Systematics, evolution and biology of the Splanchnotrophidae (Crustacea, Poecilostomatoida), a family of parasitic Copepoda

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften der Fakultät für Biologie der Ludwig-Maximilians-Universität München

Vorgelegt von

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am 24. Januar 2017

Cover: Specimen of *Cratena peregrina* with bluish egg sacs of *Splanchnotrophus angulatus* visible between the cerata on the left side of the head.

Erstgutachter: Prof. Dr. Michael Schrödl
Zweitgutachter: Prof. Dr. Gerhard Haszprunar
Tag der mündlichen Prüfung: 15. 11. 2017

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Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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List of Publications

Paper I

Anton, R. F. & Schrödl, M. (2013), The gastropod – crustacean connection: Towards the phylogeny and evolution of the parasitic copepod family Splanchnotrophidae; Zoological Journal of the Linnean Society 167(4), pp. 501-530

Paper II

Anton, R. F., Schories, D., Wilson, N. G., Wolf, M., Abad, M. & Schrödl, M. (2016), Host specificity versus plasticity: testing the morphology-based taxonomy of the endoparasitic copepod family Splanchnotrophidae with COI barcoding; Journal of the Marine Biological Association doi:10.1017/S002531541600120X

Paper III

Salmen, A., Anton, R., Wilson, N. & Schrödl, M. (2010), SEM-description of the philoblennid endoparasitic copepod *Briarella doliaris* n. sp. From Queensland, Australia; a potential link to the Splanchnotrophidae (Crustacea, Copepoda, Poecilostomatoida); Spixiana 33 (1), pp. 19-26

Paper IV

Anton, R. F., Schories, D., Joerger, K. M., Kaligis, F. & Schrödl, M. (2015), Description of four new endoparasitic species of the family Splanchnotrophidae (Copepoda, Poecilostomatoida) from nudibranch and sacoglossan gastropod hosts; Marine Biodiversity 46 (1), pp. 183-195

Paper V

Anton, R. F. & Schrödl, M. (2013), The inner values of an endoparasitic copepod - Computerbased 3D – reconstruction of *Ismaila aliena* (Copepoda; Poecilostomatoida; Splanchnotrophidae) ; Spixiana 36 (2), pp. 183-199

Declaration of author's contribution

In this thesis, I present the results from my doctoral research conducted from 2008 until 2014, carried out under the supervision of Prof. Dr. Michael Schrödl at the Bavarian State Collection of Zoology in Munich.

Contribution to paper I: Anton, R. F. & Schrödl, M. (2013)

Roland Anton and Michael Schrödl conceived and designed the project. The data collection and phylogenetic analyses were done by Roland Anton. The data were analysed by Roland Anton under the guidance of Michael Schrödl. Manuscript concept, designing of all figures and writing was done by Roland Anton under the guidance of Michael Schrödl.

Contribution to paper II: Anton, R. F., Schories, D., Wilson, N. G., Wolf, M., Abad, M. & Schrödl, M. (2016),

Roland Anton and Michael Schrödl conceived and designed the project. The molecular work and phylogenetic analyses were conducted by Roland Anton. The data were analysed by Roland Anton under the guidance of Michael Schrödl and Isabella Stöger. Dirk Schories, Nerida G. Wilson, Maya Wolf and Marcos Abad provided several specimens for DNA extraction. Manuscript concept, designing of all figures and writing was done by Roland Anton under the guidance of Michael Schrödl. Improvements on the final manuscript were made by Dirk Schories, Nerida G. Wilson, Maya Wolf and Marcos Abad.

Contribution to paper III: Salmen, A., Anton, R., Wilson, N. & Schrödl, M. (2010)

Nerida Wilson discovered the new species and provided three specimens. Scanning electron microscopy and detailed description of the new species was done by Andrea Salmen. Manuscript concept, designing of all figures and writing was done by Roland Anton under the guidance of Michael Schrödl.

Contribution to paper VI: Anton, R. F., Schories, D., Joerger, K., M. Kaligis, F. & Schrödl, M. (2015)

Roland Anton and Michael Schrödl conceived and designed the project. Collection of specimens was done by Dirk Schories, Katharina Joerger and Fontje Kaligilis. SEM work and character-based species descriptions were conducted by Roland Anton. Manuscript concept, designing of all figures and writing was done by Roland Anton under the guidance of Michael

Schrödl. Improvements on the final manuscript were made by Dirk Schories, Katharina Joerger and Fontje Kaligilis.

Contribution to paper V: Anton, R. F. & Schrödl, M. (2013)

Roland Anton and Michael Schrödl conceived and designed the project. The histological sectioning was done by Eva Lodde-Bensch. 3D-reconstruction was conducted by Roland Anton. The data were analysed by Roland Anton under the guidance of Michael Schrödl. Manuscript concept, designing of all figures and writing was done by Roland Anton under the guidance of Michael Schrödl.

I hereby confirm the above statement.

Poing, 23. 12. 2017	Roland Anton
Ort, Datum	Dipl.-Biol. Roland Anton

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1. Summary

The Splanchnotrophidae is a small family of parasitic copepods. These small arthropods living endoparasitic in nudibranch and sacoglossan gastropod hosts are currently ignored to a great extent, since their hosts have no commercial value. Taking a closer look, this host-parasite system turns out to be more complex than expected, not seeming to apply to any standard model.

Knowledge about this family is restricted mainly to historical and unfortunately often insufficient descriptions of their external morphology. This is, on the one side, owed to the fact that the importance of the morphology of mouthparts for species descriptions was recognised rather late. On the other side, a great part of the original type and museum material is no longer available for redescriptions.

The first aim was to conduct a classical, morphology-based phylogenetic analysis of the family to test the current taxonomic hypotheses. Therefore all information concerning the morphology of splanchnotrophid species was collected from the literature, and 109 characters were comparatively discussed and coded. The results of this first cladistic phylogenetic analysis of Splanchnotrophidae supported nearly all currently accepted taxonomic hypotheses, such as the monophyly of the family and most of the genera. Only the genus *Lomanoticola* Scott and Scott, 1895 was recovered paraphyletic. The Splanchnotrophidae was sister to *Briarella* Bergh, 1976, another group of copepod endoparasites of sea slugs, suggesting that the parasitic lifestyle in nudibranch and sacoglossan gastropods evolved only once. However, parts of the morphology-based topology were sensible to expansion of the taxon sampling, as shown by recent reanalyses herein, and thus the origin of Splanchnotrophidae remains open to future research.

All phylogenetic analyses indicated that splanchnotrophids ancestrally infested nudibranchs and that the switch from nudibranch to sacoglossan hosts occurred repeatedly. However, there is no discernible evidence of a coevolution between hosts and parasites. Ancestral area reconstructions suggested that the geographic origin of the Splanchnotrophidae lies in the Indo-Pacific, from where, using the Tethys-Ocean, the area of today's Mediterranean Sea; and from there, later, the American Continent was colonised.

As a second aim, morphology-based results were tested using first molecular sequence data of the Splanchnotrophidae, using the "barcoding region" of the

cytochrome oxidase I (COI). In order to gain a comprehensive dataset, as many representatives of the Splanchnotrophidae as possible are collected worldwide. Furthermore, for the first time, DNA was extracted from the egg sacs, leaving the body of the parasite intact for further studies.

Phylogenetic hypotheses based solely on morphological data were in general recognised as problematic, especially concerning highly modified groups. However, in case of the Splanchnotrophidae, most of the morphology-based hypotheses were compatible with the COI trees. On species level, current morphology-based taxonomy was supported by initial molecular species delimitation analyses. Remarkably, the variation in host specificity between the supposedly strict host-specific species of the genus *Ismaila* and the host-promiscuous species of *Splanchnotrophus* could also be confirmed. Haplotype networks and analyses of diagnostic nucleotides suggested a potential ongoing speciation within the species *Splanchnotrophus angulatus*; individuals infesting the host *Spurilla neapolitana* seem to separate from those utilising other host species.

During the collection of molecular samples, several infested slugs not yet known as potential hosts for members of the Splanchnotrophidae were discovered. Detailed examination of taxonomically relevant external features of the parasites by scanning electron microscopy revealed them as new species. In order to achieve a more realistic impression on the species diversity of the Splanchnotrophidae, all those new species were described and named. The newly obtained morphological data and tissues were included in the cladistic and molecular analyses.

Morphological knowledge on the family Splanchnotrophidae was restricted to external features. Another aim of this thesis thus was to gain new insights into internal anatomy and to shed some light on the life history of these highly adapted parasites, above all concerning questions about nutrition, reproduction and mobility. By combining semi-thin histological sectioning with modern, computer-based 3D reconstruction it was possible for the first time to give a detailed and comprehensive description of the internal anatomy of a splanchnotrophid, *Ismaila aliena*. Results supported the assumption that at least the genus *Ismaila* feeds on the haemolymph of its host, rather than being a tissue feeder, as eponymous for the family. Further, the function of the dorsal appendages can be fathomed, mainly providing space for the extremely enlarged ovaries. Another function previously discussed, the enhancement of the respiratory surface, could also be confirmed, while a former hypothesis of

gripping the inner organs of the host, in order to fix the parasite in a certain position, has to be rejected at least for *Ismaila*, since there is no musculature found inside the dorsal appendages.

Referring to this, future studies will not only provide a wealth of new information about the Splanchnotrophidae but also new insights on the influence on their host and generally on this interesting case of a host-parasite interaction.

Zusammenfassung

Die Splanchnotrophidae sind eine kleine, parasitische Copepodenfamilie. Diese endoparasitisch in nudibranchen und sacoglossen Gastropoden lebenden Kleinkrebse wurden bislang größtenteils ignoriert, da die von ihnen befallenen Wirte keinerlei kommerzielle Bedeutung haben. Bei genauerer Betrachtung ist allerdings zu erkennen, dass es sich hier um ein äußerst komplexes Wirts-Parasit System handelt, dass sich bei keinem der einfachen Standardschemata zuordnen zu lassen scheint.

Das derzeitige Wissen um diese Familie beschränkt sich fast ausschließlich auf historische und oftmals leider sehr ungenaue Beschreibungen der äußeren Anatomie. Dieser Umstand ist zum einen der Tatsache geschuldet, dass der Aufbau der Mundwerkzeuge erst relativ spät als elementares Bestimmungsmerkmal für parasitische Copepoden erkannt wurde. Auf der anderen Seite stehen große Teile des originalen Typen- und Museumsmaterials nicht mehr für eine Nachbeschreibung zur Verfügung.

Ein erstes Ziel war es, eine klassische, morphologie-basierte phylogenetische Analyse der Familie durchzuführen, um die gängigen taxonomischen Hypothesen zu überprüfen. Dazu wurden aus der Literatur alle Informationen über die Morphologie der einzelnen Arten in einer Datenmatrix zur Merkmalsausprägung zusammengetragen und 109 Merkmale wurden umfassend diskutiert und kodiert. Anhand dieser ersten phylogenetischen Analyse konnten nahezu alle gängigen taxonomischen Hypothesen bezüglich der Familie Splanchnotrophidae, einschließlich der der Monophylie der Familie und der meisten Gattungen bestätigt werden. Einzig die Gattung *Lomanoticola* Scott und Scott, 1895 erwies sich als paraphyletisch. Die Splanchnotrophidae erschienen als Schwestergruppe zu Briarella Bergh, 1976, einer anderen Gruppe von endoparasitisch in Meeresnacktschnecken lebenden Copepoden, was den Schluss nahelegt, dass der Parasitismus in nudibranchen und sacoglossen Gastropoden nur einmal evolviert ist. Allerdings waren Teile der morphologie-basierten Topologie abhängig von der Wahl der einbezogenen Taxa, wie durch die kürzlichen Nachanalysen in dieser Arbeit gezeigt wurde, weshalb der Ursprung der Splanchnotrophidae offen bleibt für zufünftige Forschungen.

Alle phylogenetischen Analysen deuten darauf hin, dass die Splanchnotrophidae ursprünglich Nudibranchia befallen haben, und dass der Wechsel auf sacoglosse Wirte mehrfach stattgefunden hat. Hinweise auf eine Koevolution zwischen Parasiten

und Wirten konnten allerdings nicht festgestellt werden. Einer Rekonstruktion der ursprünglichen Verbreitung nach liegt der geographische Ursprung der Splanchnotrophidae im Indo-Pazifik von wo aus, über das Tethis-Meer, zuerst das Gebiet des heutigen Mittelmeeres und von dort aus vermutlich später der Amerikanische Kontinent besiedelt wurden.

In einem zweiten Ansatz wurden die morphologie-basierten Ergebnisse mittels erster Sequenzdaten der Splanchnotrophidae, genauer der „Barcoding-Region" der Cytochromoxidase I (COI) getestet. Um einen möglichst umfassenden Datensatz zu erhalten, wurden weltweit so viele Vertreter der Splanchnotrophidae wie möglich gesammelt. Außerdem wurde erstmals versucht, die benötigte DNA aus den Eisäcken zu gewinnen, da so der Körper des Parasiten vollständig für anderweitige Untersuchungen erhalten bleibt.

Rein morphologisch begründete phylogenetische Hypothesen werden generell als potentiell problematisch angesehen, vor allem wenn sie hochabgeleitete Gruppen betreffen. Dennoch waren im Fall der Splanchnotrophidae die meisten getesteten taxonomischen Hypothesen mit den COI-Bäumen vereinbar. Auf Artniveau wurden die derzeitigen taxonomischen Hypothesen durch erste molekulare Artabgrenzungsanalysen gestützt. Bemerkenswerter Weise konnte sogar die abweichende Wirtsspezifität zwischen der vermutlich strikt wirtsspezifischen Gattung *Ismaila* und einer Art der Gattung *Splanchnotrophus* mit breiterem Wirtsspektrum bestätigt werden.

Haplotypen-Netzwerke und Analysen diagnostischer Nukleotide legen eine mögliche derzeit ablaufende Aufspaltung der Art *Splanchnotrophus angulatus* nah; Individuen, die die Wirtsart *Spurilla neapolitana* infizieren scheinen sich demnach von solchen, die andere Wirtsarten befallen abzuspalten.

Während der ausgedehnten Sammeltätigkeit für genetische Proben wurden mehrere infizierte Schnecken gefunden, die bislang noch nicht als Wirte für Splanchnotrophidae bekannt waren. Eine genauere Untersuchung von taxonomisch relevanten äußeren Merkmalen der Parasiten mittels Rasterelektronenmikroskopie ergab, dass es sich um neue, bislang unbekannte Arten handelte. Um ein möglichst vollständiges Bild der Diversität der Splanchnotrophidae zu bekommen, wurden diese neuen Arten detailliert anatomisch beschrieben und benannt. Die so neu erhaltenen Daten und Gewebe wurden dann in die kladistischen und molekularen Analysen integriert.

Morphologisches Wissen bezüglich der Familie Splanchnotrophidae war bislang fast vollständig auf Beschreibungen der äußeren Anatomie beschränkt. Daher war ein weiteres Ziel dieser Arbeit, neue Erkenntnisse über die innere Anatomie zu gewinnen und Licht in die Lebensgeschichte dieser hochangepassten Parasiten zu bringen, besonders was Fragen nach der Ernährung, Reproduktion und Mobilität angeht. Durch eine Kombination aus semi-dünnen histologischen Schnittserien und moderner computergestützter 3D-Rekonstruktion gelang erstmals eine detaillierte Beschreibung der inneren Anatomie eines Splanchnotrophiden, *Ismaila aliena*. Die Ergebnisse stützen die Vermutung, dass sich zumindest die Gattung *Ismaila* von der Haemolymphe des Wirtes ernährt und nicht von Gewebe, wie der Gattungsname nahelegt. Zudem konnte die Funktion der dorsalen Anhänge ergründet werden, die hauptsächlich dazu dienen, die extrem vergrößerten Ovarien aufzunehmen. Eine weitere Funktion als Vergrößerung der respirativen Oberfläche kann ebenfalls bestätigt werden, wohingegen die frühere Hypothese einer Haltefunktion zur Fixierung des Parasiten innerhalb des Wirts zurückgewiesen werden muss, da die Anhängsel zumindest bei *Ismaila* keine eigene Muskulatur aufweisen.

Diesbezüglich werden zukünftige Studien eine Fülle neuer Informationen nicht nur über die Splanchnotrophidae bereitstellen, sondern auch neue Erkenntnisse über deren Einfluss auf den Wirt und allgemein über diese interessante Wirts-Parasit Beziehung.

2. Introduction

2.1 Introduction to splanchnotrophid copepods

2.1.1 Diversity and importance of copepods

Thinking about important marine organisms, most people first would imagine either those with great body mass like whales or those being present in everyday life such as fish or crustaceans, since most people recognise their gastronomic value. However those organisms being vital to keep the entire ecosystem called ocean functioning remain mainly unrecognised, mostly because of their small body size and their low economic value. They can be found at the bottom of each ecological (or trophic) pyramid: primary producers and primary consumers (Elton 1927; Gasol et al. 1997). In marine ecosystems those two groups are usually combined in one term: plankton. Commonly the overall biomass of primary producers (phytoplankton) is thought to exceed that of primary consumers (zooplankton) (Elton 1927). Nevertheless in some cases, zooplankton biomass can equal or even surpass phytoplankton biomass (Odum 1971; Jumars 1993; Hopcroft and Roff 1996; Gasol et al. 1997; Chiba et al. 2002).

Members of the Crustacea clearly have the greatest ecological importance within the zooplankton, for example regarding all taxa included in the common term 'krill' (Nicol and Endo 1999; Nicol 2006). Copepods, together with their larvae constitute the greatest part of the marine zooplankton (Longhurst 1985; Mauchline 1998) and therefore are one of the most important organisms of marine ecosystems (Yoshikoshi 1975; Ho 2001; Turner 2004; Ikeda et al. 2007).

As a consequence, sooner or later nearly all marine organisms come into contact with some sort of copepod. In most cases this contact will be rather short and unspectacular: most marine organisms simply feed on them and given the overwhelming abundance of copepods, their position in marine food webs is pivotal (Turner 2004). But other cases are more complicated, leading to the vast group of associated copepods. Apart from the mass of free living copepods there is also a great number of copepods, which in time entered relationships to other phyla of marine organisms and therefore are referred to as associated copepods (Gotto 1979). This includes those entire species dependant on a particular host species, at least at a certain developmental stage of their life history (Gotto 1979; Gotto 2004; Boxshall 2005). The strategy of associating seems to be very successful since not only all major

copepod orders comprise associated forms but also those associated copepods inhabit members of nearly all major phyla of marine organisms including sponges (Boxshall and Huys 1994), ascidians (Gotto 1957; López-Gonzáles et al. 1997), sea anemones (Vader 1970), polychaetes (Björnberg and Radashevsky 2009), echinoderms (Dojiri and Cressey 1987; Boxshall and Ohtsuka 2001; Anton et al. 2013), crustaceans (Dvoretsky and Dvoretsky 2013), bivalves (Kim 2004), gastropods (Izawa 1976; Ho 1981; Avdeev et al. 1986; Ho and Thatcher 1989; Clarke and Klussmann-Kolb 2003; Marshall and Hayward 2006), cephalopods (Cavaleiro et al. 2013), fish (Cressey and Boyle Cressey 1980; Ho and Kim 1992; Ho 1994; Ibraheem and Izawa 2000; Ho and Nagasawa 2001; Ho and Lin 2006; Cavaleiro et al. 2010) and marine mammals (Dailey and Brownell 1972; Boxshall 2005; Danyer et al. 2014). Of all 11956 known species of marine copepods 4224 species (35.33%) belong to the group of associated copepods (Ho 2001). This species richness leads to a vast variety of different forms depending on the degree of adaptation between the respective associated copepod and its host (Gotto 1979; Boxshall 2005). While those living on the surface of their respective host often resemble their free living relatives (Gotto 1979; Suh 1993), those living inside their host may exhibit rather bizarre body forms (Gotto 1979; Gotto 2004; Boxshall 2005; Anton et al. 2013) including extreme aberrations no longer discernible as copepods (O'Donoghue 1924; Gotto 1979; Huys 2001; Haumayr and Schrödl 2003; Salmen et al. 2008a; Salmen et al. 2008b).

2.1.2 Associated copepods and parasites

Interestingly, all these associations are either commensalistic or parasitic (Gotto 1979; Ho 2001; Gamarra-Luques et al. 2004) and there has not yet been any report of mutualistic copepods (Ho 2001). However, it is relatively easy to imagine associated copepods living on the skin of some host feeding rather on some algae growing in the surface than on the hosts' skin so that the host will indeed benefit from the copepods. This example illustrates the severe lack of interest in associated copepods already mentioned by Gotto (1979). Unfortunately since then things do not seem to have improved much. Ho (2001) again complained the striking under-representation of associated copepods concerning the focus of research of most copepodologists throughout the world. The only exception are parasitic copepods infesting hosts of commercial value (Saby 1933; Cressey and Boyle Cressey 1980; Østergaard et al.

2003; Kim 2004; Østergaard 2004; Özel et al. 2004; Huys et al. 2006). But even if associated copepods are in the focus of attention, studies are often limited mainly to distribution and prevention of infestation (Berry et al. 1991; Devine et al. 2000; Ingvarsdottir et al. 2002a; Ingvarsdottir et al. 2002b).

Studying parasites in general is rather difficult since they often exhibit an intimate contact with their host and are living secretively and invisible to the outside world (Preston and Johnson 2010). This hidden lifestyle seems to have led to the assumption that parasites play only a minor part in community ecology compared to free living organisms (Preston and Johnson 2010). Also the fact that parasites in general are not included in the concepts of food webs assuming their contribution to biomass to be negligible (Preston and Johnson 2010) is pointing in this direction.

However, including parasites into food webs clearly reveals their potential importance (Preston and Johnson 2010). According to Sukhdeo and Hernandez (2005) it may even be necessary to revise the classical Eltonian pyramid (Elton 1927). Parasites feeding on a trophic level above their hosts would occupy the pinnacle of this new pyramid (Sukhdeo and Hernandez 2005) implying a significant departure from the traditional placement of top predators at the peak of the food chain (Sukhdeo and Hernandez 2005; Preston and Johnson 2010). But even assuming their role being less seminal, their influence on ecosystems can be substantial. Recent studies suggest parasites to contribute significantly to ecosystem energetics (Mitchell 2003; Kuris et al. 2008). Parasites may even influence biodiversity by affecting competitive interactions between host species, for example via parasite-mediated competition (Price et al. 1986). This term describes a tolerant host species amplifying the abundance of a certain parasite and thus causing an indirect negative effect on a second, less tolerant host species (Schall 1992; Tompkins et al. 2003; Preston and Johnson 2010). And such effects on ecological communities can be particularly pronounced if the respective hosts are keystone or dominant species (Sinclair 1979; Lessios 1988; Edmunds and Carpenter 2001; Thomas et al. 2005). Therefore parasites eminently contribute to structuring ecological communities (Preston and Johnson 2010).

Given the great number of parasites among the associated copepods and the almost infinite modes of interaction between host and associate, this group still conceals countless interesting new insights concerning the respective ecological community – especially regarding those groups usually rather unaffected by parasites or at least not

yet recognised to be. The shell-less nudibranch and sacoglossan gastropods provide a good example for such a group. Not only are there few predators known to feed on Nudibranchia (Todd 1981; Harris 1987; Piel 1991; Schrödl 2003), but moreover the only group managing to successfully establish permanent associations with nudibranch or sacoglossan gastropods are copepods (Lysaght 1941; Thieltges et al. 2009). Many of them are living on the skin of their respective host, feeding mainly on the mucus like the members of the genus *Philoblenna* (Izawa 1976; Ho 1981; Ho and Kim 1992), but there are some associated copepods that found a way inside their hosts.

2.1.3 Splanchnotrophidae - highly adapted endoparasites

Few even managed to infest hosts like opisthobranch gastropods that are normally thought to have at least very few natural enemies. The most species rich of them is the copepod family Splanchnotrophidae Norman and Scott, 1906. Members of this family are living endoparasitically inside their respective nudibranch or sacoglossan hosts (Huys 2001; Schrödl 2002; Haumayr and Schrödl 2003; Schrödl 2003). As a special feature, the abdomen of adult females is protruding through the hosts' integument, locating the in some cases brightly coloured egg sacks outside the host (Huys 2001). Furthermore, all representatives of the Splanchnotrophidae exhibit strong modifications, especially of their external morphology compared to free living copepods. This displays their high level of adaptation to the endoparasitic lifestyle (Huys 2001; Schrödl 2003) and makes adult individuals nearly unrecognisable as copepods (Fig. 1). Their peculiar shape is in particular due to the dorsal appendages, which are typical for members of Splanchnotrophidae and the enigmatic genus *Briarella doliaris* Salmen, Anton, Wilson and Schrödl, 2010, and whose potential function is still discussed.

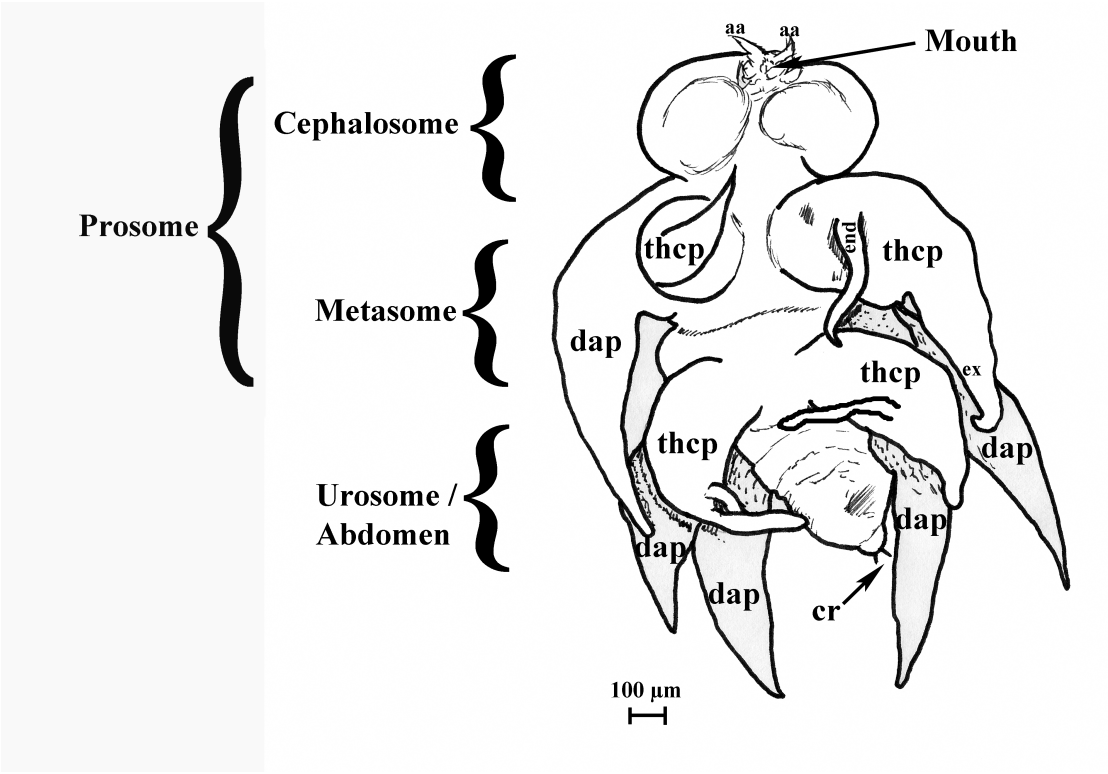


Figure 1. Line drawing of the general habitus of the splanchnotrophid *I. damnosa*; ventral view; aa antennae; cr caudal rami; dap dorsal appendages; end endopodit; ex exopodit; thcp thoracopod.

2.1.4 History of splanchnotrophid taxonomy

Hancock and Norman (1863) discovered the first representative, *Splanchnotrophus gracilis* in 1863, thus introducing the genus *Splanchnotrophus*, but it was not until 1906 when Norman and Scott introduced the family Splanchnotrophidae. The origin of the Splanchnotrophidae was disputed ever since their discovery. First the genus *Splanchnotrophus* was placed in the Chondracanthidae (Hancock and Norman 1863), while Bergh (1876) refrained from placing *Ismaila* in a particular family and questioned the placement of *Splanchnotrophus*. Canu (1898) listed the Splanchnotrophidae under the Lichomolgidae, but his reasons remained unknown (Huys 2001). Laubier (1964) examined the mouthparts in detail and re-established the Splanchnotrophidae as a distinct family. In addition, the composition of the included taxa changed several times. Monod and Dollfus (1932) included the genera *Ismaila*, *Chondrocarpus* Bassett-Smith, 1903, *Briarella* and *Splanchnotrophus* treating *Lomanoticola* Scott and Scott, 1895 as a subgenus of the latter. Laubier (1964) excluded *Briarella*, but Jensen (1987) still listed *Briarella* as a member of the Splanchnotrophidae together with the genera *Splanchnotrophus*, *Ismaila*,

Chondrocarpus, *Micrallecto* Stock, 1971, *Nannallecto* Stock, 1973 and *Megallecto* Gotto, 1986. Huys (2001) reviewed the family and included the following five genera: *Lomanoticola, Splanchnotrophus* Hancock and Norman, 1863, *Ismaila* Bergh, 1867, *Arthurius* Huys, 2001 and *Ceratosomicola* Huys, 2001. Recently, Uyeno and Nagasawa (2012) added a sixth genus, *Majimun* Uyeno and Nagasawa, 2012. Although the family is distributed worldwide, the geographic range of the included genera is usually rather limited. *Splanchnotrophus* and *Lomanoticola* are known only from the Mediterranean Sea and the European coasts of the Atlantic ocean, with the exception of two species assigned to *Splanchnotrophus* recently discovered in Japan (Uyeno and Nagasawa 2012). *Arthurius* and *Ceratosomicola* are known only from Indonesian waters and the north-western coast of Australia (Huys 2001; Salmen et al. 2008a; Salmen et al. 2008b and chapter 6), *Majimun* is known from Japan, and *Ismaila* is exclusively reported from the American continent (Huys 2001; Haumayr and Schrödl 2003; Salmen et al. 2008a; Salmen et al. 2008b; Uyeno and Nagasawa 2012 and chapter 6).

2.1.5 Splanchnotrophid life history - a book of seven seals

Although being widely distributed and - due to the externally located egg sacks - relatively easy to detect, knowledge about the Splanchnotrophidae in general is mainly limited to poorly detailed descriptions of their external morphology (Bergh 1868; Canu 1891; Hecht 1893; Delamare Deboutteville 1950; Delamare Deboutteville 1951). Questions about their life history like nutrition, reproduction or the mechanisms to infest new hosts yet remain unanswered.

For example: since their first introduction, Splanchnotrophidae were referred to as parasites due to the fact, that at least the greater part of their body was located inside their host (Hancock and Norman 1863; Huys 2001; Haumayr and Schrödl 2003). First the parasites' lifestyle was thought to be eponymous (*Splanchnotrophus* means "tissue feeder"), and the discovery of hosts displaying destroyed gonads seemed to confirm this assumption (Hancock and Norman 1863; Jensen 1987; Marshall and Hayward 2006; Wolf and Young 2014). But detailed observations during dissections of several host species did not reveal any signs of gnawing marks on the inner organs the parasite was in contact with (Schrödl 1997; Haumayr and Schrödl 2003; Schrödl

2003; Salmen et al. 2008a; Salmen et al. 2008b; Abad et al. 2011). The hypothesis thus changed and Splanchnotrophidae since then are considered as haemolymph suckers (Schrödl 1997; Schrödl 2002; Schrödl 2003). However, the impact of the parasite on its host is still unresolved. Usually only a very small number of predators feed on shell-less nudibranch and sacoglossan gastropods (Edmunds 1966; Faulkner and Ghiselin 1983; Schrödl 2003), so the influence of parasites on the population dynamics of the respective host species might be considerable.

2.2 Sourcing material

To extend the material already present in the Bavarian State Collection of Zoology and to gain a sufficient number of independent samples for a molecular based analysis, several collection trips were realised to different locations in Europe and southern America. Rovinj (Croatia) and Banyuls-sur-Mer (France) were chosen due to the presence of scientific institutions (Figs. 2C and D). In addition, Banyuls-sur-Mer is also the type locality of *S. dellachiajei*.

Since the greatest part of all recent findings of splanchnotrophids come from South America, this was made the second focus for collection. Again two locations were chosen providing excellent scientific institutions in Chile: The scientific field station of the “Fundacion San Ignacio del Huinay“ (Fig. 2A) located in the Comau fjord, Palena Province, Region X, and the Laboratorio Costero de Recursos Acuáticos Calfuco near Valdivia (Fig. 2B).

Figure 2. Sampling of specimens; **A** Huinay field station; **B** Calfuco field station; **C** "Red isle" near Rovinj, Croatia; **D** Laboratoire Arago, Banyuls-sur-Mer, France; **E** Examination of collected material to identify infected specimens; **F** Sea-water aquarium to keep infected specimens (Asterisks mark enclosures where parasite larvae were kept). Photos A-C and F taken by the author, D and E taken by Bastian Brenzinger.

Host specimens were collected alive by snorkelling or SCUBA diving. Additional material was also received from Michael Schrödl, Katharina Joerger, Vinicius Padula, Roland Melzer (all Bavarian State Collection of Zoology), Alexander Martynov (Lomonosov Moscow State University), Marcos Abad (Estación de Bioloxía Mariña da Graña, Universidade de Santiago de Compostela), Maya Wolf (Department of Biology, University of Oregon), Nerida Wilson (Molecular Systematics Unit, University of Western Australia) and Dirk Schories (Instituto de Ciencias Marinas y Limnológicas, Universidad Austral de Chile).

In the laboratory, infested specimens were identified using a stereo microscope (Fig. 2E). Infested hosts were kept in a seawater aquarium in order to gain the free-living

larval stages before being fixated. Parasites were extracted from their hosts by carefully dissecting the host under a stereomicroscope.

2.3 Phylogeny and evolution of the Splanchnotrophidae

The first step towards an initial morphocladistic analysis of the Splanchnotrophidae was to gather all existing information on any member of the family Splanchnotrophidae and potential closely related taxa, in order to lay the necessary foundations for comprehensive integrative taxonomy. The next intention was to test the status of the family and all its members, compared to the hypotheses presented by Huys (2001) in his review of the family. Creating a character state matrix containing all available data on the external morphology of endoparasitic copepod species known from sea slugs in 2013, their relationship and their affiliation to the family Splanchnotrophidae is studied in chapter 3. In addition, using the resulting phylogenetic tree a first attempt is made to resolve the biogeographic history of the family.

The second step towards recovering the phylogeny of Splanchnotrophidae was to apply modern, molecular methods. This seemed to be necessary due to the high level of morphological adaptations displayed by the parasites, causing serious problems in morphology based phylogenetic studies as already mentioned by Huys (2001). During the last years, even simple, single gene DNA-barcoding was suggested as a promising initial approach for such issues (Jörger et al. 2012; Weis and Melzer 2012; Stöger et al. 2013; Jörger et al. 2014; Jörger and Schrödl 2014; Padula et al. 2014). Since specimen samples are rather scarce for some species and even genera, DNA samples were gained using the egg sacs of mature females, because this method leaves the whole body including all its appendages available for morphological studies (Anton et al. 2013). In chapter 4 barcoding of the cytochrome oxidase I gene (COI) was applied to test the currently accepted taxonomic hypotheses. Results were compared to those from chapter 3 and were used to test the distinct difference between the European genera *Lomanoticola* and *Splanchnotrophus* and the rest of the family concerning host specificity. For that purpose two species of *Ismaila* showing supposedly strict host specificity, with only one host species respectively, were compared to *Splanchnotrophus angulatus* Hecht, 1893, which was known from at least five different host species.

In addition, a variety of up to date molecular analytical methods were applied to extend the traditional view on species boundaries in splanchnotrophids and allow for an initial integrative view on life history traits such as host specificity.

2.4 Taxonomic revisions and description of newly discovered species

During the collection of information concerning the Splanchnotrophidae a yet unknown new species of the genus *Briarella* was described, now given in chapter 5, which seemed to be important for the present study due to obvious similarities to the genus *Splanchnotrophus*. The genus *Briarella* was previously included into the Splanchnotrophidae (Monod and Dollfus 1932) but then was placed together with the genus *Philoblenna* in the family Philoblennidae (Izawa 1976; Huys 2001), and recently they were included into the Lichomolgidae (Kim et al. 2004). Jensen (1987) suggested to consolidate all associated copepods living endoparasitic in opisthobranch molluscs into the family Splanchnotrophidae. However, Huys (2001) in his review of the Splanchnotrophidae did not follow this suggestion, but excluded *Briarella* and several other taxa from the family regarding their obvious variation in life history. According to this it seems interesting to find a member of *Briarella* showing strong similarities regarding the external morphology, which could be either due to homologous evolution or due to convergent development. As part of various research and collection trips to southern Chile, Croatia and southern France as well as in the context of donations by colleagues, infested opisthobranchs were discovered which were not yet identified as potential hosts for splanchnotrophid endoparasites. Because of the usually high host specificity of the Splanchnotrophidae, those newly found hosts were examined carefully, and the extracted parasites were studied extensively. Most of the parasites obtained from hosts previously unrecognised as such indeed proved to be new species. In order to complete the knowledge about the family Splanchnotrophidae, those new species were morphologically described in detail, using scanning electron microscopy (SEM). Chapter 6 provides the respective description of those new species.

2.5 Splanchnotrophid biology and life history

In chapter 7, modern computer-based imaging procedures are used to cast a glance inside the parasites. Recently the combination of semi-thin and ultra-thin sectioning

with computer based 3D reconstruction has proven as a reliable tool from investigating the internal anatomy of specimens (Neusser et al. 2006; Neusser et al. 2007a; Neusser and Schrödl 2007; Jörger et al. 2008; Neusser and Schrödl 2009; Brenzinger et al. 2013; Brenzinger et al. 2014), the function of organs and structures (Neusser et al. 2008; Brenzinger et al. 2012) through to their regulation (Neusser et al. 2007b; Brenneis and Richter 2010; Lehmann et al. 2012; Geiselbrecht and Melzer 2013; Lehmann and Melzer 2013). Until now only Belcik (1965) studied the internal morphology of *Ismaila belciki*, but mainly regarding the musculature and not in great detail (Belcik 1965; Belcik 1981). Apart from that, nothing was known about the internal anatomy of any other member of the Splanchnotrophidae. Computer-based 3D reconstruction thus was used to create a 3D model based on histological semi-thin sections of both sexes of *Ismaila aliena*. For the first time, the definite structure and arrangement of the inner organs of these copepods was revealed and studied in detail. These newly gained insights are then used to shed some light on yet unanswered questions about the life history of these parasites. As part of that the genus *Ismaila* is proven to feed on the hosts haemolymph. Apart from that, the mobility of *Ismaila* was found to differ between the sexes, and the purpose of the dorsal appendages is clarified. As a second goal the benefit of data on the internal anatomy for future studies based on morphological data is discussed. Regarding the external morphology it is often obscure whether a certain character represents a true homology or if it is just a simple case of convergent development (Huys 2001). Knowledge about the internal anatomy could therefore not only extend the morphological dataset but also provide reliable additional data.

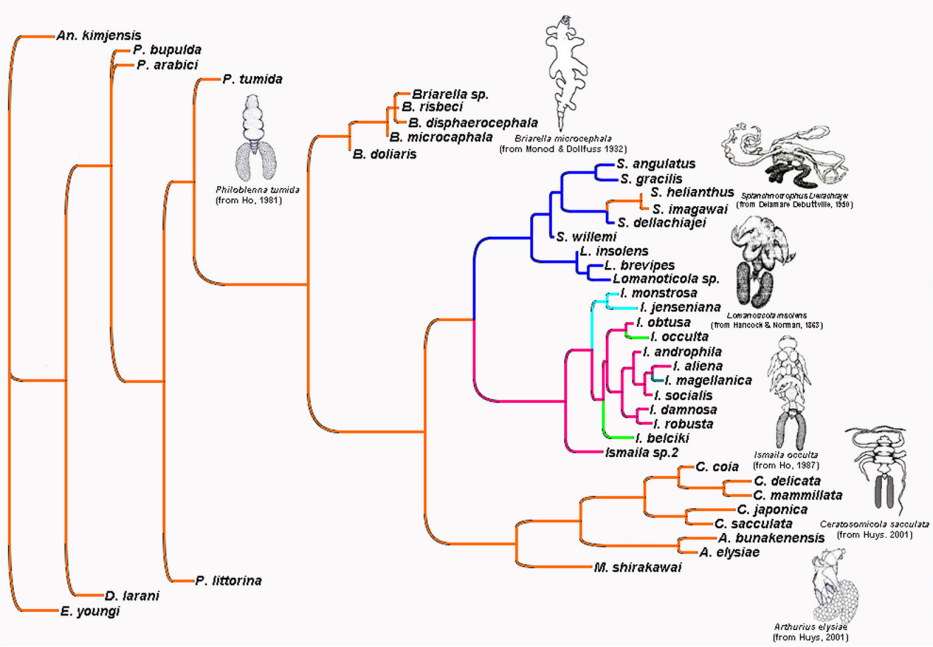
2.6 Aims of this study

To summarise, the main goals of this study are: 1) to test the currently accepted taxonomic hypotheses and to confirm the eligibility of the family Splanchnotrophidae. For this purpose a morphology-based phylogeny was established for the first time, including all currently known splanchnotrophid taxa. A comprehensive dataset was elaborated, including all available morphological data on every member included in the family Splanchnotrophidae after its revision (Huys, 2001) together with several potential outgroup taxa; 2) to create a database including molecular data on all available splanchnotrophid species and appropriate outgroup taxa. Therefore

widespread sampling efforts were made with a special focus on Europe and South America to extend knowledge not only about splanchnotrophid diversity, but also concerning potential outgroup taxa. Furthermore a non-invasive method of gaining molecular samples by using the egg sacs of mature female parasites is established. This newly gained molecular data is then used to test the hypotheses resulting from the morphology based phylogenetic analysis. In addition, the evolution of characters and the biogeographic dispersal of the respective genera are discussed in the light of the present phylogeny. 3) Since general knowledge about the Splanchnotrophidae currently is limited to acquaintance of the respective host species, the area of discovery and –unfortunately– often imprecise information on the external morphology, the third aim of this study is to research on the life history of the Splanchnotrophidae. Therefore the internal anatomy is studied in great detail, the varying host specificity between European genera on the one hand and Indonesian and American genera on the other hand is investigated and for the first time the early ontogenetic larval stages are isolated for several species.

Chapter 3

Anton, R. F. & Schrödl, M. (2013), The gastropod – crustacean connection: Towards the phylogeny and evolution of the parasitic copepod family Splanchnotrophidae; Zoological Journal of the Linnean Society 167(4): 501-530



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The gastropod–crustacean connection: towards the phylogeny and evolution of the parasitic copepod family Splanchnotrophidae

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Amongst the most significant metazoan taxa associated with gastropod molluscs is the endoparasitic copepod family Splanchnotrophidae. Currently it contains five genera with highly modified morphology and exclusively infesting nudibranch and sacoglossan sea slug hosts. The present study is a first approach towards reconstructing their phylogeny and evolution. Cladistic analysis of 109 morphological characters including 24 known splanchnotrophid species resulted in a fully resolved strict consensus tree that is discussed in morphological, functional, and geographical frameworks. Alternative topologies are also explored. Originating from paraphyletic Philoblennidae, the Splanchnotrophidae emerge as sister group to the genus *Briarella*. Unique synapomorphies, such as the bizarre body shapes and successive reduction of mouthparts, are discussed as adaptive traits to endoparasitism that evolved only once within copepods infesting shell-less heterobranch gastropods. The ancestrally Indo-Pacific Splanchnotrophidae split up into a clade of the still Indo-Pacific genera *Ceratosomicola* and *Arthurius*, sister to a clade composed of the monophyletic amphi-American genus *Ismaila* and European *Splanchnotrophus* emerging from paraphyletic *Lomanoticola*. Although initial radiation of *Briarella* and Splanchnotrophidae is likely to have involved chromodoridid nudibranch hosts, later phylogenies of parasites and their hosts are incongruent; intriguingly, host shifts from nudibranch to only distantly related sacoglossan species occurred at least two times independently. Such remarkable ecological plasticity is assumed to have driven splanchnotrophid diversification. Topological hypotheses and historical biogeographical and evolutionary scenarios inferred herein can be tested by future molecular research.

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ADDITIONAL KEYWORDS: *Briarella* – Copepoda – endoparasites – Opisthobranchia – Poecilostomatoida.

INTRODUCTION

Studying host–parasite interactions as model systems for coevolution often leads to new insights into evolutionary mechanisms as they make evolution observable (D'Ettorre & Heinze, 2001). However, the reconstruction of the macroevolutionary influence of parasites on their host groups requires sound concepts on their phylogeny, which are seldom available.

The Copepoda constitutes one of the most important groups of marine zooplankton, showing great diversity regarding species numbers, shape, and mode of life (Yoshikoshi, 1975). Besides the pelagial, copepods also successfully colonized benthic habitats (Gheerardyn *et al*., 2009), and it is possible that ectoparasitic forms originated from those benthic copepods (Itoh & Nishida, 2007). Parasitic copepods in particular are very successful, as indicated by the broad variety of possible hosts such as ascidians, fish, bivalves, polychaetes, and gastropods (Gotto, 1957; Ho, 1987a; Kim, 2001; Huys *et al*., 2006). In fact they are, besides some trematodes, the only metazoan parasites successfully infesting marine gastropod hosts (Lysaght, 1941; Thieltges *et al*., 2009), apart from several taxa living commensally with them (Vega *et al*., 2006). Parasitic copepods can be divided

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into ectoparasites, located on the skin of their hosts with only a few modifications, and endoparasitic forms, entering the body of the host more or less completely. Endoparasites are highly adapted (Østergaard, Boxshall & Quicke, 2003; Boxshall & Strong, 2006) and often specific to certain hosts, such as the Splanchnotrophidae, which exclusively infest shell-less opisthobranch hosts (Ho, 1987a; Huys, 2001; Haumayr & Schrödl, 2003; Abad, Díaz-Agras & Urgorri, 2011).

The structure of the family Splanchnotrophidae has been discussed repeatedly (Monod, 1928; Oakley, 1930; Monod & Dollfus, 1932; Laubier, 1964; Jensen, 1987). The currently accepted classification was proposed by Huys (2001). Since then, 15 new species have been discovered (Haumayr & Schrödl, 2003; Salmen *et al*., 2008a, 2010; Salmen, Wilson & Schrödl, 2008b), but in most cases knowledge of the species is restricted to the morphological information from the species descriptions. Only a very small number of studies have included life history traits (Belcik, 1981; Ho, 1987a; Schrödl, 1997, 2002) and the internal phylogeny has never been analysed in detail until now.

The family name Splanchnotrophidae was introduced in 1906 by Norman and Scott without an accompanying diagnosis (Norman & Scott, 1906), only including the type species *Splanchnotrophus gracilis* Hancock & Norman, 1863. The Splanchnotrophidae were then treated as a subfamily of the Chondracanthidae (see Gerstäcker, 1866–1879), which was used as a convenient 'catch all' taxon for modified parasites in the late 19th and early 20th centuries until it was rigorously defined by Ho's (1970) revision. Monod & Dollfus (1932) reinstated the family as a member of the Poecilostomatoida. They included the genera *Ismaila* Bergh, 1967, *Briarella* Bergh, 1976, *Chondrocarpus* Bassett-Smith, 1903, and *Splanchnotrophus* Hancock & Norman, 1863, treating *Lomanoticola* Scott & Scott, 1895, as a subgenus of the latter (Monod & Dollfus, 1932; Huys, 2001). Laubier (1964) examined the mouthparts of *Splanchnotrophus dellachiajei* Delamare Deboutteville, 1950. He diagnosed the Splanchnotrophidae by the absence of maxillipeds and the special shape of the mandible and maxilla, removing the genus *Briarella* and provisionally placing it into the Chondracanthidae (Laubier, 1964; Huys, 2001). Two more genera were included in the Splanchnotrophidae: *Micrallecto* Stock, 1971 and *Nannallecto* Stock, 1973 (see Stock, 1971, 1973). Later the genus *Megallecto* Gotto, 1986, was also placed within the Splanchnotrophidae by Gotto (1986). In 1987, Jensen included in Splanchnotrophidae all genera living endoparasitically in opisthobranch gastropod hosts, i.e. *Splanchnotrophus s.l.* (including *Lomanoticola*), *Ismaila*, *Briarella*, and *Chondrocarpus*, and excluded the ectoparasitic genera *Micrallecto*, *Nannallecto*, and *Megallecto* (see Jensen, 1987).

In the latest and most comprehensive revision of the Splanchnotrophidae Huys (2001) excluded the genera *Briarella*, *Chondrocarpus*, *Micrallecto* Stock, 1971, *Nannallecto*, and *Megallecto*. He synonymized *Micrallecto* and *Nannallecto* and recognized the only member of *Megallecto*, *Megallecto thirioti* Gotto, 1986, as a head fragment of a pelagic peracarid. Huys (2001) introduced two new genera, *Ceratosomicola* Huys, 2001, on the basis of *Ceratosomicola sacculata* (O'Donoghue, 1924), and *Arthurius* Huys, 2001, based on *Arthurius elysiae* (Jensen, 1990), including them in Splanchnotrophidae. He also upgraded *Lomanoticola* to genus level, so that the family Splanchnotrophidae now consists of the five genera *Splanchnotrophus*, *Lomanoticola*, *Ismaila*, *Arthurius*, and *Ceratosomicola* (see Huys, 2001). Concerning the internal phylogenetic relationship of the Splanchnotrophidae there is as yet only one hypothesis. Schrödl (2002) suggested that the genera *Ismaila* (eastern Pacific and Caribbean Sea) and *Splanchnotrophus* (northeastern Atlantic and Mediterranean Sea) are more closely related to each other than to Indo-Pacific *Arthurius* and *Ceratosomicola*, and *Splanchnotrophus* is referred to as *Splanchnotrophus s.l.* and contains the two sympatric subgroups *Splanchnotrophus* and *Lomanoticola*.

Even more enigmatic is the origin of the Splanchnotrophidae. The only available phylogenetic analysis of the Poecilostomatoida including Splanchnotrophidae was based on morphological characters of female specimens only (Ho, 1991), and suggested the Splanchnotrophidae to be a sister taxon to the Shiinoidae, a family of highly modified fish parasites. However, since then the concept of Splanchnotrophidae has changed substantially (Huys, 2001), towards a more rigorous and homogeneous bauplan that greatly differs from Shiinoidae.

Recently, Salmen *et al*. (2010) presented two hypotheses on the possible relationship between the genera *Briarella* and *Splanchnotrophus*. Considering the similarity between *Splanchnotrophus* and *Briarella doliaris* Salmen, Anton, Wilson & Schrödl, 2010, it was assumed that either *Briarella* is the sister group to the monophyletic Splanchnotrophidae or *B. doliaris* alone is the sister taxon to the family Splanchnotrophidae, rendering *Briarella* paraphyletic. These ideas remained to be tested in a rigorous phylogenetic framework.

Members of the genus *Briarella*, like the Splanchnotrophidae highly modified endoparasites in nudibranch gastropods, were discovered first by Bergh (1876) during dissection of host specimens from the Red Sea. Monod & Dollfus (1932) included *Briarella* in the Splanchnotrophidae. During his revision of the

Splanchnotrophidae, owing to great similarities in mouthpart morphology, Huys (2001) placed *Briarella* into the Philoblennidae, a family of ectoparasitic copepods introduced by Izawa (1976). The relationships of the family Philoblennidae are still unclear and are under discussion (Boxshall & Halsey, 2004; Kim *et al*., 2004; Walter, 2012).

The external morphology of Splanchnotrophidae reflects adaptations to their endoparasitic lifestyle (see Fig. 1), e.g. the reduction of the first pair of thoracopods (maxillipeds), of body segmentation, and of swimming legs (Huys, 2001). The cephalic limbs such as antennae and mouthparts are also highly modified and, in the case of *Arthurius*, even partly reduced (Huys, 2001; Haumayr & Schrödl, 2003; Salmen *et al*., 2008a, b; Abad *et al*., 2011). A special form of 'reduction' can be seen in the body size of males (see Fig. 2). All splanchnotrophid species possess dwarf males, which was traditionally taken as evidence for a splanchnotrophid–chondracanthid relationship (Huys, 2001).

Another putative synapomorphy of all splanchnotrophid species is the presence of long thoracic appendages, but their relevance has not been investigated conclusively (Huys, 2001; Salmen *et al*., 2010; Abad *et al*., 2011). The internal anatomy of splanchnotrophid species is largely unknown. Only Belcik (1981) has given an overview of the histology of *Ismaila belciki* Ho, 1987 (as *Ismaila monstrosa* Bergh, 1867) (Ho, 1987b). Females of Splanchnotrophidae penetrate their host at least two times: First during the initial, presumably larval, infection and second after reaching sexual maturity (Huys, 2001). The females protrude through the host's integument to bear the egg sacs outside the host's body cavity (Ho, 1987a; Huys, 2001; see also Fig. 1A). Members of other endoparasitic genera such as *Briarella* enter the host completely, and dispatch the nauplii probably via the excretory system of the host (Bergh, 1876; Monod, 1928). Many other biological traits, such as the feeding habits, are still unrecognized. Originally splanchnotrophids were thought to feed on the host tissue, but Haumayr & Schrödl (2003) assumed at least *Ismaila* species to be haemolymph suckers. Most of the species apparently do not harm the internal organs of the host. Only *Splanchnotrophus willemi* Canu, 1891, and *Ismaila damnosa* Haumayr &Schrödl, 2003, are known to attack such

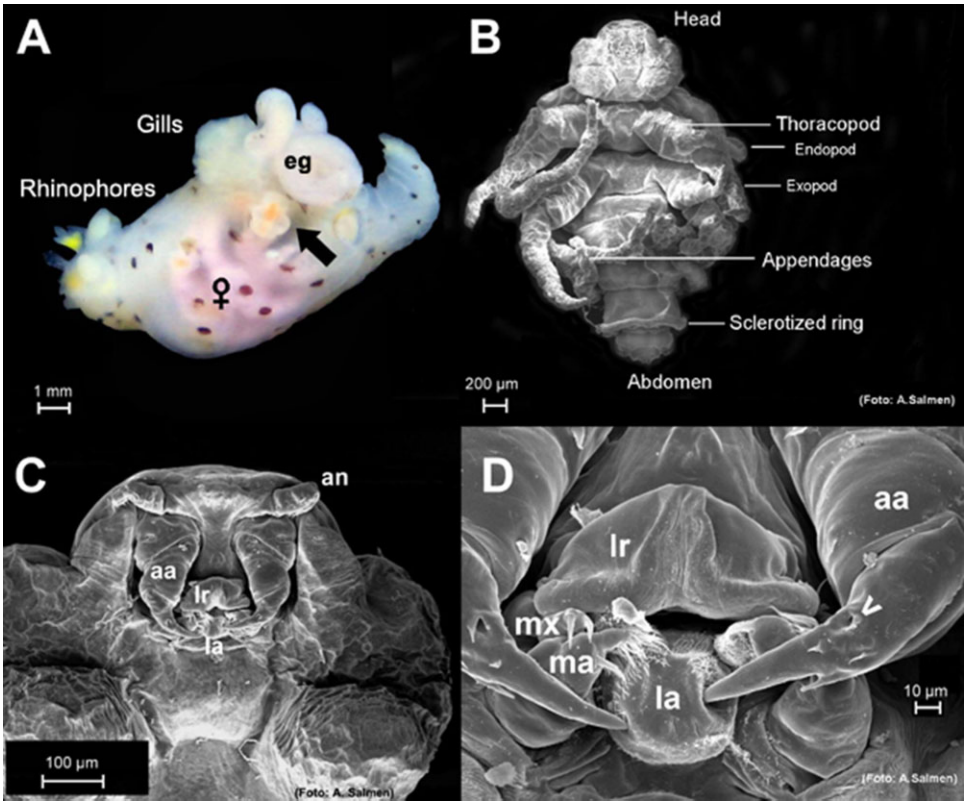

Figure 1. *Ismaila aliena*: A, dorsal view of an infected *Thecacera darwini* Pruvot-Fol, 1950 with the female parasite shining through the integument (arrow marking male parasite). B–D, scanning electron micrographs. B, habitus, ventral view. C, head with cephalic appendages, ventral view. D, mouthparts, ventral view. Abbreviations: aa, antenna; an, antennule; eg, egg sacs; ma; maxilla; mx, maxillule; la, labium; lr, labrum.

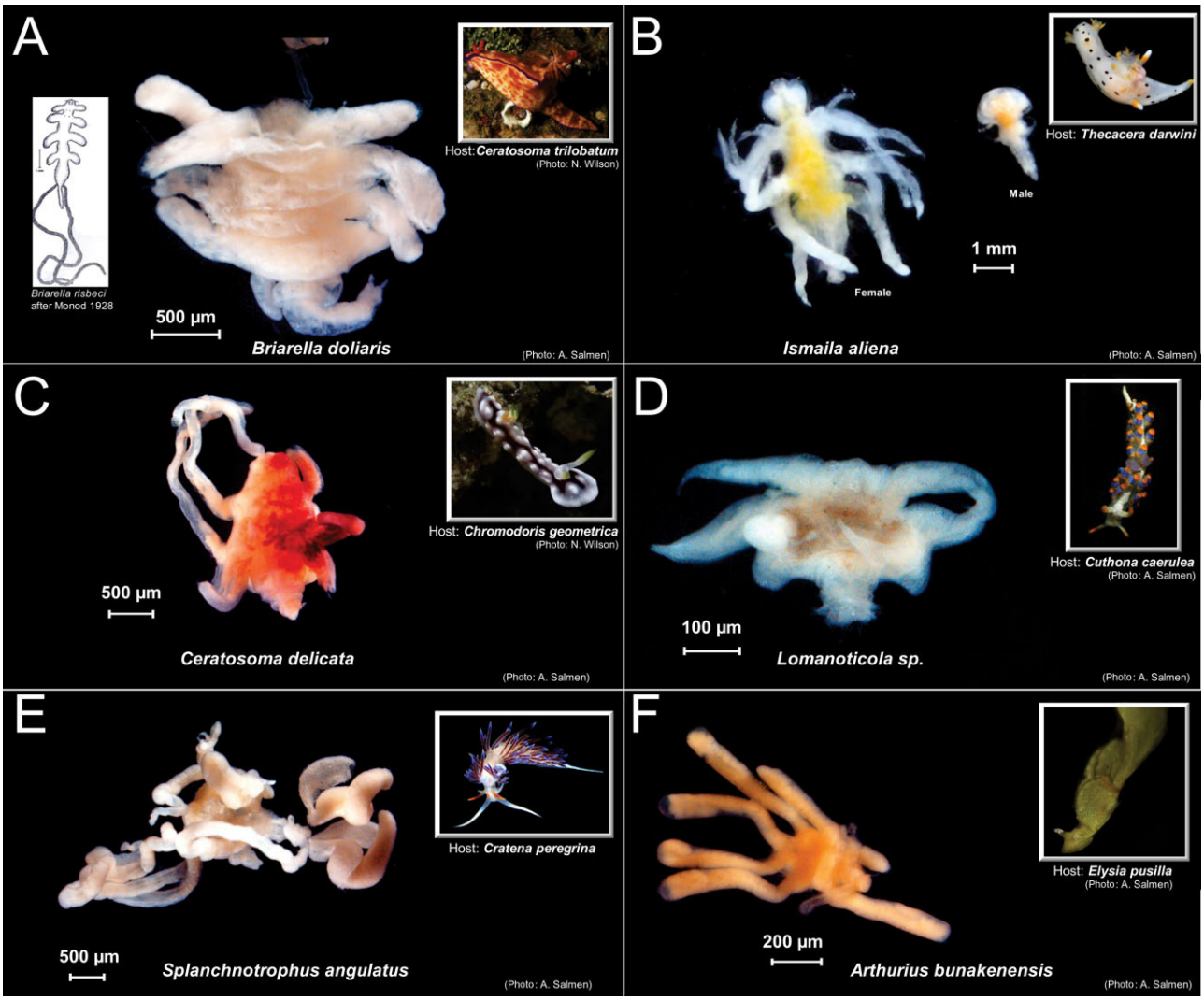

Figure 2. Endoparasitic copepods and their sea slug hosts, examples from all genera. A, *Briarella doliaris* (female) and its host *Ceratosoma trilobatum* (Gray, 1827); drawing of *Briarella risbeci* after Monod (1928). B, *Ismaila aliena* (female and male) with its host *Thecacera darwini*. C, *Ceratosomicola delicata* (female) with its host *Chromodoris geometrica* Risbec, 1928. D, *Lomanoticola* sp. with its host *Cuthona caerulea* (Montagu, 1804). E, *Splanchnotrophus angulatus* with *Cratena peregrina* (Gmelin, 1791), one of its most common hosts. F, *Arthurius bunakenensis* with its host *Elysia pusilla* Bergh, 1872.

organs. The former was observed to cause damage to the gonads and the digestive gland (Marshall & Hayward, 2006) and the latter destroys the host's gonads, but it is unclear whether these species actually feed on the gonads or just make space for themselves (Schrödl, 2002; Haumayr & Schrödl, 2003). Other important characters, such as host detection or the method of infection, are also completely unknown.

The absence of any generally agreed hypothesis on the origin of Splanchnotrophidae and internal relationships impedes the interpretation of patterns observed, such as separate geographical distributions (Schrödl, 2002), associations to different host groups or potentially different feeding strategies and other host–parasite interactions.

We present for the first time a comprehensive list of 109 morphological, phylogenetically relevant characters of both sexes of all valid splanchnotrophid species (Table 1). The main aim is to test the monophyly of the Splanchnotrophidae against similar species also infesting shell-less gastropod hosts, and especially to present a first parsimony-based hypothesis on internal splanchnotrophid phylogeny. Including all valid species, we aim to address some of the most interesting aspects of splanchnotrophid evolution, such as the

Table 1. Character-state matrix

	0		1	2		3	4	5	6	7			8		9	10
Character number	12	3456789	0123456789	0	123456789	0123456789	0123456789	0123456789	012345678	9	0	12345	6789	0123456789	0123456789	0123456789
Anthessius kimjensis	01	0005011	2010?0?00?	0	2??00?010	????00???0	?0?00??0?0	00000----0	0--00000?	0	-	????0	102?	?0?1011001	??1??00000	???000????
Philoblenna bupulda	01	0003021	00100?0010	{2,3}	100??010	???10???0?	0?????1?11	10000----0	0--00000?	0	-	?????	131?	?101010001	??00010000	??11000?0?
Philoblenna tumida	21	0013111	0?????0010	{2,3}	10010010	???101??0?	0?00??1?11	10010----0	0--001000	0	-	00?0?	130?	01?2010???	?????00000	?01010????
Philoblenna arabici	01	0003120	001?4?0010	3	100?0010?	0??100?00?	0?00??1?11	11010----0	0--000000	0	-	00?01	????	??????0???	?1????????	??????????
Philoblenna littorina	21	0012001	0010?10010	3	??0??010?	0??000?00?	0000??1---	00000----0	0--000000	0	-	00?01	0???	??????????	??????????	??????????
Briarella microcephala	01	0120??1	0????100?0	?	??0??011?	0??10?????	0?????1???	?101121110	00101-000	0	-	01??0	????	?0????????	??????0???	?????????0
Briarella sp.	02	0120??1	0????100?0	?	??0??011?	0??10?????	0?????1???	?101111110	00001-000	0	-	01?0?	?221	?0?2?10???	??1??10???	???????1??0
Briarella risbeci	02	0120??1	0????100?0	{2,3}	??01?011?	0??10?????	0?????1?11	1101111110	00001-000	0	-	01?1?	????	?0????????	???????0???	?????????0
Briarella disphaerocephala	02	0120??1	0????10000	3	??01?011?	0??101002?	0?????1???	?101111110	01001-000	0	-	01?11	?312	?0?????00?	0?1??00???	???1110??0
Briarella doliaris	11	0130131	0011210000	2	0?01?011?	0??101??2?	0200??1?11	1101121110	01101-000	0	-	01??0	1???	?0????????	???????0???	?????????0
Splanchnotrophus angulatus	11	1153??1	1011600000	1	?1012010?	0?111?30?0	0?00??1---	-101101010	03?003111	1	1	101?2	0310	21161??0??	??030?1111	?0?011?000
Splanchnotrophus gracilis	10	1153??1	1011300000	1	?0012010?	0?111?????	0000??1---	-?0?10???0	03?003111	1	1	100?2	1301	21061??001	?1030?1111	0000???000
Splanchnotrophus dellachiajei	11	01?????	1????000??	1	??01??10?	???11?????	0?????1---	-101101110	03??03111	1	1	10???	4?1?	110611????	????3?11111	0?????0?00
Splanchnotrophus willemi	11	01?????	1????000??	?	??01??10?	???11?????	???????---	-?0?10???0	011?03111	1	1	10?1?	?31?	?10?1?????	?????0????	????????00
Ceratosomicola coia	01	0132121	21-0100001	3	0?1--010?	0??110?0??	0000??1---	-101101110	02?001110	0	-	00??2	?322	21?401?001	0013?01---	10?0111220
Ceratosomicola delicata	01	1142111	11-1200000	{1,2}	0?1--010?	0??11000??	0000??1---	-101101110	02?001110	0	-	00???	?302	21?30??001	0013?01---	11?0011210
Ceratosomicola mammillata	01	0132131	1??130000?	3	011--010?	01?110????	0?00??1---	-101101110	03?001110	0	-	00?01	?312	21?40???01	0013?01---	11?0?11210
Ceratosomicola sacculata	11	1132121	1000500000	3	0?1--010?	0??11031??	0?00??1---	-101103330	020001110	0	-	00?11	?3??	?1??????01	0013?01---	1??0?112?0
Lomanoticola insolens	11	015???1	1????100?0	{1,2}	??01?010?	0??11??1?0	0?00??1---	-101111110	01100311{0,1}	1	0	00?1?	4???	??????????	???????---	?????????0
Lomanoticola brevipes	10	115???1	1????100?0	3	??01?010?	0??11??1??	0?00??1---	-101101110	00100311{0,1}	1	0	00?1?	5???	??????????	???????---	?????????0
Lomanoticola sp.	10	1131121	1011310000	3	?001?010?	01?111?0?1	0000??1---	-101101130	01100311{0,1}	1	0	00?12	6???	??????????	???????---	?????????0
Arthurius bunakenensis	10	1164??2	1????111--	-	-1--1---	1--11141??1	---?---	-?0?131310	01?002110	0	-	00??2	?300	010702?11-	01?1101---	00?0111?1
Arthurius elysiae	11	0160??2	100??111--	-	-1--010?	1--111?1??1	----1---	--01103330	1?1002110	0	-	00??{1,2}	?300	?10502?11-	1-?1101---	?0?111?131
Ismaila monstrosa	01	0153??1	2??????00??	?	??0010000	00?11120?00	000001---	-011100000	0??101110	0	-	?0???	???2	?11?0?????	??12?0?---	0?0000???0
Ismaila obtusa	12	015???1	2??????00??	?	??0?1000?	???11110?00	00?111---	-011100000	001101110	0	-	?0???	???2	?11?0?????	??12?0?---	0?0000???0
Ismaila jenseniana	01	016???0	2??????????	?	?????????	???1112??00	20?001---	-011100001	00?101110	0	-	00???	?312	?11700????	??12?01---	0?0000???1
Ismaila occulta	11	01510?1	2010200011	-	--0010100	0001112??10	0002??---	-010100000	00?101110	0	-	00??0	2212	0116011001	2?12001---	010100???0
Ismaila belciki	31	01510?1	2010400010	0	1?0010000	0001112??00	000011---	-011100000	0?0101110	0	-	?0??0	2212	?11601?001	0012?11---	0?0000???0
Ismaila androphila	31	01510?1	2010400010	0	1?0010001	00011120?00	100??1---	-?11100000	00?101110	0	-	00???	?312	?11601?000	??12?01---	0?0000??30
Ismaila aliena	01	01510?1	2010400010	0	1?0010001	00011120?00	000??1---	-011100000	00?101110	0	-	100??	?222	?11601?000	??12?01---	000000??30
Ismaila damnosa	1{0,1}	11510?1	2??????0010	0	1?0010000	0??11?00?00	100??1---	-011100000	001101110	0	-	100??	?212	?1160??000	???2001---	000000??30
Ismaila robusta	11	11510?1	2010400010	0	1?0010000	00011100?00	100???---	-?1?100000	001101110	0	-	101??	?112	?1160??000	???2001---	000000??30
Ismaila socialis	31	01510?1	2010400?10	0	1??0?000?	0001112??00	0001?1---	-?11100000	00?101110	0	-	00?0?	?312	?11601?000	??12?01---	0?0000??30
Ismaila magellanica	01	01510?1	2010400010	0	1?0010000	0001112??00	?0???1---	-?11100000	00?101110	0	-	00?0?	?302	?11?01?000	??12?01---	0?0000??31

Missing data is marked by ?; – refers to inapplicable data.

formation of geographical distribution patterns and switches from an ectoparasitic to an endoparasitic lifestyle and the adaptation to different host groups such as nudibranchs or sacoglossan heterobranchs.

