

Phylogenetic assessment and systematic revision of the acoel family Isodiametridae

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Isodiametridae is a large family of Acoela with 22 nominal genera and nearly 100 species. Unfortunately, systematics of Isodiametridae, as it stands, is highly problematic. Genera frequently have been proposed without reference to an explicit phylogenetic hypothesis, such that the current classification system holds little or no predictive power. Many taxa do not fit with the family diagnosis, and it is increasingly difficult to determine in which taxon a new species should be described. Herein, we reconstruct the phylogenetic relationships of Acoela with a focus on Isodiametridae using both previously published and new ribosomal and mitochondrial sequence data. Our dataset comprises sequences from 45 species representing 16 of the 22 isodiametrid genera. Our results recovered a well-supported Isodiametridae, but provided further evidence that the family and several genera within require revision. We have updated the classification system of Isodiametridae to be consistent with its phylogeny, including the transference of *Otocelis* to Otocelididae, *Postaphanostoma* and *Faerlea* to Mecynostomidae and *Alluna* to Actinoposthiidae. Six other genera are placed in synonymy. We review the morphological taxonomy and provide an identification key of the genera in the revised family.

ADDITIONAL KEYWORDS: Acoela – identification key – morphology – new taxa – phylogeny – taxonomy.

INTRODUCTION

Acoela comprises approximately 450 nominal species of marine worms found primarily in sediments or among algae all over the world. Through the 20th century, acoels were placed in Platyhelminthes (e.g. Ehlers, 1985), but more recent phylogenetic analyses of nucleotide sequences reject this position (e.g. Cannon *et al.*, 2016; Rouse *et al.*, 2016). Currently, Acoela together with Nemertodermatida and *Xenoturbella* Westblad, 1949 form the group Xenacoelomorpha, separate from Platyhelminthes. The phylogenetic position of Xenacoelomorpha is contentious, but the current mainstream view is that they form the sister-group of the Nephrozoa (Ruiz-Trillo *et al.*, 1999; Egger *et al.*, 2009; Cannon *et al.*, 2016; Rouse *et al.*, 2016), although they have also been considered closely related to ambulacrarians (Philippe *et al.*, 2011).

Acoels can be difficult to study since they are small (~0.2–5.0 mm), often fragile and have comparatively

few morphological characters that, nevertheless, can be extremely variable (Jondelius *et al.*, 2011). Early classification systems reflect the challenges in working with such animals, and numerous, sometimes conflicting, taxonomical changes were frequently proposed without reference to a phylogenetic hypothesis (e.g. Graff, 1912; Luther, 1912; Westblad, 1948; Faubel, 1974; Kostenko, 1989). Dörjes (1968) revised acoel classification based in large part on characters of the relatively complex hermaphroditic reproductive system and proposed a large number of new taxa at the generic, family and species levels. Modifications to Dörjes's system were based on the structure of the nervous system (e.g. Raikova *et al.*, 1998, 2004), sperm ultrastructure (Hendelberg, 1977; Raikova *et al.*, 2001), body wall musculature (Hooge, 2001; Tekle *et al.*, 2005) and on the musculature of the copulatory organs (Hooge & Tyler, 2005).

Analyses of nucleotide sequences have generated testable phylogenetic hypotheses that necessitated revisions of acoel classification (e.g. Hooge *et al.*, 2002; Raikova *et al.*, 2004; Hooge & Tyler, 2005). The hitherto largest study of acoel phylogeny combined data from two nuclear genes and one mitochondrial

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gene with 37 multistate morphological characters to assess the relationships and morphological evolution of 126 different acoel species, approximately one-third of the species known at the time, and to generate a classification consistent with the phylogenetic hypothesis (Jondelius *et al.*, 2011). The resulting classification system included a total of 21 families, with several (e.g. Convolutidae Graff, 1905; Hofsteniidae Bock, 1923; Paratomellidae Dörjes, 1966; and Solenofilomorphidae Dörjes, 1968) considered well-resolved and with clear morphological diagnoses, although the positions, interrelationships and morphology of others (e.g. Actinoposthiidae Hooge, 2001 and Otocelididae Westblad, 1948) were still ambiguous.

Isodiametridae Hooge & Tyler, 2005 was proposed by Hooge & Tyler (2005) to accommodate ‘small convolutids’: acoel species with 9 + 2 sperm axonemes and lacking symbiotic algae that were previously classified in Convolutidae. This was in response to findings by Hooge *et al.* (2002) and Petrov *et al.* (2004) that nine species of ‘large convolutids’ with 9 + 0 axonemes and symbiotic algae form a monophyletic group separate from another nine species of ‘small convolutids’ from three nominal genera in their analysis of 18S rDNA sequences. In addition, Hooge & Tyler (2005) studied the musculature of the male copulatory organ of 17 species of ‘small-bodied convolutids’ from seven nominal genera and determined that these specimens all possessed a seminal vesicle that encloses a tubular isodiametric penis with inner circular muscle fibres and outer non-anastomosing longitudinal fibres.

Hooge & Tyler (2005) decided to reclassify all ‘small convolutids’ in the new family Isodiametridae, including species that had not been included in the 18S rDNA gene tree or studied in detail with regards to the male copulatory organ. As a consequence, Isodiametridae is currently a large family comprising 22 nominal genera and nearly 100 species, but many taxa do not fit the family diagnosis provided by Hooge & Tyler (2005). For instance, a muscular seminal vesicle is absent from species of *Alluna* Faubel & Rieger, 1983, *Proconvoluta* Dörjes, 1968 and *Pseudoposthia* Westblad, 1946, while the male copulatory organ is a simple inpocketing of the epidermis in the genera *Avagina* Leiper, 1902 and *Proaphanostoma* Dörjes, 1972.

While results from the molecular analyses of Jondelius *et al.* (2011) corroborated the monophyly of Isodiametridae with high support, relationships within the family remained largely unresolved. Generic classification within Isodiametridae is problematic, because genera have been introduced without reference to an explicit phylogenetic hypothesis, often on the basis of a single morphological character (e.g. *Ancylocirrus* Kozloff, 2000; *Raphidophallus* Kozloff,

1965; *Rimicola* Bohmig, 1908), without actually assessing the prevalence of that character in other acoel species (e.g. Böhmig, 1908; Kozloff, 1965, 2000; Dörjes, 1968). The result is a confused classification system rife with monotypic genera, with little or no predictive power (Jondelius *et al.*, 2011) and with which it is difficult to determine into which taxon a new species should be referred.

Herein, we reconstruct the phylogenetic relationships of Isodiametridae and closely related groups within Acoela using both previously published and new ribosomal and mitochondrial sequence data. Our dataset comprises sequences from 45 species representing 16 of the 22 isodiametrid genera. We aim to provide a robust classificatory backbone for future work. Thus, our dataset did not include any unidentified or undescribed species. We update the classification of the family to be consistent with our phylogenetic hypothesis, review the morphological taxonomy of the genera and provide an identification key to these genera. Our goal is to clarify and simplify the taxonomy to facilitate future research into Isodiametridae diversity.

MATERIAL AND METHODS

New specimens were collected over a period of 21 years from a variety of marine sediments and aquatic vegetation (Supporting Information, Table S1). Specimens were collected through multiple methods, including by hand from the beach at low tide, via SCUBA or snorkelling or by dredging at lower depths. Samples were transported back to laboratories and processed according to type. Animals were extracted from marine sandy or mixed sediments following the anesthetization-decantation protocols of Martens (1984) and from mud or finer/silt sediments via the siphoning method detailed in Holovachov *et al.* (2017). Vegetation was simply washed through a fine mesh sieve.

Following extraction, animals were manually isolated and identified using a compound microscope equipped with DIC (differential interference contrast). When possible, images and digital video were captured and uploaded to the Acoela Scratchpad project (<https://acoela.myspecies.info/en>). Measurements were taken with an ocular micrometre. Following documentation, specimens were directly fixed in 96% ethanol, RNAsShield (Zymo Research) or Histochoice (Sigma-Aldrich) for later processing.

For histological sections, specimens fixed in Histochoice were washed in PBS, dehydrated in an ethanol series, embedded in paraffin and finally serially sectioned in 4–6 µm slices using an LKB

Bromma 2219 Historange microtome. Sections were stained with haematoxylin and eosin as counter stain following the protocols of [Aeschl *et al.* \(2010\)](#).

For molecular analyses, DNA was extracted from whole animals using the DNeasy Blood & Tissue kit (Qiagen) following the instructions of the manufacturer. Polymerase chain reaction (PCR) amplification was performed using 0.2 mL PuReTaq Ready-To-Go PCR beads (GE Healthcare) with 2 µL DNA and 5 pmol each of forward and reverse primers. Products were viewed on 1% agarose gel and then purified with ExoSAP-IT enzymes (Exonuclease and Shrimp Alkaline Phosphatase; GE Healthcare). Sequencing was performed commercially at Macrogen Europe (Netherlands).

The complete nuclear 18S rRNA and 28S rRNA genes and the ~600 bp 'Folmer' segment ([Folmer *et al.*, 1994](#)) of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene were selected as molecular markers for phylogenetic analyses following the protocols of [Jondelius *et al.* \(2011\)](#). These three gene regions are the most widely available molecular markers for acoels in GenBank and have been shown to be useful in resolving the phylogenetic relationships for Acoela ([Hooge *et al.*, 2002](#); [Jondelius & Raikova, 2002](#); [Hooge & Tyler, 2005](#); [Jondelius *et al.*, 2011](#)) and numerous other meiofaunal groups (e.g. [Kieneke & Nikoukar, 2017](#); [Zhao *et al.*, 2018](#); [Atherton & Jondelius, 2019](#)).

New DNA sequences were combined with the dataset of [Jondelius *et al.* \(2011\)](#) and acoel sequences available from GenBank. To ensure accuracy, sequences representing undescribed or uncertain species of acoels were excluded from the dataset. Further, analyses were run both including and excluding sequences from six species of Isodiametridae and Actinoposthiidae (Supporting Information, [Table S1](#)) that were represented only by 18S sequences downloaded from GenBank and that grouped together in a clade with a highly unstable position in the analyses of [Jondelius *et al.* \(2011\)](#). Overall, the final analyses included sequences from a total of 128 different acoel species, with 47 species from 16 of the 22 genera of Isodiametridae represented. [Table S1](#) in the Supporting Information lists the collection information and GenBank accession numbers of all specimens used in this study. Because *Diopisthiporidae* Westblad, 1940 is the sister-taxon to all other acoels ([Jondelius *et al.*, 2011](#)), sequences from two specimens, *Diopisthoporus longitubus* Westblad, 1940 and *Diopisthoporus psammophilus* [Dörjes, 1968](#), were defined as outgroups.

Sequences were aligned with Multiple Alignment using Fast Fourier Transformation (MAFFT; [Katoh & Toh, 2008](#)). *COI* sequences were translated to amino acids using the standard invertebrate mitochondrial genetic code, manually checked for stop codons and reading frame shifts, aligned and reverted back to

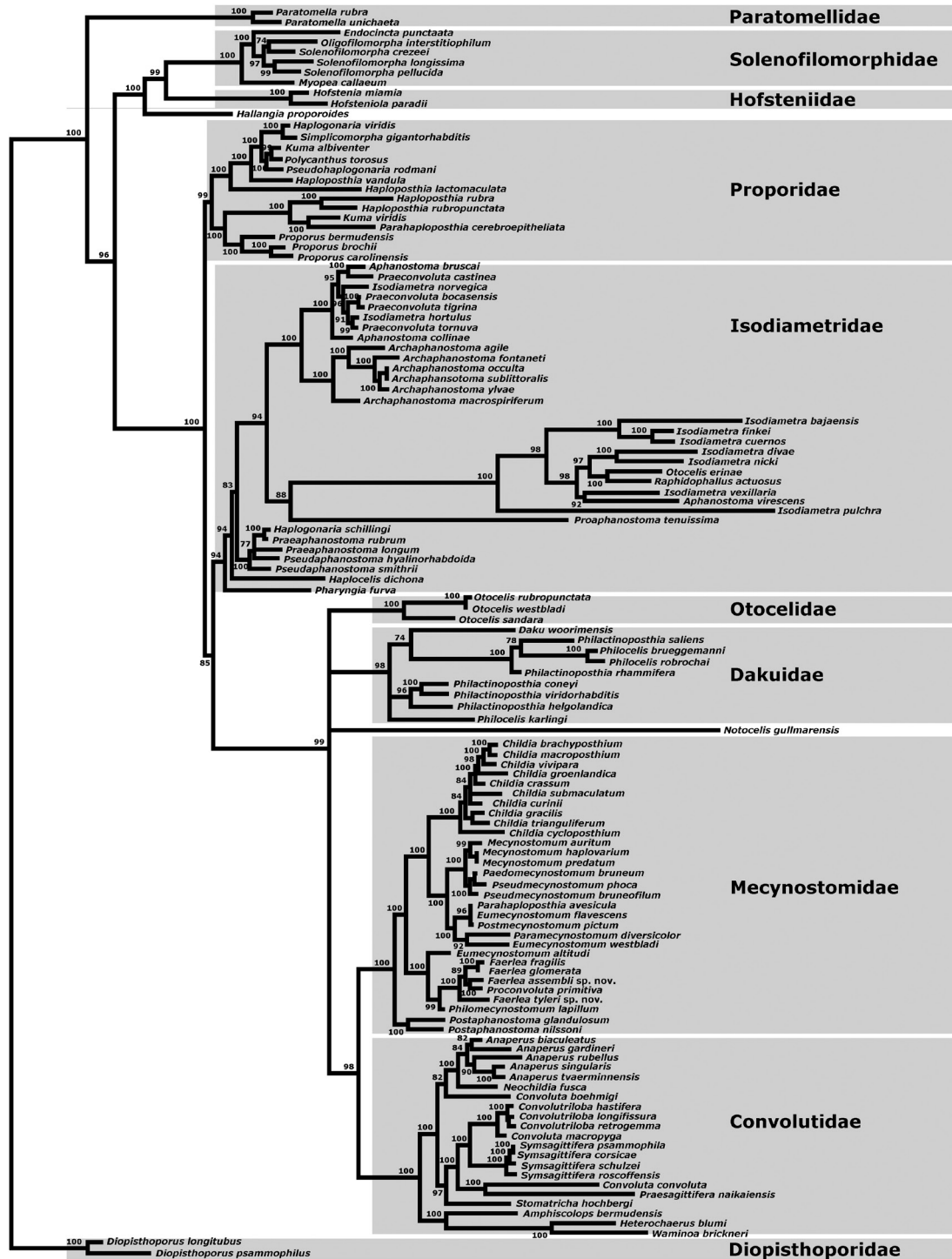
the original nucleotides. As acoels are known to have unusually variable ribosomal sequences compared with most metazoans ([Ruiz-Trillo *et al.*, 1999](#)), 18S and 28S alignments were filtered with GBlocks, available online through the CSIC-UPF webserver (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), allowing for gap positions within the final blocks, although all analyses were performed using both non-filtered and filtered alignments. Maximum likelihood (ML) analysis was performed on each marker individually and with concatenated datasets in IQTree v.1.6.12 ([Trifinopoulos *et al.*, 2016](#); <https://www.iqtree.cibiv.univie.ac.at>) with 1000 ultrafast bootstrap replicates. The best-fitting substitution models were determined using the ModelFinder Algorithm via BIC, with the GTR+G+I+G4 model selected for all gene datasets, excepting the 18S dataset filtered with GBlocks, for which the GTR+F+R4 model was selected.

