Journal of the Marine Biological Association of the United Kingdom, 2014, 94(3), 499–514. © Marine Biological Association of the United Kingdom, 2014
doi:10.1017/S0025315413001471

Molecular examination of kalyptorhynch diversity (Platyhelminthes: Rhabdocoela), including descriptions of five meiofaunal species from the north-eastern Pacific Ocean

REBECCA J. RUNDELL[1,2] AND BRIAN S. LEANDER[1]

[1]Department of Zoology, Biodiversity Research Centre, University of British Columbia, Canadian Institute for Advanced Research, Program in Integrated Microbial Diversity, Vancouver, British Columbia, V6T 1Z4 Canada, [2]Department of Environmental and Forest Biology, State University of New York, College of Environmental Science and Forestry, Syracuse, New York 13210, USA

*The spaces between sand grains are home to a myriad of microscopic marine eukaryotes, including kalyptorhynch rhabdocoels equipped with an eversible proboscis that enables them to capture microscopic prey living in these environments. The structure of the kalyptorhynch proboscis separates the two major subclades within the group: the Schizorhynchia (bifurcated proboscis) and the Eukalyptorhynchia (unbranched proboscis). A survey of meiofaunal metazoans in the Pacific north-west led to the discovery of three new schizorhynch species (*Undicola tofinoensis *gen. nov., sp. nov.,* Schizorhinos vancouverensis *gen. nov., sp. nov. and* Linguabana tulai *gen. nov., sp. nov.) and two new eukalyptorhynch species (*Thinodactylaina tlaoquiahtensis *gen. nov., sp. nov. and* Rostracilla nuuchahnulthensis *gen. nov., sp. nov.). This survey also recovered the putative cosmopolitan eukalyptorhynch (Polycystididae)* Gyratrix hermaphroditus *Ehrenberg, 1831. We performed molecular phylogenetic analyses on 18S rDNA sequences from all five novel isolates and from all available kalyptorhynch species in GenBank. The molecular data supported the monophyly of the Eukalyptorhynchia and Schizorhynchia and helped demonstrate the boundaries between different species within the Kalyptorhynchia.*

Keywords: biodiversity, DNA barcode, meiofauna, Turbellaria, 18S rDNA

Submitted 21 June 2013; accepted 25 September 2013; first published online 10 January 2014

INTRODUCTION

Marine interstitial environments are replete with microscopic detritus feeders, eukaryovores and bacterivores (i.e. meiofauna: eukaryotes between 60 and 2000 μm in length). Kalyptorhynch turbellarians are microscopic predators in this environment that are armed with an eversible, and sometimes bifurcated, proboscis. The proboscis can be equipped with hooks, toxins and mucus that aid in the entrapment of microscopic prey (e.g. harpacticoid copepods). Once prey has been captured from interstitial spaces, kalyptorhynchs convey the meal to the mid-ventral, muscular pharynx (Meixner, 1925; Karling, 1961; Martens & Schockaert, 1986; Brusca & Brusca, 2003; Uyeno & Kier, 2010). Kalyptorhynchs are simultaneous hermaphrodites, and the mutual exchange of gametes occurs through genital or through hypodermic impregnation using a stylet apparatus. There is considerable morphological variation in these often spear-like stylet apparatuses (Karling, 1983a). Internal fertilization and the evolution of complex male and female structures have likely played a role in speciation in kalyptorhynchs, as they do in many animals (Mayr, 1963). Despite a few detailed studies of these organ systems in kalyptorhynchs (e.g. Schilke, 1970a; Rieger, 1974; Schockaert, 1974; Artois & Schockaert, 2003, 2005a; see also Seth Tyler's literature database at turbellaria.umaine.edu), the diversity and evolutionary history of kalyptorhynchs remains poorly known (Karling, 1983a; Rieger, 1998).

