mollusca: Gastropoda: Opisthobranchia).** *Zoological Journal of the Linnean Society* **158**: 124-154.

Neusser: contributed data from 3D reconstructions; prepared figures 1 and 2; assisted preparing the list of characters and generating the data matrix; discussed the final manuscript.

Article XII (Chapter 3.12)

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(8)**: e23313.

Neusser: collected material, realised SEM, histological work and morphological analyses; prepared figures (except figure 11) and interactive 3D model; drafted and wrote the manuscript except the sections written by Jörger ("DNA extraction, polymerase chain reaction and sequencing", "Sequence alignment and phylogenetic analyses", "Molecular results" and "Cryptic species?").

Article XIII (Chapter 3.13)

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.

Neusser: collected material; realised SEM, histological work and morphological analyses of specimens from Vanuatu (Figs. 1D; 2A,F,F`; 4A,C; 9C-E; 10C-H); prepared interactive 3D model.

Article XIV (Chapter 3.14)

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: realised SEM, histological work and morphological analyses; prepared figures 1-10; drafted and wrote the manuscript except the sections written by Fukuda ("Habitat" and "External morphology of living specimens") and Jörger ("Molecular studies" and "Molecular phylogeny").

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Neusser Timea _____

9 LIST OF PUBLICATIONS

Peer-reviewed contributions

1. Brenzinger B, **Neusser TP**, Glaubrecht M, Haszprunar G & Schrödl M 2011. Redescription and three-dimensional reconstruction of the limnic acochlidian gastropod *Strubellia paradoxa* (Strubell, 1892) (Gastropoda: Euthyneura) from Ambon, Indonesia. *Journal of Natural History* 45(3/4): 183-209.

2. 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.

3. 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.

4. **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.

5. **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**(8): e23313.

6. Schrödl M & **Neusser TP** 2010. Towards a phylogeny and evolution of Acochlidia (Mollusca: Gastropoda: Opisthobranchia). *Zoological Journal of the Linnean Society* **158**(1): 124-154.

7. 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**(6): 1159-1170.

8. **Neusser TP**, Hess M & Schrödl M 2009. Tiny but complex - interactive 3D visualization of the interstitial acochlidian gastropod *Pseudunela cornuta* (Challis, 1970). *Frontiers in Zoology* **6**:20.

9. **Neusser TP**, Martynov AV & Schrödl M 2009. Heartless and primitive? 3D reconstruction of the polar acochlidian gastropod *Asperspina murmanica*. *Acta Zoologica* **90**(3): 228-245.

10. **Neusser TP** & Schrödl M 2009. Between Vanuatu tides: 3D anatomical reconstruction of a new brackish water acochlidian gastropod from Espiritu Santo. *Zoosystema* **31**(3): 453-469.

11. Jörger KM, **Neusser TP**, Haszprunar G & Schrödl M 2008. Undersized and underestimated: 3D-visualization of the Mediterranean interstitial acochlidian gastropod *Pontohedyle milaschewitchii* (Kowalevsky, 1901). *Organisms Diversity & Evolution* **8**(3): 194-214.

12. Jörger KM, **Neusser TP** & Schrödl M 2007. Re-description of a female *Pontohedyle brasilensis* (Rankin, 1979), a junior synonym of the Mediterranean *P. milaschewitchii* (Kowalevsky, 1901) (Acochlidia, Gastropoda). *Bonner Zoologische Beiträge* **55**(3/4): 283-290.

13. **Neusser TP**, Heß M, Haszprunar G & Schrödl M 2007. Sperm ultrastructure of *Microhedyle remanei*, an interstitial acochlidian gastropod with dermal fertilization. *Journal of the Marine Biological Association of the UK* **87**(3): 747-754.

14. **Neusser TP**, Jörger KM & Schrödl M 2007. Exploring cerebral features in Acochlidia (Gastropoda: Opisthobranchia). *Bonner zoologische Beiträge* **55**(3/4): 301-310.

15. **Neusser TP** & Schrödl M 2007. *Tantulum elegans* reloaded: a computer-based 3D-visualization of the anatomy of a Carribean freshwater acochlidian gastropod. *Invertebrate Biology* **126**(1): 18-39.

16. **Neusser TP**, Hess M, Haszprunar G & Schrödl M 2006. Computerbased 3-dimensional reconstruction of the anatomy of *Microhedyle remanei* (Marcus, 1953), an interstitial acochlidian gastropod from Bermuda. *Journal of Morphology* **267**(2): 231-247.

