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Research Article

Grappleria corona gen. et sp. nov. (Platyhelminthes: Rhabdocoela: Jenseniidae fam. nov.) and an updated molecular phylogeny of 'dalyelliid' and temnocephalid microturbellarians

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Species of rhabdocoels within the family Dalyelliidae (Platyhelminthes) generally inhabit fresh water and are rare in marine and brackish environments. Here we describe a new species of marine dalyelliid, *Grappleria corona* gen. et sp. nov., from the Pacific coast of British Columbia. Based on the distinctive morphology of its male copulatory organ and a thorough comparison of traits in all known species of marine and brackish water dalyelliids, we place this new species in a genus of its own. Phylogenetic analyses inferred from rRNA gene sequences derived from *G. corona* gen. et sp. nov., other representatives of the Dalyelliidae and the closely related Temnocephalida, provide justification for an updated taxonomic classification of these groups. In light of these molecular phylogenetic data, we establish the new family Jenseniidae fam. nov. to encompass genera previously considered dalyelliids but are found only in marine and brackish environments; the composition of the Dalyelliidae sensu novo now contains only freshwater genera. We also classify all temnocephalids into one family and into several subfamilies in order to reflect our improved understanding of rhabdocoel phylogeny.

http://www.zoobank.org/urn:lsid:zoobank.org:pub:9DA178AE-5038-4379-AAA1-BEE19565FEF6

Key words: Dalyelliidae, Dalytyphloplanida, flatworms, microturbellaria, Rhabdocoela, species discovery, Temnocephalida

Introduction

With approximately 190 species, the Dalyelliidae Graff, 1908, is among the most species-rich families of rhabdocoels. Together with representatives of the Typhloplanidae Graff, 1905, and Temnocephalida Bresslau & Reisinger, 1933, dalyelliids are part of a larger group of predominantly freshwater microturbellarians called the Limnotyphloplanida Van Steenkiste et al., 2013.

Currently, 174 species of dalyelliids occur in freshwater environments around the world (Balsamo et al., 2020); however, seven genera occur only in marine and brackish water habitats: *Alexlutheria* Karling, 1956, *Axiola* Luther, 1955, *Beauchampiola* Luther, 1955, *Halammovortex* Karling, 1943, *Jensenia* Graff, 1882, *Polliculus* Van Steenkiste et al., 2008, and *Thalassovortex* Papi, 1956. Most of these 'marine' dalyelliids also have distinct morphological characters setting them apart from the more uniform 'freshwater' genera, with only a few of these species able to tolerate brackish water conditions (Ax, 2008; Luther, 1955). This observation is supported by a molecular phylogeny of dalytyphloplanid rhabdocoels, in which *Jensenia* and *Halammovortex* are more closely related to representatives of the Temnocephalida than to the clade composed of 'freshwater' dalyelliids (Van Steenkiste et al., 2013). With the exception of one species, dalyelliids are all free-living (i.e. not living in association with another host animal). In contrast, temnocephalids are epibionts associated with freshwater invertebrates and turtles. This ectosymbiotic lifestyle is unusual among freshwater

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rhabdocoels and has resulted in several morphological adaptations including a well-developed posterior adhesive organ and anterior tentacle-like modifications in most genera. However, the phylogenetic relationship between 'marine' dalyelliids and freshwater temnocephalids is not clear in previous molecular phylogenetic analyses because only six taxa were available at the time (Van Steenkiste et al., 2013).

Here, we describe a new genus and species of marine dalyelliid from the Pacific coast of British Columbia: *Grappleria corona* gen. et sp. nov. We discuss its taxonomic position based on morphological data, including a morphological comparison among all known species of 'marine' dalyelliids. We also provide a molecular phylogeny inferred from 18S and 28S rDNA sequences from this new genus and representatives of the Dalyelliidae and Temnocephalida. Building upon these results, we update the current taxonomic classification of these groups within the Limnotyphloplanida.

Materials and methods

Collection and microscopy

Live individuals of *Grappleria corona* gen. et sp. nov. were collected in January 2020 from the rocky intertidal in Bamfield, British Columbia, Canada. Intertidal algae were handpicked from the rocks during low tide and transferred to the lab. Live animals were separated from the algae using the $MgCl_2$ decantation method (Schockaert, 1996). Individual worms were isolated and observed under a stereoscope and subsequently whole mounted alive to be studied and photographed at the Bamfield Marine Sciences Centre (BMSC) under an Olympus FluoView 300 microscope using DIC and epifluorescence optics. Some specimens were preserved with lactophenol to study the sclerotized parts of the male copulatory system, commonly known as the stylet. These preserved specimens were photographed and measured with a Zeiss Axioplan 2 microscope equipped with a Zeiss Axiocam 503 colour camera and using the Zeiss ZEN 2 software. All measurements were taken along the axis of the animal or the stylet, unless indicated otherwise in the text. The composite image of a preserved whole mount in Fig. 7 was produced in Helicon Focus (HeliconSoft) by stacking a series of micrographs to produce a greater depth of field. Micrographs were edited with Adobe Photoshop v21.1.1 for contrast and brightness and figures were put together in Adobe Illustrator v24.1, including the drawings in Figs 2 and 11 and Figs S1–S19.

The material from *G. corona* gen. et sp. nov. was thoroughly compared with the existing primary literature for a differential diagnosis (see references in Table S1). The holotype and paratypes were deposited in the Beaty Biodiversity Museum (BBM, University of British Columbia, Vancouver, Canada).