Corresponding author:
R.J. Rundell
Email: rundell@esf.edu

The kalyptorhynch proboscis

The anterior proboscis of kalyptorhynchs is a terminal invagination of the epidermis, basement membrane and subepidermal muscle sheath (Rieger, 1974). The kalyptorhynch proboscis can take on many different morphologies depending on the species; therefore, the most detailed information on these feeding structures is available in the widely scattered taxonomic literature (e.g. Noldt & Reise, 1987; Artois & Schockaert, 2000; Artois & Schockaert, 2003; Willems *et al.*, 2007 for eukalyptorhynch kalyptorhynchs; Karling, 1961; Schilke, 1970a; Karling, 1983a for schizorhynch kalyptorhynchs; see also turbellaria.umaine.edu). However, in general, the schizorhynch proboscis consists of two dorsoventrally opposed tongues, and the proboscis in eukalyptorhynchs is cone-shaped. One schizorhynch species, *Typhlorhynchus nanus* Karling 1981, lacks a proboscis altogether, suggesting that losses of this trait occurred in some lineages of kalyptorhynchs.

The proboscis is everted forcefully from the anterior end of the animal to capture prey. In eukalyptorhynchs, the proboscis is everted using prominent musculature at the base of the proboscis sheath. In schizorhynchs, the proboscis is everted by retracting the rostral wall and generating pressure through the retraction of superficial circumferential body muscles; schizorhynchs lack protractor muscles (Karling, 1961; Rieger *et al.*, 1991; Uyeno & Kier, 2010). Most kalyptorhynchs also have prominent glands at the base of the proboscis that secrete chemicals or mucus that aid in capturing and subduing prey. Other kalyptorhynchs, such as some schizorhynchs and *Smithsoniarhynches*, possess proboscis tip hooks capable of a range of motions for manipulating prey (Hochberg, 2004; Uyeno & Kier, 2010). Traits associated with the proboscis sometimes have been used to discriminate different species of kalyptorhynchs from one another.

The kalyptorhynch stylet apparatus

Differences between the male stylet apparatuses in kalyptorhynch species are scattered in the taxonomic literature (e.g. Schockaert & Karling, 1975; Dean, 1977; Karling & Schockaert, 1977; Willems *et al.*, 2006b; Timoshkin *et al.*, 2009, for eukalyptorhynchs; Dean, 1980; Doe, 1974; Karling, 1983a for schizorhynchs). There are at least four male stylet types, and in some species more than one type can occur within a single stylet atrium. For example, the polycystidid *Triaustrorhynchus armatus* Willems *et al.*, 2006b has three stylet types in its male atrium. Stylets may appear singly, or in a V-shape. The context of the stylet relative to the rest of the male copulatory apparatus is sometimes difficult to place in older reports because the stylet was separated from the body using chemicals and then photographed or drawn in isolation. Some species lack a distinct single or double stylet organ, and instead have a male copulatory organ with multiple visible points (Karling, 1983a).

Kalyptorhynch systematics

The Kalyptorhynchia is one of two subclades within the Rhabdocoela, which is a diverse, monophyletic group of free-living flatworms whose sister group is uncertain (Cannon, 1986; Littlewood *et al.*, 1999a, b; Joffe & Kornakova, 2001; Littlewood & Olson, 2001; Willems *et al.*, 2006a). There are about 530 described kalyptorhynch species (Van Steenkiste *et al.*, 2013) but as is the case for other turbellarians (Artois & Schockaert, 2005b), this is likely a vast underestimate of the true diversity of the group. The Kalyptorhynchia contain two subclades: the Schizorhynchia (bifurcated proboscis) and the Eukalyptorhychia (unbranched proboscis) (Cannon, 1986; Willems *et al.*, 2006a). The Schizorhynchia include at least 25 genera in the following four families: Diascorhynchidae Meixner, 1928, Karkinorhynchidae Meixner, 1928, Nematorhynchidae Schilke, 1969 and Schizorhynchidae Graff, 1905; the Eukalyptorhynchia include at least 100 genera (47 of which are polycystidids) in twelve families. Molecular phylogenetic analyses of 18S rDNA sequences suggest that a clade comprising Dalytyphloplanida and *Mariplanella frisia* Ax & Heller, 1970 forms the nearest sister group to the Kalyptorhynchia (Willems *et al.*, 2006a).