In order to examine the pertinence of morphological characters typically utilized to delimit genera, 21 morphological characters considered in the past (e.g. [Hooge & Tyler, 2005](#)) to be taxonomically important were coded for the 32 species positioned within the Isodiametridae clade of our molecular phylogeny, as well as nine additional outgroup species, and mapped on to the concatenated tree (Supporting Information, [Table S1](#)). The coding method followed that of [Hooge & Tyler \(2005\)](#). The morphological characters primarily comprised details of the reproductive system, although some other general morphology, such as body size and presence of pigmented eyespots, were also considered. Characters were coded following original descriptions, illustrations, photos and live observations of each species, whenever possible. The outgroup species were selected from diverse positions across the closest sister-clade of Isodiametridae (see Results; [Fig. 1](#)).

RESULTS

Results from the phylogenetic analyses can be found in [Figure 1](#) and [Figures S1–S8](#) in the Supporting Information. Tree topologies were consistent across individual genes and concatenated analyses regardless of whether or not datasets were filtered with GBlocks, and the deeper nodes were generally recovered with high support in the 18S and 28S gene trees and in the concatenated analyses. Much lower support occurred for the deep nodes of the *COI* gene tree, although this was expected given that the *COI* gene is known to be highly variable and thus less suited to assessing distant relationships ([Sanna *et al.*, 2009](#); [Tang *et al.*, 2012](#)).

[Figure 1](#) summarizes the concatenated phylogeny. Results were overall consistent with the previous



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Figure 1. Concatenated 28S, 18S and *COI* gene tree summary. Percent bootstrap values are given at each node. Sequences from six species of Isodiametridae and Actinoposthiidae represented only by 18S sequences downloaded from GenBank (Table S1) were excluded.

findings of Jondelius *et al.* (2011). Mecynostomidae Dörjes, 1968 and Convolutidae form a clade with high support. Dakuidae Hooge, 2003 is recovered, although it

does not include *Notocelis* Dörjes, 1968, and indeed the relationships between Dakuidae, *Notocelis gullmarensis* (Westblad, 1946), Mecynostomidae + Convolutidae and

a clade consisting of three species of *Otocelis* Diesing, 1862 (Otocelididae) are all unresolved. Outside this polytomy, Proporidae Graff, 1882 and Isodiametridae are both resolved; *Hallangia proporoides* Westblad, 1946 (Hallangiidae Westblad, 1946) is sister to a Solenofilomorphidae clade + Hofsteniidae clade; and Paratomellidae is sister to the remaining Bitesticulata.

Isodiametridae is recovered in all analyses, although the support varied somewhat depending on whether six species that were difficult to place in Isodiametridae/Actinoposthiidae (Supporting Information, Table S1) are included in the analyses. Each of these species are represented only by 18S sequences downloaded from GenBank. Jondelius *et al.* (2011) found that the six species formed part of a clade with a highly unstable position that grouped either within or outside Isodiametridae. When included in our analyses, they form a clade within Isodiametridae, but the overall support for Isodiametridae is low. Without these six species, Isodiametridae is highly supported. We chose not to reclassify the species of this group until more sequence data can be attained and their position inside or outside Isodiametridae can be determined with more certainty. The remaining results and discussion concerns only the analyses with these species excluded unless otherwise stated. Results from the three-gene concatenated and 18S datasets that include all species are available as Figures S1 and S3 in the Supporting Information.

There are several highly supported groups in the larger Isodiametridae clade: (1) six species of *Archaphanostoma* Dörjes, 1968 grouped together; (2) eight species of *Isodiametra* Hooge & Tyler, 2005, *Praeconvoluta* Dörjes, 1968 and *Aphanostoma* Ørsted 1845 formed the sister-group to the *Archaphanostoma* clade. *Proaphanostoma tenuissima* (Westblad, 1946) was sister to (3) a diverse clade of ten species, including *Otocelis erinae* Hooge & Rocha, 2006, *Raphidophallus actuosus* Kozloff, 1965, *Aphanostoma virescens* Ørsted, 1845 and seven species of *Isodiametra*; and (4) a clade containing *Pseudaphanostoma hyalinorhabdoida* Kånneby & Jondelius, 2013, *Pseudaphanostoma smithii* Hooge & Tyler, 2003, *Haplogonaria schillingi* Hooge & Tyler, 2015, *Praeaphanostoma rubrum* Dörjes, 1968 and *Praeaphanostoma longum* Dörjes, 1968, which is sister to the previous three clades. Finally, *Haplocelis dichona* Dörjes, 1968 followed by *Pharyngia furva* Nilsson *et al.*, 2011 are sister to the remaining species of Isodiametridae.

Several species previously considered isodiametrids did not group within Isodiametridae. As previously stated, three species of *Otocelis* grouped together outside any represented acel family. In addition, *Proconvoluta primitiva* Dörjes, 1968 nests in a clade of four species of *Faerlea* Westblad, 1945 with high support, which in turn is nested in Mecynostomidae, and two species

of *Postaphanostoma* Dörjes, 1968 form a clade that is sister to all other species of Mecynostomidae. Thus, results from our molecular analyses provide further evidence that Isodiametridae require revision.

TAXONOMIC NOTES AND NOMENCLATURE ACTS

OTOCOLIDIDAE WESTBLAD, 1948

Westblad (1948) recognized the monotypic family Otocelididae for acuels with a vagina opening posterior to the male copulatory organ. Only two species, *Otocelis rubropunctata* (Schmidt, 1852) and *O. gullmarensis* Westblad, 1946, were known at the time. Dörjes (1968) transferred the latter to a new genus, *Notocelis*, on account of its lack of a tubular penis and at the same time introduced the genera *Archocelis* Dörjes, 1968, *Haplocelis* Dörjes, 1968, *Haplotestis* Dörjes, 1968 and *Philocelis* Dörjes, 1968. Following this, Ehlers & Dörjes (1979) erected the genus *Exocelis* Ehlers & Dörjes, 1979; Kozloff (2000) recognized *Posticopora* Kozloff, 2000; and Hooge (2003) introduced *Stomatricha* Hooge, 2003. However, the presence of symbiotic algae within *Stomatricha hochbergi* Hooge, 2003 caused Hooge & Tyler (2005) to subsequently transfer the genus to Convolutidae and question the taxonomic importance of the position of the vagina and hence the validity of Otocelididae as a monophyletic family. Based on this and the morphology of the male copulatory organ, Hooge & Rocha (2006) proposed the transfer of the genera *Haplocelis* and *Otocelis* to Isodiametridae. Building on a phylogenetic hypothesis derived from a combination of nucleotide sequences and morphological characters, Jondelius *et al.* (2011) placed *Notocelis* and *Philocelis* to Dakuidae, while refraining from reclassifying the type genus *Otocelis*, due to ambiguities in its phylogenetic position. *Archocelis*, *Exocelis*, *Haplotestis* and *Posticopora* were not represented in the study by Jondelius *et al.* (2011). The results of our analyses (Fig. 1) support the inclusion of *Stomatricha* in Convolutidae and *Philocelis* in Dakuidae, but show that *Otocelis* forms a clade outside Isodiametridae, herein interpreted to represent the family Otocelididae.

The position outside Isodiametridae is somewhat problematic, since at least three species of *Otocelis* (*O. westbladi* Ax, 1959, *O. rubropunctata* and *O. erinae*) are all known to possess the diagnostic character of Isodiametridae – penis musculature with inner circular and outer non-anastomosing longitudinal fibres (Hooge & Rocha, 2006). Notably, this morphology was not found in *O. sandara* Hooge & Tyler, 2003, leading Hooge & Rocha (2006) to question the relationship between *O. sandara* and the remaining species of *Otocelis*, although they ultimately chose not to reclassify the species until further data could be attained. Results from our DNA analyses clearly

confirm the position of *O. sandara* within *Otocelis* and Otocelididae, but surprisingly do not include *O. erinae* in the family. The latter species is positioned within a diverse Isodiametridae clade containing several species of *Isodiametra*, *Raphidophallus actuosus* and *Aphanostoma virescens*. There are some morphological characters that support such a grouping, such as a vagina with a well-developed muscular sphincter, which is found in *Otocelis erinae* and the remaining species of the clade, but is lacking in all other species of *Otocelis* and Otocelididae, and the presence of a bursa with a well-developed nozzle. The position of *O. erinae* – as well as *Haplocelis dichona*, *Stomatricha hochbergi*, *Philocelis bruggemanni* Hooge & Tyler, 2003 and *P. rochbrochai* Hooge & Rocha, 2006 – outside of Otocelididae further supports Hooge & Tyler's (2005) assertion that the positioning of the vagina is taxonomically of low importance. These ambiguities perfectly illustrate the shortcomings of basing a classification on perceived morphological apomorphies without reference to an explicit, testable phylogenetic hypothesis. Inclusion of the remaining Otocelididae genera and species in a comprehensive phylogenetic analysis is clearly needed to establish their positions and to uncover any synapomorphic characters that potentially exist for the family.

REASSIGNMENT OF THE GENUS *FAERLEA*
WESTBLAD, 1945 TO THE FAMILY MECYNOSTOMIDAE
DÖRJES, 1968

Results from our phylogenetic analyses show that the species of *Faerlea*, as well as *Proconvoluta primitiva*, form a clade with high support nested within the larger Mecynostomidae clade, and indeed, the general structure of the male copulatory organ of *Faerlea* does share some similarities to those typical of species of Mecynostomidae. For instance, the copulatory organ is often circular and appears as two concentric circles with the gonopore (e.g. Fig. 2A), and numerous glands surround the distal part of the penis, particularly in, for example, *F. antora* Marcus, 1952 and *F. fragilis* Westblad, 1945. Additionally, species of *Faerlea* lack rhabdoids, a condition much more common to species of Mecynostomidae than Isodiametridae. Hooge & Tyler (2005) assigned *Faerlea* to Isodiametridae without specifying any reason beyond that the genus was previously within Convolutidae and lacked symbionts. Yet to the best of our knowledge, there has been no detailed investigation into the morphology of the musculature, male copulatory organ or sperm for any species of *Faerlea*, and so it is not certain if any, or all, of the species possess morphology consistent with Isodiametridae (= penis musculature with inner circular and outer non-anastomosing longitudinal fibres; Hooge & Tyler, 2005) or Mecynostomidae

(= sperm with 9 + 1 axonemes and distal microtubules only and four layers of dorsal body-wall musculature; Jondelius *et al.*, 2011). Regardless, the position of the four species of *Faerlea*, in particular the type species *F. fragilis*, justify the reassignment of the genus into Mecynostomidae (Fig. 1).

SYNONYMIZATION OF *FAERLEA* WESTBLAD, 1945 AND
PROCONVOLUTA DÖRJES, 1968

Results from our phylogenetic analyses found *Proconvoluta primitiva* clearly nested in the *Faerlea* clade as sister to the new species *Faerlea assembla* (Fig. 1). Dörjes (1968) separated *Proconvoluta* from *Faerlea* based on the seminal bursa, which is present in *Proconvoluta*, although small and difficult to distinguish in live specimens, and absent in species of *Faerlea*. Otherwise, the morphologies of the two genera generally overlap, in particular with the absence of rhabdoid glands and the heavily vacuolated parenchyma. *Proconvoluta primitiva* is the only species currently recognized for the genus, and so, based on the results of the phylogenetic analyses, *Proconvoluta* becomes a junior synonym of *Faerlea*.

ORDER ACOELA ULJANIN, 1870

FAMILY MECYNOSTOMIDAE DÖRJES, 1968
GENUS *FAERLEA* WESTBLAD, 1945

Unpigmented and often glassy in appearance. Parenchyma heavily vacuolated. Frontal glands well developed. Rhabdoids absent. Testes and ovaries paired. Male system includes a round seminal vesicle, a small muscular penis and a short antrum masculinum. Copulatory bursa, if present, is small, difficult to discern in live animals and includes a ventrofrontally directed cellular appendage. Gonopore(s) separate or male only. Type species: *Faerlea fragilis* Westblad, 1945.