MATERIAL AND METHODS

Phylogenetic analysis

Taxon selection

Preliminary analyses with a broad variety of outgroups showed several outgroup taxa [i.e. *Shiinoa inauris* Cressey, 1975, *Micrallecto fusii* (Stock, 1973) (as *Nannallecto fusii*), and *Acanthochondria phycidis* (Ruthbun, 1886) (as *Chondracanthus*)] nestling within the Splanchnotrophidae (trees not shown). *Acanthochondria phycidis* and *Sh. inauris* resulted as sister taxa to the Splanchnotrophidae and as derived members of the *Briarella* clade. Although these species have been discussed to have some affinity with the Splanchnotrophidae (Ho, 1991; Huys, 2001; Huys *et al*., 2006), none of them was thought to be a distinct member of the Splanchnotrophidae or the Philoblennidae (Ho, 1991; Huys, 2001; Boxshall & Halsey, 2004; Huys *et al*., 2006). *Micrallecto fusii* appeared as a basal offshoot of the *Arthurius* clade within the Splanchnotrophidae. As Huys (2001) excluded *Micrallecto* from the Splanchnotrophidae, molecular studies will have to resolve this.

We think that these topologies are artefacts of our large but still splanchnotrophid-focused character sampling, combined with high levels of convergence amongst parasitic copepod lineages, masking the true phylogenetic relationship. We thus pruned the outgroup sampling to those taxa that have been discussed to be related to splanchnotrophids in the more recent taxonomic literature. Alternatively, the inclusion of many more taxa and relevant characters would lead to a phylogeny of the Poecilostomatoida as a whole, and therefore go far beyond the scope of the present analysis. When included, the poorly described *Chondrocarpus reticulosus* Bassett-Smith, 1903, which was assumed to belong to Splanchnotrophidae, Chondracanthidae, or *Briarella*, emerged within the genus *Briarella* as assumed by Huys (2001). Unfortunately, because of many unknown or ambiguous character states, *Ch. reticulosus* adversely affects the resolution of the resulting strict consensus tree (not shown), and therefore *Ch. reticulosus* was also excluded.

For the main analysis *Anthessius kimjensis* Suh, 1993, was chosen as a putatively distant and plesiomorphic outgroup; this species is a well-described member of the ectoparasitic Anthessiidae, which is considered to be one of the most basal families within the Poecilostomatoida (see Ho, 1991; Huys, 2001; Huys *et al*., 2006). Nine additional outgroup taxa were chosen from the Philoblennidae, a taxon that was recently discussed as a close relative (Boxshall & Halsey, 2004; Huys *et al*., 2006), if not a sister group to, or even paraphyletic stem group of Splanchnotrophidae (Salmen *et al*., 2010). Owing to the similarity of the latest discovered species, *B. doliaris*, to splanchnotrophids (Salmen *et al*., 2010), all representatives of the endoparasitic genus *Briarella* were included in this analysis, together with the four members of the ectoparasitic genus *Philoblenna* Izawa, 1976.

The ingroup comprises all 24 splanchnotrophid species presently considered to be valid, regardless of their very heterogeneous state of knowledge. The species that is herein called '*Lomanoticola* sp.' was studied by Salmen (2005) using scanning electron microscopy and identified as *Lomanoticola brevipes* (Hancock & Norman, 1863) (as *Splanchnotrophus brevipes*). However, there are several differences to both *Lomanoticola insolens* Scott & Scott, 1895, and *L. brevipes*, e.g. the second thoracopod is well developed in *Lomanoticola* sp. but rudimentary in *L. brevipes* and in *L. insolens*; the fourth thoracopod is present in *Lomanoticola* sp. but absent in both *L. brevipes* and *L. insolens*; and the segmentation of the abdomen is detectable in *L. brevipes* and in *L. insolens* but not in *Lomanoticola* sp. The latter is considered as a separate species here.

Compilation of characters

Characters were selected according to the following criterion: outgroup-specific morphological characters were included only to an extent that guaranteed a reasonable framework for rooting of the Splanchnotrophidae. In contrast, for the ingroup all morphological characters discernible, available, and relevant to splanchnotrophids were collected from the literature and defined (see lists below). This was to minimize selectivity and subjectivity. Primary homology was assumed according to positional and structural similarity criteria. a priori uninformative characters, i.e. putative autapomorphies of single terminal taxa, and characters showing too much ambiguity or lack of information within the ingroup were not considered for cladistic analyses. To guarantee transparency of the selection process, all excluded characters are listed and briefly discussed in the Appendix.

Morphological information on outgroups was obtained from the original species descriptions and from recent reviews (Bergh, 1876; Bassett-Smith, 1903; Monod, 1928; Monod & Dollfus, 1932; Humes, 1954; Ho, 1971; Izawa, 1976; Cressey & Boyle Cressey, 1980; Ho, 1981b; Brunckhorst, 1985; Ho & Kim, 1992; Suh, 1993; Huys, 2001; Salmen, 2005; Tavares & Luque, 2005; Salmen *et al*., 2010).

For splanchnotrophid species, all available original and secondary literature was considered (Hancock & Norman, 1863; Bergh, 1867, 1868; Canu, 1891; Hecht,

1893; O'Donoghue, 1924; Delamare Deboutteville, 1950, 1951; Laubier, 1964; Ho, 1981a, b; Jensen, 1987; Huys, 2001; Salmen, 2005; Salmen *et al*., 2008a, b; Abad *et al*., 2011), including a diploma thesis with partly unpublished data on *Lomanoticola* sp., *S. gracilis* and *Splanchnotrophus angulatus* Hecht, 1893.

In his cladistic analysis Ho (1991) included female characters only, because of the great amount of missing data on male characters. However, splanchnotrophid males are less modified than the females (Bergh, 1876; Ho, 1981a; Huys, 2001); therefore, information from males may bear some phylogenetic signal that is not masked by convergences. Thus, male characters are considered in this analysis.

In the 19th and early 20th centuries most splanchnotrophid species described were found accidentally during dissection of the host (Bergh, 1867, 1876; Canu, 1891; Hecht, 1893; Bassett-Smith, 1903; Delamare Deboutteville, 1950). Therefore, these early descriptions are not very detailed and often limited to gross body shape, number of appendages, and their superficial shapes. Recently, some efforts have been made to complete the morphological descriptions by revising older species, including available type material (Huys, 2001; Haumayr & Schrödl, 2003). Haumayr & Schrödl (2003) started using scanning electron microscopy for detailed examination of the genus *Ismaila*, a technique that was also used for other splanchnotrophids by Salmen *et al*. (2008a, b) and Abad *et al*. (2011). Unfortunately the original material of some species was damaged or not traceable, and several splanchnotrophid species had not been recollected since their original description, although the authors mentioned their high abundance (Canu, 1891; Delamare Deboutteville, 1950). For the present analysis all splanchnotrophid species are covered, even those with only fragmentary morphological data available (see also Table 1).

The presence or condition of structures is coded only when mentioned in the literature description or shown in illustrations. In case of discrepancies, the most recent, detailed, and reliable data source was preferred, or coding was set to unknown.

The following 109 characters were used for parsimony analysis:

External morphology (female) (see also Figs 1A, B, 2)

1. *Body shape:* The body, comprising cephalothorax, thorax, and abdomen, can be elongate (see Monod, 1928: fig. 6) (0), compact or stocky (see Huys, 2001: fig. 1) (1), inflated (see Ho, 1981a: fig. 7a) (2), or delicate (see Haumayr & Schrödl, 2003: fig. 13d) (3).
2. *Body length:* The body length can be small (< 1 mm) (0), medium sized (1–9 mm) (1), or large (> 9 mm) (2). The body length is measured from the cephalothorax to the abdomen, without considering the antennae, processes, and caudal rami.
3. *Demarcation of cephalothorax in females:* A distinct border between the cephalothorax and the rest of the body may be present (seeSalmen *et al*., 2008b: fig. 3a) (0) or absent (see Huys, 2001: fig. 11a) (1).
4. *External body segmentation:* Although in all ectoparasitic outgroup taxa the external body segmentation is still detectable (0), it is no longer detectable in some ingroup species (1).
5. *Antennule (segmentation)* (see Fig. 1C): The antennule can be seven-segmented (0), six-segmented (1), five-segmented (2), four-segmented (3), three-segmented (4), two-segmented (5), or one-segmented, in which case no segment boundaries are discernible (6).
6. *Number of setae on first antennulary segment:* First antennulary segment with nine (0), five (1), four (2), three (3), two (4), or with just one seta (5).
7. *Large quantity of setae:* The quantity of setae on the second antennulary segment is considered large if there are ten or more setae (0) or low if there are fewer than ten setae (1).
8. *Number of setae on third segment of antennule:* Third segment of antennule with four (0), three (1), or two setae (2).
9. *Antenna (segmentation)* (see Fig. 1C, D): The antenna is four-segmented (0), three-segmented (1), or two-segmented (2).
10. *Shape of tip of distal segment:* The distal segment of the antenna bears two claws (see Ho & Kim, 1992: fig. 5f) (0), one strong recurved claw (see Huys, 2001: fig. 8c) (1), or a slightly curved hook (see Haumayr & Schrödl, 2003: fig. 11b) (2).
11. *First antennal segment setae present:* On the first antennal segment setae are present (0) or absent (1).
12. *Number of setae on first antennal segment:* The first antennal segment bears two setae (0) or just one (1).
13. *Second antennal segment setae present:* Second antennal segment with setae (0) or not (1).
14. *Number of setae on third antennal segment:* There are eight (0), six (1), five (2), four (3), three (4), two (5), or just one seta (6) present on the third antennal segment.
15. *Small pore on the third segment:* A small pore is present on the third antennal segment (see Haumayr & Schrödl, 2003: fig. 14b or Salmen *et al*., 2008b: fig. 3f) (0) or absent (1).
16. *Labrum* (Fig. 1C): The labrum is present (0) or absent (1).

17. *Mandible:* The mandible is present (0) or absent (1).
18. *Covered by labrum:* The mandible is covered by the labrum (0) or not (1).
19. *Blade:* The mandible has a blade (0) or not (1).
20. *Shape of blade:* The mandibular blade is small (see Haumayr & Schrödl, 2003: fig. 17c) (0), recurved (see Laubier, 1964: fig. 1f) (1), saw-like (see Suh, 1993: fig. 6) (2), or simple curved (see Izawa, 1976: fig. 10) (3).
21. *Processes:* Processes on the mandible (see Salmen *et al*., 2008b: fig. 4a) are present (0) or absent (1).
22. *Arrangement of processes:* The dentiform processes on the mandible are arranged in one or two rows (0), situated on a rounded apex (1), or there are several dentiform processes at the apex (2).
23. *Maxillule* (Fig. 1D): The maxillule is present (0) or absent (1).
24. *Number of lobes:* The maxillule bears several lobes (0), or just one (1).
25. *Number of setae:* The maxillule bears four (0), two (1), or just one seta (2).
26. *Maxilla* (Fig. 1D): The maxilla is either present (0) or absent (1).
27. *Segmentation of the maxilla:* The maxilla is three-segmented (0) or two-segmented (1).
28. *Apical elements on first segment:* The first segment of the maxilla may possess no (0) or two apical elements (1).
29. *Processes:* The maxilla bears two processes (0) or a single terminal one (1).
30. *Labium* (Fig. 1D): The labium is present (0) or absent (1).
31. *Hairs:* The labium is hairy all over the surface (0) or it has only hairy patches (1).
32. *Distal hairs:* The distal hairs of the labium are concentrated at the lateral portions (see Ho, 1981a: fig. 1e) (0) or there are several hairs all over the labium (see Huys, 2001: fig. 2b) (1).
33. *General shape of thoracopods:* The thoracopods are of the usual swimming leg shape (see Suh, 1993: figs 11–14) (0) or greatly reduced (see Haumayr & Schrödl, 2003 fig. 16B) (1).
34. *First thoracopod (maxilliped):* The first thoracopod (maxilliped) is present (see Ho & Kim, 1992: fig. 6h) (0) or absent (1).
35. *Second thoracopod outer seta present* (see also Fig. 1B): At the base of the second thoracopod one seta may be present (0) or not (1).
36. *Shape of the exopodite* (see also Fig. 1B): The exopodite of the second thoracopod is voluminous (see Monod & Dollfus, 1932: fig. 24c) (0), thick and distally flattened (1), conical (see Haumayr & Schrödl, 2003: fig. 15a) (2), lobate (see Huys, 2001: fig. 12d) (3), or spinous (see Salmen *et al*., 2008a: fig. 2a) (4).
37. *State of development:* The exopodite of the second thoracopod is well developed (see Monod & Dollfus, 1932: fig. 24c) (0) or rudimentary (see Salmen *et al*., 2008a: fig. 2a) (1).
38. *Segmentation:* The exopodite of the second thoracopod is three-segmented (0) or indistinctly two-segmented (1).
39. *Tip shape:* The tip of the exopodite of the second thoracopod bears a claw (see Haumayr & Schrödl, 2003: fig. 21a) (0) or a minute recurved element (see Haumayr & Schrödl, 2003: fig. 9a) (1).
40. *Third thoracopod:* The third thoracopod is present (0) or absent (1).
41. *Comparative length of exopodite and endopodite:* The exopodite of the third thoracopod is longer than the endopodite (0), both are of equal length (1), or the exopodite is shorter than the endopodite (2).
42. *Exopodite of third thoracopod:* The exopodite of the third thoracopod is present (0) or absent (1).
43. *Endopodite of third thoracopod:* The endopodite of the third thoracopod is present (0) or absent (1).
44. *Length of internal process:* The endopodite of the third thoracopod and its internal process have the same length (0), the internal process is shorter than the endopodite (1), or the internal process is very small and rudimentary (2).
45. *Thickness of internal process:* The endopodite of the third thoracopod and its internal process are equally thick (0) or the internal process is thinner than the endopodite (1).
46. *Fourth thoracopod:* The fourth thoracopod is present (0) or absent (1).
47. *Shape of the fourth thoracopod:* The fourth thoracopod is of normal size and is clearly separated into exo- and endopodite (0) or is very small (1).
48. *Protopodite of fourth thoracopod:* The protopodite of the fourth thoracopod is present (0) or absent (1).
49. *Exopodite:* The exopodite of the fourth thoracopod is present (0) or absent (1).
50. *Endopodite of fourth thoracopod:* The endopodite of the fourth thoracopod is present (0) or absent (1).
51. *Fifth thoracopod:* The fifth thoracopod is present (0) or absent (1).
52. *Sclerotized ring:* A sclerotized ring between the fourth and the fifth thoracic segment may be absent (0) or present (1) (see Haumayr & Schrödl, 2003: fig. 16b).

53. *Sixth thoracopod:* The sixth thoracopod is present (0) or absent (1).
54. Processes (Fig. 1B): Processes on the thorax are absent (0) or present (1).
55. *Number of thoracic processes:* Thorax with three (0), four (1), five (2), or six pairs (3) of processes.
56. *Site (or location) of the first pair:* The thoracic processes are situated dorsally (0), laterally (1), or ventrolaterally (2).
57. *Site of the second pair:* The thoracic processes are situated dorsally (0), laterally (1), or ventrolaterally (2).
58. *Site of the third pair:* The thoracic processes are situated laterally (0), dorsolaterally (1), or ventrolaterally (2).
59. *Dorsolateral process:* Nearly all members of the Splanchnotrophidae lack this process (0), but in *Ismaila jenseniana* Haumayr & Schrödl, 2003, it is present (1).
60. *Ventral processes:* Ventral processes are absent (0) or present (1).
61. *Length of thoracic processes:* The dorsal processes are relatively short (0), approximately as long as the body (1), longer than the body (2), or twice as long as the body (3).
62. *Thickness of thoracic processes:* The processes are slender compared to the body (0) (see Fig. 2C) or voluminous (1) (see Fig. 2A, D).
63. *Mediodorsal process:* Usually, a mediodorsal process between the third pair of dorsal processes is absent (0), but in all members of the genus *Ismaila* a single mediodorsal process is present (1) (see Haumayr & Schrödl, 2003: fig. 16b).
64. *Abdomen (segmentation)* (see also Fig. 1B): The abdomen has distinct segmentation (0) or not (1).
65. *Number of abdominal segments:* If segmented, the abdomen consists of four segments (0), three segments (1), two segments (2), or one segment (3).
66. *Length of abdomen:* The abdomen is long and slender (0) (Fig. 2A) or short and small (1) (Fig. 2B).
67. *Abdomen protruding through host integument* (see Figs 1A, 3): Whereas the abdomen is free in all mature females of outgroup taxa (0), in all splanchnotrophid species it protrudes through the host's integument so that the egg sacs are located outside the host's body cavity (1).
68. *Egg sacs:* The egg sacs are unilobate (0) or bilobate (1) (see Fig. 3).
69. *Terminal attachment of egg sacs:* The egg sacs are attached to the abdomen of the female at their terminal ends (see Haumayr & Schrödl, 2003: fig. 16a) (0) or not (1).

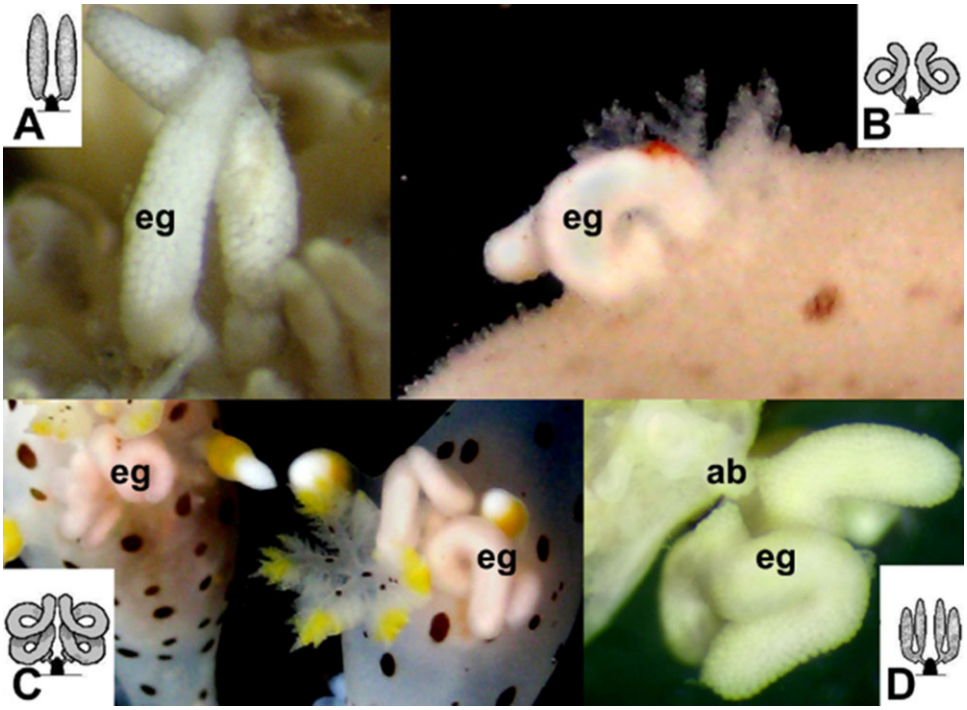

Figure 3. Egg sac morphology. A, straight. B, curled, one whorl. C, curled, two whorls. D, bilobate. Abbreviations: ab, abdomen of female parasite; eg, parasite egg sacs.

70. *Subterminal attachment of egg sacs:* If not terminal, the egg sacs may be attached at about one third of their length (see Hancock & Norman, 1863: plate 16, fig. 2) (0) or at about their middle (1), causing a bilobate appearance (see Delamare Deboutteville, 1950: fig. 1).
71. *Straight or coiled:* The egg sacs are straight (0) or coiled (1) (see also Fig. 3).
72. *Thickness of egg sacs:* The shape of the egg sacs is thick, banana-shaped (see Huys, 2001: fig. 7a) (0) or very long and slender (see Fig. 2A) (1).
73. *Whorls of egg sacs:* Coiled egg sacs form one (0) or two whorls (1) (see also Fig. 3B, C).
74. *Length:* The egg sacs are short (0) or longer than the body (1). In splanchnotrophids with information available (e.g. *Ismaila aliena* and *Spl. angulatus*; M. Schrödl, pers. observ.), egg sac maximum lengths (and specific arrangement of eggs) are consistent within populations rather than depending upon the number or size of the eggs inside.
75. *Shape of caudal rami:* The caudal rami are long and stylet-like (see Ho, 1981a: fig. 1d) (0), oval and globular knob-like (see Salmen *et al*., 2008b: fig. 12f) (1), or small and minute (see Salmen *et al*., 2008b: fig. 4g) (2).
76. *Number of setae on the caudal rami:* The caudal rami bear seven (0), six (1), five (2), three (3), two (4), or just one seta (5).

External morphology (male)

77. *Dwarf males:* In *An. kimjensis* only the male is larger than the female (0). In other taxa the body size of the males is equal to that of the females (1), there is an overlap between the size ranges of the two sexes (2), or the males are generally smaller than the females (3).
78. *Body size of male:* The body size is large (> 2 mm) (0), small (1–2 mm) (1), or very small (< 1 mm) (2). As in the female, the body was measured from the front of the cephalothorax to the end of the abdomen, without the antennae and caudal rami.
79. *Body shape of male:* The male body is cyclopiform (see Huys, 2001: fig. 3a) (0), elongate (1), or pear-shaped (see Haumayr & Schrödl, 2003: fig. 22d) (2).
80. *Cephalothorax:* The cephalothorax of the male consists of five head segments and the first thoracic segment (0), of the head and the first two thoracic segments (1), or of the head segments and the first three thoracic segments (2).
81. *Swollen segments:* The head of the male is not swollen (0) or displays swollen partitions (see Ho, 1981a: fig. 2f and Salmen *et al*., 2008b: fig. 9c) (1).
82. *Cephalothorax (demarcation):* The cephalothorax is distinctly set off from the thorax (0) or not (1).
83. *Antennule (segmentation):* The antennule is seven-segmented (0), six-segmented (1), five-segmented (2), four-segmented (3), three-segmented (4), two-segmented (5), or one-segmented (6).
84. *First segment of antennule:* First antennulary segment bears setae (0) or does not (1).
85. *Antenna (segmentation):* The antenna is four-segmented (0), three-segmented (1), or two-segmented (2).
86. *Shape of third segment of the antenna:* The third segment of the antenna is claw-shaped (0) or hook-shaped (1).
87. *Mandible:* The mandible is present (0) or absent (1).
88. *Maxillule:* The maxillule is present (0) or absent (1).
89. *Maxilla (segmentation):* The maxilla is three-segmented (0) or two-segmented (1).
90. *Labrum:* The labrum covers the mouth medially (0), is a small chitinized plate (1), or an arched plate with a smooth surface (2).
91. *State of development:* The labrum is well developed (see Salmen *et al*., 2008b: fig. 7d) (0) or very small (see Salmen *et al*., 2008a: fig. 3d) (1).
92. *Thorax (segmentation visible):* The segmentation of the thorax is well defined (0) or is not (1).
93. *Thorax (segmentation):* The thorax is seven-segmented (0), five-segmented (1), four-segmented (2), or three-segmented (3).
94. *Fused elements:* The thoracic segments are usually free (0). In *Arthurius* only the first and second pedigerous somites are fused laterally (1).
95. *Processes:* Processes on the thorax are absent (0) or present (1).
96. *First thoracopod (maxilliped):* The first thoracopod is present (0) or absent (1).
97. *First segment of the first thoracopod:* The first segment of the first thoracopod is present (0) or absent (1).
98. *Second segment of the first thoracopod:* The second segment of the first thoracopod is present (0) or absent (1).
99. *Third segment of the first thoracopod:* The third segment of the first thoracopod is present (0) or absent (1).
100. *Second thoracopod (length):* The second thoracopod is enlarged (0) or minute (1).
101. *Third thoracopod (number of rami):* The third thoracopod is biramous (0) or uniramous (1).
102. *Terminal claw on third thoracopod:* A terminal claw is present on the third thoracopod (0) or is not (1).

103. *Fourth thoracopod:* The fourth thoracopod is present (0) or absent (1).
104. *Fifth thoracopod:* The fifth thoracopod is present (0) or absent (1).
105. *Sixth thoracopod:* The sixth thoracopod is present (0) or absent (1).
106. *Abdomen (size):* The abdomen is elongated (0) or short (1).
107. *Caudal rami (shape):* The caudal rami are long, strong and cylindrical (see Huys, 2001: fig. 5e) (0), globular (see Huys, 2001: fig. 10a) (1), or small (see Salmen *et al*., 2008b: fig. 10e) (2).
108. *Number of setae:* The caudal rami bear six (0), four (1), three (2), or two setae (2).

Ecology

109. *Host:* Most splanchnotrophid species and all members of the genus *Briarella* infest nudibranchs (0) and only four species within the Splanchnotrophidae infest sacoglossan hosts (1).

Although this character refers to ecology, it was included in the analysis because of the a priori assumption that the capability of infesting such distant host taxa can be attributed to certain inherited, otherwise uncoded, morphological and/or physiological adaptations in the ontogenetic cycle, with considerable primary homology probability. None of the known splanchnotrophid species infest both sacoglossan and nudibranch hosts.

Parsimony analysis

Parsimony analyses were performed using the program PAUP 4.0b10 (Swofford, 2002). Pre-analyses used a broad set of poecilostomatoid outgroups. Thirty-four taxa (ten outgroup and 24 ingroup taxa) and 109 characters were included in the main analysis (Table 1). Additional analyses were run including *Chondrocarpus*, using female or male character sets, and constraining monophyly of *Lomanoticola*. All characters were unordered and all were given equal weight. Accelerated transformation was used for character state optimization. Trees were unrooted. The number of bootstrap replicates was set to 1000; the maximum number of trees held at each stage was set to 100 000. The Bremer decay indices were calculated using TreeRot v. 3 (Sorenson & Franzosa, 2007) and PAUP 4.0b10. The illustration of the resulting strict consensus tree was carried out using FigTree v. 1.3.1 (Rambaut, 2009). Search for homoplasies and apomorphies was performed using MESQUITE 2.0 (Maddison & Maddison, 2007). Historical biogeography was reconstructed by parsimony, treating different regions (Yellow Sea, tropical Indo-Pacific, temperate north-eastern Atlantic plus Mediterranean Sea, tropical America, and temperate north-eastern and south-eastern Pacific) as an unordered multistate character, i.e. allowing free dispersal between regions. In addition, a Bayesian binary Markov Chain Monte Carlo (MCMC) analysis implemented in the computer program RASP (Yu, Harris & He, 2011) was conducted.

RESULTS

The heuristic search produced one single most parsimonious tree with a length of 255 steps (see Fig. 4). The consistency index (CI) is 0.6118. The homoplasy index (HI) is 0.3882. The CI excluding uninformative characters is 0.6115, and the HI excluding uninformative characters is 0.3885. The retention index is 0.7871; the rescaled consistency index is 0.4815. Twelve characters are parsimony uninformative (numbers 30, 49, 50, 53, 54, 55, 56, 57, 67, 68, 97, 100). Of the 97 parsimony-informative characters, 13 (11.9 %) show homoplasies in the strict consensus tree, i.e. character states evolved more than once or show at least one reversal within the ingroup. In the fully resolved strict consensus tree, at least some nodes show adequate bootstrap support (BT > 70). There is Bremer support (BS) for several nodes, even though the values are generally low.

At the base of the strict consensus tree (Fig. 4) *An. kimjensis* and *Philoblenna littorina* Avdeev, Tzimbaljuk Lukomskaya, 1984, form a basal polytomy. After successive branching off of *Philoblenna bupulda* Ho & Kim, 1992, *Philoblenna arabici* Izawa, 1976 (BT 56, BS 2) and *Philoblenna tumida* Ho, 1981 (BT 51, BS 2) from the stem line, the Splanchnotrophidae cluster as sister group (BT 71, BS 4) to the genus *Briarella*. We infer that the family Philoblennidae and genus *Philoblenna* is paraphyletic (Fig. 4). The well-supported (BT 92, BS 3) genus *Briarella* comprises *B. doliaris* as a basal offshoot and a clade with the remaining four species (BT 54, BS 1). After the offshoot of *Briarella microcephala* Bergh, 1867. an internal clade with some support (BT 79, BS 2) is left, with *Briarella* sp. Bergh, 1867 as sister to *Briarella risbeci* Monod, 1928, and *Briarella disphaerocephala* Monod & Dollfus, 1932 (BS 1).

The Splanchnotrophidae is recovered as a monophyletic group (BT 56, BS 3). The family shows one nonhomoplastic synapomorphy in the main analysis, i.e. the absence of the first thoracopod (in adults) (see also Fig. 5). The basal splanchnotrophid dichotomy bears one clade (BS 1) of all Indo-Pacific species comprising the monophyletic genera *Arthurius* (BT 100, BS 10) and *Ceratosomicola* (BT 58, BS 1), which is supported by the synapomorphic loss of maxillules. *Arthurius* consists of the two sister species *Arthurius elysiae* and *Arthurius bunakenensis* Salmen *et al*., 2008a. The major nonhomoplastic synapomorphy

here is the loss of the mandibles in both sexes. Further, there are six nonhomoplastic synapomorphies, i.e. the two-segmented antenna, the absence of the labium in females, the five-segmented thorax, the presence of fused thoracic segments, the absence of fifth thoracopods in males and the absence of sixth thoracopods in males (Fig. 5). In the genus *Ceratosomicola*, *Ce. sacculata* emerges as most basal offshoot (BS 1), with *Ceratosomicola coia* Salmen *et al*., 2008b (BS 1), sister to *Ceratosomicola delicata* Salmen *et al*., 2008b, and *Ceratosomicola mammillata* Salmen *et al*., 2008b (BS 1).

The other branch of the basal splanchnotrophid dichotomy (Fig. 4) is formed by the strongly supported, amphi-American (i.e. Pacific and Atlantic) genus *Ismaila* (BT 99, BS 7) sister to a clade (BT 59, BS 1) with all European splanchnotrophid species, i.e. members of the paraphyletic *Lomanoticola* and monophyletic *Splanchnotrophus* (BT 65, BS 2).

The genus *Ismaila* shows three nonhomoplastic synapomorphies (the presence of a mediodorsal process, the reduction of the number of setae on the maxillule in females, and the presence of a sclerotized ring between the fifth and sixth thoracopods in both sexes) (see also Fig. 5). North-eastern Pacific *Ismaila occulta* Ho, 1981 (BS 7) and *Ismaila belciki* (BS 1) are basal offshoots. Then, two clades of south-eastern Pacific species split off the stem line; the first comprising *I. aliena* Haumayr & Schrödl, 2003 as the sister to *I. damnosa* and *Ismaila robusta* Haumayr & Schrödl, 2003 (BT 92, BS 2); the second with *Ismaila obtusa* Haumayr & Schrödl, 2003 as sister to a clade of *Ismaila socialis* Haumayr & Schrödl, 2003 and *Ismaila androphila* Haumayr & Schrödl, 2003. Finally, the Caribbean *I. monstrosa* Bergh, 1867, emerges as sister taxon to the equally Caribbean *I. jenseniana* (Jensen, 1987) and *Ismaila magellanica* Haumayr & Schrödl, 2003, known from the Atlantic side of the Magellan Strait.

The European clade of *Splanchnotrophus s.l.* (Fig. 4) receives poor node support, but is indicated by synapomorphic subterminal egg sac attachment (Fig. 5). Whereas *L. brevipes* and *Lomanoticola* sp. cluster together, *L. insolens* emerges as sister taxon (BS 1) to the *Splanchnotrophus s.s.* clade. The genus *Splanchnotrophus* is monophyletic (BT 65, BS 2),

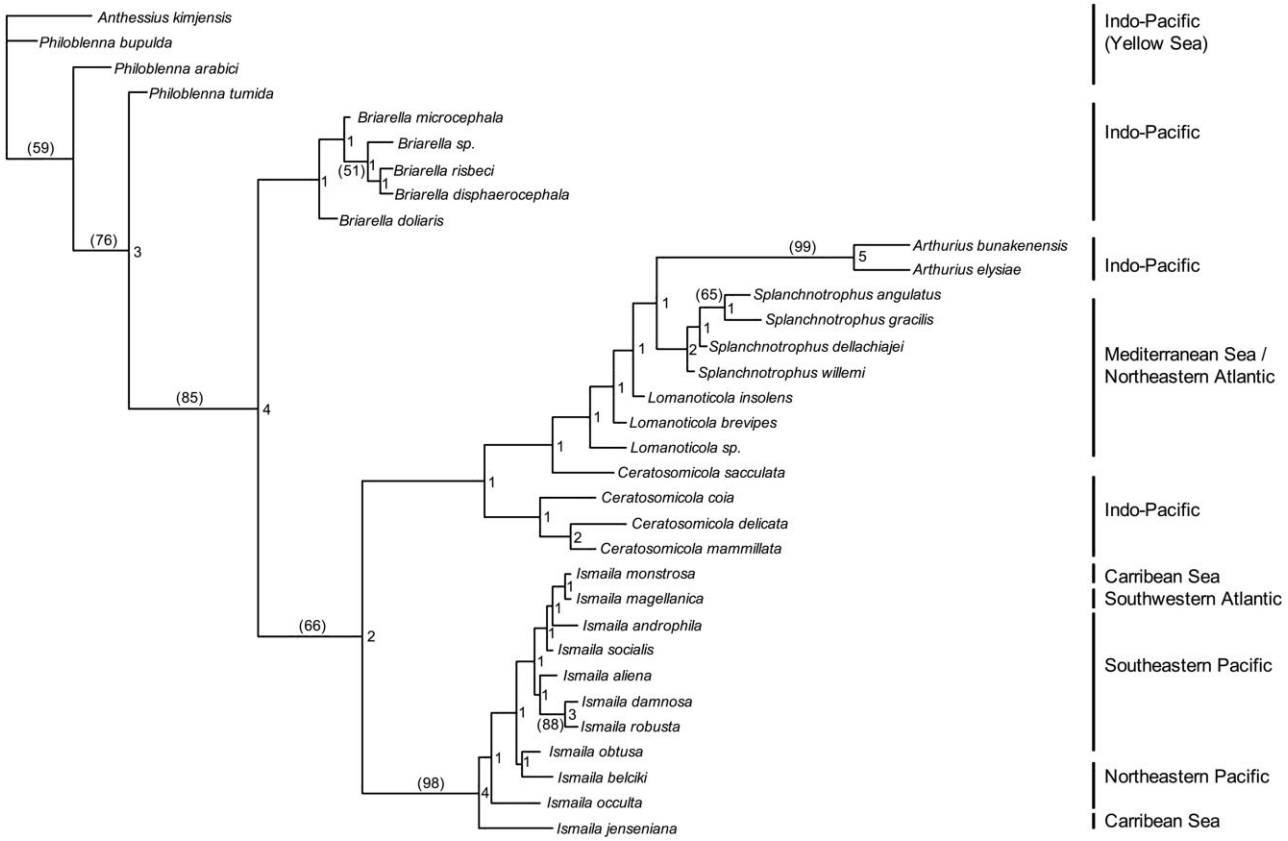

Figure 4. Phylogeny of Splanchnotrophidae. Strict consensus tree of the main parsimony analysis with bootstrap support (> 50, in parentheses) and Bremer decay values. Geographical distributions are indicated according to major regions. Branch length reflects number of character-state changes.

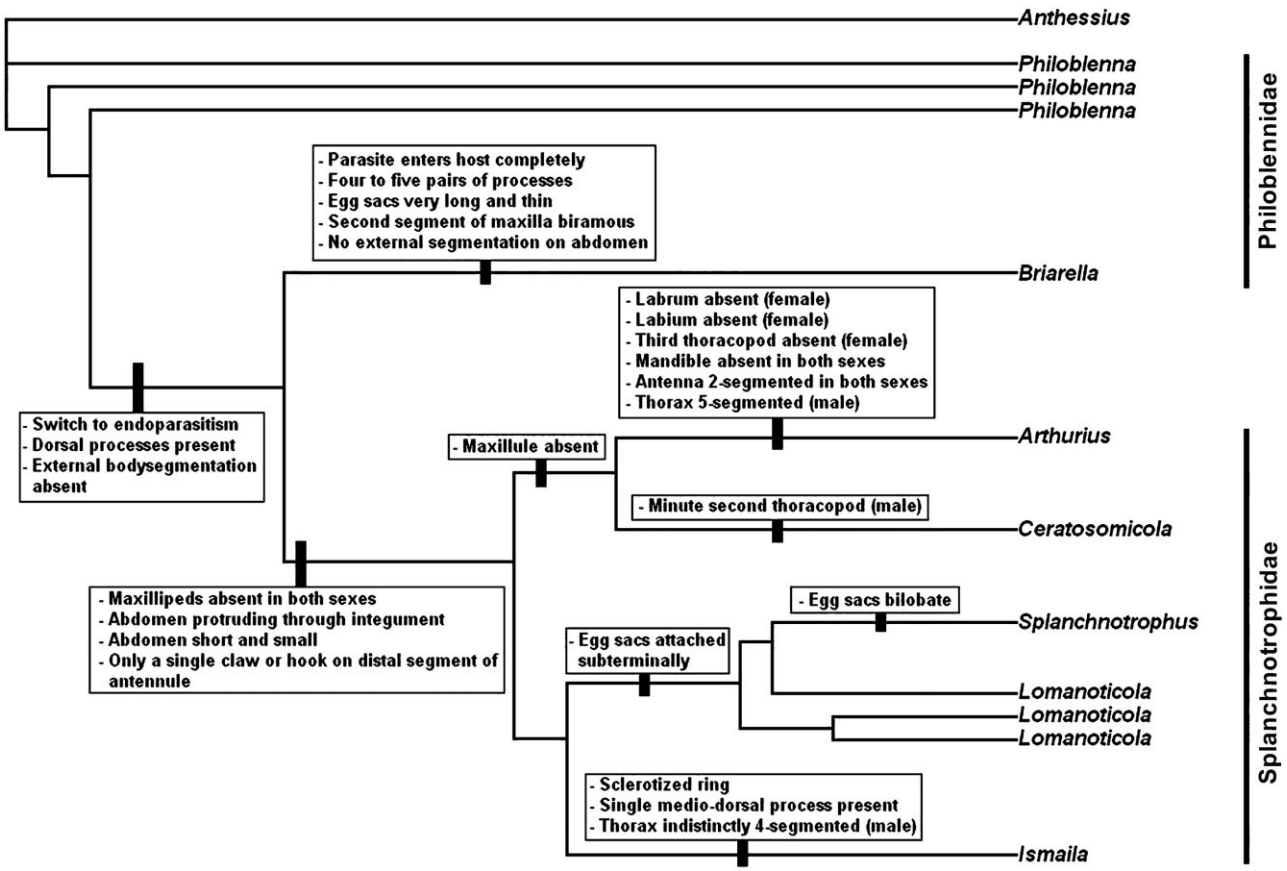

Figure 5. Character evolution. Consensus tree showing selected putative apomorphies of endoparasitic clades.

with *Spl. willemi* and *Spl. dellachiajei* as successive offshoots, leaving a clade of *Spl. angulatus* and *Spl. gracilis* (BT 52, BS 1).

DISCUSSION

This study is the first cladistic attempt to resolve the natural relationships of the Splanchnotrophidae. The ingroup sampling was optimized by including all available literature data on the morphology and biology of all described splanchnotrophid species. Outgroup sampling was problematic. Pre-analyses with a broad set of poecilostomatoid taxa showed that ectoparasitic and endoparasitic copepods of different families may be mixed without any discernible evolutionary pattern (trees not shown). Such highly improbable topologies can be explained by character selection focusing on splanchnotrophids, and the effect of multiple parallel, habitat-induced reductions, together with convergent adaptations to similar modes of life. The deficiency of numerical approaches to highly modified parasite lineages might also explain the unconventional topology of poecilostomatoid families in Ho (1991). Similarly, concerted parallelisms drowning out the true phylogenetic signal have been detected in various subclades of interstitial heterobranch gastropods that have adapted independently to life in an extreme, meiofaunal environment (Jörger *et al*., 2010; Schrödl & Neusser, 2010).

Morphology-based cladistic analyses on Splanchnotrophidae are thus sensitive to outgroup selection. To avoid artefacts, we pruned the outgroup sampling of the main analysis to all those taxa that have been considered as putative relatives of Splanchnotrophidae in current classificatory concepts (Huys, 2001) and the latest findings on the genus *Briarella* (Salmen *et al*., 2010). This approach cannot reveal the origin of splanchnotrophids amongst copepods, but instead tests the monophyly of Splanchnotrophidae against the inclusion of the morphologically and ecologically most similar, i.e. philoblennid, species. In addition, our outgroup taxon sampling was designed to root the Splanchnotrophidae appropriately in order to allow reconstruction of their internal relationships.

Origin of the Splanchnotrophidae

In the strict consensus tree both the family Philoblennidae and the genus *Philoblenna* emerge as basal

paraphyla (see Fig. 4). The genus *Briarella* robustly clusters as sister to the family Splanchnotrophidae (see Fig. 4), reflecting one of the predictions of Salmen *et al*. (2010). This node represents the switch from ectoparasitism to endoparasitism (see Fig. 5). As assumed earlier (Jensen, 1987), copepod endoparasitism in euthyneuran sea slugs apparently evolved once, in the common ancestor of Splanchnotrophidae and *Briarella*. The first indications of reductions are discernible; in the female the antennule has fewer than five segments and in the male the sixth thoracopod is absent. As soon as molecular results on a broader outgroup sampling confirm this topology, the classification of the families 'Philoblennidae' and Splanchnotrophidae can be adjusted.

None of the *Briarella* species inhabit the body cavity of the host, but all are found in the pericardium (Bergh, 1876; Monod, 1928; Monod & Dollfus, 1932, 1934; Huys, 2001). According to Monod's (1928) drawing (Fig. 6), it can be assumed that in *Briarella* the egg sac-carrying female abdomen does not protrude through the integument of the host. This condition is also certain for the latest-discovered species, *B. doliaris*, although the exact location of the incidentally found parasites is unknown (Salmen *et al*., 2010). This stands in clear contrast to all splanchnotrophids, which live in the body cavity and protrude from the host's integument with their abdomen to place their egg sacs outside the hosts' body.

Comparing the two sister clades *Briarella* and Splanchnotrophidae, it is striking that the species diversity of the Splanchnotrophidae is about five times higher than in *Briarella*. A possible explanation for this difference, apart from potential sampling bias, may be the surrounding environment of adult parasites. In the case of pericardium-inhabiting *Briarella* this is gastropod primary urine (e.g. Fahrner & Haszprunar, 2002), which is poor in cellular and dissolved substances. In contrast, all splanchnotrophids are surrounded by haemolymph, which is comparatively rich in particles and nutrients. Further, egg sacs of *Briarella* species are long and thin (see Fig. 2A), obviously to fit within pericardial and renal spaces of their hosts, whereas splanchnotrophid egg masses mature outside the host's body (Fig. 3). The latter may be advantageous for several reasons: egg masses are less limited in size and shape, oxygen supply is better when in contact with seawater rather than urine, and pelagic larvae can swim away freely rather than having to be excreted. The disadvantage of egg sacs being exposed to seawater is the lack of protection. In fact, cut egg sacs of *I. aliena* are eaten by a variety of syntopic fish, whereas infested host specimens or parts thereof are not (M. Schrödl, pers. observ.). Many opisthobranch gastropods compensate for the reduction of the shell by the development of chemical defence mechanisms (Thompson, 1960; Edmunds, 1966; Faulkner & Ghiselin, 1983), which obviously also protect the parasite inside. In most hosts, splanchnotrophid females hide the egg sacs between the cerata or under lobes of mantle tissue (Schrödl, 1997; Haumayr & Schrödl, 2003). Furthermore, the parasite is also capable of retracting the abdomen so that the egg sacs are drawn up near the host's body where they are not easily accessible to potential predators (Schrödl, 1997; R. F. Anton, pers. observ.).

Besides the lower diversity, *Briarella* in some aspects shows a lower level of reductions compared to the Splanchnotrophidae, i.e. the maxillipeds are still

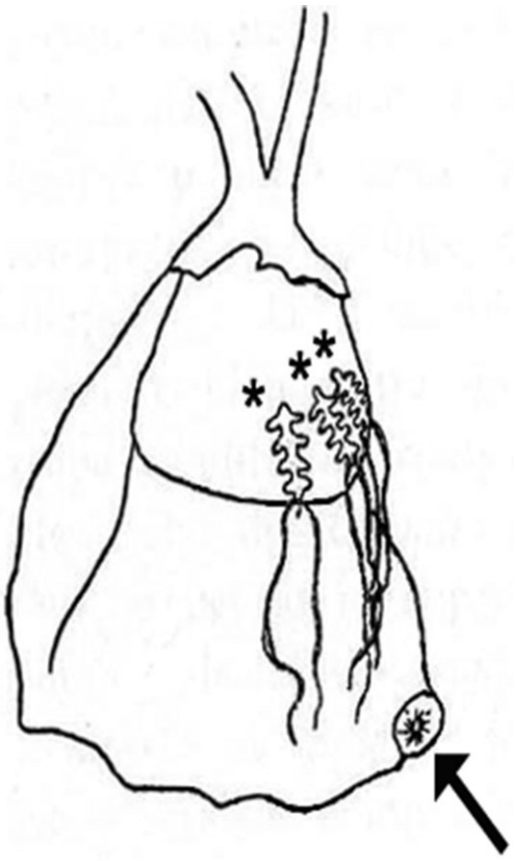

Figure 6. Sketch of *Briarella risbeci*, redrawn from Monod (1928), showing three egg sac-bearing females (asterisks) associated with the outer surface of the heart of their nudibranch host, *Hexabranchus sanguineus* (Rueppell & Leuckart, 1828) [as *Hexabranchus marginatus* (Quoy & Gaimard 1832)]. Although the sea slug anatomy (with atrium and ventricle surrounded by a pericardium that connects to the kidney) is not correctly reflected in the drawing, Monod's text explicitly mentions that the parasites are situated within the renopericardial cavity of their hosts; thus, the copepod larvae would have to exit the host via its nephroporus (arrow).

present and the antennule is six- to four-segmented, whereas in Splanchnotrophidae it is not more than four-segmented. To resolve whether this represents a different path of evolution, more information about the life histories of both taxa is needed.

Phylogeny of the Splanchnotrophidae

In the present study the family Splanchnotrophidae, as defined by Huys (2001), results as a clade (see Fig. 4). There is only moderate node support, but monophyly is indicated unambiguously by three unique synapomorphies, i.e. the abdomen of the parasite protruding from the host's integument, the presence of long, slender thoracic processes, and the absence of maxillipeds in both sexes.

The Splanchnotrophidae divide into two major clades. The first comprises the Indo-Pacific genera *Ceratosomicola* and *Arthurius*, showing a trend towards reduction of mouthparts and thoracopods (Fig. 5). *Ceratosomicola* is well defined by an elongate body shape, the absence of the maxillules and fifth thoracopods in females, and the absence of second thoracopods in males (Huys, 2001; Salmen *et al*., 2008b). Within *Ceratosomicola*, *Ce. sacculata* is sister of a clade with three recently described Indonesian species (Salmen *et al*., 2008b). The genus *Arthurius* stayed monophyletic with high bootstrap and Bremer support in all our analyses. The two species *Ar. elysiae* and *Ar. bunakenensis* show several synapomorphies, most notably the reduction of the antennule, the two-segmented antenna, and the absence of the labium in females and of the mandibles in both sexes (Huys, 2001). In addition, this genus stands out because of very distinctive sexual dimorphism. All members of the Splanchnotrophidae show sexual dimorphism concerning the body shape, but only in both species of *Arthurius* do the sexes differ also in mouthpart morphology (Huys, 2001; Salmen *et al*., 2008a).

The second major splanchnotrophid clade consists of the genus *Ismaila* sister to *Splanchnotrophus* (*s.l.*), confirming an earlier assumption by Schrödl (2002). The monophyly of *Ismaila* is strongly supported in all our analyses. The most convincing synapomorphies are the unpaired mediodorsal process (females) and the presence of a sclerotized ring between the fourth and fifth thoracopods (mature males and females), which are unique for members of the genus *Ismaila* (see Fig. 5). Although the function of the process is unknown, the sclerotized ring (Fig. 1B) fits the hole in the body wall perforated by the abdomen of mature *Ismaila* specimens, and is usually overgrown by and firmly embedded in the host's connective tissue (M. Schrödl, pers. observ.).

According to Huys (2001), within the Splanchnotrophidae the genus *Ismaila* shows the most 'primitive' character state conditions for the maxillule (distinct bisetose lobe), the maxilla (allobasis with two accessory elements), and for leg 5 (free segment with two setae), which is supported by the present analysis. The maxillule of *Ismaila* is similar to that of the basal *An. kimjensis*. Within the Splanchnotrophidae it is absent in the genera *Ceratosomicola* and *Arthurius*, whereas in *Splanchnotrophus* and *Lomanoticola* it is present in rudimentary form. The maxilla of *Ismaila* is reduced compared to that of *An. kimjensis* and *Philoblenna*, but compared to all other splanchnotrophid genera the allobasis possessing two accessory elements represents the most complex condition, which is therefore considered as basal. The fifth pair of thoracopods is still shaped as a swimming leg in *An. kimjensis*. Except in *Ismaila*, the fifth pair of thoracopods is completely lost in all splanchnotrophid genera.

Members of *Splanchnotrophus* (*s.l.*), i.e. *Lomanoticola* and *Splanchnotrophus* species, all occur in European waters and so far are the only known temperate splanchnotrophids outside *Ismaila*. The clade is supported by two synapomorphies: the abdomen is two-segmented and the egg sacs are no longer attached terminally. In contrast to its classification by Huys (2001), the rather poorly known genus *Lomanoticola* resulted as paraphyletic in our unconstrained analysis (Fig. 4). Forcing monophyly of *Lomanoticola*, the resulting strict consensus tree required three additional steps. Still, the nonmonophyly of *Lomanoticola* may be caused by missing data. To date, no male representative of any of the three species of *Lomanoticola* has been found (Huys, 2001; Salmen, 2005), and revision is overdue. The genus *Splanchnotrophus* in a strict sense is monophyletic with a BT of 65 and a BS of 2 (see Fig. 4), confirming the classification by Huys (2001). All female members of the genus *Splanchnotrophus* are characterized by unique, bilobate egg sacs (see Fig. 3D). The low node support for *Splanchnotrophus* may be explained by missing and unreliable morphological data, especially for both sexes of *Spl. willemi* and *Spl. dellachiajei*.

In summary, this study gives a first insight into the internal phylogeny of the family Splanchnotrophidae based on morphological data. The topology of the resulting strict consensus tree in large parts reflects Huys' (2001) assumptions for the taxonomy of the Splanchnotrophidae, with the exception of the paraphyly of *Lomanoticola*. As the tree is unstable in this region, probably as a result of missing data, the traditional classification is maintained until sufficient morphological or molecular information becomes available. Re-analyses of the internal splanchnotrophid phylogeny in the light of broader taxon sampling within the Poecilostomatoida are desirable, and

the inclusion of Philoblennidae and Splanchnotrophidae in molecular studies is overdue.

Splanchnotrophid evolution: phenotype and feeding mode adapted to endoparasitic life

Some evolutionary trends are evident from reconstructing character states of the strict consensus tree (Fig. 4). All species included in our main analysis are parasitic on or in molluscan hosts, and from ectoparasitic outgroups towards endoparasitic splanchnotrophids there is a clear trend of successive reduction to loss of thoracopods, head appendages, and mouthparts (Fig. 5); further reductions relate to segment borders and abdomen length. Some may reflect general consequences of endoparasitic life, such as reduction or not of further developing organs used for locomotion or for feeding, whereas others may be unique to splanchnotrophids, and a loss of function during development or functional shifts can be assumed.

Antennules are present in all taxa analysed, but show reductions regarding the number of segments towards and within splanchnotrophids. As a chemoreceptive function of the antennule has been proven by Lenz *et al*. (1996) and Boxshall (2005), it can be assumed that small splanchnotrophid antennules in the infective copepodite stage mainly serve to find a potential host, and favourable sites and mates within the hosts.

Ectoparasitic copepods usually use their antennae to grasp the host, and their mandibles to rasp host tissue. Owing to their massive, claw-like shape, splanchnotrophid antennae are probably still used to grasp host tissue. As the only large limbs available in all splanchnotrophid species, antennae are here assumed also to be used to perforate the host's integument during initial invasion. It is, however, unclear whether this happens through the host's external skin or internal digestive epithelia. During later development, splanchnotrophid antennae rather than the reduced or lost mandibles may still be used to destroy tissue in those hosts showing obvious damage (Marcus, 1959; Schrödl, 1997; Boxshall, 2005; Marshall & Hayward, 2006), or to fix the parasite's head or mouth in a certain position. Remarkably, there have been no direct observations indicating eponymous 'splanchnotrophid' tissue feeding, and no destructive effects on host organs directly related to feeding have been reported (Marcus, 1959; Schrödl, 1997, 2002; Marshall & Hayward, 2006).

In our main analysis, mouthparts showed a successively regressive pattern (Fig. 5). All genera used as outgroup taxa, including *Briarella*, still possess maxillipeds, although these structures already show reductions in the Philoblennidae, i.e. the reduction of the endopodal claw and the indistinct separation of the endopodite and the basis (Huys, 2001). In all splanchnotrophid genera this structure is absent (Huys, 2001; Haumayr & Schrödl, 2003), and thus was already lost in the ancestral splanchnotrophid (see Fig. 5). Such structural differences may reflect different feeding modes in *Briarella* and its sister clade Splanchnotrophidae, but further investigation is needed as the feeding mode is unclear for both taxa.

Living in renopericardial spaces, an adult *Briarella* cannot be assumed to feed on the thin and sensitive surrounding tissue without destroying vital host organs; rather, it may ingest and absorb primary urine that is supposedly poor in suspended particles. It is remarkable, however, that adult *Briarella* still possess all mouthparts, including maxillipeds; the close resemblance of briarellid mouthparts with those of the ectoparasitic genus *Philoblenna* (Izawa, 1976; Huys, 2001) may be explained when information on whole life cycles becomes available. In contrast, setose maxillules in some splanchnotrophids, i.e. *Ismaila*, may be used to feed on fragments of host tissue suspended in the haemolymph, and the sickle-shaped mandible may carry larger particles into the mouth. However, tissue feeding is unlikely for other splanchnotrophids because the maxillules are only represented by small lobes in the genera *Splanchnotrophus* and *Lomanoticola*, and were lost in the ancestor of *Ceratosomicola* and *Arthurius* (Fig. 5). In *Arthurius*, even the mandibles are reduced in both sexes (Fig. 5), as is the labium in the female (Huys, 2001; Salmen *et al*., 2008a). Because of the nearly complete reduction of mouthparts, tissue or particle feeding can be excluded for the latter genus. Within splanchnotrophids, a tendency away from potential tissue feeding to an endoparasitic adult life as haemolymph suckers can be assumed, a trait that may have started early during splanchnotrophid evolution or already in the common ancestor with *Briarella*.

Evidence supporting this hypothesis may come from digestive anatomy. In his family diagnosis of the Splanchnotrophidae, Huys (2001) mentioned the presence of an anus, whereas no distinct anal opening was mentioned in any of his species descriptions. In their revision of the genus *Ismaila*, Haumayr & Schrödl (2003) also did not observe any distinct anal opening, which also applies to the latest descriptions of members of the genera *Arthurius* and *Ceratosomicola* (Salmen *et al*., 2008a, b). In addition, the only existing histological examination of a splanchnotrophid, a male *I. belciki*, described the digestive system as 'incomplete' (Belcik, 1981). The absence of an anus is histologically confirmed for *I. aliena* (R. F. Anton, pers. observ.). This simplified digestive system is in line with the hypothesis that Splanchnotrophidae feed on haemolymph, which can be digested and residues may be discarded through the mouth or seques-

tered within the body of the parasite as described by Boxshall (2005) for blood-feeding copepods.

Splanchnotrophids grow fast in their hosts. In translucent hosts such as *Phidiana lottini* or *Thecacera darwini*, a mature female can emerge from an inconspicuous copepodite stage within two days in a host with no prior signs of infection (M. Schrödl, pers. observ.). The development of eggs is also fast in splanchnotrophids. When the egg sacs of a female *Spl. angulatus* infesting the aeolid *Cratena peregrina* (Gmelin, 1791) are removed, it takes only 8 to 12 h until they are completely replaced (R. F. Anton, pers. observ.). Both the fast growth and the rapid replacement of the egg sacs are traits also displayed by free-living copepods (Hopcroft & Roff, 1996). Rapid growth and high reproductive output of Splanchnotrophidae requires access to high quantities of food; consequently, where there is feeding on host organs some traces of gnawing should be detectable.

In fact, head appendages and mouthparts of adult splanchnotrophids are small relative to body size (Fig. 1B, C) and thus may represent vestigial larval organs. According to Ho (1987a), mouthparts may not grow any more after the copepodite IV stage; while the body grows, segment borders vanish and the cephalothorax in particular inflates in both sexes (Ho, 1878a; Belcik, 1981). In mature specimens of all splanchnotrophid species the gonads are huge and extend throughout the body (Huys, 2001; Schrödl, 2002; Haumayr & Schrödl, 2003). The trend of body extension thus is explained by the enhanced production of gametes. In several *Ismaila* species even the thoracopods are swollen, showing the ovaries inside (Haumayr & Schrödl, 2003). The same is true for the thoracic processes, as was assumed by Huys (2001). We thus suggest that some special and even unique structures of splanchnotrophids, such as the inflated head segments in males, inflated thoracopods in both sexes, and most obviously the long thoracic appendages (Fig. 2) especially in females (Huys, 2001; Haumayr & Schrödl, 2003), are adaptations to maximize sperm and egg production. This supports the assumption of Hancock & Norman (1863) that splanchnotrophid thoracic processes are not homologous with modified thoracopods but rather formed *de novo* (Hancock & Norman, 1863; Huys, 2001) to house branches of the ovaries (Huys, 2001). The reproductive output is lower in *Briarella* than in Splanchnotrophidae, as discussed above. Still, splanchnotrophids are hardly more inflated than briarellids, but possess much longer appendages (Fig. 2). We interpret this as an adaptation to maximize egg production under optimal nutrition conditions, but avoiding harmful effects on the host. The special elongated form of the processes allows for them to be wound around host organs without destroying or displacing them, and for all available space in the body cavity of the host to be filled.

Besides the housing of ovaries, several other possible functions of the thoracic appendages have been discussed, e.g. uptake of nutrients (O'Donoghue, 1924), positioning of the parasite within the host (Huys, 2001), and respiration (Salmen *et al*., 2008b). To clarify the true functions of these appendages, histological studies are necessary.

Possibly, the general reduction of the abdomen in all splanchnotrophids is also a result of the unique way of releasing the offspring, i.e. by simply positioning the genital openings outside of the host's integument. Any long section of abdomen outside the host could thus represent an unprotected weakness of the parasite. The egg sacs of splanchnotrophid species are also short and comparatively thick; if long, they are rolled up in spirals (see Fig. 3) or, in the case of *Splanchnotrophus*, even bilobate (Monod & Dollfus, 1932; Huys, 2001; Haumayr & Schrödl, 2003), possibly to avoid extending too far from the host. As an example, posterior to the gills and between the peribranchial appendages of *Thecacera darwini*, even the long, double-curled egg sacs of *I. aliena* are nearly inaccessible when the parasite retracts its abdomen in response to mechanical disturbance (M. Schrödl, pers. observ.).

In the Splanchnotrophidae there is a striking contrast between the body sizes of females and males (Huys, 2001; see also Fig. 2B). Initially this was considered as evidence for a relationship between Splanchnotrophidae and Chondracanthidae (Huys, 2001). However, Laubier assumed that the females are peramorphic instead of the males being paedomorphic (Laubier, 1966; Huys, 2001). Judging from the male and female mouthpart lengths in the species included in this study, Laubier's assumption seems even more likely. On the one hand males show no indications of development being stopped at a larval stage, which would be implied by paedomorphism. On the other, there are cases in which the mouthparts of the smaller male are longer than those of the larger female. For example, male mandibles of *Spl. angulatus* and *Spl. gracilis*, male maxillae of *Ar. elysiae*, and male antennae of *Ar. bunakenensis*, *Spl. gracilis*, and *Ce. coia* are longer than the corresponding structures of the respective females (Huys, 2001; Salmen, 2005; Salmen *et al*., 2008a, b).

We conclude that endoparasitic copepods of sea slugs all retain at least some larval features such as antennules that are necessary to detect and infest hosts, but they may not grow further once the parasite has established itself inside the host. Mouthparts in splanchnotrophids show clear trends of reduction already in endoparasitic copepodite stages, whereas early larval anatomy and feeding modes are virtually unknown. Adult mouthpart anatomy is best explained

by assuming adaptations to endoparasitic life as haemolymph suckers, with the genus *Arthurius* showing the most radical reductions of plesiomorphic features. Floating within an environment providing a surplus of liquid food (or particles therein), splanchnotrophid gamete production is maximized by inflating anterior body parts as well as thoracopods, and by extending body appendages that are assumed to be an efficient and harmless way to maximize parasite volume within a host; owing to their larger surfaces they could also enhance respiration. Sexual dimorphism is explained by extended growth in females, probably as a consequence of eggs needing more time and space to develop than sperm. We conclude that regressive tendencies, combined with structural and functional innovations adaptive to endoparasitic life, have transformed quite normal copepod-shaped larval splanchnotrophids into bizarre high-throughput 'breeding units'.

Host specificity and infection of new hosts

Given that *Briarella* and the Splanchnotrophidae share a common ancestor (see Fig. 4), and assuming that '*Chondrocarpus*' is a (so far unidentifiable) member of this clade (Monod & Dollfus, 1932; Huys, 2001), copepods switched to endoparasitism in euthyneuran shell-less hosts just once, i.e. within the philoblennid stemline. In the absence of any fossil record of philoblennid copepods the timing of this event is completely unknown. Exploring how closely splanchnotrophid and briarellid evolution was linked to that of their hosts, we compared whether phylogenies of parasites and hosts are congruent or not. On the parasite side, the present study provides the first topological framework 'to read history'. Hosts of *Briarella* are dorid nudibranchs, whereas splanchnotrophids infest a variety of nudibranch subgroups plus sacoglossans (see Fig. 7; O'Donoghue, 1924;

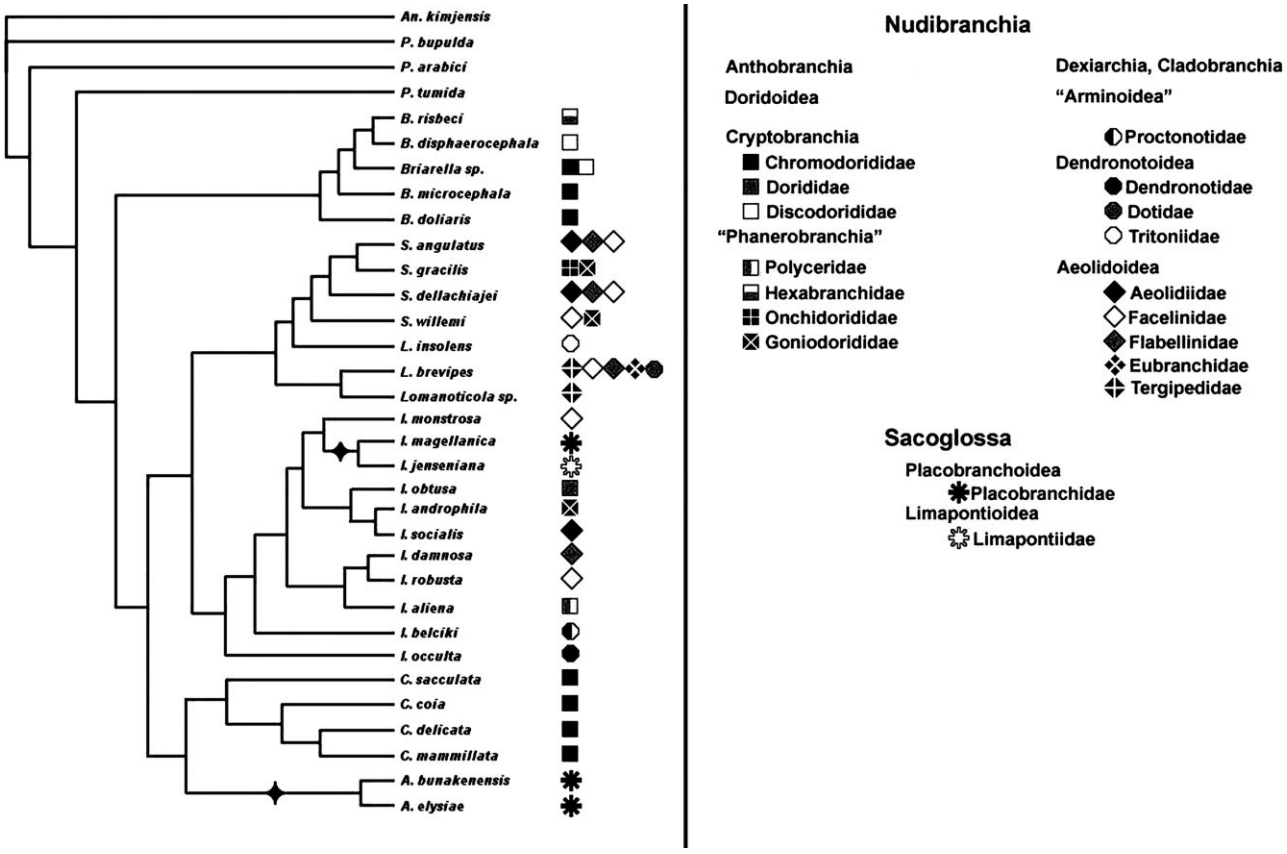

Figure 7. Parasite–host relationships. Host families of endoparasitic briarellid and splanchnotrophid species are plotted on the consensus tree and symbol coded according to a current sea slug classification (Wägele & Willan, 2000; Jörger *et al*., 2010; Schrödl *et al*., 2011a). Note that basal *Briarella* and *Ceratosomicola* species infest chromodoridid nudibranchs, suggesting the latter as the ancestral host group. Other endoparasites infest a wide range of other nudibranchs and sacoglossans. Host switches from nudibranchs to distantly related sacoglossans are likely to have occurred three times independently (marked by asterisks).