Evaluating the species diversity of kalyptorhynchs using DNA sequences is particularly useful because the availability of comparative morphological traits within the group is limited and some of the most conspicuous traits are likely subject to convergence (e.g. traits associated with the proboscis apparatus; Karling, 1983a, b). However, DNA barcoding efforts for understanding meiofaunal species diversity are limited by meagre sampling within kalyptorhynchs, and microturbellarians in general, despite the fact that turbellarians arguably rank third in abundance in meiofaunal samples, after nematodes and harpacticoid copepods (Giere, 2009). The diversity of gene regions represented in past studies is also limited, which prevented us from using COI (the common barcoding gene) here. The main goal of this study was to use DNA sequence data to survey the diversity of kalyptorhynch species in marine sand from the eastern Pacific Ocean in order to increase our understanding of the interstitial biodiversity in this region and to help build a more comprehensive phylogenetic framework for the kalyptorhynch flatworms.

MATERIALS AND METHODS

Sampling and microscopy

Sand samples were collected from the western coast of Vancouver Island and the city of Vancouver (Canada) for DNA extraction and light microscopy (LM; Figures 1–6). Fine sand (2 l) was collected from Long Beach near Tofino on the western coast of Vancouver Island (49°03′20.42″N 125°43′40.16″W) at low tide in a wave-exposed section of the beach in September 2009. *Undicola tofinoensis* gen. nov., sp. nov. and *Rostracilla nuuchahnulthensis* gen. nov., sp. nov. were isolated from this sample. Another fine sand sample (2 l) was collected near this site in a less wave-exposed pool in September 2009, and *Linguabana tulai* gen. nov., sp. nov. and *Thinodactylaina tlaoquiahtensis* gen. nov., sp. nov. were isolated from this sample. One specimen of *T. tlaoquiahtensis* sp. nov. was also isolated from a 2 l fine sand sample collected at low tide from Pachena Beach on the western coast of Vancouver Island (48°47.551′N 125°06.974′W) in June 2009. No other kalyptorhynchs were found in these samples.

Course sand (6 l) was collected by snorkelling at high tide 3 m from the shore of Stanley Park (Vancouver, 48°17′18.48″N 123°08′37.78″W) in August 2009. *Schizorhinos vancouverensis* gen. nov., sp. nov. was isolated from this sample. Shell hash, mixed with marl and silt (50 l), was collected by dredge at 15–20 m depth near Wizard Islet (48°51.580′N 125°09.659′W), Trevor Channel, Barkley Sound, on the western coast of Vancouver Island in September 2009. *Gyratrix hermaphroditus* Ehrenberg, 1831 isolates (e.g. Figure 1) were collected from these samples.

In all cases, marine sediment was kept in clean plastic containers on ice in a cooler and immediately transported to the laboratory at Bamfield Marine Sciences Centre or the University of British Columbia for sieving on the same day, or one day later. Organisms were sieved through large PVC tubes into a large plastic Petri dish using the Uhlig sea water ice method (Uhlig, 1964) and 230 μm Nitex plankton mesh.

Individual kalyptorhynchs were captured by a glass pipette under a dissecting microscope and transferred to smaller Petri dishes containing filtered autoclaved seawater. Behaviour was observed under a Leica MZ6 stereomicroscope or a Zeiss stereomicroscope using DIC settings. Live kalyptorhynchs were anaesthetized in isotonic $MgCl_2$