Book chapters

17. **Neusser TP** 2011. Marine interstitial. In: *The Natural History of Santo*. (Eds. P Bouchet, H Le Guyader & O Pascal), MNHN, Paris; IRD, Marseille; PNI, Paris. 572 p. (Patrimoines naturels; 70).

Congress contributions

18. Brenzinger B, **Neusser TP**, Jörger KM & Schrödl M 2010. 120 years after Strubell: 3D microanatomy and biology of the limnic acochlidian slug *Strubellia* Odhner, 1937. *Tropical Natural History* Suppl. **3**: 48. (Talk)

19. Kohnert P, Jörger KM, **Neusser TP** & Schrödl M. 2010. Time for sex change! 3D-reconstruction of the copulatory system of the "aphallic" acochlidian *Hedylopsis ballantinei*. In: Abstracts of the 3rd International Workshop on Opisthobranchs, Vigo, Spain (Eds. JS Troncoso, J Moreira & G Díaz-Agras): 39. (Poster)

20. **Neusser TP**, Fukuda H, Jörger KM, KanoY & Schrödl M 2010. Sacoglossa or Acochlidia? 3D micromorphology of Aitengidae. In: Abstracts of the 3rd International Workshop on Opisthobranchs, Vigo, Spain (Eds. JS Troncoso, J Moreira & G Díaz-Agras): 25. (Talk)

21. **Neusser TP**, Jörger KM & Schrödl M 2010. Reconstruyendo la historia evolutiva de un taxón antiguo: el caso de los gastrópodos Acochlidios. *Biological Research* 43 (Suplemento A): R-39. (Talk)

22. **Neusser TP**, Kano Y, Fukuda H, Jörger KM & Schrödl M 2010. "Himitsu namekuji" - the Secret Slug(s): 3D-reconstruction of Aitengidae. *Tropical Natural History* Suppl. **3**: 274. (Poster)

23. Brenzinger B, **Neusser TP** & Schrödl M 2008. The 3D microanatomy and redescription of the acochlidian *Strubellia paradoxa* (Gastropoda: Opisthobranchia). Abstracts of the 1st International Congress on Invertebrate Morphology, Copenhagen, Denmark. In: ICIM-1 Abstracts. *Journal of Morphology* **269**(12): 1488. (Poster)

24. **Neusser TP** & Schrödl M 2008. Colonizing of freshwater systems in opisthobranch gastropods: comparative 3D microanatomy of marine, brackish-water and limnic acochlidian species from the Indo-Pacific. Abstracts of the 1st International Congress on Invertebrate Morphology, Copenhagen, Denmark: In: ICIM-1 Abstracts. *Journal of Morphology* **269**(12): 1474. (Talk)

25. Schrödl M, Jörger KM & **Neusser TP** 2008. Phylogeny and evolution of Acochlidia (Mollusca: Gastropoda: Opisthobranchia): from morphology to sequences. Abstracts of the 1st International Congress on Invertebrate Morphology, Copenhagen, Denmark: In: ICIM-1 Abstracts. *Journal of Morphology* **269**(12): 1464. (Talk)

26. Jörger KM, **Neusser TP** & Schrödl M 2007a. "Mining deeper" - additional characters for reconstruction of acochlidian phylogeny. In: Abstracts from the 9th Annual Meeting of the "Gesellschaft für Biologische Systematik" (GFBS), Vienna, Austria (Eds. C Hörweg, H Sattmann): 54. (Talk)

27. Jörger KM, **Neusser TP** & Schrödl M 2007b. Cilia patterns and pores: Comparative external SEM examination of acochlidian opisthobranch gastropods. In: Abstracts from the 9th Annual Meeting of the "Gesellschaft für Biologische Systematik" (GFBS), Vienna, Austria (Eds. C Hörweg, H Sattmann): 55. Electronic supplement (abstract PDF file 1 and poster PDF file 10). *Organisms Diversity & Evolution* **7**: 252. (Poster)

28. **Neusser TP**, Martynov AV, Jörger KM & Schrödl M 2007c. The 3D microanatomy and sperm ultrastructure of the interstitial acochlidian gastropod *Asperspina murmanica* (Kudinskaya & Minichev, 1978). In: Abstracts from the 9th Annual Meeting of the "Gesellschaft für Biologische Systematik" (GFBS), Vienna,

Austria (Eds. C Hörweg, H Sattmann): 75-76. Electronic supplement (abstract PDF file 1 and poster PDF file 14). *Organisms Diversity & Evolution* **7**: 252. (Poster)

