

Molecular phylogeny of trigonostomine turbellarians (Platyhelminthes: Rhabdocoela: Trigonostomidae), including four new species from the Northeast Pacific Ocean

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Rhabdocoels comprise a species-rich group of microturbellarians in marine habitats around the globe. Knowledge of their species diversity in the Northeast Pacific Ocean is limited to a few studies. Here we present nine species within Trigonostominae and one species within Brinkmanniellinae associated with intertidal algae, sandy beaches and mudflats from the coast of British Columbia and Washington. Four species, *Trigonostomum tillicum* sp. nov., *Ceratopera cascadiensis* sp. nov., *Ceratopera complicata* sp. nov. and *Ptychopera unicornis* sp. nov., are new to science and are established based on molecular data and distinctive traits associated with the stylet and bursal appendage. Furthermore, we report on the occurrence of *Trigonostomum tori*, *Ceratopera axi*, *Ceratopera pilifera*, *Ceratopera pacifica* comb. nov., *Ptychopera japonica* and *Tvaerminea karlingi* and provide new data on their morphology. A molecular phylogenetic analysis of Trigonostominae and other Thalassotyphloplanida using 18S rRNA and partial 28S rRNA sequences from 42 species demonstrates the phylogenetic positions of the four new species in addition to other inter- and intrageneric relationships within Trigonostominae (e.g. *Messopiana* is embedded within *Ceratopera*). This molecular phylogenetic context enables us to reassess homology statements about stylet morphology and the organization of the afferent system within Trigonostominae.

ADDITIONAL KEYWORDS: British Columbia – Dalytyphloplanida – flatworms – meiofauna – microturbellaria – species discovery – Thalassotyphloplanida – Washington.

INTRODUCTION

Small marine free-living flatworms (microturbellarians) are important components of meiofaunal and seaweed-associated communities worldwide. With around 1700 species described so far, rhabdocoels encompass the most species-rich taxon of microturbellarians. Marine biodiversity studies on this group have predominantly focused on the temperate Northeast Atlantic Ocean, with the bulk of the species described from this region. Only 65

species of marine rhabdocoels are known from the temperate Northeast Pacific Ocean, seven of which are representatives of Trigonostominae. The latter were reported from only two sampling campaigns in California and Oregon by Karling (1986) and in Alaska by Ax & Armonies (1990).

Trigonostominae constitutes a diverse group of marine and brackish water rhabdocoels with 14 genera and 97 species. The vast majority of trigonostomines belong to the genera *Beklemischeviella* Luther, 1943 (3 spp.), *Ceratopera* Den Hartog, 1964 (11 spp.), *Messopiana* Den Hartog, 1966a (13 spp.), *Proxenetes* Jensen, 1878 (31 spp.), *Ptychopera* Den Hartog, 1964 (13 spp.) and *Trigonostomum* Schmidt, 1852 (19 spp.). The remaining genera (*Brederveldia* van der Velde & van de Winkel, 1975, *Cryptostiopera* Ehlers & Ax, 1974, *Feanora* De Clerck & Schockaert, 1995,

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Lutheriella Den Hartog, 1966b, *Mahurubia* Willemse et al., 2005b, *Marinellia* Riedl, 1954, *Parapharyngiella* and *Petaliella* Ehlers, 1974) are monotypic. All of these taxa are characterized by the presence of a stylet and an efferent and afferent system connecting the ovaries to the common genital atrium. The afferent system has a complex organization and has structures that play a role in the reception and processing of sperm. It usually consists of a bursa and some sort of sclerotized bursal appendage. This appendage leads to the insemination duct that in turn connects to the 'fecundatorium' close to the ovaries. With the exception of *Messoplana*, Van Steenkiste et al. (2013) included representatives of all the polytypic genera of Trigonostominae in a molecular phylogenetic analysis of Dalytyphloplanida Willemse et al., 2006 showing that these genera form a well-supported clade within a larger marine clade of dalytyphloplanids called Thalassotyphloplanida Willemse et al., 2006.

Here we report nine trigonostomine species and one species within Brinkmanniellinae collected from different intertidal habitats from the coast of British Columbia and Washington. *Trigonostomum tillicum* sp. nov., *Ceratopera complicata* sp. nov. and *Ptychopera unicornis* sp. nov. are new and are formally described here with morphological and molecular data. *Ceratopera cascadiensis* sp. nov. is also new and described with morphological data. For *Trigonostomum tori* (Karling, 1986) Willemse et al., 2004b; *Ceratopera axi* (Riedl, 1954) Den Hartog, 1964; *Ceratopera pilifera* Karling, 1986; *Ceratopera pacifica* (Karling, 1986) comb. nov.; and *Tvaerminnea karlingi* Luther, 1943, records are a northward expansion of their known distribution in the temperate Northeast Pacific Ocean. *Ptychopera japonica* Ax, 2008, originally described from Japan, is reported for the first time in this region. The 18S rRNA and 28S rRNA sequences of all species, except of *C. cascadiensis* sp. nov., are included in a phylogenetic analysis of Trigonostominae, providing more insight into the diversity and evolutionary history of this group.

MATERIAL AND METHODS

COLLECTION AND MORPHOLOGICAL EXAMINATION OF THE TAXA

Most specimens were collected in 2015–2016 from beaches, rocky intertidal zones and estuarine mudflats along the Pacific coast of British Columbia (Canada) and Washington (USA), including the Juan de Fuca Strait (Victoria), the Vancouver Island Shelf (Bamfield), the Strait of Georgia (Surrey, Friday Harbor, Nanaimo) and the North Coast Fjords (Calvert Island). *Parapharyngiella* sp. 1 from Curaçao

was included in the phylogenetic analysis because it provides additional taxon resolution within the Trigonostominae.

Live animals were isolated from either sand or algae using the MgCl₂ decantation method (Schokkaert, 1996). *Ptychopera japonica* and *T. karlingi* were collected from mud using the oxygen depletion method (Schokkaert, 1996). Specimens were studied alive with the aid of a stereoscope and DIC optics, photographed and subsequently whole mounted with lactophenol to preserve the sclerotized parts of the genital system. These hard parts were then photographed, measured and drawn to aid in the identification and description of the animals. All pictures were taken with a Zeiss Axioplan 2 microscope equipped with a Zeiss-Axiocam 503-color camera. Pictures of all whole mounts presented in the figures were produced in Helicon Focus (HeliconSoft) by stacking series of micrographs. Schematic line art diagrams were drawn freehand based on series of micrographs, scanned and retouched with Inkscape (www.inkscape.org) and GIMP (www.gimp.org). Measurements were taken from whole-mounted and live specimens using ImageJ software (www.imagej.net). All measurements were taken along the axis of the animal or its sclerotized structures (i.e. axial), unless indicated otherwise in the text. In the latter case, measurements were taken along a straight line (referred to as 'non-axial'). Clearly identifiable specimens were frozen in a few µL of seawater for DNA extraction.

Holotypes were deposited in the Swedish Museum of Natural History (SMNH, Stockholm, Sweden) and paratypes were deposited in the Beaty Biodiversity Museum (BBM, University of British Columbia, Vancouver, Canada).

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Genomic DNA was extracted from entire specimens with the DNeasy Blood & Tissue kit (Qiagen). Extractions followed manufacturer's instructions except that (1) the AE elution buffer was heated to 60 °C before elution; and (2) DNA was eluted twice for every sample in reduced volumes of 60 and 30 µL, respectively.

Nearly complete 18S (1693–1789 bp) and partial 28S rRNA (1664–1675 bp) sequences were amplified using Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare) and the primers and thermocycling conditions listed in Table S1 (Supporting Information). Amplicons were visualized on 1.5% agarose gels stained with GelRed (Biotium) and enzymatically cleaned prior to sequencing with Illustra ExoProStar S (GE Healthcare).

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

Taxa	Locality	Coordinates	18S accession #	28S accession #
Ingroup				
<i>Lituciavis serpens</i> Ax & Heller, 1970	Sylt, Germany	55°00'55"N; 08°26'19"E	AY775758	KC529552
<i>Tuaerminea karlingi</i> Luther, 1943	Nanaimo, BC	49°11'43"N; 123°57'32"W	MF321755	
<i>Cillionema hawaiiensis</i> Karling <i>et al.</i> , 1972	Oahu, Hawaii	21°19'36"N; 157°40'59"W	KC529556	
<i>Coronhelmis multispinosus</i> Luther, 1948	Hanko, Finland	59°49'21"N; 22°58'21"E	KC529427	KC529555
<i>Coronhelmis lutheri</i> Ax, 1951	Skåne, Sweden	56°25'57"N; 12°34'18"E	KC529426	KC529554
<i>Parapharyngiella</i> sp. 1	Piscaderabaai, Curáçao	12°07'20"N; 68°58'10"E	MF321750	-
<i>Parapharyngiella</i> sp. 2	Doha, Qatar	25°19'09"N; 51°32'16"E	KC529405	KC529531
<i>Trigonostomum penicillatum</i> (Schmidt, 1857) Micoletzky, 1910	Sardinia, Italy	40°49'33"N; 08°26'36"E	KC529414	KC529540
Trigonostomum tillicum sp. nov.	Victoria, BC	48°24'12"N; 123°21'03"W	MF321753	
<i>Trigonostomum armatum</i> (Jensen, 1878) Gamble, 1900	Bohuslän, Sweden	58°52'36"N; 11°06'40"E	KC529419	KC529545
<i>Trigonostomum frankii</i> Willems <i>et al.</i> , 2004b	Goa, India	15°29'38"N; 73°46'03"E	KC529416	KC529542
<i>Trigonostomum setigerum</i> Schmidt, 1852	Sardinia, Italy	40°57'12"N; 08°13'43"E	KC529418	KC529544
<i>Trigonostomum tori</i> (Karling, 1986) Willems <i>et al.</i> , 2004b	Victoria, BC	48°24'12"N; 123°21'03"W	MF321754	
<i>Trigonostomum venenosum</i> (Ujanin, 1870) Meixner, 1924	Sardinia, Italy	40°57'12"N; 08°13'43"E	KC529417	KC529543
<i>Trigonostomum watsoni</i> Willems <i>et al.</i> , 2004b	KwaZulu-Natal, South Africa	28°16'50"S; 32°29'06"E	KC529415	KC529541
<i>Trigonostomum denhartogii</i> (Karling, 1978) Willems <i>et al.</i> , 2004b	Nouméa, New Caledonia	22°18'20"S; 166°26'51"E	AY775773	-
<i>Beklemishevella contorta</i> (Beklemischev, 1927) Luther, 1943	Hanko, Finland	59°49'21"N; 22°58'21"E	KC529413	KC529539
<i>Beklemishevella angustior</i> Luther, 1943	Hanko, Finland	59°49'50"N; 23°08'33"E	KC529412	KC529538
<i>Proxenetes fasciger</i> Ehlers, 1974	Sylt, Germany	55°01'21"N; 08°26'25"E	KC529408	KC529534
<i>Proxenetes quinquespinosis</i> Ax, 1971	Sylt, Germany	55°02'06"N; 08°24'29"E	KC529406	KC529532
<i>Proxenetes simplex</i> Luther, 1948	Zwin, the Netherlands	51°21'56"N; 03°22'18"E	KC529410	KC529536
<i>Proxenetes quadrispinosus</i> Den Hartog, 1966a	Zwin, the Netherlands	55°01'21"N; 08°26'25"E	AY775766	-
<i>Proxenetes karlingi</i> Luther, 1943	Zwin, the Netherlands	51°21'56"N; 03°22'18"E	KC529409	KC529535
<i>Proxenetes trigonus</i> Ax, 1960	Sylt, Germany	55°01'21"N; 08°26'25"E	AY775768	-
<i>Proxenetes puccinellicola</i> Ax, 1960	Zwin, the Netherlands	51°21'56"N; 03°22'18"E	KC529411	KC529537
<i>Proxenetes bilioi</i> Den Hartog, 1966a	Zwin, the Netherlands	51°21'56"N; 03°22'18"E	KC529407	KC529533
<i>Proxenetes flabellifer</i> Jensen, 1878	Oostende, Belgium	-	AY775764	-
<i>Ceratopera pilifera</i> Karling, 1986	Calvert Island, BC	51°39'53"N; 128°07'44"W	MF321749	
<i>Ceratopera axi</i> (Riedl, 1954) Den Hartog, 1964	Victoria, BC	48°24'12"N; 123°21'03"W	MF321746	MF321756
<i>Ceratopera</i> sp.	Goa, India	15°34'03"N; 73°44'29"E	KC529421	KC529548
<i>Ceratopera gracilis</i> (von Graff, 1882) Den Hartog, 1964	Sardinia, Italy	40°32'39"N; 08°19'13"E	KC529422	KC529549
<i>Ceratopera complicata</i> sp. nov.	Victoria, BC	48°24'12"N; 123°21'03"W	MF321747	
<i>Ceratopera pacifica</i> comb. nov. Karling, 1986	Bamfield, BC	48°51'05"N; 125°07'19"W	MF321748	MF321758
<i>Ptychopera</i> sp.	Oahu, Hawaii	21°22'36"N; 158°00'17"W	KC529420	KC529547
<i>Ptychopera unicornis</i> sp. nov.	Victoria, BC	48°24'12"N; 123°21'03"W	MF321752	MF321761
<i>Ptychopera westbladi</i> (Luther, 1943) Den Hartog, 1964	Zealand, the Netherlands	51°35'38"N; 03°52'17"E	AY775770	KC529546