Marine, free-living or parasitic; seven species:

- *Faerlea fragilis* Westblad, 1945
- *Faerlea antora* Marcus, 1952
- *Faerlea echinocardii* Dörjes, 1972
- *Faerlea glomerata* Westblad, 1945
- ***Faerlea primitiva* (Dörjes, 1968), comb. nov.**
- ***Faerlea assembla* sp. nov.**
- ***Faerlea tyleri* sp. nov.**

***FAERLEA ASSEMBLI* SP. NOV.**

FIGS 2–3

Zoobank registration: urn:lsid:zoobank.org:act:C46A5FAF-F9E0-4C44-8B5C-D69CD8CD46EC

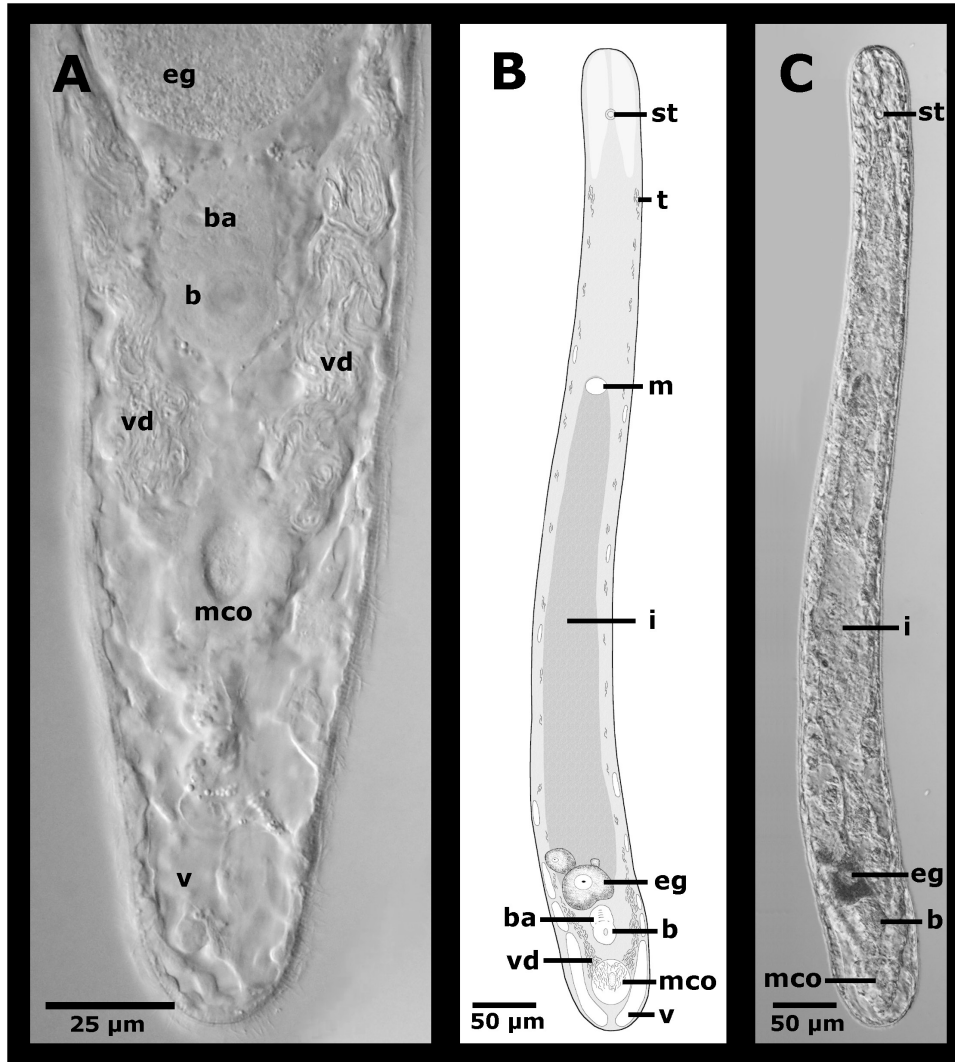


Figure 2. *Faerlea assembla*. A, microphotograph of posterior focusing on the reproductive structures. B, drawing of whole body C, microphotograph of whole body. b, bursa; ba, bursal appendage; eg, egg; I, intestine; m, mouth; mco, male copulatory organ; st, statocyst; t, testis; v, vacuole; vd, vas deferens.

Material examined: Holotype (SMNH-Type-9334) and paratypes (SMNH-Type-9335): serially sectioned specimens. Digital video and photographs of original specimens.

Type locality: SPAIN. Mutriku, Siete Playas. 43°19'41.5"N, 2°22'46.7"W

Habitat: Marine sediments. Subtidal, medium sand.

Diagnosis: Species of *Faerlea* without pigmentation, glassy. Body 0.8 mm long, vermiform with rounded anterior and posterior. Large vacuoles in posterior. Smaller vacuoles present laterally and in the anterior. Frontal glands large. Rhabdoids absent. Testes and

ovaries paired. Male system with round seminal vesicle, small antrum. Penis a simple inpocketing of the epidermis. Female system with bursa with a single small, ventrofrontally directed appendage. Gonopores separate.

Etymology: This species is named after the Assemble Plus programme, funded by the European Community, which has supported a variety of marine field studies.

Description

Living specimens approximately 0.8 mm long. Width ~50 µm at position of stylet and slightly increasing toward posterior; ~75 µm at position of largest egg. Body shape vermiform with rounded anterior and posterior

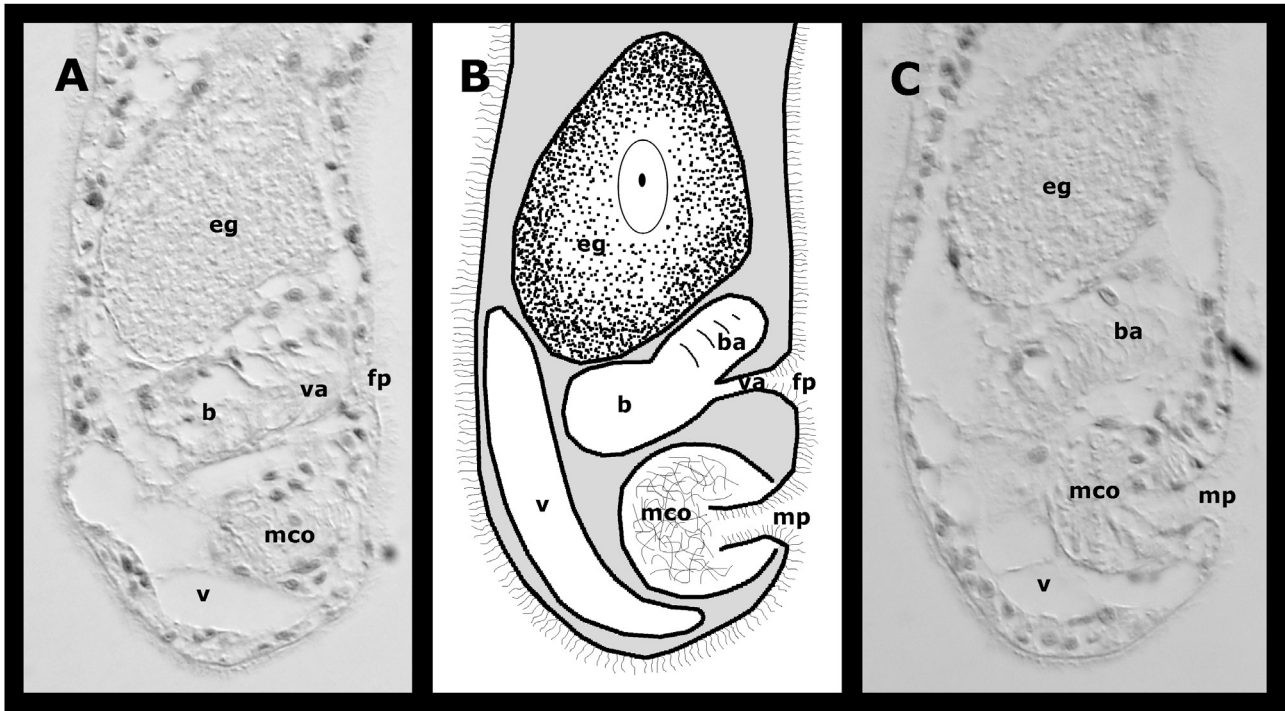


Figure 3. *Faerlea assemblis* posterior end, side view. A, sagittal section of posterior end stained with haematoxylin-eosin, displaying bursa. B, Composite drawing of posterior end. C, Sagittal section of posterior end stained with haematoxylin-eosin, displaying bursal appendage and male pore. b, bursa; ba, bursal appendage; eg, egg; fp, female pore; mco, male copulatory organ; mp, male pore; v, vacuole; va, vagina.

ends. Without body pigmentation or eyespots. Glassy in appearance. Statocyst 10–12 μm in diameter located ~50 μm from anterior end. Frontal organ present and large. Two large vacuoles present at the posterior end; smaller vacuoles present laterally and in the anterior, though more numerous in the posterior body. Epidermis 4 μm thick, uniformly covered with cilia. Cilia ~4 μm long. Rhabdoids absent. Mouth located at the second-quarter of the body, 15 μm long in fixed specimens. Ovaries paired. Female pore clearly separate from male pore, ciliated, opening ventral, ~9 μm long. Female atrium present, ~11 μm deep in fixed specimens leading to a short vagina and bursa. Bursa present 75 μm from posterior end, 28 μm long and 34 μm wide, with a single ventrofrontally directed cellular appendix. Sperm was not observed in the bursa of any specimen examined. Testes paired, located laterally approximately one-quarter of the way from the anterior end of the body. Vas deferens large and clearly evident toward male copulatory organ, with evident sperm. Male copulatory organ present ~45 μm from posterior end, spheroid, ~35 μm long and ~30 μm wide in living specimens. Penis present as a simple in-pocketing of the epidermis, 17 μm in fixed specimens, with a shallow male atrium. Male pore large, 15 μm long, ciliated.

Remarks

There are seven species of *Faerlea*, following the synonymization of *Proconvoluta* and *Faerlea*. *Faerlea assemblis* is most similar to *Faerlea primitiva* in that they both possess a bursa with a ventrofrontally directed cellular appendix. The two species can be distinguished by the size of the bursa and male copulatory organ, which are more clearly evident and larger in *Faerlea assemblis*, and through the small, brownish-black pigments that are present in *Faerlea primitiva* and absent in the new species.

FAERLEA TYLERI SP. NOV.

(FIG. 4)

Zoobank registration: urn:lsid:zoobank.org:act:D7ACB50A-70AB-40E3-ADB7-86310945FB0F

Material examined: Holotype (SMNH-Type-9336) and paratypes (SMNH-Type-9337): serially sectioned specimens. Digital video and photographs of original specimens.

Type locality: USA, Hawaii, Waimanolo Beach. 21°19'8.23"N, 157°40'11.92"W.

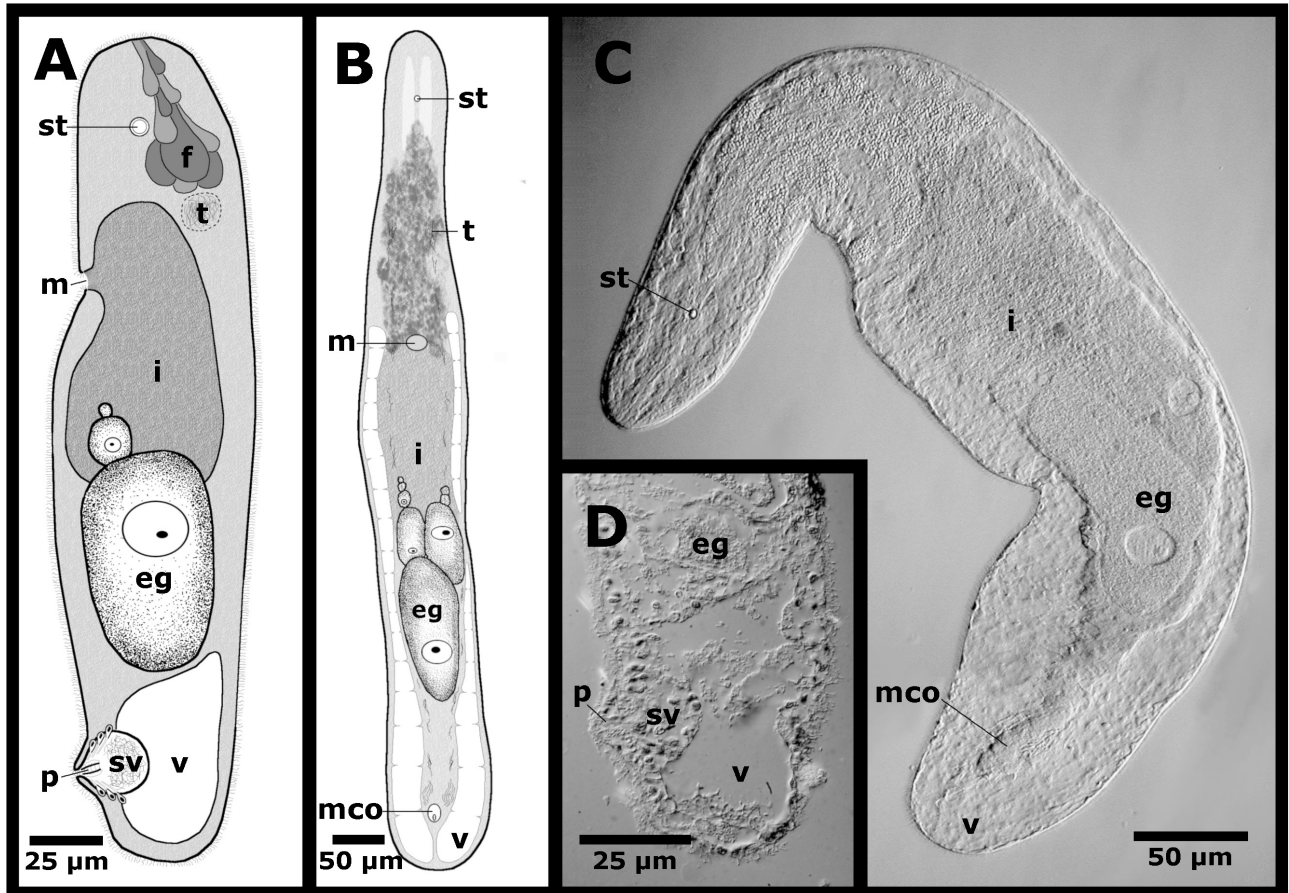


Figure 4. *Faerlea tyleri*. A, whole body drawing, sagittal view. B, whole body drawing, frontal view. C, microphotograph of whole body. D, sagittal section of posterior end stained with haematoxylin–eosin. eg, egg; f, frontal organ; I, intestine; m, mouth; mco, male copulatory organ; p, penis; st, statocyst; sv, seminal vesicle; t, testis; v, vacuole.