29. **Neusser TP**, Martynov AV, Jörger KM & Schrödl M 2007d. 3-dimensional microanatomy and sperm ultrastructure of the arctic interstitial acochlidian gastropod *Asperspina murmanica* (Kudinskaya & Minichev, 1978). In: Abstracts of the World Congress of Malacology, Antwerp, Belgium (Eds. K Jordaens, N Van Houtte, J Van Goethem & T Backeljau): 154. (Poster)

30. **Neusser TP** & Schrödl M 2007. Opisthobranchs go limnic: comparative 3D microanatomy of the marine interstitial acochlidian *Pseudunela* and the freshwater *Strubellia* from Vanuatu. In: Abstracts of the World Congress of Malacology, Antwerp, Belgium (Eds. K Jordaens, N Van Houtte, J Van Goethem & T Backeljau): 153. (Talk)

31. Schrödl M & **Neusser TP** 2007. Germany's next top model? Towards a morphological phylogeny and evolution of acochlidian opisthobranch gastropods. In: Abstracts of the World Congress of Malacology, Antwerp, Belgium (Eds. K Jordaens, N Van Houtte, J Van Goethem & T Backeljau): 198. (Talk)

32. **Neusser TP** & Schrödl M 2006. Computer-based 3D-visualization of *Tantulum elegans*, an enigmatic Caribbean freshwater acochlidian opisthobranch. In: Abstracts of the 72nd Annual Meeting of the American Malacological Society and 37th Annual Meeting of Western Malacological Society, Seattle, USA (Ed. RC Anderson): 74. (Talk)

33. Schrödl M & **Neusser TP** 2006. Towards a phylogeny and evolution of Acochlidia. Abstracts of the 2nd International Workshop on Opisthobranchs, Bonn, Germany: 14. (Talk)

34. **Neusser TP**, Haszprunar G, Hess M & Schrödl M 2004. Microanatomy and ultrastructure of *Unela sp*., an interstitial acochlidian gastropod from Bermuda. In: Abstracts of the World Congress of Malacology, Perth, Australia (Ed. F Wells): 107. (Poster)

Other non peer-reviewed publications

35. Schrödl, M, Ryall P, Gertin T & **Neusser TP** 2007. West African *Navanax aenigmaticus*. [*Message in*] *Sea Slug Forum*. Australian Museum, Sydney. Available from http://www.seaslugforum.net/find/19658.

36. Schrödl M & **Neusser TP** 2006. Phylogeny and evolution of acochlidian opisthobranchs. [*Message in*] *Sea Slug Forum*. Australian Museum, Sydney. Available from http://www.seaslugforum.net/find/15744.

10 CURRICULUM VITAE

Personal data

Name	Timea Pamela Neusser
E-mail	neusser@biologie.uni-muenchen.de
Birth	December 30, 1976 in Heidelberg, Germany

Studies (Biology)

10.2001 – 12.2003	Ludwig-Maximilians-Universität München (LMU)
08.2000 – 09.2001	Universidad de Concepción, Chile. Studies financed by a grant of the German Academic Exchange Service (DAAD)
10.1999 – 07.2000	LMU
10.1997 – 09.1999	Technische Universität München

Diploma thesis

03.2003 – 12.2003	"Anatomy, histology and ultrastructure of *Unela sp*., an interstitial acochlid from Bermuda (Gastropoda, Opisthobranchia, Acochlidia)", supervisor Prof. Dr. Haszprunar, LMU Munich, (mark 1.3)

PhD

08.2005 – 06.2012	"Systematics and evolution of the Acochlidia (Gastropoda, Euthyneura) - a microanatomical approach by means of computer-based 3D reconstruction using Amira®", supervisor Prof. Dr. Gerhard Haszprunar, LMU Munich and PD Dr. Michael Schrödl, Bavarian State Collection of Zoology, Munich (ZSM) Project financed by a grant of the German Research Foundation to MS (DFG grant SCHR 667-4)

Employments at University

2004	Voluntary helper in field work: "Recording of the diversity of fish species in Bavaria" at the ZSM
2001 – 2004	Tutor in "Course of Biodiversity" and "Basic course in Zoology" at the LMU

Organisation and realisation of workshops

08.2010	3D reconstructions with Amira®, Leibniz-Rechenzentrum Munich
11.2008	Anatomía convencional y reconstruida tridimensionalmente por computador en moluscos gastrópodos, VII Congreso Latinoamericano de Malacologia, Valdivia, Chile (in spanish)

11 APPENDIX

Table 1: **(Type) material stored in museums or institutions according to the original literature. A**, allotype; **H**, holotype; **L**, lectotype; **NHMW**, Museum of Natural History Vienna, Austria; **P**, paratype; **Pl**, paralectotype; **S**, syntypes; **sections**, section series; **SMNH**, Swedish Museum of Natural History, Sweden; **spec**, specimen; **V**, voucher; **ZSM**, Bavarian State Collection of Zoology, Germany**; +**, present; **-**, absent; **§**, synonymised according to EDER *et al.* (2011); *****, one paratype sectioned by NEUSSER *et al.* (2011a); ******, sectioned by BRENZINGER *et al.* (2011a); **?**, data not available.