Taxa		Locality	Coordinates	18S accession #	28S accession #
Outgroup					
	<i>Ptychopera plebeia</i> (Beklemishev, 1927) Den Hartog, 1964	Thessaloniki, Greece	—	AY775769	—
	<i>Ptychopera japonica</i> Ax, 2008	Surrey, BC	49°05'09"N; 122°51'39"E	MF321751	MF321760
	<i>Microvalvine corallicola</i> Karling <i>et al.</i> , 1972	Northern Territory, Australia	12°24'16"S; 130°48'49"E	KC529423	KC529550
	<i>Promesostoma marmoratum</i> (Schultze, 1851) von Graff, 1882	Hanko, Finland	59°49'10"N; 23°05'02"E	KC529430	KC529558
	<i>Brysophlebs delamarei</i> (Ax, 1956) Karling, 1985	Pyrénées-Orientales, France	—	KC529435	KC529565
	<i>Thalassoplana collaris</i> Luther, 1946	Raseborg, Finland	59°49'08"N; 23°15'27"E	KC529483	KC529614

Accession numbers in bold are new sequences.

Clean amplicons were sequenced in 10 µL reactions using the amplification primers and several internal sequencing primers (see Table S1, Supporting Information). Sequencing reactions contained 1 µL BigDye Terminator (BDT) v3.1 (Applied Biosystems), 2 µL BDT buffer, 0.5 µM primer and 1–2 µL PCR product. Sequencing products were cleaned and run on an Applied Biosystems 3730S 48-capillary DNA analyzer by the Nucleic Acid Protein Service Unit (NAPS) at the University of British Columbia. Resulting trace files were assembled into full sequences in Geneious v9.1.5 (Biomatters) and subjected to a BLAST search on the NCBI website (<http://blast.ncbi.nlm.nih.gov>) to verify the sample's taxonomic identity. All sequences were deposited in GenBank. Specimen collection data and sequence accession numbers are provided in Table 1.

MOLECULAR PHYLOGENETIC ANALYSES

The new 18S and 28S rRNA sequences were aligned with existing thalassotyphloplanid rRNA sequences downloaded from GenBank (Table 1) using the structural Q-INSL algorithm in MAFFT (Katoh & Toh, 2008). Taxa for the ingroup and outgroup were chosen based on current knowledge of the phylogenetic relationships within Thalassotyphloplanida (Van Steenkiste *et al.*, 2013). The 5' and 3' ends of the alignments were trimmed in Geneious v9.1.7 (www.geneious.com; Kearse *et al.*, 2012). Ambiguous positions were selected with Aliscore v2.2 (Misof & Misof, 2009) and removed from the alignments with Aicut v2.3 (Kueck, 2009).

Before running the phylogenetic analyses, best-fit partitioning schemes and models of molecular evolution for the concatenated data set (18S + 28S) were recovered in PartitionFinder v.1.1.0 using a greedy search with PhyML and the Bayesian information criteria (BIC) (Lanfear *et al.*, 2012). This resulted in two partitions corresponding with the 18S and 28S rRNA sequences and the GTR+GAMMA+I model for both partitions. Maximum likelihood (ML) and Bayesian analyses were conducted in RAxML v8.2.9 (Stamatakis, 2014) and MrBayes v3.2.6 (Ronquist & Huelsenbeck, 2003), respectively, using XSEDE on the CIPRES Science Gateway v3.3 (<https://phylo.org>). Best-scoring ML tree search and non-parametric bootstrapping (1000 replicates) were performed under the recommended partition scheme and models of molecular evolution. Partitioned Bayesian analyses used the same substitution models and default prior and mcmc settings in two independent simultaneous runs for 10 million generations. Trees were sampled every 100th generation after a 25% burn-in. LogL values and the average deviation of split frequencies were considered as convergence diagnostics. The remaining 75 000 trees were summarized in a 50% majority-rule consensus tree.

Table 1. Continued

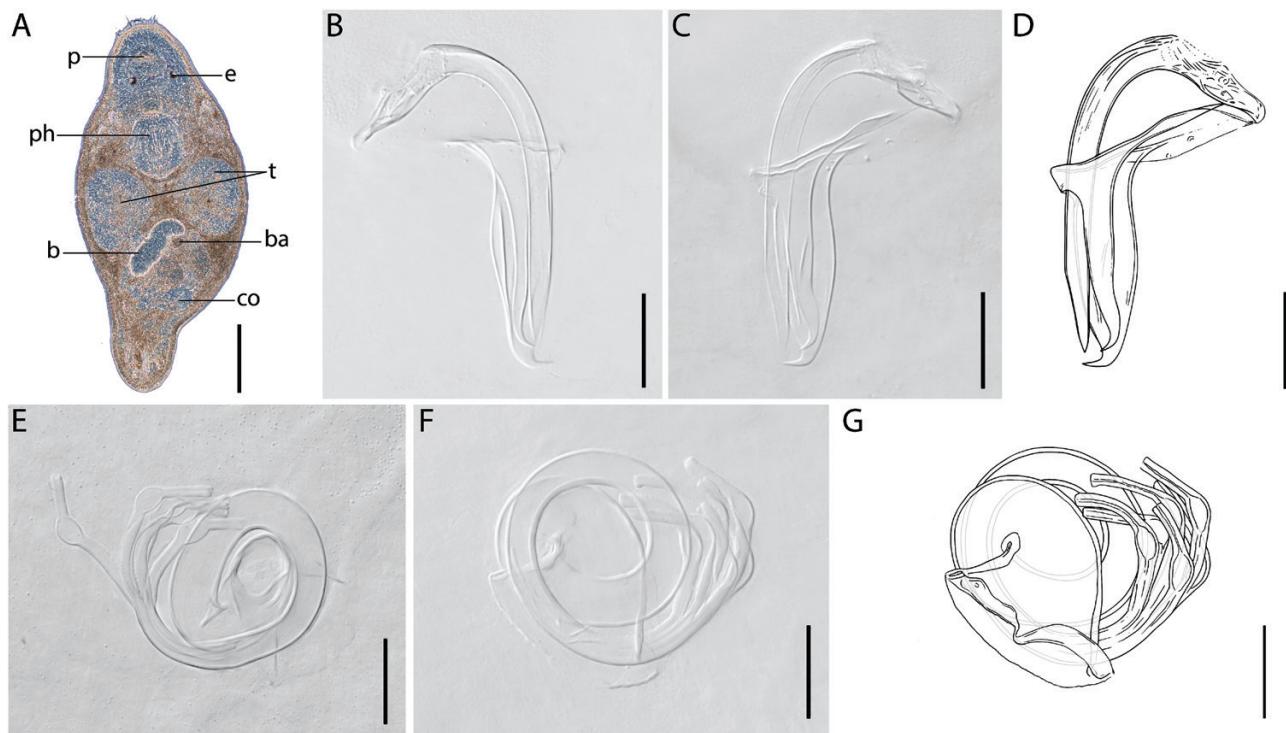


Figure 1. *Trigonostomum tillicum* sp. nov. A, live animal under cover slip. B–D, stylets from two different paratypes. E, bursal appendage from a paratype. F, G, bursal appendage from the holotype. Abbreviations: b, bursa; ba, bursal appendage; co, copulatory organ; e, eye; p, proboscis; ph, pharynx; t, testis. Scale bars: A = 200 µm; B–G = 20 µm.

The vitellaria extend dorsally on both sides of the body from behind the pharynx to the ovaries. The large female bursa is elongated and anteriorly provided with a sclerotized bursal appendage. This sclerotized appendage is a coiled, 230- to 300-µm-long tube ($\bar{x} = 261$ µm; $n = 10$) that is funnel-shaped proximally and distally splits into six slender tubes with a swollen vesicle-like part just before the distal end (Fig. 1E–G). In some specimens, these six tubes seem to be grouped in two units of three tubes for the first proximal part. Although difficult to observe in the squeezed whole mounts, the appendage seems to make two coils from the proximal funnel to the distal tubes.

Discussion: This species clearly belongs to the genus *Trigonostomum* because of the rostral integumental invagination ('proboscis'), the forwardly inclined pharynx, and the construction of the male and female genital system in general and of the stylet and bursal appendage in particular. The stylet is typical for the species group 1B (stylet with a proximal turn of 90–180°) as defined by Willems *et al.* (2004b). Furthermore, based on the morphology of the sclerotized structures, this species is almost identical to *T. vanmecheleni* from the Italian Adriatic. The

latter species belongs to the *T. lilliei* species group as designated and discussed by Artois *et al.* (2013) which now comprises four morphologically similar species: *T. lilliei* (von Graff, 1911b) Meixner, 1924; *T. pryerherchi* Kepner *et al.*, 1941; *T. vanmecheleni* and *T. tillicum* sp. nov.

A thorough re-examination of the type material of *T. vanmecheleni* revealed the stylets to be almost identical to those of the new species from British Columbia. The only notable difference is the size of the stylet, which is about 1/3 larger in *T. tillicum* sp. nov. Artois *et al.* (2013) describe a spur-like structure on the base of the funnel-like proximal opening of the stylet proper in *T. vanmecheleni*, which we think corresponds to a protruding ridge on the funnel rim as also observed in *T. tillicum* sp. nov. The bursal appendages of both species form a coiled tube that splits into six smaller tubes, but is twice as long in *T. tillicum* sp. nov. as in *T. vanmecheleni*. In addition, the six smaller tubes consistently display vesicle-like enlargements in *T. tillicum* sp. nov., which are absent in *T. vanmecheleni*.

Based on the above-mentioned differences and the disjunct geographical distribution, we assign the specimens from British Columbia to a new species. In doing so, we follow Artois *et al.* (2013) who

argue that the morphology of the bursal appendage is a good diagnostic feature within the *T. lilliei* group. It is nevertheless clear that all species of this group are very closely related, which raises interesting questions on the biogeography of this group given the disjunct localities of these species in the Northwestern Atlantic (*T. lilliei*, *T. prytherchi*), Southwestern Atlantic (*T. lilliei*), Adriatic (*T. vanmecheleni*), Southwestern Pacific (*T. prytherchi*) and Northeastern Pacific (*T. tillicum* sp. nov.). A thorough integrative taxonomic analysis including molecular data from the previously described species will be necessary in the future for a better understanding of species boundaries and diagnostic characters within this group.