Habitat: Marine sediments; mixed fine and medium sand with some organic content; 1.5 m deep.

Diagnosis: Species of *Faerlea* without pigmentation, glassy. Body up to 1 mm long, vermiform with rounded anterior and posterior. Large vacuoles in posterior and laterally. Smaller vacuoles in the anterior. Frontal glands well developed. Rhabdoids absent. Testes and ovaries paired. Male copulatory organ located close the posterior end. Small, round seminal vesicle present with a penis as a simple inpocketing of the epidermis. Bursa or female accessory organs absent.

Etymology: This species is named after Seth Tyler in recognition of his contributions to the taxonomy of Acoela.

Description

Living specimens up to 1 mm long. Glassy and without body pigmentation or eyespots. Body width ~67 µm at position of stylet and increasing toward

posterior; ~100 µm at position of largest egg. Body shape vermiform with rounded anterior and posterior ends. Statocyst to 8 µm in diameter located 67 µm from anterior end. Frontal organ well developed. Large vacuoles present at the posterior end and extending laterally, decreasing in size toward the anterior; smaller vacuoles present in the anterior. Epidermis 4–5 µm thick, uniformly covered with cilia. Cilia 3–5 µm long. Rhabdoids absent. Mouth located at the second-quarter of the body. Ovaries paired, located in third-quarter of the body. Bursa or female accessory organs absent. Testes paired, located laterally approximately ~300 µm from the anterior end the body. Vas deferens more evident toward male copulatory organ, with clear sperm. Male copulatory organ small, ~20 µm long and ~25 µm deep in fixed specimens, spheroid. Present 55 µm from posterior end in living specimens. Penis present as a simple inpocketing of the epidermis, 9 µm long and projecting outward in fixed specimens. Male pore small, ~5 µm long, ciliated.

Remarks

Faerlea tyleri is the first species of its genus to be documented from Hawaii or the Pacific. In general, the species fits well with the other known species of *Faerlea*: *Faerlea tyleri* is fragile due to its heavily vacuolated parenchyma; the position of the testes is far toward the anterior; and the position and composition of the male copulatory organ is consistent with the other species. The species is most similar to *F. fragilis* and *F. glomerata* in that it is non-parasitic and does not possess female accessory organs. However, *Faerlea tyleri* differs from either species in its general body shape, which is more strap-shaped with rounded anterior and posterior ends than either *F. fragilis* or *F. glomerata*, and the small size of the male copulatory organ. Additionally, *Faerlea tyleri* can be distinguished from *F. glomerata* by the size of the frontal organ, which, although well developed, is smaller in comparison, the more distinctly paired ovaries and wide separation between the ovaries and the male copulatory organ. *Faerlea tyleri* can be distinguished from *F. fragilis* by the relatively few vacuoles and the position of the ovaries, which begin further toward the posterior end.

REASSIGNMENT OF *POSTAPHANOSTOMA* DÖRJES, 1968
TO THE FAMILY MECYNOSTOMIDAE DÖRJES, 1968

Both species of *Postaphanostoma* included in our analyses, *P. glandulosum* Dörjes 1968 and *P. nilssoni* Kånneby & Jondelius, 2013, grouped together as the highly supported sister-group of Mecynostomidae, separate from Isodiametridae. As with *Faerlea*, once again there has not been any detailed investigation into the body and reproductive musculature or sperm ultrastructure for any species of *Postaphanostoma*, and so it cannot be definitively stated if the animals of this group possess any of the morphological characteristics of Isodiametridae, Mecynostomidae or any other group. Indeed, the position of *P. glandulosum* and *P. nilssoni* as sister of Mecynostomidae in our phylogenetic analyses suggests such detailed morphological investigations should be of high priority for future studies. Unfortunately, no gene sequences of the type species of *Postaphanostoma*, *P. atriomagnum* Dörjes, 1968, currently exist. However, there are also no significant morphological characters that distinguish *P. glandulosum* and *P. nilssoni* from the remainder of the genus with which to justify a separation, and so, rather than create confusion by forming a new genus in which to transfer the two species, we choose to reassign *Postaphanostoma* in its entirety to Mecynostomidae until such time as sequences or more detailed morphology can be assessed for the type.

REASSIGNMENT OF *ALLUNA* FAUBEL & REGIER, 1983
TO THE FAMILY ACTINOPOSTHIIDAE HOOGE, 2001

There were several inconsistencies in Faubel & Regier's (1983) original description of *Alluna*. First, they stated in the genus diagnosis that *A. sublittoralis* Faubel & Regier, 1983 was the type and only species, but entitled *Alluna vulgaris* as the caption of their illustrations. Since no other species description occurred apart from that of *A. sublittoralis*, and no illustration of *A. sublittoralis* was provided, *Alluna vulgaris* can be considered a synonym of *Alluna sublittoralis*. More importantly, Faubel & Regier (1983) stated in the diagnosis of *Alluna* that a true seminal vesicle is absent, but they described paired false seminal vesicles attached to a (true) seminal vesicle in the subsequent description of *A. sublittoralis*, a feature that was also clearly present in the following illustrations.

If the genus diagnosis is thus emended to fit its type and only species, its morphology becomes more consistent with species of Actinoposthiidae than with Isodiametridae. Following the family diagnosis of Hooge (2001), Actinoposthiidae includes those species of Acoela with a penis built of either sclerotized or muscular elements that is never invaginated into a seminal vesicle. Alternately and according to the diagnosis of Hooge & Tyler (2005), Isodiametridae includes those species of Acoela with a muscular, isodiametric penis that is partially or completely invaginated into a muscular seminal vesicle, if present. The penis of *Alluna sublittoralis* is highly muscular, not isodiametric (following the original illustrations, the proximal part of the penis swells to form a bulb), and – different to all other species of Isodiametridae – lies entirely outside of the seminal vesicle. Unfortunately, sequences of *Alluna sublittoralis* are currently not available for analysis, so the position of the species could not be tested using molecular techniques. However, in consideration of its morphology, the species and genus must be transferred to Actinoposthiidae.

SYNONYMIZATION OF *BALTALIMANIA* AX, 1959 AND
ARCHAPHANOSTOMA DÖRJES, 1968

Dörjes (1968) placed the three species of *Baltalimania* of the time into different genera, based solely on the presence or absence of a female bursa. *Archaphanostoma* was created to encompass two species [*A. agile* (Jensen, 1878) and *A. macrospiriferum* (Westblad, 1946)] with bursal tissue, leaving the one species that lacked bursal tissue (*B. kosswigi* Dörjes, 1968) to comprise its own monotypic genus. Subsequently and without mention of *Baltalimania*, Kånneby *et al.* (2014) amended the

diagnosis of *Archaphanostoma* to include both species with and without bursal tissue, following phylogenetic analysis of nucleotide sequences from three genes that demonstrated that four new species without bursa (*A. fontaneti* Kånneby *et al.*, 2015; *A. occulta* Kånneby *et al.*, 2015; *A. sublitoralis* Kånneby *et al.*, 2015 and *A. ylva* Kånneby *et al.*, 2015) nested within the *Archaphanostoma* clade. Unfortunately, the amended diagnosis caused *Baltalimania* and *Archaphanostoma* to be morphologically indistinguishable. Phylogenetic analyses oppose Dörjes' (1968) argument that bursal tissue is a good character to differentiate these genera (Figs 1, 5). Although no DNA sequences currently exist for *B. kosswigi*, the identical generic diagnoses and similarities in the morphologies of *B. kosswigi* and species of *Archaphanostoma* without bursal tissue strongly suggest that the two genera are synonymous. Therefore, *Archaphanostoma* is a junior synonym for *Baltalimania*.

SYNONYMIZATION OF *BURSOSAPHIA* DÖRJES, 1968 AND *PRAEAPHANOSTOMA* DÖRJES, 1968

Dörjes (1968) placed great emphasis on the presence and structure of the bursa in his classification system, and numerous genera were created or separated based on small differences of this organ. When Dörjes (1968) described the species *Bursosaphia baltalimaniaformis* Dörjes, 1968, he justified the creation of a new genus based on what he interpreted as bursal tissue ('*bursales Gewebe*') in which the true bursa was embedded. However, Dörjes (1968) was somewhat unclear in his description of the bursal tissue that surrounds the female system, stating only that it is a tissue that stores foreign sperm, that it is separate from the parenchyma of the rest of the body and that it similarly is present in species of *Archaphanostoma* (= *Baltalimania*), which otherwise lack a true bursa. Dörjes (1968) also stated that bursal tissue may occur in other genera, but will typically disappear after a

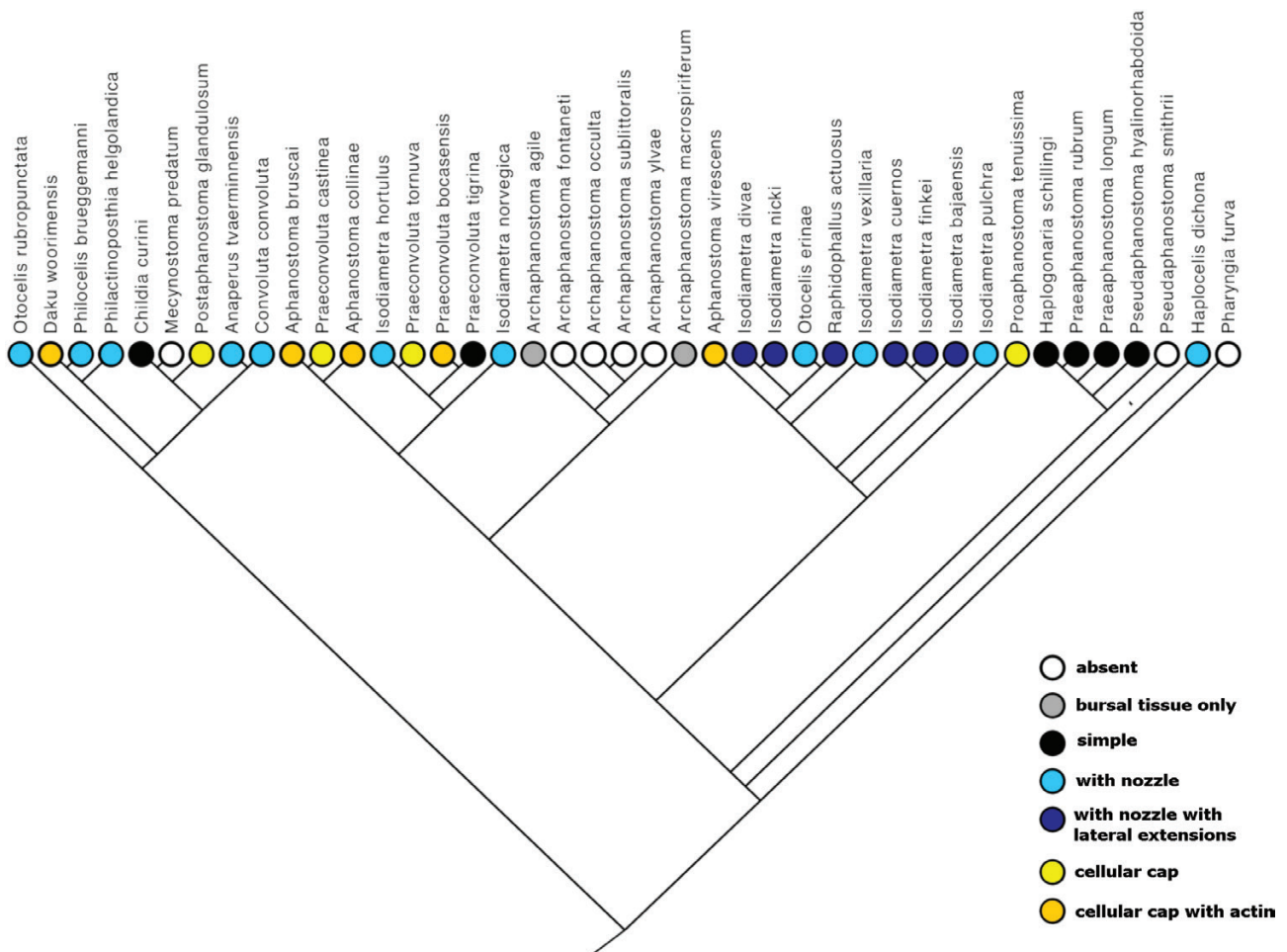


Figure 5. Bursal morphology of Isodiamedridae mapped onto the concatenated tree. The bursal morphology of each species was coded following original descriptions and illustrations, as well as photos and live observation of species where possible.

true bursa has formed. Unfortunately, this character has not been assessed for any species with a true bursa in any of the literature outside Dörjes' (1968) description of *Bursosaphia* and, therefore, it is unclear how prevalent it is within Isodiametridae.