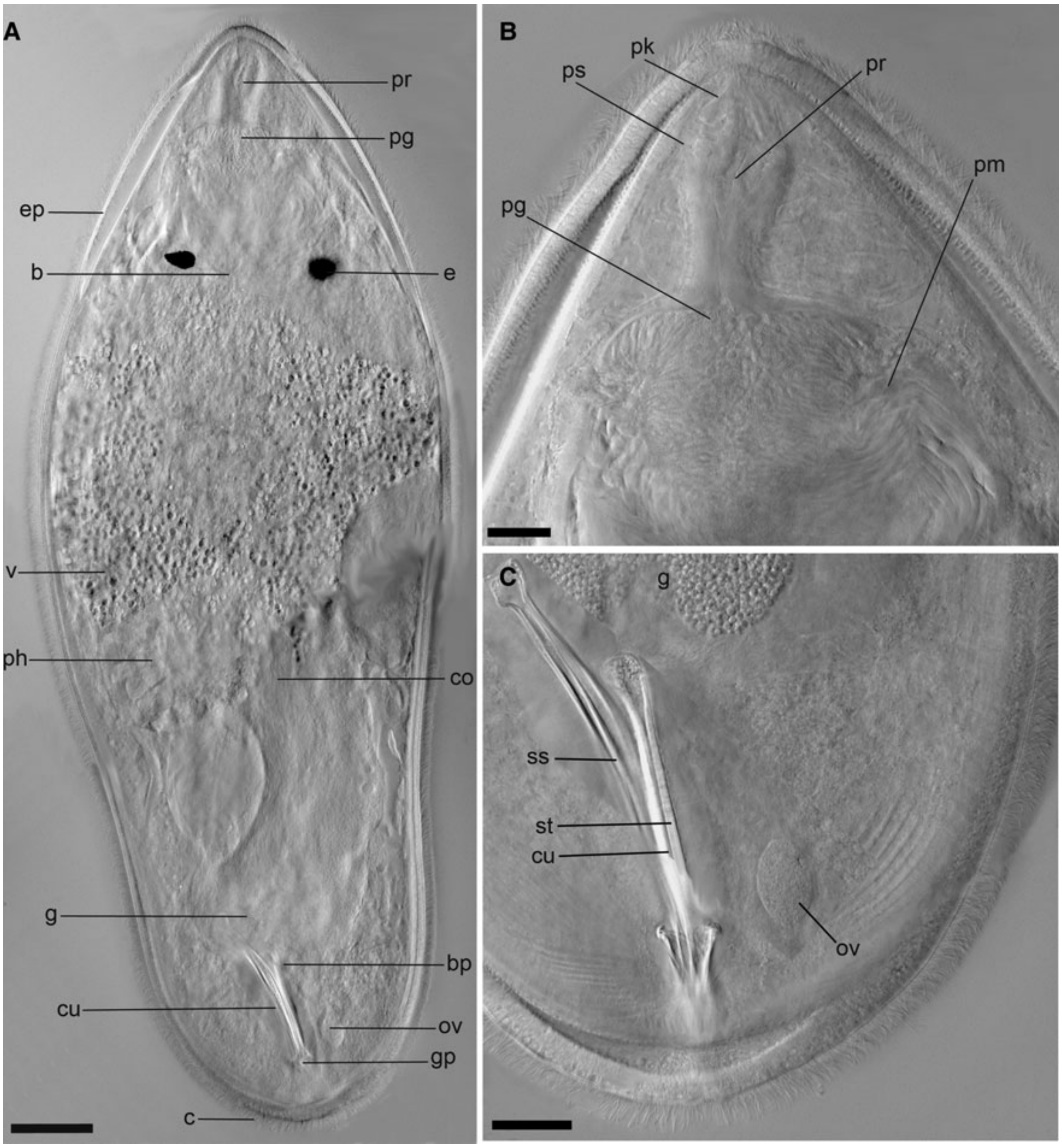

Fig. 1. (A–C) Dorsal view of a gluteraldehyde-preserved *Gyratrix hermaphroditus* specimen (squeeze preparation); (A) full body; (B) head region showing details of proboscis and associated musculature; (C) posterior region showing details of the male and female genitalia, particularly the male cuticular apparatus. Abbreviations: b, brain; bp, bursal pore; c, cilia; co, copulatory organ; cu, male cuticular apparatus; e, eye; ep, epidermis; g, germarium; gp, common gonopore; ov, ovary; pg, proboscis glands; ph, pharynx; pk, proboscis hooks; pm, proboscis muscles; pr, proboscis; ps, proboscis sheath; ss, stylet sheath; st, stylet; v, vitellaria. Scale bars: A, 50 µm; B,C, 20 µm.