TRIGONOSTOMUM TORI (KARLING, 1986) WILLEMS ET AL., 2004b
(FIG. 2)

'*Trigonostomum setigerum* Schmidt, 1852' in Karling, 1986: 209–210, figs 45, 46.
Trigonostomum tori (Karling, 1986) Willems et al., 2004b: 292, figs 5F, 9F, 10E, table 1.

New localities: Clover Point, Victoria, British Columbia, Canada (48°24'12"N, 123°21'03"W), algae in rocky lower intertidal (06/05/2015; 02/09/2015; 14/11/2016). Wizard Island, Bamfield, British Columbia, Canada (48°51'30"N, 125°09'33"W), algae in rocky lower intertidal (01/06/2015). Dixon Island, Bamfield, British Columbia, Canada (48°51'05"N, 125°07'19"W), algae in rocky lower intertidal (02/06/2015). Grappler Inlet, Bamfield, British Columbia, Canada (48°50'17"N, 125°08'04"W), algae in rocky lower intertidal (30/08/2015). West Beach boulders, Calvert Island, British Columbia,

Canada (51°39'07"N, 128°08'33"W), algae in rocky lower intertidal (09/04/2016). Friday Harbor, San Juan Island, Washington, USA (48°32'42"N, 123°00'44"W), algae on the dock of the marine station (08/10/2016).

Known distribution: Northeast Pacific Ocean: California (Karling, 1986).

Material: Observations on about 15 live animals. Ten whole mounts (BBM MI4032–MI4041). 18S rRNA (GenBank accession # MF321754), 28S rRNA (GenBank accession # MF321763).

Remarks: Animals about 0.6–1.0 mm long. Specimens from Victoria and Calvert Island often with a parenchymatous brownish coloration (Fig. 2A). General appearance typical of species of *Trigonostomum*, but larger and plumper than *T. tillicum* sp. nov. also found at the Victoria locality. Rostral integumental invagination ('proboscis') and forwardly inclined pharynx in the first third of the body. Epidermis packed with oblong to slightly falcate rhabdites and rostral sensory bristles.

Internal organization similar to other species of *Trigonostomum* with paired testes, and paired seminal vesicles entering the prostate vesicle. Bursal canal slightly sclerotized, extremely long and narrow, and partly curled and twisted (Fig. 2D). Its distal part is somewhat broadened with a constriction right before entering the large oval bursa (arrow in Fig. 2D). The latter is provided with a sclerotized bursal appendage. Paired ovaries and vitellaria.

Stylet and bursal appendage as described by Karling (1986) and Willems et al. (2004b) (Fig. 2B–D). The spiral stylets of the specimens from British Columbia and Washington measure 748–896 µm ($\bar{x} = 807$ µm;

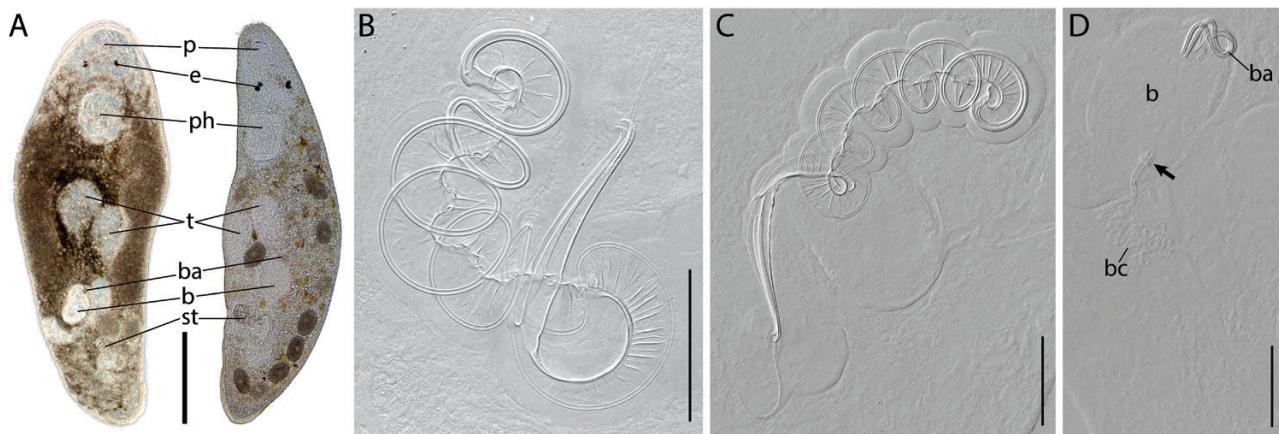


Figure 2. *Trigonostomum tori*. A, live animals under cover slip. B, C, stylets from two whole-mounted specimens. D, afferent system with bursal canal, bursa and bursal appendage in a whole-mounted specimen. Abbreviations: b, bursa; ba, bursal appendage; bc, bursal canal; e, eye; p, proboscis; ph, pharynx; st, stylet; t, testis. Scale bars: A = 200 µm; B–D = 50 µm.

$n = 10$) and have five to six coils. In several specimens, the flexible spiral is partly unwound missing one to two full coils. This results in most specimens having five full coils and an unwound part corresponding to roughly one coil. In two specimens from Bamfield only four full coils were counted with one and two unwound sections, respectively. A third specimen from Bamfield has exactly five coils. The bursal appendage measures 75–101 µm ($\bar{x} = 90$ µm; $n = 10$) (Fig. 2D). In some specimens, the distal ends of the tubes are slightly funnel-shaped instead of straight. Our measurements for the stylet and bursal appendage are consistent with those for the Californian specimens (683–853 and 80–106 µm, respectively; Willems *et al.*, 2004b).

Karling (1986) considered the Californian specimens of *T. tori* to be representatives of *T. setigerum*; the latter taxon formerly included all populations with two or more stylet coils. Willems *et al.* (2004b) analysed the stylet morphology of all representatives of *Trigonostomum* from species group 2 (i.e. with a proximal mantle rim closely adhering to the proximal rim of the stylet and enveloping the stylet over its entire length, diverging distally into two spiny plates with a terminal hook) and concluded that the number of stylet coils is constant in different populations. Consequently, Willems *et al.* (2004b) split *T. setigerum* into a number of different species, keeping populations with two coils in *T. setigerum* and erecting *T. australis* Willems *et al.*, 2004b, for the populations with three coils from Queensland and New South Wales, *T. galapagoensis* (Ehlers & Ax, 1974) Willems *et al.*, 2004b, for the population with four coils from the Galapagos and *T. tori* for the population with five coils from California. Willems *et al.* (2004b) report that care should be taken when counting the number of coils depending on the angle the stylet is observed from and also mention the occurrence of unwound sections in the longer stylets of the Pacific species. Although it is clear that both these issues are also present in the specimens from British Columbia and Washington, all our specimens have at least five coils when including the unwound portions. In addition to the number of coils, also the size of the stylet and the bursal appendage, and its distribution in the Northeastern Pacific are consistent with the stylet morphology and distribution of *T. tori*. Therefore, we assign the populations from British Columbia and Washington to this species.

CERATOPERA DEN HARTOG, 1964

Mesostoma Levinsen, 1879: 175.

Proxenetes von Graff, 1882: 277; Vaillant, 1890: 642;

Pereyaslawzewa, 1893: 255–259; von Graff, 1905: 112; von Graff, 1908: 2531–2532; von Graff, 1913:

181; Meixner, 1938: 11, fig. 10A; Luther, 1943: 61, 63; Luther, 1948: 63–64; Ax, 1953: 238.

Ceratopera Den Hartog, 1964: 377–382, 399, fig. 2; Ax, 1971: 146–150, fig. 1.

Messoplana Den Hartog, 1966a: 147; Den Hartog, 1964: 377–382, fig. 2; Ax, 1971: 146–150, 173, fig. 1.

New diagnosis: Trigonostominae with afferent system differentiated into a bursa and a sclerotized bursal appendage. Bursa usually with slender bursal canal. Bursal appendage very variable, consisting of a funnel-shaped tube, two slender tubes or a combination of both, with or without a sclerotized ring, sometimes partly protruding into the bursa. Sclerotized parts of the male copulatory organ consisting of a proximally curved stylet proper connected to an accessory mantle at its base. Mantle differentiated into one or several pieces, either plates, tubes, or a combination of both. Accessory mantle either partly sheathing the stylet proper or completely separate from it.

Type species: *Ceratopera gracilis* (von Graff, 1882) Den Hartog, 1964.

Remarks: The genus *Messoplana* is suppressed based on the results of our phylogenetic analysis and the ambiguity of the morphological characters differentiating the representatives of *Messoplana* and *Ceratopera* sensu Den Hartog (see discussion *C. complicata* sp. nov.). All species of *Messoplana* are transferred to *Ceratopera*, which now has 26 valid species. A new diagnosis for *Ceratopera* is provided.

CERATOPERA CASCADIENSIS SP. NOV. (FIG. 3)

Etymology: The species epithet refers to Cascadia, the Pacific Northwest of North America.

Type locality: Clover Point, Victoria, British Columbia, Canada (48°24'12"N, 123°21'03"W), algae in rocky lower intertidal (16/03/2015).

Type material: One whole mount which is designated as the holotype (SMNH Type-8919).

Other material: Observations on a live animal.

Diagnosis: Species of *Ceratopera* with a 158-µm-long stylet. Stylet composed of a stylet proper that makes a 90° proximal turn, and a mantle consisting of a proximal girdle bearing two 100-µm-long, plate-like

tubes with pointed tips. Distal end of the stylet proper pointed. Plate-like tubes provided with a ridge of which at least one has a combed edge. Bursal appendage 91 µm long, with broad base and spirally curled funnel that distally splits into two short insemination tubes.

Description: Animal 1.2 mm long with eyes and some parenchymatous brownish coloration (Fig. 3A). General appearance much plumper than the more typical fusiform *C. axi* found at the same locality (Figs 3A, 4A). Pharynx slightly anterior to the midpoint of the body. Epidermis packed with oblong rhabdites. Rhabdite tracks present in the anterior body half.

Internal organization identical to *C. axi* with paired testes posterior to the pharynx, paired seminal vesicles entering the prostate vesicle, an elongated bursa with a sclerotized bursal appendage, paired ovaries and vitellaria.

The sclerotized parts of the male copulatory organ measure 158 µm (non-axial: 117 µm) and consist of (1) a 158-µm-long stylet proper that is proximally curved over 90° and distally straight with a pointed tip; and (2) a mantle modified into a proximal girdle that modifies into two 100-µm-long, plate-like tubes with pointed tips (Fig. 3B, C). The girdle connects to and surrounds the proximal curved part of the stylet. The distal tubes run adjacent to the stylet and bear a ridge of which at least one has a combed edge (arrow in Fig. 3B, C).

The bursal appendage is typical of *Ceratopera* and consists of a broad, striated proximal base and a spirally curled, more sclerotized funnel that distally splits

into two short insemination tubes (Fig. 3D, E). The spiral length of the bursal appendage measures 91 µm.