Monod, 1928; Monod & Dollfus, 1932, 1934; Jensen, 1990; Huys, 2001; Haumayr & Schrödl, 2003; Salmen *et al*., 2008a). Phylogenetic hypotheses concerning these major euthyneuran gastropod taxa have been revolutionized recently by multilocus molecular studies (e.g. Dinapoli & Klussmann-Kolb, 2010; Jörger *et al*., 2010; Göbbeler & Klussmann-Kolb, 2011; Schrödl *et al*., 2011a; Schrödl, Jörger &Wilson, 2011b), which have shown that Nudipleura (including Nudibranchia) divided from a clade consisting of Euopisthobranchia and Panpulmonata (including Sacoglossa), perhaps as early as the late Palaeozoic, at least *c*. 250 Mya. Intranudibranch relationships remain dubious; however, there is agreement that nudibranchs divided into Anthobranchia (including dorids) and Dexiarchia (including nonmonophyletic arminoids, Dendronotoidea, and Aeolidioidea) (Wägele & Willan, 2000; Schrödl, Wägele & Willan, 2001; Wägele *et al*., 2009). Whereas Schrödl (2003) estimated that split to be younger than *c*. 40 Mya, according to Göbbeler & Klussmann-Kolb (2010) nudibranchs started to diverge earlier, i.e. between 50 and 140 Mya, and Chromodorididae may be younger than 37 Myr old.

Comparing our strict consensus tree for the parasites with a classification of higher host groups (see Fig. 7) that reflects natural relationships as discussed above, splanchnotrophid radiation does not appear to be directly correlated with the early divergence of host lineages. Instead, some basal offshoots of *Briarella*, and all *Ceratosomicola* species parasitize chromodoridid nudibranchs, whereas other splanchnotrophids apparently do not. Therefore, that host family is a likely candidate to have also hosted the endoparasitic ancestor. If so, the first sea slug endoparasite, and the split between *Briarella* and Splanchnotrophidae, cannot pre-date the very approximate 37 Myr age (95% confidence range of about 5 to 60 Myr; Göbbeler & Klussmann-Kolb, 2010) of the Chromodorididae, giving a first timing estimate. The European clade shows a switch to dexiarchian hosts, and back to dorids (but not Chromodorididae). In contrast, the *Ismaila* clade shows a mix of hosts of different sea slug groups, but dexiarchians are in the majority (Fig. 7).

The most remarkable intrasplanchnotrophid host shifts are those from nudibranch to sacoglossan hosts, which occurred at least twice independently: in the common ancestor of *I. jenseniana* and *I. magellanica*, and in the common ancestor of *Arthurius* species (Fig. 7). Striking is the phylogenetic distance between Nudibranchia and Sacoglossa and their at least early Mesozoic separation (Jörger *et al*., 2010) that has, however, not led to firm barriers to infestation. Surprisingly, the many other marine euthyneuran lineages between nudibranchs and sacoglossans (Jörger *et al*., 2010), nearly all of which have shells reduced to at least some extent or none at all, apparently are not infected by briarellids and splanchnotrophids. An interesting exception is *Chondrocarpus*. The two known specimens are the only copepod endoparasites found in a pleurobranchoidean species (Bassett-Smith, 1903). That they inhabited the host's kidney may point toward a relationship with *Briarella* rather than with splanchnotrophids (or other taxa) as assumed by Monod & Dollfus (1932) and supported herein. This special host preference of *Chondrocarpus* may allow this dubious parasite species to be found again in spite of its inadequate original description.

Most splanchnotrophids, such as all members of *Ismaila*, seem to be specific to a single host species (Huys, 2001; Haumayr & Schrödl, 2003), whereas other species, especially those infesting aeolid nudibranchs, e.g. species of *Lomanoticola* and *Splanchnotrophus*, apparently are not (Fig. 7; Hancock & Norman, 1863; Monod & Dollfus, 1932; Huys, 2001). The possibility of narrow versus broader host ranges amongst closely related parasite species cannot be neglected based on available knowledge, but detailed redescriptions are needed in order to clarify the taxonomy of dubious species and to confirm hardly substantiated records from different hosts. Molecular studies are also needed to exclude the existence of cryptic, ecologically specialized species.

An already more evident aspect is that members of different parasite clades infest the same host taxa or even species. For example, the Mediterranean aeolid species *Spurilla neapolitana* and *Flabellina affinis* are both parasitized by *Spl. angulatus* and *Spl. dellachiajei*, and *L. brevipes* and *Spl. dellachiajei* are both reported from the aeolid host *Facelina bostoniensis* (Huys, 2001). The phylogenetically most distant parasites associated with the same host are *Spl. angulatus* in the Mediterranean Sea and *I. socialis* from central Chile both infecting the aeolid *Aeolidia papillosa* (Monod & Dollfus, 1932; Haumayr & Schrödl, 2003). In the case of the closely related *Spl. angulatus* and *Spl. dellachiajei*, the ability to infect the same spectrum of host species could be inherited, or simply be a taxonomic artefact if the poorly described *Spl. dellachiajei* proves to be a synonym of *Spl. angulatus*. *Lomanoticola brevipes* and *Spl. angulatus*, however, are not so closely related (Fig. 4) and taxonomically distinct beyond any doubt (Huys, 2001; Salmen, 2005). Possibly the host *Fa. bostoniensis* has some traits that facilitate infection. Another, perhaps especially tolerant, nudibranch species (or cryptic species complex) is *Aeolidia papillosa*; it is parasitized by two different splanchnotrophid species that are significantly divided morphologically and geographically. *Cuthona caerulea* (Montagu, 1804) is an especially small and not very abundant aeolid that

becomes infested, whereas the sympatric aeolid *Dondice banyulensis* Portmann & Sandmeier, 1960, is large and often abundant but is not infested (Calado *et al*., 2003; Urgorri *et al*., 2011). Whereas the abundant Chilean aeolid *Flabellina* sp. 1 was frequently infested (and sterilized by the parasite) (Schrödl, 1997), the sympatric and almost syntopic, equally abundant and similar sized *Flabellina* sp. 2 was never infested (Schrödl, 2003). However, assuming a high level of parasite adaptations to certain hosts conflicts with the inference of host switches between very different sea slug taxa. Remarkably, only about 1% of the roughly 3000 nudibranch species known worldwide are reported to be infested by splanchnotrophids (Huys, 2001; Haumayr & Schrödl, 2003; Salmen *et al*., 2008a, b), but some hosts are attacked by various, not necessarily closely related, parasite species. To explain such remarkable patterns we need molecular data elucidating the taxonomy of the parasites and observational data on interactions between parasites and hosts, especially on mechanisms of host detection and infection.

In summary, phylogenetic reconstruction (Fig. 7) has revealed frequent historic switches between different hosts, and even between very old and phylogenetically distant sea slug taxa. It can be concluded that high ecological plasticity has allowed for numerous successful host switches during splanchnotrophid evolution, driving diversification. This contrasts with the current opinion that most (but apparently not all) extant Splanchnotrophidae species are strictly host specific, suggesting at least some level of adaptation to these hosts (Huys, 2001; Schrödl, 2002; Haumayr & Schrödl, 2003). In addition to other preconditions such as host detection or overcoming potential defences, splanchnotrophid host switches may be limited to a small number but a wide taxonomic range of sea slug species by their tolerance against infection with comparatively large endoparasites perforating their integument once (*Briarella*) or twice (Splanchnotrophidae). Molecular studies will show whether certain splanchnotrophid species with a broader spectrum of hosts, if any, are in the process of adapting to certain hosts that may lead to speciation. How and why certain hosts are infected, while others are not, remains to be explored in more detail.

Distribution patterns and historical biogeography

In the absence of any clear correlation between parasite and major host lineages we may assume that the diversification of the parasites is old but that signatures have vanished, or that parasite radiation is relatively young and signatures have never existed. The latter alternative is more parsimonious, and is supported by the relatively young age of the supposed ancestral host group, the Chromodorididae (discussed above). Therefore we might expect to see current geographical distribution patterns to be reflected in the topology presented here. Almost all nudibranch and all sacoglossan hosts are marine shallow-water species and thus confined to coasts, but the dispersal abilities of both hosts and parasites may be considerable having pelagic larvae (Thompson, 1958; Mileikovsky, 1968; Kempf, 1981).

In fact, we see clear patterns in the distribution of extant parasite taxa (Fig. 8). Members of the basal, paraphyletic genus *Philoblenna* are only known from the Yellow Sea (Izawa, 1976; Avdeev, Tsimbalyuk & Lukomskaya, 1986; Ho & Kim, 1992; Kim, 2004). The more derived genus *Briarella* has a wider distribution range within the Indo-Pacific (Fig. 8): *B. risbeci* and *B. disphaerocephala* were found in New Caledonia (Monod, 1928; Monod & Dollfus, 1932), *B. doliaris* at the eastern coast of Australia (Salmen *et al*., 2010), and *B. microcephala* and *Briarella* sp. are known from the Red Sea (Bergh, 1876). The family Splanchnotrophidae is distributed worldwide, but the distribution areas of the individual genera are limited to certain oceans or continental coasts (Fig. 8). The Neotropical genus *Ismaila* shows a wide distribution from the Pacific coast of northern America via the coast of Chile to the Atlantic eastern part of the Magellan Strait (Schrödl, 1997, 2002; Haumayr & Schrödl, 2003). No tropical eastern Pacific *Ismaila* species are known, but *I. monstrosa* and *I. jenseniana* were found in the Caribbean Sea (Bergh, 1867; Haumayr & Schrödl, 2003). The genera *Ceratosomicola* and *Arthurius* are known from relatively few records from subtropical and tropical waters forming a narrow distribution range (Fig. 8). The type species of the two genera were discovered in Western Australia (O'Donoghue, 1924; Jensen, 1990; Huys, 2001), and recently three new species of *Ceratosomicola* and one new *Arthurius* species were discovered in Sulawesi (Salmen *et al*., 2008a, b). *Splanchnotrophus* and *Lomanoticola* inhabit temperate waters of the Mediterranean Sea and the European coast of the Atlantic Ocean (Hancock & Norman, 1863; Canu, 1891; Hecht, 1893, 1895; Delamare Deboutteville, 1950; Gotto, 2004; Salmen, 2005).

According to these distribution patterns and the results of our phylogenetic analysis (Fig. 4), it is parsimonious to infer that the geographical origin of endoparasitic copepods infesting sea slugs probably lies in tropical waters of the Asian/Australian region of the Indo-Pacific, with *Briarella* still occurring there (see Fig. 8). The ancestral splanchnotrophid probably inhabited the Palaeogenic tropical Indo-Pacific, as members of the basal *Ceratosomicola* and *Arthurius*

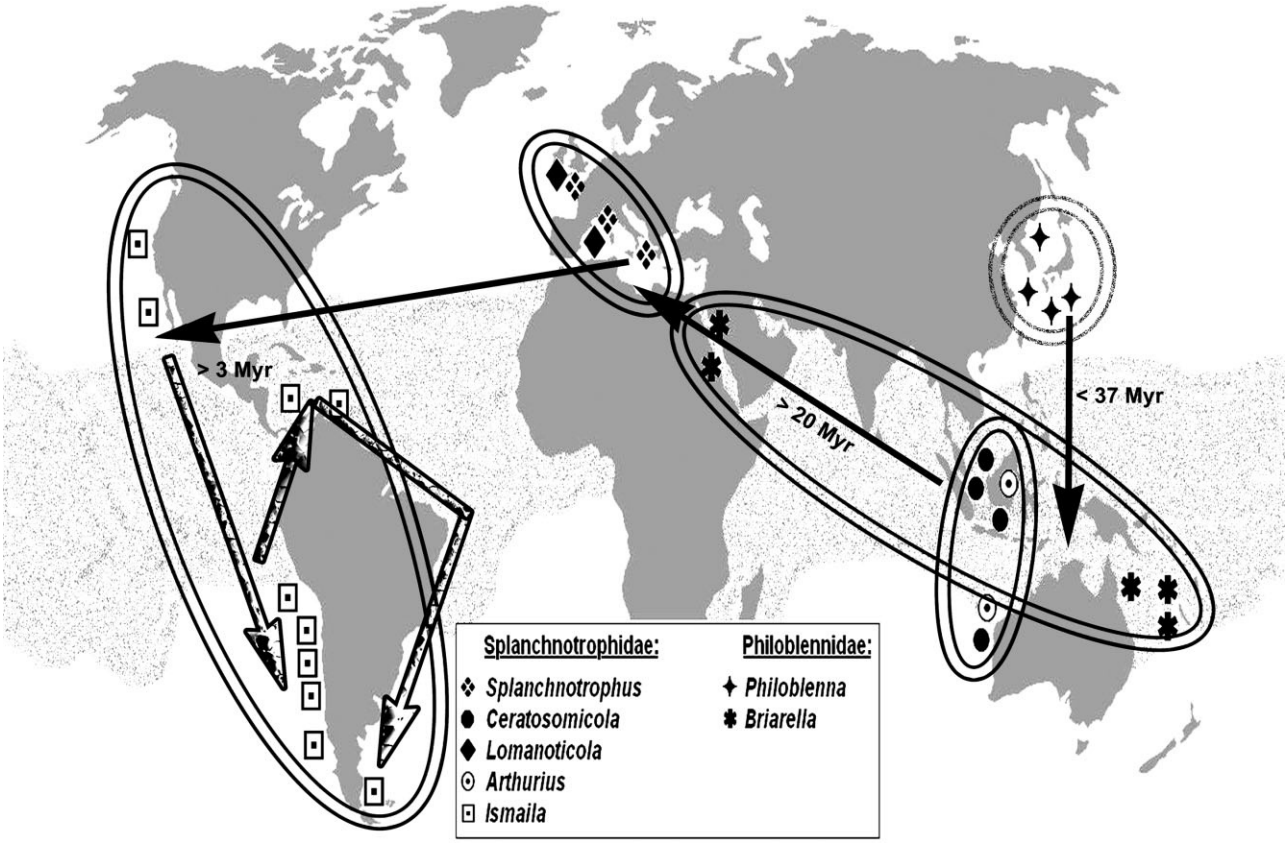

Figure 8. Hypothesis on historical biogeography of philoblennid and splanchnotrophid genera. Double circles mark assumed recent and ancestral areas of distribution of members of monophyla, whereas the dashed circles of *Philoblenna* indicate paraphyly. Inferred migration events in the stem lineages are represented by bold arrows; potential migration within the widespread genus *Ismaila* is represented by dashed white arrows. Time scales are estimated from molecular clock dating of host lineages (split between *Philoblenna* and *Briarella* plus Splanchnotrophidae) and from closure dates for the Tethys and Panamanian seaways (see text for details). The grey-shaded area is determined by the 20 °C isochrymes (Forkel, 2008) and indicates tropical versus temperate waters.

still do. This scenario is strongly supported by our Bayesian (binary MCMC) ancestral area analysis (Fig. 9; Table 2). The colonization of the American coasts by the common ancestor of *Splanchnotrophus s.l.* and *Ismaila* could have occurred (20.8% support) by crossing the Pacific (Fig. 9; Table 2); however, there is no evidence of any corresponding signature left near any central or eastern Pacific islands or along the northern Pacific coast. Because of the much shorter distance to travel, and considering that all known *Splanchnotrophus* (*s.l.*) species occur in European waters, it is more plausible to assume that the common ancestor of *Splanchnotrophus* (*s.l.*) and *Ismaila* migrated westwards into the Atlantic (Fig. 8) before the closing of the Tethys seaway approximately 18–19 Mya (Malaquias & Reid, 2009). The latter scenario is supported by Bayesian analyses (Fig. 9), which favour an Indo-Pacific (50.6%) over a north-eastern Atlantic and Mediterranean (26.4%) ancestral area; however, during Tethys times these ocean areas were connected and migration was possible. Since then, European splanchnotrophids have radiated, adapting to successively cooler, i.e. temperate, water conditions (Fig. 8). In contrast, the ancestral *Ismaila* crossed the developing Atlantic Ocean and colonized American waters. The two known Caribbean species, *I. monstrosa* and *I. jenseniana*, may be remnants of an initial tropical radiation. However, these are derived *Ismaila* species according to our topology, and Bayesian analyses strongly support a different scenario. As indicated by the basal stem offshoots *I. occulta* and *I. belciki*, the radiation of *Ismaila* may have started in the north-eastern Pacific (Fig. 9), with further radiation of *Ismaila* occurring in temperate south-eastern Pacific waters (Figs 8, 9). Our phylogenetic hypothesis (Fig. 4) confirms that the many

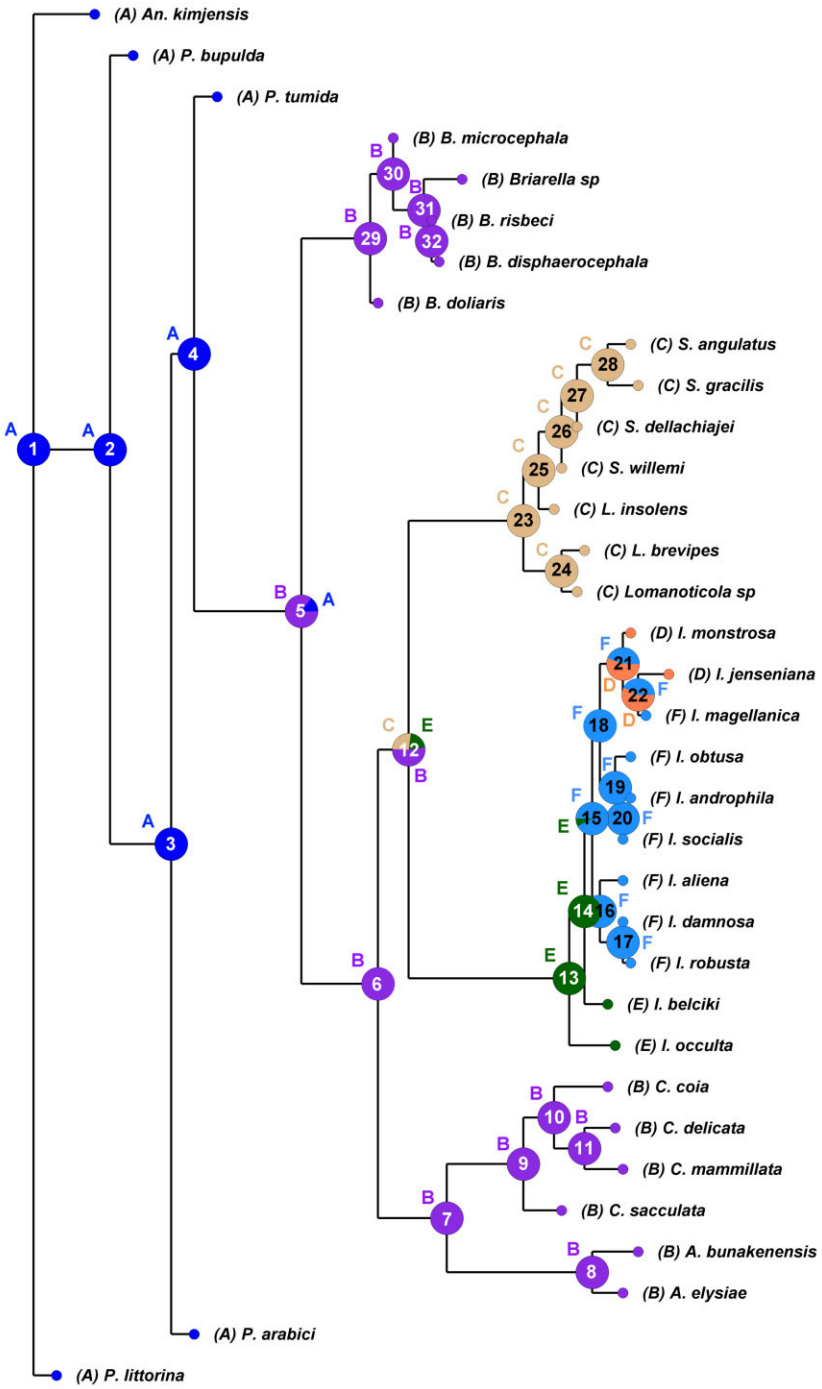

Figure 9. Ancestral area reconstruction for Splanchnotrophidae. The tree shown in Figure 4 was analysed via the Bayesian Markov Chain Monte Carlo model included in the computer program RASP. Default values were used, but the number of generations was increased to 2 000 000. The maximum number of areas per node was set to 1, reflecting actual distribution ranges of species and genera. Areas were defined according to present-day oceans and continental coasts showing similar hydrographical conditions. *Ismaila magellanica*, although occurring in the Atlantic part of the Magellan Strait, was conservatively coded as 'south-eastern Pacific' because of similar hydrographical conditions and unknown total distribution range of this species. Areas are indicated by capital letters (A, Yellow Sea; B, Indo-Pacific; C, Mediterranean Sea and northern Atlantic; D, Caribbean Sea; E, north-eastern Pacific; F, south-eastern Pacific) and colour coded. Nodes are numbered, and support values for competing ancestral areas are visualized as coloured pie-charts; for exact values, see Table 2.

Table 2. Results of the Bayesian binary Markov Chain Monte Carlo analysis. Probability values for the particular distribution areas (A, Yellow Sea; B, Indo-Pacific; C, Mediterranean Sea; D, Caribbean Sea; E, north-eastern Pacific; F, south-eastern Pacific) as they occur on the respective node (see Fig. 9)

	A	B	C	D	E	F
Node 32:	0.000496	0.997955	0.000233	0.000233	0.000233	0.000233
Node 31:	0.000789	0.997684	0.000261	0.000261	0.000261	0.000261
Node 30:	0.002383	0.992587	0.000332	0.000331	0.000332	0.000330
Node 29:	0.010644	0.952183	0.000658	0.000635	0.000655	0.000636
Node 28:	0.000219	0.000401	0.998158	0.000219	0.000219	0.000218
Node 27:	0.000258	0.000544	0.997956	0.000258	0.000258	0.000259
Node 26:	0.000281	0.000897	0.996449	0.000281	0.000281	0.000280
Node 25:	0.000524	0.002264	0.986582	0.000524	0.000551	0.000525
Node 24:	0.000454	0.002042	0.984413	0.000454	0.000479	0.000454
Node 23:	0.000707	0.016417	0.866029	0.000693	0.002141	0.000706
Node 22:	0.001203	0.001203	0.001443	0.313047	0.001566	0.248680
Node 21:	0.000746	0.000746	0.001192	0.400330	0.001486	0.345188
Node 20:	0.000270	0.000270	0.000270	0.000291	0.001030	0.992251
Node 19:	0.000351	0.000351	0.000353	0.001425	0.003627	0.948456
Node 18:	0.001439	0.001439	0.001523	0.044773	0.014086	0.630765
Node 17:	0.000286	0.000286	0.000286	0.000288	0.001745	0.994215
Node 16:	0.000612	0.000612	0.000612	0.000707	0.007849	0.966728
Node 15:	0.000623	0.000634	0.000634	0.005147	0.107744	0.610690
Node 14:	0.001369	0.001652	0.001510	0.002654	0.705789	0.044101
Node 13:	0.000933	0.005843	0.003344	0.001440	0.654385	0.007780
Node 12:	0.002528	0.137095	0.076628	0.001966	0.061316	0.002310
Node 11:	0.000429	0.998119	0.000220	0.000220	0.000220	0.000220
Node 10:	0.000645	0.997498	0.000256	0.000256	0.000256	0.000256
Node 09:	0.001395	0.990044	0.000329	0.000321	0.000328	0.000320
Node 08:	0.001207	0.990435	0.000294	0.000287	0.000293	0.000286
Node 07:	0.004505	0.927508	0.000785	0.000418	0.000743	0.000428
Node 06:	0.015726	0.527524	0.016070	0.001765	0.013845	0.001990
Node 05:	0.140810	0.484475	0.001777	0.000605	0.001601	0.000621
Node 04:	0.912566	0.018464	0.000752	0.000640	0.000736	0.000644
Node 03:	0.991539	0.002039	0.000329	0.000316	0.000328	0.000316
Node 02:	0.997665	0.000481	0.000273	0.000271	0.000273	0.000272
Node 01:	0.998022	0.000283	0.000259	0.000259	0.000259	0.000258

central Chilean *Ismaila* species all descend from a common ancestor (Schrödl, 2002; Haumayr & Schrödl, 2003), and radiated in sympatry (92.5 to 100% support at the respective nodes, see Fig. 9). The temperate south-eastern Pacific *Ismaila* radiation has also given rise to the tropical Caribbean species (Fig. 8) before the closing of the Isthmus of Panama 3 Mya. Probably from a Caribbean ancestor, *I. magellanica* then spread to the Magellan Strait (Figs 8, 9). The historical biogeographical scenario of Splanchnotrophidae reconstructed herein is remarkably well supported by Bayesian analyses, and the inferred migration processes are plausible. Recent distribution patterns and historical biogeography thus are consistent with the backbone of our phylogenetic topology, which can be tested and refined by future molecular systematic studies.

NOTE ADDED IN PROOF

While this paper was in press, a relevant study was published by Uyeno & Nagasawa (2012), with results that are not yet considered in our analyses.

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APPENDIX

The following characters were excluded from the analysis because they were exclusively present in outgroup taxa not considered in the final analysis.

Ecology

110. *Host:* Copepods are parasitic in many groups of organisms. *Anthessius kimjensis* infests bivalves (0), whereas all philoblennid and splanchnotrophid genera are parasitic in gastropod hosts (1).

Female characters

111. *Rostrum formed as grasping structure:* Only in *Shiinoa inauris* is the rostrum formed as a grasping structure (see Cressey & Boyle Cressey, 1980: fig. 38a, b).
112. *Antennule:* Only in *Chondrocarpus reticulosus* is the antennule absent.
113. *Grasping spines:* On the third antennal segment there are grasping spines present in *Micrallecto fusii* (see: Huys, 2001: fig. 13d).
114. *Paragnath lobes:* Only in *Acanthochondria phycidis* do the paragnath lobes on the mandible consist of one naked basal lobe and a spinous terminal lobe (see Ho, 1971: fig. 9b).
115. *Grasping appendage:* A grasping appendage on the maxillule is present in *M. fusii* and absent in all other species included.
116. *Shape of endopodite of the first thoracopod:* The endopodite of the first thoracopod is enlarged in *M. fusii*.
117. *Segmentation of the endopodite of the first thoracopod:* The endopodite of the first thoracopod is three-segmented in *Sh. inauris* and two-segmented in *Ergasilus youngi* Tavares & Luque, 2005.
118. *Second thoracopod:* Only in *Ch. reticulosus* and *M. fusii* is the second thoracopod absent.
119. *Exopodite distinguishable from protopodite:* On the second thoracopod the exopodite is clearly distinguishable from the protopodite in all included species except *Acanthochondria phycidis*, where it is indistinguishable from the protopodite.
120. *Segmentation of the second thoracopod:* The exopodite of the second thoracopod is two-segmented only in *Sh. inauris*.
121. *Dorsal shield:* Only *M. fusii* possesses such a structure (see Huys, 2001 fig. 15).
122. *Surface:* The abdomen has integumental pores in *Acanthochondria phycidis*.

Male characters

123. *Number of setae:* The sixth segment of the antennule is armed with seven setae in *Ergasilus youngi*.

Characters listed below were excluded from the main analysis because too little comparative information was available. However, for future cladistic analysis these characters are likely to be useful once additional information becomes available on character states both in outgroups and within the Splanchnotrophidae.

Female characters

124. *Number of setae on fifth antennulary segment:* Fifth segment of antennule with five setae in *Anthessius kimjensis*.
125. *Quantity of setae:* For cases in which the number of setae on the second antennulary segment is considered to be large, this means 16 (0), 14 (1), or 11 setae (2).
126. *Low quantity of setae:* For cases in which the number of setae on the second segment of the

antennule is considered to be low, this means eight (0), seven (1), four (2), three (3), two (4), or just one seta (5).

127. *Number of setae on the fourth antennulary segment:* Fourth segment of antennule with eight setae (0), six (1), four (2), three (3) or two setae (4).
128. *Number of spines on the second segment of the antenna:* The second segment of the antennule is armed with three (0) or two spines (1).
129. *Distal segment with two constrictions:* Two constrictions on the distal segment of the antennule (see Salmen, 2005: fig. 4d) are absent (0) or present (1).
130. *Second segment of the antenna:* All included species possess an antenna that is at least two-segmented.
131. *Shape of setae:* The seta on the second antennal segment is stubby (see Haumayr & Schrödl, 2003: fig. 14b) (0) or short and thin (see Huys, 2001: fig. 11c) (1).
132. *Shape of antenna:* The antenna is large and bent forwards (0), robust (1), or small, plump, and fleshy (2).
133. *Number of setae on the first segment of the antenna:* First segment of antenna with three setae (0) or one seta (1).
134. *Number of setae on the second segment of the antenna:* Second segment of antenna with two setae (0) or one seta (1).
135. *Spine present:* There is a spine present on the second antennal segment (0) or not (1).
136. *Shape of third segment:* The third segment of the antenna is claw-shaped (see Huys, 2001: fig. 8c) (0) or hook-shaped (see Haumayr & Schrödl, 2003: fig. 11b) (1).
137. *Shape of setae:* The setae on the third segment of the antenna are described as long in *S. angulatus* and in *Lomanoticola* sp.
138. *Armament of hole:* Several spines surround the small hole on the third segment of the antenna.
139. *Size of labrum:* The labrum is large (0) or small (1).
140. *Shape of labrum:* The labrum is triangular (0), elongate (1), or inverted U-shaped (2).
141. *Morphology of surface:* The labrum is an arched plate with a smooth surface (0) or swollen bearing an irregular pattern of pores (1).
142. *Direction of curved labrum:* In *Ceratosomicola sacculata* O'Donoghue, 1924 (as *Splanchnotrophus sacculatus*) the labrum is curved ventrally.
143. *Second ramus:* The second ramus of the mandible has a minute terminal claw in all *Ismaila* species.
144. *Shape of processes:* The processes on the mandible are formed as teeth (dentiform) (0), as thorns (1), or as spinules or bristles (2).
145. *Shape of maxillule:* The maxillule is thick in *Sp. gracilis* and small in *B. doliaris*.
146. *Terminal elements:* The maxillule bears terminal elements (0) or does not (1).
147. *Shape of second segment:* The second segment of the maxilla is long (0), slender (1), or short (2).
148. *Number of setae:* On the second segment of the maxilla there are three (0) or two setae (1), or just one seta (2).
149. *Tip shape:* The tip of the maxilla has a terminal claw (see Ho & Kim, 1992: fig. 5j) (0) or it is formed as a hook (see Monod, 1928: fig. 8d) (1).
150. *Labium shape:* The labium is either tongue-shaped (see Salmen *et al*., 2008a: fig 11d) (0) or it is produced into paired spinose lobes (see Huys, 2001: fig 12a) (1).
151. *Sides of labium:* The sides of the labium have small triangular processes (0) or they are large with a hairy patch (1).
152. *Slit:* There is a zig-zag-shaped slit running over one third of the labium (see Salmen *et al*., 2008b: fig. 4a) (0) or a deep vertical slit at the posterior edge of the labium (see Salmen *et al*., 2008b: fig. 7d, f) (1).
153. *Armament of second segment:* The second segment of the maxilla is armed with spines and setae in *Anthessius kimjensis* and in *Mytillicola porrecta*.
154. *Number of spinules on the protopodite:* The protopodite of the second thoracopod has only one spinule in *An. kimjensis*.
155. *Number of setae on the second segment:* The second exopodal segment of the second thoracopod bears one seta in *B. doliaris* and in *An. kimjensis*.
156. *Number of rami:* The exopodite of the second thoracopod is biramous (0) or uniramous (1).
157. *Lengths of exopodite and endopodite:* The exopodite and the endopodite of the second thoracopod are of equal length (0) or the exopodite is longer (1).
158. *Number of spines on second segment:* The second segment of the second thoracopod bears four spines (0) or just one (1).
159. *First segment:* The first segment of the endopodite of the second thoracopod has three setae (0), one seta (1), or it is unarmed (2).
160. *Setae on third segment:* The third segment of the second thoracopod bears five (0) or just two setae (1).
161. *Second segment:* The second endopodal segment of the second thoracopod bears one seta in *An. kimjensis*.

162. *Spines on third segment:* On the third segment of the endopodite of the second thoracopod only one spine is present in *An. kimjensis*.
163. *Constrictions:* There are multiple constrictions on the exopodite of the second thoracopod and one at about midlength of the endopodite (0) or there are two indistinct constrictions (1) (see Huys, 2001: figs 1d, 5d).
164. *First segment of second thoracopod:* The first segment of the endopodite of the second thoracopod bears one seta in *An. kimjensis*.
165. *Second segment of second thoracopod:* The second segment has two setae in *An. kimjensis*.
166. *Third segment of second thoracopod:* On the third segment there are three spines and three setae in *An. kimjensis*.
167. *Exopodite (segmentation):* In *An. kimjensis* the exopodite is three-segmented.
168. *First segment:* In *An. kimjensis* the first segment of the third thoracopod bears one spine.
169. *Second segment:* The second segment of the third thoracopod bears one spine and one seta in *An. kimjensis*.
170. *Segmentation:* The third thoracopod is three-segmented (0) or one-segmented (1).
171. *Shape of tip of exopodite:* The tips of the elements forming the exopodite of the third thoracopod are three short elements that taper into a terminal claw (0) or the tips are hook-shaped (1).
172. *Appendages:* The exopodite of the third thoracopod has two apical, claw-shaped elements (see Salmen, 2005: fig. 22c) (0), one single seta on a faint ridge (see Huys, 2001: fig. 12e) (1), or it has one hyaline element along its internal margin (see Huys, 2001: fig. 2d) (2).
173. *Armament:* On the endopodite of the third thoracopod there are two minute subapical spines (0) or a basal, small, thin process (1).
174. *First segment (setae present):* The first segment of the endopodite of the third thoracopod bears setae (0) or does not (1).
175. *Armament:* The first segment of the third thoracopod bears two setae (1) or just one seta (2).
176. *Spines on the exopodite:* There are three pointed spines on the exopodite of the third thoracopod (0), one recurved spine apically (1), or one small spine at the base (2).
177. *Endopodite (segmentation):* The endopodite of the third thoracopod is three-segmented in *Acanthochondria phycidis* and one-segmented in *Sp. angulatus* and *S. gracilis*.
178. *Thickness (compared to second thoracopod):* The exopodite of the third thoracopod is thicker than the exopodite of the second thoracopod (0) or it is less voluminous than that of the second thoracopod (1).
179. *Spines:* The exopodite of the third thoracopod bears three short spines (0) or only one minute spine (1).
180. *Armament:* The third segment of the third thoracopod bears two setae (0) or just one seta (1).
181. *Armament (third segment):* The third segment of the fourth thoracopod bears four spines and two setae in *An. kimjensis*.
182. *State of development (fifth thoracopod):* The fifth thoracopod is very small and rudimentary in many *Ismaila* species.
183. *State of development (sixth thoracopod):* The sixth thoracopod is represented by two small elements in *Ismaila occulta*.
184. *Number of segments:* The thorax is two-segmented in *Ceratosomicola delicata* Salmen, 2008.
185. *Bulges:* There are two transversal bulges on the thorax (0), three transversal bulges dorsally (1), or there are bulges on the sides of the thorax (2).
186. *Sites of the fourth pair:* The thoracic processes are situated dorsally (0), laterally (1), or dorsolaterally (2).
187. *Sites of the fifth pair:* The thoracic processes are situated laterally (0) or dorsolaterally (1).
188. *Pores:* Both urosomites have numerous integumental pores (0) or there are two pores along the outer margin (1).
189. *Shape of setae:* The setae of the caudal rami are enlarged (0), pinnate (1), or small (2).
190. *Number of spines:* On the caudal rami there are five spines (0), three (1), two spines (2), or one minute spine on each ramus (3).

Male characters

191. *Swelling:* Dorsally on the cephalothorax there is a large, hemisphere-shaped swelling (see Salmen *et al*., 2008b: fig. 5a).
192. *Armament of first segment:* The first segment of the antennule bears one seta in *An. kimjensis* and in *I. occulta*.
193. *Armament of second segment:* The second segment of the antennule bears 16 (0), 14 (1), 13 (2), or just one seta (3).
194. *Armament of third segment:* The third segment of the antennule bears four setae in *An. kimjensis*.
195. *Armament of fourth segment:* The fourth antennular segment bears four setae (0) or one seta (1).
196. *Armament of fifth segment:* The fifth antennular segment bears five (0) or three setae (1).

197. *Armament of sixth segment:* The sixth antennular segment bears eight (0), three (1), or two setae (2).
198. *Armament of seventh segment:* The seventh segment of the antennule bears eight setae in *An. kimjensis*.
199. *Size of antenna:* The antenna is large (0) or slender (1).
200. *Second segment:* The second antennal segment bears one seta in *I. occulta*.
201. *Morphology:* In *Arthurius bunakenensis* the second segment of the antenna is drawn out into a claw.
202. *Dentiform processes:* Dentiform processes on the mandible are present (see Suh, 1993: fig. 6) (0) or absent (1).
203. *Mandible:* The mandible is styliform with an enlarged base and a recurved blade (0), it has setiform elements (1), or it consists of a small rod tipped with a short tooth and a slender spine (2).
204. *Segmentation:* The maxillule is two-segmented in *I. belciki* and in *Ce. delicata*.
205. *Second segment:* The second segment of the maxillule is present (0) or absent (1).
206. *Shape of body processes:* In *I. belciki* and *Arthurius elysiae* there are two pairs of ventral appendages.
207. *State of exopodite:* The exopodite of the second thoracopod has five (0) or four spines (1).
208. *Segmentation of exopodite:* The exopodite of the second thoracopod is two-segmented (0) or one-segmented (1).
209. *Exopodite:* The exopodite of the second thoracopod is incompletely two-segmented in *Ar. elysiae*.
210. *Surface:* The surface of the second thoracopod has a spinular pattern in both species of *Arthurius*.
211. *Third thoracopod:* The third thoracopod of *An. kimjensis* is three-segmented.
212. *Length of exopodite:* The exopodite of the third thoracopod is long (0) or minute (1).
213. *Length of podites:* The exopodite of the third thoracopod is much longer than the endopodite (0) or the endopodite is nearly as long as the exopodite (1).
214. *Bases of processes:* The processes arise from separate bases (0) or from a common base (1).
215. *First segment:* The first abdominal segment forms the genital lobes armed with two spinous setae (0) or it has paired apertures forming a common median genital slit without armature (1).
216. *Setae of caudal rami:* The caudal rami bear seven (0), four (1), two or three (2), or one single seta (3).
217. *Anal opening:* The anal opening is present between the caudal rami (0) or absent (1).

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Zoological Journal of the Linnean Society, 2015, **175**, 439. With 1 figure

ERRATUM

The gastropod–crustacean connection: towards the phylogeny and evolution of the parasitic copepod family Splanchnotrophida

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In the article by Anton and Schrödl (2013), which appeared in the April 2013 issue of *Zoological Journal of the Linnean Society*, Figure 4 was found to contain errors.

The correct figure should be:

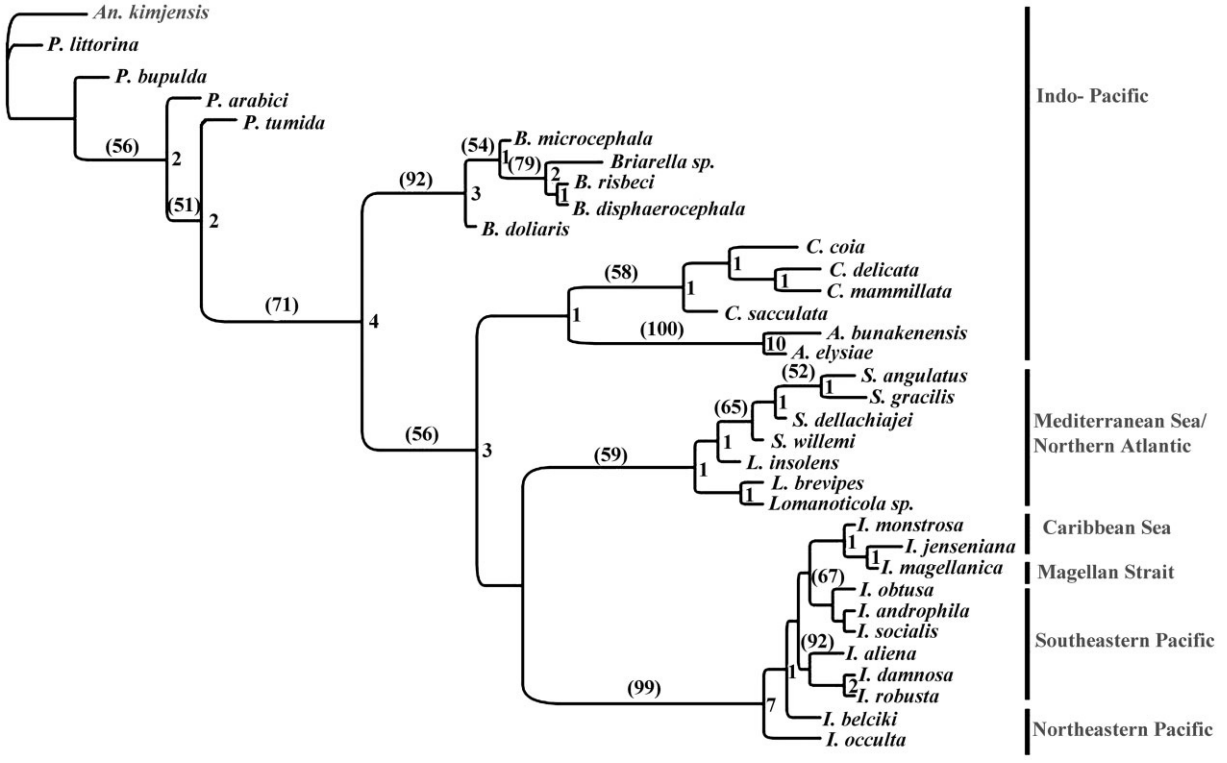


Figure 4. Phylogeny of Splanchnotrophidae. Strict consensus tree of the main parsimony analysis with bootstrap support (> 50, in parentheses) and Bremer decay values. Geographical distributions are indicated according to major regions. Branch length reflects number of character-state changes.

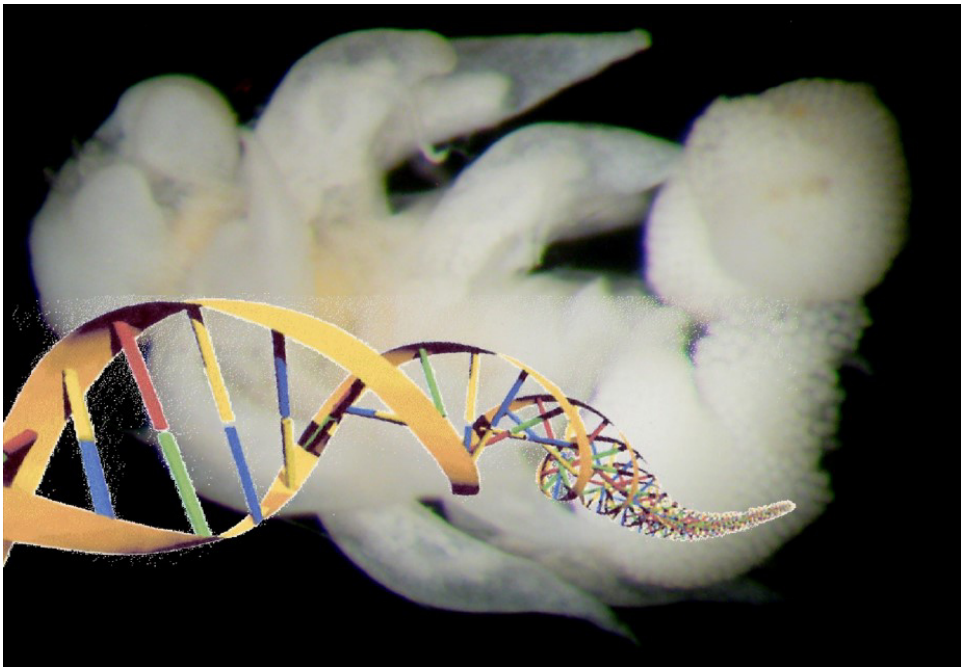
The Publisher apologizes for this error.

REFERENCE

Anton RF, Schrödl M. 2013. The gastropod–crustacean connection: towards the phylogeny and evolution of the parasitic copepod family Splanchnotrophida. *Zoological Journal of the Linnean Society* **167:** 501–530.

Chapter 4

Anton, R. F., Schories, D., Wilson, N. G., Wolf, M., Abad, M. & Schrödl, M. (2016), Host specificity versus plasticity: testing the morphology-based taxonomy of the endoparasitic copepod family Splanchnotrophidae with COI barcoding; Journal of the Marine Biological Association doi:10.1017/S002531541600120X



Journal of the Marine Biological Association of the United Kingdom, page 1 of 13.
doi:10.1017/S002531541600120X

Host specificity versus plasticity: testing the morphology-based taxonomy of the endoparasitic copepod family Splanchnotrophidae with COI barcoding

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The Splanchnotrophidae is a family of highly modified endoparasitic copepods known to infest nudibranch or sacoglossan sea slug hosts. Most splanchnotrophid species appear to be specific to a single host, but some were reported from up to nine different host species. However, splanchnotrophid taxonomy thus far is based on external morphology, and taxonomic descriptions are, mostly, old and lack detail. They are usually based on few specimens, with intraspecific variability rarely reported. The present study used molecular data for the first time to test (1) the current taxonomic hypotheses, (2) the apparently strict host specificity of the genus Ismaila *and (3) the low host specificity of the genus* Splanchnotrophus *with regard to the potential presence of cryptic species. Phylogenetic analyses herein used sequences of the barcoding region of the cytochrome oxidase I (COI) gene from 40 specimens representing 13 species of five genera. Species delimitation approaches include distance and barcoding gap analyses, haplotype networks and diagnostic nucleotides. Molecular results are largely compatible with the commonly accepted, morphology-based taxonomy of the Splanchnotrophidae. Strict host specificity could be confirmed for two* Ismaila *species. COI analyses also supported the idea that* Splanchnotrophus angulatus *is host-promiscuous. In* Ismaila*, morphology seems more suitable than barcoding to display speciation events via host switches in a recent Chilean radiation. In* Splanchnotrophus*, some genetic structure suggests ongoing diversification, which should be investigated further given the inadequate morphology-based taxonomy. The present study thus supports the presence of two different life history strategies in splanchnotrophids, which should be explored integratively.*

Keywords: species delimitation, molecular phylogeny, DNA taxonomy, speciation, Copepoda, sea slugs, parasite

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INTRODUCTION

Copepods are the most abundant and speciose group in marine habitats (Yoshikoshi, 1975; Ho, 2001; Blanco-Berical *et al.*, 2014) and they also display the greatest variety of forms (Gotto, 1979, 2004; Ho, 2001; Blanco-Berical *et al.*, 2014). Endoparasitic copepods often exhibit extremely aberrant body forms due to the high level of adaptation to their respective host (Gotto, 1979, 2004; Huys, 2001; Haumayr & Schrödl, 2003; Anton *et al.*, 2015). Such is the case in Splanchnotrophidae Hancock & Norman, 1863, a family of bizarre endoparasitic copepods exclusively infesting nudibranch and sacoglossan hosts. The family is distributed worldwide in temperate and warm coastal waters and currently comprises six genera: *Splanchnotrophus* Hancock & Norman, 1863, *Ismaila* Bergh, 1867, *Lomanoticola* Scott & Scott, 1895, *Arthurius* Huys, 2001, *Ceratosomicola* Huys, 2001 and *Majimun* Uyeno & Nagasawa, 2012, with a total of now 32 species (Anton *et al.*, 2015). All members are characterized by an enhanced body size in females, the possession of dorsal appendages (with one exception, see Anton *et al.*, 2015), the reduction of the maxillipeds, and the abdomen of females protruding through the host's integument (Huys, 2001; Anton & Schrödl, 2013a, b).

The taxonomy of Splanchnotrophidae is exclusively based on external morphology, with descriptions offering a highly heterogeneous level of detail and reliability. In addition, the use of external morphological characters in highly modified endoparasitic taxa has to be regarded as problematic at best (Huys, 2001). In such a case, the differentiation between true homoplasies and convergent evolution is rather complex. Most splanchnotrophids (i.e. 25 species; 78%) are

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considered to be highly host specific, and usually each host species is infested by a single parasite species (Schrödl, 1997, 2003; Huys, 2001; Haumayr & Schrödl, 2003; Anton & Schrödl, 2013a, b); identification of an infested host thus may permit identification of their parasite. Interestingly, all members of the species-rich and recently reviewed genera *Ismaila* and *Ceratosomicola* are strictly host specific. A recent radiation of Chilean *Ismaila* species via host shifts was proposed (Schrödl, 2003; Anton & Schrödl, 2013a, b). However, some splanchnotrophids are reported from multiple hosts (Figure 1A). The recently revised or described genera *Arthurius* and *Majimun* (Huys, 2001; Salmen *et al.*, 2008; Uyeno & Nagasawa, 2012) comprise a few species that are host specific and others that infest multiple host species. Similarly, five of nine species of the taxonomically obscure genera *Splanchnotrophus* and *Lomanoticola* are reported from more than one (i.e. up to nine) different species (Figure 1A) of not necessarily closely related sea slug groups (Anton & Schrödl, 2013a). For example, *Lomanoticola brevipes* (Hancock & Norman, 1863) was reported infesting members of the dexiarchian nudibranch family Dotidae, but was also found in representatives of the aeolid families Flabellinidae, Tergipedidae, Facelinidae and Eubranchidae. Different splanchnotrophid genera and species thus display different patterns of host specificity, possibly reflecting phylogenetic constraints on their ability to detect, colonize or survive in different hosts (Anton & Schrödl, 2013a, b). It is also striking that five of the seven splanchnotrophids known from more than one host species occur exclusively in the Mediterranean Sea and along the European coasts of the Atlantic ocean (Figure 1B). These areas are among the earliest and most intensely studied with regard to marine invertebrates. However, neither the parasites nor their hosts are of apparent commercial value, and original or subsequent descriptions of European splanchnotrophids are typically old and usually based on single individuals with no adequate vouchers deposited for later study (Canu, 1891; Hecht, 1895; Bassett-Smith, 1903; O'Donoghue, 1924; Delamare Debouteville, 1950).

Estimates of host specificity in splanchnotrophid copepods, and conclusions on the presence, ecology and evolution of highly heterogeneous specificity in different genera and geographic areas entirely depend on taxonomic identifications of parasites and hosts. On the host side, taxonomy appears straightforward, although the existence of cryptic species has only been tested by molecular data for two complexes. Both the *Cratena peregrina* (Gmelin, 1791) (Padula *et al.*, 2014) and the *Spurilla neapolitana* (Delle Chiaje, 1841) (Carmona *et al.*, 2014) complexes were split up using integrative taxonomic evidence. To date, splanchnotrophid taxonomy is exclusively based on (external) morphology, and little is known about intrapopulational variation (Anton & Schrödl, 2013a, b); taxonomically relevant features such as special details of mouth parts are unknown for several species, i.e. several but not all of the species described to inhabit different hosts (Huys, 2001; Haumayr & Schrödl, 2003; Anton & Schrödl, 2013a, b). In general, the morphology of endoparasites can be especially adapted to their environment, i.e. conditions in their hosts (Gotto, 1979; Huys, 2001). For example, large-sized hosts may allow for longer body lengths, and the morphology of the host may affect the position of the parasites inside the hosts. Therefore, it is a crucial task to evaluate phenotypic splanchnotrophid taxonomy using genetic data, testing the assumption of narrowly adapted parasite species against host-induced plasticity. Anton & Schrödl (2013a, b) provided a morphocladistic hypothesis on the phylogeny of splanchnotrophids and also proposed a preliminary scenario of character evolution and coevolution of splanchnotrophids with certain host groups. Since parts of the tree were not robustly supported, investigating historic and recent coevolution requires molecular analyses. DNA sequence data for splanchnotrophids has been lacking entirely, due to the difficulty of collecting and preserving a variety of rare or at least sporadic endoparasites.

The present study for the first time uses molecular data to (1) test the current taxonomic hypotheses on Splanchnotrophidae introduced by Huys (2001) and recently confirmed by morphocladistic analysis (Anton & Schrödl, 2013a, b); (2) test the strict host specificity reported for the genus *Ismaila* (potentially leading to the highest species diversity of all splanchnotrophid genera) against undiscovered host-induced phenotypic plasticity; and (3) evaluate the supposedly low host specificity of *Splanchnotrophus* against the possibility of the presence of cryptic species.

To test general taxonomic hypotheses, phylogenetic analyses were conducted, using 38 novel barcode sequences of the cytochrome oxidase I (COI) gene from 12 morphospecies, covering four splanchnotrophid genera. To further study host specificity, species delimitation analyses were performed focusing on two supposedly strictly host-specific species of *Ismaila* (*Ismaila aliena* Haumayr & Schrödl, 2003, *Ismaila robusta* Haumayr & Schrödl, 2003) and on *Splanchnotrophus angulatus* Hecht, 1893, a species currently known from five different host species. Here, a variety of molecular methods complement and extend the traditional view on species boundaries in splanchnotrophids, and allows for a preliminary integrative view on life history traits such as host specificity.

MATERIALS AND METHODS

Species sampling

For molecular analyses all ethanol-fixed splanchnotrophid samples available in the collection of the Bavarian State Collection of Zoology (ZSM) were used to obtain genetic material. Additional samples of *I. aliena, I. robusta* and *S. angulatus* were gathered during several collection trips to southern Chile in 2008 and 2010, and to southern France in 2010. Wherever possible, egg sacs were carefully removed from the host using forceps as soon as possible after collection. Samples were then stored in 96% ethanol and kept chilled until the DNA extraction was performed. A detailed list of all included specimens is given in Table 1.

DNA extraction, amplification and sequencing

We used a NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) and extraction procedures followed manufacturers' instructions. Universal primers LCO-1490 (forward) and HCO-2198 (reverse) (Folmer *et al.*, 1994) were used to amplify a ~650 bp segment of the cytochrome oxidase I (COI) gene. For amplification Illustra PuRe Taq Ready-To-Go PCR beads (GE Healthcare) were used. A mix of 0.5 µl of each primer (conc. 10 pm, Metabion) plus 23 µl of molecular water was added to 1.0 µl of raw DNA. For PCR conditions

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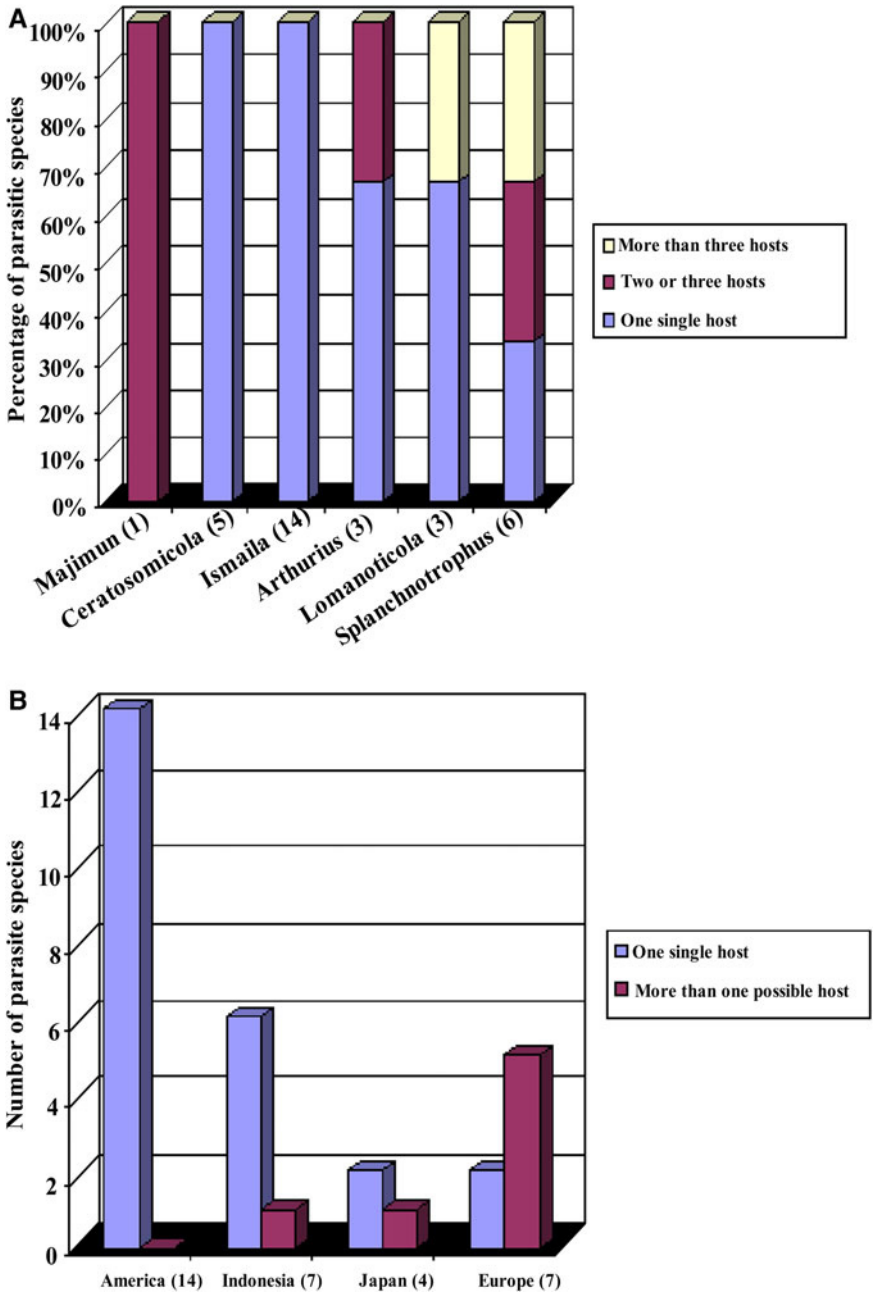

Fig. 1. Overview of the number of host species (A) per splanchnotrophid genus and (B) given the geographic distribution area following Anton & Schrödl (2013a, b). Total number of species given in parentheses.

we applied 94°C – 300 s for the initial step, then 94°C – 45 s, 45°C – 50 s, 72°C – 200 s for 40 cycles, with a final elongation of 72°C – 600 s. For purification of the PCR-product a NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) was used following the manufacturer's instructions. The complete sequencing process was carried out on an ABI 3730 48 capillary sequencer by the Sequencing Service Unit of the Ludwig-Maximilians-University Munich. All sequence amplicons were subjected to a nucleotide BLAST search to test for contamination.

Phylogenetic analysis

COI fragments of 38 splanchnotrophid specimens (12 species from four genera) were obtained. Outgroups included *Pionodesmotes domhainfharraigeanus* Anton, Stevenson & Schwabe, 2013 (GenBank accession no. KF652042) and *Cyclopoida* sp. (JX948803.1) (see also Table 1). Consensus sequences were generated with BioEdit (Hall, 1999), edited, translated into amino acid sequences using the invertebrate mitochondrial genetic code, checked for stop codons and frame shifts, and aligned with Muscle using the Mega 5.0 software (Tamura *et al.*, 2011). The alignment then was masked by Gblocks (Castresana, 2000; Talavera & Castresana, 2007) applying less stringent options; substitutional saturation was statistically tested using Dambe (Xia *et al.*, 2003; Xia & Lemey, 2009); base pair frequencies and p-distances were calculated with Mega 5.0.

A maximum likelihood (ML) analysis with 1000 bootstrap (BS) replicates was conducted with RAxML (Stamatakis,

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Table 1. Overview of all included specimens giving the registration number, the host specimens and the country and exact location of the collection site respectively.

Voucher ID	GenBank accession number	Species	ZSM-ID	Host	ZSM-ID	Country/Region	Latitude	Longitude	Depth (m)
G 001	KT122805	*Splanchnotrophus angulatus*	ZSMA20142906	*Flabellina ischitana*	ZSM-Mol10100477	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 002	KT122806	*Splanchnotrophus angulatus*	ZSMA20142907	*Spurilla neapolitana*	ZSM-Mol20100409	Croatia/Mala Portic	44°46′45.15″N	13°55′10.84″O	2–5
G 003	KT122807	*Splanchnotrophus angulatus*	ZSMA20142908	*Spurilla neapolitana*	ZSM-Mol20100409	Croatia/Mala Portic	44°46′45.15″N	13°55′10.84″O	2–5
G 004	KT122808	*Splanchnotrophus angulatus*	ZSMA20142909	*Cratena peregrina*	ZSM-Mol20130874	Islote 5.6.1998			
G 005	KT122809	*Splanchnotrophus angulatus*	ZSMA20142910	*Aeolidiella alderi*	ZSM-Mol20070272				
G 006	KT122810	*Ismaila robusta*	inside host	*Phidiana lottini*	ZSM-Mol20110432	Southern Chile/Playa Chica	39°43′10″S	73°24′12″W	2
G 011	KT122812	*Splanchnotrophus angulatus*	ZSMA20142912	*Cratena peregrina*	host lost	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 012	KT122813	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130849	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 013	KT122814	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130850	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 015	KT122815	*Ismaila genalis*	ZSMA20142903	*Holoplocamus papposus*	ZSM-Mol20130872	Southern Chile/Isla Carmen	43°01′08.80″S	72°49′44.79″W	1–20
G 016	KT122816	*Ismaila belciki*	ZSMA20142916	*Janolus fuscus*	host lost	USA/Oregon	43°21′32.4″N	124°18′45.36″W	0–2
G 017	KT122817	*Ismaila volatilis*	ZSMA20142900	*Janolus* spec.	ZSM-Mol20130847	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–20
G 019	KT122818	*Ismaila aliena*	ZSMA20142918	*Thecacera darwini*	ZSM-Mol20130851	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 020	KT122819	*Ismaila aliena*	ZSMA20142919	*Thecacera darwini*	ZSM-Mol20130851	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 021	KT122820	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130852	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 022	KT122821	*Ismaila robusta*	ZSMA20142921	*Phidiana lottini*	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 023	KT122822	*Ismaila robusta*	ZSMA20142921	*Phidiana lottini*	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 024	KT122823	*Ismaila robusta*	ZSMA20142923	*Phidiana lottini*	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 025	KT122824	*Splanchnotrophus angulatus*	inside host	*Spurilla neapolitana*	ZSM-Mol20110684	Italy/Bastione Conca	38°01′03″N	12°30′14″E	2–5
G 028	KT122825	*Ismaila robusta*	ZSMA20142925	*Phidiana lottini*	ZSM-Mol20130855	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 029	KT122826	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130856	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 030	KT122827	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130856	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 031	KT122828	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130857	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 032	KT122829	*Ismaila chaihuiensis*	ZSMA20142902	*Diaulula punctuolata*	ZSM-Mol20130858	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 034	KT122830	*Ismaila damnosa*	ZSMA20142905	*Flabellina* sp. 1	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	12
G 035	KT122831	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130860	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 036	KT122832	*Splanchnotrophus angulatus*	ZSMA20142930	*Cratena peregrina*	host lost	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 038	KT122833	*Lomanoticola* spec.	ZSMA20142931	*Cuthona cerulea*	ZSM-Mol20130862	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 042	KF652042	*Pionodesmotes domhainfharraigeanus*	ZSMA20130004	*Sperosoma grimaldii*	host lost	Ireland/Whittard Canyon	48.491°N	10.692°W	2000
G 044	KT122834	*Ceratosomicola mammilata*	inside host	*Chromodoris geometrica*	ZSM-Mol20130863	Indonesia/Sulawesi	5°28′29″S	123°45′40″E	4
G 046	KT122835	*Splanchnotrophus gracilis*	ZSMA20142933	*Trapania tartanella*	host lost	Spain/Ria de Ferrol	43°28′02.16″N	8°14′47.70″W	20
G 055	KT122836	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130864	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 056	KT122837	*Splanchnotrophus angulatus*	ZSMA20142935	*Cratena peregrina*	ZSM-Mol20130865	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 057	KT122838	*Ismaila robusta*	ZSMA20142936	*Phidiana lottini*	host lost	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 058	KT122839	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130867	Southern France/Banyuls	42°28′56.20″N	3°08′13.19″O	2–5
G 059	KT122840	*Ismaila robusta*	ZSMA20142938	*Phidiana lottini*	ZSM-Mol20130868	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 060	KT122841	*Ismaila robusta*	inside host	*Phidiana lottini*	ZSM-Mol20130869	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–10
G 082	KT122842	*Ismaila volatilis*	inside host	*Janolus* sp.	ZSM-Mol20130866	Southern Chile/Valdivia	39°57′25.94″S	73°36′10.15″W	6–20
G 100	KT122811	*Ismaila* spec.	inside host	*Eubranchus* sp. 2	ZSM-Mol20130871	Southern Chile/Isla Traiguen	45°11′26.11″S	73°30′49.69″W	6

2014) using the GTRCAT model. Bayesian inference (BI) with MrBayes (Ronquist & Huelsenbeck, 2003) used the invertebrate mitochondrial code, the codon nucleotide model, and 2 million generations, with a sampling frequency of 500 generations. In addition neighbour network graphs were calculated using SplitsTree4 (Huson & Bryant, 2006) to check for incompatibilities within the data.

Detection of barcode gaps, haplotype networks and diagnostic nucleotides

For the genera *Splanchnotrophus* and *Ismaila* a search for barcode gaps was performed using alignments of all sequences of the respective genera and the Abgd-software (Puillandre *et al.*, 2011, 2012), which sorts the sequences into hypothetical species based on the barcode gap, which can be observed whenever the divergence among organisms belonging to the same species is smaller than divergence among organisms from different species. A second approach, Species Identifier (Meier *et al.*, 2006), was used to calculate pairwise distances (see Table 2) and clusters that identify potential species. A third approach was also used, a Poisson Tree Processes (PTP) model (Zhang *et al.*, 2013) provided on the webserver of The Exelixis Lab (URL: http://sco.h-its.org/exelixis/web/software/PTP/index.html), with default settings of 100,000 MCMC generations and a burn-in of 0.1. Furthermore, a statistical parsimony network was conducted on all 13 sequences of *S. angulatus* and on the 19 sequences representing the genus *Ismaila* using the Tcs 1.2 software (Clement *et al.*, 2009). Diagnostic characters were obtained through searching the overall alignment following the definition given by Sarkar *et al.* (2008) for single pure and single private characters.