Species	Data source	Museum	Museum N°	Preparation	Type	Status
Hedylopsis spiculifera	pers. comm. Warén A	SMNH	985A/B 27209-27211	sections	L/Pl	+ +
	WAWRA (1989)	NHMW	?	sections	?	+
Hedylopsis ballantinei	SOMMERFELDT & SCHRÖDL (2005)	ZSM	20040549 20040550 20040552 20004766/1 20004767-69	spec spec spec sections sections	H P P P P	+ + + + +
	KOHNERT *et al.* (2011)	ZSM	20100855, 856	sections	V	+
Pseudunela cornuta	CHALLIS (1970)	The Natural History Museum, UK	? ? ?	spec 20 spec radula	H P P	- - -
		Museum of New Zealand Te Papa Tongarewa, New Zealand	? ?	10 spec radula	P P	- -
	NEUSSER *et al.* (2009a)	ZSM	20071911 20071809	sections sections	V V	+ +
Pseudunela eirene	WAWRA (1988a)	NHMW	84500/166	crush preparation of radula	H	+
Pseudunela marteli	NEUSSER *et al.* (2011b)	ZSM	20071803 20090418 20071851 20071864, 865 20071061 20090416 20071826 20080105	spec 2 spec sections sections sections sections radula radula	H P P V V V V V	+ + + + + + + +

Pseudunela viatoris	NEUSSER *et al.* (2011b)	ZSM	20061954	spec	H	+
			20061945	20 spec	P	+
			20080492, 493	sections	V	+
			20090422, 423	sections	V	+
			20062048	radula	V	+
			20071120	radula	V	+
Pseudunela espiritusanta	NEUSSER & SCHRÖDL (2009)	ZSM	20080115	spec	H	+
			20070968	sections	P	+
			20080791	sections	P	+
			20080116	visceral sac	P	+
			20080117-118	mol	P	+
Aiteng ater	SWENNEN & BUATIP (2009)	Zoological Reference Collection of the Raffles Museum of Biodiversity Research, National University of Singapure	?	spec	H	+
			?	3 spec	P	+
		Zoological Museum, University of Amsterdam	?	3 spec *	P	+
	NEUSSER *et al.* (2011a)	Zoological Museum, University of Amsterdam	409068	sections	P	+
		ZSM	20110189	radula	V	+
Aiteng mysticus	NEUSSER *et al.* (2011a)	ZSM	20110185	spec	H	+
			20110186, 188	sections	P	+
			20110187	radula	P	+
		National Museum of Nature and Science, Tokyo, Japan	77319	spec	P	+
		Laboratory of Conservation of Aquatic Biodiversity, Okayama University, Japan	M21473	spec	P	+
			M21474	spec & radula	P	+
Tantulum elegans	RANKIN (1979)	Royal Ontario Museum, Canada	1118	spec	H	+
			1118	spec	P	+
			1118	radula	P	+
			1118	sections	P	+

Strubellia paradoxa	pers. comm. Glaubrecht M	Museum für Naturkunde, Berlin, Germany	90761	spec **	P	+
	BRENZINGER *et al.* (2011a)	Museum für Naturkunde, Berlin, Germany	193943 193944	sections radula		+ +
Strubellia wawrai	WAWRA (1988b) (*as S. paradoxa*)	NHMW	78000/167-173	sections		+
	BRENZINGER *et al.* (2011b)	ZSM	20100718 20071797 20071881 20071883 20071886 20071892 20071894 20071895	spec 9 spec sections sections sections sections sections sections	H P P P P P P P	+ + + + + + + +
Strubellia sp.	HAYNES (2000)	Australian Museum Sydney, Australia	C 204278	spec	?	+
Acochlidium amboinense	pers. comm. Glaubrecht M	Museum für Naturkunde, Berlin, Germany	90762	2 spec	?	+
Acochlidium bayerfehlmanni	BAYER & FEHLMANN (1960)	National Museum of Natural History, USA	575737	4 spec	V	+
	WAWRA (1980)	NHMW	81232 81233 81233/161 81233/162 81233/163 81234 81234/164	spec dissected radula radula gonad dissected gonad	H P P P P P P	+ + + + + + +
		Natural History Museum Basel, Switzerland	11117a	spec	P	+