Discussion: This species closely resembles representatives of *Ceratopera* sensu Den Hartog because of the typical construction of the stylet with a mantle surrounding the stylet proximally and the funnel-shaped bursal appendage. *Ceratopera* sensu Den Hartog encompassed 11 recognized species, which are discussed in Den Hartog (1964), Ehlers & Ax (1974) and Karling (1986) (but see also the discussion on *C. complicata* sp. nov. and the general discussion). The mantle associated with the stylet is modified into two structures in only three species of this group: *C. sellai* (Steinböck, 1933) Den Hartog, 1964, *C. levinseni* Den Hartog, 1964 and *C. reisingeri* (Riedl, 1959) Den Hartog, 1964. In the first two species, these modifications consist of elongated plates ('lamellae' in Den Hartog, 1964) proximally originating on a girdle surrounding the stylet, while in the latter species a girdle is absent. The stylet of *C. cascadiensis* sp. nov. mostly resembles the one of *C. sellai* as one plate is also tubular and runs closely adjacent to the stylet. However, the other plate in *C. sellai* is curved and distally modified into a sheet (Den Hartog, 1964), thus differing from the situation in *C. cascadiensis* sp. nov.

Ax (1995) reports on a Greenlandic specimen of *Ceratopera* he provisionally attributes to *C. cfr. levinseni*. The elongated stylet of the live specimen he observed (Ax, 1995: figs 14D, 16) shows a general resemblance to the stylet of *C. cascadiensis* sp. nov. Although Ax (1995) did not notice any division of the mantle into two 'lamellae',

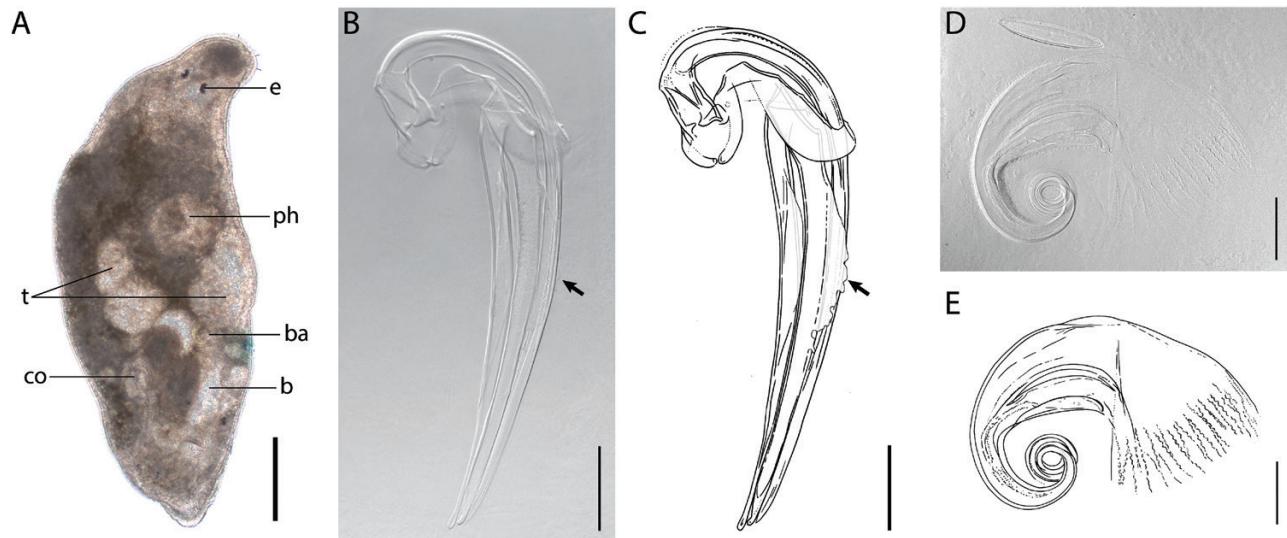


Figure 3. *Ceratopera cascadiensis* sp. nov. A, live animal under cover slip. B, C, stylet from the holotype. D, E, bursal appendage from the holotype. Abbreviations: b, bursa; ba, bursal appendage; co, copulatory organ; e, eye; ph, pharynx; t, testis. Scale bars: A = 200 µm; B–E = 20 µm.

two elongated plates or tubes seem to be present (Ax, 1995: fig. 16B). Unfortunately, no further details on the morphology of the mantle or the bursal appendage of the Greenlandic specimen are available. As such, this individual remains without a formal species designation.

Ceratopera cascadiensis sp. nov. further differs from all other species of *Ceratopera* sensu Den Hartog by the presence of a combed edge on at least one of its mantle tubes. In all other species the edges of the mantle plates or tubes are smooth. With four species of *Ceratopera* known from the Northeast Pacific Ocean (*C. axi*, *C. pacifica* comb. nov., *C. ehlersi* Karling, 1986, and *C. pilifera*), *C. cascadiensis* sp. nov. is the fifth species described from this region.

**CERATOPERA AXI (RIEDL, 1954) DEN HARTOG, 1964
(FIG. 4A, C, D)**

Proxenetes axi Riedl, 1954: 217–220, figs 26–27; Riedl, 1953: 133, 137.

Ceratopera bifida Ehlers & Ax, 1974: 656–660, figs 8, 9; considered a synonym of *C. axi* by Karling, 1986: 212.

Ceratopera axi (Riedl, 1954) Den Hartog, 1964: 401, 406–407, fig. 11K; Karling, 1986: 211–212, figs 50–57; Artois *et al.*, 2000: 107; Willems *et al.*, 2004a: 334, table 1; Willems *et al.*, 2005a: 88, 96, table 2; Willems *et al.*, 2005b: 1565–1566; Van Steenkiste *et al.*, 2008: 28–29, fig. 11D–E.

New locality: Clover Point, Victoria, British Columbia, Canada (48°24'12"N, 123°21'03"W), algae in rocky lower intertidal (06/05/2015; 02/09/2015; 03/03/2016).

Known distribution: Northeast Pacific Ocean: Oregon and California (Karling, 1986). Central East Pacific Ocean: Galapagos Islands (Ehlers & Ax, 1974). Southwest Pacific Ocean: New South Wales (Willems *et al.*, 2004a), New Caledonia (Willems *et al.*, 2005a). East Indian Ocean: La Réunion (Artois, Vermin & Schockaert, 2000). South Indian Ocean: Kerguelen (Willems *et al.*, 2005b). Southern Ocean: Weddell Sea (Artois *et al.*, 2000). Southwest Atlantic Ocean: Falkland Islands (Karling, 1986), Uruguay (Van Steenkiste *et al.*, 2008). Mediterranean: Gulf of Naples and Sicily (Riedl, 1953, 1954).

Material: Observations on seven live animals. Four whole mounts (BBM MI4042–MI4045). 18S rRNA (GenBank accession # MF321746), 28S rRNA (GenBank accession # MF321756).

Remarks: Animals fusi- or filiform, between 0.8 and 1.8 mm long (Fig. 4A). The stylet proper is 118–129 µm long ($\bar{x} = 123 \mu\text{m}$; $n = 4$; non-axial: 89–96 µm) (Fig. 4C). The typical accessory mantle piece of the stylet is S-shaped, with a funnel, and 74–81 µm long ($\bar{x} = 78 \mu\text{m}$; $n = 4$; non-axial: 70–77 µm). In three out of four whole mounts, the accessory mantle piece connects to the elongated edge of the proximal asymmetrical stylet opening through a proximal plate with a thickened outer edge (arrow in Fig. 4C). The bursal appendage measures 100–118 µm ($\bar{x} = 108 \mu\text{m}$; $n = 4$) and has an enlarged mid part in at least two specimens (Fig. 4D). In some individuals, the appendage bifurcates distally and a weakly sclerotized ring could be observed just proximal from this bifurcation.

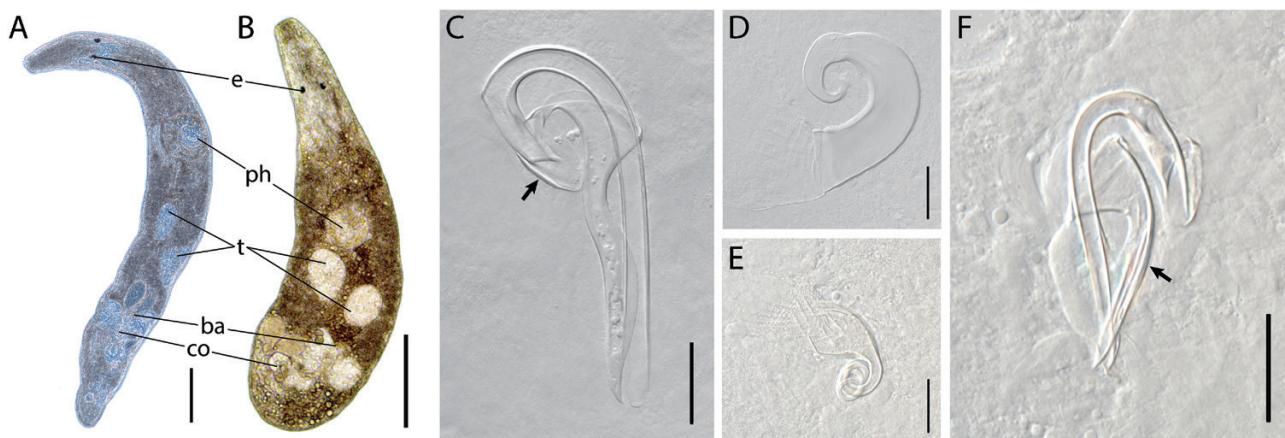


Figure 4. *Ceratopera axi* and *Ceratopera pilifera*. A, live specimen of *C. axi* under cover slip. B, live specimen of *C. pilifera* under cover slip. C, stylet from a whole-mounted specimen of *C. axi*. D, bursal appendage from a whole-mounted specimen of *C. axi*. E, bursal appendage from a whole-mounted specimen of *C. pilifera*. F, stylet from a whole-mounted specimen of *C. pilifera*. Abbreviations: ba, bursal appendage; co, copulatory organ; e, eye; ph, pharynx; t, testis. Scale bars: A, B = 200 µm; C–F = 20 µm.

Ceratopera axi has been found in very disjunct geographic locations around the globe. The general appearance and the length of the stylet and bursal appendage differ somewhat among populations (see Willems *et al.*, 2004a). Measurements on the stylet and bursal appendage from the specimens from British Columbia correspond to those from the population from California. The proximal plate of the accessory mantle piece is only mentioned in the specimens from the Galapagos (Ehlers & Ax, 1974), but is also clearly present in specimens from California and Oregon (figs 50, 51 in Karling, 1986). In addition, specimens from British Columbia also have large adenal rhabdites in the atrial region as is also reported in specimens from California and the Galapagos (Ehlers & Ax, 1974; Karling, 1986).

Some rhabdocoels and other microturbellarians with wide geographic distributions are now recognized as complexes of cryptic species (e.g. Curini-Galletti & Puccinelli, 1998; Casu & Curini-Galletti, 2004; Delogu & Curini-Galletti, 2009; Tessens, 2012; Scarpa *et al.*, 2016). Cryptic species within these complexes often correspond with distinct morphotypes. Possibly, *C. axi* also consists of such a complex (Willems *et al.*, 2005a, b; Van Steenkiste *et al.*, 2008), and the above-mentioned morphological similarities suggest the existence of a Pacific morphotype. A thorough taxonomic revision integrating molecular and morphological data from these disjunct populations is necessary to test this hypothesis.

**CERATOPERA PILIFERA KARLING, 1986
(FIG. 4B, E, F)**

Ceratopera pilifera Karling, 1986: 212–214, figs 58, 65–67.

New localities: Clover Point, Victoria, British Columbia, Canada ($48^{\circ}24'12''N$, $123^{\circ}21'03''W$), algae in rocky lower intertidal (14/11/2016). Little Wolf Beach, Calvert Island, British Columbia, Canada ($51^{\circ}39'53''N$, $128^{\circ}07'44''W$), algae in rocky lower intertidal (07/04/2016). Friday Harbor, San Juan Island, Washington, USA ($48^{\circ}32'42''N$, $123^{\circ}00'44''W$), algae on the dock of the marine station (08/10/2016).