Unfortunately, no sequences of *B. baltalimaniaformis* are currently available, and so the relationship between this species and other acoels cannot be directly tested at this time. Regardless, results from our phylogenetic analyses suggest that bursal tissue is not a good character for distinguishing genera, since the only species other than *B. baltalimaniaformis* known to possess it – *Baltalimania macrospiriferum* and *B. agile* – do not on their own form a clade, but instead group with species of *Baltalimania* without bursal tissue (Fig. 1). Given these results, and that there is no difference in the morphology of *Bursosaphia* and *Praeaphanostoma* apart from the bursal tissue, we propose to synonymize the two genera, with *Bursosaphia* the junior synonym for *Praeaphanostoma*.

REASSIGNMENT OF *HAPLOGONARIA SCHILLINGI*
HOOG & TYLER, 2015 TO THE GENUS
PRAEAPHANOSTOMA DÖRJES, 1968

Our results show *Haplogonaria schillingi* to be nested in *Praeaphanostoma* and closely related to *Praeaphanostoma rubrum*. Sequences of the 18S, 28S and COI genes were published for *H. schillingi* and used in several phylogenetic analyses before the species was formally described. Jondelius *et al.* (2011) found the species (denoted there as *Haplogonaria 'schillingi'*) positioned closely to *Pseudaphanostoma smithrii* and transferred the species to *Pseudaphanostoma* on this basis. Later, Nilsson *et al.* (2011) and Kånneby & Jondelius (2013) used the sequences in their phylogenetic analyses with results congruent to Jondelius *et al.* (2011), but still listing the species as *Haplogonaria schillingi*. Of note, at the time of all three publications, no sequences of any species of *Praeaphanostoma* were available and, indeed, results from our analysis show that *P. smithrii* is the closest sister to all other species of *Praeaphanostoma*. Hooge & Tyler (2015) eventually described the species, but found that the male copulatory organ lacks a muscular, tubular penis. Because such morphology is incongruous with the diagnostic character of Isodiametridae, Hooge & Tyler (2015) dubbed the species *Haplogonaria schillingi* and assumed the molecular data resulted from an error in handling specimens.

Our results, with the additional sequence data of *Praeaphanostoma longum* and especially *P. rubrum*, contradict the assertion that the position of *H. schillingi* in the phylogenetic analyses was due to some error. The morphology of *H. schillingi* is generally consistent with species of *Praeaphanostoma*. *Praeaphanostoma*

is in part characterized by a moderate to small frontal organ, a muscular seminal vesicle, a seminal bursa with well-defined walls but without nozzles and a well-defined vagina with a sphincter, all characters that *H. schillingi* also possesses. *Praeaphanostoma longum* is additionally morphologically similar to *H. schillingi* in the shared possession of an unpaired ovary, common gonopore, diagonal muscles restricted to the anterior end and a characteristic bright-red pigmentation. The two species differ only in the size of the rhabdoids (small in *P. longum*, large in *H. schillingi*), possession of a genital atrium (absent in *P. longum*, present in *H. schillingi*) and, of course, the penis (present in *P. longum*, absent in *H. schillingi*). Given such similarities, the position of *H. schillingi* is conceivable, and the absence of a penis could potentially represent a secondary loss. *Praeaphanostoma* includes several species – including those in our analysis that were the closest to *H. schillingi* – where the penis is a simple inpocketing of the epidermis, a condition that in Isodiametridae is only otherwise present in *Proaphanostoma*.

Thus, based on the positioning of the species in our phylogenetic analyses and the morphological similarity, we formally reassign *Haplogonaria schillingi* to *Praeaphanostoma* in Isodiametridae.

REDESCRIPTION OF *PSEUDAPHANOSTOMA*
HYALINORHABDOIDA KÅNNEBY & JONDELIUS, 2013
AND REASSIGNMENT TO *PRAEAPHANOSTOMA* DÖRJES,
1968

Following a re-examination of the original collection notes and materials, it is clear from both photographs of live animals and from fixed, sectioned animals that Kånneby & Jondelius (2013) incorrectly interpreted the morphology of the reproductive system of *Pseudaphanostoma hyalinorhabdoidea* in their original description. The female bursa was described as the seminal vesicle; the actual seminal vesicle and connecting vas deferens were dismissed as patches of free sperm outside the male copulatory organ, and the penis was not identified. Kånneby & Jondelius (2013) based the inclusion of the species in *Pseudaphanostoma* on the mistaken lack of female accessory organs and the relationship between this species and *Pseudaphanostoma smithrii* in their phylogenetic tree.

It is clear that this species does possess a female bursa, ciliated vagina and gonopore common to both the male and female system (Figs 6–7). The presence of an unadorned female bursa is a characteristic of species of *Praeaphanostoma*, while species of *Pseudaphanostoma* lack all female accessory organs. Further, results from our DNA analyses confirm a close relationship between this species with *Praeaphanostoma longum* and *P. rubrum* (Fig. 1), neither of which had sequences available when

Kånneby & Jondelius (2013) performed their analysis. The morphological inaccuracies of the original description require that the species be redescribed, and the updated morphology and phylogenetic relationships support the transference of the species from *Pseudaphanostoma* to *Praeaphanostoma*.

FAMILY ISODIAMETRIDAE HOOGE & TYLER, 2005

GENUS *PRAEAPHANOSTOMA* DÖRJES, 1968

PRAEAPHANOSTOMA HYALINORHABDOIDA
(KÅNNEBY & JONDELIUS, 2013), COMB. NOV.

FIGS 6–7

Material examined: Holotype (SMNH Type-8468) and paratypes (SMNH Type-8469–8471): serially sectioned specimens. Digital video and photographs of original living specimens.

Type locality: CHILE. El Quisco, Marina, 33°23'35.664"S, 71°41'53.088"W.

Habitat: Marine sediments; fine sand at 10 m depth.

Diagnosis: Species of *Praeaphanostoma* without body pigmentation or eyespots. Body 0.5 mm long, vermiform with rounded anterior and more pointed posterior end. A single, large vacuole present in the posterior end with additional smaller vacuoles present laterally and in the anterior. Rhabdoid glands distinct in longitudinal rows, more numerous towards body ends. Paired ovaries and testes. Female system includes muscular seminal bursa without adornments and a short, ciliated vagina with muscular sphincter. Male system includes short, cylindrical penis invaginated in seminal vesicle. Penis with two distal, fingerlike lobes that reach outward toward genital pore. Gonopore common.

Description

Living specimens approximately 500 µm long and 150–200 µm wide. Fixed specimens up to 300 µm in body length. Body shape vermiform with a tapering,

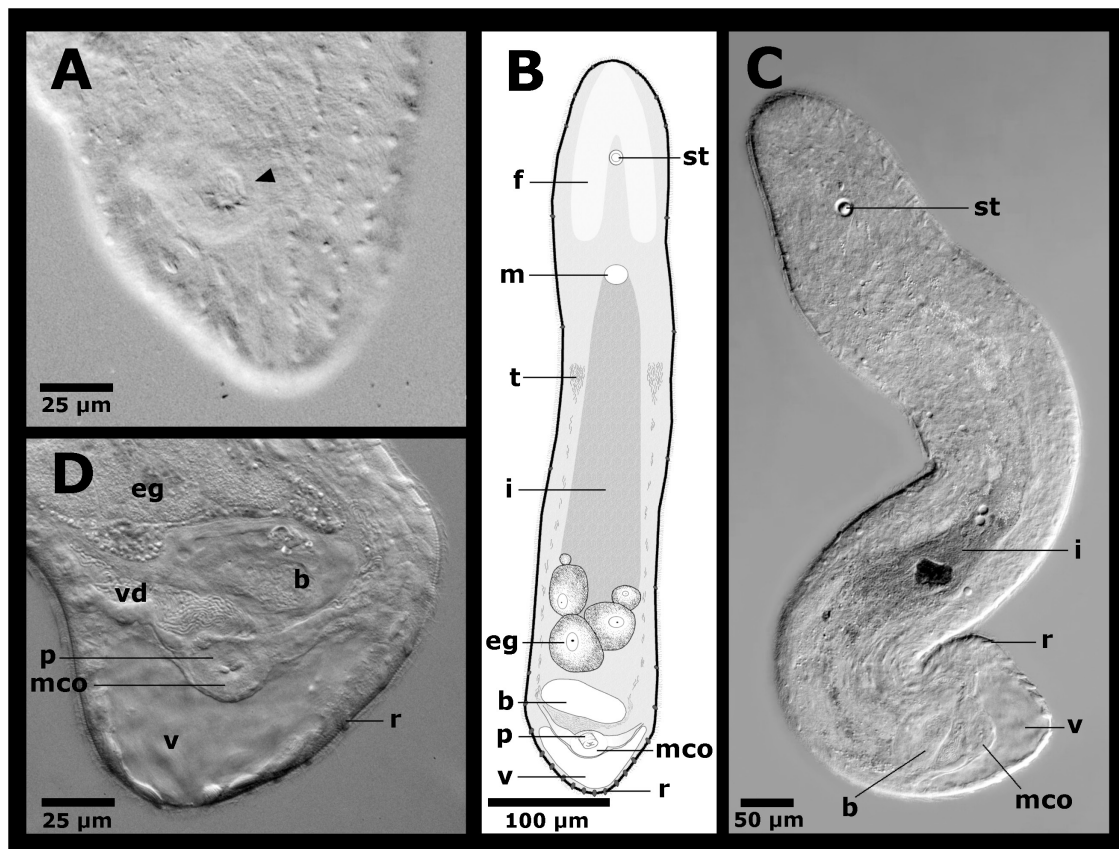


Figure 6. *Praeaphanostoma hyalinorhabdoidea*. A, micropictograph of posterior end, male pore indicated by arrow. B, drawing of whole body. C, micropictograph of whole body. D, micropictograph of posterior end, focus on reproductive anatomy. b, bursa; eg, egg; f, frontal organ; i, intestine; m, mouth; mco, male copulatory organ; p, penis; r, rhabdoid glands; st, statocyst; t, testis; v, vacuole; vd, vas deferens.

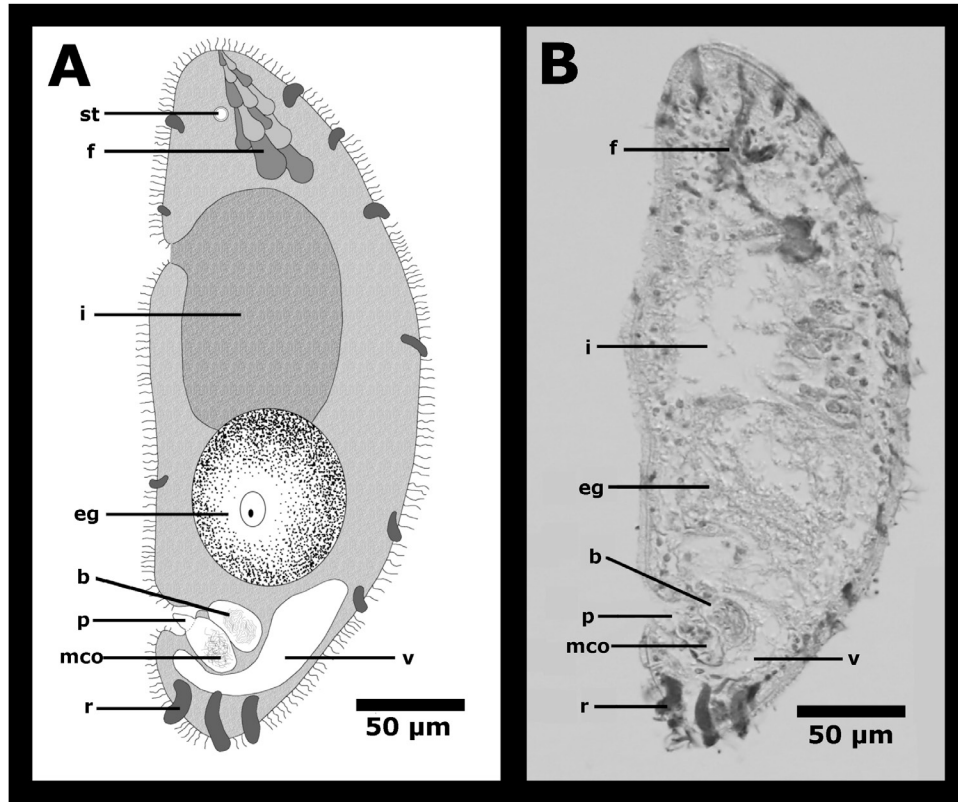


Figure 7. *Praeaphanostoma hyalinorhabdoida*, sagittal view. A, drawing of whole body. B, sagittal section of whole body stained with haematoxylin–eosin. b, bursa; eg, egg; f, frontal organ; i, intestine; mco, male copulatory organ; p, penis; r, rhabdoid glands; st, statocyst; v, vacuole.

conically rounded anterior end and a somewhat more sloped and pointed posterior end. Without body pigmentation or pigmented eyespots. Central parenchyma may appear yellow-orange from food. Statocyst and statolith 15 µm and 9 µm in diameter, respectively, located 75 µm from anterior end. Frontal organ present, extending from the frontal pore to the level of the mouth. Mouth 25 µm long in fixed specimens, located ventrally approximately one-quarter of the way from the anterior of the body. A single, large vacuole present in the anterior end; other smaller vacuoles may also be present laterally and in the anterior. Epidermis 3–4 µm thick, uniformly covered with cilia, and penetrated by numerous well-developed rhabdoids. Rhabdoid glands present in distinct longitudinal rows, more numerous dorsally and towards the anterior and especially posterior ends. Ovaries paired, ventral. Thick-walled, muscular seminal bursa clearly present, usually filled with allosperm. Bursa shape an irregular prolate spheroid, ~20 µm long and ~60 µm wide in living specimen (Fig. 6D). A short, ciliated vagina connects the bursa ventrally to a ciliated common antrum and gonopore. Muscular sphincter present at the junction between the vagina and bursa. Testes paired, diffuse,

located laterally just anterior to the midpoint of the body and extending to the seminal vesicle. Male copulatory organ present closely between the bursa and the posterior vacuole, consisting of a glandular, short penis invaginated into a seminal vesicle. Penis cylindrical with two distal, fingerlike lobes. The twin lobes of the penis reach outwards into the common antrum and are clearly visible in living specimens (Fig. 6C, D).