Acochlidium fijiense	HAYNES & KENCHINGTON (1991)	Natural History Museum Los Angeles County, USA	2457 2458	spec 2 spec	H P	+ +
		NHMW	84901	10 spec (5 spec & 5 sections)	P	+
		Biological Department, University of the South Pacific, Suva	?	7 spec radula, penis and gonads	P	-
	pers.comm. Seeto J	Marine Studies Programme Collection Room, University of the South Pacific, Suva	5437	sections, radula, penis	? ? 	+ +
	HAASE & WAWRA (1996); pers. comm. Eschner A	NHMW	81125/MP/240 81125/MP/244 81125/MP/245	3 sections 1 section 1 section	P P P	+ + +
Palliohedyle sutteri	WAWRA (1979a)	NHMW	81230 81125/157-160 81231 81125/155-156	penis, CNS radula spec 2 sections	H H P P	+ + + +
		Natural History Museum Basel, Switzerland	5819	5 spec	P	+
		Zoological Museum Amsterdam, Netherlands	?	2 spec	P	+
Palliohedyle weberi	pers. comm. Glaubrecht M	Museum für Naturkunde, Berlin, Germany	?	?	H	-
Asperspina loricata	SWEDMARK (1968b)	?	?	?	?	?
Asperspina brambelli	SWEDMARK (1968b)	SMNH	?	spec	?	-
Asperspina rhopalotecta	VON SALVINI-PLAWEN (1973)	NHMW	78703	?	H	+

Asperspina murmanica	KUDINSKAYA & MINICHEV (1978)	Zoological Institute of the Russian Academy of Sciences, Russia	? ?	spec sections	S V	+ +
	NEUSSER *et al.* (2009b)	ZSM	20062163-165 20062167	sections sections	V V	+ +
Asperspina riseri	MORSE (1976)	National Museum of Natural History, USA	710910 710911	spec 5 spec	H P	+ +
		SMNH	2675	spec	P	?
		Museum of Comparative Zoology, Harvard University, USA	288014	2 spec	P	+
Microhedyle glandulifera	KOWALEVSKY (1901)	?	?	?	?	?
"Microhedyle glomerans" §	VON SALVINI-PLAWEN (1973)	NHMW	78001	section	H	-
Microhedyle nahantensis	DOE (1974)	National Museum of Natural History, USA	708380 708381 708382	spec spec spec	H P A	+ + +
		SMNH	2580 2581	spec spec	P A	- -
Microhedyle odhneri	MARCUS & MARCUS (1955)	?	?	?	?	?
Microhedyle remanei	pers. comm. Magenta C	Zoological Museum, Sao Paulo, Brazil	?	4 spec	?	+
	KIRSTEUER (1973)	American Museum of Natural History, NY, USA	?	5 spec	V	-
	pers. comm. Voss N	Rosenstiel School of Marine and Atmospheric Science	301800	radula	V	+
	NEUSSER *et al.* (2006)	ZSM	20070079-84	6 sections	V	+
Ganitus evelinae	pers. comm. Magenta C	Zoological Museum, Sao Paulo, Brazil	?	30 spec	?	+

Paraganitus ellynnae	CHALLIS (1968)	The Natural History Museum, UK	?	?	H	-
		Museum of New Zealand Te Papa Tongarewa, New Zealand	?	?	P	-
		Bernice Bishop Museum, Hawaii	?	?	P	-
Parhedyle cryptophthalma	WESTHEIDE & WAWRA (1974)	NHMW	79100	crush preparation	H	+
Parhedyle tyrtowii	ODHNER (1952)	Musée National d`histoire naturelle, France	?	?	?	?
Parhedyle gerlachi	MARCUS & MARCUS (1959)	?	?	?	?	?
Pontohedyle milaschewitchii	KOWALEVSKY (1901)	?	?	?	?	?
	JÖRGER *et al.* (2008)	ZSM	20060522–525	sections	V	+
Pontohedyle verrucosa	CHALLIS (1970)	The Natural History Museum, UK	?	spec 10 spec radula	H P P	- - -
		Museum of New Zealand Te Papa Tongarewa, New Zealand	?	5 spec radula (slide)	P P	- -

Table 2: **Material loaned for re-examination. H**, holotype ; **L**, lectotype; **P**, paratype ; **Pl**, paralectotype; **S**, syntype ; **spec**, specimen.