Known distribution: Northeast Pacific Ocean: Oregon (Karling, 1986).

Material: Observations on five live animals. Three whole mounts (BBM MI4046–MI4048). 18S rRNA (GenBank accession # MF321749), 28S rRNA (GenBank accession # MF321759).

Remarks: Animals are 0.7–1 mm long and appear plumper and more *Proxenetes*-shaped than described

by Karling (1986) (Fig. 4B). Internal organization typical for species of *Ceratopera* with paired lenticular eyes, rostral rhabdite tracks, a pharynx located mid-body, paired testes situated behind the pharynx, paired ovaries and vitellaria. Two large ovate seminal vesicles are connected to the rounded copulatory bulb.

The stylet is 97–106 µm long ($\bar{x} = 100 \mu\text{m}$, $n = 3$; non-axial: 52–57 µm) and resembles the stylet of *C. pilifera* as described by Karling (1986). It consists of a curved stylet proper, measuring 74–80 µm ($\bar{x} = 76 \mu\text{m}$, $n = 3$), with a 23- to 26-µm-long ($\bar{x} = 24 \mu\text{m}$, $n = 3$) proximal base, and a curved, funnel-shaped, 46- to 47-µm-long ($\bar{x} = 46 \mu\text{m}$, $n = 3$) accessory mantle piece (Fig. 4F). These measurements correspond to the ones from the Oregonian specimens. The hair-like eponymous protrusion of the accessory mantle piece could not be observed in our specimens. However, a thickened part of the convex edge of the mantle piece (arrow in Fig. 4F) corresponds to this structure. Moreover, the needle-like structure on the accessory piece in figure 67 in Karling (1986) could easily be interpreted as a thickened edge rather than a hair-like protrusion.

The funnel-shaped and distally curled bursal appendage measures 78–88 µm ($\bar{x} = 82 \mu\text{m}$; $n = 3$) and is typical for species of *Ceratopera* sensu Den Hartog. Distally it splits into two 29- to 32-µm-long tubes ($\bar{x} = 31 \mu\text{m}$; $n = 3$).

Based on the strong resemblance of the stylet and bursal appendage with those of *C. pilifera*, we attribute our specimens to this species.

**CERATOPERA PACIFICA (KARLING, 1986) COMB. NOV.
(FIG. 5A, C, D)**

Messoplana pacifica Karling, 1986: 210–211, figs 41–44, 49.

New localities: Dixon Island, Bamfield, British Columbia, Canada ($48^{\circ}51'05''N$, $125^{\circ}07'19''W$), algae in rocky lower intertidal (02/06/2015). Little Wolf Beach, Calvert Island, British Columbia, Canada ($51^{\circ}39'53''N$, $128^{\circ}07'44''W$), algae in rocky lower intertidal (07/04/2016). West Beach boulders, Calvert Island, British Columbia, Canada ($51^{\circ}39'07''N$, $128^{\circ}08'33''W$), algae in rocky lower intertidal (09/04/2016).

Known distribution: Northeast Pacific Ocean: California (Karling, 1986).

Material: Observations on three live animals. One whole mount (BBM MI4049). 18S rRNA (GenBank accession # MF321748), 28S rRNA (GenBank accession # MF321758).

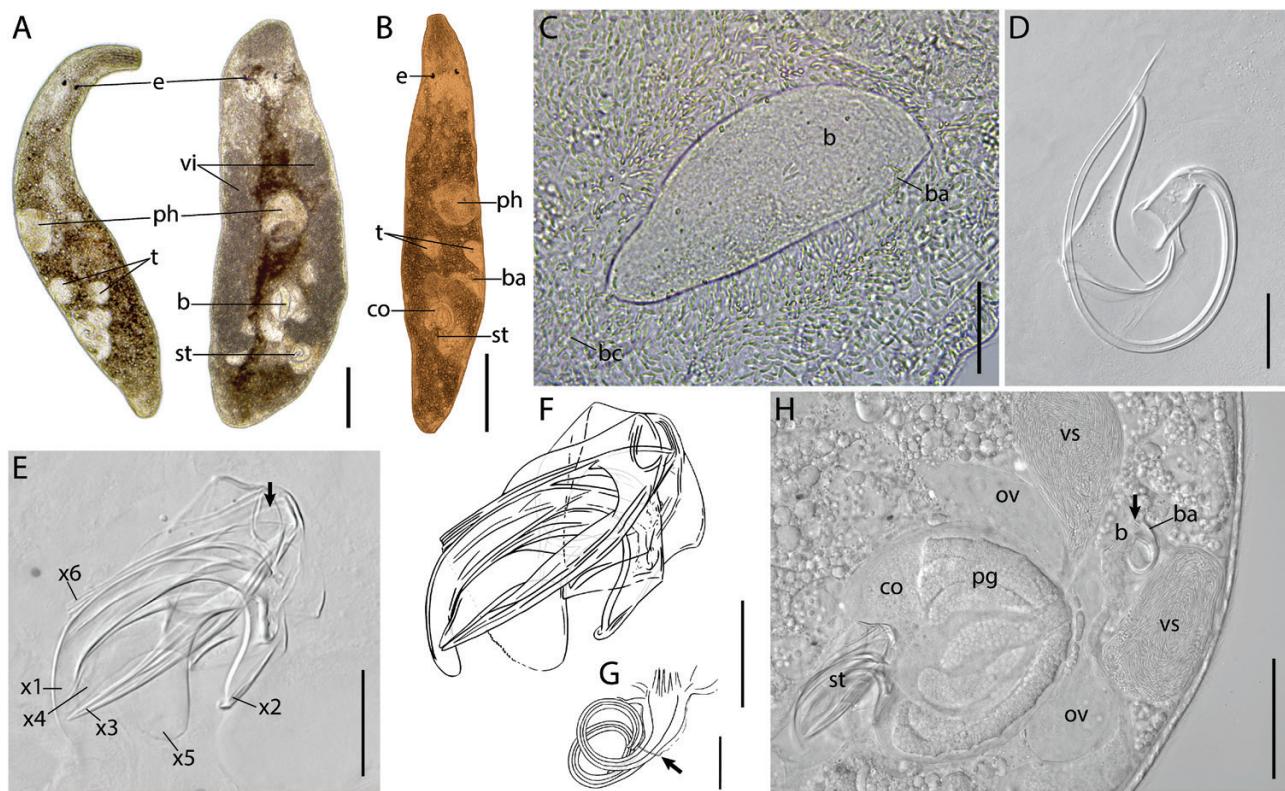


Figure 5. *Ceratopera pacifica* comb. nov. and *Ceratopera complicata* sp. nov. A, free-swimming live specimen (left) and squeezed live specimen (right) of *C. pacifica* comb. nov. under cover slip. B, live specimen of *C. complicata* sp. nov. under cover slip. C, bursa, bursal canal and bursal appendage in a live specimen of *C. pacifica* comb. nov. D, stylet from a whole-mounted specimen of *C. pacifica* comb. nov. E, F, stylet from the holotype of *C. complicata* sp. nov. G, bursal appendage from the holotype of *C. complicata* sp. nov. H, detail of the atrial organs in a live specimen of *C. complicata* sp. nov. Abbreviations: b, bursa; ba, bursal appendage; bc, bursal canal; co, copulatory organ; e, eye; ov, ovary; pg, prostate glands; ph, pharynx; st, stylet; t, testis; vi, vitellaria; vs, seminal vesicle; x1–x6, see description of *C. complicata* sp. nov. Scale bars: A, B = 200 µm; C = 50 µm; D–F = 20 µm; G = 10 µm; H = 50 µm.

Diagnosis: Species of *Ceratopera* with partly coiled stylet. Stylet composed of a 120- to 184-µm-long, filiform stylet proper with a cup-shaped proximal part, and a 58- to 64-µm-long, funnel-shaped accessory mantle piece. Cup of the stylet proper connected to the mantle piece by a sclerotic string; filiform part partially enclosed by the mantle piece. Bursa with bursal canal. Bursal appendage 20 µm long and consisting of a proximal tube that protrudes into the bursa and distally splits in two smaller tubes.

Remarks: Animals about 1.3 mm long, fusiform, with some specimens displaying a parenchymatous brownish coloration (Fig. 5A). General appearance, internal organization and the morphology of the stylet and bursal appendage correspond with those of ‘*Messoplana pacifica*’ (see Karling, 1986). The stylet proper and the accessory mantle piece of the mounted specimen measure 184 µm (non-axial:

82 µm) and 58 µm (non-axial: 56 µm), respectively (Fig. 5D). Although the stylet is a bit larger, this is still in line with the measurements for the Californian specimens (120–170 µm and 58–64 µm, respectively). Bursa, bursal canal and small bursal appendage are faintly visible in the live animal (Fig. 5C).

Karling (1986) attributes his specimens to the genus *Messoplana*. Although he does not explicitly mention why, he lists some of the features also found in other representatives of this genus such as the mid-body position of the pharynx, a sclerotic string that connects the stylet proper and its accessory piece, a small, bitubular bursal appendage (‘insemination apparatus’) and a bursa with a bursal canal. Based on the ambiguity of the morphological characters differentiating *Messoplana* from *Ceratopera* sensu Den Hartog and the results of our phylogenetic analysis, all species of *Messoplana* are transferred to *Ceratopera*. See the discussion on *C. complicata* sp. nov.

CERATOPERA COMPLICATA SP. NOV.
 (FIG. 5B, E–H)

Etymology: The species epithet refers to the complex stylet.

Type locality: Clover Point, Victoria, British Columbia, Canada ($48^{\circ}24'12''N$, $123^{\circ}21'03''W$), algae in rocky lower intertidal (14/11/2016).

Type material: One whole mount which is designated as the holotype (SMNH Type-8920).

Other material: Observations on two live animals. 18S rRNA (GenBank accession # MF321747), 28S rRNA (GenBank accession # MF321757).

Diagnosis: Species of *Ceratopera* with a very complex stylet. Stylet proper 66 µm long with a long base and rounded tip. Mantle with numerous plates and a tubular spine. Base of stylet with window-like opening and several ridges. Bursal appendage 48 µm long, slightly protruding in the bursa, with proximal funnel, slightly sclerotized ring, and two coiled insemination ducts.

Description: Animal about 1 mm long, robust with large rostral rhabdite tracks, lenticular eyes and some parenchymatous brownish coloration (Fig. 5B). Pharynx slightly anterior to the midpoint of the body. Epidermis packed with oblong rhabdites.

Internal organization typical for *Ceratopera* and other Trigonostominae with paired testes posterior to the pharynx, paired seminal vesicles entering the prostate vesicle, an elongated bursa with a sclerotized bursal appendage, paired ovaries and vitellaria.

The stylet is 66 µm long (non-axial: 65 µm). It is very complex and consists of (1) a curved 66-µm-long stylet proper with a rounded tip (x1) and a very long base (x2); and (2) a mantle enveloping and connecting to the stylet (Fig. 5E, F). This mantle is modified into a 52-µm-long slender tubular spine with a pointed tip (x3), a 33-µm-long sickle-shaped plate (x4) running more or less parallel to the stylet proper, and a broad plate with a rounded, slightly combed edge (x5). Where this combed edge connects to the stylet proper, some smaller overlapping plates (x6) are present. All these structures come together in the proximal part of the stylet, which has a window-like opening (arrow in Fig. 5E) and on which several ridges originate.

The bursa is long and slender in one animal, likely because of the absence of sperm. In the other animal, the distal part of the bursa is swollen. The latter part is provided with the 48-µm-long bursal appendage consisting of a proximal 12-µm-long funnel that splits into two spirally curled, 36-µm-long insemination tubes

(Fig. 5G). The thick wall of the proximal funnel is confluent with the bursa wall through several sclerotized ridges, but also partly extends into the bursa (arrow in Fig. 5H). A ring marks the transition between the funnel and the insemination tubes (arrow in Fig. 5G). This ring seems to be an outward extension of the bursa wall.