DNA sequencing

Genomic DNA was extracted from entire individuals using the DNeasy Blood & Tissue kit (Qiagen). Manufacturer's instructions were followed, with the exception that DNA was eluted in 60 µL of preheated AE elution buffer (60 °C). Fragments of the nearly complete 18S (1787 bp) and partial 28S rRNA (1669 bp) genes were PCR amplified using Illustra^{TM} PuReTaq^{TM} Ready-To-Go^{TM} PCR beads (GE Healthcare), and the primer pairs TimA (5′-AMCTGGTTGATCCTGCCAG-3′) and TimB (5′-TGATCCATCTGCAGGTTCACCT-3′), and LSU5 (5′-TAGGTCGACCCGCTGAAYTTA-3′) and LSUD6.3B (5′-GCTGTTCACATGGAACCCT TCTC-3′), respectively. Thermocycling conditions were as follows: 95 °C for 3 min, touch down in 9 cycles (94 °C for 30 s, 60 °C down to 56 °C for 30 s, 72 °C for 1 min 30 s), 31 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min 30 s), 72 °C for 5 m. Amplicons were visualized on 1.5% agarose gels stained with GelRed^{TM} (Biotium), enzymatically cleaned with Illustra^{TM} ExoProStar S (GE Healthcare), and subsequently sequenced by Genewiz (Brooks Life Sciences) through standard Sanger DNA sequencing using the amplification primers and the following internal sequencing primers: 600 F (5′-GGTGCCAGCAGCCGCGGT-3′), 600 R (5′-ACCGCGGCTGCTGGCACC-3′), 1100 F (5′-CAG AGGTTCGAAGACGATC-3′), 1100 R (5′-GATCGTC TTCGAACCTCTG-3′), 18S7F (5′-GCAATAACAGGT CTGTGATGC-3′), and 18S7FK (5′-GCATCACAGA CCTGTTATTGC-3′) for the 18S rRNA amplicon; L300F (5′-CAAGTACCGTGAGGGAAAGTTG-3′), L300R (5′-CAACTTTCCCTCACGGTACTTG-3′), L1600F (5′-GCAGGACGGTGGCCATGGAAG-3′), and L1600R (5′-CTTCCATGGCCACCGTCCTGC-3′) for the 28S rRNA amplicon.

Resulting trace files were assembled into full sequences in Geneious v10.2.3 (www.geneious.com, Kearse et al., 2012) and subjected to a BLAST search on the NCBI website (http://blast.ncbi.nlm.nih.gov) to verify the samples' taxonomic identity. Both sequences were deposited in GenBank and sequence accession numbers are provided in Table 1.

Molecular phylogenetic analyses

The new 18S and 28S rDNA sequences from *G. corona* gen. et sp. nov. were aligned with rDNA sequences from 18 other representatives of free-living

Table 1. 18S and 28S GenBank accession numbers of the taxa used in the phylogenetic analyses.

Taxa	18S accession number	28S accession number
‘Dalyelliidae’		
Castrella truncata	KC529439	KC529570
Gieysztoria knipovici	KC529463	KC529594
Dalyellia viridis	KC529444	KC529575
Pseudodalyellia alabamensis	KC529440	KC529571
Microdalyellia fusca	KC529453	KC529584
Jensenia angulata	–	KC529568
Halammovortex sp.	KC529437	KC529567
Grappleria corona gen. et sp. nov.	MW052803	MW052802
Temnocephalida		
Scutariella sinensis	MF773690	MF773687
Scutariella sinensis	MF773692	MF773689
Didymorchis sp.	AY157182	AY157163
Decadidymus sp.	MG345101	MG345102
Diceratocephala boschmai	KC517073	–
Diceratocephala boschmai	MG725689	MG725695
Temnocephala sp.	AF051332	–
Temnocephala sp.	AJ012520	–
Craspedella pedum	MN073837	–
Temnosewellia fasciata	–	KC869888
Temnosewellia minor	AY157183	AY157164

dalyelliids and symbiotic temnocephalids downloaded from GenBank (Table 1). The 18S and 28S rDNA sequences were aligned in MAFFT using the Q-INSI algorithm (Katoh & Toh, 2008). Ambiguous positions were selected in Aliscore v2.2 (Misof & Misof, 2009; window size 6), and removed from the alignments with Alicut v2.3 (Kueck, 2009) to produce curated alignments. These curated 18S rDNA and 28S rDNA alignments were concatenated in Geneious v10.2.3.

Best-fit partitions and models of molecular evolution corresponding to the 18S and 28S rDNA datasets and GTR + GAMMA + I were recovered for the concatenated dataset (18S + 28S) in PartitionFinder v.1.1.0 using a greedy search with PhyML and the Bayesian information criteria (BIC) (Lanfear et al., 2012). These partition schemes and models were subsequently used in the phylogenetic analyses of the concatenated dataset. The maximum likelihood (ML) analysis was performed with the RAxML v8.2.11 plugin (Stamatakis, 2014) in Geneious v10.2.3 selecting the algorithm for best-scoring ML tree search and non-parametric bootstrapping (1000 replicates). Five freshwater dalyelliids, *Castrella truncata*, *Gieysztoria knipovici*, *Dalyellia viridis*, *Pseudodalyellia alabamensis*, and *Microdalyellia fusca*, function as the outgroup based on current knowledge of the phylogenetic relationships within Dalytyphloplanida in general and Limnotyphloplanida in particular (Van Steenkiste et al., 2013). A Bayesian analysis was done on the same dataset in MrBayes v3.2.7a (Ronquist & Huelsenbeck, 2003) through XSEDE in the CIPRES Science Gateway v3.3 (https://www.phylo.org), using default prior and mcmc settings, in two independent simultaneous runs for 100 million generations. Trees were sampled every 1000th generation after a 25% burn-in. Convergence was assessed through the LogL values and the average deviation of split frequencies. The remaining 75,000 trees were summarized in a 50% majority-rule consensus tree. Branch support was evaluated with the ML bootstrap values (bs) and Bayesian posterior probabilities (pp) for the ML and Bayesian trees, respectively.

Results

Taxonomic account

Rhabdocoela Ehrenberg, 1831
Dalytyphloplanida Willems et al., 2006
Neotyphloplanida Willems et al., 2006
Limnotyphloplanida Van Steenkiste et al., 2013
Jenseniidae Van Steenkiste et al., fam. nov.