Species	Type locality	Legit	Museum	Loan
Hedylopsis spiculifera (as *H. suecica*)	Bonden, Gullmarfjord, Sweden	Odhner N	Swedish Museum of Natural History, Sweden	sections and spec for re-examination (L, Pl)
Asperspina murmanica	Dalniye Zelentsy, Barents Sea, Russia	Kudinskaya & Minichev	Zoological Institute of the Russian Academy of Sciences, Russia	sections for re-examination (S)
Asperspina murmanica	Dalniye Zelentsy, Barents Sea, Russia	Smirnov AV	Zoological Institute of the Russian Academy of Sciences, Russia	1 spec for semithin sectioning
Tantulum elegans	Golden Grove, St. Vincent Island, West Indies	Harrison AD & Rankin JJ	Royal Ontario Museum, Canada	4 section series (P), 2 spec for semithin sectioning
Strubellia paradoxa	Batu gatja River, Ambon, Indonesia	Strubell AD	Museum für Naturkunde, Berlin, Germany	1 spec for semithin sectioning (P)
Strubellia paradoxa	Matanikau River, Guadalcanal, Solomon Island	Starmühlner F	Museum of Natural History Vienna, Austria	sections for re-examination
Strubellia sp.	La Marona River, Efate Island, Vanuatu	Haynes A	Australian Museum Sydney	2 spec for semithin sectioning (P)
Acochlidium amboinense	Batu gatja River, Ambon, Indonesia	Strubell AD	Museum für Naturkunde, Berlin, Germany	spec for re-examination
Acochlidium bayerfehlmanni	Arakitaoch River, Island Babelthuap, Palau Islands	Bayer F & Fehlmann H	National Museum of Natural History, USA	4 spec (P), 2 for semithin sectioning
Acochlidium fijiense	Nasekawa River, Vanuau Levu, Fiji	Haynes A	Natural History Museum Los Angeles County, USA	2 P, 1 for semithin sectioning
Acochlidium sutteri	Lai Bondokodi, Kodi, West Sumba	Sutter E	Museum of Natural History Vienna, Austria	sections for re-examination
Aiteng ater	Pak Phanang Bay, Gulf of Thailand	Swennen C	Zoological Museum Amsterdam, Netherlands	1 P for semithin sectioning

Table 3: Sampling localities for acochlidian species. AM, Alexander Martynov (Zoological Museum, Moscow, Russia); **KJ**, Katharina Jörger (ZSM); **MG**, Matthias Glaubrecht (Naturkundemuseum, Berlin, Germany); **MS**, Michael Schrödl (ZSM), **TN**, Timea Neusser (ZSM).

(Type) locality	Species	Date	Legit
Secche della Meloria/Livorno, Italy	*Asperspina rhopalotecta* (Salvini-Plawen, 1973) *Microhedyle glandulifera* (Kowalevsky, 1901) *Hedylopsis spiculifera* (Kowalevsky, 1901) *Pontohedyle milaschewitchii* (Kowalevsky, 1901)	2005	MS, TN
Vila, Ilhabela, Brazil	*Ganitus evelinae* Marcus, 1953 *Pontohedyle brasilensis* Rankin, 1979	2010 2005	MS
Dalniye Zelentsy, Barents Sea, Russia	*Asperspina murmanica* (Kudinskaya & Minichev, 1978)	2005	AM
Savai`i Island and Upolu Island, Samoa	*Paraganitus ellynnae* Challis, 1968 *Microhedyle* cf. n. sp	2005	MS
Rovinj, Istria, Croatia	*Pontohedyle milaschewitchii* (Kowalevsky, 1901) *Microhedyle glandulifera* (Kowalevsky, 1901)	2005-2007	KJ
Viti Levu, Fiji	*Acochlidium fijiense* Haynes & Kenchington, 1991 *Hedylopsis* cf n. sp. *Asperspina* cf. n. sp. *Pontohedyle* cf. v*errucosa* *Microhedyle* cf. n. sp. *Paraganitus ellynnae*	2006	MS
Naples, Italy	*Microhedyle cryptophthalma* (Westheide & Wawra, 1974)	2006	MS
San Juan de Marcona, Punta Sal and Laguna Grande, Peru	*Asperspina* cf. n. sp. *Pontohedyle* cf. n. sp. *Microhedyle* cf. n. sp.	2006	MS
Oyster Island and Espiritu Santo Island, Vanuatu	*Pseudunela marteli* Neusser, Jörger, Schrödl, 2011 *Pseudunela espiritusanta* Neusser & Schrödl, 2009 *Paraganitus* sp. *Microhedyle* sp. *Strubellia wawrai* Brenzinger, Neusser, Jörger, Schrödl, 2011	2006	TN
Guadalcanal, Solomon Islands	*Strubellia wawrai* Brenzinger, Neusser, Jörger, Schrödl, 2011 *Pseudunela cornuta* (Challis, 1970) *Paraganitus ellynnae* Challis, 1968 *Pontohedyle verrucosa* (Challis, 1970) *Pseudunela marteli* Neusser, Jörger, Schrödl, 2011 *Asperspina* sp. *Acochlidium* sp.	2007	KJ