Discussion: The morphological characters in *C. complicata* sp. nov. are reminiscent of several taxa within Trigonostominae including *Ceratopera* sensu Den Hartog, *Messoplana*, *Trigonostomum* and *Ptychopera*. The stylet is very complex as in some species of *Trigonostomum* and *Ptychopera*. This is unusual for species of *Ceratopera* sensu Den Hartog and *Messoplana* of which most species only have a curved, tubular stylet proper of varying length connected to a tubular spine or plate. These are also the two most prominent features in the stylet of *C. complicata* sp. nov., but additional plates and ridges result in a very ornate stylet. The bitubular appendage is typical for *Messoplana*, but the thick-walled, funnel-shaped proximal part is rather wide at its base and connects to the bursa through sclerotized ridges, not unlike species of *Ceratopera* sensu Den Hartog (e.g. *C. pilifera*).

Den Hartog (1966a) and Ax (1971) mention a close relationship between *Messoplana* and *Ceratopera* sensu Den Hartog (see also general discussion). Both authors clearly define *Messoplana* by the position of the pharynx in the mid or hind part of the body, the construction of the stylet and its accessory mantle piece, and the morphology of the bursal appendage. However, the position of the pharynx is a dubious feature as species in both genera have representatives with a pharynx in or close to the middle of the body.

The morphology of the stylet can be very similar between representatives of *Ceratopera* sensu Den Hartog and *Messoplana*. For instance, some species of *Ceratopera* sensu Den Hartog also have the accessory mantle piece modified into either a separate duct or a tubular spine as in *Messoplana*; however, according to Den Hartog (1964) the accessory mantle piece in *Ceratopera* sensu Den Hartog distally connects to and sheathes the straight part of the stylet. This distal connection is not always present in several species of *Ceratopera* sensu Den Hartog, including *C. axi*, *C. ehlersi*, *C. pilifera* and *C. steinboecki* (Riedl, 1959) Den Hartog, 1964. Moreover, in several species of *Messoplana*, such as *M. elegans* (Luther, 1948) Den Hartog, 1966a, *M. canariensis* Ehlers & Ehlers, 1980 and '*M. pacifica*' (now *C. pacifica* comb. nov.), the stylet proper is also sheathed by the mantle.

The bursal appendage is another important diagnostic character used to separate *Ceratopera* sensu Den Hartog and *Messoplana*. It consists of a single,

funnel-shaped, curved or coiled and strongly sclerotized tube in *Ceratopera* sensu Den Hartog, while *Messoplana* typically has two slender ducts surrounded by a sclerotized ring confluent with the bursa wall. The latter type of bursal appendage is shared with *Proxenetes*; however, *Proxenetes* differs from *Messoplana* by its typical stylet surrounded by a closed, funnel-shaped mantle. When looking at the bursal appendage in several species of *Ceratopera* sensu Den Hartog and *Messoplana* more closely, the difference in its morphology becomes less apparent. In some species of *Ceratopera* sensu Den Hartog, including *C. cascadiensis* sp. nov., *C. axi* and *C. pilifera*, the bursal appendage distally bifurcates into two smaller tubes, sometimes with a faintly sclerotized ring around the transition zone (e.g. *C. axi*). In most species of *Messoplana*, the proximal part is undivided and partly extends into the bursa. This undivided proximal part can be longer, shorter or the same length as the two tubes. In 'M. globulifera' Artois *et al.*, 2000', a distal bifurcation is lacking altogether.

The examples and discussion above show that the characters separating *Messoplana* from *Ceratopera* sensu Den Hartog are equivocal. Not surprisingly, the phylogenetic position of *C. pacifica* comb. nov. and *C. complicata* sp. nov. (Fig. 7A), two species that could either be placed within *Ceratopera* sensu Den Hartog or *Messoplana*, reflects the ambiguous taxonomic status of these two genera. It is clear that *Ceratopera* sensu Den Hartog is not monophyletic when assigning *C. complicata* sp. nov. to *Messoplana* and keeping *C. pacifica* comb. nov. in its original genus. Therefore, the genus *Messoplana* is suppressed and its representatives are transferred to *Ceratopera*.

PTYCHOPERA DEN HARTOG, 1964

Proxenetes von Graff, 1882: 277; Vaillant, 1890: 642; Pereyaslawzewska, 1893: 255–259; von Graff, 1905: 112; von Graff, 1908: 2531–2532; von Graff, 1913: 181; Meixner, 1938: 11, fig. 10A; Luther, 1943: 61, 63; Luther, 1948: 63–64; Ax, 1953: 238.
Ptychopera Den Hartog, 1964: 377–382, 386, fig. 2; Ax, 1971: 146–150, 152–153, fig. 1; Ax, 2008: 353.

Diagnosis (amended from Den Hartog, 1964): Trigonostominae with pharynx in the first half of the body. Afferent system differentiated into a large bursa and a small seminal receptacle connected by a narrow spermatic duct. Seminal receptacle connects to the female system through a long, slightly sclerotized duct. Wall of the bursa provided with sclerotized structures (folds or teeth). Sclerotized parts of the male copulatory organ consisting of a stylet proper surrounded by a mantle. Mantle provided with folds, spines or plates.

Type species: *Ptychopera westbladi* (Luther, 1943) Den Hartog, 1964.

PTYCHOPERA JAPONICA Ax, 2008 (FIG. 6A, F)

Ptychopera japonica Ax, 2008: 356–358, figs 160–161.

New locality: Mud Bay Park, Surrey, British Columbia, Canada (49°05'09"N, 122°51'39"W), mud and algae in intertidal mudflat (29/07/2015; 26/10/2015).

Known distribution: Northwest Pacific Ocean: Japan (Ax, 2008).

Material: Observations on eight live animals. Five whole mounts (BBM MI4050–MI4054). 18S rRNA (GenBank accession # MF321751), 28S rRNA (GenBank accession # MF321760).

Remarks: Animals about 0.5 mm long. Live specimens with a parenchymatous brownish to reddish coloration (Fig. 6A). General appearance typical of species of *Ptychopera* with the pharynx in the first third of the body right behind the lenticular eyes. Epidermis with oblong rhabdites.

Internal organization similar to other species of *Ptychopera* with paired testes and paired seminal vesicles entering the large, globuliform copulatory bulb. The large bursa appears striated and its basement membrane is slightly sclerotized (Fig. 6F). It connects to the seminal receptacle through a slender, slightly sclerotized spermatic duct provided with a sphincter. The seminal receptacle in turn connects to the female system through another longer, slightly sclerotized duct ('bursal appendage' in Den Hartog, 1964). Paired ovaries and vitellaria.

The stylet measures 67–78 µm ($\bar{x} = 70$ µm; $n = 5$) and consists of two curved, funnel-shaped and digitiform tubes surrounded by a folded sclerotized mantle that distally ends in a large triangular plate with a serrated edge (Fig. 6F). The digitiform tubes are of equal length, but differ in width with the wider tube being the stylet proper for the evacuation of sperm and prostate secretion. The more narrow tube connects to the sclerotized mantle at its base. The triangular plate folds over in its proximal half and continues as a weakly sclerotized girdle surrounding and connecting to the stylet in its middle portion. The proximal part of the stylet consists of the bases of the two digitiform plates and has several folds. A slightly curved, slender spur protrudes from the outer edge of one of these folds on the outer side of the larger digitiform tube (arrow in Fig. 6F).

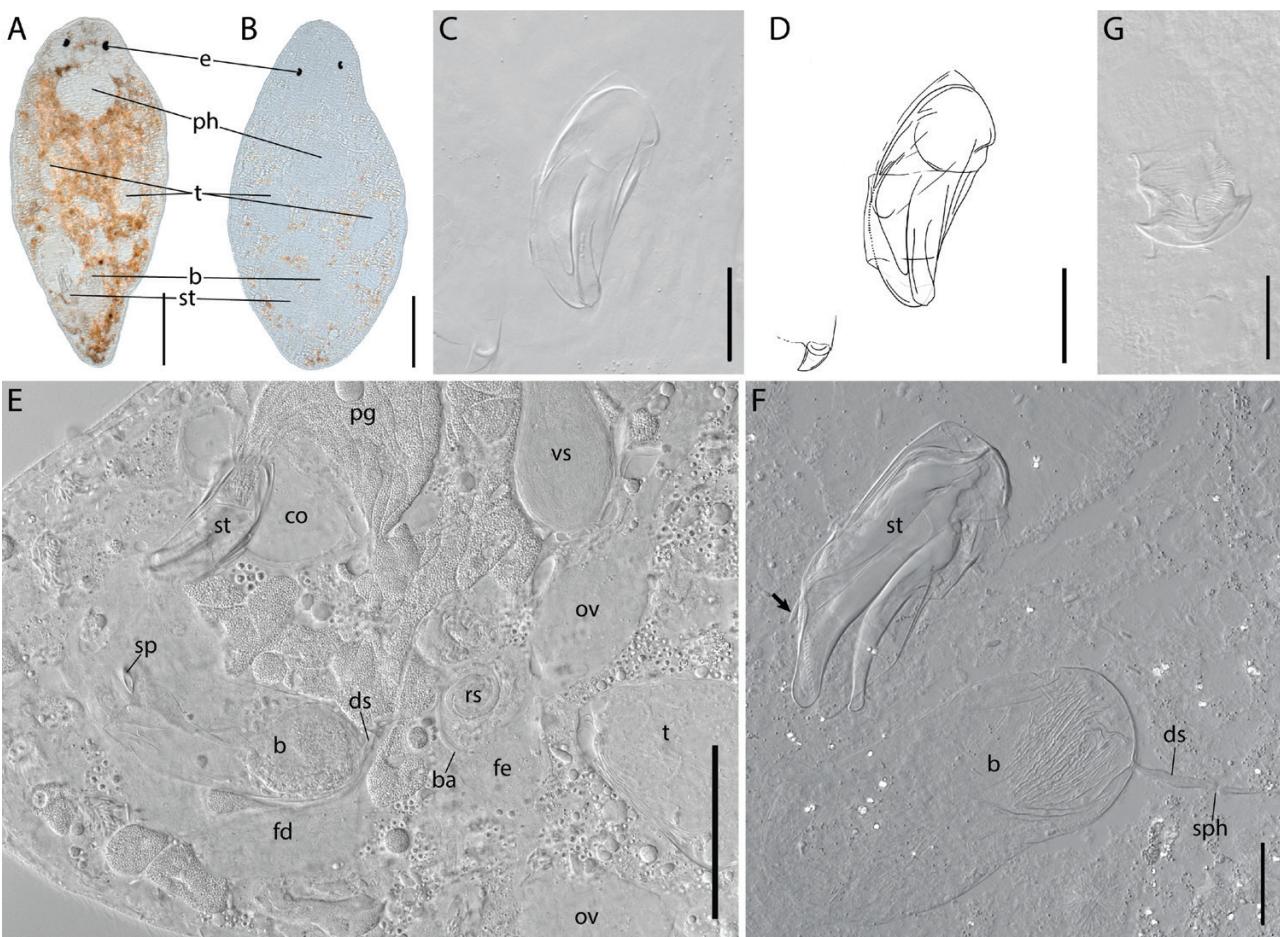


Figure 6. *Ptychopera japonica*, *Ptychopera unicornis* sp. nov. and *Tvaerminnea karlingi*. A, live specimen of *P. japonica* under cover slip. B, live specimen of *P. unicornis* sp. nov. under cover slip. C, D, stylet and bursal spine of the holotype of *P. unicornis* sp. nov. E, detail of the atrial organs in a live specimen of *P. unicornis* sp. nov. F, stylet and detail of afferent system in whole-mounted specimen of *P. japonica*. G, stylet from a whole-mounted specimen of *T. karlingi*. Abbreviations: b, bursa; ba, bursal appendage; co, copulatory organ; ds, spermatic duct; e, eye; fd, female duct; fe, fecundatorium; ov, ovary; pg, prostate glands; ph, pharynx; rs, seminal receptacle; sp, bursal spine; sph, sphincter; st, stylet; t, testis; vs, seminal vesicle. Scale bars: A, B = 100 µm; E = 50 µm; C, D and F, G = 20 µm.