solution and photographed using a squeeze preparation technique to enable observation of internal morphology. It was necessary to photograph kalyptorhynchs through several planes of focus, because their bodies were rounded even under squeeze preparations. According to Cannon (1986) fixed whole mounts for microturbellaria (in contrast to some macroscopic forms) are not useful, except for visualizing cuticular structures, because fixation can render most morphological features unobservable. Thus, we aimed to photograph live specimens. When necessary, we used fixed specimens preserved at 4°C in a dilute gluteraldehyde and autoclaved filtered seawater solution. Vouchers were deposited in the Beaty Biodiversity Museum Marine Invertebrates Collection (BBMMI).

DNA extraction, PCR and sequencing

Kalyptorhynchs were dissolved in MasterPure kit buffer plus proteinase K solution at 55°C for >24 h and DNA was subsequently extracted according to the kit protocol (MasterPure Complete DNA and RNA Purification Kit, Epicentre Biotechnologies). One individual was used in each extraction. 18S rDNA sequences were amplified using illustra PuReTaq Ready-to-Go PCR beads (GE Healthcare), 22 µl dH_2o, 1 µl genomic DNA and the primer pairs (1 µl each of 1F-5R; 3F-9R) and PCR protocol described by Giribet *et al.* (1996). The PCR products were purified with ExoSAP-IT enzyme (USB, Affymetrix, Inc.) and sequenced using Big Dye chemistry (Applied Biosystems) and an ABI 3730 DNA

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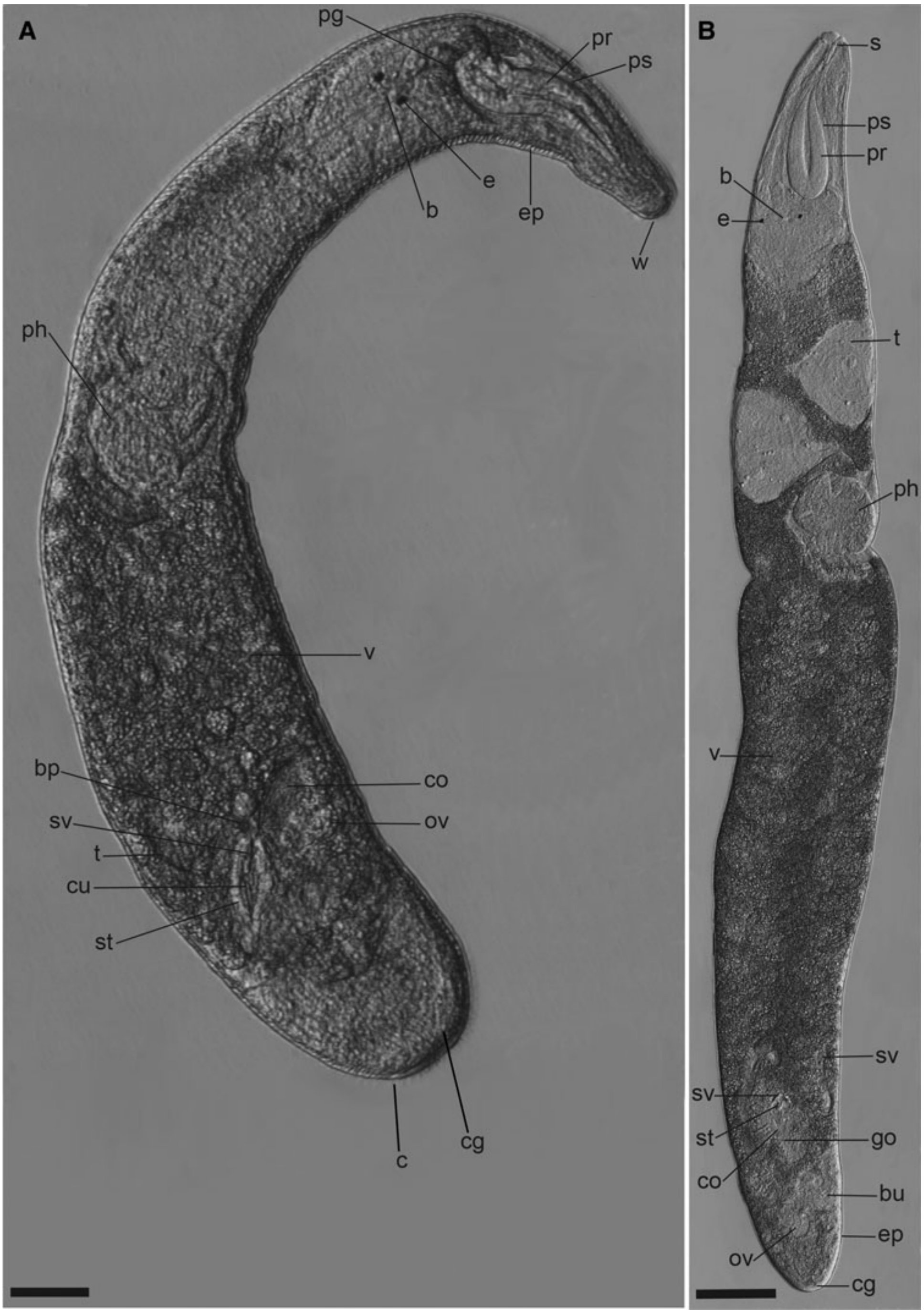