Remarks

Praeaphanostoma hyalinorhabdoida can be easily distinguished from all known species of *Praeaphanostoma* by its larger, distinct rhabdoids and small penis with distal, fingerlike lobes that are clearly visible in live specimens (Fig. 6C, D). Apart from these characters, *P. hyalinorhabdoida* is perhaps most similar to *P. foramivora* Hooge & Tyler, 2008 and *P. thalassophilum* Ehlers & Dörjes, 1979 in general body shape and size, and in that they all possess a single, large, posterior vacuole and a common gonopore. However, *P. foramivora* has a much larger seminal vesicle and smaller bursa, and *P. thalassophilum* has a longer vagina and unpaired ovary.

SYNONYMIZATION OF *ANCYLOCIRRUS* KOZLOFF, 2000
AND *PRAEAPHANOSTOMA* DÖRJES, 1968

Kozloff (2000) proposed the genus *Ancylocirrus* after finding secretory granules in the lumen of the cirrus and in the vagina of its one species, *A. ornatus* Kozloff, 2000. The only details presented about these granules were that they were colourless, ~3–5 µm in diameter and likely secreted by the male system, since they were associated with sperm masses and present in the female system only after insemination. Kozloff (2000) stated that such secreted granules had not been reported in any other genera at the time and was thus sufficient for the establishment of a new genus.

Sequence data is not available for *Ancylocirrus ornatus* at this time. However, while secretory granules such as those described by Kozloff (2000) may not be a character that has typically been assessed in the past (although, see e.g. *Aphanostoma album* Dörjes, 1968; *Baltalimania histobursalium*; *Diatomovora amoena* Kozloff, 1965), we can confirm that it is present across multiple species of Acoela (Fig. 8), including in at least one species of *Praeaphanostoma* (*P. rubrum*; Fig. 8D). Further, based on our own personal observations, we hypothesize that far from being unique to one species, such secretions are probably common, especially in species with larger and more glandular male copulatory organs, such as can be found in species of, for example, *Baltalimania* (Fig. 8B, C).

Ancylocirrus is further characterized by the morphology of the reproductive system: a long, curved cirrus invaginated into a seminal vesicle; a small, unciliated antrum masculinum; a long, muscular vagina; and, finally, a conspicuous seminal bursa without adornment. This morphology, along with the general body shape and pigmentation, fits in its entirety with species of *Praeaphanostoma*. While *Ancylocirrus ornatus* does differ in that it has pigmented eyespots, genera with species both with and without eyespots are widespread throughout Acoela (e.g. *Amphiscolops* Graff, 1904; *Isodiametra*; *Nadina* Ulajnin, 1870; *Otocelis*; *Proporus* Schmidt, 1848) and the character is unlikely to be of much taxonomic importance. Thereby, *Ancylocirrus* is designated a junior synonym of *Praeaphanostoma*.

APHANOSTOMA ØRSTED 1845, *ISODIAMETRA* HOOGE & TYLER, 2005 AND *PRAECONVOLUTA* DÖRJES, 1968

Species of *Aphanostoma*, *Isodiametra* and *Praeconvoluta* are united by the possession of a single bursa with some type of bursal appendage, the morphology of which, following the classification systems of Dörjes (1968), Faubel (1974) and Hooge & Tyler (2005), in large part distinguishes each genus (for a list of bursa terminology, see also: Petrov *et al.*, 2006). *Isodiametra* encompassed those species of Isodiametridae with a

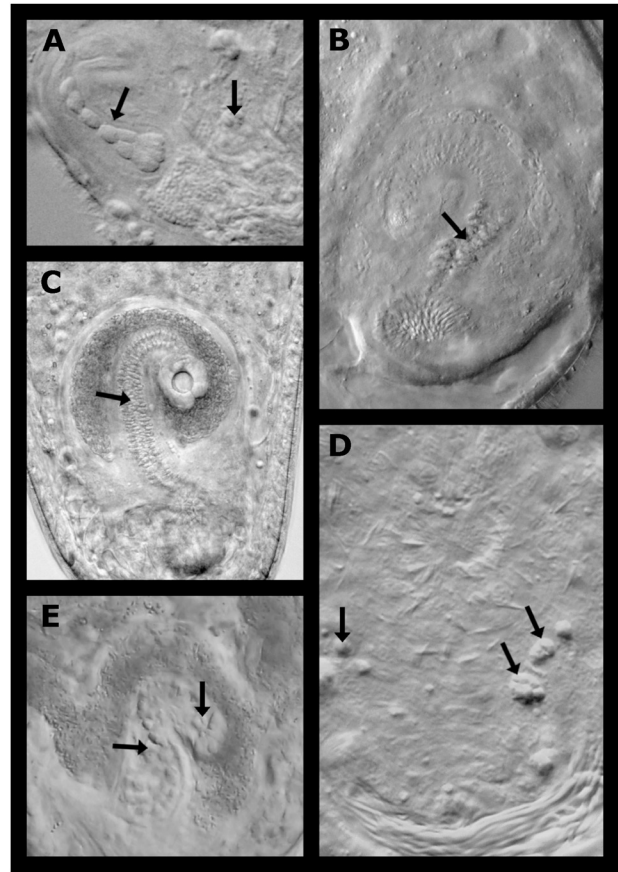


Figure 8. Micropictographs of the male copulatory organ of various species of Acoela depicting widespread presence of secretory granules (arrow). A, *Aphanostoma finkei*. B, *Baltalimania ylvae*. C, *Baltalimania macrospiriferum*. D, *Praeaphanostoma rubrum*, E, *Otocelis rubropunctata*.

single sclerotized bursal nozzle, and *Aphanostoma* comprises species with a cellular bursal cap, which may also include small actin-sclerotized bodies (Petrov *et al.*, 2006; Hooge & Tyler, 2008). For *Praeconvoluta*, Dörjes (1968) and later Faubel (1974) specified a ‘simple bursa’ in the genus diagnosis. However, all species, except *P. tigrina* Hooge & Tyler, 2003, were described with a bursal cap, and one species, *P. bocasensis* Hooge & Tyler, 2008, further possesses a bursal cap with several spots of concentrated actin, and Hooge & Tyler (2005) listed *Praeconvoluta* and *Aphanostoma* as morphologically indistinguishable in their identification table. There was some discussion of combining *Aphanostoma* and *Praeconvoluta* (Nilsson *et al.*, 2011; Zauchner *et al.*, 2015), since the general morphologies of the species of the two genera are the same, although no formal synonymization ever actually occurred.

All species of *Praeconvoluta* in our analyses form a clade with *Aphanostoma bruscai* Dörjes, 1968 and

A. collinae Hooge & Tyler, 2008, a result that is not surprising, given that the genera are morphologically indistinguishable from each other (Hooge & Tyler, 2005; Zauchner *et al.*, 2015). This clade also included *Isodiametra norvegica* (Westblad, 1946), the type species of *Isodiametra*, and *I. hortulous* (Hooge & Tyler, 2003) (Fig. 1). Thus, this clade includes species with all forms of bursa morphology (Fig. 5): simple without adornments (*P. tigrina*), with a cellular cap reported with (*A. bruscai*, *A. collinae* and *P. bocasensis*) or without (*P. castinea* Hooge & Tyler, 2003 and *P. tornuva* Hooge & Tyler, 1999) spots of actin and with a fully sclerotized nozzle (*I. norvegica* and *I. hortulous*). The type species of *Aphanostoma*, *A. virescens*, was not included in this group, but formed a clade with *Otocelis erinae*, *Raphidophallus actuosus* and seven other species of *Isodiametra* (Fig. 1). The bursal appendages in the species of this clade are more similar to each other, since all species, except for *A. virescens*, possess a bursa with a sclerotized nozzle (Fig. 5), and the morphology of the bursal cap of the latter is somewhat unique. *Aphanostoma virescens* has a large and highly muscular bursal cap with numerous (six to eight according to Steinböck, 1931, although Graff, 1905, recorded as many as 14) sclerotic spines. Both clades were maximally supported.

Our results indicate that, while the presence or absence of a walled/true bursa may be useful to distinguish genera of Isodiametridae, the details and composition of the bursal appendage is not (Fig. 5). This is perhaps not so surprising given the similarities in the composition of the different bursal appendages. Each sclerotized bursal nozzle in Acoela is composed of a sperm duct with actin-reinforced inner edges, while the bursal cap of at least some species includes disjunct spots of intracellular actin localized along a sperm duct (Petrov *et al.*, 2006). Further, because intracellular actin is not always visible in squeeze preparations and living specimens, it can easily be missed if the bursal cap of a species has not been properly examined with phalloidin-labelled probes and fluorescence microscopy (Hooge & Tyler, 2008). Petrov *et al.* (2006) warned that actin-sclerotized sections of bursal caps could be confused with muscle fibres following conventional staining by iron haematoxylin and, in fact, they were highly skeptical that muscles were a component of any bursal appendage in Acoela at all. Thus, the analysis of bursal morphology may be incomplete or incorrect in some species considered to have only muscular or cellular caps, especially for species with older descriptions.

Below, we attempt to update the current classification system to reflect as best as possible their phylogenetic relationships as represented by the results of our molecular analyses (Fig. 1), and we reassign species to, and emend, the genera accordingly. Information

gathered from live observations and original descriptions of each species included in the analyses form the basis for the morphological summary of each genus, while those species for which sequence data are currently unavailable, were classified based on morphology. Specifically, our results supported the existence of two clades representing two genera with walled bursas (Figs 1, 5), which appeared to be best distinguished morphologically based on the presence or absence of one or more distinct muscular vaginal sphincters (Fig. 9). This result notably concurs with the findings of Jondelius *et al.* (2011) that details of the musculature have higher potential to be taxonomically informative at the genus level for Isodiametridae. Nevertheless, we must underscore the need for further detailed morphological study using modern methodology to clarify any potential discrepancies that may exist in the reproductive anatomy and musculature, as well as the importance of obtaining DNA sequences of the remaining species of the genera.

EMENDED DIAGNOSIS OF *APHANOSTOMA*
ØRSTED, 1845 AND REASSIGNMENT OF SPECIES;
SYNONYMIZATION OF *RAPHIDIOPHALLUS* KOZLOFF,
1965 AND *APHANOSTOMA* ØRSTED, 1845

Aphanostoma is hereby emended to include the species that form a clade with its type species, *A. virescens*. *Isodiametra bajaensis* Hooge & Eppinger, 2005, *I. cuernos* Hooge & Tyler, 2008, *I. divae* (Marcus, 1950), *I. finkei* Kånneby & Jondelius, 2013, *I. nicki* Hooge & Tyler, 2008, *I. pulchra* (Smith & Bush, 1991), *Otocelis erinae* and *Raphidophallus actuosus* are all transferred to *Aphanostoma*.

Raphidophallus was distinguished from other genera solely based on the delicate cuticularized rods that occurred in the lumen of the penis. Otherwise, Kozloff (1965) noted that its morphology was highly similar to other species of *Isodiametra*, particularly with reference to the bursa with nozzle, which the majority of the species in the clade do also possess (although see notes above). *Raphidophallus actuosus* is the type and only species of its genus and, therefore, *Raphidophallus* is considered a junior synonym of *Aphanostoma*.