Ptychopera japonica was first described by Ax (2008) from brackish salt marshes on the Pacific coast of Japan. Our specimens clearly belong to this species based on the morphology of the stylet. While the different parts of the stylet are discernable in the pictures of the Japanese specimens (see Ax, 2008: 357, fig. 161), Ax did not recognize the triangular plate as a separate structure and wrongly attributes the serrated edge to the larger digitiform tube. Furthermore, specimens from Japan lack pigment and have a slightly shorter stylet (up to 62 µm).

As already mentioned by Ax (2008), *P. japonica* is probably closely related to *P. westbladi* and *P. plebeia* (Beklemishev, 1927) Den Hartog, 1964. The stylets of *P. westbladi* and *P. plebeia* also consist of two more

or less funnel-shaped tubes and a sclerotized mantle with several folds, plates or protrusions. Particularly, the stylet of *P. westbladi* is similar to the stylet of *P. japonica*, but it lacks a serrated triangular plate and it also has a large projection that extends to the tip of the stylet. This structure is possibly homologous with the smaller spur-like projection of the stylet in *P. japonica*. The stylet of *P. plebeia* lacks a projection and serrated mantle edges.

PTYCHOPERA UNICORNIS SP. NOV. (FIG. 6B, C–E)

Etymology: The species epithet refers to the single, horn-like spine in the bursa.

Type locality: Clover Point, Victoria, British Columbia, Canada (48°24'12"N, 123°21'03"W), algae in rocky lower intertidal (02/09/2016; 03/03/2016).

Type material: One whole mount which is designated as the holotype (SMNH Type-8921).

Other material: Observations on three live animals. 18S rRNA (GenBank accession # MF321752), 28S rRNA (GenBank accession # MF321761).

Diagnosis: Species of *Ptychopera* with 46-μm-long stylet. Stylet consists of two slightly curved tubes. One tube is longer and club-shaped, the other one is shorter and triangular. Mantle with rounded plate and serrated edge. Bursa with sclerotized folds and an 8-μm-long spine.

Description: Live animals mostly transparent and measuring about 0.5–0.7 mm (Fig. 6B). General appearance typical of species of *Ptychopera* with the pharynx in the first half of the body and oblong rhabdites in the epidermis.

Internal organization (Fig. 6E) almost identical to *P. japonica* and other species of *Ptychopera*, the only difference being the somewhat more posterior position of the pharynx, the size and construction of the stylet and the presence of a spine in the proximal half of the bursa. The slightly curved stylet consistently measures 46 μm ($\bar{x} = 46 \mu\text{m}$; $n = 4$) and is composed of two slightly curved tubes or plates surrounded by a folded sclerotized mantle of which the distal half forms a large rounded plate with a slightly serrated convex edge (Fig. 6C, D). The two curved tubes differ in length and form. The longer tube on the concave side of the stylet is club-shaped with a rounded distal end while the shorter tube on the convex side of the stylet is more triangular and pointed. The bursa has a slightly sclerotized bursal membrane with sclerotized folds and an 8-μm-long spine at its base (Fig. 6C–E).

Discussion: Although the stylet of *P. unicornis* sp. nov. resembles the stylet of *P. japonica*, it differs by its smaller size, the more rounded serrated plate, the lack of an extended spur on the mantle, and a marked difference between the form and length of the two slightly curved stylet tubes. *P. unicornis* sp. nov. also has a spine in the proximal part of the bursa while *P. japonica* clearly lacks this. Sclerotized folds and ridges in the bursa are common in *Ptychopera*, but only three other species, *P. avicularis* Karling, 1974; *P. spinifera* Den Hartog, 1966b; and *P. purasjokii* Ax, 1971, have a single proper spine or teeth at the basis of the bursa. However, these species differ from *P. unicornis* sp. nov. by their stylet morphology.

In addition to differences in stylet morphology, *P. unicornis* sp. nov. and *P. japonica* seem to prefer

different kinds of intertidal habitats. *P. unicornis* sp. nov. was found on algae in the marine rocky intertidal, while *P. japonica* has a preference for slightly brackish intertidal mudflats.

PROMESOSTOMIDAE DEN HARTOG, 1964

BRINKMANNIELLINEAE LUTHER, 1948

TVAERMINNEA LUTHER, 1943

Tvaerminnea Luther, 1943: 81; Luther, 1948: 36; Luther, 1962: 44.

Diagnosis (amended from Luther, 1962): Brinkmanniellinae with long, stretchy body. Copulatory organ consists of a proximal copulatory bulb filled with sperm, a middle part containing the ejaculatory duct, and a distal stylet. Stylet is a short tube, with transversal folds and a complex hook-shaped distal end. Bursa with proximal, tooth-like folds of the basal membrane.

Type species: *Tvaerminnea karlingi* Luther, 1943.

TVAERMINNEA KARLINGI LUTHER, 1943

(FIG. 6G)

Tvaerminnea karlingi Luther, 1943: 80–84, figs 91–104; Ax, 1951: 369, table 11; Ax, 1956: 112–114, 172, 179, fig. 27, tables 5, 8; Ax, 1959: 47, 99; Luther, 1962: 44–46, fig. 18; Straarup, 1970: 191, 198, table 1; Karling, 1974: 29, 60, table 1, figs 68–70; Hellwig, 1987: 175, 198, table 6; Ax, 2008: 307–309, fig. 135.

Tvaerminnea karlingi pacifica Karling, 1986: 208–209, figs 33–38; Ax, 2008: 308, fig. 135D.

Tvaerminnea karlingi karlingi Karling, 1986: 208.

New localities: Mud Bay Park, Surrey, British Columbia, Canada (49°05'09"N, 122°51'39"W), mud and algae in intertidal mudflat (29/07/2015). Departure Bay, Nanaimo, British Columbia, Canada (49°11'43"N, 123°57'32"W), coarse sand and shell hash in the low intertidal (12/04/2015).

Known distribution: Northeast Atlantic Ocean: Baltic Sea (Luther, 1943, 1962; Ax, 1951; Straarup, 1970), Irish Sea (Boaden, 1963), North Sea (Hellwig, 1987). Mediterranean: Gulf of Lion (Ax, 1956). Bosphorus (Ax, 1959). Northeast Pacific Ocean: California (Karling, 1986).

Material: Observations on two live animals. Three whole mounts (BBM MI4055–MI4057). 18S rRNA (GenBank accession # MF321755), 28S rRNA (GenBank accession # MF321764).

Remarks: Animals as described by Luther (1943, 1962) and Karling (1986). The stylet measures 22–27 µm ($\bar{x} = 25$ µm; $n = 3$) and consists of a tube, transversal folds and a crescent-shaped plate (Fig. 6G). The bursa is only visible in the live animals and appears to have one or two tooth-like sclerotized folds. Based on the morphology of this ‘bursa comb’, Karling (1986) distinguishes two morphotypes: *T. karlingi karlingi* from the Northeastern Atlantic and Mediterranean, and *T. karlingi pacifica* from California. Unfortunately, we cannot attribute our specimens to one of these morphotypes, because the bursa comb is not visible in the whole-mounted specimens from British Columbia. Given the disjunct distributions of the Atlantic and Pacific population and the recognition of different morphotypes, it is not unlikely *T. karlingi* consists of two or more cryptic species (see also discussion on *C. axi*).

MOLECULAR PHYLOGENETIC RELATIONSHIPS

The final 18S and 28S rRNA sequence data sets comprised 42 taxa and 1723 bp and 36 taxa and 1727 bp, respectively. This results in a concatenated data set (18S + 28S) of 42 taxa and 3450 bp for our phylogenetic analyses. Bayesian and ML topologies were congruent.

Results of the phylogenetic analyses are summarized in Figure 7A. The ingroup consists of two clades: (1) a clade with *Litucivis serpens* Ax & Heller, 1970 (*Adenorhynchinae* Ax & Heller, 1970), and a polytomy of some representatives of *Brinkmanniellinae*, including *T. karlingi*, *Cilionema hawaiiensis* Karling *et al.*, 1972, and two species of *Coronhelmis* Luther, 1948; and (2) a clade with several genera of Trigonostominae, including *Parapharyngiella* Willems *et al.*, 2005b, *Trigonostomum*, *Beklemischeviella*, *Proxenetes*, *Ceratopera* and *Ptychopera*. The outgroup consists of *Microvahine corallicola* Karling *et al.*, 1972 (*Paramesostominae* Luther, 1948); *Promesostoma marmoratum* (Schultze, 1851) von Graff, 1882 (*Promesostominae* Luther, 1948); *Byrsophlebs delamarei* (Ax, 1956) Karling, 1985 (*Byrsophlebidae* von Graff, 1905); and *Thalassoplanella collaris* Luther, 1946 (*Typhloplanidae* von Graff, 1905).

Within Trigonostominae, all genera are monophyletic with high support values (bs = 100; pp = 1). *Ceratopera* sensu Den Hartog is paraphyletic because of the position of *C. pacifica* comb. nov. (formerly *M. pacifica*) and *C. complicata* sp. nov., which are deeply embedded within *Ceratopera*. *Parapharyngiella* is the sister taxon of all other genera of Trigonostominae. Phylogenetic relationships among the remainder genera remain partly unresolved except for a sister group relationship between *Beklemischeviella* and *Proxenetes*.

DISCUSSION

INTERGENERIC RELATIONSHIPS

Trigonostominae and some of its polytypic genera have been the subject of a number of taxonomic reviews (Luther, 1948; Den Hartog, 1964, 1966b; Ax, 1971; Willems *et al.*, 2004b). Different hypotheses on the taxonomic relationships between and within most genera were presented in these works based on morphological characters such as the position of the pharynx, the presence or absence of a ‘proboscis’, the structure of the male copulatory organ and the organization of the afferent system. However, the topology of our tree and position of the different genera suggest that the assumptions on the homology and plesio- or apomorphy of certain morphological character states are in need of revision, a reservation already mentioned by Den Hartog (1964). In this discussion, we will focus on those genera that are also included in our phylogenetic analysis.

The basal position of *Parapharyngiella* to all other genera within Trigonostominae was already uncovered in a previous molecular phylogenetic analysis (Van Steenkiste *et al.*, 2013). This genus was only recently described (Willems *et al.*, 2005b), and never included in any of the aforementioned morphological reviews. It is characterized by a stylet surrounded by a mantle, and an afferent system with a sclerotized bursal stalk and a weakly sclerotized, club-shaped bursal appendage leading into a simple insemination duct. A mantle in the form of a sclerotized part of the male atrium surrounding and/or fusing with the stylet also occurs in all other genera of Trigonostominae, except for *Beklemischeviella*. As such, the presence of a mantle seems to be plesiomorphic while its absence in *Beklemischeviella* is most likely a secondary reduction. This challenges the phylogenetic system of Den Hartog (1964) and Ax (1971), who considered a stylet without a mantle the plesiomorphic condition.