Fig. 2. (A) Dorsal view of live anaesthetized *Undicola tofinoensis* gen. nov., sp. nov. specimen (squeeze preparation); (B) dorsal view of live anaesthetized *Linguabana tulai* gen. nov., sp. nov. specimen (squeeze preparation). Abbreviations: b, brain; bp, bursal pore; bu, bursa; c, cilia; cg, caudal glands; co, copulatory organ; cu, male cuticular apparatus; e, eye; ep, epidermis; go, genital opening; ov, ovary; pg, proboscis glands; ph, pharynx; pr, proboscis; ps, proboscis sheath; s, sphincter; st, stylet; sv, seminal vesicle; t, testis; v, vitellaria; w, whiskers. Scale bars: A, B, 50 μm.

Analyzer. The new 18S rDNA sequences generated here were edited in Sequencher (Gene Codes).

Multiple sequence alignment and molecular phylogenetic analyses

GenBank was used to obtain additional kalyptorhynch samples and non-kalyptorhynch rhabdocoels for molecular phylogenetic analysis. All species included in the analyses and their GenBank Accession numbers are listed in Table 1. Novel sequences were aligned with the GenBank sequences using ClustalX and by eye using MacClade 4 (Maddison & Maddison, 2001) (Thompson *et al.*, 1997), resulting in a 27-taxon alignment.

Maximum likelihood (ML) analyses were conducted using two non-kalyptorhynch rhabdocoels as outgroups (JN205119-JN205122, JN205125), based on previous molecular work demonstrating the monophyly of Kalyptorhynchia (Willems *et al.*, 2006a). jModelTest v.01.1 (Guindon & Gascuel, 2003; Posada, 2008) was run using the Akaike information criterion