We propose the new family Jenseniidae based on the results of the molecular phylogenetic analyses and the occurrence of its representatives in marine and brackish water habitats. Currently no anatomical synapomorphies can be assigned to this clade, but the following combination of characters is helpful to identify its representatives: only found in either marine or brackish water environments, often with broadened anterior end, with or without eyes, round to barrel-shaped pharynx in the first body half and anterior to the gut, paired testes and extracapsular seminal vesicles, male copulatory organ always with a sclerotized proximal girdle or membranes and distal spines (all but one genus) or a retort-shaped stylet (*Axiola*), with or without copulatory bursa, almost always with seminal receptacle, with or without posterior adhesive glands/plate (see also Table S1). The family includes all species of the following genera: *Alexlutheria*, *Axiola*, *Beauchampiola*, *Grappleria* gen. nov., *Halammovortex*, *Jensenia*, and *Thalassovortex*.

Grappleria Van Steenkiste et al., gen. nov.

Type species. *Grappleria corona* Van Steenkiste et al., sp. nov., by monotypy.

Etymology. The genus name refers to the type locality Grappler Inlet in Bamfield, British Columbia, Canada.

Diagnosis. Genus of Jenseniidae fam. nov. with a broadened anterior end, a pair of lenticular eyes, small round to ovoid pharynx (approx. 1/6 of body length), paired testes in middle body third on both sides of and behind pharynx, paired extracapsular and single intracapsular seminal vesicles, male copulatory organ with complex sclerotized structures consisting of an inner elongated funnel-shaped tube surrounded by a proximal half-open girdle and several rows of distal spines attaching to the girdle. Copulatory bursa absent, separate seminal receptacle with at least two compartments and decomposed sperm.

Grappleria corona Van Steenkiste et al., sp. nov.
(Figs 1–11)

Type material. Holotype: One whole mount (BBM MI4181). Paratypes: Three whole mounts (BBM MI4182–MI4184).

Other material. Observations on and images of four live specimens.

Type locality. Mouth of Grappler Inlet opposite the BMSC, Bamfield, British Columbia, Canada (48°50′17.6″N, 125°08′02.1″W). Date of collection: 30 January 2020.

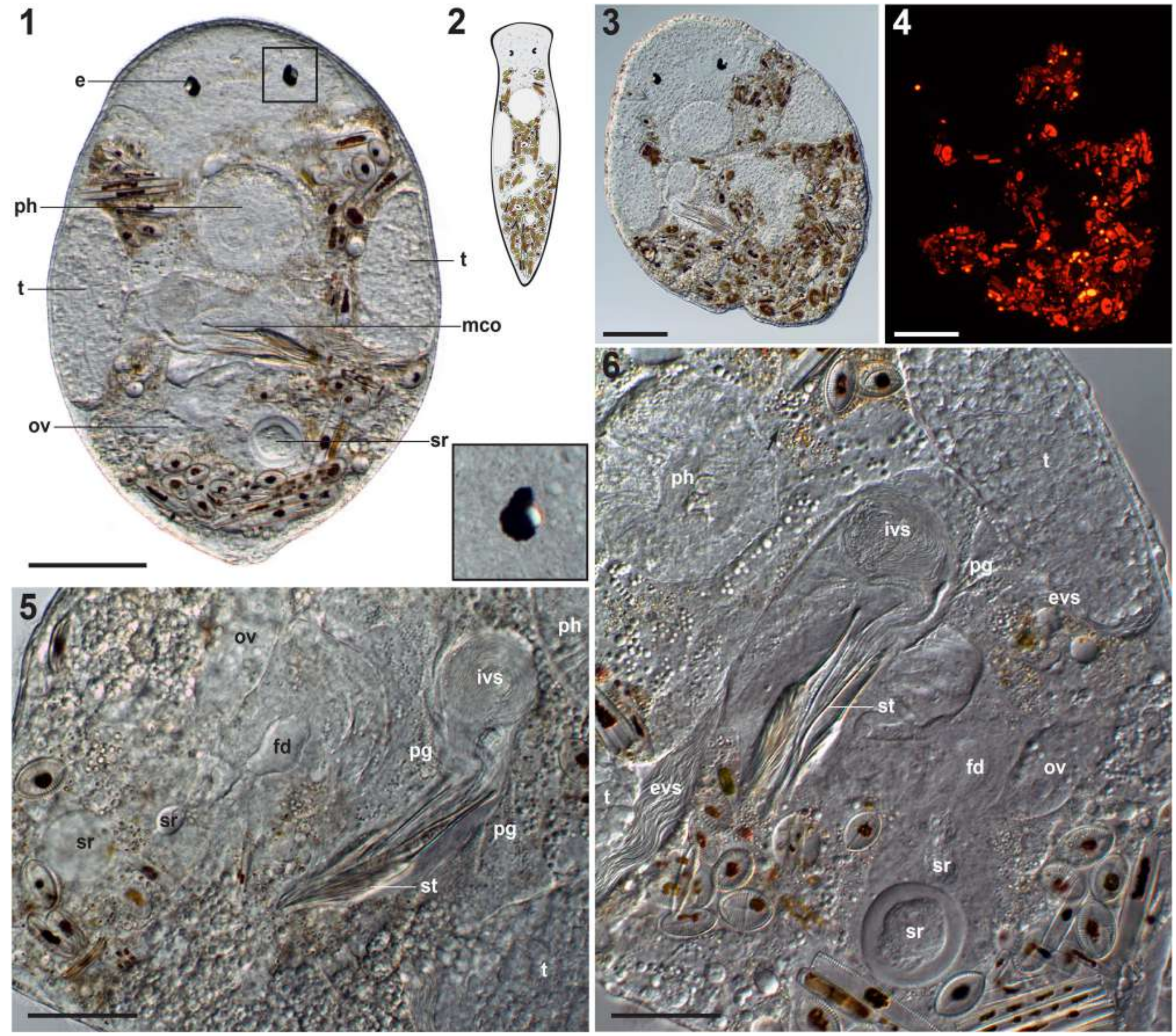
Distribution. Marine algae at type locality. Salinity of 30‰, but some freshwater input from a small creek flowing into the ocean.

Etymology. The species epithet refers to the covid-19 virus that caused a global pandemic in 2020.