Our phylogeny suggests the afferent system should also be reinterpreted (Fig. 7B). Ax (1971) considered the differentiation of the afferent system of *Beklemischeviella* and *Ptychopera* (and *Lutheriella*) in a large bursa, a slender sclerotized spermatic duct, a small seminal receptacle, an undifferentiated, weakly sclerotized ‘mouthpiece’ (or bursal appendage) and an insemination duct, to be symplesiomorphic. The spermatic duct and seminal receptacle are absent in *Parapharyngiella*, *Trigonostomum*, *Ceratopera* sensu Den Hartog and *Messoplana*, while the ‘mouthpiece’ was considered homologous with the bursal appendage of these genera. However, the phylogenetic position of *Parapharyngiella*, *Beklemischeviella* and *Ptychopera* questions this assumed homology. Alternatively, the spermatic duct of *Ptychopera* could be homologous

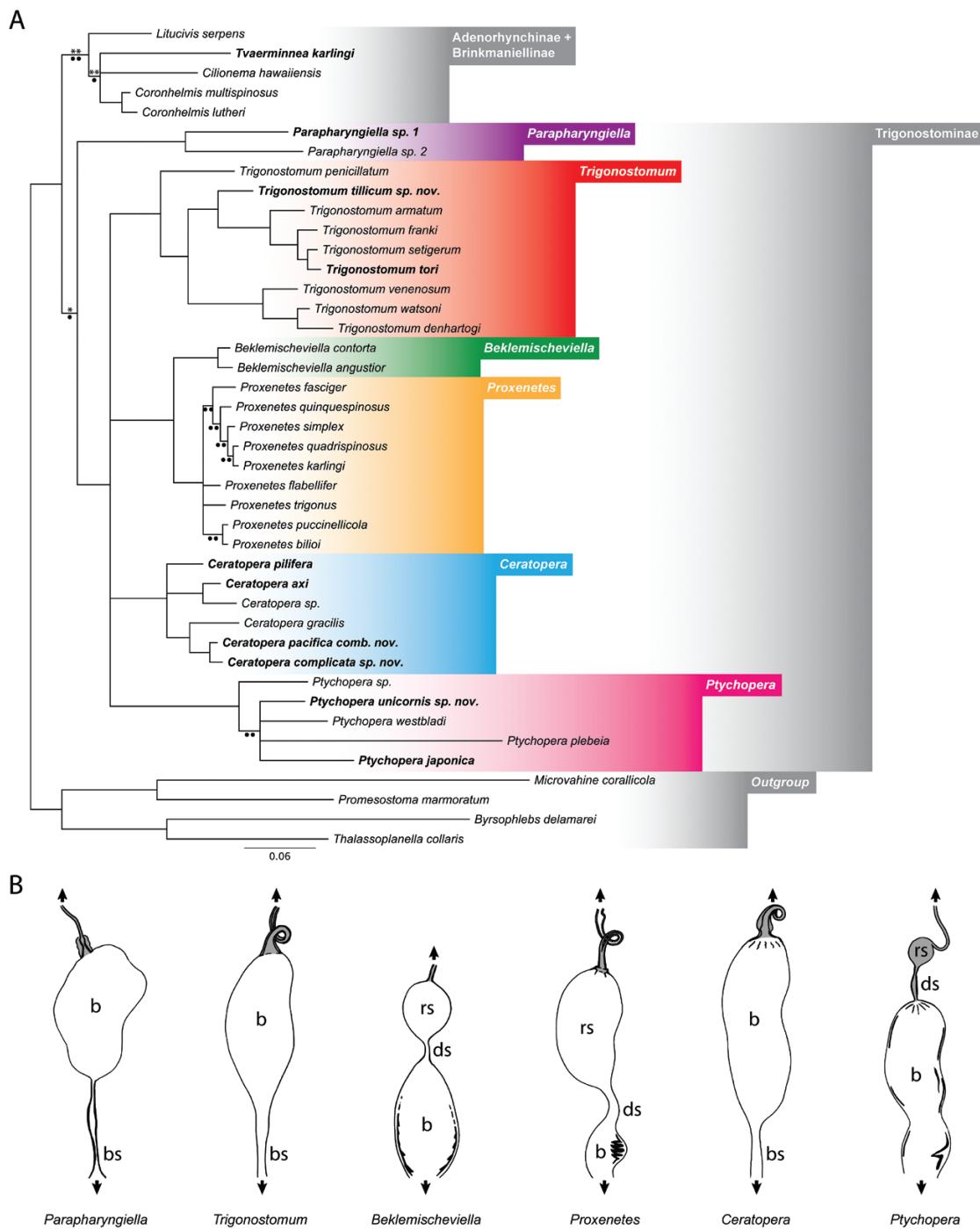


Figure 7. A, Bayesian majority-rule consensus tree of the concatenated 18S + 28S alignment. Symbols above branches indicate posterior probabilities (pp) from the Bayesian analysis ($0.95 \leq * < 0.98$, $0.98 \leq ** < 1$). Symbols below branches represent bootstrap (bs) values from the maximum likelihood analysis ($70 \leq \bullet < 85$, $85 \leq \bullet\bullet < 100$). Branches without symbols are fully supported (pp = 1, bs = 100) while unsupported branches (pp < 0.95, bs < 70) have been collapsed. Taxa in bold have been sequenced for this study. Scale bar represents substitutions per site. B, schematic representation of the afferent system in different trigonostomine genera. The bursa or bursal stalk connects to the common genital atrium (downward pointing arrow). The bursal appendage and insemination duct run to the fecundatorium and the ovaries of the female system (upward pointing arrow). Bursal appendage and structures homologous with the bursal appendage are in grey. Sclerotized basal membrane and its derived structures (spines, folds) are in bold. Abbreviations: b, bursa; bs, bursal stalk; ds, spermatheca; rs, seminal receptacle.

with the bursal appendage. This spermatic duct is sclerotized, striated, proximally somewhat enlarged and often provided with a constriction and a ring-like structure (most likely the position of one or two sphincters), not unlike the bursal appendage in *Parapharyngiella* and some species of *Trigonostomum*. Consequently, the ‘seminal receptacle’ of *Ptychopera* could be a vesicular portion of the ‘bursal appendage’ or the insemination duct. Similarly, new hypotheses on the homologies between the afferent system of *Beklemischeviella* and other genera could be put forward, although descriptions of the different species of *Beklemischeviella* generally lack detailed information on the morphology of the spermatic duct, seminal receptacle and ‘mouthpiece’. In several species of *Proxenetes*, a constriction divides the bursa into two parts. Some authors describe the part carrying the bursal appendage as a seminal receptacle. Given the sister group relationship of *Proxenetes* and *Beklemischeviella*, the common ancestor of these genera also could have had a bipartite bursa. Reinterpreting homologies in the afferent system also affects interpretations about the form of the bursal appendage in the most recent ancestor of Trigonostominae. Possibly, it resembled that of *Parapharyngiella*, i.e. a slightly sclerotized tubular or club-shaped mouthpiece in the proximal part of the insemination duct. As such, this simple, undivided, sclerotized tube would have been retained as the ‘spermatic duct’ in *Ptychopera* and the striated bursal appendage in some species of *Trigonostomum*. It developed into a bitubular appendage with a cingulate base in *Proxenetes*, some species of *Messoplana* and the majority of species of *Trigonostomum*.

The bursal appendage of *Ceratopera* sensu Den Hartog and *Messoplana*, now united in *Ceratopera*, shows mixed characteristics and could offer us some insight in the transition to a bitubular organization (see discussion *C. complicata* sp. nov.). Given the position of *C. pacifica* comb. nov. and *C. complicata* sp. nov. in our tree (i.e. embedded within *Ceratopera* with *C. gracilis* as a sister species), a large undivided proximal part is probably the plesiomorphic condition. Miniaturization of this part, enlargement of the distal bifurcation to form two long tubes and possibly sclerotization of the ring-like structure could explain the morphology of the bursal appendage in some species of the suppressed genus *Messoplana*.

INTRAGENERIC RELATIONSHIPS

Intrageneric relationships within Trigonostominae were not discussed by *Van Steenkiste et al.* (2013), mainly because of a lack of taxa and

the large phylogenetic scope (i.e. phylogeny of Dalytyphloplanida). The addition of new representatives of Trigonostominae and improved phylogenetic resolution allows us to make some inferences on the interspecific relationships and morphological characters within the species-rich genera *Proxenetes* and *Trigonostomum*.

Proxenetes was subdivided into different species groups by *Den Hartog* (1964, 1965, 1966a) and *Ax* (1971). *Den Hartog* established his three species ‘sections’ based on the structure of the bursal appendage while *Ax* created five ‘subgenera’ based on the construction of the stylet and mantle. While these groupings are not supported by our molecular data, some synapomorphies based on stylet morphology could be attributed to the two clades of *Proxenetes* displayed in our tree: (1) *P. puccinellicola* Ax, 1960, and *P. bilioi* *Den Hartog*, 1966a, both have a conspicuous thickening and striation of the wall of the stylet proper; and (2) part of the mantle in *P. fasciger* Ehlers, 1974; *P. quinquespinosus* Ax, 1971; *P. simplex* Luther, 1948; and *P. karlingi* Luther, 1943, is modified into two or more spines.

The morphology and taxonomy of *Trigonostomum* was reviewed by *Willems et al.* (2004b). These authors recognize two different species groups based on the morphology of the stylet and bursal appendage. Representatives of both groups are present in our tree. Species group 1 consists of *Trigonostomum penicillatum* (Schmidt, 1857) Micoletzky, 1910; *T. venenosum* (Uljanin, 1870) Meixner, 1924; *T. watsoni* *Willems et al.*, 2004b; *T. denhartogi* (Karling, 1978) *Willems et al.*, 2004b; and *T. tillicum* sp. nov. They form a paraphyletic assemblage and have a proximally curved stylet distally surrounded by a mantle with one or more spiny plates. *Willems et al.* (2004b) considered this condition plesiomorphic and it is also retrieved as such in our tree. The bursal appendage of *T. penicillatum* consisting of around 12 tubules, tightly packed together (*Artois et al.*, 2013), suggests a striated appendage with multiple tubules is plesiomorphic. The remainder taxa of group 1 retain a striated undivided proximal part but the appendage has undergone a reduction in the number of distal tubes: six in *T. tillicum* sp. nov., two in *T. venenosum*, *T. watsoni* and *T. denhartogi*. Species in group 2 have a coiled stylet completely surrounded by a mantle ending in two spiny plates. This condition is considered synapomorphic and defines the clade that includes *T. armatum* (Jensen, 1878) Gamble, 1900, *T. franki* *Willems et al.*, 2004b, *T. setigerum* and *T. tori*. The bursal appendage in this group is always bitubular.

CONCLUSIONS

Increased taxon sampling of Trigonostominae, including four new species, in a comprehensive molecular

phylogeny inferred from 18S and 28S rRNA sequences allowed us to demonstrate inter- and intrageneric relationships within this group. This phylogenetic context enabled us to reassess homology statements on some key morphological traits associated with the stylet and the afferent system. Our results show that (1) species of *Ceratopera* and *Messoplana* cannot be clearly separated based on morphological and molecular data leading to the suppression of the latter genus in favour of *Ceratopera*; (2) traditional species groups within *Trigonostomum* are partly supported by molecular data, while traditional species groups within *Proxenetes* are not; (3) the presence of a mantle accompanying the stylet proper reflects the plesiomorphic condition of the Trigonostominae; (4) the spermatic duct and seminal receptacle in *Ptychopera* is inferred to be homologous with (parts of) the bursal appendage and the insemination duct in other genera; and (5) the ancestral bursal appendage resembled a simple, slightly sclerotized tube. Further taxon sampling and additional molecular markers will be necessary to more fully resolve the phylogeny of Trigonostominae and refine our inferences about the evolution of their highly complex reproduction structures.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1. Amplification primers and internal sequencing primers used in this study.