All species in this clade are united by the possession of a muscular bursa with evident actin in the form of a nozzle or spines, a distinct male or common atrium and one or more well-developed vaginal sphincters. Of the species for which DNA is currently unavailable, *Isodiametra earnhardti* (Hooge & Smith, 2004), *I. helgolandica* (Dörjes, 1968), *I. karpredi* (Hooge & Tyler, 2003), *I. vexillaria* (Marcus, 1948) and *I. westbladi* (Marcus, 1949) all also possess these characters and are, therefore, transferred to *Aphanostoma*. In addition, *Aphanostoma album* and *A. rhomboides* Jensen, 1878 both possess strong vaginal sphincters that are characteristic of the

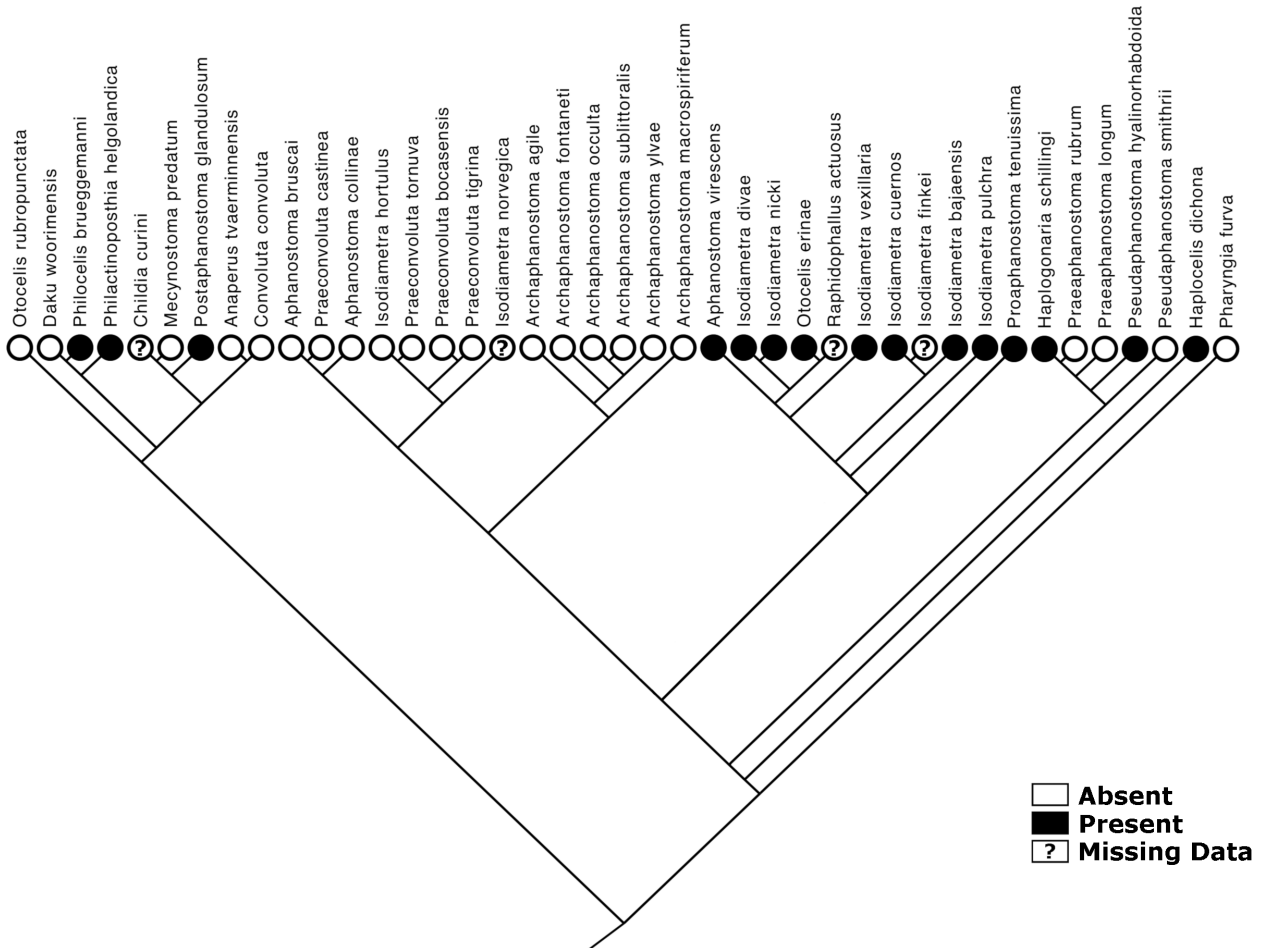


Figure 9. Vaginal sphincter presence or absence in species of Isodiometridae mapped on to the concatenated tree. The morphology of each species was coded following original descriptions and illustrations, as well as photos and live observation of species where possible. ? indicates that the presence or absence of a vaginal sphincter was not examined/could not be determined.

genus, although neither have the evident spots of actin in their bursal caps. Neither species has been examined with phalloidin-labelled probes.

SYNONYMIZATION OF *PRAECONVOLUTA* DÖRJES, 1968 AND *ISODIAMETRA* HOOGE & TYLER, 2005; EMENDED DIAGNOSIS OF *PRAECONVOLUTA* DÖRJES, 1968 AND REASSIGNMENT OF SPECIES

All four species of *Praeconvoluta* group with *Aphanostoma bruscai* Hooge & Tyler, 2003, *A. collinae* Hooge & Tyler, 2008, *Isodiametra norvegica* and *I. hortulous* in our analyses to form a second clade of species of Isodiometridae with true/walled bursas (Fig. 5). The species of this clade are united together and distinguished from *Aphanostoma* through a lack of well-defined vaginal sphincter muscles (Fig. 9). Nine of the 16 species with true bursas without DNA sequences currently available all lack well-defined

vaginal sphincters, including all remaining four species of *Praeconvoluta*, *Aphanostoma piscae* Zauchner *et al.*, 2015, *Isodiametra colorata* (Ehlers & Dörjes, 1979), *I. marginalis* (Ivanov, 1952), *I. urua* (Marcus, 1954) and *I. variomorpha* (Dörjes, 1968).

Isodiametra norvegica is the type species of *Isodiametra*, which was proposed by Hooge & Tyler (2005) for species with a single bursa nozzle. According to our phylogenetic hypothesis, this genus is polyphyletic with the majority of the species grouping separately from the type species. *Isodiametra norvegica* groups with the four species of *Praeconvoluta* included in the analyses. Although DNA sequences are not available for the type species *Praeconvoluta karinae*, it and other species of the genus lack well-defined vaginal sphincters. Based on our phylogenetic hypothesis, *Praeconvoluta* and *Isodiametra* are synonymized such that *Isodiametra* is a junior synonym of *Praeconvoluta*.

Isodiametra was the type genus of Isodiametridae. Nevertheless, no name replacement for the family is necessary in this instance since replacing Isodiametridae with another name would be exclusively due to the type genus being considered a junior synonym (see ICZN section 40.1). Hence Isodiametridae is retained and *Praeconvoluta* becomes the type genus.

CONCLUSIONS

The classification system of Isodiametridae has been updated to be consistent with its phylogeny following the results of molecular analyses that included sequence data from 128 species of Acoela and 16 of the 22 genera of Isodiametridae. Following the transfer of *Otocelis* to Otocelididae, *Postaphanostoma* and *Faerlea* to Mecynostomidae and *Alluna* to Actinoposthiidae, as well as the synonymisation of six other genera, Isodiametridae now includes 12 genera and 78 nominal species. Below we provide a morphological summary of the genera of Isodiametridae and a family key.

TAXONOMIC SUMMARY OF ISODIAMETRIDAE

APHANOSTOMA ØRSTED, 1845

With or without orange pigmented eyespots in the anterior. Rhabdoids present in rows. Frontal glands often prominent. Testes paired. Ovaries paired or unpaired. Male system includes curved, muscular penis invaginated into a muscular seminal vesicle and a distinct male or common atrium. Penis glandular, may have granule secretions or delicate cuticular rods in lumen. Female system includes distinct vagina with one or more well-developed sphincter muscles and walled bursa with cap or sclerotized single nozzle. Bursal cap may have multiple spines. Gonopore common or separate; if separate, female pore may be either anterior or posterior to male pore. Type species: *Aphanostoma virescens* Ørsted, 1845.

Marine, free-living, 16 species:

- *Aphanostoma virescens* Ørsted, 1845
- *Aphanostoma actuosus* (Kozloff, 1965), **comb. nov.**
- *Aphanostoma album* Dörjes, 1968
- *Aphanostoma bajaensis* (Hooge & Eppinger, 2005), **comb. nov.**
- *Aphanostoma cuernos* (Hooge & Tyler, 2008), **comb. nov.**
- *Aphanostoma divae* (Marcus, 1950), **comb. nov.**
- *Aphanostoma earnhardti* (Hooge & Smith, 2004), **comb. nov.**
- *Aphanostoma erinae* (Hooge & Rocha, 2006), **comb. nov.**

- *Aphanostoma finkei* (Kånneby & Jondelius, 2013), **comb. nov.**
- *Aphanostoma helgolandica* (Dörjes, 1968), **comb. nov.**
- *Aphanostoma kapredi* (Hooge & Tyler, 2003), **comb. nov.**
- *Aphanostoma nicki* (Hooge & Tyler, 2008), **comb. nov.**
- *Aphanostoma pulchra* (Smith & Bush, 1991), **comb. nov.**
- *Aphanostoma rhomboides* Jensen, 1878
- *Aphanostoma vexillaria* (Marcus, 1948), **comb. nov.**
- *Aphanostoma westbladi* (Marcus, 1949), **comb. nov.**

AVAGINA LEIPER, 1902

Frontal glands weakly developed. Rhabdoids absent. Paired testes and paired or unpaired ovaries. Male system includes a muscular seminal vesicle, an eversible muscular penis and a ventral or subterminal gonopore. Antrum masculinum and female reproductive accessory organs absent. Type species: *Avagina incola* Leiper, 1902.

Marine, free-living or parasitic; six species:

- *Avagina incola* Leiper, 1902
- *Avagina glandulifera* Westblad, 1953
- *Avagina marci* Dörjes & Karling, 1975
- *Avagina polyvacuola* Ehlers & Dörjes, 1979
- *Avagina sublitoralis* Faubel, 1976
- *Avagina vivipara* Hickman, 1956

Notes

Avagina incola was first described by Leiper in 1902. Graff subsequently moved it to *Haplodiscus* in 1905 arguing that the description was not thorough enough to warrant another genus. Westblad then moved it back to *Avagina* in 1948 based on the paired testes and parasitic lifestyle, where it has remained since. At the time of writing, *Avagina incola* is incorrectly listed as a synonym of *Haplodiscus incola* in WoRMS (Tyler *et al.*, 2006–21).

Three genera of Isodiametridae include species that lack a seminal bursa yet have a muscular seminal vesicle (*Baltalimania*, *Pharyngia* and *Pseudaphanostoma*). *Avagina* differs from all three genera through the absence of rhabdoids and a distinct antrum masculinum. Otherwise, *Avagina* is similar to *Alluna*, a genus of Actinoposthiidae, and *Faerlea*, a genus that molecular analyses suggests belongs in Mecynostomidae. The former is distinguishable by the morphology of the seminal vesicle, which is muscular in

Avagina and non-muscular with paired, false seminal vesicles in *Alluna*, while the sole defining morphological difference for the latter is the small antrum masculinum in species of *Faerlea* that is absent in species of *Avagina*. Further investigation, including obtaining DNA sequences for, especially, *Avagina incola*, is needed to assess the validity of the genus and the position within Isodiametridae and Acoela.

BALTALIMANIA Ax, 1959

Frontal organ well developed. Rhabdoid glands present in longitudinal rows. Yellow-orange lipid globules often present. Testes paired; ovaries paired or unpaired. Male system includes a muscular seminal vesicle, a muscular penis, a distinct antrum masculinum and a gonopore. Penis distinctly curved, typically relatively large, completely or incompletely invaginated into the seminal vesicle. Antrum masculinum ciliated or unciliated. Gonopore terminal or subterminal. Female bursa absent or, if present, indistinct without walls and without any adornments. Type species: *Baltalimania kosswigi* Ax, 1959.

Marine, free-living; nine species:

- *Baltalimania kosswigi* Ax, 1959
- *Baltalimania agile* Jensen, 1878
- *Baltalimania fontaneti* (Kånneby *et al.*, 2015), **comb. nov.**
- *Baltalimania histobursalium* (Dörjes, 1968), **comb. nov.**
- *Baltalimania macrospiriferum* Westblad, 1946
- *Baltalimania marcusi* (Hooge & Rocha, 2006), **comb. nov.**
- *Baltalimania occulta* (Kånneby *et al.*, 2015), **comb. nov.**
- *Baltalimania sublittoralis* (Kånneby *et al.*, 2015), **comb. nov.**
- *Baltalimania ylvae* (Kånneby *et al.*, 2015), **comb. nov.**

DIATOMOVORA KOZLOFF, 1965

Rhabdoids present in distinct longitudinal rows or scattered. Paired or unpaired ovary and paired testes. Male system includes a highly muscular, curved penis partially invaginated into a seminal vesicle. Female system includes a heavily muscularized vagina and one or two muscular seminal bursae with two to six cuticularized nozzles each. Common gonopore ventral. Common, ciliated genital atrium may be present. Type species: *Diatomovora amoena* Kozloff, 1965.

Marine, free-living; two species:

- *Diatomovora amoena* Kozloff, 1965
- *Diatomovora jacki* Hooge & Tyler, 2008

Notes

Species of *Diatomovora* have been reported with a wide amount of morphological variability in the female anatomy. *Diatomovora amoena*, as originally described by Kozloff (1965), was characterized by a single bursa with two bursal nozzles, a highly muscular vagina and a common ciliated antrum. Dörjes & Karling (1975) re-examined materials of the same species deposited in SMNH and found specimens with one or two bursae and three nozzles per bursa. Hooge & Tyler (2008) described *Diatomovora jacki* with a single bursa with ‘~6 bursal nozzles’ that were not visible in squeeze preparations or live materials. The nozzles were visible in the actin-stained whole mounts viewed with fluorescence microscopy.

The only other species of Isodiametridae that possesses a bursa with multiple nozzles is *Haplocelis dichona*. Species of *Diatomovora* and *Haplocelis* are easily distinguished through the positioning of the gonopore (ventral in *Diatomovora*, terminal in *Haplocelis*) and the positioning of the vagina (anterior to the male copulatory organ in *Diatomovora*, dorsal to the male copulatory organ in *Haplocelis*).