Diagnosis. Provisionally with the same diagnosis as the genus.

Description. General habitus: Animals between 0.3–0.5 mm long. With a pair of lenticular eyes composed of a kidney-shaped eye cup with black pigment granules partly wrapped around a large, spherical transparent lens (e and inset, Fig. 1). Live free-swimming animals are spindle-shaped, but the anterior end conspicuously widened creating a ‘head’ and ‘neck’ region (Figs 1–6). Ciliated cellular epithelium with evenly distributed groups of two to three rhabdites on the dorsal side of the animal. Forward-directed globular pharynx at about 30% of the body length (ph, Fig. 1). Mouth opening in the body wall at the level of the anterior end of the pharynx. Intestine filling most of the animal behind the ‘head’ and between the atrial organs. In several animals, the intestine was completely occupied by ingested diatoms of which the plastids were in various states of digestion (Figs 1–4). One individual was observed while regurgitating diatom frustules.

Male genital system: Paired ellipsoidal testes on each side of the body, lateral to and behind the pharynx (t, Fig. 1). Vasa deferentia originate from the posterior end of the testes, of which a portion is enlarged to form paired extracapsular seminal vesicles (evs, Fig. 6) filled with sperm. The vasa deferentia enter the proximal part of the male copulatory organ (mco, Fig. 1), where they merge into a large proximal intracapsular seminal vesicle (ivs, Figs 5–6). The elongated male copulatory organ is situated behind the pharynx and between the testes. It receives at least two types of extracapsular prostate glands (pg, Figs 5–6) – coarse-grained and medium-grained – just distally from the intracapsular seminal vesicle. The intracapsular gland necks of the prostate glands fill the middle part of the copulatory organ – thus forming a prostate vesicle – and release their granular secretion in a complex of sclerotized structures, commonly called the stylet (st, Figs 5–6). The latter is composed of an elongated funnel-shaped tube, a half-open girdle and multiple rows of spines (Figs 7–11). The funnel-shaped tube (tu, Figs 7–11) measures 78–93 µm ($\bar{x}$ = 87 µm; $n = 3$) in length and receives the medium-grained prostate secretion. It is slightly S-shaped in its distal half (tu, Figs 7, 9, 11). Its proximal half is surrounded by a 34–46-µm-long ($\bar{x}$ = 40 µm; $n = 4$) half-open girdle with a very fine reticulate pattern (gi, Figs 7–8, 10–11). The girdle continues into multiple rows of long straight spines that gradually shorten towards the distal end (sp, Figs 7–11). Together, these spines also form a second half-open girdle measuring 44–50 µm ($\bar{x}$ = 48 µm; $n = 4$) in length, surrounding the distal half of the funnel-