Miamia, Ghana	*Hedylopsis* cf n. sp. *Asperspina* cf n. sp. *Microhedyle* cf n. sp. *Pontohedyle* cf n. sp.	2007	MS, TN
Gullmarfjord, Bonden Island, Sweden	*Hedylopsis spiculifera* (Kowalevsky, 1901) *Microhedyle glandulifera* (Kowalevsky, 1901)	2008	MS, KJ, TN
Flores and Sumba, Indonesia	*Palliohedyle weberi* (Bergh, 1895) *Acochlidium sutteri* (Wawra, 1979) *Pontohedyle* spp. *Paraganitus* sp.	2008	KJ
Ambon, Indonesia	*Acochlidium amboinense* (Strubell, 1892) *Strubellia paradoxa* (Strubell, 1892) *Strubellia* sp.	2008	MG
Starichkov Island, Russia	*Asperspina* spp.	2008	AM
Sebastopol, Black Sea, Ukraine	*Pontohedyle milaschewitchii* (Kowalevsky, 1901)	2008	AM
Totoralillo, Coquimbo, Chile	*Microhedyle sp.*	2008	TN
St. Vincent Island and St. Lucia Island	*Paraganitus* sp., *Pontohedyle* sp. *Asperspina* sp.	2009	KJ

Table 4: **Serial semithin sections prepared and used in the present dissertation.** All preparations were conducted by TN except as noted otherwise. **EL**, Eva Lodde (ZSM); **eth**, ethanol**; form**, formalin; **glu**, 4 % glutardialdehyde; **KJ**, Katharina Jörger (ZSM); **MH**, Martin Heß (LMU); **NMNH**, National Museum of Natural History, USA; **R**, staining after RICHARDSON *et al*. (1960); **ROM**, Royal Ontario Museum/Canada; **Sp**, Spurr`s low viscosity resin (SPURR 1969); **TN**, Timea Neusser (ZSM); **ZIN RAS**, Zoological Institute of the Russian Academy of Siences, St. Petersburg/Russia; **ZMA**, Zoological Museum, University of Amsterdam/Netherlands; **ZSM**, Zoologische Staatssammlung München/Germany; **?**, no data available.

Museum	Museum N°	Species	Locality	GPS data	Fixation; embedding medium	Section thickness; staining
ZSM	20062163	*Asperspina murmanica*	Dalniye Zelentsy, Russia	69°7′5′′ N, 36°3′30′′ E	glu; Sp	1,5 µm; R
ZSM	20062164	*Asperspina murmanica*	Dalniye Zelentsy, Russia	69°7′5′′ N, 36°3′30′′ E	glu; Sp	1,5 µm; R
ZSM	20062165	*Asperspina murmanica*	Dalniye Zelentsy, Russia	69°7′5′′ N, 36°3′30′′ E	glu; Sp	1,5 µm; R
ZSM	20062167	*Asperspina murmanica*	Dalniye Zelentsy, Russia	69°7′5′′ N, 36°3′30′′ E	glu; Sp	1,5 µm; R. 0,9 µm (MH)
ZIN RAS	?	*Asperspina murmanica*	Dalniye Zelentsy, Russia	69°7′5′′ N, 36°3′30′′ E	form 4 %; Sp	1,5 µm
ZSM	20070391	*Hedylopsis spiculifera*	Secche della Meloria, Livorno, Italy	43°32′46.50′′ N 10°13′06.75′′ E	glu; Sp	1,5 µm; R
ZSM	20071809	*Pseudunela cornuta*	Komimbo Bay, Guadalcanal, Solomon Islands	09°15.843′ S, 159°40.097′ E	eth 75 %; Sp	1,5 µm; R
ZSM	20071911	*Pseudunela cornuta*	Komimbo Bay, Guadalcanal, Solomon Islands	09°15.843′ S, 159°40.097′ E	eth 75 %; Sp	1,5 µm; R
ZSM	20071851	*Pseudunela marteli*	Honiara, Guadalcanal, Solomon Islands	-	glu; Sp	1,5 µm; R
ZSM	20071864	*Pseudunela marteli*	Honiara, Guadalcanal, Solomon Islands	-	glu; Sp	1,5 µm; R
ZSM	20071865	*Pseudunela marteli*	Honiara, Guadalcanal, Solomon Islands	-	glu; Sp	1,5 µm; R
ZSM	20071061	*Pseudunela marteli*	Mounparap Island, Vanuatu	15°22.588′ S, 167°11.619′ E	glu; Sp	1,5 µm; R
ZSM	20090416	*Pseudunela marteli*	Mounparap Island, Vanuatu	15°22.588′ S, 167°11.619′ E	glu; Sp	1,5 µm; R