HAPLOCELIS DÖRJES, 1968

Without body pigmentation. Rhabdoids present, scattered across body. Paired testes and ovaries. Vagina dorsal to the male copulatory organ. Male system includes a muscular, glandular penis invaginated into a muscular seminal vesicle. Female system includes ciliated, muscular vagina and a bursa with one or two coiled bursal nozzles. Common gonopore terminal. Type species: *Haplocelis dichona* (Marcus, 1954).

Marine, free-living; one species:

- *Haplocelis dichona* (Marcus, 1954), Dörjes 1968

PHARYNGIA NILSSON ET AL., 2011

Pharynx present. With dark-brown body pigmentation. Testes paired; ovary unpaired. Male system includes small penis invaginated into muscular seminal vesicle, well-developed ciliated antrum masculinum and ventral gonopore. Seminal bursa or any female accessory organs absent. Type species: *Pharyngia furva* Nilsson *et al.*, 2011.

Marine, free-living; one species:

- *Pharyngia furva* Nilsson *et al.*, 2011

Notes

Two other species of Isodiametridae have a pharynx (*Isodiametra helgolandica* and *Praeaphanstoma*

longum). Species of *Pharyngia* can be separated from both based on the absence of a seminal bursa.

PRAEAPHANOSTOMA DÖRJES, 1968

With or without body pigmentation. With small frontal organ. Rhabdoids small, often inconspicuous, in rows. Testes paired. Ovaries paired or unpaired. Male system includes a straight, tubular penis partially or completely invaginated into a muscular seminal vesicle. Female bursa present without appendage or cellular cap. Vagina with sphincter typically present. Gonopore(s) separate or common, ventral. Type species: *Praeaphanostoma chaetocaudatum* Dörjes, 1968.

Marine, free-living; 16 species:

- *Praeaphanostoma chaetocaudatum* Dörjes, 1968
- *Praeaphanostoma baltalimaniaformis* (Dörjes, 1968), **comb. nov.**
- *Praeaphanostoma brevifrons* Dörjes, 1968
- *Praeaphanostoma foramivora* Hooge & Tyler, 2008
- *Praeaphanostoma gusana* Hooge & Eppinger, 2005
- *Praeaphanostoma hyalinorhabdoida* (Känneby & Jondelius, 2013), **comb. nov.**
- *Praeaphanostoma longum* Dörjes, 1968
- *Praeaphanostoma musculosum* Ehlers & Dörjes, 1979
- *Praeaphanostoma ornatus* (Kozloff, 2000), **comb. nov.**
- *Praeaphanostoma parvum* Rieger & Ott, 1971
- *Praeaphanostoma rubrum* Dörjes, 1968
- *Praeaphanostoma schillingi* (Hooge & Tyler, 2015), **comb. nov.**
- *Praeaphanostoma sizilianum* (Riedl, 1954) Dörjes, 1968
- *Praeaphanostoma thalassophilum* Ehlers & Dörjes, 1979
- *Praeaphanostoma vitreum* Ehlers & Dörjes, 1979
- *Praeaphanostoma wadsworthi* Hooge & Tyler, 2003

PRAECONVOLUTA DÖRJES, 1968

Rhabdoids absent or sparsely present and small, scattered or in longitudinal rows. Frontal organ present. Testes paired. Ovary single or paired. Male system includes a muscular penis capped with a prostatic vesicle and invaginated into a muscular seminal vesicle. Female system includes a bursa with distinctly thick walls. Bursal appendage may be absent or present; if present may be a distinct nozzle or a cellular cap with or without one or more spots of concentrated actin. Vagina typically present without a well-developed sphincter. Sphincter always absent or weak. Gonopore common or male only, ventral. Type species: *Praeconvoluta karinae* Dörjes, 1968.

Marine, free-living; 17 species:

- *Praeconvoluta karinae* Dörjes, 1968
- *Praeconvoluta bocasensis* Hooge & Tyler, 2008
- *Praeconvoluta bruscai* (Hooge & Tyler, 2003), **comb. nov.**
- *Praeconvoluta castinea* Hooge & Tyler, 2003
- *Praeconvoluta collinae* (Hooge & Tyler, 2008), **comb. nov.**
- *Praeconvoluta colorata* (Ehlers & Dörjes, 1979), **comb. nov.**
- *Praeconvoluta hortulous* (Hooge & Tyler, 2003), **comb. nov.**
- *Praeconvoluta marginalis* (Ivanov, 1952), **comb. nov.**
- *Praeconvoluta minor* Faubel, 1974
- *Praeconvoluta norvegica* (Westblad, 1946), **comb. nov.**
- *Praeconvoluta piscae* (Zauchner *et al.*, 2015), **comb. nov.**
- *Praeconvoluta schmidti* Faubel, 1977
- *Praeconvoluta stephania* Faubel & Regier, 1983
- *Praeconvoluta tigrina* Hooge & Tyler, 2003
- *Praeconvoluta tornuva* Hooge & Tyler, 1999
- *Praeconvoluta urua* (Marcus, 1954), **comb. nov.**
- *Praeconvoluta variomorpha* (Dörjes, 1968), **comb. nov.**

PROAPHANOSTOMA DÖRJES, 1972

Numerous vacuoles present. With frontal organ. Rhabdoids absent. Paired testes and ovaries. Male system includes penis invaginated into a seminal vesicle. Penis an inpocketing of epidermis. Antrum masculinum absent. Seminal bursa present with cellular-muscular appendage and entrance sphincter. Gonopores separate. Type species: *Proaphanostoma tenuissima* Dörjes, 1972.

Marine, free living; one species:

- *Proaphanostoma tenuissima* Dörjes, 1972

PSEUDAPHANOSTOMA WESTBLAD, 1946

Frontal organ present. Rhabdoids small and in longitudinal rows. Ovaries and testes paired or unpaired. Male system consists of a straight, eversible penis fully invaginated into a muscular seminal vesicle and a distinct, ciliated antrum masculinum. The seminal vesicle attaches directly to the antrum masculinum. False seminal vesicle present or absent. Male genital opening usually terminal or subterminal. Female accessory organs absent. Type species: *Pseudaphanostoma variabilis* Westblad, 1946.

Marine, free-living; eight species:

- *Pseudaphanostoma variabilis* Westblad, 1946
- *Pseudaphanostoma brevicaudatum* Dörjes, 1968
- *Pseudaphanostoma divae* Marcus, 1952

- *Pseudaphanostoma herringi* Hooge & Rocha, 2006
- *Pseudaphanostoma murmanicus* (Mamkaev, 1967) Dörjes, 1968
- *Pseudaphanostoma pelophilum* Dörjes, 1968
- *Pseudaphanostoma psammophilum* Dörjes, 1968
- *Pseudaphanostoma smithi* Hooge & Tyler, 2003

PSEUDOPOSTHIA WESTBLAD, 1946

Without body pigmentation. With frontal organ and numerous small rhabdoid glands. Testes and ovaries paired. Male system includes a muscular penis associated with a separate glandular organ, a false seminal vesicle and a gonopore. False seminal vesicle not connected to the penis. True seminal vesicle absent. Glandular organ opens together with the penis at the gonopore. Male gonopore ventral, located at mid-body. Female accessory organs absent. Type species: *Pseudoposthia macrogonopora* Westblad, 1946.

Marine, free-living; one species:

- *Pseudoposthia macrogonopora* Westblad, 1946

RIMICOLA BÖHMIG, 1908

Without body pigmentation. Frontal organ present; rhabdoid glands absent. With paired testes and ovaries. Male system includes a well-developed penis, paired false seminal vesicles, a short antrum masculinum and ventral gonopore. True seminal vesicle absent. Female accessory organs absent. Type species: *Rimicola glacilis* Böhmig, 1908.

Marine, free-living; one species:

- *Rimicola glacilis* Böhmig, 1908

Notes

Rimicola can be distinguished from most of the other genera of Isodiametridae by the absence of rhabdoid glands, a muscular seminal vesicle and female accessory organs. It is closest in morphology to *Pseudoposthia*, which also lacks a true seminal vesicle and bursa, but *Pseudoposthia* can be differentiated through the absence of an antrum masculinum and the presence of rhabdoids

KEY TO ISODIAMETRIDAE

- | | |
|---|--|
| 1a. Seminal bursa absent | 2 |
| 1b. Seminal bursa present | 7 |
| 2a. Pharynx absent | 3 |
| 2b. Pharynx present | <i>Pharyngia</i> |
| 3a. Penis distinct. True seminal vesicle absent | 4 |
| 3b. Penis at least partially invaginated into muscular seminal vesicle | 5 |
| 4a. Rhabdoid glands absent. Male system includes false seminal vesicle, short antrum masculinum and muscular penis | <i>Rimicola</i> |
| 4b. Rhabdoid glands present. Male system includes muscular penis associated with a separate glandular organ and a separate false seminal vesicle | <i>Pseudoposthia</i> |
| 5a. Rhabdoids absent | <i>Avagina</i> |
| 5b. Rhabdoids present, usually in longitudinal rows | 6 |
| 6a. Penis well developed and distinctly curved, only partially invaginated into seminal vesicle | <i>Baltalimania</i> (<i>B. fontaneti</i> , <i>B. occulta</i> , <i>B. sublitoralis</i> , <i>B. ylvae</i>) |
| 6b. Penis straight or nearly so, fully invaginated into seminal vesicle | <i>Pseudaphanostoma</i> |
| 7a. Bursa with distinct muscular walls | 8 |
| 7b. Bursa tissue only | <i>Baltalimania</i> (<i>B. agile</i> , <i>B. histobursalim</i> , <i>B. macrospiriferum</i> , <i>B. marcusii</i>) |
| 8a. Common gonopore terminal; vagina dorsal to male copulatory organ; bursa with one or two large, coiled nozzles | <i>Haplocelis</i> |
| 8b. Gonopore usually ventral or subterminal. If terminal, gonopores separate, with female pore anterior (ventral) to male pore (terminal). Vagina never dorsal to male copulatory organ | 9 |
| 9a. One or two bursas present, each with multiple sclerotized nozzles | <i>Diatomovora</i> |
| 9b. Only a single bursa present, always without multiple sclerotized nozzles | 10 |
| 10a. Rhabdoids absent. Antrum masculinum absent. Penis an inpocketing of the epidermis | <i>Proaphanostoma</i> |
| 10b. Rhabdoids present, scattered or in longitudinal rows | 11 |
| 11a. Vaginal sphincter weak or absent | <i>Praeconvoluta</i> |
| 11b. Vaginal sphincter muscles well developed | 12 |
| 12a. Frontal glands prominent. Penis curved. Bursal appendage present as nozzle or cap | <i>Aphanostoma</i> |
| 12b. Frontal glands weak. Penis straight. Bursa always simple without appendage | <i>Praeaphanostoma</i> |

and a glandular organ associated with the male copulatory organ.

Rimicola is also similar in morphology to other species of *Faerlea* (Mecynostomidae). Species of both lack rhabdoids and a seminal bursa and have a small antrum masculinum. Unlike *Rimicola*, species of *Faerlea* are characterized, among other things, by a short penis with associated glands and a muscular seminal vesicle. Further investigation, including obtaining DNA sequences, is needed to assess the position of the genus within Isodiametridae and Acoela.

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ETHICS STATEMENTS

All necessary permits for field study and sampling have been obtained by the authors from the competent authorities. The study is compliant with the United Nations Convention of Biological Diversity (CBD) and Nagoya protocols.

DATA AVAILABILITY

The data underlying this article are available in the GenBank Nucleotide Database at <https://www.ncbi.nlm.nih.gov/genbank/>, and can be accessed with accession numbers MZ518796-816, MZ519760-776, and MZ520441-463.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Table S1. List of species used in this study along with the corresponding collection country and GenBank accession number, where available. New genetic sequences are highlighted in bold. **the five species of Isodiametridae and Actinoposthiidae with unstable positions that were excluded from analyses.

Table S2. Morphological character matrix. The morphology of each species was coded following original descriptions and illustrations, as well as photos and live observations where possible. ? indicates the presence or absence of the character could not be determined or was not examined.

Figure S1. Concat tree full. Concatenated 28S, 18S and COI gene tree summary. Percent bootstrap values are given at each node. Sequences from all species in the study were included.

Figure S2. 18S tree full. 18S gene tree summary. Percent bootstrap values are given at each node. Sequences from all species in the study were included.

Figure S3. 18S tree full GBlocks. 18S gene tree summary. The alignment was filtered with GBlocks allowing for gap positions within the final blocks. Percent bootstrap values are given at each node. Sequences from all species in the study were included.

Figure S4. 18S tree part. 18S gene tree summary. Percent bootstrap values are given at each node. Sequences from six species of Isodiametridae and Actinoposthiidae represented only by 18S sequences downloaded from GenBank ([Table 1](#)) were excluded.

Figure S5. 18S tree part GBlocks. 18S gene tree summary. The alignment was filtered with GBlocks allowing for gap positions within the final blocks. Percent bootstrap values are given at each node. Sequences from six species of Isodiametridae and Actinoposthiidae represented only by 18S sequences downloaded from GenBank ([Table 1](#)) were excluded.

Figure S6. COI tree. Mitochondrial Cytochrome C Oxidase (COI) gene tree summary. Percent bootstrap values are given at each node.

Figure S7. 28S tree. 28S gene tree summary. Percent bootstrap values are given at each node.

Figure S8. 28S tree GBlocks. 28S gene tree summary. The alignment was filtered with GBlocks allowing for gap positions within the final blocks. Percent bootstrap values are given at each node.