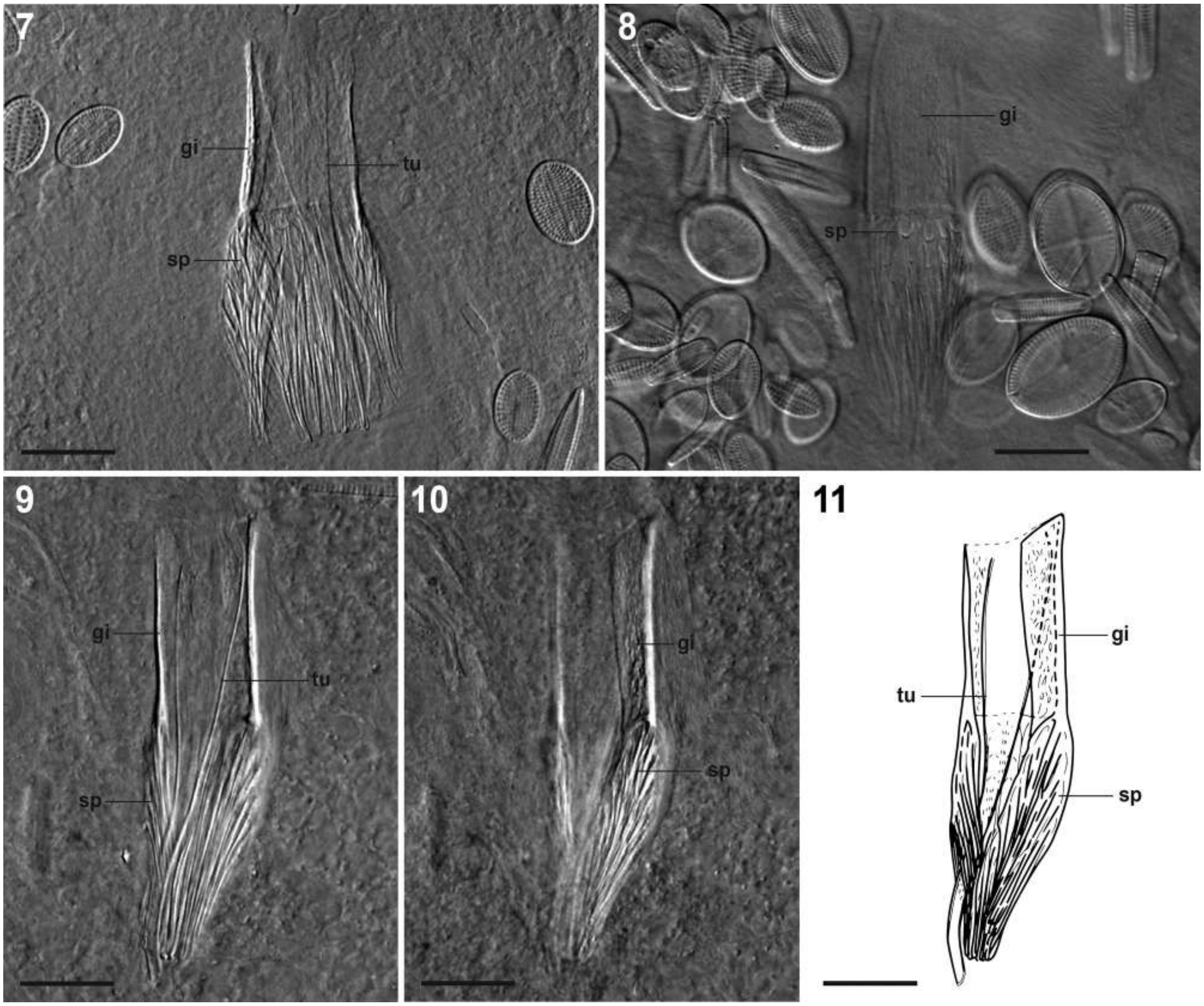


Figs. 1–6. Live specimens of *Grappleria corona* gen. et sp. nov. (1) Specimen slightly squeezed under cover slip. Inset shows lenticular eye in detail. (2) Drawing of free-swimming specimen showing the broadened anterior end. (3) Specimen with a large number of ingested diatoms as food items. (4) Autofluorescence of diatom plastids in the same specimen as in 3. (5–6) Detail of the atrial organs. Abbreviations: e, eye; evs, external seminal vesicle; fd, female duct; ivs, internal seminal vesicle; mco, male copulatory organ; ov, ovary; pg, prostate glands; ph, pharynx; sr, seminal receptacle; st, stylet; t, testis. Scale bars: 1–4 = 100 µm; 5–6 = 50 µm.

shaped tube. The space between the girdles and the funnel-shaped tube is filled with the coarse-grained prostate secretion. The total length of the stylet amounts to 84–99 µm ($\bar{x} = 90$ µm; $n = 4$).

Female genital system: Detailed connections within the female system are difficult to observe in the live animals. A bipartite female duct (fd, Figs 5–6) connects the common genital atrium with the single ovary (ov, Figs 5–6) and paired vitellaria. The proximal part of the female duct is filled with intact sperm and receives the oviduct, at least one vitelloduct, and possibly some female glands. It also connects to a seminal receptacle (sr, Figs 5–6) which consists of a tissue mass with a sperm-filled lumen and a separate, large spherical compartment with degraded sperm. Sphincters guard the connections between the proximal part of the female duct and the oviduct, seminal receptacle and distal part of the female duct.

Common genital atrium: The female duct connects to the common genital atrium through a sphincter, but the connection between the male atrium containing the stylet and the common atrium could not be observed. The common gonopore lies at about 60% of the body length.



Figs. 7–11. Sclerotized parts of the male copulatory organ in whole-mounted specimens of *Grappleria corona* gen. et sp. nov. (7) Slightly squeezed stylet composed of proximal girdle and distal spines surrounding an internal tube. (8) Stylet in different focal plane showing fine reticulate pattern of the proximal girdle and the bases of multiple rows of distal spines. Large amounts of diatom frustules from ingested prey cells. (9–10) Stylet in different focal planes showing the half-open proximal girdle and rows of distal spines folding around the internal tube. (11) Drawing of the same stylet as in 9–10. Abbreviations: gi, girdle; sp, spines; tu, tube. Scale bars = 20 μm.

Remarks. *Grappleria corona* gen. et sp. nov. is the fourth species of marine Jenseniidae fam. nov. recorded from the Pacific Ocean. *Jensenia parangulata* Ax & Armonies, 1990, *Halammovortex nigrifrons* (Karling, 1935) and *Halammovortex promacropharynx* Ax, 2008, have all been found in Alaska. However, these three species also occur in other oceans including the Atlantic Ocean (all three species) and the Arctic Ocean (*H. nigrifrons*). The distribution records of the other species of Jenseniidae fam. nov., which mainly include localities in marginal seas of the Atlantic Ocean and to a lesser extent the Arctic Ocean, exemplify a historical sampling bias in these areas that is typical for rhabdocoels (Table S1).

Phylogeny

The final 18S rDNA sequence alignment has 17 sequences belonging to 15 taxa and is 1726 bp long. The final 28S rDNA sequence alignment has 15 sequences belonging to 14 taxa and is 1644 bp long. The concatenated dataset (18S + 28S) for phylogenetic analyses has 19 sequences belonging to 17 taxa and is 3370 bp long.

Results of the phylogenetic analyses including support values are summarized in Fig. 12. Topologies were congruent for the ML and Bayesian trees except for the *Craspedella–Temnosewellia* clade. Two well-supported sister clades were recovered for the ingroup: (a) a clade