RESULTS

Phylogenetic hypothesis

The final COI alignment consisted of 615 bp, including 38 splanchnotrophid specimens (12 morphologically defined species from four genera) and two outgroup taxa. In Splanchnotrophidae, the mean base pair frequencies for T (34.8%), C (19.5%), A (25.2%) and G (20.5%) reflected the bias towards adenosine and thymine which is characteristic for arthropods (Weis & Melzer, 2012). The index of substitution saturation (Iss) was tested for the whole alignment after Xia & Lemey (2009) with an estimated proportion of invariant sites of 0.54; this was significantly lower than the critical Iss.c value, indicating no substitutional saturation.

Although the neighbour network built with the SplitsTree4 software revealed some conflict within the clades of *Ismaila* and *S. angulatus*, there were very few incompatible splits within the data (Figure 2A). Regarding *Splanchnotrophus*, the specimens parasitizing the nudibranchs *S. neapolitana* and *A. alderi* were recovered as strictly separated to a group including all those utilizing *C. peregrina* or *F. ischitana* as hosts (see Figure 2B). On the other hand, *I. belciki* was recovered as the most basal sister taxon to all other members of the genus. In addition there was split support for a group comprising *I. volatilis, Ismaila* sp. and *I. damnosa*, with *I. chaihuiensis* as a basal offshoot (see Figure 2C).

Both ML and BI analyses led to two similar trees, only differing in two regions. In both analyses the Splanchnotrophidae are recovered as a clade with high support (BS 100/BI 1). *Ceratosomicola mammilata* Salmen, Wilson & Schrödl, 2008 formed the highly supported (BS 100/BI 1) sistergroup to the rest, followed by *Splanchnotrophus gracilis* Hancock & Norman, 1863; then all members of *S. angulatus* was recovered as the sister clade to a poorly supported clade formed by *Lomanoticola* and the monophyletic genus *Ismaila* (BS 100/BI 1). Inside the monophyletic (BS 100/BI 1) *S. angulatus* most of the sequences from specimens found in the aeolid nudibranch host *Cratena peregrina* (Facelinidae) clustered together with one sequence from a specimen extracted from the aeolid *Flabellina ischitana* Hirano & Thompson, 1990 (Flabellinidae). However in the ML analysis the clade resulted as a trichotomy consisting of sequence G11, a clade comprising of the three sequences G02, G03 and G25 (infesting the aeolid *Spurilla neapolitana*; Aeolidiidae) together with the sequence G05 (infesting *Aeolidiella alderi* (Cocks, 1852); Aeolidiidae) and a clade with the rest of the sequences as described above (Figure 3). In contrast, the BI analysis recovered a subclade consisting of the sequences G02, G03, G05, G11 and G25 originating from a polytomy formed by the rest of the sequences as described above (Figure 4).

The topologies recovered for the *Ismaila* clade were similar in both analyses with *I. aliena* and *I. robusta* both strongly supported individually and as a sister group. However, the results of the ML analysis suggested a clade with *Ismaila chaihuiensis* Anton, Schories, Jörger, Kalagis & Schrödl, 2015 as its most basal offshoot to a dichotomy of a clade consisting of undescribed *Ismaila* sp. and *Ismaila damnosa* Haumayr & Schrödl, 2003 and a clade comprising *Ismaila volatilis* Anton *et al.*, 2015 and *Ismaila genalis* Anton *et al.*, 2015, forming the sister to the clade of *I. aliena* and *I. robusta* (Figure 3), but with only low support values. In contrast, BI favoured a polytomy of *I. volatilis, I. genalis*, a clade comprising *I. damnosa* and *Ismaila* sp. and a dichotomy of *I. aliena* and *I. robusta*. Within *I. robusta* three sequences (G22, G24 & G28) formed a subclade with moderate support (BS62/BI96, see also Figure 4) in both analyses.

Distances and barcode gaps

P-distances between the included splanchnotrophid genera are given in Table 2. Within genera the ABGD-analyses revealed strong barcode gaps. In *Ismaila*, ABGD favoured five groups: group 1 consists of *Ismaila* sp., *I. genalis, I. volatilis* and *I. damnosa*; group 2 represents *I. robusta*; group 3 represents *I. aliena*; group 4 *I. belciki* and group 5 *I. chaihuiensis*. For the genus *Splanchnotrophus* the ABGD-analyses also revealed a strong barcode gap between *S. angulatus* and *S. gracilis*, but between $P = 0.0010$ and $P = 0.0046$ the sequences formed three different groups, with two sequences separated from the rest of the *S. angulatus* group. Excluding *S. gracilis*, ABGD still favoured this split within *S. angulatus*; however, there is no clear detectable barcode gap.

The software Species Identifier found 12 clusters, under a threshold of 2.42%, calculated from a pairwise summary. Clusters 1 and 10 represent the two outgroup taxa. Cluster 2 included all *S. angulatus* sequences and cluster 3 represented *I. robusta*. Cluster 4 included all sequences of *I. volatilis, Ismaila* sp. and *I. damnosa*. Clusters 5, 6, 7, 8 and 9 represented the species *I. aliena, I. genalis, I. belciki, I. chaihuiensis*

Table 2. Data output of pairwise distances calculated with SPECIES IDENTIFIER.

Sequence name	Largest conspecific match	Distance	Overlap	Closest congeneric. interspecific match	Distance	Overlap
Cyclopoida sp.	No matching conspecific sequence	N/A	N/A	No matching congeneric, interspecific sequence	N/A	N/A
G01 *Splanchnotrophus angulatus* F	G03 *Splanchnotrophus angulatus* S	2.19	638	G46 *Splanchnotrophus gracilis*	15.55	643
G02 *Splanchnotrophus angulatus* S	G12 *Splanchnotrophus angulatus* C	2.73	657	G46 *Splanchnotrophus gracilis*	16.19	667
G03 *Splanchnotrophus angulatus* S	G12 *Splanchnotrophus angulatus* C	2.73	657	G46 *Splanchnotrophus gracilis*	16.46	662
G04 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus* angulatus S	2.42	660	G46 *Splanchnotrophus gracilis*	15.6	660
G05 *Splanchnotrophus angulatus* A	G03 *Splanchnotrophus angulatus* S	1.81	662	G46 *Splanchnotrophus gracilis*	14.39	667
G06 *Ismaila robusta*	G57 *Ismaila robusta*	0.62	639	G13 *Ismaila aliena*	4.06	639
G100 *Ismaila* sp.	No matching conspecific sequence	N/A	N/A	G34 *Ismaila damnosa*	1.48	672
G11 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	2.11	662	G46 *Splanchnotrophus gracilis*	15.14	667
G12 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	2.73	657	G46 *Splanchnotrophus gracilis*	15.67	657
G13 *Ismaila aliena*	G21 *Ismaila aliena*	0.44	671	G23 *Ismaila robusta*	4.01	672
G15 *Ismaila genalis*	No matching conspecific sequence	N/A	N/A	G06 *Ismaila robusta*	5.63	639
G16 *Ismaila belciki*	No matching conspecific sequence	N/A	N/A	G100 *Ismaila* sp.	12.2	672
G17 *Ismaila volatilis*	G82 *Ismaila volatilis*	2.1	666	G34 *Ismaila damnosa*	1.63	671
G19 *Ismaila aliena*	G21 *Ismaila aliena*	0.74	669	G23 *Ismaila robusta*	4.33	669
G20 *Ismaila aliena*	G21 Ismaila aliena	0.59	671	G23 *Ismaila robusta*	4.17	671
G21 *Ismaila aliena*	G19 *Ismaila aliena*	0.74	669	G59 *Ismaila robusta*	4.39	592
G22 *Ismaila robusta*	G57 *Ismaila robusta*	0.59	672	G13 *Ismaila aliena*	4.16	672
G23 *Ismaila robusta*	G57 *Ismaila robusta*	0.44	672	G13 *Ismaila aliena*	4.01	672
G24 *Ismaila robusta*	G57 *Ismaila robusta*	0.89	671	G13 *Ismaila aliena*	4.17	671
G25 *Splanchnotrophus angulatus* S	G03 *Splanchnotrophus angulatus* S	2.41	662	G46 *Splanchnotrophus gracilis*	15.59	667
G28 *Ismaila robusta*	G57 *Ismaila robusta*	0.74	672	G13 *Ismaila aliena*	4.31	672
G29 *Ismaila aliena*	G21 *Ismaila aliena*	0.44	671	G23 *Ismaila robusta*	4.01	672
G30 *Ismaila aliena*	G21 *Ismaila aliena*	0.44	671	G23 *Ismaila robusta*	4.01	672
G31 *Ismaila aliena*	G21 *Ismaila aliena*	0.74	670	G06 *Ismaila robusta*	4.22	639
G32 *Ismaila chaihuiensis*	No matching conspecific sequence	N/A	N/A	G82 *Ismaila volatilis*	3.74	667
G34 *Ismaila damnosa*	No matching conspecific sequence	N/A	N/A	G100 *Ismaila* sp.	1.48	672
G35 *Splanchnotrophus angulatus* C	G03 Splanchnotrophus *angulatus* S	1.96	662	G46 *Splanchnotrophus gracilis*	15.14	667
G36 *Splanchnotrophus angulatus* C	G03 Splanchnotrophus *angulatus* S	2.11	662	G46 *Splanchnotrophus gracilis*	14.99	667
G38 *Lomanoticola* sp.	No matching conspecific sequence	N/A	N/A	No matching congeneric, interspecific sequence	N/A	N/A
G42 *Pionodesmotes domhainfharraigeanus*	No matching conspecific sequence	N/A	N/A	No matching congeneric, interspecific sequence	N/A	N/A
G44 *Ceratosomicola mammillata*	No matching conspecific sequence	N/A	N/A	No matching congeneric, interspecific sequence	N/A	N/A
G46 *Splanchnotrophus gracilis*	No matching conspecific sequence	N/A	N/A	G05 *Splanchnotrophus angulatus* A	14.39	667
G55 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	2.26	661	G46 *Splanchnotrophus gracilis*	14.86	666
G56 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	1.96	662	G46 *Splanchnotrophus gracilis*	15.14	667
G57 *Ismaila robusta*	G24 *Ismaila robusta*	0.89	671	G13 *Ismaila aliena*	4.46	672
G58 *Splanchnotrophus angulatus* C	G03 *Splanchnotrophus angulatus* S	2.43	658	G46 *Splanchnotrophus gracilis*	15.23	663
G59 *Ismaila robusta*	G24 *Ismaila robusta*	0.5	592	G13 *Ismaila aliena*	4.22	592
G60 *Ismaila robusta*	G57 *Ismaila robusta*	0.44	672	G13 *Ismaila aliena*	4.01	672
G82 *Ismaila volatilis*	G17 *Ismaila volatilis*	2.1	666	G34 *Ismaila damnosa*	2.24	667

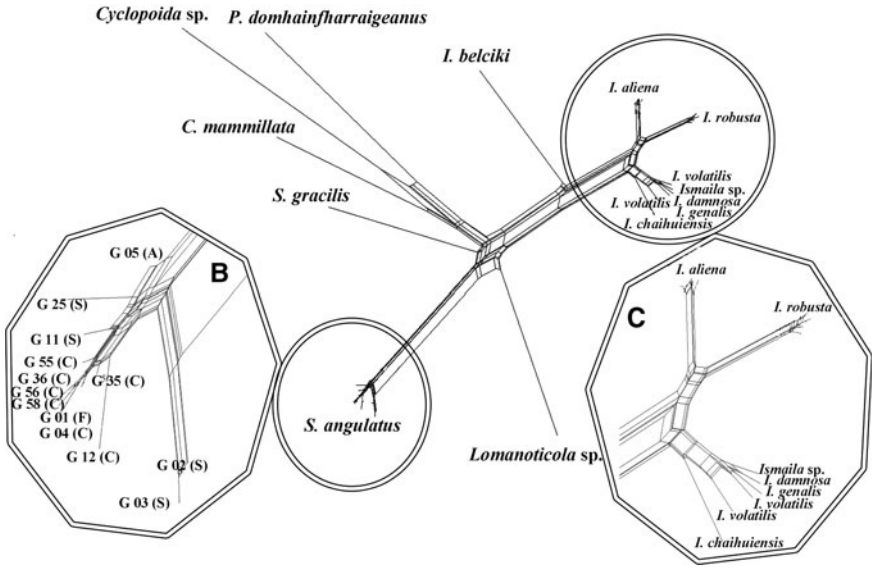

Fig. 2. Neighbour network computed by SplitsTree (A) with magnifications for the regions of interest inside (B) the *Ismaila* and (C) the *Splanchnotrophus* cluster. Capitals before or following species name refer to respective hosts: C: *Cratena peregrina*; S: *Spurilla neapolitana*; F: *Flabellina ischitana*; A: *Aeolidiella alderi.*

and *Lomanoticola* sp. respectively. Cluster 11 included *C. mammillata* and cluster 12 *S. gracilis* (see Figure 5G).

The PTP-analysis indicated outgroup taxa, *C. mammillata, S. gracilis, Lomanoticola* sp. and *I. belciki* as independent species with high support values. Good support was recognized for *S. angulatus, I. aliena* and *I. robusta*. However, all recently discovered *Ismaila* species form one cluster, although this is poorly supported (Figure 5F). Results are mostly

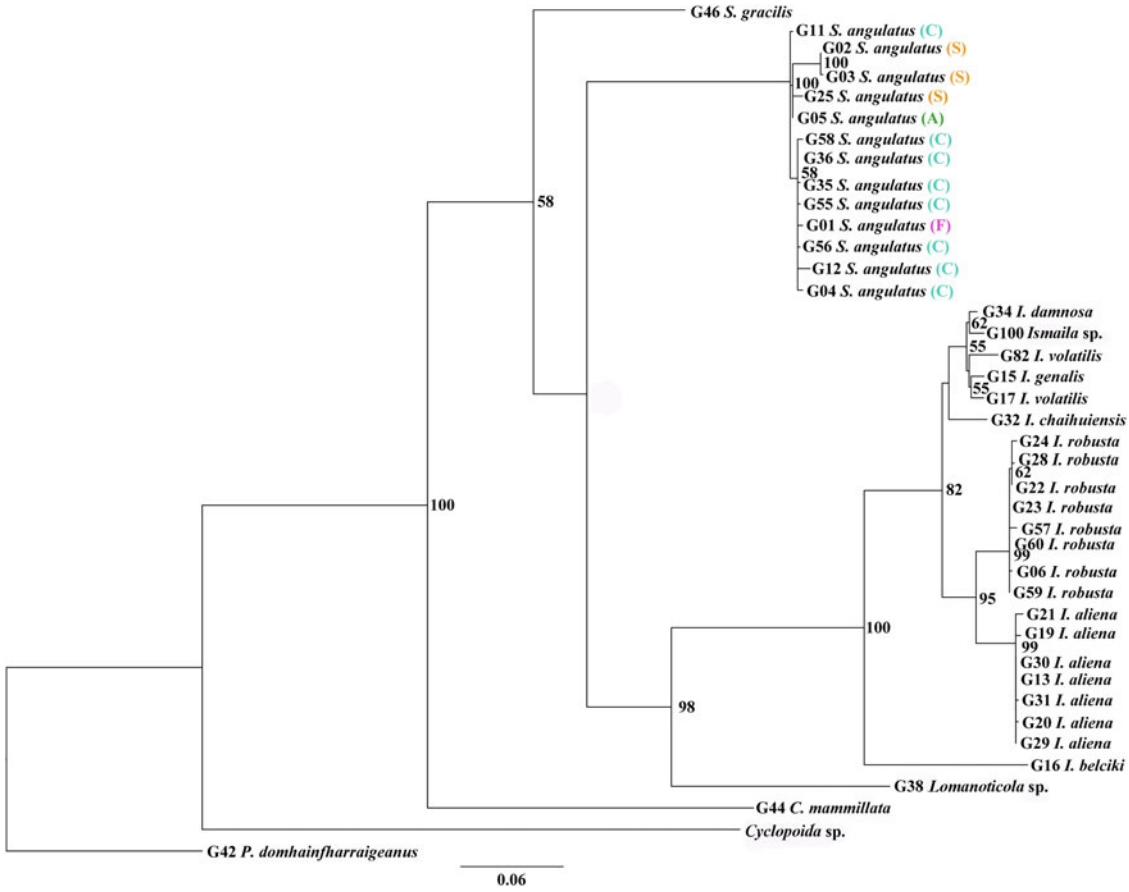

Fig. 3. Maximum likelihood consensus tree of the cytochrome c oxidase I (COI) sequences of 38 splanchnotrophids and two outgroup taxa. Numbers above branches show bootstrap values (>55%); branch length indicates substitutions per site. Capitals in parentheses refer to respective hosts: C: *Cratena peregrina*; S: *Spurilla neapolitana*; F: *Flabellina ischitana*; A: *Aeolidiella alderi.*

http://dx.doi.org/10.1017/S002531541600120X

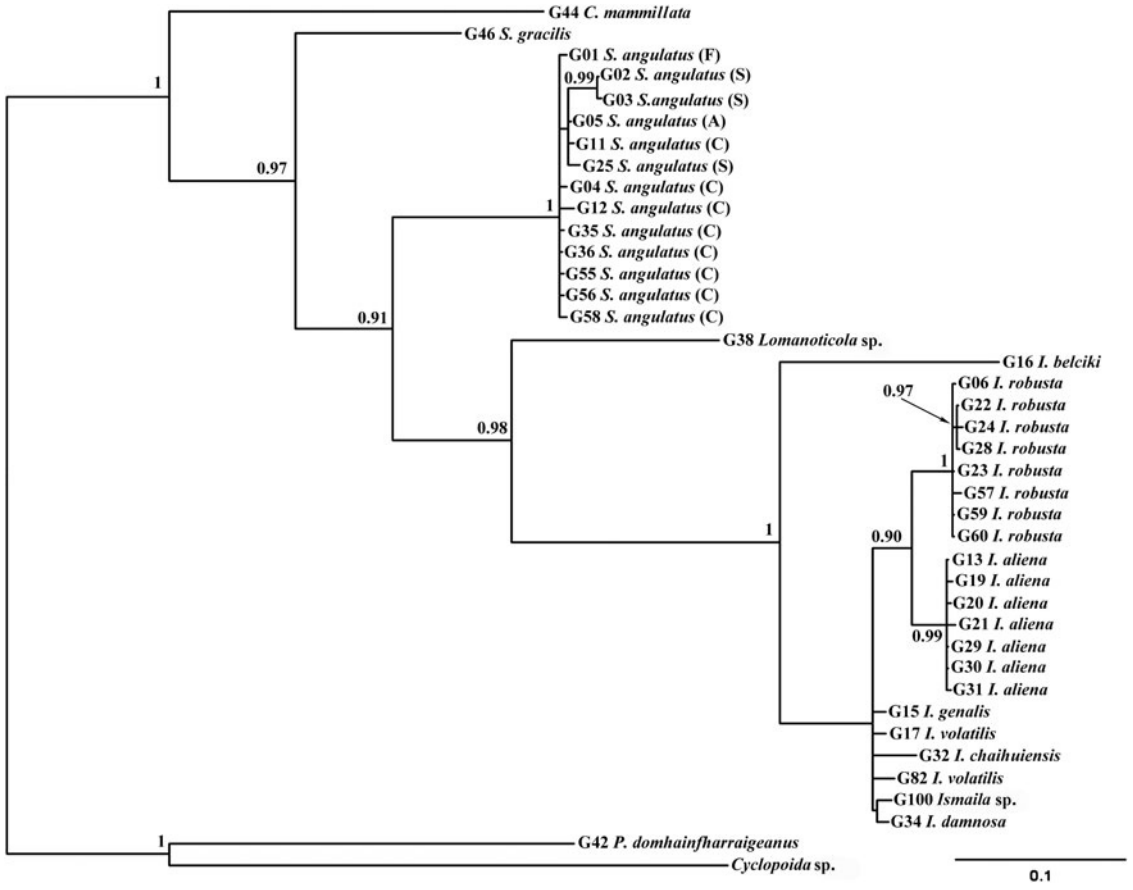

Fig. 4. Bayesian inference consensus tree of the cytochrome c oxidase I (COI) sequences of 38 splanchnotrophids and two outgroup taxa. Numbers above branches show posterior probability of BI (>0.90); branch length indicates substitutions per site. Capitals in parentheses refer to respective hosts: C: *Cratena peregrina*; S: *Spurilla neapolitana*; F: *Flabellina ischitana*; A: *Aeolidiella alderi.*

congruent regarding the ML and BI approach implemented in the PTP-analysis. Differences include the clade containing *Ismaila* sp. emerging as one species in the ML approach, while *I. genalis, I. chaihuiensis* and one sequence of *I. volatilis* (G17) are recovered as distinct species in the BI approach (see Figure 5F).

Haplotype networks

Each of the 13 *S. angulatus* sequences represented a distinct haplotype. The analysis using TCS software with a 90% statistical parsimony connection limit led to one network linking all haplotypes. In this network the inferred ancestral haplotype was from the host *Cratena peregrina*. Other haplotypes from this host were connected nearby (except G4 and G12), whereas those infesting other host species occupied more derived positions (Figure 6). However, setting the statistical parsimony connection limit to 95%, as is usually applied, resulted in three separate networks (see Figure 7). The first consisted of two sequences from the host *Cratena peregrina* and the second consisted of the two haplotypes G02 and G03 (infesting *Spurilla neapolitana*). The third network comprised the rest of sequences, with all sequences from haplotypes infesting *Cratena peregrina* inferred to be more ancestral and the haplotypes of three specimens infesting other hosts occurred in the more derived positions (Figure 7).

For the genus *Ismaila*, i.e. *I. belciki, I. aliena, I. genalis* and *I. chaihuiensis* were recovered as independent networks under a 95% statistical parsimony connection limit. Although most haplotypes of *I. robusta* emerged as a single network, there were two haplotypes (G06 and G59) that separated into an independent haplotype network. Another independent network consisted of a single haplotype shared by *I. volatilis, Ismaila* sp. and *I. damnosa*. However the second included haplotype of *I. volatilis* formed a separate network (Figure 8).

Diagnostic nucleotides

Splanchnotrophus gracilis differed from *S. angulatus* in 81 single pure characters (following Sarkar *et al.*, 2008; Jörger & Schrödl, 2014). *Lomanoticola* sp. differed from the genus *Splanchnotrophus* in 40 single pure characters. Within the genus *Ismaila, I. belciki* showed the highest divergence with 31 single pure characters differing from other *Ismaila* species. *Ismaila robusta* differed in nine, *I. aliena* in six, *I. chaihuiensis* in five, *I. genalis* in four and *I. damnosa, Ismaila* sp. and *I. volatilis* in one single pure character respectively.

Inside *S. angulatus* there were no differing single pure characters discernable; however, those parasites extracted from the host *C. peregrina* differed in nine single private characters from those infesting other host species. In addition, the *S.*

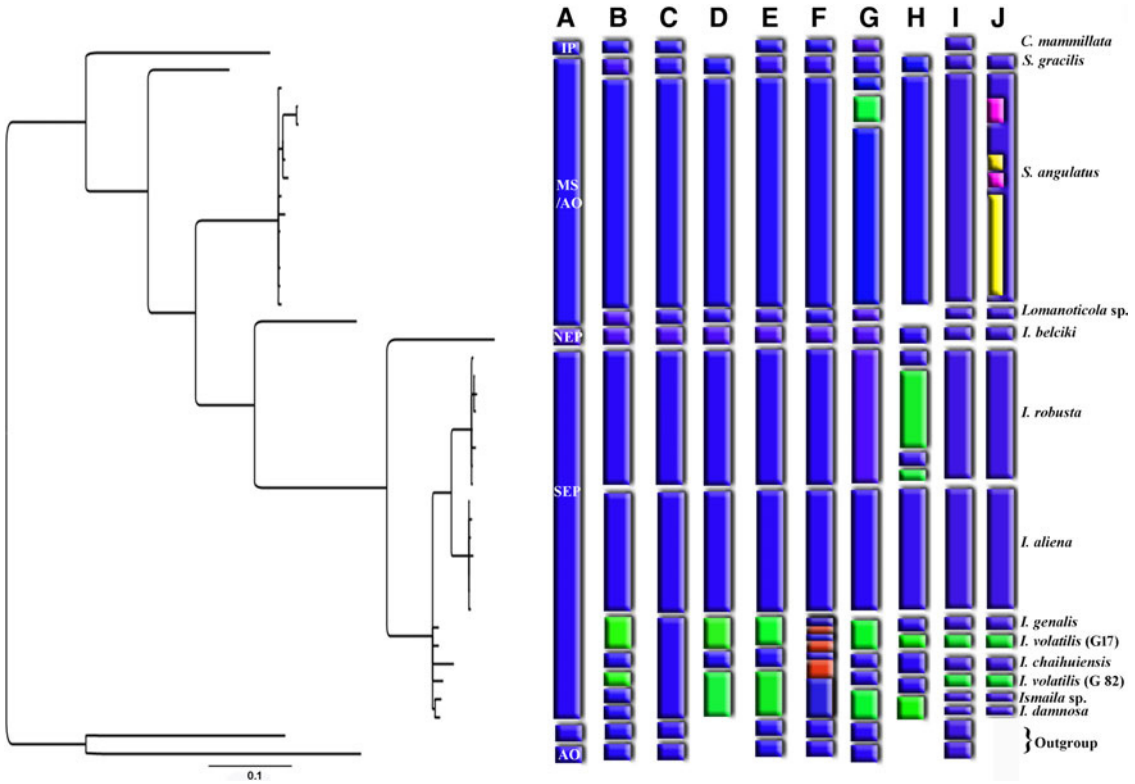

Fig. 5. Geographic distribution, sequence clusters and potential species obtained with the respective methods plotted in the Bayesian Inference tree. (A) Geographic distribution: IP, Indo-Pacific; MS, Mediterranean Sea; AO, Atlantic Ocean; NEP, north-eastern Pacific; SEP, south-eastern Pacific; (B) Maximum likelihood; (C) Bayesian inference; (D) ABGD; (E) Species Identifier; (F) PhyloMap – Poisson Tree Processes (PTP). The blue bars represent congruent results of the ML/BI approach, while the red bar indicates the differing results of the ML/BI approach; (G) Splitstree; (H) Tcs independent parsimony haplotype networks; (I) traditional species hypotheses based on morphological characters; (K) diagnostic nucleotides. Bars represent clades. Green bars represent clades in the respective analysis, which are not represented in the Bayesian Inference tree. Yellow and pink bars indicate groups within *S. angulatus* infesting *S. neapolitana* (pink) and *C. peregrina* (yellow) differing only in single private characters.

angulatus found in *S. neapolitana* also differed in nine single private characters from all other conspecifics. These nine single private characters did not overlap.

DISCUSSION

The high species diversity of copepods makes morphological identification and quantification of species a challenging task (Blanco-Berical *et al.*, 2014). In such cases DNA barcoding can be a simple but suitable tool to help identify species and to shed at least some light at the respective relationships (Blanco-Berical *et al.*, 2014; Jörger *et al.*, 2014; Padula *et al.*, 2014). However, barcoding identification requires that the taxonomy of the group is known, and that these taxonomic units correspond to a clade of COI sequences. This is the first attempt to apply molecular techniques to members of the Splanchnotrophidae to test the current morphology-based species hypotheses and to study the host specificity of selected members of the family.

Phylogeny of the Splanchnotrophidae

The resulting molecular trees are generally congruent with the current morphocladistic hypotheses on splanchnotrophid phylogeny (Anton & Schrödl, 2013a, b). The traditionally accepted monophyly of Splanchnotrophidae (e.g. Huys, 2001) is supported here, as is the monophyly of the Panamerican genus *Ismaila*. *Splanchnotrophus*, another morphology-based genus represented herein with multiple individuals, appeared paraphyletic. Surprisingly, *S. gracilis*, infesting the dorid nudibranch *Trapania tartanella* (Ihering, 1886), was recovered sister to all splanchnotrophids but *Ceratosomicola*. The COI topologies (Figures 3 & 4) suggested *Ceratosomicola* as earliest splanchnotrophid offshoot, which is also in accord with the results of the morphocladistic analyses of Anton & Schrödl (2013a, b). Interestingly, *Ismaila* is sister to *Lomanoticola* in the molecular trees, while morphological data usually suggested a clade of *Splanchnotrophus* and *Lomanoticola*. This supports Huys (2001) who elevated *Lomanoticola*, which was previously considered a subgenus of *Splanchnotrophus* (Hecht, 1895; Monod & Dollfus, 1932; Delamare Deboutteville, 1950; Jensen, 1990), to genus rank. Obviously, future molecular analyses should include further splanchnotrophid species, covering the entire generic, morphological and geographic diversity of the family, and representatives of *Briarella*, the putative sister of Splanchnotrophidae. As indicated by high support values, the barcoding fragment of COI appears informative for resolving splanchnotrophid genus level phylogeny.

On a species level, molecular phylogenetic trees are compatible with traditional taxonomy, but do not resolve all of the valid parasite species based on morphology. COI trees confirm the monophyly of *S. angulatus* and its separation from *S. gracilis* (Figures 2 – 5) as already suggested by previous studies based on morphological data (Huys, 2001; Abad *et al.*,

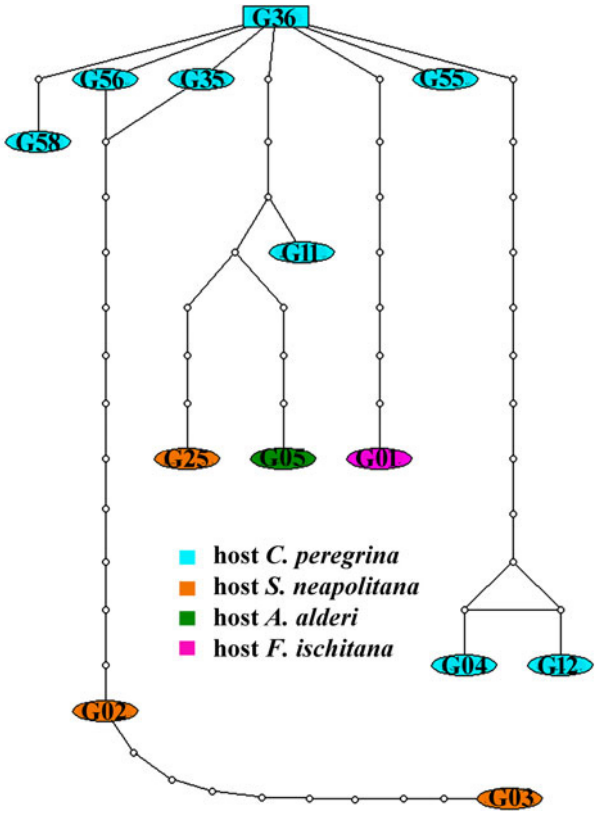

Fig. 6. Statistical parsimony network of 13 COI haplotypes in *Splanchnotrophus angulatus* with a connection limit set to 90%; white dots represent intermediate haplotypes missing in the sample set.

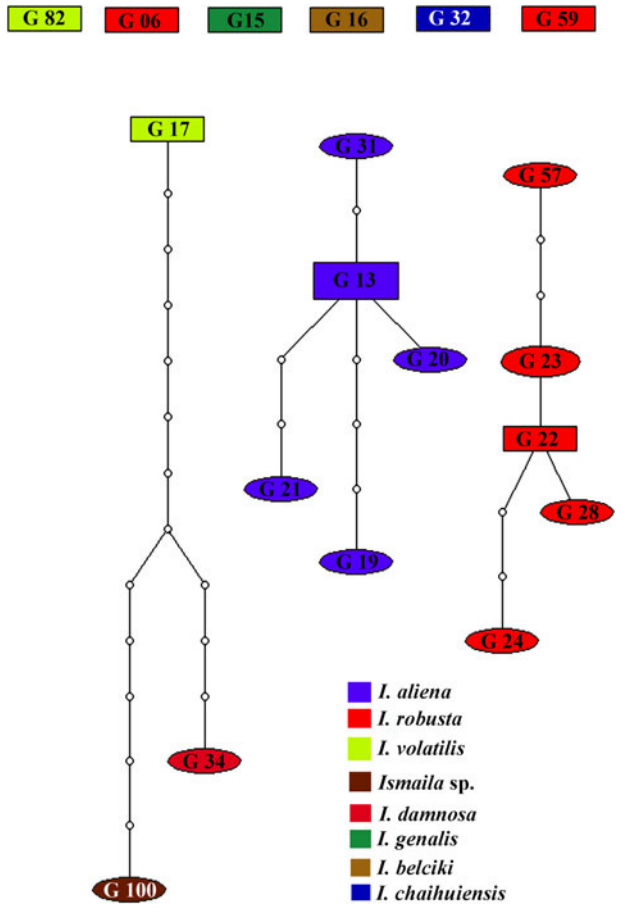

Fig. 8. Statistical parsimony network of 19 haplotypes of the genus *Ismaila* with a connection limit of 95%; white dots represent intermediate haplotypes missing in the sample set.

2011; Anton & Schrödl, 2013a, b). Within *Ismaila*, the morphologically clearly distinct species *I. robusta, I. aliena* and *I. belciki* were recovered monophyletic, while the recently described and similarly characteristic *I. volatilis* was not. The remaining species *I. genalis, I. chaihuiensis, Ismaila* sp. and *I. damnosa* emerged as a common clade in the ML analysis but paraphyletic in the BI analysis.

Phylogenetic trees showing a characteristic branching pattern with long internodes leading to well-supported shallow nodes with a couple of short terminals are often believed to be suggestive for species units, although there is no objective way to interpret the meaning of such units and their potential substructure appropriately by eye. In current barcoding practice, even a distance-based, quickly calculated COI genealogy, combined with some genetic threshold value, may deliver a first approximation on potential species (e.g. Layton *et al.*, 2014), and this may be useful to get a rough estimate on species diversity, e.g. when dealing with rare(ly sampled) groups or remote habitats (Jörger *et al.*, 2010, 2014; Padula *et al.*, 2014). However, gene histories may differ, and splanchnotrophid species level relationships appear to be complicated. Our initial phylogenetic, species delimitation and network analyses herein are based on a single gene and on an incomplete taxon and population sampling, and are inevitably preliminary.

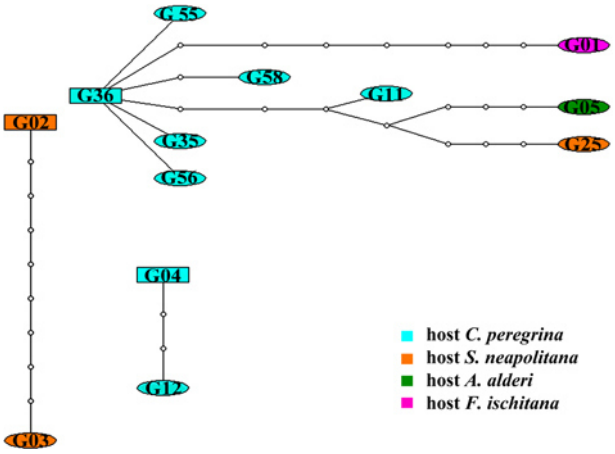

Fig. 7. Statistical parsimony network of 13 COI haplotypes in *Splanchnotrophus angulatus* with a connection limit of 95%; white dots represent intermediate haplotypes missing in the sample set.

Molecular species delimitation

Regarding *Splanchnotrophus*, both Species Identifier and ABGD basically confirmed the two morphological species *S. gracilis* and *S. angulatus* (Figure 5), showing considerable minimum interspecific p-distance of 16.4%. This is also supported by the presence of 81 single pure diagnostic characters and the results of the PTP-analysis (Figure 5F). However, two of the three *Spurilla* infesting *S. angulatus* animals isolated from the same host individual were separated under certain ABGD permutations. The hypothesis of a third,

morphologically cryptic *Splanchnotrophus* species is supported by the haplotype network analysis (Figures 6–8), since recovering separate networks using a 95% connection limit is sometimes used as a predictor of speciation; e.g. Miralles *et al.* (2011) considered species as distinct if showing separate mtDNA haplotype networks and unshared nDNA haplotypes. Unfortunately there are no reliable data from nuclear markers available for the Splanchnotrophidae.

According to the presence and number of diagnostic nucleotides both *S. gracilis* and *Lomanoticola* sp. receive good support. Regarding *Ismaila, I. belciki* is clearly separated from *I. robusta* and *I. aliena* also supported by differences in 31 single pure diagnostic characters. Within the genus, however, there is only poor support for the included species regarding diagnostic nucleotides. With a maximum of nine single pure characters *I. robusta* gains the highest support, but *I. damnosa, Ismaila* sp. and *I. volatilis* differ only in one single pure character respectively. Regarding *S. angulatus* there are no differences in single pure characters detectable according to the respective host species, supporting the hypothesis of one species displaying a lower level of host specificity. However the nine independent single private characters found for those individuals infesting *S. neapolitana* and those infesting *C. peregrina* respectively seem to indicate some kind of autocorrelation between gene flow and host.

In contrast to the ambiguous phylogenetic analyses, ABGD indicates *I. chaihuiensis* as a distinct species also (Figure 5D).

Ismaila aliena, *I. chaihuiensis, I. belciki* and *I. genalis* are supported as distinct species by the results of the TCS analysis (Figures 5H–8), since they all were recovered as independent networks or independent haplotypes, respectively. *Ismaila robusta* is also supported, nevertheless two sequences emerged as independent haplotypes (Figure 5H). In the case of Go6 a possible explanation for this separation could be the geographic origin of the sample, which is quite distant to the location of all the other samples of *I. robusta* (see Table 1). G59, however, was collected in the same location as the rest of the specimens, so the separation from the other haplotypes remains unexplained. Neither changing the connection limit nor excluding any other haplotype had any influence on the result. The large number of inferred extinct or unsampled haplotypes suggests the data set is highly undersampled, which can result in inferring more structure than is actually present.

Ismaila damnosa, Ismaila sp. and *I. volatilis* emerging in the same haplotype network might initially seem to contradict the hypothesis of independent species. However, these three species are each represented only by a single sequence, rendering any attempt of estimating the intra- or interspecific variation impossible. Only a single pure diagnostic character supports these three species respectively, but this may also change as data increase. At the present time, at least some diagnostic nucleotides were found for all included *Ismaila* species; future exploration of the quantity and significance of diagnostic characters needs more genetic material, and the validity of these species remains somewhat equivocal.

Host specificity: *Ismaila* versus *Splanchnotrophus*

Of the morphology-defined *Ismaila* species included in the molecular analyses, the specific status of *I. aliena* and *I. robusta* was unambiguously confirmed. Both *Ismaila aliena* and *I. robusta* were previously assumed to be strictly host specific (to the dorid nudibranchs *Thecacera darwini* Pruvot-Fol, 1950 and *Okenia luna* Millen, Schrödl, Vargas & Indacochea, 1994, repectively), and this is supported herein. Assessing the specificity of the remaining *Ismaila* species is much harder since there are so few observations. The limited barcoding data to date remains compatible with assuming strict host specificity of the herein included *I. belciki, I. damnosa, I. genalis, I. volatilis, I. chaihuiensis* and *Ismaila* sp. (Figure 5). This null hypothesis of specificity was generated by the state being plesiomorphic in the phylogenetic hypothesis of Anton & Schrödl (2013a), and in light of our initial molecular data, there is no reason yet to assume host-induced morphological plasticity in *Ismaila*. We conclude that the earlier hypothesis of a species-rich neotropical clade *Ismaila* showing a rather rapid and recent radiation via host switches (Schrödl, 2003; Anton & Schrödl, 2013a, b) remains a plausible evolutionary scenario.

Splanchnotrophus angulatus was recovered as a single species in both phylogenetic analyses (Figures 3 & 4). There is no genetic substructure suggestive of a hidden species complex according to the ABGD analysis, which showed no distinct barcode gap for *S. angulatus*. In the light of barcoding data, *S. angulatus* is a single species infesting various host species, including the aeolids *Spurilla neapolitana, Aeolidia alderi, Cratena peregrina* and *Flabellina ischitana*, comprising three different host families. Interestingly, two of three members of *S. angulatus* infesting *Spurilla neapolitana* cluster together in both phylogenetic analyses. This subgroup is also supported by the results of the ABDG- and TCS analyses (Figures 6–8). This genetically derived group may reflect some reproductive isolation due to distinct host species and represents a beginning state of speciation. According to the results of the TCS analysis there is also another group separating from the rest, consisting of two haplotypes infesting *Cratena peregrina*. Nevertheless, divergences are low, ABGD analyses show no distinct barcode gap, and only single private characters were found, suggestive of early divergence or limited gene flow due to ecological host differences. Morphological comparisons thus are overdue to scrutinize current taxonomy, and they need to be on a broader basis, i.e. revising all relevant *Splanchnotrophus* type material and specimens from a broad range of hosts.

The different life-history strategies and their potential reasons

All members of the Splanchnotrophidae capable of infesting more than two host species were reported from the Mediterranean Sea and the European coasts of the Atlantic Ocean (Figure 1), and all belong to the genus *Splanchnotrophus* in a broad sense. Huys (2001) split *Lomanoticola* from *Splanchnotrophus*, and both were considered either sister taxa or *Splanchnotrophus* deriving from paraphyletic *Lomanoticola* (Anton & Schrödl, 2013a). Regardless, the ability to infest several, not necessarily closely related hosts, appeared phylogenetically and geographically correlated. Morphocladistic and molecular tree hypotheses all support a scenario in which ancestral splanchnotrophid lineages, *Ceratosomicola, Ismaila* and *Arthurius* are highly specific to a single host. Assuming diversification via

host switch in *Ismaila* (Anton & Schrödl, 2013a, b), infestation of a new host seems to invariably reduce or lose the ability to infest the original host, thus creating a bottleneck leading to a reproductive barrier. An obvious consequence of this scenario, if confirmed, is that strictly host-specific lineages can radiate in sympatry, adapting to different hosts. Strict dependence on certain sea slug hosts, which may be highly sporadic or rare (Schrödl, 2003), means higher risk of rapid extinction of newly diversified parasites. In contrast, host-promiscuous *Lomanoticola* and *Splanchnotrophus*, if confirmed by morphology-based taxonomy, may need allopatry to diverge permanently, and would have a lowered extinction risk.

CONCLUSION

The present study successfully extracted genetic material from the egg sacs of female parasites, with minimal damage of rare specimens (Anton *et al.*, 2013). Our preliminary molecular study on splanchnotrophids included 11 of the currently 32 known species and a new *Ismaila* sp., many with single or few specimens; the need for more samples and markers thus is obvious. These first molecular-based analyses are largely but not fully congruent with morphology-based taxonomic hypotheses on Splanchnotrophidae (Figure 5). In addition, host specificity reported *Splanchnotrophus*, could be confirmed. Amphi-American *Ismaila* appears to radiate via host switches, losing connection to ancient populations, while individuals of *Splanchnotrophus angulatus* infesting different hosts may maintain some gene exchange. Uncovering details, reasons and consequences of these substantially different ecological and evolutionary strategies in the family Splanchnotrophidae provides an interesting field of research. In addition to morphology-based taxonomic revisions, we need more information on the life cycles of splanchnotrophids, on mechanisms of infections and on population dynamics of parasites and hosts to understand coevolution.

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CONFLICT OF INTEREST

None.

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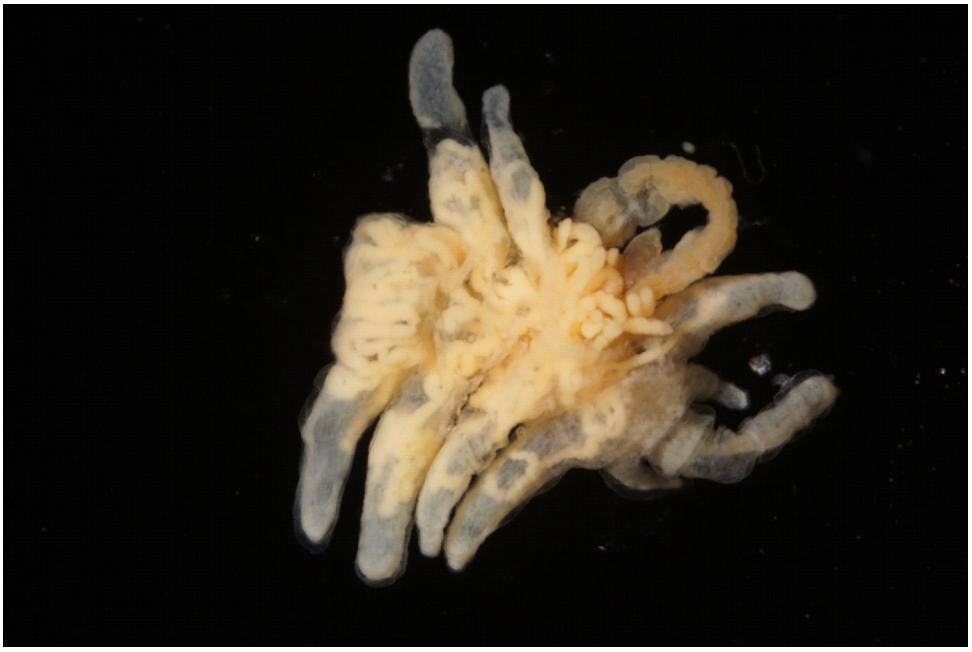
Chapter 5

Salmen, A. Anton, R. Wilson, N. & Schrödl, M. (2010), SEM-description of the philoblennid endoparasitic copepod *Briarella doliaris* n. sp. From Queensland, Australia; a potential link to the Splanchnotrophidae (Crustacea, Copepoda, Poecilostomatoida); Spixiana 33 (1), pp. 19-26



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Briarella doliaris spec. nov., a new philoblennid copepod parasite from Australia: a potential link to the Splanchnotrophidae

(Copepoda, Poecilostomatoida)

Andrea Salmen, Roland Anton, Nerida G. Wilson & Michael Schrödl

Salmen, A., Anton, R., Wilson, N. G. & Schrödl, M. 2010. *Briarella doliaris* spec. nov., a new philoblennid copepod parasite from Australia: a potential link to the Splanchnotrophidae (Copepoda, Poecilostomatoida). Spixiana 33 (1): 19-26.

Members of the quite common and diverse copepod family Splanchnotrophidae are specialised endoparasites of shell-less opistobranch gastropod hosts. Another less well-known group of endoparasites also infesting opistobranch sea slugs is the genus *Briarella* Bergh, 1876 that is currently placed within the Philoblennidae.

A new species of *Briarella* from Queensland, Australia, infesting the chromodorid nudibranch *Ceratosoma trilobatum* Gray, 1827 is described using scanning electron microscopy (SEM). The new species differs from the four currently known species *Briarella microcephala* Bergh, 1876, *Briarella risbeci* Monod, 1928, *Briarella disphaerocephala* Monod & Dollfus, 1932, and the unnamed *Briarella* sp. Bergh, 1876, by having a stocky rather than a vermiform body and longer lateral processes. Of all the members of this genus, *Briarella doliaris* most resembles splanchnotrophids due to the stocky body. It is thus possible, that *Briarella* and the Splanchnotrophidae share a common ancestor which switched to an endoparasitic lifestyle. If so, *Briarella doliaris* could represent the most basal offshoot of a clade of secondarily vermiform *Briarella* species, or it could be a direct sister taxon to splanchnotrophids, rendering the genus *Briarella* paraphyletic.

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Introduction

Traditionally, all endoparasitic copepods parasitizing in opistobranch gastropods were considered to belong to the family Splanchnotrophidae Norman & Scott, 1906 (see review by Jensen 1987). Revising the family, Huys (2001) only recognised five genera, *Splanchnotrophus* Hancock & Norman, 1863 (4 species), *Ismaila* Bergh, 1867 (11 species), *Lomanoticola* Scott & Scott, 1895 (2 species), *Ceratosomicola* Huys, 2001 (4 species), and *Arthurius* Huys, 2001 (2 species), all of them highly modified endoparasites in shell-less sea slugs (Schrödl 2002; Haumayr & Schrödl 2003; Schrödl 2003; Marshall & Hayward 2006; Salmen et al. 2008a,b).

Since the genus *Briarella* was first established, the gross-morphological similarity to the Splanchnotrophidae was emphasised (Bergh 1876; Jensen 1987; Huys 2001). The systematic placement of *Briarella*, however, was in a state of flux: Bergh (1876) claimed a relationship to the phylichthyids, but did not integrate it there. His original descriptions unfortunately are quite inadequate and lack any information on mouthpart morphology (Huys 2001). First, *Briarella*

was placed within the Chondracanthidae (see Monod 1928), then it was included into the Splanchnotrophidae (see Monod & Dollfus 1932). In 1964, the genus *Briarella* was removed from the Splanchnotrophidae due to the presence of maxillipeds (Laubier 1964). Together with the genus *Philoblenna* Izawa, 1976, it was placed into the newly established Philoblennidae Izawa, 1976, because of obvious similarities such as two strong claws on the distal margin of the antenna, a long blade of the mandible and the maxilla displaying a subapical element on the allobasis (Izawa 1976).

Philoblenna, however, comprises ectoparasites that are attached to the gills of prosobranch gastropods, including littorinids and cowries (Izawa 1976; Ho 1981; Avdeev et al. 1986; Ho & Kim 1992; Huys 2001). Recently, both genera *Briarella* and *Philoblenna*, i.e. the Philoblennidae, were transferred to the Lichomolgidae considering several similarities in mouthpart morphology of the copepodite I of *Philoblenna* and *Critomolgus* (see Kim et al. 2004), but were later separated again (Boxshall & Huys 2007).

Herein, the Philoblennidae thus are treated as an independent family. Based on mouthpart morphology, it includes the genera *Briarella* and *Philoblenna*. Although mouthparts are unknown yet, Huys provisionally also included the poorly described genus *Chondrocarpus* into the Philoblennidae due to general body facies such as the presence of four pairs of lobate processes (Bassett-Smith 1903; Huys 2001). In contrast to the endoparasitic *Briarella* and *Chondrocarpus*, all members of *Philoblenna* possess swimming-legs and are considered to be more "primitive" (Huys 2001). Phylogenetic studies on splanchnotrophids and Philoblennidae still are impeded by the absence of suitable material for molecular analysis. Morphological knowledge on many species is restricted to old and inadequate original descriptions of a single or a few female specimens. Especially information concerning the mouthparts is often missing in older descriptions and thus, the taxonomy is unclear.

The genus *Briarella* currently consists of four species (Huys 2001). *Briarella microcephala* Bergh, 1876 parasitizes *Ceratosoma trilobatum* Gray, 1828 (see Bergh 1876; Hecht 1893; Jensen 1987). Monod (1928) found *Briarella risbeci* Monod, 1928 in *Hexabranchus sanguineus* (Rüppell & Leuckart, 1828) (as *Hexabranchus marginatus*), *Briarella disphaerocephala* Monod & Dollfus, 1932 utilises the host slugs *Platydoris cruenta* (Quoy & Gaimard, 1832) and *Kentrodoris inframaculata* (Abraham, 1877) (as *Doris inframaculata*) (Monod & Dollfus 1932; Jensen 1987), and *Briarella* sp. Bergh, 1876 was found in *Chromodoris elisabethina* Bergh, 1877 and in *Asteronotus cespitosus* (van Hasselt, 1824) (see Bergh 1876; Jensen 1987). Thus far, all *Briarella* species are exclusively known as infesting dorid nudibranchs in the Indo-Pacific (Huys 2001). It is unclear whether the similarity of *Briarella* species with other species in the genus *Splanchnotrophus* is due to common ancestry or, as implied by their classification in different families by more recent studies (e.g. Huys 2001; Kim et al. 2004), evolved convergently by adaptations to similar hosts.

In order to gain supplementary data for an analysis of relationships within the Poecilostomatoida, an additional, endoparasitic copepod species from the dorid nudibranch *Ceratosoma trilobatum* is described here using scanning electron microscopy (SEM) and is assigned to the genus *Briarella*. Based on even greater structural similarity than previously known from congeners, the new species is discussed as a potential link to splanchnotrophids.

Material and methods

Infection with female splanchnotrophids can usually be recognised due to the presence of external egg sacs, and sometimes endoparasites can be seen shining through host integument. In this case, no external signs were noted, and the parasites were discovered during routine dissection.

The infected sea slug analysed in this study was collected at Amity, North Stradbroke Island, Moreton Bay, Queensland, Australia and determined by N. Wilson. The host slug was deposited in the South Australian Museum (SAMD 19256).

The two female parasite specimens were relaxed in an isotonic $MgCl_2$ solution, the body was preserved in 75 % and the egg sacs in 90 % ethanol and given to the Zoologische Staatssammlung München (ZSM). Photographs of parasites were taken with a "Jenoptic ProgRes C12 plus" camera connected with an Olympus SZX 12 binocular. For SEM examination the copepods were dehydrated in an acetone series and critical-point dried in a BAL-TEC CPD 030 device. They were mounted on SEM stubs and coated with gold in a POLARON SEM COATING SYSTEM for 120 seconds. A LEO1430 VP scanning electron microscope was used for ultra-structural analysis and digital documentation.

The descriptive terminology used herein is adopted from Huys & Boxshall (1991), Gruner (1993), Huys (2001) and Haumayr & Schrödl (2003). The following terms are used to describe body segmentation: Cephalothorax (five head segments fused with a variable number of thorax segments), thorax and abdomen. In all postlarval Splanchnotrophidae the first pair of thoracopods is reduced (Huys 2001). The counting of thoracopods is adopted from Haumayr & Schrödl (2003).

The SEM is suitable to identify and document very fine and tiny structures. However, it is hardly possible to examine each sample from all sides. Due to the delicate nature of the parasites, host tissue and dirt cannot always be removed completely and may cover certain parasite structures.

Taxonomy

Class Copepoda H. M. Edwards, 1840
Order Poecilostomatoida Thorell, 1859
Family Philoblennidae Izawa, 1976

Genus *Briarella* Bergh, 1878

Briarella doliaris spec. nov.

Material. Holotype (♀, ZSMA20092004 mounted on SEM stub) and paratypes (1♀ ZSMA20092005, mounted on SEM stub and 1♀ ZSMA20092006 in ethanol) partly damaged, collected together by Nerida Wilson, 9m, Amity, North Stradbroke Island, Moreton Bay, Queensland, Australia, 27°24'13.81"S, 153°26'11.49"E, 07 December 2002. Host: *Ceratosoma trilobatum* Gray, 1828. 2♀♀ examined by SEM.

Etymology. The Latin species name *doliaris* refers to the barrel-shaped body.

Description (Figs 1-3)

Female. Body length 3.0-4.7 mm, (measurements were made from the anterior end of the cephalothorax to the posterior end of the abdomen, including the caudal rami and excluding antennae and the setae on caudal rami), width 1.1-1.4 mm, body stocky. Ratio of length to width about 1.71 : 1. Parasites whitish, slightly translucent (Fig. 1A). Cephalothorax distinctly set off from trunk; thorax enlarged with five pairs of lateral processes; abdomen long and slender (Fig. 1B). Segmentation of all body parts unclear.

Cephalothorax consisting of head with five pairs of cephalic appendages and first thoracic segment bearing maxillipeds (Fig. 1C, 3F). Antennule (Fig. 1D) long and unbranched, indistinctly 4-segmented; first segment long, bearing nine setae, five short ones and four long ones; second segment with three long setae and one short one; third segment with two long setae; fourth segment with six long setae at apex. Antenna (Fig. 1C, 3A) unbranched, 3-segmented; first and second segment with small spine on proximal edge; third segment with at least five minute spines, apex with two subequal strong claws. Labrum (Fig. 1C) well developed, bilobate; lobes very long. Mandible (Fig. 1E, 3B) with broad and thick base, tapering into long and flat blade with thorns on both edges like a saw blade. Mandible palp very thick with blunt tip (Fig. 1E, 3E). Maxillule (Fig. 1F, 3C) thick, bearing two small spines at apex and a triangular bulge laterally. Maxilla (Fig. 2A, 3D) 2-segmented; first segment enlarged, second segment biramous, longer ramus with two apical elements. Labium tongue-shaped. Maxilliped posterior to maxilla (Fig. 1C, 3F).

Second thoracopod biramous, located on second thoracic segment, close to cephalothorax (Fig. 1B). Exopodite indistinctly 2-segmented with one strong spine at proximal edge of first segment; second segment with 4 strong spines increasing in size distally, one seta at level of longest spine, one seta at base of thoracopod (Fig. 2B). Endopodite about as long as exopodite, blunt apex bearing one seta. Third thoracopod biramous. Exopodite as in second thoracopod; endopodite longer than exopodite, apex split in two short elements; one seta at base of third thoracopod (Fig. 2C). No further thoracopods detected.

Thorax with deep transversal furrows demarcating four pairs of lateral processes. Processes shorter than whole body; stout with round tip. Fifth pair of lateral processes shorter than all others and more slender, situated posterior to enlarged part of thorax, slightly bent medially.

Abdomen long and slender with four indistinct constrictions; genital openings not detected; egg sacs slender, slightly bent with pointed tip. Caudal rami long and stout; each ramus with two pinnate setae laterally and four pinnate ones at apex, latter with small bulge bearing one long pinnate seta (Fig. 2D).

Male. Not found.

Biology

For the present study, no biological information on *B. doliaris* was available, e. g. on the specimens' positions inside the host, or the colour of the egg sacs. Both parasites were damaged (see Fig. 1A,B) during their incidental discovery; egg sacs were removed, fixed in ethanol, and given to the ZSM separately. No males were found, despite considerable effort dissecting the host specimen.

Remarks

The females resemble each other regarding the size and shape of the body. The morphology of mouthparts is nearly identical; differences only exist with regard to number and position of setae. Thoracopods were only detectable in one specimen, in the second one they were covered with host tissue. Genital openings could not be detected in both specimens, but it is likely that they are situated on the first slightly swollen abdominal segment as it is usual for copepods (Gruner et al. 1993).

The specimens examined herein are members of the genus *Briarella* Bergh, 1876. Diagnostic features refer to the morphology of the mouthparts, especially the long mandible, the two claws on the third segment of the antenna (see Fig. 3A) and the shape of the maxilla, the five pairs of lateral processes on

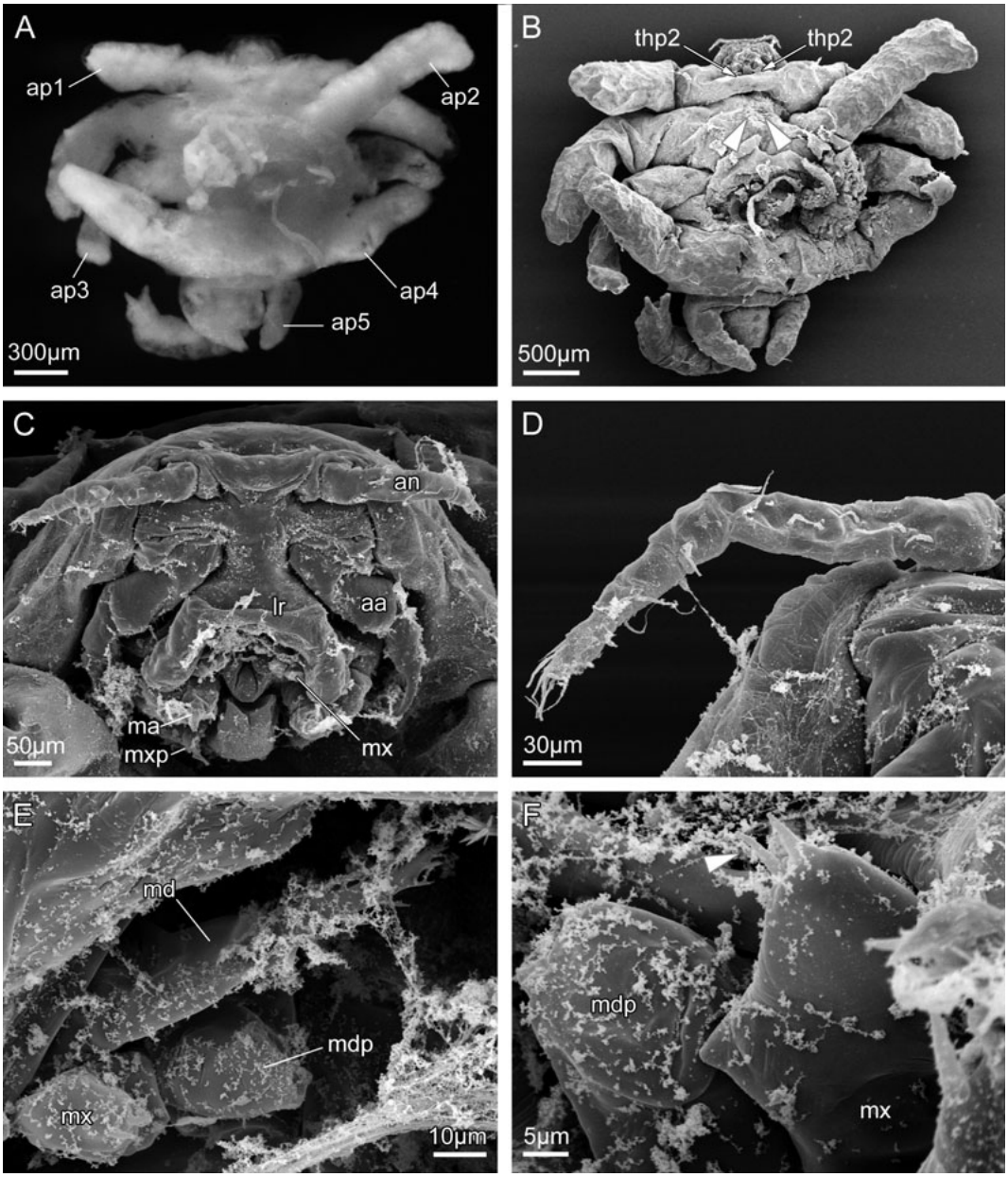

Fig. 1. *Briarella doliaris*, ♀. **A.** Habitus, ventral view (light microscope picture). **B-F.** SEM-micrographs. **B.** Habitus, ventral view. Position of 3rd thoracopods (arrows). **C.** Cephalic appendages. **D.** Antennule (right). **E.** Oral area (right side), mandible blade and palp, maxillule. **F.** Maxillule (right) with apical spines (arrow), mandible palp. Abbreviations: **aa**, antenna; **an**, antennule; **ap1-5**, appendages 1-5; **lr**, labrum; **md**, mandible; **mdp**, mandible palp; **mx**, maxillule; **ma**, maxilla; **mxp**, maxilliped; **thp2**, thoracopod 2.

the thorax and the presence of only two pairs of thoracopods, i. e., second and third ones (Monod 1928; Huys 2001). According to Huys (2001), four other species belong to this genus: *B. microcephala* (type species), *B. risbeci*, *B. disphaerocephala* and an unnamed *Briarella* sp. (see also Monod 1928). *Briarella risbeci* has a very elongate body with four pairs of short lateral processes ("lobes" according to Monod

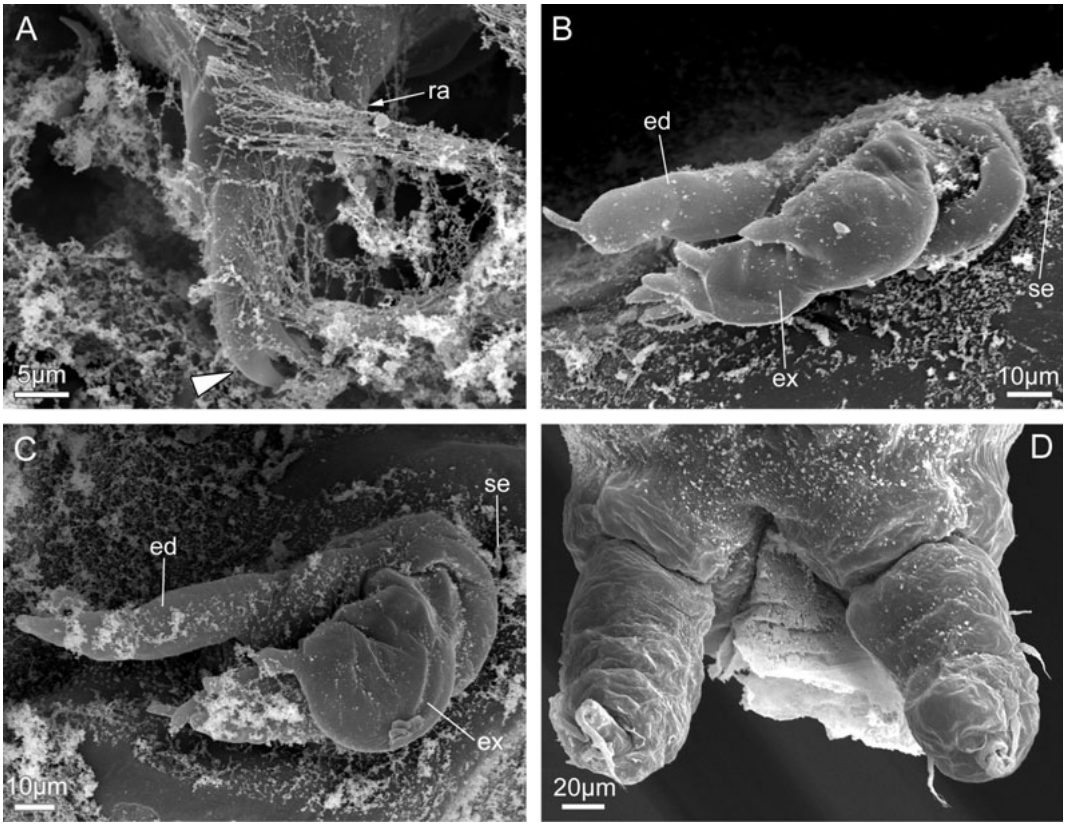

Fig. 2. *Briarella doliaris*, ♀. SEM-micrographs. **A.** Maxilla (left) with two apical elements (arrow). **B.** 2nd thoracopod (left). **C.** 3rd thoracopod (left). **D.** Caudal rami. Abbreviations: **ed**, endopodite; **ex**, exopodite; **ra**, ramus; **se**, seta.

1928), while *B. doliaris* shows a stocky body with an enlarged thorax and a slender abdomen, and five pairs of longer lateral processes. Furthermore, *B. risbeci* possesses three setae on the maxillule (Huys 2001), whereas *B. doliaris* has only two spines at the apex of the maxillule. Monod labelled a mandibular palp for *B. risbeci* (Monod 1928). Huys re-examined *B. risbeci and B. disphaerocephala*. In his drawings he reproduced a structure similar to the mandibular palp of Monod, but did not mention it in the text (Huys 2001). Nevertheless the presence of a mandibular palp can be confirmed in this study (Fig. 1E). In the specimens examined herein the antennule is indistinctly 4-segmented, while in *B. risbeci* it shows 5-6 segments (Monod 1928). Further differences concern the thoracopods. In *B. doliaris* both pairs of thoracopods are biramous, with a 2-segmented exopodite bearing 5 strong spines, whereas in *B. risbeci* the thoracopods are uniramous, with the second thoracopod bearing 5 spines and the third thoracopod bearing none (Monod 1928). In contrast to *B. risbeci*, which has egg sacs longer than the whole body, *B. doliaris* has short egg sacs. *Briarella* thus far was exclusively found in dorid nudibranchs (Huys 2001), what also applies for *B. doliaris*, which infests *Ceratosoma trilobatum*. The latter is already known as host for *B. microcephala* (see Monod 1928).