ZSM		*Pseudunela viatoris*	Viti Levu, Nukumbutho Island, Laucala Bay, Fiji	18°10.47′ S, 178°28.34′ E	glu; Sp	1,5 µm; R
ZSM	20080493	*Pseudunela viatoris*	Viti Levu, Nukumbutho Island, Laucala Bay, Fiji	18°10.47′ S, 178°28.34′ E	glu; Sp	1,5 µm; R
ZSM	20090422	*Pseudunela viatoris*	Gili Lawa Laut, Indonesia	-	glu; Sp (EL)	1,5 µm; R
ZSM	20090423	*Pseudunela viatoris*	Gili Lawa Laut, Indonesia	-	glu; Sp (EL)	1,5 µm; R
ZSM	20070968	*Pseudunela espiritusanta*	Vanuatu, Espiritu Santo Island	15°30′58″ S, 167°11′52″ E	glu; Sp	1,5 µm; R
ZSM	20080791	*Pseudunela espiritusanta*	Vanuatu, Espiritu Santo Island	15°30′58″ S, 167°11′52″ E	glu; Sp (EL)	1,5 µm; R
ZSM	20071106	*Strubellia wawrai*	Puelapa River, Espiritu Santo, Vanuatu	15°34.664′ S, 167°01.902′ E	glu; Sp	2 µm; R
ZSM	20071105	*Strubellia wawrai*	Wounaouss River, Espiritu Santo, Vanuatu	15°34.320′ S, 167°00.159′ E	glu; Sp	2 µm; R
ZSM	20071105	*Strubellia wawrai*	Wounaouss River, Espiritu Santo, Vanuatu	15°34.320′ S, 167°00.159′ E	glu; Sp	2 µm; R
ROM	?	*Tantulum elegans*	Golden Grove, St. Vincent, West Indies	13°11′30′′ N 61°11′30′′ W	eth 70 %; Sp	1,5µm; R
ROM	?	*Tantulum elegans*	Golden Grove, St. Vincent, West Indies	13°11′30′′ N 61°11′30′′ W	eth 70 %; Sp (TN, KJ)	1,5µm; R
ZSM	20110188	*Aiteng mysticus*	Matsubara, Hirara, Miyako Island, Okinawa, Japan	24°47′01″ N, 125°16′05″ E	form 10 %; Sp	2 µm; R
ZSM	20110186	*Aiteng mysticus*	Shimozaki, Nikadori, Hirara, Miyako Island, Japan	24°49′49″ N, 125°16′42″ E	form 10 %; Sp	2 µm; R
ZMA	409068	*Aiteng ater*	Pak Phanang Bay, Gulf of Thailand	24°47′01″ N, 125°16′05″ E	eth 70 %; Epon (EL)	2 µm; R
NMNH	575737	*Acochlidium bayerfehl-manni*	Arakitaoch River, Island Babelthuap, Palau Islands	-	SP	2 µm; R

12 STATUTORY DECLARATION AND STATEMENT

Eidesstattliche Versicherung:

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt wurde.

Erklärung:

Diese Dissertation wurde im Sinne von § 12 der Promotionsordnung von PD Dr. Michael Schrödl und Prof. Dr. Gerhard Haszprunar betreut. Ich erkläre hiermit, dass die Dissertation nicht einer anderen Prüfungskommission vorgelegt worden ist und dass ich mich nicht anderweitig einer Doktorprüfung ohne Erfolg unterzogen habe.

_____

Neusser Timea

München, den 05. Juni 2012