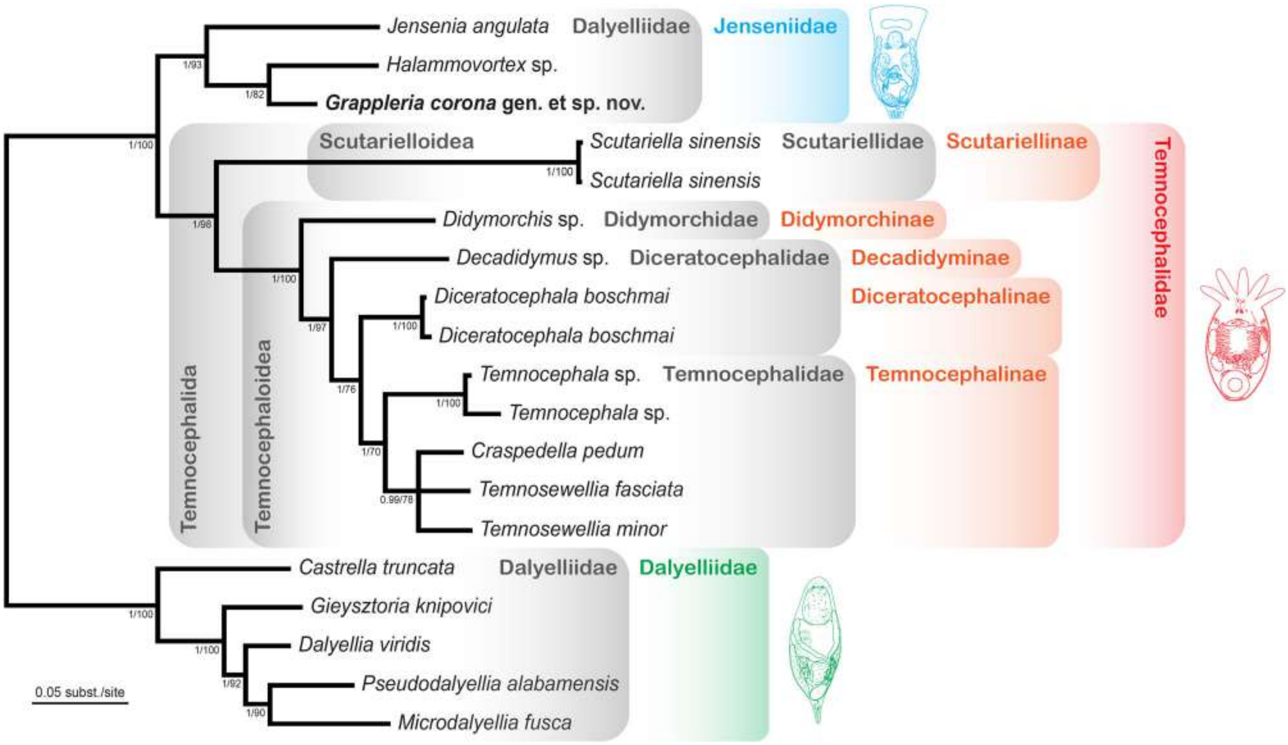


Fig. 12. Phylogeny based on 18S and 28S rRNA sequences of dalyelliid and temnocephalid microturbellarians. The Bayesian majority-rule consensus tree of the concatenated alignment is shown. Branch support values of the Bayesian (pp, posterior probabilities) and ML (bs, bootstrap) analyses are also included: pp/bs. The *Craspedella–Temnosewellia* clade has conflicting topologies between trees and is collapsed. The traditional taxonomic classification based on morphology is depicted in grey. The newly proposed taxonomic classification reflecting the molecular phylogenetic hypothesis is indicated in colour. Illustrations of *Jensenia angulata* (blue), *Temnosewellia neqae* (red), and *Gieysztoria garudae* (green) are based on habitus drawings in Ehlers (1990), Cannon (1993), and Van Steenkiste et al. (2012), respectively.

of marine and brackish water dalyelliids including *Jensenia angulata*, *Halammovortex* sp. and *Grappleria corona* gen. et sp. nov., with *J. angulata* as the most basal taxon (bs = 93; pp = 1); and (b) a clade with all temnocephalids (bs = 98; pp = 1). Interrelationships within Temnocephalida are largely resolved. The scutariellid *Scutariella sinensis* is the most basal temnocephalid taxon. The families Scutariellidae, Didymorchidae, and Temnocephalidae are monophyletic, while the Diceratocephalidae is paraphyletic. The relationship between *Temnosewellia* and *Craspedella* is unresolved with conflicting topologies and low support values (bs < 70; pp < 0.95).

Discussion

Morphological comparison

The combination of several characters including a forwardly directed pharynx and mouth opening in front of the intestine and in the anterior body half, paired compact testes, a complex and partly sclerotized male copulatory organ, a compact single ovary, paired vitellaria, and a single gonopore in the posterior body half, suggest *Grappleria corona* gen. et sp. nov. is closely related to other marine and brackish water genera of the Dalyelliidae sensu Luther, 1955 (see Luther, 1955 for a monograph).

The seven genera only found in marine and brackish water, *Alexlutheria*, *Axiola*, *Beauchampiola*, *Halammovortex*, *Jensenia*, *Polliculus*, and *Thalassovortex*, can be distinguished from each other based on the presence/absence of (lenticular) eyes, the pharynx size and morphology, the overall organization of the atrial organs, and particularly the detailed morphology of the sclerotized parts of the male copulatory organ. Each genus has a unique mix of diagnostic characters and most characters are shared between two or more genera (Table S1). Based on these characters, the specimens from Canada are most closely related to the two representatives of *Alexlutheria*, *A. acrosiphoniae* Karling, 1956 and *A. psammophila* Ax, 2008, and to a lesser extent to *Thalassovortex tyrrhenicus* Papi, 1956, and the two species of *Axiola*, *A. luetjohanni* (Ax, 1952) and *A. remanei* (Ax, 1952). The species of *Alexlutheria* and *Thalassovortex* also have a pair of lenticular eyes, a relatively small pharynx, testes in the first half partly lateral to and/or behind the pharynx, a clearly separate seminal receptacle, and a male copulatory organ with a sclerotized girdle and distal spines. The two species of *Axiola* also have lenticular eyes but possess a retort-shaped stylet very different from other marine and brackish water dalyelliids. The species of *Axiola* further differ from *G. corona* gen. et sp. nov. by their pear-shaped pharynx and the position of the testes (lateral and anterior to pharynx). A broadened anterior end seems to be a common feature among all genera except for *Halammovortex* and *Polliculus*.