Monod (1928) described *B. microcephala* with five pairs of lateral lobes, but with a very vermiform body shape; this stands in clear contrast to the stocky body of *B. doliaris*. *Briarella disphaerocephala* is considered to be similar to *B. risbeci* (see Monod 1928; Monod & Dollfus 1932; Huys 2001). In *B. disphaerocephala* the maxillule possesses three setae like in *B. risbeci* (see Huys 2001) and *B. disphaerocephala* possesses two more pairs of lateral lobes. One pair is situated on the sides of the head and one pair is located in the pregenital area (Monod & Dollfus 1932). Thus *B. disphaerocephala* is also different to *B. doliaris*.

Bergh's unnamed *Briarella* sp. (see illustration in Monod & Dollfus 1932: fig. 17E) externally is very similar to *B. microcephala* (see Bergh 1876) and to *B. risbeci* (see Monod 1928), and thus differs from the stocky body shape of *B. doliaris*. The egg sacs of

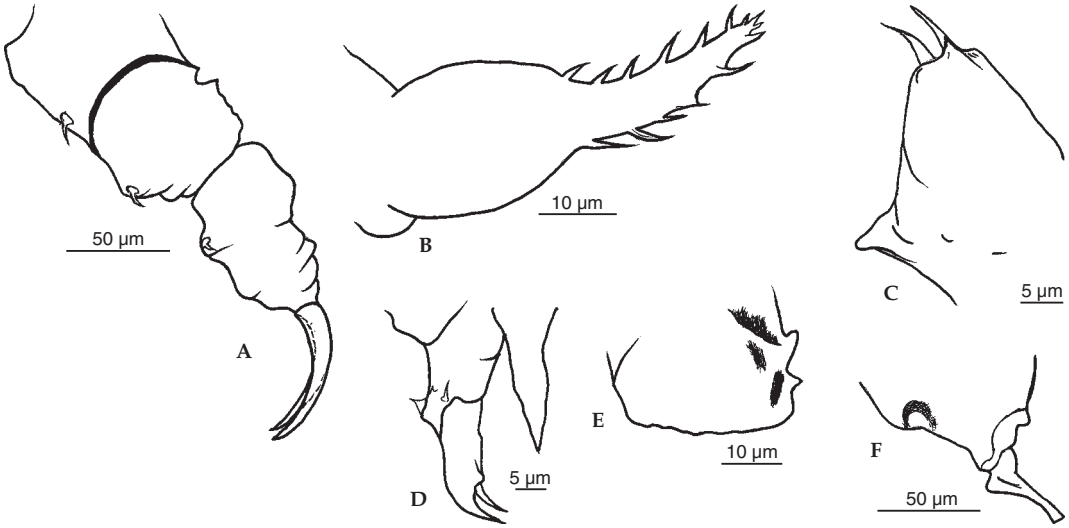

Fig. 3. *Briarella doliaris*, ♀. Cephalic appendages. **A.** Antenna. **B.** Mandible. **C.** Maxillule. **D.** Maxilla. **E.** Mandible palp. **F.** Maxilliped.

Briarella sp. are only half as long as the whole body, and thus more similar to those of *B. doliaris*. Bergh (1876) also describes the antennule of *Briarella* sp. as 5-segmented, while in *B. doliaris* it is indistinctly 4-segmented. *Briarella* sp. is only known from the Philippines, where it infests *Chromodoris elisabethina* and *A. cespitosus* as hosts (Monod 1928).

Our material examined thus differs from all known congeners, and the new species *B. doliaris* is established.

Discussion

On the one hand, according to our results, there is no doubt that *Briarella doliaris* spec. nov. belongs to the genus *Briarella*. The cephalic appendages of the new species *B. doliaris* fit exactly with the general description of *Briarella* and *Philoblenna* mouthparts by Huys (2001), supporting the common placement within the Philoblennidae (see Izawa 1976; Ho 1981; Huys 2001). On the other hand *B. doliaris* shows several novel features observed for the genus *Briarella* such as a maxillule possessing two instead of three setae (Fig. 2F), and the second and third thoracopods being biramous (Fig. 3B,C) instead of uniramous as described by Monod (1928) for *B. risbeci*. Furthermore, *Briarella doliaris* has a stocky body with four pairs of long and one pair of short lateral processes, whereas all other four *Briarella* species are vermiform with a varying number of short lateral processes (Monod 1928). More than other congeners, adult *B. doliaris* thus resemble female splanchnotrophids, in particular the genus *Splanchnotrophus*, concerning the shape of the body and egg sacs and the biramous thoracopods (Huys 2001). The lateral processes are, concerning their length, also in a stage between *B. risbeci* and *Splanchnotrophus angulatus* Hecht, 1893 (see Monod 1928; Huys 2001). The fifth short lateral process of *B. doliaris* is similar to the lateral outgrowth of *S. angulatus* as described by Huys (2001). It is thus possible, that *B. doliaris* represents a "missing link" between the two genera *Briarella* and *Splanchnotrophus*. However, there are some major differences between *B. doliaris* and *S. angulatus*. One is the presence of maxillipeds and of a mandibular palp in *Briarella* which are generally missing in Splanchnotrophidae (see Huys 2001). Also, in *Briarella* the head is distinctly set off from the thorax, whereas in *Splanchnotrophus* there is no such distinct border (Huys 2001). Another difference is the presence of a very reduced fourth pair of thoracopods in splanchnotrophids like *S. angulatus* (see Huys 2001). Unfortunately such appendages could not be found in *B. doliaris*, possibly due to remainders of host tissue covering that particular area. This last point will need further investigation as soon as more material is available.

Accepting that *Briarella*, *Chondrocarpus* and *Philoblenna* belong to a monophyletic group (Izawa 1976; Ho 1981; Huys 2001), the strong similarity between *Briarella* and *Splanchnotrophus* may be explained by common ancestry. In this scenario, the Philoblennidae would include a plesiomorphic ectoparasitic

genus *Philoblenna* retaining features such as swimming legs (Izawa 1976; Ho 1981; Avdeev et al. 1986; Ho & Kim 1992; Huys 2001). The common ancestor of *Chondrocarpus*, *Briarella* and splanchnotrophids switched to an endoparasitic life in sea slug hosts, reducing swimming legs and evolving a stocky body with long lateral processes and evolving dwarf males.

If *Briarella* is monophyletic, then a stocky body with long lateral processes has evolved in the common ancestor with splanchnotrophids (and possibly *Chondrocarpus*), and *B. doliaris* would represent the most basal offshoot of a clade of secondarily vermiform *Briarella* species. If *Briarella doliaris* is the direct sister to splanchnotrophids (and perhaps *Chondrocarpus*), rendering the genus *Briarella* paraphyletic; potential synapomorphies of such a clade include the stocky body shape, reduced body size, the possession of a fifth lateral appendage (only four in *Chondrocarpus* and several splanchnotrophids), and a successive reduction of antennule segments.

Although the herein described *B. doliaris* is more similar to *Splanchnotrophus* than any of its congeners, such similarities still may reflect convergent adaptations to an endoparasitic mode of life in the same group of hosts. Available morphology-based phylogenetic analyses are not conclusive yet. Analyses by Ho (1991) resulted in the Splanchnotrophidae (in the old, much broader sense; current usage applies to Huys 2001) as sister to Shiinoidae, a group of ectoparasites on fish that is highly dissimilar to endoparasitic Splanchnotrophidae in the modern, strict sense.

In conclusion, morphological studies on more material including males are necessary. Future phylogenetic studies should explore whether *Briarella* (or a subset thereof) is the sister group to Splanchnotrophidae and/or *Chondrocarpus*. The traditional inclusion of all endoparasitic copepods of sea slugs in the Splanchnotrophidae may ultimately remain the preferred arrangement (Monod & Dollfus 1932; Jensen 1987).

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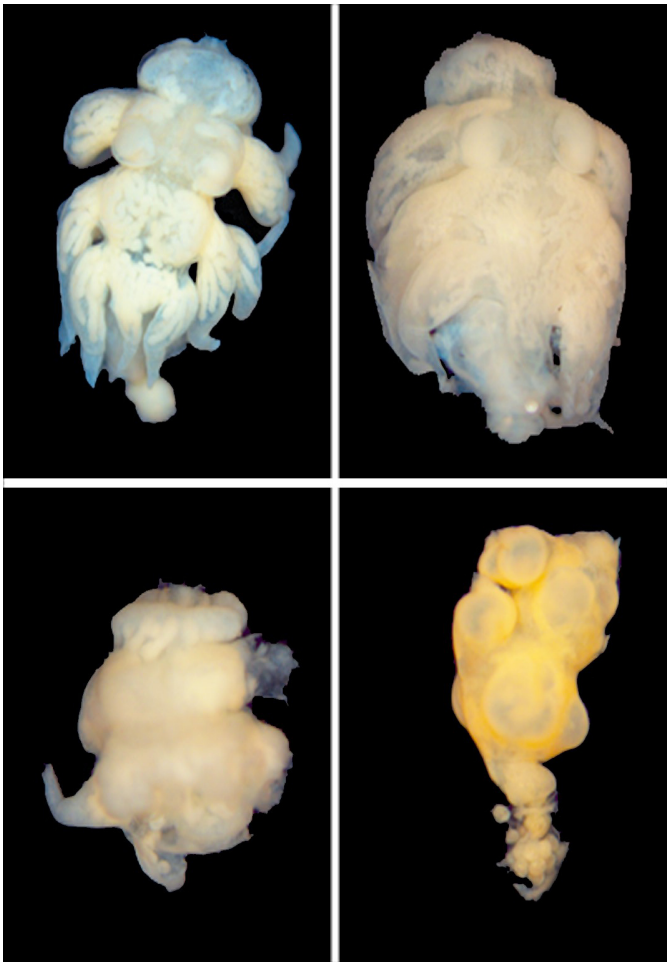
Chapter 6

Anton, R. F. Schories, D. Joerger, K. M. Kaligis, F. & Schrödl, M. (2015), Description of four new endoparasitic species of the family Splanchnotrophidae (Copepoda, Poecilostomatoida) from nudibranch and sacoglossan gastropod hosts; Marine Biodiversity 46 (1), pp. 183-195



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SENCKENBERG

ORIGINAL PAPER

Description of four new endoparasitic species of the family Splanchnotrophidae (Copepoda, Poecilostomatoida) from nudibranch and sacoglossan gastropod hosts

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Abstract The Splanchnotrophidae are a worldwide-distributed family of endoparasitic copepods, utilising shell-less opisthobranch gastropod hosts. Using scanning electron microscopy, we describe three new *Ismaila* Bergh, 1867 species infesting nudibranch hosts from southern Chile. *Ismaila volatilis* spec. nov. infests the proctonotid *Janolus sp.* and differs from all congeners by the size and number of dorsal bulges, the number of processes on the maxilla, and the thickness of thoracic appendages. *Ismaila chaihuiensis* spec. nov. was found in the doridoidean *Diaulula punctuolata* (D'Orbigny, 1837) and is diagnosed by a pore situated on a prominent bulge above the labrum. *Ismaila genalis* spec. nov. from a polycerid *Holoplocamus papposus* Odhner, 1926 host differs from its congeners in the size and form of the ventral bulges present on the head. These discoveries further broaden the range of splanchnotrophid host taxa; they are in line with earlier hypotheses of strict host specificity of *Ismaila* species and support Chile as a hotspot for *Ismaila* radiation. Herein, we present a key to the identification of all 14 *Ismaila* species. In Sulawesi (tropical Indo-West Pacific), *Arthurius gibbosa* spec. nov. infests the sacoglossan *Elysia macnaei* Marcus, 1982. The new species differs from both congeners by the short and stubby dorsal bulges. Uniquely among splanchnotrophids, long thoracic appendages are absent. *Arthurius* Huys, 2001 thus is the morphologically most divergent genus of the Splanchnotrophidae, but it is well characterized by the loss of several mouthparts and the preference for sacoglossan hosts. Currently, the distribution of the genus is limited to the Indo-West Pacific.

Keywords Parasitic copepods · Sea slugs · *Ismaila* · *Arthurius*

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Introduction

Among the vast variety of copepods, those associated with other taxa often are distinguished by exceptional morphological adaptations (Boxshall and Strong 2006; Gotto 1979; Østergaard et al. 2003). Members of the family Splanchnotrophidae Norman & Scott, 1906 live as endoparasites exclusively in nudibranch and sacoglossan hosts (Huys 2001; Schrödl 2003). Their high level of adaptation is reflected by the aberrant body shapes displayed in all genera of the family (Abad et al. 2011; Anton and Schrödl 2013a; Haumayr and Schrödl 2003; Huys 2001; Salmen et al. 2008a, b). Since its last taxonomic revision (Huys 2001), the family includes five recognized genera: *Arthurius* Huys, 2001, *Ceratosomicola* Huys, 2001, *Lomanoticola* Scott and Scott, 1895, *Splanchnotrophus* Hancock and Norman, 1863 and *Ismaila* Bergh, 1867. Recently, it was enlarged by a sixth genus—*Majimun* Uyeno and Nagasawa, 2012—based on a

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single new species discovered in Japan (Uyeno and Nagasawa 2012).

The currently best-studied splanchnotrophid genus *Ismaila* is known only from the coasts of the American continent (Anton and Schrödl 2013a; Haumayr and Schrödl 2003; Ho 1981; Huys 2001; Schrödl 2003). The type species *Ismaila monstrosa* Bergh, 1867 was first discovered by Bergh (1867) in a specimen of the cladobranch *Phidiana lynceus* Bergh, 1867 from Saint Thomas, U.S. Virgin Islands. More than a hundred years later, further species were scientifically recognized, e.g. *I. occulta* Ho, 1981 by Ho (1981) and few years later a third species *I. belciki* Ho, 1987 (Ho (1987) based on material of Belcik (1981), who did not, however, recognize it as separate species. In 2003 the genus was revised by Haumayr and Schrödl (2003) and eight new species were described. With 11 known species, *Ismaila* is currently the most diverse genus among splanchnotrophids (with 28 species contained in the family in total). All *Ismaila* species have been described from single host species, and thus appeared strictly host-specific (Anton and Schrödl 2013a). While the other splanchnotrophid genera infest exclusively either nudibranch (*Splanchnotrophus, Lomanoticola, Majimun*, and *Ceratosomicola*) or sacoglossan (*Arthurius*) hosts, *Ismaila* is the only genus currently known to infest both host groups (Anton and Schrödl 2013a; Haumayr and Schrödl 2003; Ho 1991; Huys 2001).

In contrast to *Ismaila*, *Arthurius* represents one of the smallest genera among the Splanchnotrophidae including only two species, *A. elysiae* (Jensen, 1990) and *A. bunakenensis* Salmen, Kaligis, Mamangkey, and Schrödl, 2008. The most prominent common features of the genus are remarkable reductions of the mouthparts (Anton and Schrödl 2013a; Huys 2001; Salmen et al. 2008a) and the fact that all known *Arthurius* species parasitize exclusively sacoglossan hosts.

In the present paper three new species of the genus *Ismaila* and one new species of *Arthurius* are described, and an identification key to *Ismaila* species is presented.

Material and methods

Nudibranchs were collected by SCUBA diving during several trips to southern Chile (in 2007 and 2010) and to Indonesia (in 2003). Infected specimens were photographed and identified by external characters using standard field guides (Coleman 2001; Schrödl 2003, 2009). The hosts were then preserved in 96 % ethanol. Egg sacs were separated for molecular analysis and stored in 96 % ethanol. In the lab, the hosts were dissected to extract all parasites, which were then photographed using a Olympus SZX 12 Stereomicroscope with an Olympus DF PLAPO 1× PF objective and a mounted Sony NEX 5N camera. For examination via scanning electron microscopy (SEM) the copepods were dehydrated in a graded acetone series and then critical-point dried in a BAL-TEC CPD 030 device. After mounting on SEM stubs they were coated with gold in a POLARON SEM COATING SYSTEM for 180 s under argon atmosphere. For the ultra structural analysis, a LEO1430 VP scanning electron microscope was used.

Descriptive terms used herein are adopted from Gruner et al. (1993), Huys (2001), Huys and Boxshall (1991), and Haumayr and Schrödl (2003).

Taxonomy

Subclass Copepoda H. M. Edwards, 1840
Order Poecilostomatoida Thorell, 1859
Family Splanchnotrophidae Norman & Scott, 1906
Genus *Ismaila* Bergh, 1867

***Ismaila volatilis* spec. nov.**

Material Holotype, SEM-mounted female (ZSMA20142900) with two attached males, one paratype, SEM-mounted male (ZSMA20142901), extracted from the host *Janolus* sp.1 in (Schrödl 2009 p. 531), (ZSM Mol 20130774, see Fig. 1a) collected by Dirk Schories, in 1–20 m depth, Chaihuin (lat.: 39°57′25.94″S long.: 73°36′10.15″ W), Southern Chile, 11.2010.

Distribution So far only known from the type locality

Etymology The name refers to the large dorsal bulges reminiscent of stubby wings.

Description of female (see Fig. 2)

Body delicate, measuring 3.2 mm in length, no external body segmentation detected. **Head** (cephalosome) distinctly set off from thorax, bearing two voluminous ventral bulges. **Thorax** with three pairs of dorsal processes and one unpaired mediodorsal process. **Processes** shorter than whole body, mediodorsal process slightly thinner and shorter than paired processes. One pair of voluminous dorsal bulges between the 1st pair of processes and two smaller swellings between the 2nd pair of processes (Fig. 2a). **Antennule** 2-segmented, unbranched, 1st segment with three lateral setae, 2nd segment with two ventral setae, one dorsal seta and seven terminal setae (Fig. 2d, fI). **Antenna** 3-segmented, unbranched, 1st segment voluminous with one strong serrate spine, 2nd segment with one spine and small integumental pore, 3rd segment claw-like with pointed tip and with five spines (Fig. 2c, fII). **Labrum** crescent moon-shaped with median furrow (Fig. 2c). **Mandible** sickle-shaped, partly covered by labrum (see Fig. 2e). **Maxillule** curved inwards, distal third bilobate, one lobe very thin, unarmed, 2nd lobe thick with numerous terminal setae (see Fig. 2c, fIV). **Maxilla** 3-segmented, 1st segment trapezoidal and enlarged, 2nd segment much smaller with one

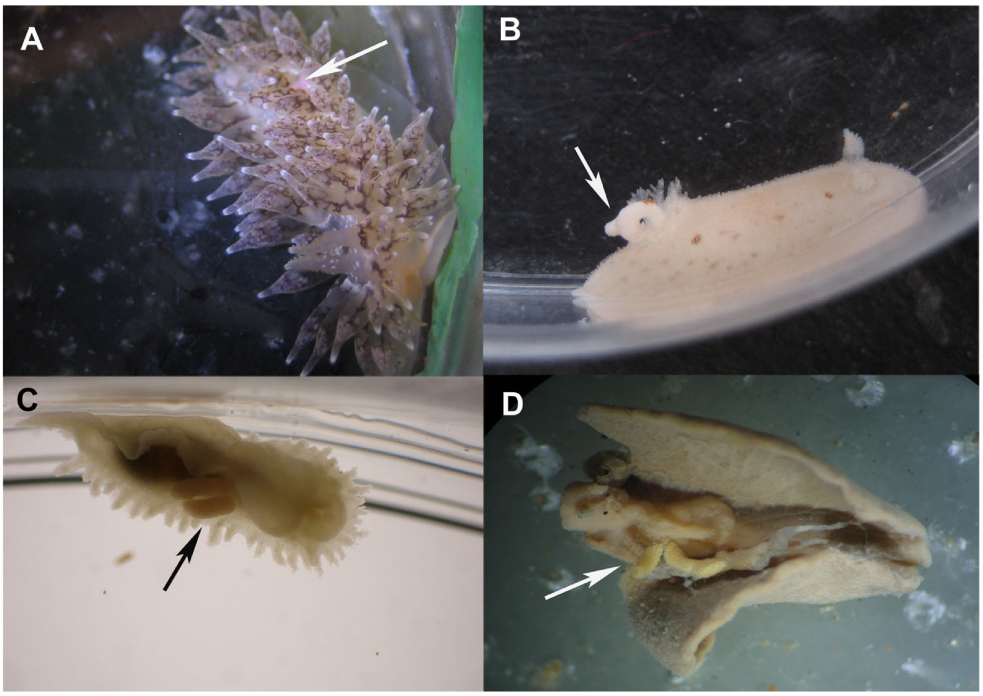

Fig. 1 Photographs of infected host specimens, arrows mark the egg sacs of the parasites. **a** *Janolus* sp.1, **b** *Diaulula punctuolata*, **c** *Holoplocamus papposus* (ventral view), **d** *Elysia macnaei* (fixed specimen, photograph courtesy A. Salmen)

spinous element, bearing 3rd segment and single additional process, 3rd segment with 18 long, thick hairs ordered brush-like in circles around long, sickle-shaped, pointed tip. Additional process with 19 shorter setae at inner edge (Fig. 2c, fV). **Labium** tongue-shaped with three triangle-shaped hairy patches, frontal section without hairs (Fig. 2c). **Maxilliped**: absent. **1st thoracopod** biramous, exopodite conical with blunt tip, endopodite much thinner and shorter than exopodite (Fig. 2b). **2nd thoracopod** biramous, both endo- and exopodite similar to 1st thoracopod (Fig. 2b, fVI). No further thoracopods detected due to host tissue. **Sclerotized ring** present where parasite embedded in integument of host. **Egg sacs** long straight or coiled, sausage-shaped, when coiled then forming one whorl, pink in living specimens. **Abdomen** 2-segmented, 1st segment with genital opening, 2nd segment bearing caudal rami. **Caudal rami** strong, stylet-like, two setae at base.

Description of male (see Fig. 3)

Body pear shaped, no external segmentation, dorsal processes absent. Cephalic and first two thoracic segments enlarged, testes visible through integument (Fig. 3a). **Head** voluminous, indistinctly set off from thorax (Fig. 3b). **Antennule** 2-segmented, 1st segment bent inwards with two long setae at distal end, 2nd segment about half length of 1st segment with ten long terminal setae (see Fig. 3d, and fI). **Antenna** 3-segmented, 1st segment with strong, pinnate spine, 2nd segment with one spine, 3rd segment double length of other segments, hook-like with three spines, small integumental pore covered by one spine (see Fig. 3d, fII). **Labrum** with median furrow (Fig. 3d). **Mandible** sickle-shaped, with two short processes at base (Fig. 3e, fIII). **Maxillule** as in female (Fig. 3e, fIV). **Maxilla** as in female (Fig. 3e, fV). **Labium** as in female. **Maxilliped** absent. **1st thoracopod** uniramous, no endopodite detected, exopodite conical with single terminal claw (Fig. 3b). **2nd thoracopod** uniramous, exopod resembling that of 1st thoracopod with terminal hook (Fig. 3b, c, fVI). No further thoracopods detected. **Sclerotized ring** present. **Abdomen** 4-segmented, with one pair of caudal rami, circle of pores around caudal rami. **Caudal rami** conical with three long basal setae.

Biology

The female was found laterally close to the head of the host with its abdomen protruding through the host's integument. No damage to the internal organs of the host could be detected. The egg sacs were hidden between the cerata. One single male was lying freely inside the host.

Remarks

Ismaila volatilis spec. nov. is assigned to the genus *Ismaila* due to the presence of the diagnostic single mediodorsal process in the female (Haumayr and Schrödl 2003; Anton and Schrödl 2013a), the 2-segmented antennulae, the 3-segmented antennae and maxillae, the bilobate maxillulae, and due to the presence of a small integumental pore in the 3rd segment of the antennae.

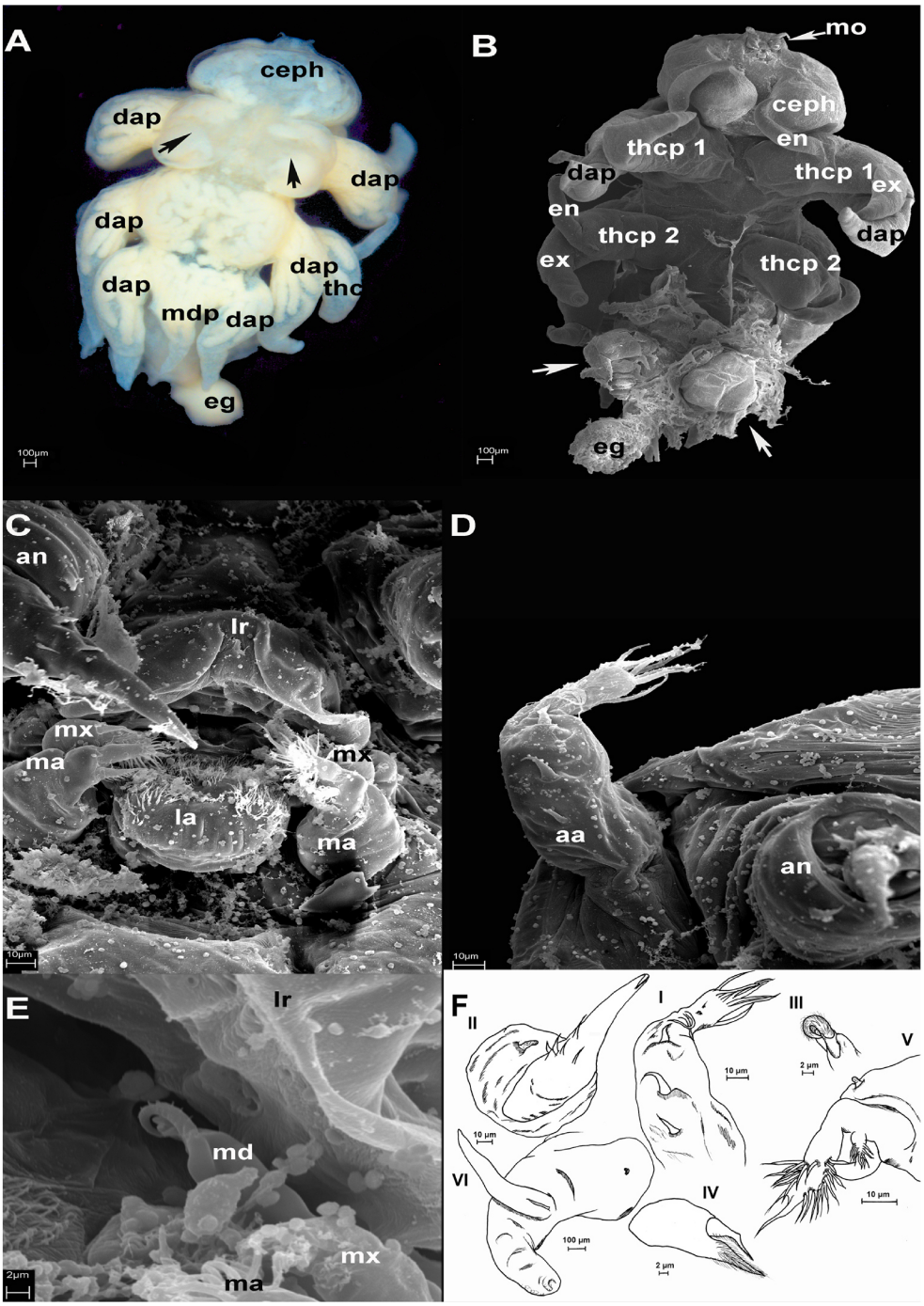

Fig. 2 *Ismaila volatilis* spec. nov. female, **a** light microscope picture, **b**–**e** SEM pictures, **a** dorsal view, prominent dorsal bulges marked by arrows, **b** ventral view, arrows indicate attached males, **c** mouth area, **d** left antennule, **e** mandible, **f** drawings of head appendages, **i** left antennule, **II** right antenna, **III** left mandible, **IV** right maxillule, **V** left maxilla, **VI** right 2nd thoracopod **aa** antennule, **an** antenna, **ceph** cephalosome, **dap** dorsal appendage, **eg** egg sac, **en** endopodite, **ex** exopodite, **la** labium, **lr** labrum, **ma** maxillule, **md** mandible, **mdp** medio-dorsal process, **mo** mouth, **mx** maxilla, **thcp** thoracopod

I. volatilis spec. nov. can be clearly distinguished from its congeners morphologically: e.g. it lacks the additional pair of processes described for *I. jenseniana* Haumayr and Schrödl, 2003. The body of *I. volatilis* spec. nov. is delicate and not stocky as described for *I. damnosa* Haumayr and Schrödl, 2003, *I. obtusa* Haumayr and Schrödl, 2003, and *I. robusta* Haumayr and Schrödl, 2003.

Ismaila volatilis spec. nov. also differs from congeners with slender bodies. The maxilla of *I. occulta* and *I. belciki* has two processes, while only one process is present in *I. volatilis* spec. nov. *Ismaila magellanica* Haumayr and Schrödl, 2003, *I. monstrosa*, *I. socialis* Haumayr and Schrödl, 2003, and *I. aliena* have long, thin thoracopods and dorsal processes, while those of *I. volatilis* spec. nov. are voluminous. The process on the maxilla has a connection to the 3rd segment in *I. androphila* Haumayr and Schrödl, 2003, but is separate from the 3rd segment in the new species (see Figs. 2c and 3e). The terminal lobes of the maxillule are equally thick in all known *Ismaila* species while in *I. volatilis* spec. nov. the inner lobe is much thicker than the outer (see Fig. 2c).

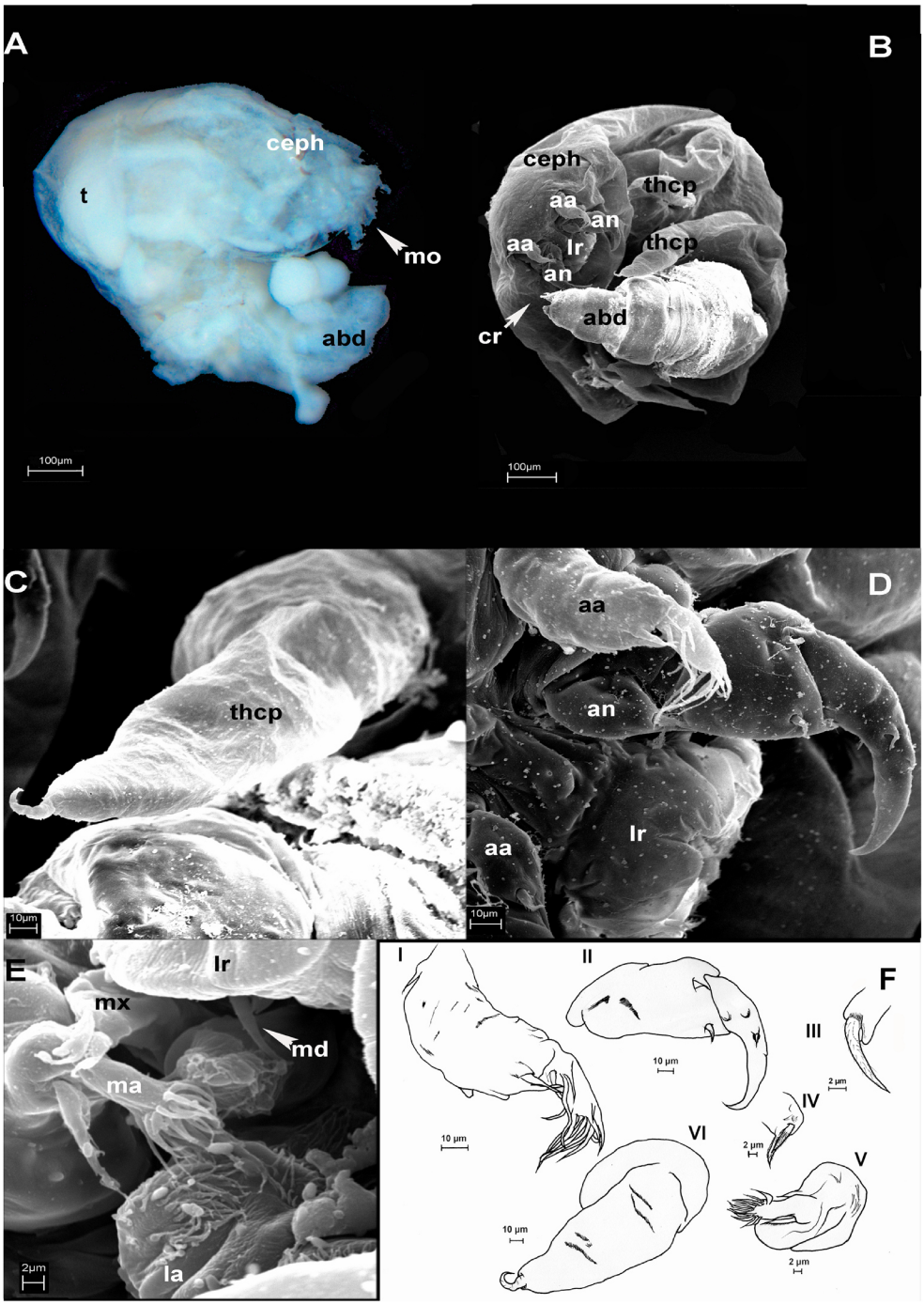

Fig. 3 *Ismaila volatilis* spec. nov. male, **a** light microscope picture, **b**–**e** SEM pictures, **a** lateral view, **b** ventral view, **c** 2nd thoracopod, **d** left antennule and antenna, **e** mouth, **f** drawings of head appendages, **I** left antennule, **II** left antenna, **III** right mandible, **IV** left maxillule, **V** left maxilla, **VI** left 2nd thoracopod **aa** antennule, **abd** abdomen, **an** antenna, **ceph** cephalosome, **cr** caudal rami, **la** labium, **lr** labrum, **ma** maxillule, **md** mandible, **mo** mouth, **mx** maxilla, **t** testis, **thcp** thoracopod

Ismaila volatilis spec. nov. also is distinctive from the other new species described herein. *Ismaila chaihuiensis* spec. nov. has thinner exopodites of the 1st thoracopods and lacks the pore above the labrum. In *I. genalis* spec. nov. the ventral bulges on the head are more pronounced and indistinctly branched, in contrast to *I. volatilis* spec. nov.

I. volatilis spec. nov. is the second splanchnotrophid species known to infest a member of the nudibranch family Proctonotidae Gray, 1853. But in contrast to *I. belciki*, which was found in Oregon (Ho, 1987) infesting *Janolus fuscus* O'Donoghue, 1924, *I. volatilis* spec. nov. was found in temperate waters of the southern hemisphere in the probably undescribed species *Janolus* sp.1.

Ismaila chaihuiensis sp. nov.

Material Holotype, SEM-mounted female (ZSMA20142902), extracted from a *Diaulula punctuolata* (D'Orbigny, 1837) host (ZSM Mol 20130775) (see Fig. 1b) collected by Dirk Schories, in 5–20 m depth, Chaihuin (lat.:

39°57′25.94″S log.:73°36′10.15″ W), Southern Chile, 11.2010.

Distribution So far only known from the type locality

Etymology The name refers to the collection site, and honours the Laboratorio Costero de Recursos Acuáticos Calfuco (Valdivia, Chile) and its entire team.

Description female (see Fig. 4)

Body very stocky, voluminous, measuring 2.9 mm in length, no segment borders visible, two dorsal bulges between 1st pair of dorsal processes, all remaining processes originating from posterior triangle-shaped area (see Fig. 4a). **Head** distinctly set off from thorax (Fig. 4a, b). Three pairs of dorsal **processes** voluminous, shorter than body with pointed tips. Single mediodorsal process equal in form and length to third pair of dorsal processes. **Antennule** 2-segmented, 1st segment bent inwards with strong spine at inner edge and two long terminal setae, 2nd segment with two short and one long seta at base and 15 long terminal setae (Fig. 4c, fI), area between antennulae with several integumental pores. **Antenna** 3-segmented, 1st segment unarmed, 2nd segment with one distal spine and bulbous, pinnate structure, 3rd segment with two small and one strong spine, segment hook-shaped with pointed tip, strong spine partly covering integumental pore in 3rd segment (Fig. 4c, fII). **Labrum** trapezoidal with median furrow, above labrum one intermediate bulge with single median pore (see Fig. 4c). **Mandible** short inward bent lobes without sickle-shaped blade (Fig. 4fIII). **Maxillule** bent inwards, distal third bilobate, each lobe with pointed tip and 10 setae at inner edge (Fig. 4d, fIV). **Maxilla** 3-segmented, 1st segment unarmed, 2nd segment bearing third segment and one single process, thinner than 2nd segment and with 12 setae at inner margin. 3rd segment of maxilla carrying approximately 15 long terminal setae (see Fig. 4d, fV). **Labium** trapezoidal with two hairy patches (Fig. 4c). **Maxilliped** absent. **1st** and **2nd** pair of **thoracopods** biramous with blunt tips, endopodite shorter and thinner than exopodite (Fig. 4b). **3rd thoracopod** reduced, seta like, no further thoracopods detected. **Egg sacs** long, sausage-shaped, coiled, forming two whorls. **Abdomen** 2-segmented, 1st segment, broad, T-shaped, bearing genital openings, 2nd segment smaller, knob-like, bearing caudal rami (Fig. 4e). **Caudal rami** short, small, seta-like, three pores at base with one seta arising from each pore (Fig. 4e).

Male not found.

Biology

The single female specimen was found posteriorly in the host with the abdomen protruding dorsally through the integument positioning the egg sacs directly behind the gills of the host. No internal damage to the host was detected.

Remarks

Ismaila chaihuiensis spec. nov. is assigned to the genus *Ismaila* due to the presence of the diagnostic single medio-dorsal process in the female (Haumayr and Schrödl 2003; Anton and Schrödl 2013a), the 2-segmented antennulae, the 3-segmented antennae and maxillae, the bilobate maxillulae and the presence of a small integumental pore in the 3rd segment of the antennae.

The additional pair of dorso-lateral processes unique to *I. jenseniana* is absent in *I. chaihuiensis* spec. nov. *Ismaila chaihuiensis* spec. nov. has a stocky body and, therefore, differs from all congeners except *I. obtusa, I. damnosa,* and *I. robusta.* There are two large dorsal bulges present in *I. chaihuiensis* spec. nov. at the level of the first pair of appendages, while in *I. obtusa* there are two pairs of dorsal bulges, one pair at the level of the 1st and 2nd pair of processes, respectively. There are two additional processes present on the maxilla of *I. robusta* and *I. damnosa,* while in *I. chaihuiensis* spec. nov. there is only one single process detected (Fig. 4d). The mandible of *I. chaihuiensis* spec. nov. is unique compared to all congeners since there is no sickle-shaped blade present (see Fig. 4fIII).

Ismaila chaihuiensis spec. nov. is not only the first splanchnotrophid species found in *Diaulula punctuolata,* but also the first record of a splanchnotrophid infesting a representative of the nudibranch family Discodoridiidae Bergh, 1891.

Ismaila genalis spec. nov.

Material Holotype, SEM-mounted female, (ZSMA20142903), one paratype, SEM-mounted male, (ZSMA20142904), extracted from *Holoplocamus papposus* (ZSM Mol 20130776; Fig. 1c) collected by Katharina Jörger, in 1–20 m depth, Isla Carmen, Chaitén, (lat.: 43°01′ 08.80″S log.:72°49′44.79″W), Southern Chile, 2007.

Distribution So far only known from the type locality

Etymology The name (Latin, meaning cheek) refers to the prominent ventral bulges present on the cephalosome.

Description female (see Fig. 5)

Body very stocky, voluminous, measuring 2.2 mm, no dorsal bulges present between 1st and 2nd pair of dorsal processes (Fig. 5a). **Head** not distinctly set off from thorax, bearing two voluminous ventral bulges with prominent constriction each (Fig. 5b, c). Three pairs of dorsal **processes** with one additional medio-dorsal process. **Antennule** 2-segmented, 1st segment with four strong setae, 2nd segment with five long terminal setae (Fig. 5fI). **Antenna** 3-segmented, 1st segment with two strong spines on inward margin, 2nd segment with two setae, one covering a small integumental pore, 3rd drawn out into strong hook (Fig. 5c, fII). **Labrum** triangular, scalloped, small pore concentric of v-shaped bulge above

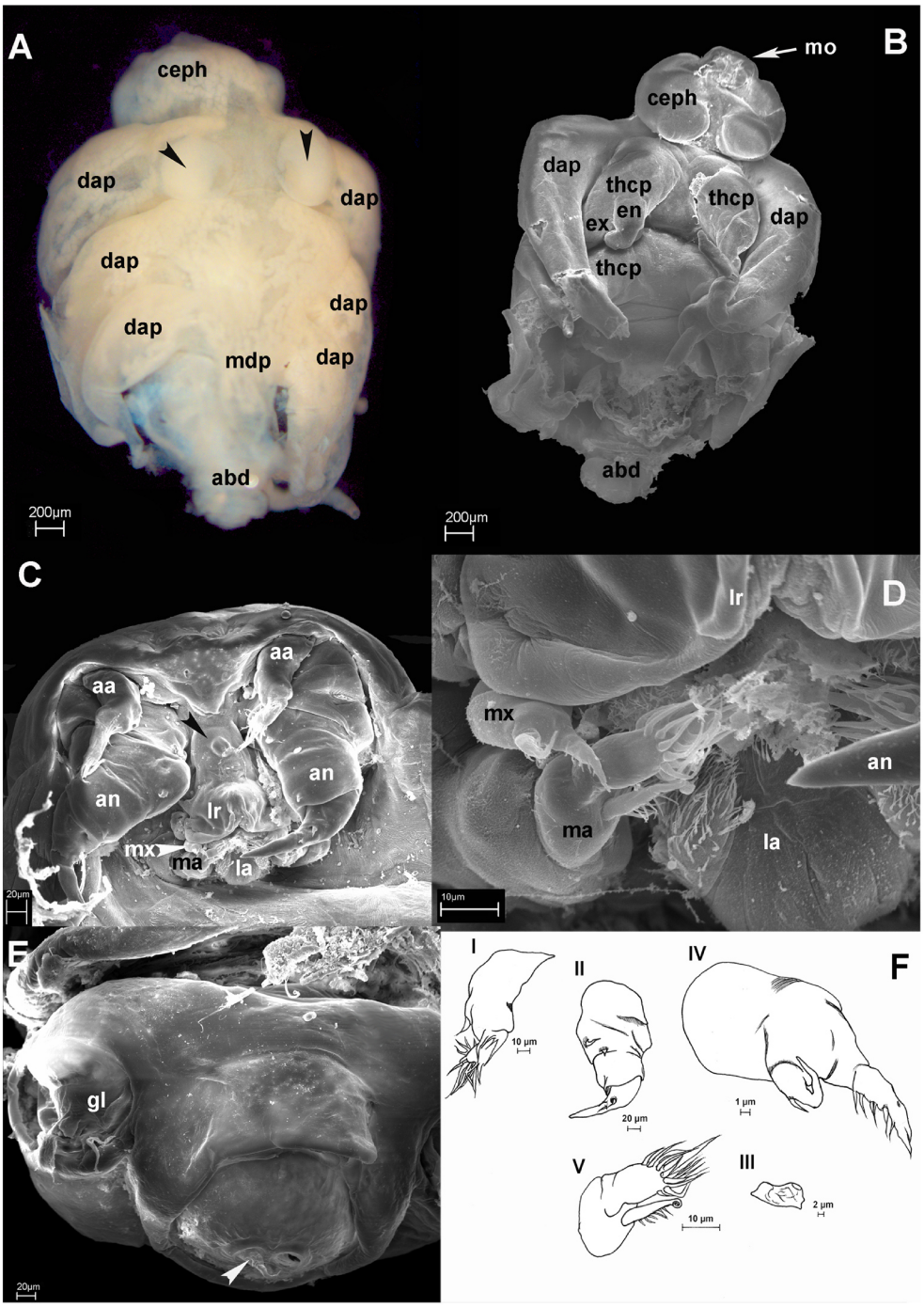

Fig. 4 *Ismaila chaihuiensis* spec. nov. female, **a** light microscope picture, **b**–**e** SEM pictures, **a** dorsal view (dorsal bulges marked by *arrows*), **b** ventral view, **c** mouth area (*arrow* indicates prominent bulge with pore present above the labrum), **d** mouthparts, **e** abdomen (*arrow* indicates caudal rami), **f** drawings of head appendages, **I** left antennule, **II** left antenna, **III** left mandible, **IV** right maxillule, **V** right maxilla **aa** antennule, **abd** abdomen, **an** antenna, **ceph** cephalosome, **dap** dorsal appendage, **en** endopodite, **ex** exopodite, **gl** genital lobe, **la** labium, **lr** labrum, **ma** maxillule, **mdp** medio-dorsal process, **mo** mouth, **mx** maxilla, **thcp** thoracopod

labrum. **Mandible** long, sickle-shaped with few fine hairs. **Maxillule** inwards bent, distal third bilobate, both lobes equally thick with pointed tips and approximately ten setae at inner edge (Fig. 5d, fIII). **Maxilla** 3-segmented, with approximately 15 long setae, additional long thin process with several setae on inner margin (Fig. 5d, fIV). **Labium** voluminous, triangular, two hairy patches connected posteriorly (Fig. 5d). **Maxilliped** absent. 1st and 2nd pair of **thoracopods** voluminous, conical, biramous, endopodite longer and thinner than exopodite (Fig. 5b, e). No further thoracopods detected. **Egg sacs** thick straight, sausage-shaped. **Abdomen** not visible. **Caudal rami** not detected.

Description of male (see Fig. 6)

Body without distinct segmentation, 1st thoracic segment enlarged (Fig. 6a). **Head** not distinctly set off from trunk. Thoracic **processes** absent (Fig. 6a, b). **Antennule** 2-

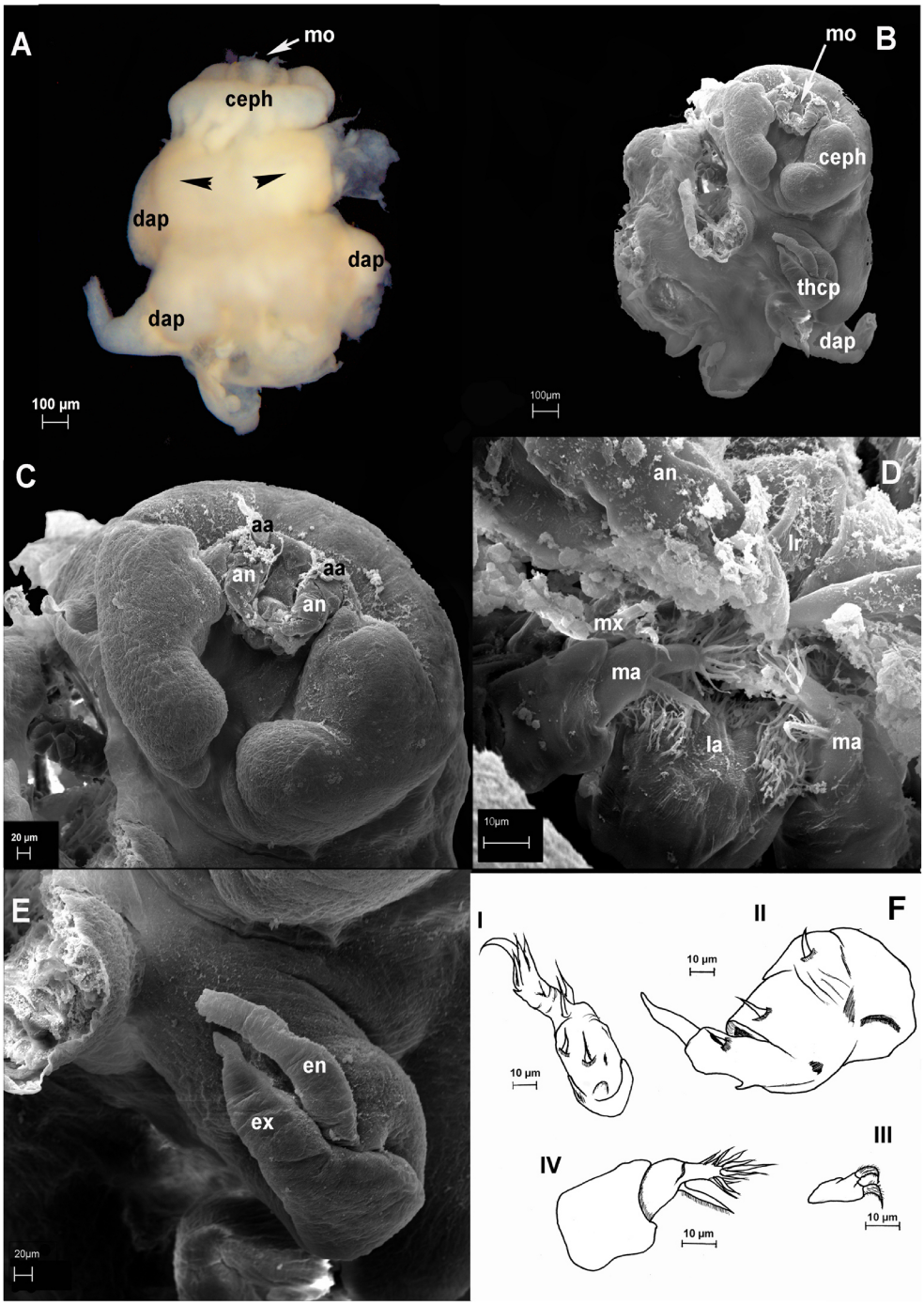

Fig. 5 *Ismaila genalis* spec. nov. female, **a** light microscope picture, **b**–**e** SEM pictures, **a** dorsal view, dorsal bulges marked by *arrows*, **b** ventral view, **c** cephalosome, **d** mouth, **e** left 1st thoracopod, **f** drawings of head appendages, **I** right antennule, **II** left antenna, **III** right maxillule, **VI** right maxilla **aa**, antennule; **an**, antenna; **ceph**, cephalosome; **dap**, dorsal appendage; **en**, endopodite; **ex**, exopodite; **la**, labium; **lr**, labrum; **ma**, maxillule; **mo**, mouth; **mx**, maxilla; **thcp**, thoracopod

segmented, 1st segment with three setae, 2nd segment bearing eight setae (Fig. 6d, gI). **Antenna** 3-segmented, 1st segment with one seta at inner margin on small bulge, 2nd segment with three setae, one covering small integumental pore, 3rd drawn out into strong claw (Fig. 6c, e, gII). **Labrum** as in female but pore not detected. **Mandible** not detected. **Maxillule** not detected. **Maxilla** as in female (Fig. 6f, gIII). **Labium** as in female (Fig. 6f). **Maxilliped** absent. **Thoracopods** conical, covered with host tissue, no endopodite detected, exopodite long and slender with terminal hook (Fig. 6b). **Abdomen** 2-segmented and short. **Caudal rami** long, stylet-like.

Biology

The two specimens were found lying close to each other on the right side in the body cavity of the host. The abdomen of the female was protruding through the integument and the egg sacs were hidden laterally under the mantle of the host. No internal damage to the host was detected.

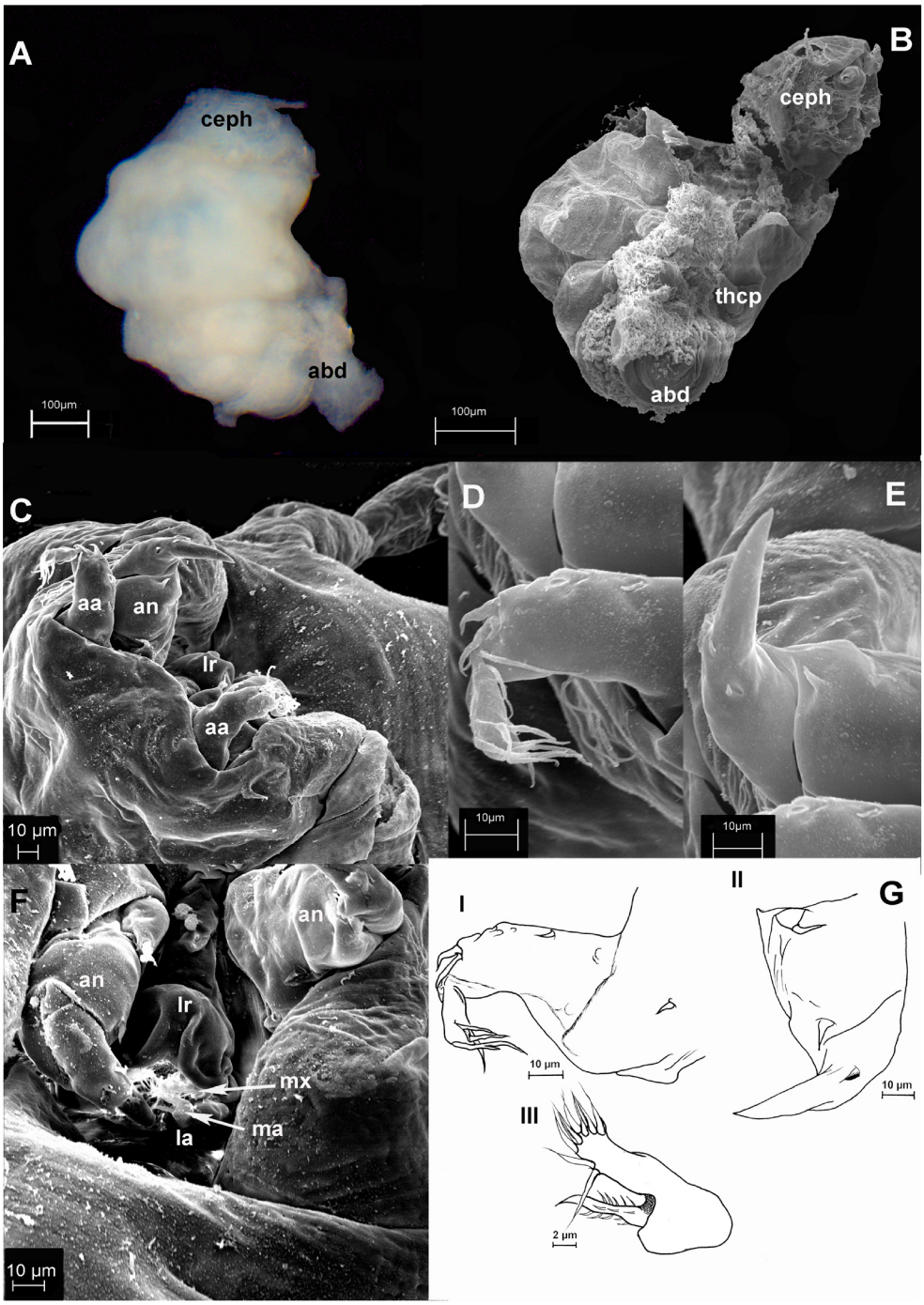

Fig. 6 *Ismaila genalis* spec. nov. male, **a** light microscope picture, **b**–**f** SEM pictures, **a** dorso-lateral view, **b** ventral view, **c** cephalosome, **d** left antennule, **e** left antenna, **f** mouth, **g** drawings of head appendages, **I** left antennule, **II** left antenna, **III** left maxilla. **aa** antennule, **abd** abdomen, **an** antenna, **ceph** cephalosome, **la** labium, **lr** labrum, **ma** maxillule, **mx** maxilla, **thcp** thoracopod

Remarks

I. genalis spec. nov. was assigned to the genus *Ismaila* due to the presence of the diagnostic single medio-dorsal process in the female (Haumayr and Schrödl 2003; Anton and Schrödl 2013a), the 2-segmented antennulae, the 3-segmented antennae and maxillae, the bilobate maxillulae, and due to the presence of a small integumental pore in the 3rd segment of the antennae.

Ismaila monstrosa, *I. jenseniana*, *I. androphila*, *I. aliena*, *I. socialis*, *I. magellanica,* and *I. belciki* differ from the new species by having a delicate body, while *I. genalis* spec. nov. has a stocky, voluminous body. The thoracopods and the processes of all former species and *I. damnosa* are much thinner than in *I. genalis* spec. nov. While there are two additional processes present on the maxilla in *I. occulta*, *I. socialis*, *I. robusta*, *I. belciki*, and *I. damnosa*, there is only one process present in *I. genalis* spec. nov. (see Fig. 5d). In contrast to

I. obtusa, *I. volatilis* spec. nov., and *I. chaihuiensis* spec. nov. there are no dorsal bulges present in *I. genalis* spec. nov. (see Fig. 5a). Most of all, *I. genalis* spec. nov. differs from all other species in the form of the ventral bulges on the head, which are nearly bilobate due to the constriction (Fig. 5c). These bulbs are present on the ventral side of the head in all females of the genus *Ismaila,* but especially those of *I. genalis* spec. nov. being suggestively bilobate might appear to be remnants of the maxillipeds, which are thought to be lost in all species of the Splanchnotrophidae.

In addition to *I. aliena*, which is associated with the nudibranch *Thecacera darwini* Pruvot-Fol, 1950 (Polycerinae Alder & Hancock, 1845), *Ismaila genalis* spec. nov. is the second species of *Ismaila* infesting a host of the family Polyceridae Alder & Hancock, 1845. It represents the first species found in *Holoplocamus papposus* and, therefore, also the first splanchnotrophid record from a member of the nudibranch subfamily Triophinae Ohdner, 1941.

Genus Arthurius Huys, 2001

***Arthurius gibbosa* spec. nov.**

Material Holotype, SEM-mounted female, (holotype deposited at the MZB; catalog number: MZB Cru Cop. 134.), extracted from *Elysia macnaei* Marcus, 1982 (ZSM20034046, see Fig. 1d) collected by Michael Schrödl for the Sam Ratulangi University, in 1–15 m depth, north-eastern-Sulawesi, Lembeh strait, "nudi retreat" (lat.: 01°29′ 07.4″N, log.: 125°14′ 27.6″E), Indonesia, 3.8.2003.

Distribution So far only known from the type locality

Etymology The name refers to the voluminous constricted bulges on the dorsal side of the thorax.

Description female (see Fig. 7)

Body: measuring 2.4 mm, no external segmentation visible (Fig. 7a). **Head** not distinctly set off from thorax (Fig. 7a). **Processes** represented by six voluminous bulges, two in mid-dorsal position four of them smaller and more lateral, middle ones each with one small knob-like structure terminating in two short tips, no internal structures visible (Fig. 7a, b, d). **Antennule** 1-segmented, small without setae (Fig. 7fI). **Antenna** 2-segmented, unarmed, 2nd segment forming strong hook (Fig. 7fII). **Labrum** absent. **Mandible** absent. **Maxillule** absent. **Maxilla** 2-segmented, 1st segment with one seta, 2nd segment forming strong outward bent hook (Fig. 7c, fIII). **Labium** absent. **Maxilliped** absent. **Thoracopods** greatly reduced, 1st and 2nd pair represented by small ventral bulges (Fig. 7b). **Egg sacs** long and straight, sausage-shaped. **Abdomen** 1-segmented, large, broad with genital openings (Fig. 7e). **Caudal rami** not detected.

Male not found.

Biology

Abdomen dorsally protruding through the integument with egg sacs located outside the body cavity but hidden under the parapodia of the host. No internal damage detected in host.

Remarks

Arthurius gibbosa spec. nov. was assigned to the genus *Arthurius* due to the absence of labrum, mandible, maxillule, and labium. *Arthurius gibbosa* spec. nov. differs from its congeners by having only six bulbous structures (see Fig. 7a) on the dorsal side while *A. elysiae* has eight and *A. bunakenensis* has 12 long voluminous dorsal processes. The maxilla is absent in *A. bunakenensis* while it is present in *A. gibbosa* spec. nov.

Discussion

The genus *Arthurius* shows the most heterogeneous external morphology among the Splanchnotrophidae, according to illustrations provided by Huys (2001) for *A. elysiae* and the morphology of *A. bunakenensis* given by Salmen et al. (2008a). Especially, the appearance of the dorsal appendages varies between the two previously known species. In particular, *A. gibbosa* spec. nov. clearly expands the known range of splanchnotrophid morphology. It is the only member of the Splanchnotrophidae missing the typical long dorsal appendages, but has unique dorsal bulges with forked tips instead (Fig. 7a, d). According to observations made during the dissection of the host, the absence of long appendages, as are typical for the Splanchnotrophidae, might result from an adaptation to the small space available inside the tiny host body cavity. Most interestingly, *A. gibbosa* spec. nov. thus shows a remarkable similarity with most members of the genus *Briarella*, also having only small, bulbous processes. This might support the hypothesis of Salmen et al. (2010) suggesting a closer relationship between *Briarella* and the *Splanchnotrophidae* based on *Briarella doliaris* Salmen, Anton, Wilson, and Schrödl, 2010, which was found to be morphologically very similar to the Splanchnotrophidae. However, according to the phylogeny of the Splanchnotrophidae given by Anton and Schrödl (2013a), the loss of appendages rather seems to be autapomorphic for the new species, while autapomorphies for the genus currently include the losses of the labrum, mandibles, maxillulae and of the labium, and the infestation of sacoglossan hosts (Anton and Schrödl 2013a; Huys 2001; Salmen et al. 2008a). For comparison with the results of Anton and Schrödl (2013b) on *Ismaila*, we predict that future studies on the internal anatomy of other Splanchnotrophidae will also unravel new and potentially diagnostic morphological characters. In the absence of high numbers of specimen available for studying in

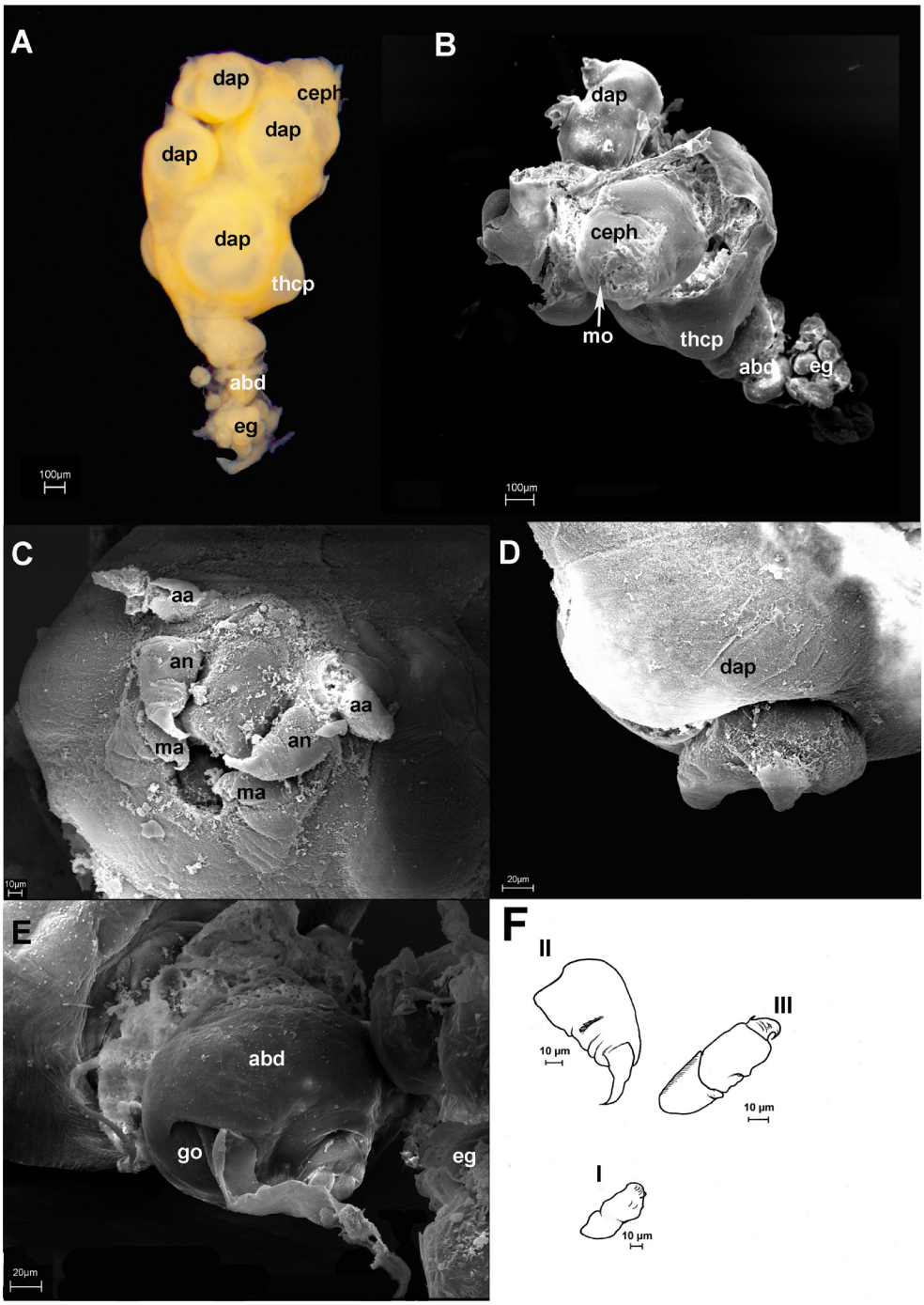

Fig. 7 *Arthurius gibbosa* spec. nov. female, **a** light microscope picture, **b**–**e** SEM pictures, **a** dorso-lateral view, **b** ventral view, **c** cephalosome, **d** dorsal appendage, **e** abdomen, **f** drawings of head appendages, **I** right antennule, **II** left antenna, **III** right maxilla, **aa** antennule, **abd** abdomen, **an** antenna, **ceph** cephalosome, **dap** dorsal appendage, **eg** egg sacs, **go** genital opening, **ma** maxilla, **mo** mouth, **thcp** thoracopod

most of the taxa of the Splanchnotrophidae obtained so far, non-invasive microanatomical approaches like micro-computed tomography (µCT) are favourable as they allow for multi-method approaches on a single specimen. So far resolution of detailed microanatomical structures is, however, still limited to two members of the genus *Ismaila* (Anton and Schrödl 2013b; Belcik 1965).

Together with the three new species described herein the genus *Ismaila* now includes 14 species, which are identifiable externally using the key given below. Based on morphological evidence, *Ismaila* is thus the most species-diverse genus of the Splanchnotrophidae. All previously known and newly found *Ismaila* species are reported from one single host species, so far confirming the hypothesis of strict host specificity within the genus (Haumayr and Schrödl 2003; Schrödl 2002). The three new species *I. volatilis* spec. nov., *I. chaihuiensis* spec. nov., and *I. genalis* spec. nov. were all found in nudibranchs yet unrecognised as possible hosts, thus further broadening the spectrum of host taxa. Remarkably, around the American continents there are at least twice as many splanchnotrophid

species than in any other region worldwide and infesting the greatest range of possible host species and families, but all belong to just one single genus. Anton and Schrödl (2013a) proposed historical biogeographic scenarios with an ancestral *Ismaila* colonizing the neotropics, and then diverging in different areas and water masses. With nine *Ismaila* species reported, temperate southeastern Pacific waters appear to host an especially rich and ecologically diverse splanchnotrophid fauna, a pattern that is further strengthened herein. Only a single *Ismaila* species has been reported from Peru (Schrödl and Hooker 2014), none from northern Chile and another single species is known from southernmost Patagonia (Haumayr and Schrödl 2003). The vast majority of *Ismaila* species occurs in a quite narrow coastal area off central Chile (Schrödl 2002) towards the northern part of the Chilean fjord area investigated herein. Schrödl (2002) already proposed a scenario of *Ismaila* radiation in Chilean waters, driven by potential host switching events. This assumption was confirmed by morphocladistic analyses by Anton and Schrödl (2013a), however, prior to the new discoveries reported here. In the future, the species level taxonomy, the implied assumption of host specifity within *Ismaila,* and the hypothesis of—potentially quite recent—adaptive radiation in Chile should be tested using molecular markers.

Key to species of *Ismaila*, based on females

1– Three pairs of dorso-lateral processes	**2**
– Additional pair of dorso-lateral processes	***I. jenseniana***
2– Body stocky (as in *I. chaihuiensis* spec. nov., see Fig. 4a)	**3**
– Body delicate (as in *I. genalis* spec. nov., see Fig. 5a)	**5**
3– Exopodites of thoracopods very thick, dorsal processes flattened	***I. obtusa***
– Dorsal processes voluminous and conical	**4**
4– 2nd thoracopod: endopodite longer than exopodite, both equally thick	***I. damnosa***
– 2nd thoracopod: exopodite and endopodite equally long, exopodite thicker than endopodite	***I. robusta***
5– Dorsal bulges present (as in Fig. 4a)	**7**
– Dorsal bulges absent (as in Fig. 5a)	**6**
6– Head: ventral pair of bulges with constriction	***I. genalis***
– Head: ventral pair of bulges without constriction	***I. socialis***
7– 1st thoracopod: exopodite and endopodite equal in length	**8**
– 1st thoracopod: exopodite longer than endopodite	**11**
8– 2nd thoracopod: exopodite and endopodite equal in length	***I. androphila***
– 2nd thoracopod: exopodite longer than endopodite	**9**
9– 1st thoracopod: exopodite and endopodite equally thick	***I. belciki***
– 1st thoracopod: exopodite thicker than endopodite	**10**
10– 2nd thoracopod: inner process of endopodite as long as endopodite	***I. monstrosa***
– 2nd thoracopod: inner process of endopodite shorter than endopodite	***I. aliena***
11– Maxillulae: inner terminal lobe much thicker than outer lobe	***I. volatilis***
– Maxillulae: terminal lobes equally thick	**12**
12– Single pore present on a prominent bulge above the labrum	***I. chaihuiensis***
– Area above labrum without pore on a bulge	**13**
13– 3rd thoracopod: inner endopodal process rudimentary; four dorsal bulges	***I. occulta***
– 3rd thoracopod: inner process small and thin; two dorsal bulges	***I. magellanica***

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Declaration of Ethical Standards The authors hereby declare that all expereiments were complient with the current laws.

Conflict of interest The authors hereby declare that there is no conflict of interest.

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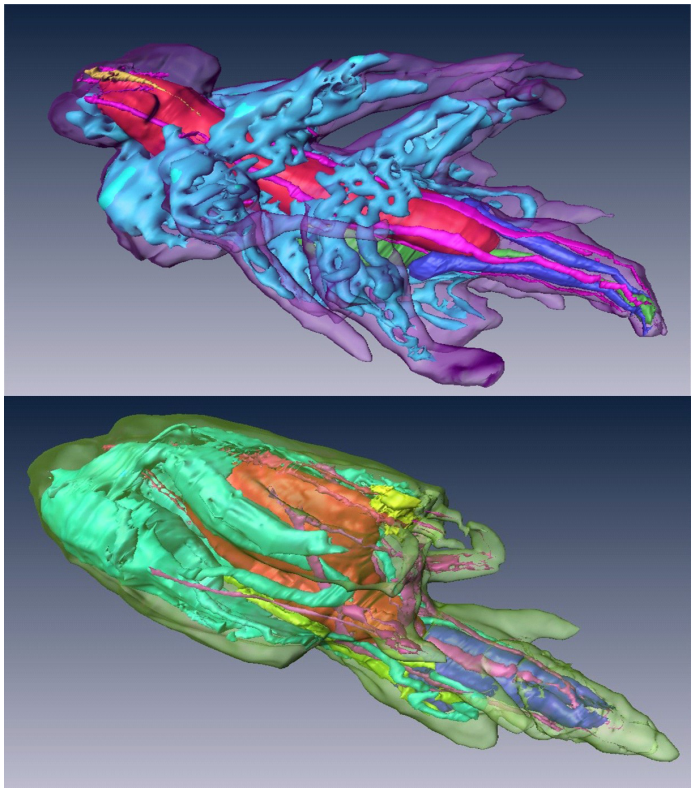
Chapter 7

Anton, R. F. & Schrödl, M. (2013), The inner values of an endoparasitic copepod - Computerbased 3D – reconstruction of *Ismaila aliena* (Copepoda; Poecilostomatoida; Splanchnotrophidae) ; Spixiana 36 (2), pp. 183-199



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The "inner values" of an endoparasitic copepod – computer-based 3D-reconstruction of *Ismaila aliena*

(Copepoda, Poecilostomatoida, Splanchnotrophidae)

Roland F. Anton & Michael Schrödl

Anton, R. F. & Schrödl, M. 2013. The "inner values" of an endoparasitic copepod – computer-based 3D-reconstruction of *Ismaila aliena* (Copepoda, Poecilostomatoida, Splanchnotrophidae). Spixiana 36 (2): 183–199.

Knowledge about the Splanchnotrophidae, a family of endoparasitic copepods infesting opisthobranch sea slugs, currently is restricted to the external morphology. In contrast, their internal anatomy is still largely unknown and many questions concerning life-history traits remain unanswered. Therefore, the microanatomy of both sexes of *Ismaila aliena* Haumayr & Schrödl, 2003, a splanchnotrophid infesting the nudibranch *Thecacera darwini* Pruvot-Fol, 1950 in Chile, was studied using computer-based 3D-reconstruction methods on serial semithin histological sections. The body musculature comprises three paired longitudinal strands. Regarding the cephalic and thoracic appendages, besides the antennae only the first pair of male thoracopods is supplied with strong musculature. The digestive system consists of an oesophagus and a voluminous, sack-like midgut, while hindgut and anus are lacking. Structural, functional and observational evidences suggest that *I. aliena* and at least some other splanchnotrophids are body fluid rather than – eponymous – tissue feeders. The gonad of *I. aliena* is large in both sexes and neither antrum nor seminal receptacle was detected in the female. Compared to ectoparasitic copepods, the central nervous system of *I. aliena* is modified, especially in males. Microanatomical results of the present study are compared with available literature results on *I. belciki* Ho, 1987 (as *I. monstrosa* Bergh) and discussed regarding potential functions. Within an emerging functional and evolutionary framework we provide some new insights in the life history of the splanchnotrophid parasites.

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Introduction

Copepods constitute one of the most important groups of marine zooplankton showing great diversity considering morphological characters (Yoshikoshi 1975). Especially parasitic copepods display diverse stages of adaptation, ranging from morphologically rather typical (ectoparasitic) forms like *Pararchinotodelphys gurneyi* Illg, 1955 to extremely specialised endoparasites like *Sacculina carcini* Thompson, 1836, which is in its adult stage hardly recognisable as a crustacean. However, the internal anatomy of parasitic copepods is mostly unstudied except for species that have at least some economic relevance due to their respective hosts (Saby 1933, Østergaard 2004, Özel et al. 2004). Therefore members of the families Chondracanthidae and Lernanthropidae, parasites of commercially important fishes, were histologically analysed in detail in order to gain insight into their life history (Saby 1933, Rigby & Tunnell 1971, Clarke

& Klussmann-Kolb 2003, Molnár & Székely 2004). But for the majority of parasitic species causing no commercial damage comparable data are yet missing (Schrödl 1997, Özel et al. 2004). In the case of the small endoparasitic family Splanchnotrophidae, which specialises upon opisthobranch sea slugs, available morphological data are largely restricted to external characters (Huys 2001, Haumayr & Schrödl 2003, Anton & Schrödl 2013), which may be insufficient due to widespread organ reductions (Huys 2001). Our recent phylogenetic analysis of Splanchnotrophidae based on external morphological data provided a first testable phylogenetic hypothesis, but suffered from homoplasy, i. e. convergent developments reflecting the high level of adaptation in these endoparasitic species (Anton & Schrödl 2013). Histological studies may provide important additional data for a more detailed phylogenetic analysis and therefore advancing the understanding of copepod evolution.

In particular, there are some fundamental biological questions like nutrition or respiration, which cannot be answered by just looking at the external morphology (Clarke & Klussmann-Kolb 2003). Life-history traits of free-living or ectoparasitic copepods can be studied simply by cultivation under laboratory conditions or by analysing stomach contents (Gotto 1957, Nival & Nival 1976, Saiz & Kiorboe 1995, Wu et al. 2004). Unfortunately these methods seem rather unfitting for endoparasitic species. Although it is possible to see parasitizing females through the integument of the host in some splanchnotrophid species (Schrödl 1997, Abad et al. 2011), a detailed analysis especially of life-history traits from outside is impossible. Consequently, the mode of nutrition of the Splanchnotrophidae was discussed ever since the family was introduced. Hancock & Norman (1863) first suggested them to feed on inner organs and tissues of their hosts, which is reflected by how they named the type genus *Splanchnotrophus*. Huys (2001) also adopted this hypothesis of nutrition in his revision of the family. More recently, the absence of gnawing marks on the inner organs of the hosts led to the hypothesis that splanchnotrophids rather seem to be haemolymph suckers (Schrödl 2003, Anton & Schrödl 2013).

Rather than dissections the implementation of histological methods seems promising to gain reliable anatomical data from small-sized copepods. However, until today only one member of the family Splanchnotrophidae was subject of histological studies. Belcik (1965) examined the internal morphology of North-Eastern Pacific *Ismaila belciki* Ho, 1987 (as *I. monstrosa* Bergh), but despite bringing up interesting results further studies were never undertaken.

The present study analyses the internal anatomy of both sexes of *Ismaila aliena* Haumayr & Schrödl, 2003 (see Fig. 1) using histological semithin-sections of resin-embedded specimens. Computer-based microanatomical 3D-reconstruction techniques have been successfully applied on small molluscs such as sea slugs (e. g. Rückert et al. 2008, Brenzinger et al. 2013) and on arthropods (Brenneis & Richter 2010). Advantages of this method include better structural resolution, analytical scrutiny and efficiency to visualise the anatomy of highly complex organ systems of small specimens (Neusser et al. 2006, DaCosta et al. 2007). Exploring the internal anatomy and functions of both sexes of *Ismaila aliena*, chief purposes of the present study are to shed some light on the debated life history of the parasite, above all with respect to nutrition, respiration and mobility.

Material and methods

During a collection trip to Valdivia, southern Chile, in 2010 several infested nudibranchs were kept in the laboratory and observed for several days. Infested host specimens of the nudibranch sea slug *Thecacera darwini* Pruvot-Fol, 1950 were collected in the Bahía de Coliumo, central Chile in 2005. One male (ZSMA20130020) and one female (ZSMA20130021) of *I. aliena* were dissected from the 70 % ethanol preserved hosts. After removing the egg sacs they were dehydrated in an acetone series and were embedded in Spurr's low viscosity resin (Spurr 1969). Both specimens were serially sectioned (thickness 1.5 µm) following Ruthensteiner (2008) using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) and sections were stained with methylene-azure II (Richardson et al. 1960). To obtain suitably sized sections, the tips of dorsal processes of the female were trimmed. Every section was photographed using a Jenoptic Prog Res C3 microscope camera (Jenoptic Laser, Optic, Systems GmbH, Jena, Germany) on a Leica DMB-RBE microscope (5×/0.15) (Leica Microsystems, Wetzlar, Germany). For the reconstruction of all major organ systems every section was photographed for the male and every second section for the female specimen. The computer-based 3D-reconstruction was performed with the software AMIRA 4.1 (TGS Europe, Mercury Computer Systems, Mérignac, France) according to the protocol of Neusser et al. (2006) and Ruthensteiner (2008).

Results

An overview of the general external habitus is given in Figure 1. Ethanol-fixation allowed analysis of tissues rather than at cellular level.

Body

The body comprises of a cephalosome, bearing the head appendages and the mouthparts, the thorax

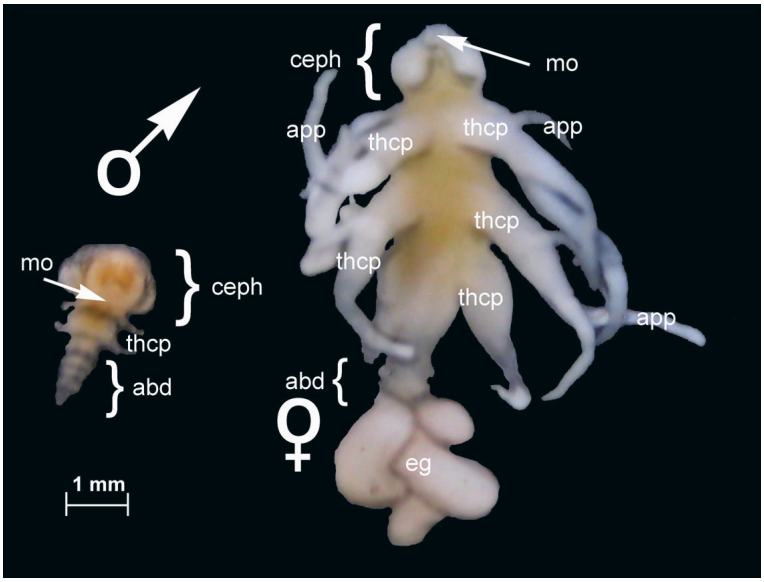

Fig. 1. General habitus of *I. aliena*. Ventral view of male and egg-bearing female. **abd**, abdomen; **app**, dorsal appendages; **ceph**, cephalosome; **eg**, egg sacs; **mo**, mouth; **thcp**, thoracopods.

bearing the modified thoracopods and the dorsal appendages in females, and of the short abdomen (Fig. 1). The body possesses three orifices: The mouth lying ventral in the head region and the paired genital openings lying laterally on the first abdominal segment. In both sexes no traces of an internal segmentation were detectable. The body wall shows an epidermis covered by a thin chitinous layer (see Figs 2 and 3).

Musculature

The female body shows three pairs of longitudinal muscles (see Fig. 4C). The dorsal strands lie close together and originate from the anterior part of the head. The strands of the ventral pair run further apart from each other, starting from the mouth area of the head. From these ventral muscles two lateral strands originate at the level of the third thoracic segment. All six resulting strands extend to the most posterior part of the abdomen (Fig. 4C), which can be retracted telescope-like in living animals. Concerning the mouthparts, the antennae are equipped with the strongest muscles. Mandibles and maxillae only possess thin strands of musculature. Further two strands of musculature, originating directly from the starting point of the ventral longitudinal muscles, run transversely along the ventral border of the head region (see Fig. 4C). At the level of the second thoracic segment a pair of V-shaped muscles reaches from the dorsal side of the body into the first segment of the thoracopods (Fig. 4C). Neither in the thoracopods nor in the dorsal appendages any musculature could be detected.

In the male, two pairs of longitudinal muscles – e. g. the lateral and the ventral pairs – reach from the mouth region of the cephalosome to the posterior end of the abdomen (see Fig. 5C). The lateral and the ventral strand are connected by a transverse strand of muscle at the level between the first and second pair of thoracopods. Transverse and longitudinal strands are directly connected in areas attached to the cuticle. An additional dorsal pair of longitudinal muscle strands reaches from the frontal area of the cephalosome to the level of the first thoracic segment and is divided into four parts by connections to the body wall (Fig. 5C). The antennae and the first pair of thoracopods show strong musculature. In the first two pairs of thoracopods each first segment is equipped with a single muscle strand reaching to the lateral sides of the respective segment (Fig. 5C).

Digestive system

The female digestive system comprises a mouth and a short oesophagus followed by a voluminous tube-like midgut (see Fig. 4D). The midgut reaches from the head region to the level of the third pair of dorsal appendages (Fig. 4). The walls of the midgut are straight and show no lumen-sacs as mentioned by Saby (1933) for *Parabrachiella insidiosa* (Heller, 1865). In the whole lumen of the midgut, gland cells

as described by Saby (1933) can be found either isolated and free in the lumen or attached to the inner wall of the midgut (Figs 2 and 3). In the female, the gland cells are more or less homogeneously dispersed (Fig. 2), while in males they are aggregated at the anterior and posterior end of the midgut and in the area around the mouth (Fig. 3). In females, a small sac-like structure is present on the right side at the posterior end of the midgut (see Fig. 4D). Additional digestive organs (e. g. gut or rectum) or an anal opening are absent.

The digestive system of the male largely resembles that of the female; the large, triangle-shaped sack-like midgut also fills great parts of the body cavity but reaches only to the level of the second pair of thoracopods (see Fig. 5D). No traces of either hindgut or anal opening were detectable.

Reproductive system

The reproductive system of the female consists of an unpaired ovary, paired oviducts and an unpaired cement-gland (Fig. 4B). The ovary stretches through the whole body – including dorsal processes and thoracopods – and sends branches even into the head region (cephalosome). At the level of the third thoracic segment the ovary is connected to the paired oviducts by thin and short ducts (Fig. 2C). The voluminous oviducts lead to the genital openings located on the ventro-lateral sides of the first abdominal segment. Dorsally, close to the midgut, an unpaired cement-gland is present which tapers into one single duct reaching to the beginning of the abdomen; there, it separates into two ducts, which are enlarged at the level of the last thoracic segment (see Fig. 4B). Although the ducts run partly alongside the oviducts, they do not fuse with them until reaching the genital openings on the first abdominal segment. A receptaculum seminis or an antrum as described by Schminke (2007) could not be detected.

In the male, the paired testes are located dorsally in the cephalosome, filling its lumen to the greatest part (see Fig. 5B). The paired vasa deferentia are long and entwined. They are enclosed by strong musculature and lead to paired seminal vesicles, where the spermatophores are formed (as described by Schram 1986) and which are connected to the genital openings in the first abdominal segment (Fig. 5B).

Nervous system

The nervous system of the female consists of a supra-oesophageal ganglion and an elongated infra-oesophageal ganglion tapering into a ventral nerve cord (see Fig. 6A). Both ganglia are connected by two massive circum-oesophageal connectives. The ventral nerve cord appears unpaired with five small ganglia and terminates at the level of the first thoracic segment (Fig. 6A). The organisation of the nervous system shows the highest level of variation between male and female *I. aliena* apart from the gonads. The nervous system of the male consists of a circum-oesophageal nerve ring (gullet-ring), and a supra-oesophageal ganglion, which is drawn out ventrally (see Fig. 6B). A ventral nerve cord like in the female could not be detected. In both sexes the supra-oesophageal ganglion seems to represent the largest part of the brain (Fig. 6). A nauplius eye was neither found in the female nor in the male. The reconstruction of the nervous system is limited to the most conspicuous parts, since for a detailed reconstruction especially of the branching nerves other methods (e. g. Bundy & Paffenhöfer 1993, Geiselbrecht & Melzer 2013) are necessary. Hence the terminology of the distinct parts refers to Saby (1933) since for the identification of proto-, deutero- and tritocerebrum (Lowe 1935) the exact innervation of all cephalic and thoracic appendages is required.

Excretion and circulation

In both sexes, a rather small, paired structure was found on the ventral sides of the cephalosome, which we assume to represent the antennal glands (see Figs 4D, 5D and 6). Neither a heart or other circulatory organs nor any special respiratory organs could be detected.

Discussion

One of the few authors dealing with the internal anatomy of parasitic copepods is Saby (1933) who examined and described six species of the ectoparasitic families Chondracanthidae and Lernaeopodidae in great histological detail. Belcik was the first to study the internal anatomy of members of the Splanchnotrophidae, i. e. describing both sexes of *I. belciki* (as *I. monstrosa*) in his doctoral thesis (Belcik 1965) and publishing on the male later (Belcik 1981). Although providing many new insights, such studies were limited by the paraffin-based methodology used at those times. Both specimens available for examination herein were fixed in 70 % ethanol; preservation was good enough to distinguish tissues and recognise organs. Here we compare and supplement the initial data provided by Belcik (1965, 1981) using modern semi-thin histological and serial 3D microanatomical reconstruction techniques for the first time for splanchnotrophids.

Body wall

In *I. aliena* the chitinous outer layer of the body wall is thin throughout the whole body, which was also mentioned for *I. belciki* by Belcik (1965, 1981); therefore the parasite appears soft and fragile during macroscopic preparation. Papillae or other structures related to respiration could not be detected. This indicates that the entire body surface is involved in exchange of gases, confirming earlier assumptions (Salmen et al. 2008b, Anton & Schrödl 2013). The body wall is slightly thickened only in the area around the mouth and at the mouthparts. In addition, the distal part of the abdomen, which becomes ingrown in the body wall of the host or is in contact with the seawater outside of the host, is equipped with a very strong chitinous layer and a thick epithelium, obviously for stability and protective reasons.

Body musculature

The musculature found in *I. aliena* greatly resembles *I. belcik,* but Belcik (1965, 1981) mention only four strands of longitudinal muscles. However, in his drawings six strands of longitudinal muscles can be seen (Belcik 1965 figs 13B and 14A) so he may simply not have counted the lateral ones as independent strands. In contrast, the musculature of *I. aliena* shows considerable differences compared to the results of Saby (1933). For example in *I. aliena* longitudinal and cephalic muscles are not originating from a ring of strong musculature and all species examined by Saby (1933) are equipped with only four strands of longitudinal muscles.

Female *I. aliena* have additional muscles running transverse from the starting point of the ventral longitudinal muscles to the ventral side of the cephalosome. Neither Saby (1933) nor Belcik (1965) mentioned any such muscles and their function remains unclear. The strong longitudinal musculature present in *I. aliena* seems to serve particularly to retract the parasite's abdomen. This behaviour can easily be observed in egg-bearing females by touching them with a forceps. The female will retract its abdomen and create a fold in the integument of the host to protect the eggs (personal observation). The antagonist of this retraction may be the internal body pressure of the parasite on the one hand and on the other hand the elastic mantle tissue of the host. Both sexes need to extend their abdomen trough the hosts' body tissue and telescope-like extension may help with penetrating; then their abdomen, with special abdominal ring in *Ismaila* (see Anton & Schrödl 2013), is firmly embedded in the host tissue.

Male *I. aliena* are equipped with stronger longitudinal musculature than females. One reason could be that in *I. aliena* the abdomen of anchored males is not in direct contact with that of the female. Therefore the retraction of the abdomen is possibly needed to make contact between the male and female genital porus. An additional explanation refers to the assumed higher mobility of male Splanchnotrophidae (Ho 1987, Schrödl 1997, Anton & Schrödl 2013).

Musculature of the thoracopods

Following the drawings provided by Saby (1933), the v-shaped muscles found in female *I. aliena* represent the only remnants of the thoracopod musculature (Fig. 4C). According to muscle arrangement, females seem to be able to move the first pair of thoracopods only as a whole, i.e. we could not detect any significant musculature indicating that the thoracopods are able to perform complex movements like grasping.

It has been assumed that in parasitic copepods males often represent the lesser-modified state of development (Saby 1933, Huys 2001). Considering the strong thoracopodal musculature of *I. aliena* (Fig. 5C), this hypothesis is supported. Possible reasons for this sexual dimorphism refer to locomotion and mating behaviour and will be discussed below.

Locomotion

The only musculature remaining for the purpose of locomotion in female *I. aliena* are the longitudinal muscles. The different strands are contracted alternately in the female resulting in a kind of movement similar to nematode worms such as *Ascaris* (personal observation). During this movement, the strong antennae may be used for grasping so that the rest of the body can be pulled forward; however, female *I. aliena* are no longer capable of any efficient directional movements (Schrödl 1997, personal observation).

The high degree of adaptation to semi-sessile endoparasitic live is revealed most obviously when observing the efforts made by a female parasite leaving its host (Schrödl 1997). Splanchnotrophid parasites may try to escape, if their host is in bad physical condition (e.g. injured or undernourished). In female *Ismaila* this escaping-behaviour is restricted to opening the host's integument and crawling out of the body cavity but the abdomen will remain embedded in host tissue (Schrödl 1997, personal observation). The reason for this behaviour is still unknown since both individuals (parasite and host) will die shortly after the parasite emerges from its host (Schrödl 1997, Abad et al. 2011). Males, however, are capable of freeing their abdomen. There is little chance to find and infest a new host, but male *Ismaila*

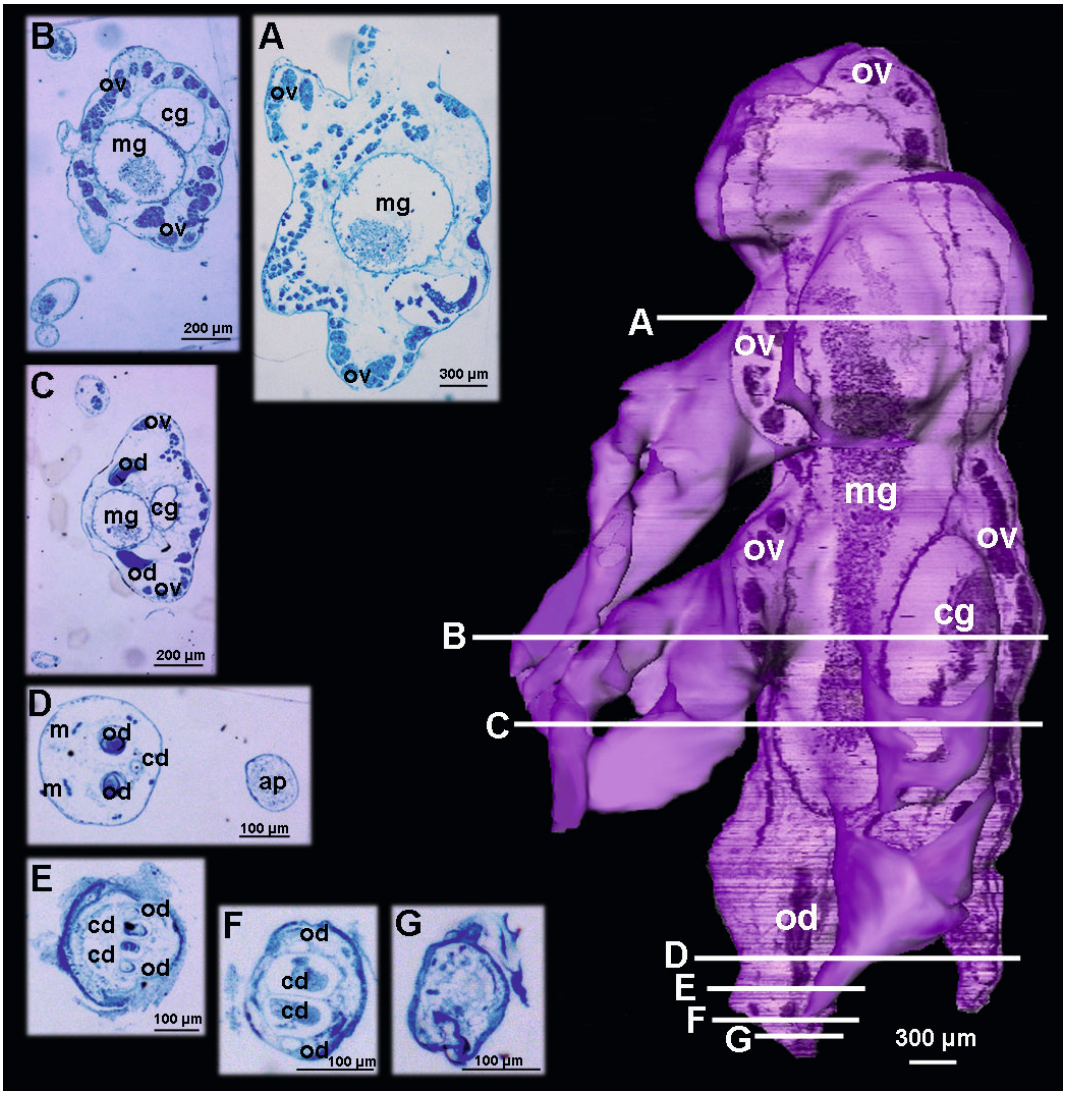

Fig. 2. Lateral view of the female with the indication of the inner organs as they can be seen on a medial section (digital reslice). Bars indicate the levels of the particular original sections **A** to **G**. **ap**, medio-dorsal appendage; **cd**, cement gland duct; **cg**, cement gland; **m**, musculature; **mg**, midgut; **od**, oviduct; **ov**, ovary.

may be able to leave a certain position at the body wall, move inside their host and mate with other females.

The higher complexity and stronger body musculature in males (Fig. 5C) suggests that mature males retain a higher degree of mobility than females from larval stages which we observed migrating freely inside the body cavity of the host. Males often are found "swimming freely" in the body cavity of the host (Ho 1981, 1987, Haumayr & Schrödl 2003, Abad et al. 2011), and need to get in touch with a female for copulation. The body cavity of nudibranchs is not very spacious but rather tightly packed with inner organs (e.g. Martynov et al. 2011), moving larvae and males thus may rather "crawl" inside the body cavity than actually "swim", e.g. by using the antennae and the first pair of thoracopods. We also observed such specimens of *I. aliena* penetrating the body wall of the host quite quickly, exiting (but not really leaving the host) with cephalosome first (Schrödl 1997). There should be a way to cut or destroy host tissue with the head.

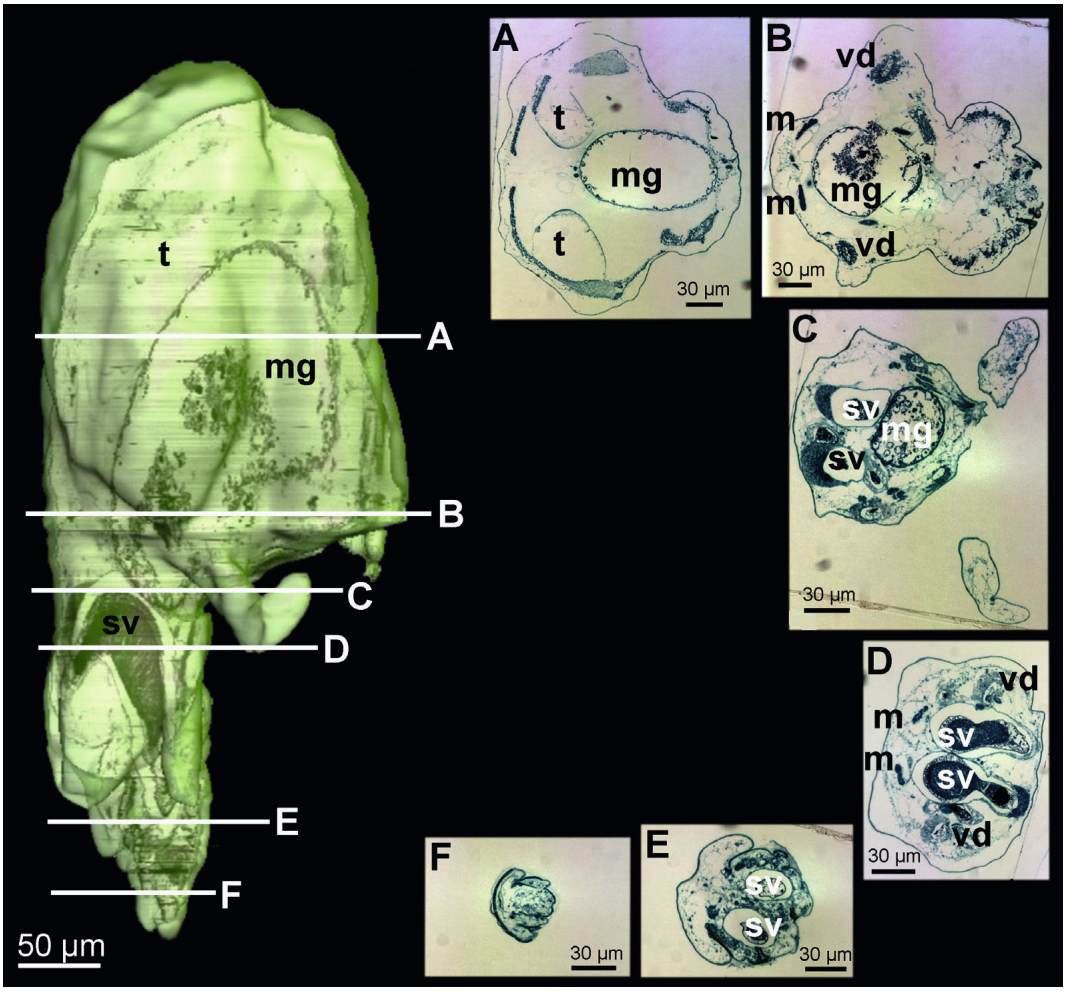

Fig. 3. Lateral view of the male with the indication of the inner organs as they can be seen on a medial section (digital reslice). Bars indicate the levels of the particular original sections **A** to **F**. **m**, musculature; **mg**, midgut; **sv**, seminal vesicle; **t**, testes; **vd**, vas deferens.

Cephalic muscles

In *I. aliena* no musculature could be detected in the antennulae, which is consistent with the assumed function of a sensoric device (Schram 1986). In ectoparasitic species the antennae are mainly used for attachment to the host (Schram 1986, Boxshall 2005) and therefore it is most interesting that the antennae are still present in endoparasitic Splanchnotrophidae, even in genera like *Arthurius*, where the mouthparts are already partially reduced. Hence the antennae still seem to have an important function. In fact, the strongest musculature found in *I. aliena* concerning cephalic appendages serves the claw-like antennae (Figs 4C and 5C). We thus assume that antennae are used as a device for anchoring the parasite during movements or copulation. Antennae may have further functions, such as destroying host tissue during migration and for perforating the body wall of the host. Although most of splanchnotrophid species do not harm their hosts, Bergh (1867), Jensen (1987) and Schrödl (1997) described the gonads of host individuals of *Ismaila monstrosa* Bergh, 1867, *I. jenseniana* Haumayr & Schrödl, 2003 and *I. damnosa* Haumayr & Schrödl, 2003 as partly destroyed or damaged. All three authors did not assume this to be for the purpose of feeding, but rather to gain space. We conclude that in these cases the antennae are used to dissect and remove the particular organ.

All species examined by Saby (1933) are blood-sucking fish parasites located on the gills of their respective host. However, all these species still have strong muscles serving the mouthparts and the oesophagus (Saby 1933) to rasp off tissue and gain access to blood vessels. Even species like *Brachiella obesa* (Krøyer, 1837) and *Clavella uncinata* (Müller O. F., 1776) (synonym of *Clavella adunca* (Strøm, 1762)), which show strong modifications of the head, still have strong muscles around the oesophagus (Saby 1933). Such musculature is missing in *I. aliena*, contradicting a similar tissue-feeding mode.

In the tiny, sickle-shaped mandible of *I. aliena* no strong muscle strands were detectable, whereas in the maxillulae and the maxillae several strands of musculature were found. This supports the hypothesis that the latter two pairs of mouthparts rather than mandibles play an active role during feeding in *Ismaila*.

Digestive system and feeding mode

In his study on *I. belciki*, Belcik (1965, 1981) describes the digestive system as incomplete since no intestine, rectum or anal opening was evident. The present analysis on both sexes of *I. aliena* confirms the absence of any hindgut or anal opening in *Ismaila* species. Several histological studies on tissue-feeding ectoparasitic copepods showed that their digestive systems are complete, i. e. form a flow-through system with separate intestine, which is the general copepod pattern (Najarian 1952, Hartmann 1986). In contrast, parasitic copepods with females showing an incomplete digestive system are known to feed on body-fluids – mostly haemolymph – of their invertebrate hosts (Gotto 1979 and references cited therein). We conclude that finding of a blind-ending digestive system in both sexes of *I. aliena* and *I. belciki* also indicates feeding on fluids rather than tissue. This supports our hypothesis that at least some splanchnotrophids feed on haemolymph (Schrödl 2003, Anton & Schrödl 2013).

In his revision of copepods associated with marine invertebrates, Gotto (1979) mentions three species lacking any sign of an alimentary canal. Since the chitinous layer in all these copepods was found to be very thin, the uptake of nutrients by absorption was the accepted explanation in all three cases (Paterson 1958, Bresciani & Lützen 1960, Vader 1970, Gotto 1979). *Ismaila aliena* has a thin body cuticle and a midgut; thus some nutritional uptake through their thin body cuticle as suggested by O'Donoghue (1924) is possible but an additional rather than main food source.

In the case of fish-parasitizing, blood feeding *Chondracanthus lophii* Johnston, 1836, only the male possesses an incomplete digestive system with a midgut ending as a blind sac (Østergaard 2004). Østergaard (2004) assumed that those males feed on special secretions produced in the glands of the female nuptial organ. In contrast, the digestive system of both sexes of *I. aliena* is quite similar in structure, suggesting a rather similar mode of nutrition. Furthermore the remaining parts of the digestive system in *I. aliena* show no signs of enhanced functionality. The sac-like midgut has straight walls instead of foldings or eversions like the lumen sacs described by Saby (1933) to maximise the surface (Figs 2 and 3). This indicates that the food of *I. aliena* is nutrient rich and rather easy to digest.

Observations on a female individual of *Ismaila* sp. found infesting a living though heavily distorted host, i. e. an aeolid nudibranch *Flabellina* species (*Flabellina* sp. 1 according to Schrödl 2003) collected in Chile in 2010 may shed further light on the question of nutrition of Splanchnotrophidae. The female parasite was enclosed by a sack-like evagination of the host's mantle tissue, having only one narrow tube-like connection to the body cavity and therefore to the haemolymph of the host (see Fig. 7), but not to any visceral organs. This very unusual position of the parasite may have resulted from the smallness of the host individual into which the fully-grown female parasite simply would not fit. Since access to other food sources than haemolymph probably has ceased earlier, and the parasite appears fully functional, i. e. reached maturity and even developed egg sacs; the only possible resource of nutrition is the haemolymph.

Accepting that *Ismaila* feeds on haemolymph of sea slugs – a fluid with dissolved nutrients and some cellular contents – explains why important parasite structures like parts of the gut and digestive glands could be reduced. Brooker et al. (2007) pointed out that digestion in blood-feeding parasites is slow but complete; however, indigestible residues also could be disgorged through the mouth or embedded in the parasite's body in form of crystals, as has been found for blood-feeding parasites of other taxa (Perkins 1985, Boxshall 2005). In the case of *I. aliena* no traces of such crystals could be detected, but they might have been dissolved and/or lost during the embedding process. In *I. aliena* a small structure was detected at the posterior end of the midgut which, according to the drawing provided by Schram (1986), could be an oil sack (see Fig. 4D). Its poor development may reflect the endoparasitic life of splanchnotrophids, i. e. living in an environment of constant food supply they are not as dependent on stored nutrients as for example free-living predatory species.

Feeding on a fluid is consistent with the reduction of mouthparts in some genera of the Splanch-

notrophidae, such as *Arthurius* Huys, 2001 and *Ceratosomicola* Huys, 2001 (see Huys 2001, Salmen et al. 2008a,b). Anton & Schrödl (2013) assume that splanchnotrophid antennulae and claw-like antennae rather than mouthparts are involved into larval host detection and penetration. Indeed, the mandible of splanchnotrophids is rather inconspicuous, but maxillae and maxillules can be well developed. Inferring their function one has to consider that according to Schminke (2007), the viscosity of haemolymph is, for small animals like copepods, more similar to thick honey than to water. In such an environment the brush-like maxillae found in all *Ismaila* species (Haumayr & Schrödl 2003) would rather function as some sort of spoon to shovel the viscous haemolymph into the mouth and hereby fill the midgut. After the absorption of nutrients compression of the midgut through a contraction of the whole body, i. e. by the longitudinal muscle strands, could simply disgorge its content. The only conceivable alternative to suck in the haemolymph would require strong strands of musculature surrounding both oesophagus and midgut (Kaestner 1967). But no traces of such muscles could be found in either sex of *I. aliena* (Figs 2 and 3) and Belcik (1965) also mentions no such musculature in his study of *I. belciki*.

In her study on several members of the Splanchnotrophidae using scanning electron microscopy Salmen (2005) indicates the presence of an anal opening in *Splanchnotrophus angulatus* Hecht, 1893, *S. gracilis* Hancock & Norman, 1863 and *Ceratosomicola mammillata* Salmen, Wilson & Schrödl, 2008. Whether this means that the nutrition of these species differs from that described herein yet needs to be clarified. The same uncertainty applies to *Arthurius*, since in this genus all mouthparts except the maxillae are reduced. At first glance reduction of mouthparts seems to be consistent with our hypothesis of fluid rather than tissue feeding, but in *Arthurius* the maxillae are claw-like (Salmen 2008a) and do not have the brush-like appearance described above. Therefore an alternative method of feeding may be possible. Concluding, there is still need of further investigation to finally resolve the exact mechanisms of feeding across members of the family Splanchnotrophidae, with genera showing considerable variation regarding mouthparts and possibly digestive systems.

Female reproductive anatomy

In female *I. aliena* there are two bulbous structures, which may be determined as paired ovaries, as is typical for parasitic copepods (Kaestner 1967, Schminke 2007). But according to the drawings provided by Saby (1933), the ovaries are distinctly set off from the oviduct. However, in *I. aliena* there is no clear distinction between these bulbs and the highly branched structures traversing the whole body of the female, which therefore are also determined as parts of the ovaries. Since the ovaries merge at several locations the entire structure may have derived from a secondary fusion of once paired ovaries. These anastomosing ovaries occupy the greatest part of the entire body lumen, reaching as far as into the dorsal appendages and even the thoracopods (see Fig. 4B), optimising ovary volume and surface in a bizarrely shaped endoparasite.

In *I. aliena* the ovaries connect to a pair of oviducts. The oviducts and the cement-gland duct stay separate until the genital opening (Fig. 4B). Since in addition no receptaculum seminis could be detected, an antrum as described by Schminke (2007) must be considered absent. The ectoparasitic copepods described by Saby (1933) and *I. belciki* studied by Belcik (1965) also lacked an antrum, but all these species still showed a receptaculum seminis. The most striking difference between *I. aliena* and the ectoparasites studied by Saby (1933) is the morphology of the cement-gland, which produces the egg sacs. While in the ectoparasitic species a pair of lateral cement-glands is always present, the cement-gland of *I. aliena* is unpaired and lies dorsal to the midgut (Fig. 4B). Interestingly not only glands are fused in *I. aliena*, but also the cement-gland ducts at least until reaching the genital segment (see Fig. 4B). In the histological sections a structure is visible inside the cement-gland duct, which is also separating at the level of the genital segment and follows the ducts until the genital openings (Fig. 2). Probably this structure represents the tissue secreting the envelope of the egg sacs.

Belcik's (1965) description of the female reproductive system differs from the present one regarding the nomenclature of its distinct parts. He described the reproductive system of *I. belciki* as consisting of paired ovaries, paired but fused oviducts, paired cement-glands and an unpaired receptaculum seminis (Belcik 1965). Although the general morphology of both reproductive systems shows great similarities, we tend to a different interpretation. Belcik (1965) assumed that the oviduct is leading through the paired cement-glands. But in the histological section of *I. aliena* it is clearly visible that there is no trace of glandular tissue enwrapping the oviduct (Fig. 2). On the contrary, the structures as a whole have a connection to the highly branched ovary (see Fig. 2C); therefore, and according to the appearance of its content (see Fig. 2), they should be regarded as paired oviducts.

The results of the present study also favour a different interpretation of what Belcik (1965) assumed to be an unpaired receptaculum seminis. First of

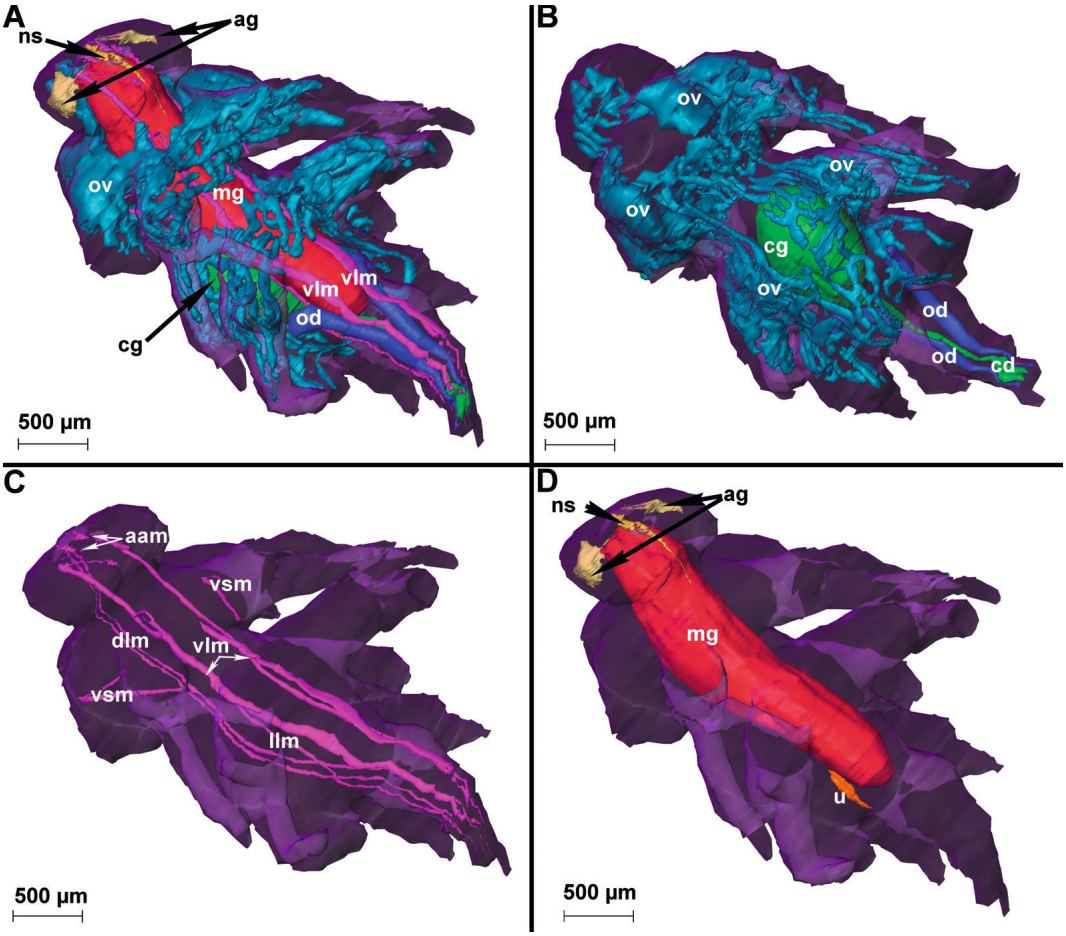

Fig. 4. 3D-reconstructed model of the internal anatomy of female *I. aliena* (ventro-lateral view). **A.** Overview of the complete internal morphology; **B.** reproductive system (dorsal view); **C.** musculature; **D.** digestive system, nervous system and excretory glands. **aam**, antennal muscles; **ag**, antennal gland; **cd**, cement gland duct; **cg**, cement gland; **dlm**, dorsal longitudinal muscles; **llm**, lateral longitudinal muscles; **mg**, midgut; **ns**, nervous system; **od**, oviduct; **ov**, ovary; **u**, unidentified structure (potential oilsac); **vlm**, ventral longitudinal muscles; **vsm**, v-shaped muscles.

all the content found inside the relevant structure looks different compared to the one found inside the seminal vesicles of the male and, therefore, do not seem to represent male gametes or spermatophores (see Figs. 2 and 3). In addition, the posterior region of the duct seems to contain a glandular structure possibly to synthesise the material of the egg sacs (Fig. 2), and this structure seems to be rather voluminous compared to the relative body size. Even in ectoparasitic species where males are not present all the time the receptaculum seminis usually is a tiny structure (Saby 1933, Najarian 1952). Considering that in *I. aliena* males and therefore male gametes are constantly available for the female makes the presence of a receptaculum seminis of this size rather unlikely. In summary, we assume that this structure rather represents an unpaired cement-gland than a receptaculum seminis.

Male reproductive anatomy

In male *I. aliena* the paired testes are located in the posterior region of the cephalosome, filling it to the biggest part (see Fig. 5B). In copepods the testes usually are situated in a similar position (Kaestner 1967, Schminke 2007), but are rather small, sometimes even unpaired and the vasa deferentia provide a rather straight connection to the seminal vesicle where the spermatophores are produced (Saby 1933, Schminke 2007). In his study on male *I. belciki*, Belcik (1981)

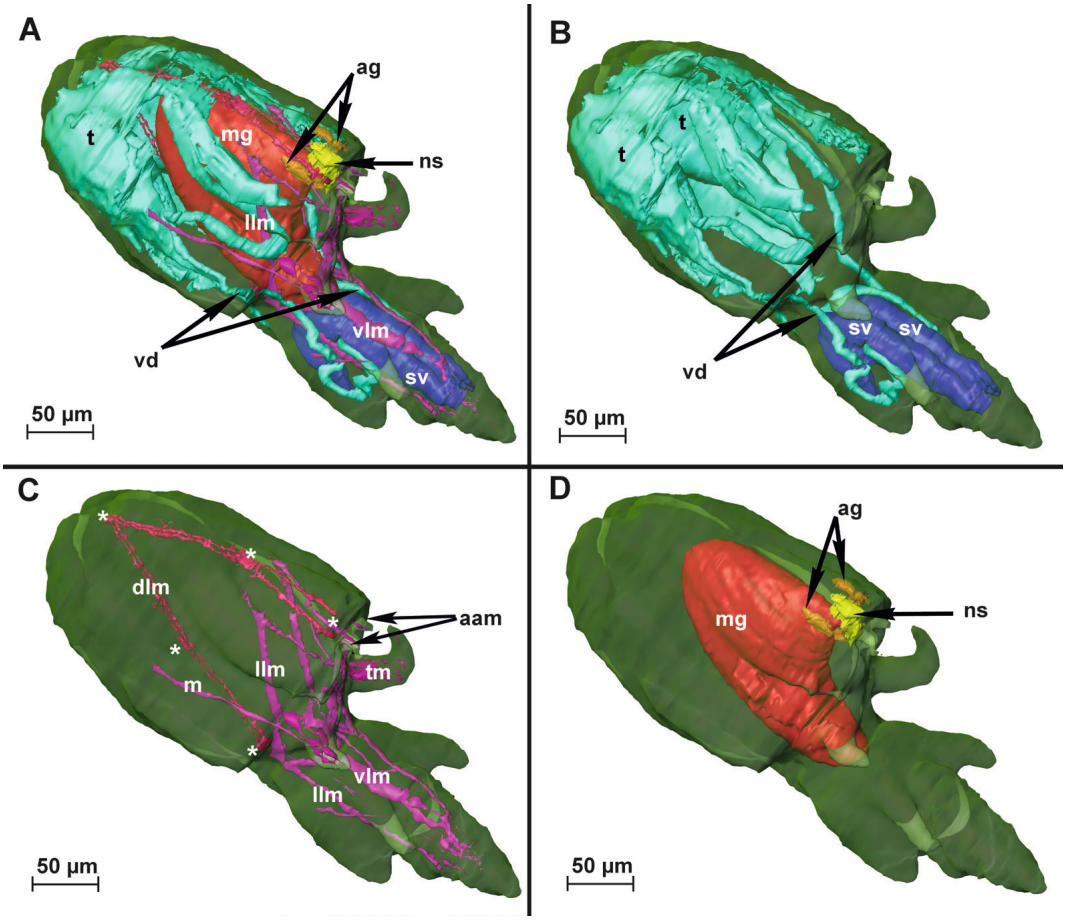

Fig. 5. 3D-reconstructed model of the internal anatomy of male *I. aliena* (ventro-lateral view). **A.** Overview of the complete internal morphology; **B.** reproductive system; **C.** musculature; **D.** digestive system, nervous system and excretory glands. **aam**, antennal muscles; **ag**, antennal gland; **dlm**, dorsal longitudinal muscles; **llm**, lateral longitudinal muscles; **m**, muscle strand connected to the thoracopods; **mg**, midgut; **ns**, nervous system; **sv**, seminal vesicle; **t**, testes; **tm**, internal thoracopodal muscles; **vd**, vas deferens; **vlm**, ventral longitudinal muscles. Asterisks indicate the areas where the dorsal longitudinal muscles are connected to the body wall, thus forming four parts.

already noted that the testes extend dorsolaterally into the swollen segments of the cephalothorax, with the vasa deferentia running along them laterally in an uneven or convoluted manner (Belcik 1965, 1981). We found that a layer of strong muscles enwraps the meandering vasa deferentia in *I. aliena*, possibly to transport the gametes from the testes to the seminal vesicles by peristaltic movement (see Fig. 3).

Mating biology

The act of copulation still is completely unknown for splanchnotrophids and therefore it is possible that sperm transfer takes place before the female anchored its abdomen in the hosts' integument. In that case males would be expected to search for migrating females rather than joining already anchored females. However, in nearly all splanchnotrophid species males were found anchored close to females (Ho 1981, Schrödl 1997, Huys 2001, Haumayr & Schrödl 2003, Salmen et al. 2008b, Abad et al. 2011). We thus assume that copulation takes place continuously between males and females anchored close together and therefore a constant supply of male gametes is provided. In the genus *Ismaila* the female is usually flanked by two or three anchored males (Haumayr & Schrödl 2003) in a way that the genital opening of the males are near those of the female,

but males also are found freely inside the host (Ho 1987, Haumayr & Schrödl 2003). According to the arrangement of anchored males and females, the offspring of one female may have different fathers, depending on whether they hatch from the left or the right egg sac. This would be remarkable, since usually copepod males are anxious to ensure to be the only one the female copulates with and many free-living species therefore show mate-guarding behaviour (Anstenrud 1992, Todd et al. 2005, Titelman et al. 2007). Further studies are necessary to confirm and explain the variable number of males aggregated to females in different species (Schrödl 1997, Huys 2001, Haumayr & Schrödl 2003, Salmen et al. 2008a,b, Abad et al. 2011) and to explore the genetic diversity of splanchnotrophids.

Circulation

As it is typical for cyclopoid copepods (Kaestner 1967, Schram 1986, Schminke 2007), a heart or other circulatory organs are missing in both sexes of *I. aliena*. It is assumed that the movement of the body itself maintains the circulation of the haemolymph (Saby 1933, Kaestner 1967, Schram 1986, Schminke 2007). This could either be by passive movement, which is induced by the movements of the host, or actively by the parasite itself. Indeed it has been observed that the retraction of the abdomen is performed by female *I. aliena* without any visible tactile stimulus (personal observation).

Excretory glands

In both sexes of *I. aliena* a paired structure in the head region was detected (see Figs 4D, 5D and 6), which is assumed to represent a pair of antennal glands as described by Schram (1986), since their ducts are leading outwards at the level of the antennae. This interpretation needs reconfirmation, however, since usually a pair of maxillary glands maintains the excretion in copepods (Claus 1880, Saby 1933, Kaestner 1967, Schminke 2007). In *I. aliena* the size of these antennal glands in relation to the body size is similar in both sexes, which is in accordance with the major function of these glands being excretion of metabolic waste. Neither frontal glands nor maxillipedal glands, structures that are otherwise indicated to be involved in excretion as described by Saby (1933), could be found in *I. aliena*.

Nervous system and sensory functions

The nomenclature of the nervous system refers to Saby (1933). For the identification of proto-, deutero- and tritocerebrum (Lowe 1935) the exact innervation of all cephalic and thoracic appendages is required, which we could not resolve in our specimens due to inadequate fixation. The nervous system of *I. aliena* could be reconstructed in both sexes, and it shows a significant sexual dimorphism. This is quite remarkable, since according to Saby (1933) and Weatherby et al. (2000), the nervous systems of male and female copepods are usually rather similar. In *I. aliena* the supra-oesophageal ganglion appears to be the largest part of the nervous system (Fig. 6A). In males it is not only enlarged but also distinctly set off from the circum-oesophageal nerve ring. Such structural differences may refer to different functions that are relevant to male and female *Ismaila*.

In general the nervous system of cyclopoid copepods consists of a supra-oesophageal ganglion, strong connectives encircling the oesophagus and a ventral nerve cord, which reaches to the end of the thorax (Saby 1933, Schram 1986). The supra-oesophageal ganglion mainly innervates the antennulae and antennae (Saby 1933, Schram 1986). The antennulae are assumed to function as major sensoric devices (Schram 1986) in copepods. Especially in splanchnotrophid copepods antennulae are assumed to play an important role during locating and identifying suitable hosts in the infective copepodite I stage (Ho 1987). In this context it is interesting that only two conditions have been observed in splanchnotrophids: either one or more female(s), or male(s) and female(s) (Huys 2001, Schrödl 2002, Haumayr & Schrödl 2003, Marshall & Hayward 2006, Abad et al. 2011). This would imply that either male copepodite I exclusively are attracted by or infest hosts already bearing a female, or that in the copepodite I state the sexual determination is not yet permanent and the first larvae entering a new host always develops into a female. Antennulae thus could play a role in male determination in an already infested host. Even a facultative sex reversal as described by Dharani & Altaff (2002) and Fleminger (1985) could be possible; unfortunately the distinct mechanisms of infection or sex determination are yet unrecognised (Ho 1987, Schrödl 1997, Anton & Schrödl 2013). Migratory larval stages and premature males and females may use antennulae for orientation in the host and monitoring the chemical environment, and also trigger the escaping behaviour of the parasites discussed above.

In adult males the antennulae may be used to find a mate. In nearly all splanchnotrophid species there have been reports of adult males lying freely in the body cavity (Ho 1987, Huys 2001, Haumayr & Schrödl 2003, Abad et al. 2011), which need to detect a female, then move towards it and anchor its abdomen nearby. However, positions and time scales of larval maturation in the hosts are still unclear.

In female *I. aliena* the supra-oesophageal ganglion is connected to the infra-oesophageal ganglion through strong connectives. In general the infra-oesophageal ganglion innervates the mouthparts (Schram 1986). According to our fluid-feeding hypothesis discussed above, in *I. aliena* this should mainly be maxillulae and maxillae. In the male the infra-oesophageal ganglion and the connectives are fused to a circum-oesophageal nerve ring. There are no obvious differences between female and male mouthparts, thus the reasons for this fusion remain unclear.

In the female a ventral nerve cord with five distinct bulbs – possibly ganglia – could be detected (see Fig. 6A). According to Schram (1986) the ventral nerve cord mainly serves the thoracopods and the abdomen but usually shows no distinct ganglia. Since none of the female thoracopods bears any strong musculature the purpose of these five ganglia remains unclear. Main purpose of the ventral nerve cord thus could be the innervation of the abdomen. As discussed above, females are sensitive to tactile stimuli at the abdomen and egg sacs, which are responded to by the retraction of the abdomen (Schrödl 1997, personal observation).

In contrast to the female, at least the first thoracopod of the male *I. aliena* shows strong musculature, but no distinct ventral nerve cord could be detected. Unfortunately in this area a few sections were lost in the male and therefore it is only concluded preliminarily that no well-developed ventral nerve cord exists in males. Since Belcik did not observe the nervous system (Belcik 1965, 1981), there is no comparable data of other splanchnotrophids available yet.

Purpose of the dorsal appendages

One of the most characteristic features of female splanchnotrophids are their dorsal appendages and several possible functions were suggested: O'Donoghue (1924) assumed an absorption of nutrients through the body wall with the appendages increasing the body surface. This was discussed by Huys (2001) and Anton & Schrödl (2013) to be rather unlikely because of the presence of functional mouthparts. As discussed above, the thin body cuticle may allow the absorption of dissolved nutrients, but we do not consider it as a major function of the dorsal appendages. Later Huys (2001) assumed the appendages to house parts of the ovary, which are visible looking at the translucent parasites. Herein we confirm the ovary reaching into the appendages by histological data (see Fig. 2). Optimising the space available for egg production is clearly a major function of splanchnotrophid appendages, especially when considering that rather soft and slender appendages can fill available space in the body cavity of hosts without necessarily destroying certain organs or competing for space too fiercely (Anton & Schrödl 2013).

Huys (2001) also suggested the dorsal appendages to enwrap the viscera on which the female feeds. Anton & Schrödl (2013) discussed this function to be rather unlikely, since the dorsal appendages as newly built structures (Hancock & Norman 1863) would take over the function of the thoracopods, which are in turn reduced. We show herein that there are no muscles detectable inside the appendages and therefore no active movement is possible, making them rather inappropriate to maintain a feeding position. Evidence against tissue feeding is summarised above. Nevertheless, splanchnotrophid appendages can grow extremely long in some species and more or less irregularly enwrap viscera of hosts. This might result in acting as counterpart to body contraction, e.g. when an anchored female *I. aliena* retracts its abdomen with egg sacs creating a fold within the usually tough integument of the host (personal observation).

More recently, Salmen et al. (2008b) suggested the dorsal appendages to increase the respiratory surface. As it is characteristic for cyclopoid copepods (Kaestner 1967, Schram 1986, Schminke 2007) respiration is generally achieved by the gradient of oxygen concentration throughout the body integument (Wolvekamp & Waterman 1960, Ikeda et al. 2007). The present study confirms the absence of any respiratory organs in both sexes of *I. aliena*, which raises the question, why this increase of respiratory surface is only necessary in females. One reason could refer to ramified gonads themselves having large surfaces, possibly for ensuring supply with nutrients and oxygen. Another factor is size, of gonads and of the entire body: Kaestner (1967) stated that a parasitic lifestyle often leads to an excessive growth in female copepods due to an oversupply of food. Such a growth would – according to Kaestner – be restricted to the body and would leave the cephalic limbs remaining as small appendages to the allometrically enlarged body (Kaestner 1967). Size increase in females is exactly the case in all splanchnotrophid genera with thoracopods and dorsal appendages being also enlarged. Providing additional surface for respiration and volume for ovaries while minimizing harm to hosts would explain why these appendages are found in all female splanchnotrophids (Anton & Schrödl 2013) but never in males. In case of the Splanchnotrophidae thus the term "dwarf male" may be misleading as already assumed by Laubier (1966) and it may be more correct to speak of giant females.

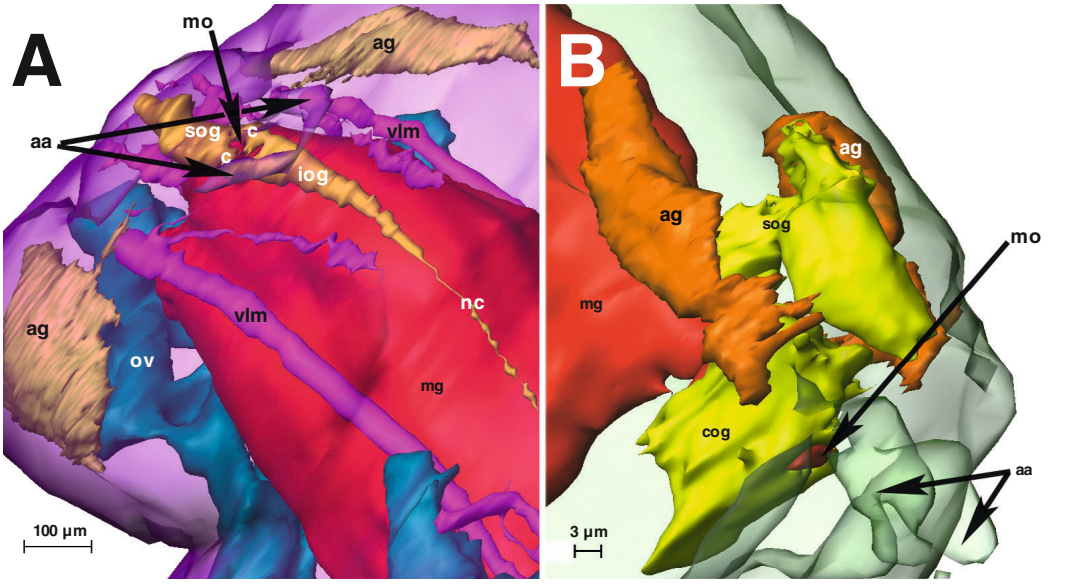

Fig. 6. Nervous system of both sexes of *I. aliena*. **A.** Female, ventral view; **B.** male latero-ventral view. **aa**, antennae; **ag**, antennal gland; **c**, connective; **cog**, circum-oesophageal ganglion; **iog**, infra-oesophageal ganglion; **mg**, midgut; **mo**, mouth; **nc**, nerve cord; **sog**, supra-oesophageal ganglion.

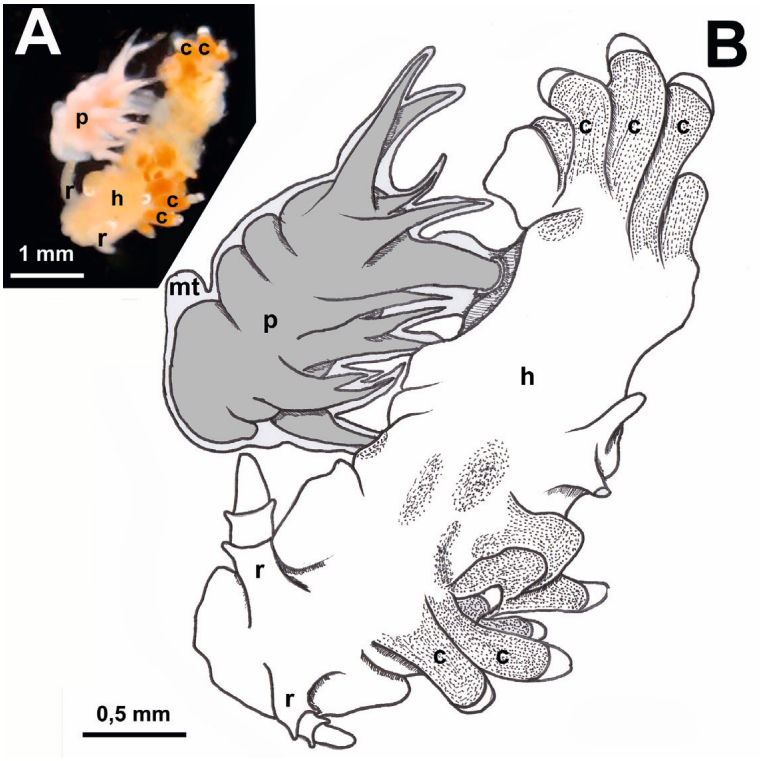

Fig. 7. Specimen of *Flabellina* sp. 1 (sensu Schrödl 2003) infected with one female *Ismaila*. The egg sacs of the parasite were removed for molecular analysis. **A.** Picture of the living animals. **B.** Drawing to clarify the position of the parasite inside the host (since the parasite is encapsulated by the integument of the host it has to be considered inside the host). Dorsal view of the host with a lateral view of the parasite flipped to the right. The abdomen of the female parasite is protruding the integument of the host and emerges on the ventral surface of the mantle. **c**, cerata; **h**, host; **mt**, mantle tissue surrounding the parasite; **p**, parasite; **r**, rhinophores.