As in most other rhabdocoels, the morphology of the male copulatory organ, and specifically its sclerotized parts, are crucial for the identification and taxonomic classification of dalyelliids. Representatives of *Alexlutheria*, *Thalassovortex*, *Jensenia*, and *Halammovortex* all have a male copulatory organ with sclerotized parts consisting of a proximal tube or girdle and distal spines. In *Jensenia*, the lateral sides and distal rim of the relatively long and slender, cone-shaped, half-open proximal tube are heavily fortified creating the impression of proximal stalks connected by a cross-bar. The distal spines are more pronounced and concentrated on two distal continuations of these proximal fortifications. A central distal spine associated with a distal papilla of *J. angulata* is missing in *J. parangulata* (see Ax, 2008; Ax & Armonies, 1990; Ehlers, 1990). Representatives of *Halammovortex* and *Thalassovortex* have a relatively broad, slightly funnel-shaped, closed proximal girdle and a closed distal crown of multiple rows (*Halammovortex*) or a single row (*Thalassovortex*) of spines (Ax, 2008; Papi, 1956). The sclerotized parts of the copulatory organ in *G. corona* gen. et sp. nov. mostly resemble those of *Alexlutheria acrosiphoniae* and *A. psammophila*. Both species have a proximal girdle which distally continues in rows of spines and a central stylet. However, in *Alexlutheria*, both the central stylet and the surrounding distal spines – described as a cirrus in *A. acrosiphoniae* and single row in *A. psammophila* – are a continuation of a closed proximal girdle (Ax, 2008; Karling, 1956). In *G. corona* gen. et sp. nov., the central stylet is completely separated from and surrounded by a half-open proximal girdle, originating close to the proximal edge of the girdle and ending just past the distal edge of the spines. Another notable difference between *G. corona* gen. et sp. nov. and *A. acrosiphoniae* is the presence of abundant caudal glands arranged in an adhesive plate in the latter species. Caudal glands were not observed in the live individuals of *G. corona* gen. et sp. nov., and if present would be inconspicuous. The sclerotized parts of the male copulatory organ in representatives of *Axiola*, *Beauchampiola*, and *Polliculus* strongly deviate from this basic girdle-and-spines model or, in the case of *Beauchampiola*, is at least strongly modified in paired proximal stalks or fortified vertical edges of paired membranes, a distal ring with spikes or lamellae that distally continue into spines, and several distal groups of heteromorphic spines and/or teeth (Ax, 2008; Van Steenkiste et al., 2008).

The specimens from Bamfield share a few other features with *Alexlutheria* that are not present in *Thalassovortex*. Both *G. corona* gen. et sp. nov. and *A. acrosiphoniae* lack a copulatory bursa and have paired extracapsular seminal vesicles as part of the vasa deferentia (in addition to the intracapsular vesicle) and a resorbent compartmentalized seminal receptacle in the female system (Karling, 1956; Table S1). The Mediterranean *T. tyrrhenicus* has a simple sacciform seminal receptacle and a copulatory bursa, and only an intracapsular seminal vesicle – as is the case in all other marine and brackish water dalyelliids except for *J. angulata* (Papi, 1956). In freshwater dalyelliids, the copulatory bursa is a separate pouch, often with a thick, fortified wall, originating from the common genital atrium. A copulatory bursa is also present in *Halammovortex*, *Jensenia*, and *Beauchampiola*, with a bursal stalk in *H. nigrifrons* and *J. angulata*. A copulatory bursa is absent in *Axiola* and *Polliculus*. The seminal receptacle is usually described as a separate sacciform pouch in the female system for the storage of sperm. Notable exceptions are *Jensenia angulata*, in which the seminal receptacle is part of the oviduct (Luther, 1955), and *Halammovortex nigrifrons*, in which the seminal receptacle consists of two compartments and contains decomposed sperm (Karling, 1943). A seminal receptacle is present in all marine and brackish water dalyelliids except for *Beauchampiola oculifera* (de Beauchamp, 1927) and possibly *Halammovortex lewisi* (Jones & Ferguson, 1948).

The unique combination of characters (Table S1), especially the structure of the sclerotized male copulatory organ, in the specimens from British Columbia justify the establishment of a new genus *Grappleria* gen. nov. The morphological comparisons discussed above suggest that this new genus is most closely related to *Alexlutheria* and *Thalassovortex*.

Phylogeny and taxonomic implications

Few molecular phylogenetic studies include marine and brackish water dalyelliids. The phylogeny of the Dalytyphloplanida based on 18S and 28S rDNA sequences by Van Steenkiste et al. (2013) contains *Jensenia angulata* and *Halammovortex* sp., as well as four temnocephalids and a large number of freshwater dalyelliids.

Members of Dalyelliidae sensu Luther, 1955 and Temnocephalida belong to a larger clade called Limnotyphloplanida, which also comprises nearly all freshwater and limnoterrestrial representatives of the family Typhloplanidae. While Van Steenkiste et al. (2013) showed that *Jensenia* and *Halammovortex* are more closely related to the temnocephalids than to the 'freshwater' dalyelliids, the exact phylogenetic position of the 'marine' dalyelliids remained unresolved. A reduced number of taxa was used for the study of Chiesa et al. (2015), in which a molecular phylogeny aimed at identifying a species of introduced temnocephalid associated with invasive crayfish, shows that *Jensenia* and *Halammovortex* cluster together as the sister clade of Temnocephalida, although with relatively low support.

The molecular phylogeny we present here – supplemented with several recently published rDNA sequences of temnocephalids and rDNA sequences of the new species and genus *Grappleria corona* gen. et sp. nov. – corroborates that 'marine' dalyelliids form the sister clade to the Temnocephalida. While the resulting paraphyly of the family Dalyelliidae sensu Luther, 1955 was already clear in the molecular phylogeny of Van Steenkiste et al. (2013), the taxonomic implications were never formalized into an updated classification. Therefore, we propose to create a new family, Jenseniidae fam. nov. (Fig. 12), for all marine and brackish water dalyelliids belonging to the genera *Jensenia*, *Halammovortex*, and *Grappleria* gen. nov., and provisionally to the closely related genera *Alexlutheria*, *Axiola*, *Beauchampiola*, and *Thalassovortex*. The only marine dalyelliid we do not classify within Jenseniidae fam. nov. is *Polliculus.* Several morphological characters of this monotypic genus are aberrant from the other genera including a single testes and vas deferens, a tubular stylet surrounded by an accessory stylet, and the presence of a separate uterus with uterine glands (Van Steenkiste et al., 2008). Therefore, we currently treat *Polliculus* as incertae sedis within the Dalytyphloplanida.

The family Dalyelliidae sensu novo retains all 'freshwater' dalyelliids consisting of representatives of *Castrella* Fuhrmann, 1900, *Dalyellia* Gieysztor, 1938, *Gieysztoria* Ruebush & Hayes, 1939, *Microdalyellia* Gieysztor, 1938, and *Pseudodalyellia* Van Steenkiste et al., 2011. Nearly all species in these genera occur in fresh water except for a few species of *Microdalyellia* and *Gieysztoria* that can tolerate various levels of salinity or are confined to brackish water (Ax, 2008; Therriault & Kolasa, 1999). Provisionally, the other freshwater dalyelliid genera *Austrodalyellia* Hochberg & Cannon, 2002, *Fulinskiella* Gieysztor & Szynal, 1939, *Sergia* Nasonov, 1923, and *Vaillantiella* Luther, 1955 also remain within Dalyelliidae, based on morphological similarities with the aforementioned 'freshwater' genera. However, future analyses including molecular data of all the genera provisionally placed in Jenseniidae fam. nov. and Dalyelliidae sensu novo are needed to corroborate this hypothesis.

We are still doubtful about the phylogenetic position of the enigmatic species *Varsoviella kozminskii* Gieysztor & Wiszniewski, 1947. While traditionally considered a freshwater dalyelliid, some of its morphological characters – such as a well-developed posterior adhesive plate – and its symbiotic association with freshwater crustaceans (Gieysztor & Wiszniewski, 1947), are very reminiscent of temnocephalids. Therefore, we suggest considering this species as incertae sedis within the clade composed of jenseniids, dalyelliids, and temnocephalids until molecular data and a more detailed morphological assessment are available.

Several hypotheses on the phylogeny of Temnocephalida have been suggested based on morphological data (Iomini et al., 1999; Joffe et al., 1998 and references therein). The clades and relationships in our molecular phylogenetic analyses mostly correspond with those proposed by Joffe et al. (1998) (Fig. 12). As such, the morphological apomorphies and synapomorphies for most of the main temnocephalid families and genera presented in that study remain unchanged. Although the close relationship between scutariellids and other temnocephalids was previously doubted based on ultrastructural data (Falleni et al., 2001, 2002), our results confirm that Scutariellidae Annandale, 1912 is indeed the sister taxon to all other temnocephalids (Chen et al., 2018; Joffe et al., 1998). The only exception to the scheme of Joffe et al. (1998) is the family Diceratocephalidae – erected by the same authors for the genera *Diceratocephala* Baer, 1953 and *Decadidymus* Cannon, 1991 – which appears paraphyletic in our trees. Therefore, we propose separate subfamilies, Diceratocephalinae and Decadidyminae, to classify these two genera, respectively. Moreover, because Temnocephalida is deeply embedded within Limotyphloplanida, we propose to bring this clade to the family level and rename it to Temnocephalidae sensu novo in order to better reflect its phylogenetic position. With inclusion of the above-mentioned changes, the temnocephalid families Scutariellidae Annandale, 1912, Didymorchidae Bresslau & Reisinger, 1933, Diceratocephalidae Joffe et al., 1998, Temnocephalidae Monticelli, 1899, and Actinodactylellidae Benham, 1901 are consequently reclassified into the following subfamilies: Scutariellinae, Didymorchinae, Decadidyminae, Diceratocephalinae, Temnocephalinae, and Actinodactylellinae. The latter taxon has only one representative, *Actinodactylella* Haswell, 1893, but no

DNA sequences are available for this genus, so its inferred phylogenetic position as the sister group to the Temnocephalinae (‘Temnocephalidae’ in Joffe et al., 1998) remains to be tested.

Towards integrative taxonomy

As a result of our updated classification, the Limnotyphloplanida is now composed of four families: Typhloplanidae, Dalyelliidae sensu novo, Jenseniidae fam. nov., and Temnocephalidae senus novo. While representatives of Temnocephalidae sensu novo possess several synapomorphies including an epibiotic life style, a multisyncytial epidermis, a posterior adhesive organ, genito-intestinal communication and a split sperm shaft (Joffe et al., 1998; Van Steenkiste et al., 2013; Watson, 2001), no anatomical synapomorphies can currently be assigned to Jenseniidae fam. nov. and Dalyelliidae sensu novo. Characters present in all dalyelliid genera such as a pair of lenticular eyes, a barrel-shaped pharynx anterior to the gut, and a copulatory bursa, can also be found in several jenseniid genera (see Table S1). However, based on a combination of features it should be possible to assign almost any encountered limnotyphloplanid with the diagnostic characters of Dalyelliidae sensu Luther, 1955 to either Jenseniidae fam. nov. or Dalyelliidae sensu novo with a relatively high degree of certainty.

Occurrence in either marine or freshwater habitats is often a reliable predictor of a shared evolutionary history. Habitat transitions are not common within Dalytyphloplanida, which have their origin in the marine environment (Van Steenkiste et al., 2013). Transitions from marine to freshwater environments are exemplified by the common ancestor of Limnotyphloplanida and a few incursions by single taxa within Thalassotyphloplanida and Neodalyellida. Transitions from fresh water back to the marine environment in Limnotyphloplanida seems to have occurred only among jenseniids. Therefore, any freshwater dalyelliid sensu Luther, 1955 is most likely a dalyelliid sensu novo, while any marine dalyelliid sensu Luther, 1955 is most likely a jenseniid. Both families have a number of representatives that are only found in or can tolerate brackish water conditions. However, all brackish water dalyelliids sensu novo belong to the genera *Microdalyellia* and *Gieysztoria* and can easily be recognized as such (Ax, 2008).

Finally, some of the features apparently shared between representatives of Jenseniidae fam. nov. and Dalyelliidae sensu novo are in fact differential diagnostic characters upon more detailed examination and comparison. For instance, eyes with lenses are only present in representatives of the jenseniid genera *Grappleria*, *Alexlutheria*, *Thalassovortex*, and *Axiola*. In all of these genera, a conspicuously large lens protrudes outward from the eye cup (for descriptions and illustrations see the references in Table S1). In contrast, the eye lens of *Dalyellia* and *Gieysztoria* consists of inward projecting laminae of the eye cup rim and is thus completely surrounded by the eye cup (Bedini et al., 1973). The latter type of pigment cup eyes is also thought to be present in the other representatives of Dalyelliidae sensu novo based on light microscopic observations (Luther, 1955). This illustrates how detailed investigations of morphological characters, both on a histological and ultrastructural level, are sorely needed to integrate comparative morphological data with molecular phylogenetic data in order to advance a more accurate classification for the Limnotyphloplanida.

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