Conclusion

The present study provides new insights concerning morphology, organ functions and life history of the genus *Ismaila*. To gain a comprehensive overview of the family Splanchnotrophidae it will nevertheless be inevitable to analyse the internal anatomy of representatives of the remaining genera, in addition to studying the genus *Briarella* Bergh, 1876. Especially *B. doliaris* Salmen, Anton, Wilson & Schrödl, 2010 shares many external features with the genus *Splanchnotrophus* (see Salmen et al. 2010) and may be of importance to this task. Detailed but time-consuming histology-based microanatomical 3D modelling as applied herein should be complemented by µCT scanning; once histological structures are reliably correlated to µCT scans the latter technique may prove efficient. In particular, tomography should provide comprehensive data on number, position and external morphology of cuticularised parasites and their larva within the hosts. Together with direct observations and experiments on e. g. potential chemotaxis and infection of hosts, which have proven difficult, conclusions from structural and functional evidence may be the key to understanding splanchnotrophid life cycles and behaviour. Molecular studies will allow for testing the current morphology-based species delimitations, and help to unravel the evolutionary history of such highly adapted parasites as the Splanchnotrophidae.

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8. Discussion

8.1 Phylogeny of the Splanchnotrophidae

For the first time a comprehensive character state matrix of the family Splanchnotrophidae, potential allies and outgroups was created (see chapter 3). The morphology-based cladistic analysis confirmed the monophyly of the family as well as the structure of the family proposed by Huys (2001). In a second attempt these new insights on splanchnotrophid relationships were supported in great parts by a molecular-based analysis (chapter 4). In addition, COI trees were compatible with traditional species level taxonomy. General interest regarding the family Splanchnotrophidae was dormant until recently, when several new species were discovered (Uyeno and Nagasawa 2012; chapter 6). This new data extended the matrix for the morphological-based analysis presented in chapter 3 rendering revision of the former analysis inevitable.

8.1.1 New cladistic analysis

The analysis described in chapter 3 was repeated including all additional species, namely *Ceratosomicola japonica*, *Splanchnotrophus helianthus*, *Splanchnotrophus imagawai* and *Majimun shirakawai* from Japan, *Arthurius gibbosa* from Indonesia and *Ismaila volatilis*, *Ismaila chaihuiensis* and *Ismaila genalis* from southern Chile. A newly discovered parasitic copepod *Pionodesmotes domhainfharraigeanus* Anton, Stevenson, Schwabe, 2013 parasitizing the echinoderm *Sperosoma grimaldii* Koehler, 1897, together with its congener *Pionodesmotes phormosomae* Bonnier, 1898 were included as outgroup taxa. The analysis was based on the updated character state matrix used for the original analysis; the matrix was extended by several characters that became relevant and potentially informative. The final matrix thus includes 199 characters and 46 taxa (for complete character state matrix and character state descriptions please see the supplementary material 1 and 2). The analysis was done with PAUP version 4.0b10 (Swofford 2002), using the same specifications as for the analysis described in chapter 3, with 1000 bootstrap replicates calculated. In addition, Bremer decay indices were calculated using TREROT (Sorenson and Franzosa 2007) and PAUP corresponding to chapter 3.

8.1.2 Comparison to the current morphology-based phylogeny

The resulting strict consensus tree was created from 24 equally parsimonious trees. It has a length of 761 steps, a consistency index (CI) of 0.4665, a homoplasy index (HI) of 0.5335, a retention index (RI) of 0.6773 and a rescaled consistency index (RC) of 0.3159. The strict consensus tree is almost fully resolved (Fig. 3) and is partially compatible with the tree given in chapter 3, but also shows some differences.

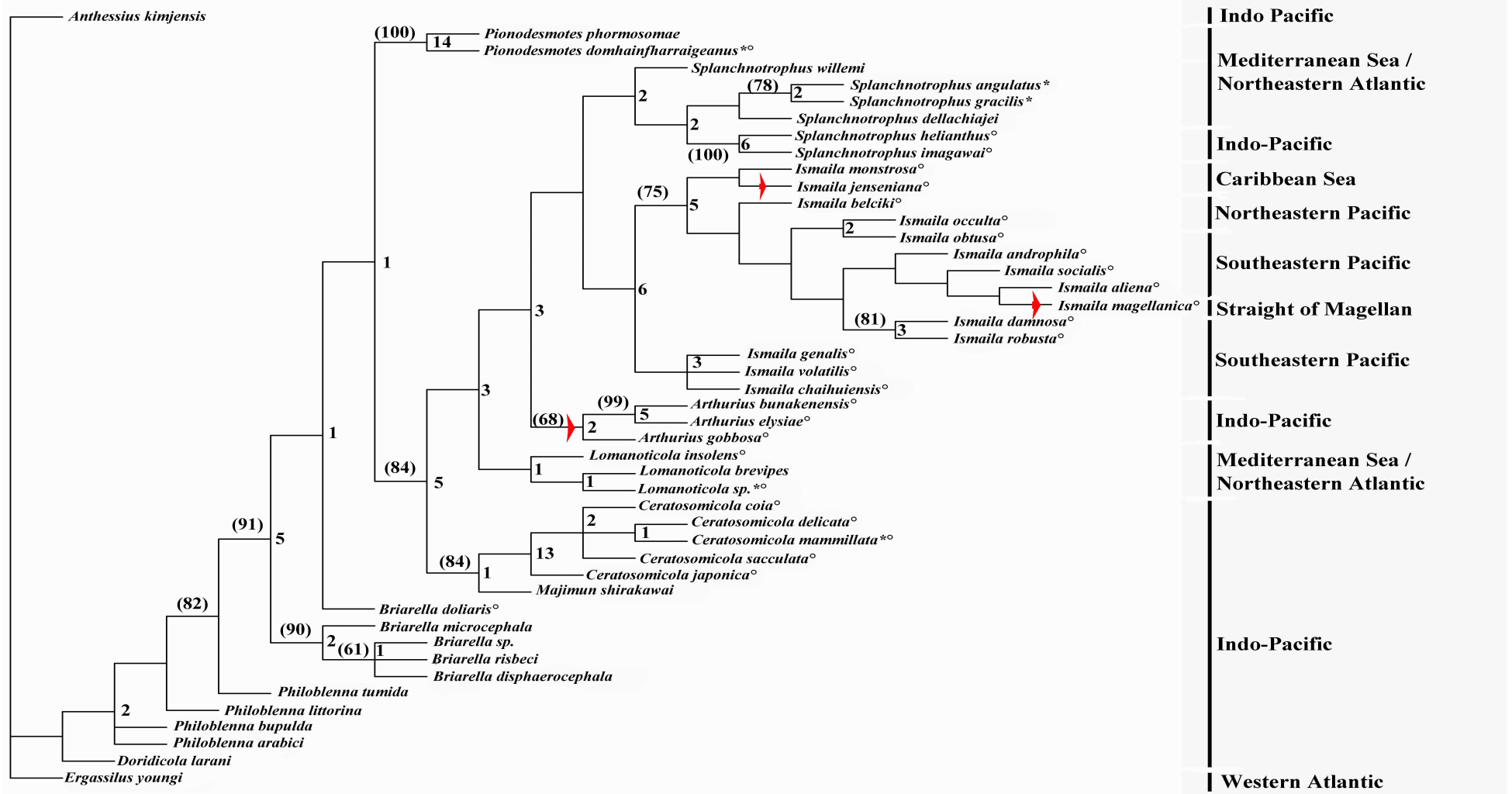

Figure 3. Phylogeny of the Splanchnotrophidae. Strict consensus tree of the new parsimony analysis with bootstrap support (>50, in parentheses) and Bremer decay values. Geographical distributions are indicated according to major regions. Branch length does not reflect number of character-state changes. Red arrows mark the (independent) switches from nudibranch to sacoglossan hosts.

Congruences between both morphology-based analyses refer to the paraphyly of the genus *Philoblenna*, the monophyly of the family Splanchnotrophidae, the monophyly of *Ceratosomicola* together with the basal position of the genus, and the monophyly of *Arthurius*, *Splanchnotrophus* and *Ismaila* (see table 1) as well as the genera *Splanchnotrophus* and *Ismaila* being sister taxa in terminal positions (see chapter 3). The differences compared to chapter 3 are the genus *Briarella* being no longer monophyletic; the separation of *Arthurius* from *Ceratosomicola* with *Arthurius* now being basal to the sister taxa *Splanchnotrophus* and *Ismaila*; the genus *Lomanoticola* now being monophyletic and separated from *Splanchnotrophus*, now resulting as a basal offshoot sister to the clade including *Arthurius*, *Splanchnotrophus* and *Ismaila*. Furthermore the internal phylogeny of the genus *Ismaila* changes dramatically. The newly discovered *Ismaila*-species are forming a basal sister clade to all originally included species. But in contrast to the phylogeny described in chapter 3, now *I. monstrosa* with *I. jenseniana* together result in the most basal position followed by *I. belciki* and a clade comprising of *I. occulta* and *I. obtusa* being sister to a clade comprising of the sister taxa *I. damnosa* and *I. robusta* on the one side, and a clade consisting of *I. androphila* splitting off followed by *I. socialis*, which is sister to the combined clade of *I. aliena* and *I. magellanica* (see also Fig. 1).

8.1.3 Comparison to the molecular-based phylogeny

Comparing the strict consensus tree of the new morphology-based analysis (Fig. 3) with the trees resulting from the analysis based on COI genealogy given in chapter 4 there are also some congruences and some differences (table 1).

Table 1: Overview of monophyly of key genera (✓= monophyletic; - = paraphyletic; ? = missing data)

	Briarella	*Pionodesmotes*	*Splanchnotrophus*	*Lomanoticola*	*Ismaila*	*Majimun*	*Ceratosomicola*	*Arthurius*
Molecular-based analysis	?	?	-	?	✓	?	?	?
New morphology-based analysis	-	✓	✓	✓	✓	✓	✓	✓
Initial morphology-based analysis	✓	?	✓	-	✓	?	✓	✓

The results of both analyses are compliant regarding *Ceratosomicola* as the most basal genus of the monophyletic family Splanchnotrophidae, the division of the genera *Lomanoticola* and *Splanchnotrophus* and the terminal position of the genus *Ismaila*. The inconsistencies of the trees involve A) the paraphyly of the genus *Splanchnotrophus* in the molecular-based analysis, while the genus results monophyletic in both morphology-based analyses (see also table 1); B) the genera *Lomanoticola* and *Ismaila* being sister taxa only in the molecular-based analysis; and C) *I. belciki* resulting as most basal taxon and *I. aliena* and *I. robusta* being sister taxa in the molecular-based analysis, which clearly differs from the new morphology-based phylogeny of *Ismaila* presented in Fig. 3.

8.1.4 Consequences for the concept of Splanchnotrophidae

8.1.4.1 Origin

Jensen (1987) suggested the inclusion of all copepods living endoparasitically in opistobranch gastropod hosts into a combined group. This was supported by morphocladistics (Anton and Schrödl, 2013). However, the results of the present study rather contradict this suggestion. The family Pionodesmotidae, living endoparasitically in echinothurids, appears as direct sister taxon to the Splanchnotrophidae, endoparasitic in gastropod hosts, with the genus *Briarella* as basal offshoot to the clade (Fig 3). According to Ho (2001), a parasitic lifestyle has evolved many times independently within the Poecilostomatoida, and families of parasitic copepods utilising the same host group are usually not clustered together in a monophyletic manner; thus a sister group relationship between Pionodesmotidae and Splanchnotrophidae cannot be easily refused at present. Unfortunately, Ho (1991) did not consider the Pionodesmotidae in his phylogeny of the Poecilostomatoida, leaving their taxonomic position within the order unresolved. Molecular data will be needed to confirm or reject the close relationship between the Pionodesmotidae and Splanchnotrophidae.

The genus *Briarella* was recovered paraphyletic, with the recently discovered species *B. doliaris* sister to the clade comprising the Pionodesmotidae and the Splanchnotrophidae. *Briarella doliaris* is highly similar to the members of the genus *Splanchnotrophus* (see chapters 3 and 5); however, again confirmation by molecular data is not yet possible due to missing data.

Despite of different outgroup selection and varying coverage of the respective genera, the family Splanchnotrophidae results monophyletic in all three analyses (see chapters 3 and 4 and Fig. 3). Therefore, the hypothesis concerning the monophyly of the Splanchnotrophidae introduced by Huys (2001) and the classification of the newly discovered species from Japan (Uyeno and Nagasawa 2012) are confirmed so far. As a consequence this means that the endoparasitic lifestyle using shell-less opisthobranch gastropods as hosts has developed only once in copepod evolution as it was suggested in chapter 3. The results also indicate that the switch from nudibranch to sacoglossan hosts occurred three times independently within the Splanchnotrophidae; in particular once in the ancestor of the genus *Arthurius*, once in *I. magellanica* and once in *I. jenseniana* as it also was proposed in chapter 3.

8.1.4.2 Inner splanchnotrophid relationships

The genus *Ceratosomicola*, although only represented by a single sequence in the molecular approach, resulted as (part of the) most basal offshoot within the Splanchnotrophidae in all conducted analyses (see chapters 3 and 4 and Fig. 3). Regarding the new morphology-based analysis, *Ceratosomicola* clustered together with *Majimun*, which could not yet be tested by molecular data.

Ismaila currently is the only genus supported by enough molecular data to provide reliable results (see chapter 4). The genus resulted monophyletic in all analyses, implying not only *Ismaila* being the only splanchnotrophid genus inhabiting the American continent, but also, that the colonisation of America occurred just once in splanchnotrophid evolution. However, the phylogenetic position of *Ismaila* as a derived taxon in all analyses presented herein stands in clear contrast to the discovery of *Ismaila* displaying the “most primitive” splanchnotrophid character states concerning maxillulae, maxillae and fifth thoracopod as reported by Huys (2001).If not plesiomorphically retained one could suspect heterochronic re-establishment of pseudoancestral features. Future molecular studies may recover trees that are comprehensive and robust enough to reconstruct character evolution, and ontogenetic studies of other splanchnotrophid genera than *Ismaila* (see below) are overdue.

Regarding the results of both morphology- and molecular-based analyses, it is interesting to see that all favour the strict division of the genera *Splanchnotrophus* and *Lomanoticola* (see chapters 3 and 4 and Fig. 3). In the beginning of splanchnotrophid

research, all species being now included into the genus *Lomanoticola* were first assigned to the genus *Splanchnotrophus* (Hancock and Norman 1863; Scott and Scott 1895; Delamare Deboutteville 1950). Even after the introduction of *Lomanoticola*, it was assumed to be only a subgenus to *Splanchnotrophus* and it was not until 2001 when Huys established *Lomanoticola* as an independent genus including only the two species *L. insolens* and *L. brevipes* (Huys 2001).

The position of the genus *Lomanoticola* within the family is unresolved. According to the new morphology-based analysis the genus is no longer sister taxon to *Splanchnotrophus* (see chapter 3) but results after the branching of the *Majimun*/*Ceratosomicola* clade as most basal member of other Splanchnotrophidae (Fig. 3). However, based on molecular-based analyses, *Lomanoticola* was the direct sister taxon to *Ismaila* (see chapter 4), with *Splanchnotrophus* in a more basal position. Internal relationships of the genus *Lomanoticola* are also problematic. Recent recollections of *L. brevipes* revealed a high level of supposedly intraspecific morphological variability requiring further investigation. The species is distributed in the Mediterranean Sea, the European coasts of the Atlantic Ocean and around the coasts of the Great Britain and Ireland (Huys, 2001). It was initially discovered infesting *Doto coronata* Gmelin, 1791 and *Flabellina verrucosa* (M. Sars, 1829) but currently is known from nine different host species (Huys, 2001) and therefore displays the lowest known host specificity within the Splanchnotrophidae. However, recent SEM examinations of specimens recollected from the host *Cuthona caerulea* Montagu, 1804 revealed serious deviation from the original description (see chapter 3). Unfortunately the original description of *L. brevipes* by Hancock and Norman (1863) is not very detailed, especially concerning the mouthparts and the reduced thoracopods. Since the deviations from the original description should be visible even using standard light-microscopical techniques, the specimen parasitizing *C. caerulea* is assumed to represent a distinct new species (this study). Interestingly, a second case of recently recollected specimens showing obvious nonconformities compared to the original description was reported from Portugal (Marcos Abad, personal communication). The respective specimens were found in *Doto coronata*, one of the hosts mentioned in the original description of the species. Therefore, *L. brevipes* has to be regarded as a potential candidate for a species complex, and revision of the genus is overdue (see also chapter 3).

The genus *Arthurius* is no longer sister taxon to *Ceratosomicola* as discussed in chapter 3, but in Fig. 3 resulted as basal offshoot to the clade formed by *Ismaila* and *Splanchnotrophus*. Since no genetic material of any representative of *Arthurius* is available, testing by molecular data is not possible as yet. The topology shown in Fig. 3 is suggestive that the reduction of mouthparts, as proposed in chapter 3, occurred more than once within the Splanchnotrophidae. Most interestingly both events appeared at different times in different clades, but in the same geographic region (Indo-Pacific), possibly indicating an environmental reason for this special adaptation. Unfortunately the lack of any data concerning the life history of both *Arthurius* and *Ceratosomicola* renders further explanation impossible.

The newly conducted analysis still suffers from the same problems as already discussed in chapter 3 and 4. The greatest problem is the incompleteness of the data set. Regarding the morphological data, many original descriptions are insufficient and not all affected species have been yet re-collected (see chapter 3). In addition, even highly accurate methods like SEM may not always easily reveal all necessary details as mentioned in chapters 5 and 6. Apart from that, the problem of distinguishing between homologous structures and convergent developments regarding highly adapted parasites as discussed in chapter 3 also still exists. Due to these reasons the analysis is still sensitive to outgroup selection. Wrong sister group hypotheses may of course affect inner splanchnotrophid topologies, and thus obscure evolutionary reconstructions.

Another explanation for sensitivity to outgroup selection is the problem of selecting appropriate outgroups itself. Although there is a morphology-based phylogenetic analysis conducted by Ho (1991), the composition of the respective families and genera was changed extensively thereafter (see chapter 3). Especially concerning the Splanchnotrophidae during Huys' (2001) revision and the inclusion of the genus *Briarella* into the Philoblennidae (Laubier 1964; Izawa 1976), followedby the transfer of the Philoblennidae from the Chondracanthidae into the Lichomolgidae (Kim, Ohtsuka et al. 2004) as described in chapter 3. Therefore, hypotheses on phylogenetic relationships within the Poecilostomatoida have to be regarded with caution, considerably impeding the selection of suitable outgroup taxa.

8.1.4.3 Species level

Molecular phylogenetic analyses are promising, but for most splanchnotrophid species there is currently no molecular data available (see chapter 4). Type material in general is no longer available or, due to fixation, impracticable for DNA-extraction. Therefore generating a comprehensive database including sequence data of all splanchnotrophid species will require extensive recollection of most of the species. This is complicated by the fact that endoparasitic species may be rare or just sporadically found. Multilocus rather than single locus barcoding needs to be established and species delimitation programs benefit from multiple independent sequence samples for each species to produce reliable results. However, this first attempt to combine COI-barcoding with morphological evidences revealed largely compatible results, such as the monophyly of the Splanchnotrophidae, the monophyly of the genus *Ismaila* and *Ceratosomicola* as earliest splanchnotrophid offshoot. On species level, molecular phylogenetic trees seem to suggest a slower rate of divergence than those based on morphology; therefore the morphologically clearly distinct species *I. volatilis* was not recovered monophyletic in the COI trees.

Our initial attempt to apply integrative taxonomy on a family of endoparasitic copepods already shows that such an approach provides distinct advantages and might prove inevitable when working with highly modified taxa like the Splanchnotrophidae.

8.2. Life history

8.2.1 Larval stages

The developmental cycle of the Splanchnotrophidae has not yet been studied extensively, although larval stages of copepods are generally well-researched (Conley 1991; Ferrari and Dahms 2007; Chullasorn et al. 2009). Among Splanchnotrophidae, the knowledge of the larval stages is currently limited to one report of several post-infective stages inside an individual of *Dendronotus iris* Cooper, 1863 (Ho 1987). Apart from that, Belcik (1981) assumed that there are at least two nauplius stages of which the first clearly is planktotrophic. The early developmental stages like the nauplii or copepodite I, the latter thought to be the infective stage (Ho 1987), however are yet completely unknown for all splanchnotrophid species. As a consequence, the mechanisms of infection including questions about how the infective stage detects

potential hosts and how it manages to enter the host were unexplored (see chapter 7). This study contributes to fill some of these fundamental gaps in knowledge.

During collection trips to southern France and southern Chile attempts were made to shed some light into the developmental stages of the genera *Ismaila* and *Splanchnotrophus*. Infested specimens were kept in small enclosures inside a bigger aquarium (Fig.2). Those enclosures enabled the exchange of water but prevented any particle bigger than 500 µm from leaving or entering the enclosure. As an alternative, egg sacs removed from a female parasite were also kept in enclosures.

During both experiments (in France and in Chile) the reproductive output observed was considerably high, and removed egg sacs were completely regrown within 12-16 hours.

After two or three days a great number of tiny, moving nauplii were visible inside the enclosures, obviously the early nauplius stages of the parasite. Samples were taken every day and fixed in glutaraldehyde for examination using scanning electron microscopy. The short time span between the samples was chosen in due consideration of the fact that no previous data exists, allowing to estimate the periods between molts.

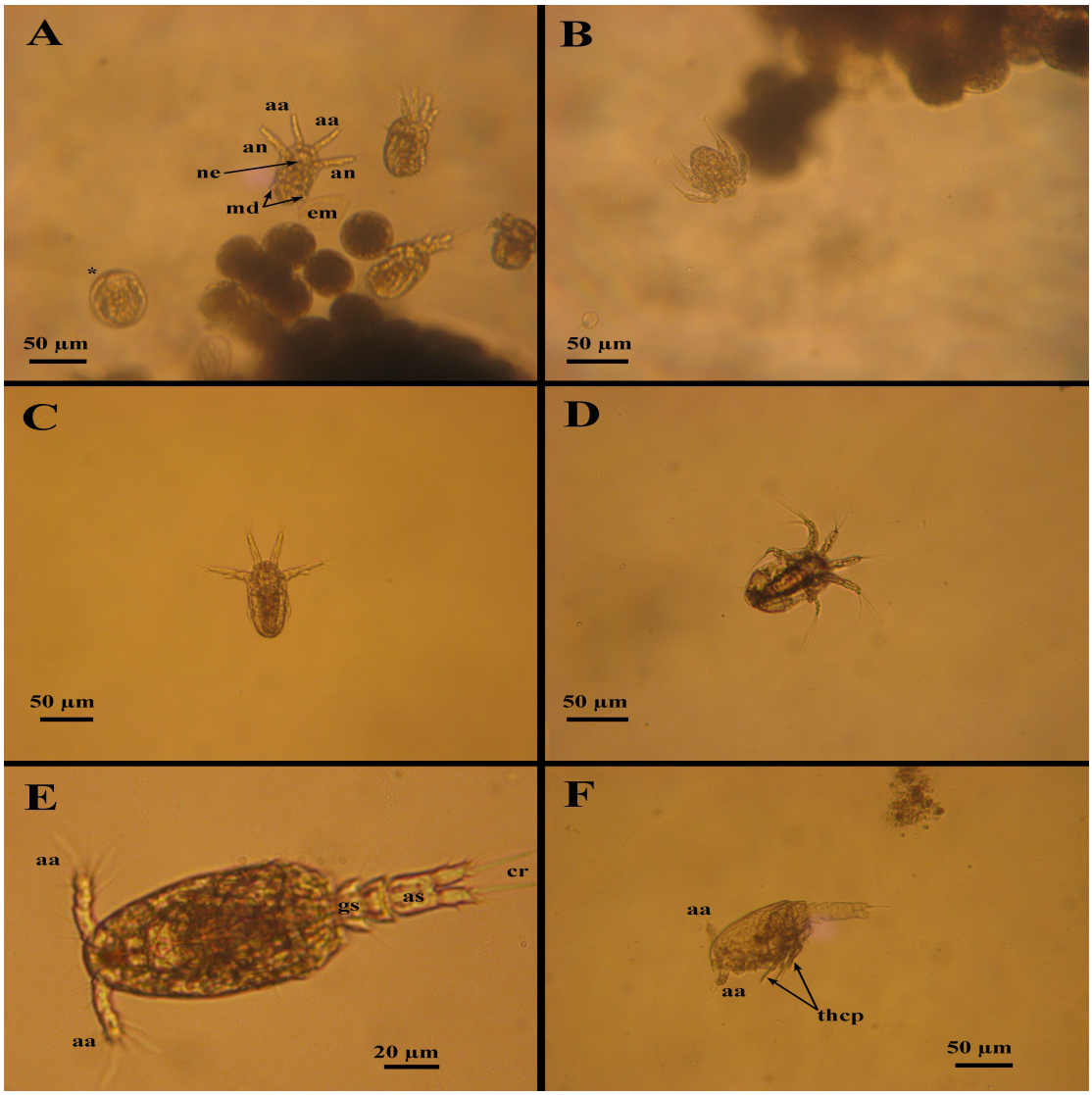

Figure 4. Light microscopical images of larval stages of *S. angulatus*. **A** Newly hatched nauplii together with eggs (The asterisk marks a fully developed nauplius still encapsulated in the egg); **B** Nauplius immediately after hatching, dorsal view; **C** Nauplius, one day after hatching, dorsal view; **D** Nauplius, two days after hatching, ventral view; **E** Copepodite I, dorsal view, **F** Copepodit I, lateral view; **aa** antennulae; **an** antennae; **as** anal somite; **cr** caudal rami; **em** egg membrane; **gs** genital somite; **md** mandibles; **ne** nauplius eye

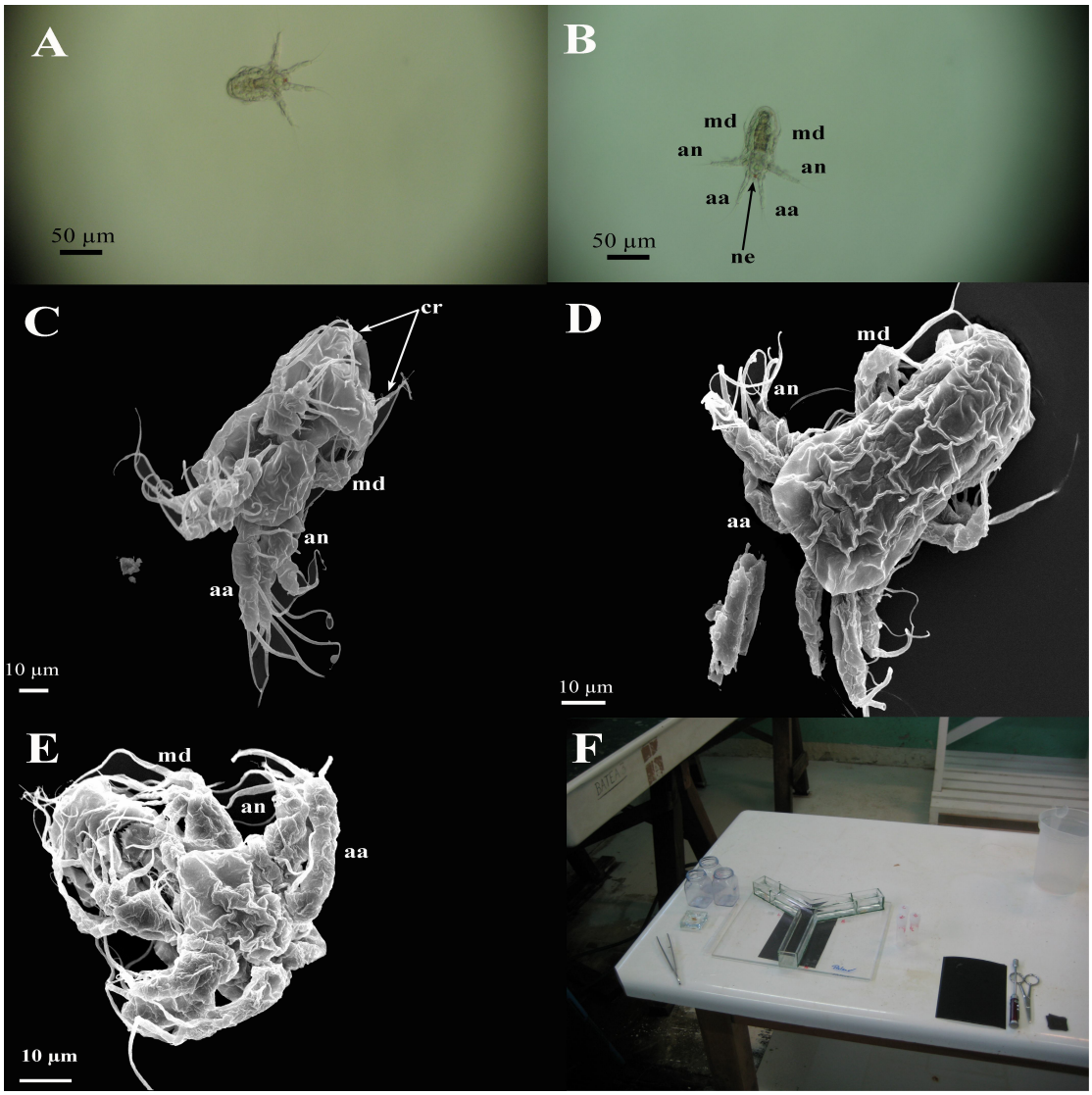

Figure 5. Nauplius stages of *I. aliena*; **A** and **B** light microscopical images, **C – E** SEM images. **A** Nauplius, ventral view, one day after hatching; **B** Nauplius, dorsal view, two days after hatching; **C** Nauplius, three days after hatching, ventro-lateral view; **D** Nauplius, three days after hatching, dorsal view; **E** Nauplius, one day after hatching, ventral view; **F** Experimental setup for testing host-preference; **aa** antennulae; **an** antennae; **cr** caudal rami; **md** mandibles; **ne** nauplius eye

Exact determination of potentially different stages will require detailed SEM-analyses since the number of nauplius stages in the Splanchnotrophidae is yet unknown. Therefore all nauplius stages are only referred to by the time span they were fixated after hatching (Figs. 4 and 5).

8.2.2 Larval development

Abad et al (2011) already assumed a correlation between the temperature and the reproductive activity of *S. gracilis*. In my experiments with Chilean and Mediterranean splanchnotrophids infested host species were identified by the

presence of egg sacs at the time they were collected from the field. Therefore it can be assumed, that the natural reproductive period had already started and the presence of egg sacs is not an artefact of the cultivation under laboratory conditions.

Observations during the first experiments of this study suggest that the temperature has a major impact on the speed of development and on the ability to reach higher developmental stages. For example regarding the results from Chile the copepodite I stage could never be detected although a great number of nauplii from different *Ismaila* species were kept in enclosures for 21 days. However, since these experiments were conducted in late spring, water temperature never exceeded 10°C. In contrast, the experiments in France were conducted in late summer with water temperature reaching 23°C and the first copepodite I appeared after only five days. However, the low temperature might not be the sole reason for slower development of Chilean *Ismaila* nauplii into copepodite stages.

Since in both cases infested specimens and larvae were kept under similar conditions, the initial prevalence of food can be regarded as equal. However, since the growth rate of algae is temperature dependant, food supply during the experiment may have influenced the larval development.

Nonetheless knowledge about the free-living larval stages of the parasite is crucial to unravel the mechanisms of splanchnotrophid infection. The different life history strategies of *Splanchnotrophus* and *Ismaila*, as discussed in chapter 4, may be correlated with or even caused by the different larval development of both genera.

8.2.3 Behavioural experiments

During the collection trip to Valdivia in 2010, first attempts were made trying to resolve the mechanisms of infestation. For this purpose, parasite larvae were given the choice either between a potential host individual or an empty compartment, or between two different potential host individuals using a Y-maze (see Fig. 5F). In one of the two arms of the Y-maze an already visibly infested specimen was offered and in the other arm one showing no sign of infestation. Test-specimens were kept in small compartments, which were connected to the main area by a piece of 500 µm gauze allowing only water transfer. For each run between five and ten nauplii were transferred into the remaining compartment of the maze. After 10, 30 and 60 minutes respectively, the number of nauplii in each arm of the maze was counted. Throwing a

coin before each rerun of the experiment chose the arrangement of infested and non-infested specimens, and both compartments housing the hosts were cleansed and the seawater in the maze was changed. Wherever possible host specimens were used only once, otherwise they were not used again directly after one experiment. In addition, since splanchnotrophid nauplii show phototaxis, the experimental setup was illuminated consistently.

According to Ho (1987) the infective stage is the copepodite I. Hence the experiments with the nauplii were conducted as a check plot and there was no sign of directional movement towards any potential host. However since splanchnotrophid larvae died before reaching the copepodit I stage experiments were ceased at that point and statistical evaluation was abandoned.

8.3 Parasitic Impact

Besides the larval stages, the actual impact of the parasite on its host also remains unresolved in great parts. Right since their first discovery Splanchnotrophidae were suggested to have a negative influence on their host, e.g. by feeding on host tissue (Hancock and Norman 1863), and therefore regarded as parasites.

Although there were some reports of splanchnotrophid species destroying their hosts' gonads (Jensen 1987; Schrödl 1997; Haumayr and Schrödl 2003; Marshall and Hayward 2006; Wolf and Young 2014) most of the examined infested hosts showed no signs of gnawing marks (see chapter 6) suggesting the damaging of the host being more of an exception or restricted to certain parasite-host species. The host *Flabellina* sp.1 showed high infestation rates and all infested individuals were sterilised by the parasite (Schrödl 1997). In other species, such as *Thecacera darwini*, no sterilisation was observed; infection rates were extremely high in several localities, including high numbers of parasite individuals per host in otherwise prospering host populations (Schrödl 2003), so the impact of the parasite was considered to be rather low. Infested individuals of *T. darwini* and *P. lottini* observed during this study produced egg-masses although they were carrying up to four adult female parasites.

However Jensen (1990) mentioned an infested specimen of *Elysia australis* (Quoy and Gaimard, 1832) to have lost the ability or will to copulate, Schrödl (1997) and Abad (2015) reported a higher mortality for starved infected host specimens, Wolf and Young (2014) describes a significant influence of infection with *I. belciki* on the

reproductive activity of its host *Janolus fuscus* and Marshall and Hayward (2006) reported significant damage to digestive and reproductive viscera resulting in sterilisation of the host caused by *S. willemi*. All these findings indicate that the negative influence of splanchnotrophid parasites might be more general than expected.

The results presented in chapters 7 suggest among others that at least *Ismaila* is feeding mainly on the haemolymph of its respective host. Accepting this, the actual impact of the parasite on its host may be difficult to detect and therefore underestimated so far. Imagining an infested host population inhabiting an area where the hosts at all times find sufficient amounts of food, the impact of the parasite even at high rates of infection and parasitic load (up to six mature females and eight males found in a single specimen of *T. darwini*) may be nearly undetectable. However if food supply becomes insufficient due to whatever reasons the situation might become more severe for highly infested populations, since the parasites then would exacerbate the situation by extracting additional resources from the hosts. Taken to the extreme, this might cause the extinction of a whole population due to a shortage in food supply an uninfected population would have easily endured.

Most interestingly, starvation experiments conducted by Abad et al. (2011) seem to confirm this hypothesis. Also the fact that infested host populations often seem to occur in a rather characteristic pattern seems to point in the same direction (see below).

All these patterns could be explained by the hypothesis that high infection rates with splanchnotrophid parasites cause extinction events, during which both host and parasite population cease. The host population is then regenerated by immigration events from neighbouring populations, causing the parasite to vanish from that distinct location until it may be re-colonised by parasitic larvae.

This hypothesis would provide an adequate explanation for the distribution pattern reported for several splanchnotrophid species from different locations worldwide (see also chapter 3). Schrödl already reported heavily infested host population being neighboured by completely uninfected populations (Schrödl 1997; Schrödl 2002; Schrödl 2003), and recently the same pattern was found in southern France (own observation). Moreover in Chile as well as in Croatia not even the host species could be found in areas where prior large populations of parasites and hosts were reported (Michael Schrödl and Roland Melzer, personal Communication). As opposed to this,

in a location near Valdivia (Chile) two co-occurring host populations showing extremely high infection rates were discovered, where previous only small numbers of infested specimens were reported (Schrödl 2002). Yet another conceivable explanation would include the assumption of hosts developing an effective defence against infection, causing the population of the parasite to collapse. But this would not explain the evidence of host populations vanishing along with the parasite, though it could also be caused by ecological or environmental reasons. Both assumptions would require a strong negative impact of the parasite, since the development of an effective defence will constrain the host to invest recourses. Therefore further research on this topic might not only reveal new insights about the influence of the parasite and shed some light on splanchnotrophid dispersal but might also reveal new data on the biology of the hosts.

8.4 Biogeography

The results of all analyses presented herein support the hypothesis of the origin of the Splanchnotrophidae lying in the Indo Pacific (see also chapter3). The recent discovery of two new species of *Splanchnotrophus* in Japan also indicates, following the hypothesis of chapter 3, the development of the genus *Splanchnotrophus* dating back to the time when the Tethys Sea provided a direct connection between present Mediterranean Sea and the Indo-Pacific region. However the topology varies regarding the respective analyses as discussed above, and therefore some differences also affect the biogeography of the family.

Figure 6. Hypothesis on historical biogeography of philoblennid, pionodesmotid and splanchnotrophid genera. Inferred migration events in the stem lineages are indicated by the order of the respective numbers

Considering the newly discovered *Splanchnotrophus* species in Japan, the biogeographic dispersal hypothesis presented in chapter 3 has to be modified. Thus the genus *Splanchnotrophus*, otherwise known only from Europe, must have colonised today´s Japanese coast before the closing of the Tethys Sea. The same seems to be true for the ancestor of the genus *Arthurius*, regarding the position of the genus in the new morphology-based analysis. Therefore the family could be much older than first anticipated in chapter 3, i.e. 18-19 mya at minimum (closure of the Tethys Sea according to Malaquias and Reid (2009)).

In chapter 3 the ancestors of *Ismaila* were supposed to first have colonised the north-eastern Pacific coast, then the south-eastern coast, and from there Caribbean waters and the Strait of Magellan. The new topology of the genus *Ismaila* (Fig. 3) now favours a first colonisation of the south-eastern Pacific coast radiating from there into the Strait of Magellan on the one side and towards the north-eastern Pacific coast on the other side, continuing from there into Caribbean waters. According to the results of the new analysis the genus *Splanchnotrophus* is supposed to share a common ancestor with *Ismaila*, strengthening the hypothesis that the colonisation of the American continent by *Ismaila* was by crossing the Atlantic Ocean (and not the

Pacific). The results of the molecular-based analysis presented in chapter 4 also favours this migration from the East, suggesting a sister taxon relationship for the European *Lomanoticola*, and *Ismaila*. Therefore both these results support the hypothesis introduced in chapter 3, suggesting that colonisation of the American continent occurred before the closing of the Isthmus of Panama and from the East, not the West. However, *Splanchnotrophus* and *Lomanoticola* both exclusively inhabit northern temperate waters. Since the origin of *Ismaila* is assumed to lie in southern temperate waters, this would involve a crossing of the entire tropical zone without leaving any record being detected so far.

Such a radiation pattern could be explained by a high parasitic impact as discussed above. During the crossing of the tropical zone only small, extremely localised infested populations would have been established. Those infested populations then would have been exterminated by the parasite, and since the parasite was not yet fully adapted to tropical habitats, those areas were never again re-colonised until now.

This hypothesis would not only be applicable for the crossing of the tropical zone, but it may also provide an explanation for missing records of parasites as an argument against colonisation of the American continent via the Pacific ocean (as discussed in chapter 3). Thus an adult parasite inside its host may have been able to reach the southwestern coast of South America by several intermediate stations where at least the populations of the parasite died out in time. Still, there are no coastal stepstones in the Eastern Pacific, and this so-called Eastern Pacific barrier exists longer than the 37 mya estimated age of splanchnotrophids (see chapter 3). A crossing of the Pacific Ocean without a host would implicate an endurance stage of splanchnotrophid nauplii to bridge the time. As shown herein, *Ismaila* nauplii may survive 21 days at least. It is unlikely though that single long-dispersal copepod larvae find suitable host species; sea slug faunas of Australasia and the southern Pacific do not overlap with the South American one.

The results presented in chapter 3 and 4 together with the gross topology of the newly conducted analysis are not contradicting the hypothesis of splanchnotrophid radiation as discussed in chapter 3 the “crossing Atlantic” hypothesis is still regarded the most probable. Especially since in all three analyses either *Splanchnotrophus* or *Lomanoticola* results as a direct sister taxon to *Ismaila*, suggesting the common ancestor to be indigenous in European waters.

At any rate, resolving the actual path of radiation will require an integrative study consisting of a comprehensive morphology based analysis (using an extended character state matrix including data on larval stages and internal anatomy) combined with an analysis based on molecular data comprising at least all splanchnotrophid genera. Possibly such a combined analysis might even provide an answer to the question why - especially if it is supposed to be relatively easy to cross the Atlantic Ocean - only *Ismaila* has managed the colonisation of the American continent.

9. Conclusions

9.1 Phylogeny

Until now, all taxonomic hypotheses concerning the Splanchnotrophidae were based solely on data referring to the external morphology (see chapter 3). Most of the currently accepted taxa (Huys 2001; Haumayr and Schrödl 2003) can be confirmed by the present morphocladistic study, although the genus *Lomanoticola* was recovered paraphyletic and *L. brevipes* might include several species.

Ismaila remains the most species-rich genus within the family. However, the exact relationship between the included species remains unresolved due to contradicting results of the morphology-based and the molecular-based analyses. *Ismaila genalis* and *I. volatilis* will require verification concerning their position in the molecular-based analyses as soon as more genetic material is available.

The usage of morphological data of highly adapted endoparasitic taxa in morphocladistic studies is known to be problematic at best (Ho 1991; Ho 2001; Huys 2001) as discussed in chapter 3; however, in case of the highly modified Splanchnotrophidae, morphology-based phylogenetic approaches seem to be suitable with the one constraint that as many data as possible are needed. Furthermore the morphology-based phylogeny even sustains the comparison with the first phylogenetic analysis using molecular data (see chapters 3 and 4).

Application of standard primers for the cytochrome oxidase I (COI) work quite well for the Splanchnotrophidae, as do also standard procedures for DNA extraction, PCR and sequencing (see chapter 4). Pre-experiments suggest that the application of 16S, 18S and 28S primers designed for other arthropod groups are generally suitable for the Splanchnotrophidae and some of their related taxa. However, there were quality

problems especially with the nuclear markers, so the primers might need optimisation. In addition, old collection material in general proved to be insufficient for molecular sampling, mainly due to age and initial fixation of the respective samples. The effort of gathering the required number of samples will be considerable, since even freshly collected samples do not always lead to convenient sequence data. It is therefore recommended to keep the specimens in an aquarium for several days before fixation. Thus aged egg sacs with nauplii standing close before hatching can be used for the molecular analysis, increasing the probability of getting significant sequence data.

9.2 Morphological queries

Unfortunately the number of species that have already been examined using SEM is still low, and the results already revealed some queries. In his family diagnosis Huys (2001) mentioned an anal opening being present on the terminal segment of the abdomen, between the caudal rami. However, in neither of the following genus diagnoses nor in any of his species descriptions the detection of an anal opening is documented. Also for *B. doliaris* no anal opening could be detected (see chapter 5), and in the species descriptions of *C. coia*, *C. delicata* and *C. mammillata* no anal opening was mentioned or is visible on the respective figures (Salmen et al. 2008b). Detailed descriptions of several species of the genus *Ismaila* also confirm the absence of an anal opening (see Haumayr and Schrödl 2003 and chapter 6). For the genera *Lomanoticola* and *Arthurius* no reliable figures showing whether there is an anal opening or not are available. However, pictures of the abdomen of *S. angulatus* and *S. gracilis* clearly confirm the presence of an anal opening (Salmen 2005; Abad et al. 2011). This seems quite remarkable since the presence of an anal opening would suggest the digestive system being either still complete or the level of reduction being at least lower than in *Ismaila*. This may even indicate the presence of two different strategies of nutrition within the Splanchnotrophidae, i.e. *Ismaila* as a supposedly haemolymph sucker and *Splanchnotrophus* with yet unknown feeding mode. This might be even more interesting, since it indicates a parallel to the different level of host specificity of these genera discussed in chapter 4.

Another query relates to the dorsal appendages. Especially the genus *Arthurius* displays an exceptional pattern concerning their shape (see chapters 3 and 6). Their morphology is usually rather persistent, at least on genus level. However, comparing

the existing figures of the three currently known species *A. elysiae*, *A. bunakenensis* and *A. gibbosa* it is obvious that length, morphology and even number of dorsal appendages is quite variable in *Arthurius* (see chapter 6; Huys 2001; Salmen et al. 2008a). This might somehow be connected to their hosts. Apart from *I. magellanica* and *I. jenseniana* the members of *Arthurius* are the only parasites known so far infesting sacoglossan hosts. Interestingly the morphology of the dorsal appendages of *I. magellanica* and *I. jenseniana* seem to slightly differ from those of their congeners by being shorter and more delicate, but these differences are not as obvious as in the case of *Arthurius*. Possibly the internal morphology of these hosts impedes the formation of "typical" splanchnotrophid dorsal appendages.

9.3 Life history

Although until now only two species of the genus *Ismaila* were studied histologically, the amount of new insights is considerable. As discussed in chapter 7, revealing the internal anatomy of other members of the Splanchnotrophidae will not only contribute to extend the knowledge about splanchnotrophid morphology, but also shed more light on the life history of this remarkable endoparasitic family. Studying the histology of *Ismaila* could not only clarify questions about nutrition or the function of the dorsal appendages, but also gave new insights in other sections of life history like the mobility of both sexes and it revealed new morphological features like the possession of an unpaired cement gland (see chapter 7).

Apart from that, life history features like the parasitic impact on the host are influencing hypotheses concerning the geographic dispersal and therefore also affecting the knowledge about host evolution. Life history is connected to all other fields and instantly affected by new discoveries. For example, the presence or absence of an anal opening might implicate the presence of two different nutrient strategies. Therefore the importance of life history cannot be overestimated.

Studying of the early ontogenetic stages may provide very interesting morphological data, especially for extremely adapted parasites like the Splanchnotrophidae. Apart from that, studying the infective mechanisms seems only possible by conducting behavioural experiments involving those larval stages assumed to be infective.

However, in order to succeed in that, basic knowledge about those larvae is needed. Since in Chile the development seems to be slower than, for example, in southern

France, housing conditions have to be improved in order to keep enough larvae alive until the required developmental stages are reached.

10. Outlook

For future studies it will be inevitable to recollect all currently included species for both morphological and molecular analyses. Apart from acquiring the necessary genetic material, this would simultaneously allow detailed re-descriptions by SEM in order to extend and maybe even revise the original descriptions. Especially problematic cases like the genus *Lomanoticola*, showing difficulties even assigning recently collected specimens to one of the actual species as mentioned above, are in desperate need of reconsideration. In addition, attention should be directed to complete the missing morphological datasets on males, especially for the genus *Lomanoticola* where data about males is as yet completely absent.

Combining both morphological and molecular data sets will enable comprehensive integrative taxonomy, recognition of potential cryptic speciation and testing of conventional species level taxonomy. Molecular phylogeny is expected to not only resolve inner splanchnotrophid relationships but also shed some light on potential sister groups such as the genus *Briarella* or the Pionodesmotidae. Eventually it will lay the foundation for the reclassification of the Splanchnotrophidae within the, itself disputed, Poecilostomatoida.

Since knowledge concerning the Splanchnotrophidae is currently restricted to descriptions of the external morphologies, further research about the life history has to become a focus of attention. The application of new histological methods might provide a vast variety of new possibilities. For example studying splanchnotrophid endoparasites using µCT would not only provide detailed 3D-models in a fraction of the time, but it will also allow histological studies on the parasite inside its host, perhaps revealing ways of infesting the host, as well as abundance, growth and numbers of larval stages and sex ratios of mature parasites.

Apart from that, infection experiments with parasitic larvae may reveal the mechanisms of infection and the development of the parasite inside its host, and thus extend knowledge on parasitic impact and geographic dispersal not only of the Splanchnotrophidae but also of their hosts.

Knowledge of the Splanchnotrophidae is still far from complete. Recent discoveries prove that there are still unknown members of this family yet to be discovered, and this is not only true for areas already known to be inhabited by the Splanchnotrophidae as proven by many photos of obviously infested Nudibranchia (see Rudman 1998). Instead, it can be assumed, that in areas that have not yet been considered, like the coasts of Africa, of the Arabian Peninsula or of India, still undiscovered members of the family lay hidden.

In the context of the first taxonomic studies in chapter 3 and 4 no direct correlation between the evolution of Splanchnotrophidae and their hosts could be found. However, there are reports of behavioural changes in hosts infected with Splanchnotrophidae (Jensen, 1987). It is therefore still possible that there are individual cases of coevolution between Splanchnotrophids and their hosts.

Thus the family Splanchnotrophidae remains an interesting field of research still offering the possibility to study a unique host-parasite relationship.

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Education and scientific work

Since 11.2015	**Pest controller** at the **APC-AG** Ostendstr 132, 90482 Nürnberg
11. 2008 – 12.2016	**PhD Thesis:** „Systematics, evolution and biology of the Splanchnotrohidae (Crustacea, Poecilostomatoida) a family of parasitic copepods“ (Supervisor Prof. Dr. Michael Schrödl, Bavarian State Collection of Zoology)
01.2008 – 03.2008	Traineeship at the **Museum Mensch und Natur** in Munich
10. 2001 – 12. 2007	Studies of Biology (Ludwig-Maximilians-University, Munich) Abschluss: **Diplom der Biologie** (Gesamtnote: sehr gut)
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11. 1999 – 08. 2000	**Millitary service** at the 3./Flugabwehrraketengruppe 33; Prinz-Heinrich-Kaserne, Lenggries
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05. 2009 - 05. 2012	Graduate Funding by the Universität Bayern e.V.

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Publications

Salmen, A. Anton, R. Wilson, N. & Schrödl, M. (2010), SEM-description of the philoblennid endoparasitic copepod *Briarella doliaris* n. sp. From Queensland, Australia; a potential link to the Splanchnotrophidae (Crustacea, Copepoda, Poecilostomatoida); Spixiana 33 (1), pp. 19-26

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Anton, R. F. Schories, D. Wilson, N. G. Wolf, M. Abad, M. & Schrödl, M. (2016), Host specificity versus plasticity: testing the morphology-based taxonomy of the endoparasitic copepod family Splanchnotrophidae with COI barcoding; Journal of the Marine Biological Association, doi:10.1017/S002531541600120X

Talk

Anton, R. & Schrödl, M. (2010), Towards the understanding the phylogeny and evolution of the Splanchnotrophidae (Copepoda, Poecilostomatoida), a family of endoparasitic copepods in opisthobranch gastropods; Third International Workshop on Opisthobranchs, Vigo (Vortrag)

Poster

Anton R. F. & Schrödl M. (2013), The enemy inside your sea slug - endoparasites of the family Splanchnotrophidae (Copepoda); World congress of Malacology, Ponta Delgada (Poster)

Skills

Languages: German (native), English (written and spoken), French (basic knowledge)
Gaschromatography
Light- and scanning electron microscopy

Molecular Biology (DNA-extraction, PCR, DNA-purification, Sequencing)
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Hobbies

Photography, SCUBA-diving, Speed Badminton, scale modelling

14 Supplementary material

Supplementary material 1: Character state description

Ecology:

1) **Host:** Copepods can be parasitic in many groups of organisms. Taxa included into this analysis may infest worms (0), sea urchins (1) or bivalves (2); all splanchnotrophid genera are parasitic in gastropods (3) and three taxa are parasitic on fishes (4).
2) **Gastropod host:** Philoblennidae infest gastropods of different orders (0) while most Splanchnotrophidae are parasitic in nudibranchs (1) only some splanchnotrophid species have sacoglossan hosts (2).
3) **Type of parasitism:** *Ventricolina*, *Micrallecto* and *Doridicola* are ectoparasitic (0) while all splanchnotrophid species are endoparasitic (1)
4) **Ectoparasitic infection sites:** Ectoparasites are located on the skin (0) or on the gills (1) of their host.
5) **Endoparasitic infection sites:** *Pionodesmotes* lives in galls in sea urchins (0). *Briarella* is located in the pericard (1). All splanchnotrophid species are located within the body cavity of their hosts (2). *Chondracarpus* and *Mytillicola* infect the intestines of their hosts (3).
6) **Geographic origin:** Members of the Splanchnotrophidae can be found in the Indo-Pacific (0), in European waters (1) and arround the American continent (2).

External morphology (female):

7) **Body shape:** The body comprising cephalothorax, thorax and abdomen can be elongate (0), compact and stocky (1), inflated (2), delicate (3), or depressed dorsoventrally (4).
8) **Colour:** The colour of the living animal can be brown (0), reddish (1), bright orange (2), yellowish (3), whitish (4), slightly pink (5) or ink blue (6).
9) **Length:** The species used for the analysis are divided into three groups. The species are considered as small if their body length is less than 1mm (0), mediumsized if the body length ranges between 1 and 9 mm (1), and large if the body is longer than 9 mm

(2) The body length is measured from the cephalothorax to the abdomen, without considering the antennae, processes and caudal rami.

10) **Demarcation of cephalothorax:** The cephalothorax can be clearly set off from the rest of the body (0) or there is no distinct border discernable (1)

11) **External segmentation:** While in some parasitic copepod genera including *Arthurius* an indistinct external segmentation is still detectable (0), there are species within the Splanchnotrophidae where there is no external segmentation detectable (1).

12) **Segmentation:** The antennule can be 7-segmented (0), 6-segmented (1), 5-segmented (2), 4-segmented (3), 3-segmented (4), 2-segmented (5) or it may be 1-segment , respectively there are no segment boundaries discernable (6)**.**

13) **Number of spines:** On the first antennual segment there can be four spines (0), three spines (1), two spines (2) or the segment is unarmed (3).

14) **Number of setae:** The first segment can be armed with nine setae (0), with five setae (1), with four setae (2) with three setae (3), two setae (4), with just one seta (5) or the segments can be unarmed (6).

15) **2nd segment of antennule:** The 2nd antennual segment can be present (0) or absent (1)

16) **Quantity of setae:** The quantity of setae is considered high if there are more than 9 setae (0) or low if there are 9 or less setae (1).

17) **Many Setae:** If the quantity of setae is considered high, there can be18 setae (0) 16 setae (1), 14 (2), 13 (3), 11 (4) or 10 (5).

18) **Few Setae:** If there are only few setae this can mean that there are nine setae (0), eight (1), seven (2), four (3), three (4), two (5) or just one seta (6) present on the 2nd segment of the antennule.

19) **3rd segment:** The 3rd segment of the antennulae can be present (0) or absent (1).

20) **Number of setae:** The 3rd segment can be armed with 11 setae (0), six (1), four (2), three (3) or with just two setae (4) or the segment is unarmed (5).

21) **4th segment of antennule:** The 4th antennual segment can be present (0) or absent (1).

22) **Number of setae:** There can be eight setae on the 4th segment (0) seven (1), six setae (2), five (3) four setae (4), three setae (5) or just two setae (6).

23) **5th segment of antennule:** The 5th segment of the antennule can be present (0) or it can be completely absent (1).

24) **6th segment:** The 6th segment of the antennule can be present (0) or absent (1)

25) **7th antennual segment:** The antennula can have a 7th segment (0) or the segment is absent (1).

26) **Distal segment with two constrictions:** There may be two constrictions on the distal segment of the antennule (1) or not (0).

27) **Segmentation:** The antenna can be 4-segmented (0), 3-segmented (1) or 2-segmented (2).

28) **Shape:** The antenna can be long and slender (0), large and robust (1) or it can be small and fleshy (2).

29) **Shape of distal segment:** The distal segment of the antenna can bear two claws (0), be small with three setiform elements (1), formed as a claw (1) or as a hook (2) or it can be within the curvature of the rostrum (3).

30) **Setae:** On the first segment there may be setae present (0) or not (1).

31) **Number of setae:** The segment can be armed with three setae (0), two setae (1), with one seta (2).

32) **Spines:** Spines may be present on the first antennular segement (0) or not (1).

33) **Number of spines:** The 1st segment may bear two spines (0), one spine (1).

34) **Setae:**There may be setae present on the second segment of the antenna (0) or not (1).

35) **Number of Setae:** On the 2nd segment there can be three (0), two setae (1)or one seta (2).

36) **Shape of seta:** The seta can be stubby (0), short (1), curved (2) or naked (3).

37) **Spine present:** There may be a spine present on the 2nd segment (0) or the spine may be absent (1).

38) **Shape of spine:** The spine can be strong (0) or small (1).

39) **3rd segment:** The antenna can have a third segment (0) or the segment can be absent (1).

40) **Number of setae:** There can be eight setae (0), seven setae (1) five setae (2), four setae (3), three setae (4), two setae (5) or just one seta present on the segment (6) or it may be unarmed (7).

41) **Shape of setae:** The setae can be long (0) or stubby (1).

42) **Number of spines:** The 3rd antennal segment bears six spines (0), five spines (1), four spines (2), three spines (3), two spines (4) or no spines (5).

43) **Small hole in the 3rd segment:** There can be a small hole visible on the third segment (0) or this hole may be absent (1).

44) **Surrounding of hole:** The small hole can be surrounded by several spines (0) or it can be covered by the edge of a spine (1).

45) **4th segment:** A fourth segment may be present (0) or absent (1).

46) **Labrum:** The labrum can be present (0) or absent (1).

47) **Shape:** The shape of the labrum can be triangular (0), elongate (1) or inverted U-shaped (2).

48) **Mandible:** The mandibles can be present (0) or absent (1).

49) **2nd ramus:** The second ramus of the mandibles can be well developed (0), it may have a minute terminal claw (1) or it can be sickle-shaped with a pointed tip (2).

50) **Covered by labrum:** The mandibles can be covered by the labrum (0) or not (1).

51) **Blade:** The mandibles can have a blade (0) or not (1).

52) **Processes:** On the mandibles there can be processes present (0) or not (1).

53) **Dentiform processes:** The dentiform processes on the mandibles can be arranged in one row or two (0) or they can be situated at the apex (1).

54) **Maxillule:** The maxillulae can be present (0) or absent (1).

55) **Number of lobes:** The maxillulae may bear several lobes (0), or just one lobe (1).

56) **Number of setae:** The maxillulae may bear four setae (0), two (1) or just one seta (2).

57) **Segmentation:** The maxillae can be 3-segmented (0) 2-segmented (1) or 1-segmented (2).

58) **Processes:** The maxillae may bear two processes (0) or it has a single terminal one (1).

59) **Shape of 2nd segment:** The 2nd segment of the maxillae can be robust (0), slender (1) or short (2).

60) **Tip shape:** The tip of the maxillae can have a terminal claw (0), it can be formed as a hook (1) or it can be shovel-like (2).

61) **Labium:** The labium can be present (0) or absent (1).

62) **Hairs:** On the labium there may no hairs be present (0), the labium can be hairy all over the surface (1) or it can have only hairy patches (2).

63) **Paragnath lobes:** Paragnath lobes may be present (0) or they may be absent (1).

64) **Shape:** The labium either is tongue-shaped (0), simple and rounded (1), steep and membranous (2), it can be produced into paired spinolous lobes (3).

65) **Distal hairs:** The distal hairs of the labium can be limited to the lateral portions (0) or there can be several short or long hairs (1).

66) **Slit:** Despite from being absent (0) there may be a zig-zag-shaped slit running over 1/3 of the labium (0).

67) **Thoracopods designed as swimming legs:** The thoracopods can be shaped as swimming legs (0) or not (1).

68) **Maxilliped:** A maxilliped can be present (0) or absent (1).

69) **Shape of maxilliped:** The maxilliped can be large and well developed (0) it can bear spine-like processes (1), or it can be small (2).

70) **2nd thoracopod:** The second thoracopod can be present (0) or it can be absent (1).

71) **Number of elements:** The second thoracopod can consist of three elements (0), of two elements (1) or of just one element (2).

72) **Shape of the exopodit:** The exopodit of the second thoracopod may be long and voluminous (0), thick and distally flattened (1), conical (2) or minute and spinous (3).

73) **State of development:** The exopodit of the second thoracopod can be of regular shape (0), it can be rudimentary (1) or it can be represented by an outer basal seta (2).

74) **Comparing length of exopod and endopod:** The exopodit and the endopodit can be of equal length (0) or the exopodit can be longer (1).

75) **Exopod distinguishable from protopodit:** The exopod of the second thoracopod can be clearly distinguishable from the protopodit (0) or indistinguishable from the protopodit (1).

76) **Exopodit:** The exopodit of the second thoracopod can be present (0) or absent (1).

77) **Number of rami:** The exopod of the second thoracopod can be biramous (0) or uniramous (1).

78) **Segmentation:** The exopodit of the second thoracopod can be 3-segmented (0), 2-segmented (1) or unsegmented (2).

79) **Tip shape:** The tip of the exopod of the second thoracopod may bear a claw (0) or a minute recurved element (1) or it may be blunt (2).

80) **Endopodit:** The endopodit of the second thoracopod can be present (0) or it can be reduced (1).

81) **1st segment:** The first segment of the endopod of the second thoracopod can have three setae (0), it may bear one seta (1) or it can be unarmed (2).

82) **2nd segment:** The second endopodal segment of the second thoracopod may bear five setae (0), four setae (0) two setae (1), it can have only one seta (2) or be unarmed (4).

83) **Setae on 3rd segment:** The third segment of the second thoracopod may bear five setae (0), four setae (1) or just two setae (2) or the segment can be unarmed (3).

84) **Spines on 3rd segment:** On the third segment of the second thoracopod of the endopod there can be two spines (0) or just one spine (1) or the segment can be unarmed (2).

85) **Number of spines on the exopodit:** There can be five (0), four (1), three setae (2) or just one single seta (3) on the exopodit of the second thoracopod.

86) **3rd thoracopod:** The third thoracopod can be present (0) or absent (1).

87) **Number of elements:** The third thoracopod can consist of three separate elements (0), it can be biramous (1) or uniramous (2).

88) **Segmentation:** The third thoracopod can be 3-segmented (0), 2-segmented (1) or it can be 1-segmented (2).

89) **Tip-shape of exopodit:** The tips of the elements forming the exopodit can be three short elements which are tapering into a terminal claw (0), the apex can have two strong elements (1),the tip can be hook-shaped (2) or it can have a blunt tip (3).

90) **Comparing length of exopod and endopod:** The exopodit of the 3rd thoracopod can be longer than the endopodit (0), both can be of equal length (1) or the exopodit can be shorter than the endopodit (2).

91) **Exopodit:** The exopod of the third thoracopod can be present (0) or absent (1)

92) **Exopod distinguishable from protopodit:** The expopod of the third thoracopod can be distinguishable from the protopod (0) or not (1).

93) **Thickness (compared to 2nd thoracopod):** the exopodit of the third thoracopod can be thicker than the exopodit of the 2nd thoracopod (0) or it can be less voluminous than that of the 2nd thoracopod (1).

94) **Number of rami:** The exopodit of the third thoracopod can be biramous (0) or uniramous (1).

95) **Endopodit:** The endopodit of the third thoracopod can be present (0) or it can be absent (1).

96) **Endopodit:** The endopodit of the third thoracopod can be 3-segmented (0), 2-segmented (1) or unsegmented (2).

97) **State of development:** The endopodit of the third thoracopod can be fully developed (0) or it can be rudimentary (1).

98) **Armament:** There can be sensoric hairs present on the endopod (0) or not (1)

99) **First segment setae present:** There may be setae at the first segment of the endopod (0) or not (1).

100) **Length of inner process:** The endopodit and its inner process can have the same length (0), the inner process can be shorter than the endopodit (1) or the inner process can be very small, rudimentary (2).

101) **Thickness of inner process:** The endopodit and its inner process can be equally thick (0) or the inner process can be thinner than the endopodit (1).

102) **4th thoracopod:** The 4th thoracopod may be present (0) or absent (1).

103) **Shape:** The 4th thoracopod can be widely seperated into exo- and endopod (0), it may be very small (1), a minute element (2) or just a small lobe (3).

104) **Protopodit:** the protopodit of the 4th thoracopod may be present (0) or absent (1).

105) **1st segment:** The first segment may be present (0) or absent (1).

106) **2nd segment:** The 2nd segment may be present (0) or absent (1).

107) **Exopodit:** The exopod can be present (0) or absent (1).

108) **3rd segment:** The third segment can be present (0) or absent (1).

109) **Endopodit:** The endopodit can be present (0) or absent (1).

110) **1st segment:** The first segment can be present (0) or absent (1).

111) **2nd segment:** The second segment can be present (0) or absent (1).

112) **3rd segment:** The third segment can be present (0) or absent (1).

113) **5th thoracopod:** The 5th thoracopod can be present (0) or absent (1).

114) **Sclerotized Ring:** The border between the 4th and the 5th thoracopod can be marked by a sclerotized ring (0) or not (1).

115) **6th thoracopod:** The 6th thoracopod can be present (0) or absent (1).

116) **Thorax segmentation visible:** The segmentation of the thorax may be clearly visible (0), or the segment borders may be indiscernable (1).

117) **Thorax (Number of segments):** The thorax can consist of five segments (0), four segments (1) or three segments (2).

118) **Bulges:** The cephalothorax may be flat (0) or there may be bulges present (1).

119) **Number of bulges:** There may be two bulges (0), three bulges (1), four bulges (2), six bulges (3) or eight bulges present on the dorsal side of the thorax (4).

120) **Processes:** There can be processes on the thorax present (1) or such processes may be absent (0)

121) **Number of processes:** On the thorax there can be one pair (0), two pairs (1), three pairs (2) or six pairs (3) of processes.

122) **Situation of first pair:** The thoracic processes can be situated dorso-laterally (0) or ventrolaterally (1).

123) **Situation of second pair:** The thoracic processes can be situated dorso-laterally (0) or ventrolaterally (1).

124) **Situation of third pair:** The thoracic processes can be situated dorso-laterally (0) or ventrolaterally (1).

125) **Situation of fourth pair:** The thoracic processes can be situated dorso-laterally (0) or ventrolaterally (1).

126) **Situation of fith pair:** The thoracic processes can be situated dorso-laterally (0) or ventrolaterally (1).

127) **Dorso lateral process**: All members of the Splanchnotrophidae are lacking this process (0) except I. jenseniana (1).

128) **Length of processes:** The dorsolateral processes can be relatively short (0), they can be as long as the body (1), longer than the body (2) or twice as long as the body (3).

129) **Thickness:** The processes may be slender, relatively thin compared to body (0) or voluminous (1).

130) **Medio-dorsal processes:** While usually absent (0), between the 3rd pair of dorsal processes there can be one single medio-dorsal process (1)

131) **Abdomen (segmentation visible):** The abdomen can be clearly segmented (0) or the segmentation can be no longer visible (1).

132) **Abdomen (number of segments)**: The abdomen can consist of five segments (0), four segments (1), three segments (2) or two segments (3).

133) **Length of abdomen:** The abdomen can be long and slender (0) or short and small (1).

134) **Abdomen protruding though integument:** In all splanchnotrophid species the abdomen is protruding through the integument of the host (1) while in all other parasitic species it is not (0).

135) **Egg sacs:** The Egg sacs can be unilobate (0) or bilobate(1).

136) **Attachement:** Egg sacs can be attached to the abdomen at their terminal ends (0) at about 1/3 of their length (1) or at about their middle (2).

137) **Egg sacs (straight or coiled):** The egg sacs can be straight (0), or coiled (1).

138) **Egg sacs (shape):** The shape of the egg sacs can be typically cyclopoid (0), elongate and slender (1) or sausage-shaped (2).

139) **Egg sacs (whorls):** The egg sacs can form one whorl (0), or they can form two whorls (1).

140) **Colour:** The colour of the egg sacs of living animals can be white (0), brown (1) pink (2), orange (3), lilac (4), yellow (5), red (6) or greenish (7).

141) **Length:** The egg sacs can be short (0) or large, longer than the body (1).

142) **Shape of caudal rami:** The caudal rami may be long (0), they may be globular (1), small and minute (2).

143) **Number of setae:** The caudal rami may bear, seven setae (0), six (1), five (2), four (3), three (4), or two setae (5) or there can be just one seta (6).

144) **Shape of setae:** The setae of the caudal rami can be enlarged (0), pinnate (1) or small (2).

External morphology (male):

145) **Dwarf-male:** Only in *A. kimjensis* the male is bigger than the female (0). The size can be equal to that of the female (1), there may be an overlap of the size of both sexes (2) or the males are smaller than the females (3).

146) **Body (size):** The size of the body is considered to be very small if it measures less than 1 mm (0), small if it measures between 1mm and 2mm (1) and large if the body measures more than 2mm (2).

147) **Body shape:** The shape of the body can be cyclopiform (0), elongate (1), pear-shaped (2) or inflated (3).

148) **Setup:** The cephalothorax can consist of five head segments and the first thoracic segment (0), include the head and the first two thoracic segments (1), or the head segments and the first three thoracic segments (2).

149) **Swollen segments:** Either the segments of the cephalothorax are of similar size (0) or the head and the 1st and 2nd thoracic segment may be swollen (1) or the 2nd and 3rd cephalic segments may be enlarged (2).

150) **Cephalothorax (demarkation):** The cephalothorax may be distinctly set off from the thorax (0), or not (1).

151) **Antennule:** The antennule can be cylindrical and elongate (0) or small (1).

152) **Segmentation:** The antennule may be 8-segmented (0), 7-segmented (1), 6-segmented (2), 5-segmented (3), 4-segmented (4), 3-segmented (5), 2-segmented (6) or 1-segmented (7).

153) **1st segment:** the first segment of the antennulae may bear eight rudimentary elements (0), six vestigial setae (1), five setae (2), three setae (3) or just one seta (4).

154) **2nd segment:** The second segment of the antennulae can be present (0) or absent (1).

155) **Armament:** The second segment of the antennulae may bear 16 setae (0), 14 setae (1), 13 setae (2), eight (3), seven (4), two (5) or just one single seta (6).

156) **3rd segment:** The third segment of the antennulae can be present (0) or absent (1).

157) **4th segment:** The 4th segment of the antennulae can be present (0) or absent (1).

158) **Armament:** The 4th segment of the antennulae may bear four setae (0), three (1), two setae (2) or one seta (3).

159) **5th segment:** The 5th segment of the antennulae can be present (0) or absent (1)

160) **Armament:** The 5th segment of the antennulae may bear eight setae (0), five setae (1), three (2) or just one seta (3).

161) **6th segment:** The 6th segment of the antennulae can be present (0) or absent (1).

162) **7th segment:** The 7th segment of the antennulae can be present (0) or absent (1).

163) **Antenna (size):** The antennae may be large (0) or slender (1).

164) **Segmentation:** The antennae can either be 4-segmented (0), 3-segmented (1) or 2-segmented (2).

165) **1st segment:** The first segment of the antennae may bear two setae (0) or just one seta (1) or the segment can be unarmed (2).

166) **3rd segment:** The third segment of the antennae can be present (0) or absent (1).

167) **Shape of 3rd segment:** The 3rd segment of the antennae can be claw-shaped (0) or hook-shaped (1).

168) **4th segment:** The 4th segment of the antennae can be present (0) or absent (1).

169) **Labium:** The labium can be present (0) or absent (1).

170) **Mandible:** The mandible can be present (0) or absent (1).

171) **Mandible (shape):** The mandible may be styliform with an enlarged base and a recurved blade (0), it can have setiform elements (1) or it can be largely fused to the lateral margin of the oral cavity (2).

172) **Dentiform processes:** Dentiform processes on the mandible may be present (0) or absent (1).

173) **Maxillule:** The maxillule can be present (0) or absent (1).

174) **Segmentation:** The maxilla can be 3-segmented (0), 2-segmented (1) or unsegmented (2).

175) **Labrum:** The labrum can cover the mouth medially (0), it may be a small chitinized plate (1) or it can be an arched plate with a smooth surface (2).

176) **State of development:** The labrum can be triangular (0), it can be very small and bilobate (1) or it can be rudimentary with paired spinous projections (2).

177) **Segmentation:** The segmentation of the thorax can be well defined (0) or indistinct (1).

178) **Thorax (segmentation):** The thorax can be 11-segmented (0), 7-segmented (1), 6-segmented (2), 5-segmented (3), 4-segmented (4), 3-segmented (5) or 2-segmented (6).

179) **Fused elements:** The thoracal segments can be free (0) or there may be fused elements (1).

180) **Processes:** Processes on the thorax can be absent (0) or present (1).

181) **Maxilliped:** The first thoracopod can be present (0) or absent (1).

182) **1st segment:** The first segment of the second thoracopod can be present (0) or absent (1).

183) **2nd segment:** The second segment of the second thoracopod can be present (0) or absent (1).

184) **3rd segment:** The third segment of the second thoracopod can be present (0) or absent (1).

185) **2nd thoracopod (number of rami):** The 2nd thoracopod can be biramous (bilobate) (0) or uniramous (1).

186) **Length:** The second thoracopod can be enlarged (0) or minute (1).

187) **State of exopodit:** The exopodit of the third thoracopod can have five spines (0), or four spines (1) or just one seta (2).

188) **3rd thoracopod (number of rami):** The third thoracopod can be biramous (0) or uniramous (1).

189) **Length of exopod:** The exopodit of the third thoracopod can be long (0) or minute (1).

190) **Terminal claw:** A terminal claw may be present (0) on the third thoracopod or not (1).

191) **4th thoracopod:** The 4th thoracopod can be present (0) or absent (1).

192) **5th thoracopod:** The 5th thoracopod can be present (0) or absent (1).

193) **6th thoracopod:** The 6th thoracopod can be present (0) or absent (1).

194) **Abdomen (size):** The abdomen can be elongated (0) or short (1).

195) **Abdomen (segmentation):** The abdomen may be 6-segmented (0), 4-segmented (1), 3-segmented (2), 2-segmented (3) or unsegmented (4).

196) **Surface:** The abdomen can have a flat surface (0) or bear a pair of setules on its dorsal surface (1), there can be two lateral ridges below the gonadal lobes (2).

197) **Caudal rami (shape):** The caudal rami can be long, strong and cylindrical (0), globular (1), flaccid (2) or small (3).

198) **Setae on caudal rami:** The caudal rami can have seven setae (0), six setae (1), five setae (2), four setae (3), each ramus may bear two or three setae (4) or just one single seta (5).

199) **Anal opening:** The anal opening can be present between the caudal rami (0) or an anal opening may be absent (1).

The following characters were excluded from the analysis, since they were rendered parsimony uninformative.

200) **Habitat:** All splanchnotrophid species are marine (0), only *Ergasilus youngi* inhabit brackish (1) waters.

201) **State of development:** The labrum can be well developed only in *Arthurius elysiae* it is not (1).

202) **Number of setae on the 2nd segment:** The 2nd exopodal segment of the second thoracopod is unarmed only in *Majimun*.

203) **Number of spines on the 2nd segment:** The 2nd segment of the second thoracopod bears four spines only in *Briarella doliaris*.

204) **Armament:** The first segment of the third thoracopod is armed with two setae only in *Ceratosomicola sacculata*.

205) **Spines on the exopodit:** Only in *Creatosomicola japonica* there are no spines present on the third thoracopod.

206) **Number of rami:** The 4th thoracopod is biramous only in *Doridicola larani*.

207) **State of development:** Only in *Doridicola larani* the 5th thoracopod is not very small and rudimentary.

208) **Ventral processes:** Ventral processes are present only in *Arthurius elysiae*.

209) **Additional processes:** An additional pair of dorsal processes is present only in *Arthurius elysiae.*

210) **Armament:**The 6th segment of the antennulae may bear eight setae (0), three (1), two setae (2) or just one single seta (3) but is absent in all known splanchnotrophid males.

211) **2nd segment:** The second antennaul segment is unarmed only in *Splanchnotrophus helianthus*.

212) **Maxilla:** The maxillae are absent only in *Arthurius bunakenensis*.

213) **1st segment:** The first segment of the maxillulae is absent only in *Arthurius bunakenensis*.

214) **2nd segment:** The second segment of the maxillulae is absent only in *Arthurius bunakenensis*.

215) **Length of podites:** The exopodit of the third thoracopod is nearly as long as the exopodit of the second thoracopod only in *Ceratosomicola mammillata*.

216) **Base of processes:** The processes arise from a common base only in *Ismaila occulta*.

These characters were excluded, since they are constant in the present matix.

217) **Antennule:** The antennulae are always present.

218) **1st segment of antennule:** The first segment of the antennule is always present.

219) **Antenna:** The antennae are always present.

220) **1st segment:** The first segment of the antennae is always present.

221) **2nd segment:** The second segment of the antennae is always present.

222) **Shape of processes:** The processes on the mandibles are formed as spinules or bristles in *Ergasilus youngi*.

223) **Terminal elements:** The maxillulae bear always terminal elements.

224) **Caudal rami:** The caudal rami are always present.

225) **8th segment:** The 8th segment of the antennulae is always absent.

226) **Segmentation:** The maxillulae are always 2-segmented.

227) **Maxilla:** The maxillae are always present in males.

Supplementary material 2: Character-state matrix

Nr. aktuelle Liste	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Anthessius kimjensis	2	?	0	0	?	0	?	1	0	0	0	3	5	0	0	1	?	0	2	0	4	0	0	1	0	1	0	3	0	2	?	?	0	1	?	?
Ergasilus youngi	4	?	0	1	?	0	?	1	0	0	1	3	5	0	0	4	?	0	3	0	5	0	0	1	0	0	0	0	0	2	?	?	0	2	2	?
Doridicola larani	3	0	0	0	?	1	6	1	0	0	0	3	3	0	0	3	?	0	1	0	6	0	0	0	0	0	1	0	0	2	?	?	0	2	3	?
Pionodesmotes phormosomae	1	?	1	?	0	2	?	1	1	1	0	3	6	0	1	?	5	0	3	0	5	0	0	0	0	0	1	1	1	?	1	?	0	2	1	1
Pionodesmotes domhainfharraigeanus	1	?	1	?	0	2	?	1	1	1	1	3	2	0	1	?	3	0	2	0	3	0	0	1	0	0	1	1	0	2	1	?	0	2	1	1
Philoblenna bupulda	3	0	0	0	?	{0,2}	?	1	0	0	0	3	3	0	0	4	?	0	3	0	5	0	0	0	0	1	1	0	0	0	?	?	0	2	?	?
Philoblenna tumida	3	0	0	1	?	{0,2}	?	1	0	0	1	3	3	0	1	?	2	0	2	0	6	0	0	1	0	1	1	0	?	?	?	?	?	?	?	?
Philoblenna littorina	3	0	0	1	?	{0,2}	?	1	0	0	1	3	3	0	0	5	?	0	1	0	2	0	0	1	0	0	1	0	0	2	1	?	0	0	?	1
Philoblenna arabici	3	0	0	0	?	{0,2}	3	1	0	0	0	1	6	0	1	?	1	0	5	0	?	0	0	0	0	0	1	0	?	?	0	1	?	?	?	0
Briarella microcephala	3	1	1	?	1	0	3	1	0	1	2	?	?	0	?	?	?	0	?	0	?	0	1	1	?	1	?	0	?	?	?	?	?	?	?	?
Briarella sp.	3	1	1	?	1	0	3	2	0	?	2	?	?	0	?	?	?	0	?	0	?	0	1	1	?	1	?	0	?	?	?	?	?	?	?	?
Briarella risbeci	3	1	1	?	1	0	?	2	0	?	{1,2}	3	5	0	?	?	?	0	5	0	?	0	0	1	?	1	1	0	?	?	?	?	?	?	?	?
Briarella disphaerocephala	3	1	1	?	1	0	?	2	0	?	2	?	?	0	?	?	?	0	5	0	?	0	1	1	?	1	1	0	?	?	?	?	?	?	?	?
Briarella doliaris	3	1	1	?	1	1	4	1	0	1	3	3	0	0	1	?	3	0	4	0	2	1	1	1	0	1	1	0	?	?	0	1	1	?	?	0
Splanchnotrophus angulatus	3	1	1	?	2	1	4	1	1	1	5	1	6	0	?	?	?	1	?	1	?	1	1	1	1	1	1	2	?	?	0	1	1	?	?	0
Splanchnotrophus gracilis	3	1	1	?	2	1	4	0	1	1	5	0	6	0	?	?	?	1	?	1	?	1	1	1	1	1	1	2	?	?	0	1	1	?	?	0
Splanchnotrophus helianthus	3	1	1	?	2	1	?	1	0	1	5	2	6	0	0	2	?	1	?	1	?	1	1	1	1	1	1	2	0	1	0	1	1	?	?	0
Splanchnotrophus imagawai	3	1	1	?	2	1	?	{0,1}	0	1	5	2	6	0	0	3	?	1	?	1	?	1	1	1	1	1	1	2	0	1	0	1	1	?	?	0
Splanchnotrophus dellachiajei	3	1	1	?	2	?	4	?	0	1	5	0	6	0	1	?	1	1	?	1	?	1	1	1	1	1	1	2	?	?	?	?	?	?	?	?
Splanchnotrophus willemi	3	1	1	?	2	1	4	1	0	?	5	0	6	0	?	?	?	1	?	1	?	1	1	1	1	1	1	?	?	?	?	?	?	?	?	?
Ceratosomicola coia	3	1	1	?	2	0	{3,1}	1	0	1	3	0	6	0	1	?	4	0	3	0	0	1	1	1	0	1	2	3	1	?	1	?	0	2	1	?
Ceratosomicola delicata	3	1	1	?	2	0	{4,5}	1	1	1	4	0	6	0	1	?	5	0	2	1	?	1	1	1	0	1	2	2	1	?	1	?	1	?	?	0
Ceratosomicola mammillata	3	1	1	?	2	0	{3,4,0}	1	0	0	3	0	6	0	1	?	6	0	4	0	?	1	1	1	0	1	2	2	?	?	?	?	1	?	?	0
Ceratosomicola japonica	3	1	1	?	2	1	?	1	0	1	3	0	6	0	1	?	3	0	3	0	1	1	1	1	1	1	2	2	1	?	1	?	0	2	?	1
Ceratosomicola sacculata	3	1	1	?	2	1	?	1	1	1	3	0	6	0	1	?	3	0	3	0	2	1	1	1	0	1	2	2	?	?	0	0	0	2	1	0
Lomanoticola insolens	3	1	1	?	2	1	?	1	1	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?
Lomanoticola brevipes	3	1	1	?	2	1	?	0	1	1	5	?	?	0	?	?	?	0	?	0	?	0	0	1	?	1	?	?	?	?	?	?	?	?	?	?
Lomanoticola sp.	3	1	1	?	2	1	{0,4}	0	1	0	3	2	5	0	1	?	6	0	3	0	0	1	1	1	0	1	1	2	?	?	0	1	1	?	?	0
Arthurius bunakenensis	3	2	1	?	2	1	2	0	1	1	6	3	5	1	?	?	?	1	?	1	?	1	1	1	0	2	1	?	?	?	?	?	?	?	?	?
Arthurius elysiae	3	2	1	?	2	1	2	1	0	?	6	3	0	1	?	?	?	1	?	1	?	1	1	1	0	2	1	2	?	?	0	0	?	?	?	?
Arthurius gibbosa	3	2	1	?	2	0	?	1	1	1	6	3	6	1	?	?	?	1	?	1	?	1	1	1	0	1	1	3	1	?	1	?	1	?	?	1
Ismaila monstrosa	3	1	1	?	2	0	?	1	0	0	5	0	6	0	1	?	0	1	?	1	?	1	1	1	0	1	1	3	?	?	?	?	?	?	?	?
Ismaila obtusa	3	1	1	?	2	1	?	2	0	1	5	?	?	0	?	?	?	1	?	1	?	1	1	1	0	1	1	3	?	?	?	?	?	?	?	?
Isamila jenseniana	3	2	1	?	2	0	?	1	0	1	6	?	?	1	?	?	?	1	?	1	?	1	1	1	0	0	1	3	?	?	?	?	?	?	?	?
Ismaila occulta	3	1	1	?	2	1	?	1	0	1	5	0	1	0	0	2	?	1	?	1	?	1	1	1	0	1	1	3	0	2	1	?	0	2	0	1
Ismaila belciki	3	1	1	?	2	3	?	1	0	1	5	1	3	0	1	?	0	1	?	1	?	1	1	1	0	1	1	3	0	2	?	?	0	2	0	?
Ismaila androphila	3	1	1	?	2	3	?	1	0	1	5	2	1	0	1	?	0	1	?	1	?	1	1	1	0	1	1	3	0	2	?	?	0	2	0	?
Ismaila alienea	3	1	1	?	2	0	?	1	0	0	5	1	3	0	1	?	0	1	?	1	?	1	1	1	0	1	1	3	0	2	?	?	0	2	0	?
Ismaila damnosa	3	1	1	?	2	1	?	{0,1}	1	1	5	0	5	0	1	?	0	1	?	1	?	1	1	1	0	1	1	3	?	?	?	?	?	?	?	?
Ismaila robusta	3	1	1	?	2	1	?	1	1	0	5	2	1	0	1	?	0	1	?	1	?	1	1	1	0	1	1	3	0	2	?	?	0	2	0	?
Ismaila socialis	3	1	1	?	2	3	?	1	0	0	5	2	2	0	1	?	0	1	?	1	?	1	1	1	0	1	1	3	0	2	?	?	0	2	0	?
Ismaila magellanica.	3	2	1	?	2	0	?	1	0	0	5	2	2	0	1	?	0	1	?	1	?	1	1	1	0	1	1	3	0	2	?	?	0	2	0	?
Ismaila genalis	3	1	1	?	2	1	?	1	0	1	5	3	3	0	1	?	3	1	?	1	?	1	1	1	0	1	1	3	0	1	1	?	0	1	1	1
Ismaila volatilis	3	1	1	?	2	1	?	1	0	1	5	1	6	0	0	5	?	1	?	1	?	1	1	1	0	1	1	3	1	?	1	?	1	?	?	0
Ismaila chaihuiensis	3	1	1	?	2	1	?	1	0	1	5	1	4	0	0	0	?	1	?	1	?	1	1	1	0	1	1	3	1	?	1	?	1	?	?	0
Majimun shirakawai	3	1	1	?	2	3	?	1	0	1	4	0	6	0	1	?	4	0	0	1	?	1	1	1	0	1	1	0	1	?	0	1	1	?	?	0

37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81
?	0	0	0	?	1	?	1	0	2	0	0	?	0	?	?	0	0	?	1	1	1	?	1	0	?	?	?	?	0	0	0	0	?	?	0	?	?	0	?	0	?	0	1	3
?	0	6	1	?	1	?	0	1	?	0	0	0	0	0	?	0	1	1	2	1	2	0	1	?	?	?	?	?	0	0	0	0	0	1	0	?	?	0	?	0	?	0	1	1
?	0	5	?	?	1	?	0	1	?	0	?	?	?	?	?	0	1	?	?	?	?	0	1	?	?	?	?	?	0	0	0	0	0	1	0	?	?	0	?	0	?	0	1	2
?	0	7	?	5	1	?	0	0	2	0	2	1	1	1	?	1	?	?	1	0	2	2	0	0	1	0	?	0	1	0	0	1	?	?	?	?	?	1	?	?	?	1	?	?
?	0	5	1	5	1	?	0	0	0	0	2	0	1	1	?	1	?	?	2	0	2	2	1	0	1	?	?	?	1	0	0	1	?	?	?	?	?	1	?	?	?	1	?	?
?	0	0	?	?	1	?	1	0	0	0	0	1	0	1	0	0	?	?	1	1	1	0	1	?	0	?	?	?	1	0	0	0	0	1	0	?	0	0	?	0	?	0	1	?
?	0	?	?	?	1	?	1	0	0	0	0	1	0	1	0	0	1	0	1	1	1	1	1	?	0	?	?	?	1	0	0	0	0	1	0	?	0	0	?	0	?	0	1	2
?	0	4	1	?	1	?	0	0	2	0	0	1	0	1	0	0	1	0	1	?	1	0	0	?	0	0	?	0	1	0	0	0	0	1	0	1	0	0	1	0	?	0	2	0
?	0	?	?	3	1	?	0	0	0	0	0	1	0	1	0	0	?	0	1	?	1	0	1	?	0	?	?	?	1	0	0	0	0	1	0	?	0	0	?	0	?	0	2	1
?	0	?	?	?	?	?	1	0	?	?	?	?	?	?	?	?	0	?	?	?	?	1	0	?	?	1	?	0	1	0	1	?	1	0	1	?	?	?	?	?	?	0	?	?	?
?	0	?	?	?	?	?	1	0	?	?	?	?	?	?	?	?	0	?	?	?	?	1	0	?	?	1	?	0	1	0	1	?	1	0	1	?	?	?	?	?	?	0	?	?	?
?	0	?	?	?	1	?	1	0	2	0	0	?	?	?	?	0	0	?	1	0	1	?	0	0	?	1	?	0	1	0	1	0	1	0	1	?	?	?	?	?	0	?	?	?
?	0	?	?	?	?	?	1	0	2	0	0	0	0	?	?	0	0	?	1	0	1	?	0	0	?	1	?	0	1	0	1	0	1	0	1	?	?	0	?	1	0	?	?	?
0	0	?	?	1	1	?	1	0	2	0	0	0	0	?	0	0	1	?	1	0	1	?	0	0	?	0	1	0	1	0	0	0	1	0	1	1	?	0	?	1	0	0	?	?
?	0	6	0	?	0	1	1	0	2	0	2	0	1	?	1	0	1	2	1	1	0	?	0	2	0	0	1	0	1	1	?	0	1	3	1	0	0	0	?	2	0	0	?	?
0	0	?	?	2	0	1	1	0	2	0	2	0	1	?	0	0	1	2	1	1	0	?	0	2	?	0	1	0	1	1	?	0	1	3	1	0	?	0	?	2	0	0	1	?
?	0	0	?	4	?	?	1	0	0	0	2	0	1	1	?	1	1	?	1	1	0	?	0	2	0	0	0	0	1	1	?	0	0	0	1	1	0	0	1	2	0	1	?	?
?	0	0	?	4	?	?	1	0	0	0	2	0	1	1	?	1	1	?	1	1	0	?	0	2	0	0	0	0	1	1	?	0	0	0	1	1	0	0	1	2	0	1	?	?
?	0	?	?	?	0	?	1	0	0	0	2	0	1	?	?	0	1	?	?	1	?	?	0	?	?	0	?	?	0	1	1	?	0	?	?	?	?	?	?	?	2	?	?	?	?
?	0	?	?	?	0	?	1	0	?	0	2	?	1	?	?	0	1	?	?	1	?	?	0	?	?	0	?	?	1	1	?	0	?	?	?	?	?	?	?	?	?	?	?	?
?	0	?	?	0	0	0	1	0	2	0	2	0	1	0	?	1	?	?	2	1	1	0	0	2	0	3	?	1	1	1	?	0	0	3	1	?	?	0	0	2	?	0	?	?
0	0	?	?	1	0	?	1	0	2	0	2	0	1	0	?	1	?	?	1	1	1	0	0	?	?	3	?	1	1	1	?	0	0	3	1	?	0	0	0	2	?	0	?	?
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?	0	1	?	5	0	?	1	0	2	0	2	1	1	0	1	1	?	?	2	1	1	0	0	2	?	2	?	0	1	1	?	0	2	3	2	?	1	1	?	2	?	1	?	?
0	0	?	?	4	0	?	1	0	2	0	2	0	1	0	?	1	?	?	2	1	2	0	0	2	?	3	?	?	1	1	?	0	2	3	2	?	0	0	?	2	?	0	?	?
?	?	?	?	?	?	?	?	?	?	0	2	?	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?	1	1	?	0	1	3	?	?	?	?	?	?	0	?	?	?
?	0	?	?	?	?	?	1	0	?	0	2	?	1	?	?	0	?	?	1	?	?	?	0	?	?	0	?	0	1	1	?	0	1	3	1	?	?	?	?	2	1	?	?	?
?	0	3	0	?	0	?	1	0	2	0	2	0	1	?	0	0	1	?	1	1	2	?	0	2	0	0	?	0	1	1	?	0	1	3	1	0	?	0	?	2	1	0	?	?
?	1	?	?	?	1	?	1	1	?	1	2	?	?	?	?	1	?	?	?	?	2	?	1	?	?	?	?	?	1	1	?	0	1	3	1	0	0	0	?	2	?	0	?	?
?	1	?	?	?	1	?	1	0	?	1	2	?	?	?	?	1	?	?	1	1	2	0	1	?	1	?	?	?	1	1	?	0	1	?	1	0	?	0	?	?	?	0	?	?
?	0	?	?	?	1	?	1	0	0	1	?	?	?	?	?	1	?	?	0	?	0	0	1	?	?	?	?	?	1	1	?	0	1	3	1	?	1	1	?	?	?	1	?	?
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?	0	?	?	?	?	?	1	0	0	0	2	?	1	?	?	0	?	1	0	?	?	?	?	?	0	?	?	?	1	1	?	0	1	1	1	0	0	0	?	2	0	0	?	?
?	0	?	?	?	?	?	0	?	0	?	2	?	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1	1	?	0	1	2	1	0	?	0	?	2	0	0	?	?
?	0	2	?	?	0	?	1	0	0	0	2	1	1	?	?	0	0	1	1	0	0	1	0	1	0	0	0	0	1	1	?	0	1	2	1	0	?	0	?	2	1	0	0	?
?	0	4	?	?	0	?	1	0	0	0	2	1	1	1	?	0	0	1	0	0	0	1	0	1	0	0	0	0	1	1	?	0	1	2	1	1	?	0	?	2	0	0	?	?
?	0	4	?	?	0	?	1	0	0	0	2	1	1	?	?	0	0	1	0	1	0	1	0	1	0	0	0	0	1	1	?	0	1	2	1	1	0	0	?	2	0	0	?	?
?	0	4	?	?	0	?	1	0	0	0	2	1	1	?	?	0	0	1	0	1	0	1	0	1	0	0	0	0	1	1	?	0	1	2	1	0	0	0	?	2	0	0	?	?
?	0	?	?	?	0	?	1	0	0	0	2	?	1	?	?	0	0	1	0	0	0	1	0	1	0	?	?	0	1	1	?	0	1	0	1	1	0	0	?	2	0	0	?	?
?	0	4	?	?	0	?	1	0	0	0	0	2	1	1	?	?	0	0	1	0	0	0	1	0	1	0	0	0	0	1	1	?	0	1	0	1	0	0	0	?	2	0	0	?	?
?	0	4	?	?	0	?	1	0	0	0	0	2	1	1	?	?	0	0	1	0	0	0	1	0	1	0	0	0	0	1	1	?	0	1	2	1	0	?	0	?	2	0	0	?	?
?	0	4	?	?	0	?	1	0	0	0	0	2	1	1	?	?	0	0	1	0	0	0	1	0	1	0	0	0	0	1	1	?	0	1	2	1	1	?	0	?	2	0	0	?	?
?	0	7	?	?	0	1	1	0	0	?	?	0	?	?	?	0	0	1	0	1	1	0	0	2	0	0	0	0	1	1	?	0	1	1	1	0	1	0	?	2	2	0	2	4
1	0	7	?	3	0	0	1	0	2	0	2	0	1	1	?	0	0	?	0	0	0	1	0	1	0	0	0	0	1	1	?	0	1	2	1	0	0	0	?	2	2	0	2	4
?	0	7	?	3	0	1	1	0	0	0	2	0	0	1	?	0	0	1	0	1	0	1	0	2	0	0	0	0	1	1	?	0	1	0	1	1	0	0	1	2	2	0	2	4
1	0	2	1	?	0	?	1	0	2	0	1	0	0	1	1	1	?	?	1	?	1	1	0	2	1	0	0	0	1	1	?	0	0	3	2	1	0	0	1	1	0	0	2	?

83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127
1	0	0	0	0	?	?	0	0	?	?	0	0	0	?	0	?	?	0	?	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	0	?	?	?	?	?	?	0	?
1	3	0	0	0	?	?	0	0	?	?	0	?	?	?	0	?	?	0	0	?	?	?	0	1	0	0	0	0	0	0	1	0	0	0	?	0	?	?	?	?	?	?	0	?
1	1	0	0	?	?	?	0	0	?	?	0	0	0	?	0	?	?	0	0	?	?	?	0	0	0	0	1	0	0	0	0	1	0	?	0	?	?	?	?	?	?	?	0	?
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?	?	1	?	?	?	?	?	?	?	?	1	?	?	?	?	?	?	1	?	1	1	1	1	1	1	1	1	1	1	0	1	1	?	0	?	1	?	?	?	?	?	?	0	?
?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1	?	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	?	0	?	?	?	?	?	?	0	?
?	?	0	1	?	?	?	0	0	?	?	0	1	0	?	0	?	?	1	?	1	1	1	1	1	1	1	1	1	0	0	1	0	1	0	?	0	?	?	?	?	?	?	0	?
?	0	0	0	1	1	0	0	0	0	1	0	1	0	0	0	?	?	1	?	?	?	?	?	?	?	?	?	?	0	?	0	0	1	0	?	0	?	?	?	?	?	?	0	?
?	?	0	0	?	?	?	0	0	?	?	0	1	0	?	1	?	?	1	?	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	?	0	?	?	?	?	?	?	0	?
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0	?	1	?	?	?	1	2	0	0	0	0	0	0	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0	?	1	?	?	?	1	1	0	0	0	0	?	0	0
?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1	?	1	1	1	1	1	1	1	1	1	1	0	1	1	?	0	?	1	1	0	0	0	0	?	0	0
?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0	?	?	?	?	?	?	?	?	?	?	0	0	1	1	?	?	?	1	1	0	0	0	0	?	0	1
?	0	0	?	?	?	2	0	0	?	0	0	?	?	?	?	?	?	1	?	1	1	1	1	1	1	1	1	1	?	0	?	1	?	0	?	1	2	0	0	0	0	0	0	1
?	?	0	2	2	0	?	0	1	0	?	0	?	?	0	?	?	?	1	?	1	?	1	1	?	1	1	1	1	?	0	1	1	?	1	0	1	0	0	0	0	?	?	0	3
?	2	0	2	2	0	0	0	1	?	?	0	?	?	?	0	?	?	0	2	?	?	?	1	?	1	1	1	1	?	0	?	1	?	1	0	1	0	?	?	?	?	?	0	3
?	?	0	2	2	0	?	0	1	?	1	1	?	?	?	?	?	?	1	?	?	?	?	1	?	1	1	1	1	1	0	1	1	?	1	1	1	2	0	0	0	?	?	0	3
?	?	0	2	2	0	?	0	1	?	1	1	?	?	?	?	?	?	1	?	?	?	?	1	?	1	1	1	1	1	0	1	1	?	0	?	1	2	0	0	0	?	?	0	3
?	?	0	2	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1	?	?	?	?	1	?	1	1	1	1	1	0	1	1	?	0	?	1	0	0	0	0	?	?	0	3
?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1	?	?	?	?	1	?	1	1	1	1	1	0	1	1	?	0	?	1	0	0	0	0	0	0	0	1
?	3	0	1	2	2	0	0	1	?	0	0	?	1	?	?	?	?	0	2	?	?	?	1	?	1	1	1	1	1	0	1	1	?	1	0	1	0	1	1	1	?	?	0	{1,2}
?	?	0	1	2	2	0	0	1	?	1	0	?	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0	?	1	?	1	2	1	0	1	1	1	?	?	0	2
?	3	0	1	2	2	?	0	1	?	1	0	?	?	?	?	?	?	0	2	?	?	?	?	?	?	?	?	?	1	0	1	1	?	1	3	1	0	1	1	1	?	?	0	3
?	?	0	1	1	2	0	0	1	0	0	0	?	1	0	?	?	?	0	3	0	0	0	0	1	1	1	1	1	1	0	1	0	2	1	4	1	2	1	1	1	1	1	0	3
?	?	0	1	?	2	?	0	1	?	?	0	?	?	?	?	?	?	0	0	?	?	?	1	?	1	1	1	1	?	0	?	1	?	1	3	1	0	1	1	1	?	?	0	2
?	?	0	?	?	2	?	?	?	?	?	?	?	?	?	?	?	?	1	?	?	?	?	1	?	1	1	1	1	1	0	1	1	?	?	?	1	0	0	0	0	?	?	0	1
?	?	0	1	2	2	?	?	1	?	?	?	?	?	?	?	?	?	1	?	?	?	?	?	?	?	?	?	?	1	?	1	1	?	1	0	1	0	?	?	?	?	?	?	1
?	?	0	1	2	2	0	0	1	?	?	0	?	?	?	?	?	?	0	3	?	?	?	1	?	1	1	1	1	?	0	?	1	?	1	0	1	0	0	0	3	?	?	0	1
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1	?	0	?	1	3	1	1	1	1	1	1	1
?	?	1	?	?	?	?	1	?	?	?	1	?	?	?	?	?	?	1	?	1	1	1	1	1	1	1	1	1	1	1	1	1	?	0	?	1	0	1	1	1	?	?	0	?
?	?	0	2	2	3	?	1	1	?	?	1	?	?	?	?	?	?	1	?	?	?	?	?	?	?	?	?	?	1	1	1	1	?	1	3	1	2	0	0	0	0	0	0	0
?	?	0	2	?	3	0	0	?	?	?	0	?	?	?	?	0	0	1	?	?	?	?	?	?	?	?	?	?	0	1	1	1	?	1	2	1	2	0	0	0	?	?	0	1
?	?	0	2	?	3	0	0	0	0	?	?	?	?	?	?	1	1	1	?	?	?	?	?	?	?	?	?	?	0	1	1	1	?	1	2	1	2	0	0	0	?	?	0	1
?	?	0	2	?	3	2	0	0	?	?	?	?	?	?	?	0	0	1	?	?	?	?	?	?	?	?	?	?	0	1	1	1	?	1	0	1	2	0	0	0	?	?	1	1
?	?	0	2	2	3	0	0	0	?	?	0	?	?	?	?	2	?	?	?	?	1	1	1	1	1	1	1	1	0	1	0	1	?	1	2	1	2	0	0	0	?	?	0	1
?	?	0	2	?	3	0	0	0	?	?	0	?	?	?	?	0	1	1	?	?	?	?	?	?	?	?	?	?	0	1	1	1	?	1	0	1	2	0	0	0	?	?	0	1
?	?	0	2	?	3	1	0	0	?	?	0	?	?	0	?	?	?	0	1	?	?	?	?	?	?	?	?	?	?	1	1	1	?	1	1	1	2	0	0	0	?	?	0	1
?	?	0	2	?	3	0	0	0	?	?	0	?	?	0	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?	0	1	1	?	1	0	1	2	0	0	0	?	?	0	1
?	?	0	2	?	3	1	0	0	?	?	0	?	?	?	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?	0	1	1	?	1	0	1	2	0	0	0	?	?	0	1
?	?	0	2	?	3	1	0	0	1	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1	?	1	?	0	?	1	2	0	0	0	?	?	0	1
?	?	0	2	?	3	0	0	0	?	?	0	?	?	?	?	1	?	0	?	?	?	?	?	?	?	?	?	?	?	1	1	1	?	0	?	1	2	0	0	0	?	?	0	1
?	?	0	2	?	3	?	0	?	?	?	?	?	?	?	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?	1	1	1	?	1	0	1	2	0	0	0	?	?	0	1
?	?	0	1	2	3	1	0	0	1	0	0	2	1	1	?	?	?	1	?	?	?	?	?	?	?	?	?	?	?	1	1	1	1	?	1	2	1	2	0	0	0	?	?	0	1
2	?	0	1	2	3	1	0	0	1	1	0	?	1	?	1	?	?	1	?	?	1	1	1	1	1	1	1	1	1	1	1	1	?	1	0	1	2	0	0	0	?	?	0	1
2	?	0	1	2	3	0	0	0	0	1	0	2	1	1	1	?	?	0	2	1	1	1	1	1	1	1	1	1	1	?	1	1	?	0	0	1	2	0	0	0	?	?	0	1
?	?	0	0	1	0	0	0	0	?	1	0	2	1	1	1	?	?	0	3	0	1	1	1	1	1	1	1	1	1	1	1	1	?	0	?	1	2	0	0	0	?	?	0	3

129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173
0	0	0	0	0	?	0	0	0	?	?	0	0	1	?	0	2	?	?	0	?	?	1	3	0	0	0	0	0	0	1	0	0	?	1	1	0	1	1	1	0	1	0	0	1
0	0	2	0	1	?	0	0	0	?	?	0	0	3	?	?	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	0	2	0	1	?	0	0	1	?	?	0	1	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	1	?	1	0	0	0	0	0	?	?	0	1	5	2	3	1	3	2	{1,2}	1	0	1	?	0	5	0	0	1	0	3	0	0	0	0	2	0	0	0	1	0	2	1	1	1
0	1	?	1	0	0	0	0	0	?	?	0	1	6	?	3	1	3	2	{1,2}	1	0	3	3	0	3	0	0	3	0	3	1	1	0	0	2	0	0	0	1	0	2	1	1	1
0	0	1	1	0	?	0	0	2	?	?	0	?	1	?	3	1	?	?	0	0	?	1	3	0	2	0	0	0	0	1	0	0	?	1	1	0	0	1	1	0	?	?	0	1
0	0	2	0	0	0	0	0	2	?	?	0	?	1	?	3	0	?	0	0	?	?	2	3	0	4	0	0	3	0	2	0	1	?	1	?	0	0	1	1	?	?	?	?	?
0	0	1	0	0	0	0	0	2	?	?	0	0	2	?	?	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	0	1	0	0	0	0	0	2	?	2	0	0	?	?	?	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	1	?	0	0	0	0	0	1	?	?	1	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	1	?	0	0	0	0	0	1	?	?	1	?	?	?	2	2	1	?	?	0	2	?	0	?	0	0	?	0	?	1	?	1	?	0	0	1	?	?	?	?	?	?	?	?	?
0	?	?	0	0	0	0	0	1	?	?	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	?	?	0	0	0	0	0	1	?	?	1	0	?	?	3	1	2	?	1	?	0	?	?	?	?	?	?	?	?	?	?	?	?	0	?	?	?	?	?	1	0	?	?	0	?
0	1	?	0	0	0	0	0	1	?	?	0	0	1	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	0	3	1	1	1	2	1	2	0	?	1	1	0	2	3	1	0	2	2	1	?	6	?	0	?	1	1	?	1	?	1	1	?	?	?	?	1	?	0	0	0	0	?	?
0	1	?	1	1	1	2	1	2	0	?	1	1	1	?	3	0	1	2	2	0	?	6	?	0	?	1	1	?	1	?	1	1	1	?	?	?	1	?	0	0	0	0	0	1
0	0	3	1	1	1	2	1	2	1	0	1	2	1	0	3	0	3	1	1	0	0	6	?	0	1	1	1	?	1	?	1	1	0	1	2	0	1	1	0	0	0	0	1	1
0	0	3	1	1	1	2	1	2	1	0	1	2	1	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	?	?	1	1	1	2	1	2	0	?	?	?	4	2	?	1	?	1	?	0	?	6	?	0	?	1	1	?	1	?	1	1	?	1	?	0	?	1	?	?	?	?	?	?	?
0	0	3	1	1	1	2	0	2	0	2	1	?	?	?	3	1	?	?	1	0	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	0	2	1	1	0	0	0	2	?	3	0	1	?	?	?	2	1	2	1	?	?	4	?	0	?	0	0	?	1	?	1	1	?	1	?	?	0	?	0	0	0	?	0	1
0	0	2	1	1	0	0	0	2	?	2	0	?	?	?	3	0	2	2	2	?	?	3	?	0	?	0	1	?	1	?	1	1	?	?	?	?	0	?	0	0	0	?	0	1
0	0	2	1	1	0	0	0	2	?	4	0	0	?	?	3	1	2	2	2	?	?	4	0	0	?	0	0	?	1	?	1	1	?	?	?	?	0	?	0	?	0	?	?	1
0	0	2	1	1	0	0	0	2	?	6	0	1	2	{1,2}	3	2	3	?	1	0	1	4	?	0	6	0	0	3	1	?	1	1	1	1	2	0	0	1	0	0	0	0	1	2
0	0	2	1	1	0	0	0	2	?	5	1	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
?	?	?	1	1	{0,1}	1	0	2	?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
?	0	3	1	1	{0,1}	1	0	2	?	5	0	?	5	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	1	?	1	1	{0,1}	1	0	2	?	{1,3}	0	1	6	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	0	3	1	1	0	0	0	2	?	7	0	1	?	?	3	0	0	0	?	0	1	7	1	1	?	1	1	?	1	?	1	1	0	2	?	1	?	1	?	1	?	1	1	?
0	1	?	0	1	0	0	0	2	?	?	0	{0,1}	?	?	3	0	0	?	?	0	1	5	0	0	?	0	1	?	1	?	1	1	0	2	?	1	?	1	0	1	0	?	1	?
0	0	3	1	1	0	0	0	2	?	5	0	1	7	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
1	0	2	1	1	0	0	?	?	?	?	?	?	?	?	?	?	2	?	?	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
1	0	2	1	1	0	0	?	?	?	?	?	?	?	?	?	?	2	?	?	1	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
1	0	2	1	1	0	0	0	2	?	?	?	?	?	?	?	3	1	2	?	1	1	?	7	?	1	?	1	1	?	1	?	1	1	?	0	?	0	1	0	0	?	0	?	?	?
1	0	2	1	1	0	0	0	2	?	?	?	0	2	?	2	1	2	0	1	1	1	6	2	0	1	1	1	?	1	?	1	1	?	1	1	0	1	1	0	0	0	?	0	1
1	0	2	1	1	0	0	?	?	?	?	?	0	2	?	2	1	2	?	?	1	?	6	?	0	?	1	1	?	1	?	1	1	?	1	?	0	1	1	0	0	0	?	0	1
1	0	2	1	1	0	0	0	2	?	?	0	?	?	?	3	1	2	?	1	1	?	6	?	0	?	1	1	?	1	?	1	1	?	1	?	0	1	1	0	0	0	?	0	0
1	0	2	1	1	0	0	1	2	0	{0,2}	0	?	?	?	2	2	2	?	1	1	?	6	?	0	?	1	1	?	1	?	1	1	?	1	?	0	1	1	0	0	0	?	0	0
1	0	2	1	1	0	0	1	2	0	0	0	?	?	?	2	1	2	?	1	1	?	6	?	0	?	1	1	?	1	?	?	?	?	?	?	0	1	1	0	0	0	?	0	0
1	0	2	1	1	0	0	1	2	1	0	0	?	?	?	1	1	2	?	1	1	?	6	?	0	?	1	1	?	1	?	1	1	?	?	?	0	1	1	0	0	0	?	0	0
1	0	2	1	1	0	0	0	2	0	?	0	?	?	?	3	1	2	?	1	1	?	6	?	0	?	1	1	?	1	?	1	1	?	1	?	0	1	1	0	0	0	?	0	0
1	0	2	1	1	0	0	0	2	?	?	0	?	?	?	3	0	2	?	1	1	?	?	?	?	?	?	?	?	?	?	?	?	?	1	?	0	1	1	0	0	0	?	0	0
1	0	3	1	1	0	0	0	2	?	0	0	?	?	?	3	1	2	?	1	1	1	6	2	0	3	1	1	?	1	?	1	1	0	1	1	0	1	1	0	?	?	?	?	0
1	0	2	1	1	0	0	0	2	?	?	0	2	?	?	3	1	2	?	1	1	1	6	?	0	4	1	1	?	1	?	1	1	0	1	0	0	1	1	0	0	0	0	0	0
1	0	3	1	1	0	0	0	2	?	0	0	0	4	2	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
0	0	2	1	1	0	0	1	2	1	?	1	2	1	2	3	0	3	1	1	1	1	5	?	0	?	0	1	?	1	?	1	1	0	1	?	0	1	1	0	0	0	0	1	1

175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199
?	1	?	?	0	0	0	0	0	?	?	?	?	?	?	0	0	0	?	0	?	?	?	0	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
0	0	1	0	1	0	0	0	0	?	?	?	?	?	?	1	1	1	1	4	0	1	4	0	0
0	0	3	0	1	0	0	0	0	?	?	?	?	?	?	1	1	1	1	4	0	1	5	0	0
?	0	0	0	1	0	0	0	0	?	?	?	?	?	?	1	0	0	0	1	?	?	3	0	0
?	?	?	?	0	0	0	0	0	0	?	?	0	?	1	0	1	0	?	1	?	?	?	0	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
?	1	?	?	1	?	?	?	?	?	?	?	?	?	?	?	?	?	1	?	?	?	?	?	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
?	1	?	?	0	0	?	?	?	0	?	?	?	?	?	1	1	1	0	4	?	?	?	0	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
?	0	5	0	?	1	1	1	1	0	?	?	0	?	?	0	1	1	?	3	2	2	5	0	1
1	0	5	0	?	1	1	1	1	0	0	0	0	0	0	0	?	?	?	3	2	0	0	0	1
0	1	?	1	0	1	?	?	?	0	0	0	0	0	0	0	1	1	0	2	0	0	2	0	1
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1
?	?	5	?	1	1	1	1	1	?	0	?	?	?	?	?	?	?	0	3	2	?	?	?	1
?	?	?	?	0	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1
0	1	5	?	0	1	1	1	1	?	?	2	0	?	?	0	1	1	1	4	1	3	5	1	0
0	1	5	?	0	1	1	1	1	?	1	?	1	1	?	?	0	?	?	4	1	3	4	1	0
0	1	?	?	0	1	1	1	1	?	1	?	1	?	?	0	?	?	?	4	1	3	?	1	0
1	0	2	0	1	1	1	1	1	1	1	2	0	1	1	0	1	1	1	1	0	1	2	1	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1	?	?	?	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
2	0	3	1	0	1	1	1	1	0	0	1	0	?	?	0	1	1	1	?	?	1	?	?	0
?	0	3	1	0	1	1	1	1	0	?	1	0	?	?	1	1	1	?	2	?	1	?	?	0
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	0
?	1	5	?	0	1	?	?	?	?	0	?	?	?	0	0	0	0	?	?	?	?	?	?	2
?	1	5	?	0	1	?	?	?	?	0	?	?	?	0	0	0	0	?	?	?	?	?	?	2
?	1	5	?	0	0	?	?	?	?	0	?	?	?	0	0	0	0	?	3	?	?	?	?	2
?	1	5	0	0	0	0	?	?	0	0	?	1	?	0	1	0	0	?	3	?	?	?	1	2
0	1	6	?	1	1	?	?	?	?	0	?	?	?	0	0	0	0	?	3	?	?	5	1	2
?	1	5	?	0	1	?	?	?	0	0	?	?	?	0	0	0	0	?	3	?	?	?	1	2
?	1	5	?	0	1	?	?	?	0	0	?	0	0	0	0	0	0	?	3	?	?	?	1	2
?	0	5	0	0	1	?	?	?	0	0	?	0	0	0	0	0	0	?	3	?	?	?	1	2
?	0	5	0	0	1	?	?	?	0	0	?	0	0	0	0	0	0	?	3	?	?	?	1	2
?	1	5	?	0	1	?	?	?	0	0	?	?	?	0	0	0	0	?	3	?	?	?	1	2
?	1	5	?	0	1	?	?	?	0	0	?	?	?	0	0	0	0	?	3	?	?	?	1	2
0	1	5	1	0	1	?	?	?	1	0	?	?	?	?	1	1	1	1	3	0	0	?	1	2
0	1	?	1	0	1	?	?	?	1	1	?	?	?	?	1	1	1	1	3	0	0	4	1	2
?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	1	2
0	1	?	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1	1	3	?	1	1	1	2

Supplementary material 3: Molecular data generated within this study

Endoparasites

DNA voucher ID	Parasite Species	ZSM-ID (Parasite)	Host species	ZSM-ID (host)	Collection date	Location	Latitude	Longitude	Depth	16S	CO I	18S	28S
G001	*Splanchnotrophus angulatus*	ZSMA20142906	*Flabellina ischitana*	ZSM-Mol10100477	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	Seq	Seq	PCR	Seq
G002	*Splanchnotrophus angulatus*	ZSMA20142907	*Spurilla neapolitana*	ZSM-Mol20100409	2009	Mala Portic/Croatia	44°46'45.15"N	13°55'10,84"O	0-5m	Seq	Seq	PCR	
G003	*Splanchnotrophus angulatus*	ZSMA20142908	*Spurilla neapolitana*	ZSM-Mol20100409	2009	Mala Portic/Croatia	44°46'45.15"N	13°55'10,84"O	0-5m	Seq	Seq	Seq	Seq
G004	*Splanchnotrophus angulatus*	ZSMA20142909	*Cratena peregrina*	ZSM-Mol20130874	1998	Islote				PCR	Seq	PCR	Seq
G005	*Splanchnotrophus angulatus*	ZSMA20142910	*Aeolidiella alderi*	ZSM-Mol20070272						Seq	Seq		Seq
G006	*Ismaila robusta*	inside host	*Phidiana lottini*	ZSM-Mol20110432	2011	Playa Chica/Chile	39°43'10"S	73°24'12"W	0-2m	PCR	Seq	PCR	PCR
G011	*Splanchnotrophus angulatus*	ZSMA20142912	*Cratena peregrina*	host lost	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	Seq	Seq	PCR	
G012	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130849	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	Seq	Seq	PCR	PCR
G013	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130850	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G015	*Ismaila genalis*	ZSMA20142903	*Holoplocamus papposus*	ZSM-Mol20130872	2007	Isla Carmen/Huinay/Chile	43°01'08.80"S	72°49'44.79"W	0-10m	Seq	Seq	PCR	PCR
G016	*Ismaila belciki*	ZSMA20142916	*Janolus fuscus*	host lost	Aug-10	Oregon/USA				Seq	Seq		Seq
G017	*Ismaila volatilis*	ZSMA20142900	*Janolus sp.*	ZSM-Mol20130847	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G018	*Ismaila volatilis*	ZSMA20142951	*Janolus sp.*	ZSM-Mol20130847	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G019	*Ismaila aliena*	ZSMA20142918	*Thecacera darwini*	ZSM-Mol20130851	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G020	*Ismaila aliena*	ZSMA20142919	*Thecacera darwini*	ZSM-Mol20130851	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G021	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130852	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G022	*Ismaila robusta*	ZSMA20142921	*Phidiana lottini*	host lost	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G023	*Ismaila robusta*	ZSMA20142921	*Phidiana lottini*	host lost	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	
G024	*Ismaila robusta*	ZSMA20142923	*Phidiana lottini*	host lost	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	
G025	*Splanchnotrophus angulatus*	inside host	*Spurilla neapolitana*	ZSM-Mol20110684	2011	Bastione Conca/Italy	38°01'03"N	12°30'14"E	2-5m	Seq	Seq	PCR	PCR
G026	*Ismaila volatilis*	inside host	*Janolus sp.*	ZSM-Mol20070580	2010					PCR	PCR		PCR
G027	*Ismaila volatilis*	inside host	*Janolus sp.*	ZSM-Mol20070580	2010								PCR
G028	*Ismaila robusta*	ZSMA20142925	*Phidiana lottini*	ZSM-Mol20130855	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G029	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130856	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G030	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130856	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G031	*Ismaila aliena*	inside host	*Thecacera darwini*	ZSM-Mol20130857	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G032	*Ismaila chaihuiensis*	ZSMA20142902	*Diaulula punctuolata*	ZSM-Mol20130858	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G033	*Ismaila chaihuiensis*	ZSMA20142902	*Diaulula punctuolata*	ZSM-Mol20130858	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G034	*Ismaila damnosa*	ZSMA20142905	*Flabellina sp.1*	host lost	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G035	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130860	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	Seq	Seq	PCR	PCR
G036	*Splanchnotrophus angulatus*	ZSMA20142930	*Cratena peregrina*	host lost	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	Seq	Seq	PCR	
G037	*Splanchnotrophus angulatus*	ZSMA20142950	*Facelina fusca*	ZSM-Mol20130875	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	Seq		PCR	Seq
G038	*Lomanoticola sp.*	ZSMA20142931	*Cuthona cerulea*	ZSM-Mol20130862	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	PCR	Seq		Seq
G042	*Pionodesmotes domhainfharraigeanus*	ZSMA20130004	*Sperosoma grimaldii*	host lost	2011	Irish Sea/Ireland	48.491°N	10.692°W	2000m	Seq	Seq	PCR	Seq
G044	*Ceratosomicola mammillata*	inside host	*Chromodoris geometrica*	ZSM-Mol20130863	2008	Lembeh strait, „nudi retreat“	5°28'29"S	123°45'40"E	4m	PCR	Seq		Seq
G045	*Briarella doliaris*	ZSMA20142945	*Ceratosoma trilobatum*	host lost	1999	Stradbroke Island/ Australia	27°23.967 S	153°26.234 E		PCR			Seq
G046	*Splanchnotrophus gracilis*	ZSMA20142933	*Trapania tartanella*	host lost	2011	Ria de Ferrol/Spain	43°28'02.16"N	8°14'47.70"W	20m	Seq	Seq		Seq
G055	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130864	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	Seq	Seq	PCR	PCR
G056	*Splanchnotrophus angulatus*	ZSMA20142935	*Cratena peregrina*	ZSM-Mol20130865	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	Seq	Seq	PCR	PCR
G057	*Ismaila robusta*	ZSMA20142936	*Phidiana lottini*	host lost	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G058	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130867	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m		Seq		
G059	*Ismaila robusta*	ZSMA20142938	*Phidiana lottini*	ZSM-Mol20130868	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G060	*Ismaila robusta*	inside host	*Phidiana lottini*	ZSM-Mol20130869	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	Seq	Seq	PCR	PCR
G061	*Splanchnotrophus angulatus*	inside host	*Cratena peregrina*	ZSM-Mol20130873	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m				
G062	*Splanchnotrophus angulatus*	ZSMA20142948	*Cratena peregrina*	host lost	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m				
G063	*Splanchnotrophus angulatus*	ZSMA20142949	*Cratena peregrina*	host lost	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m				
G033B	*Ismaila chaihuiensis*	ZSMA20142902	*Diaulula punctuolata*	ZSM-Mol20130858	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-5m	Seq	Seq		PCR
G015B	*Ismaila genalis*	ZSMA20142903	*Holoplocamus papposus*	ZSM-Mol20130872	2007	Huinay/Chile	42°21'25.49"S	72°26'29.20"W	0-10m	Seq	Seq		PCR
G073	*Ismaila belciki*	ZSMA20142916	*Janolus fuscus*	host lost	Aug-10	Oregon/USA				PCR		PCR	PCR

G074	*Ismaila belciki*	ZSMA20142916	*Janolus fuscus*	host lost	Aug-10	Oregon/USA				PCR		PCR	PCR
G075	*Ismaila belciki*	ZSMA20142916	*Janolus fuscus*	host lost	Aug-10	Oregon/USA				PCR		PCR	PCR
G076	*Ismaila belciki*	ZSMA20142916	*Janolus fuscus*	host lost	Aug-10	Oregon/USA				PCR	PCR	PCR	PCR
G077	*Ismaila belciki*	ZSMA20142916	*Janolus fuscus*	host lost	Aug-10	Oregon/USA				PCR		PCR	PCR
G078	*Splanchnotrophus angulatus*	ZSMA20142942	*Spurilla neapolitana*	host lost	2007	Rovinj/Croatia	45°07'5.51"N	13°36'51.05"E	0-5m			PCR	PCR
G079	*Arthurius gibbosa*	ZSMA20142905	*Elysia macanei*	ZSM-Mol20034046	2003	Lembeh strait, „nudi retreat“	5°28'29"S	123°45'40"E	1-15m			PCR	PCR
G082	*Ismaila volatilis*	inside host	*Janolus sp.*	ZSM-Mol20130866	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m	PCR	Seq		PCR
G083	*Splanchnotrophus gracilis*	ZSMA20142943	*Trapania tartanella*	kein wirt	2011	Ria de Ferrol/Spain	43°28'02.16"N	8°14'47.70"W	20m	PCR		PCR	
G084	*Splanchnotrophus gracilis*	ZSMA20142944	*Trapania tartanella*	kein wirt	2011	Ria de Ferrol/Spain	43°28'02.16"N	8°14'47.70"W	20m				PCR
G085	*Ismaila damnosa*	Reg.Nr. 20010018	*Flabellina sp.*	kein wirt	1994	Bahia de Coliumo/Chile	36°42'08.90"S	73°02'49.04"W	0-20m				
G086	*Ismaila damnosa*	Reg.Nr. 20010018	*Flabellina sp.*	kein wirt	1994	Bahia de Coliumo/Chile	36°42'08.90"S	73°02'49.04"W	0-20m				
G087	*Ismaila damnosa*	Reg.Nr. 20010018	*Flabellina sp.*	kein wirt	1994	Bahia de Coliumo/Chile	36°42'08.90"S	73°02'49.04"W	0-20m				
G088	*Ismaila damnosa*	Reg.Nr. 20010018	*Flabellina sp.*	kein wirt	1994	Bahia de Coliumo/Chile	36°42'08.90"S	73°02'49.04"W	0-20m				
G089	*Ismaila androphila*	Reg.Nr. 20010014	*Okenia luna*	kein wirt	1994	Bahia de Coliumo/Chile	36°42'08.90"S	73°02'49.04"W	0-20m				
G090	*Ismaila androphila*	Reg.Nr. 20010014	*Okenia luna*	kein wirt	1994	Bahia de Coliumo/Chile	36°42'08.90"S	73°02'49.04"W	0-20m				
G091	*Ismaila androphila*	Reg.Nr. 20010014	*Okenia luna*	kein wirt	1994	Bahia de Coliumo/Chile	36°42'08.90"S	73°02'49.04"W	0-20m				
G092	*Ismaila androphila*	Reg.Nr. 20010014	*Okenia luna*	kein wirt	1994	Bahia de Coliumo/Chile	36°42'08.90"S	73°02'49.04"W	0-20m				
G094	*Splanchnotrophus angulatus*	inside host	*Spurilla neapolitana*	ZSM-Mol 20130067	2010	Banyuls (Mole)/ France	42°28'56.12"N	3°08'13.71"E	1-5m	PCR		PCR	PCR
G095	*Ismaila robusta*	inside host	*Phidiana lottini*	ZSM-Mol20110432	2011	Playa Chica/Chile	39°43'10"S	73°24'12"W	0-2m	PCR	Seq	PCR	Seq
G096	*Ismaila volatilis*	inside host	*Janolus sp.*	ZSM-Mol20130870	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-5m	PCR			
G097	*Ismaila volatilis*	inside host	*Janolus sp.*	ZSM-Mol20130870	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-5m			PCR	
G098	*Ismaila volatilis*	inside host	*Janolus sp.*	ZSM-Mol20130870	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-5m	PCR	Seq	PCR	PCR
G100	*Ismaila sp.*	inside host	*cf. Eubranchus sp.2*	ZSM-Mol20130871	2003	Isla Traiguen/Chile	45°11'26.11"S	73°30'49.69"W	6,2m		PCR		
G102	*Ismaila sp.2*	inside host	*Zephyrinidae n.sp.x*	ZSM-Mol20130851	2013	Muro Roberto			20m				
Ectoparasites													
G 39	*Ektoparasit von Hypselodoris*	ZSMA20142947	*Hypselodoris tricolor*	ZSM-Mol20130876	2010	Banyuls le Troc/ France	42°28'56.20"N	3°08'13.19"O	0-5m	Seq		PCR	
G 40	*Ektoparasit von Tritonia*	ZSMA20142947	*Tritonia odhneri*	ZSM-Mol20070576		Huinay/Chile	42°21'25.49"S	72°26'29.20"W	0-5m	PCR		PCR	Seq
G 41B	*Ekto von Berthella platei*	ZSMA20142947	*Berthella platei*	host lost	2009	Huinay/Chile	42°21'25.49"S	72°26'29.20"W	0-5m			PCR	Seq
G 49	*Ectoparasit (Nr. 86)*	ZSMA20142952	*Pleurobranchus aerulatus*			Mancora/Peru	4°06'9.43"S	81°03'24.77"W				PCR	Seq
Hosts													
G047			*Diaulula punctuolata*	ZSM-Mol20130858	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-5m	PCR	Seq		
G 48			*Janolus sp.*	ZSM-Mol20130847	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-5m	PCR	Seq		
G 50			*Flabellina sp.1*	ZSM-Mol20130877	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-5m	PCR	Seq		
G 51			*Diaulula punctuolata*	ZSM-Mol20130878	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-5m	PCR	Seq		
G81			*Diaulula punctuolata*	ZSM-Mol20130879	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-10m		Seq		
G99			*Gargamella immaculata*	ZSM-Mol20130880	2010	Chaihuin/Valdivia/Chile	39°57'25.94"S	73°36'10.15"W	0-5m		PCR		
G101			*cf. Eubranchus sp.2*	ZSM-Mol20130871	2003	Isla Traiguen/Chile	45°11'26.11"S	73°30'49.69"W	6,2m		PCR		
G103			*Zephyrinidae n.sp.x*	ZSM-Mol20130851	2013	Muro Roberto			20m				

15 Appendix

Relevant Poster

Anton R. F. & Schrödl M. (2013), The enemy inside your sea slug - endoparasites of the family Splanchnotrophidae (Copepoda); World congress of Malacology, Ponta Delgada

The enemy within your sea slug - endoparasites of the family Splanchnotrophidae (Copepoda)

staatliche naturwissenschaftliche sammlungen bayerns

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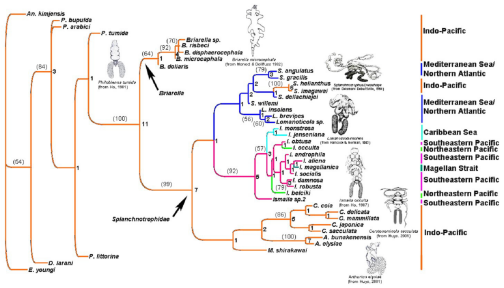
Splanchnotrophidae are a family of locally abundant marine endoparasites exclusively infesting the body cavity of nudibranch or sacoglossan sea slugs. In contrast to the considerable interest and advances in sea slug research, little was known on biological interactions with parasites and potential coevolution. Updating morphology-based phylogenetic analyses by Anton & Schrödl (2013), here we present new results on:

Infested hosts and the respective parasites (egg sacs marked by arrows)

1) Phylogeny and distribution of known splanchnotrophids



Strict consensus tree of the morphology-based maximum parsimony analysis of 182 characters (bootstrap support >50 is given in brackets; Bremer decay index is given in bold numbers). Geographic distributions and ancestral areas (RASP, BBMCMC analysis) are color-coded.

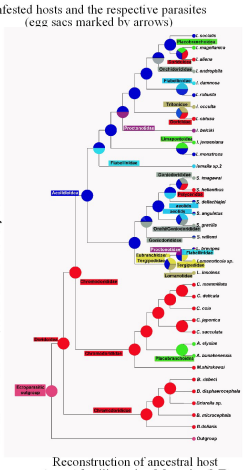
Splanchnotrophidae and *Briarella* are sister taxa; the switch towards copepod endoparasitism occurred just once.

Ancestral area reconstruction suggests an origin of the family in the tropical Indo-Pacific, with subsequent colonisation of temperate areas of Europe and the Americas, and later migration to the Caribbean and back to the Indo-Pacific.

2) Ancestral hosts

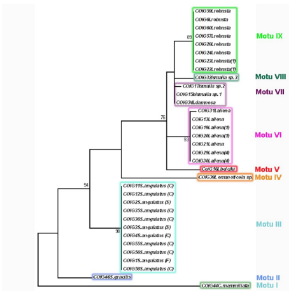
According to our ancestral host reconstruction, members of Chromodorididae (doridoidean nudibranchs; red color code) were the first hosts for endoparasitic copepods. Ancestral, temperate *Splanchnotrophus*/ *Ismaila* invaded aeolid hosts. Descendents diversified to various sea slug hosts.

Invasion of sacoglossan hosts (green color code) occurred at least three times independently.



Reconstruction of ancestral host (super)families using Mesquite (MP character state analysis); hosts are color-coded and states plotted on the MP tree.

3) Molecular systematics and species delimitation

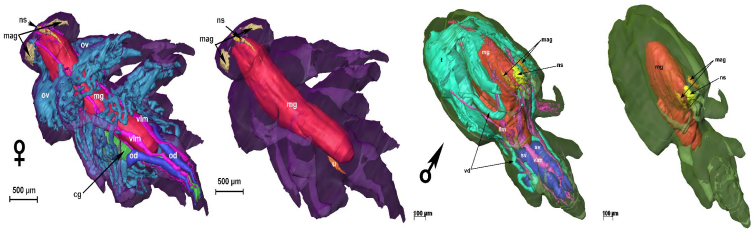


Maximum likelihood COI tree (bootstrap values). Colored boxes refer to results of the ABGD species delimitation analysis (Motu: **M**olecular **o**perational **t**axonomic **u**nit).

Most COI-Motu's correspond to morphology-based species, confirming general host specifity of *Ismaila* species. However, Motu VII contains three morphologically clearly distinct *Ismaila* specimens from three different nudibranch host species (all southern Chile); we assume recent speciation via host switch and ongoing genetic diversification.

Preliminary COI and morphology-based trees are partly compatible, but *Splanchnotrophus* and the combined clade with *Lomanoticola* are not recovered with limited available data (yet?).

4) First 3D-microanatomical data on sea slug parasites



3D reconstruction of *Ismaila aliena*. **cg**: cement gland, **llm**: lateral longitudinal muscles, **mag**: maxillary gland, **mg**: midgut, **ns**: nervous system, **od**: oviduct, **ov**: ovary, **sv**: seminal vesicle, **t**: testes, **u**: unknown structure, **vd**: vas deferrence, **vlm**:ventral longitudinal muscles.

The digestive system of *Ismaila aliena* is sac-like without anus in both sexes. The incomplete nature of the gut supports our hypothesis that haemolymph rather than host tissue serves as primary food source.

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