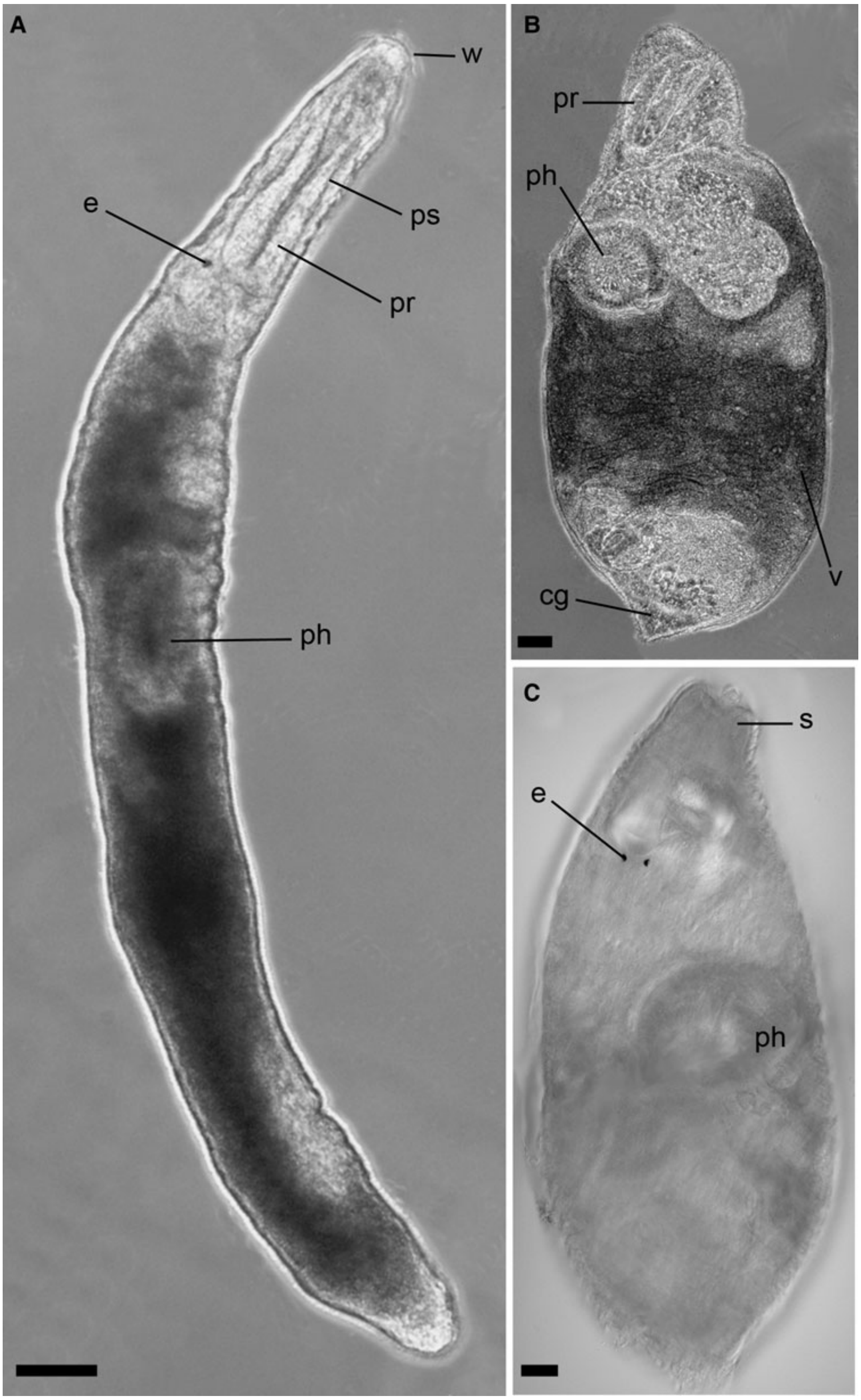

Fig. 3. *Schizorhinos vancouverensis* gen. nov., sp. nov.: (A) dorsal view of fully extended live anaesthetized specimen; (B, C) dorsal views of retracted live anaesthetized specimens. Abbreviations: cg, caudal glands; e, eye; ph, pharynx; pr, proboscis; ps, proboscis sheath; s, sphincter; v, vitellaria; w, whiskers. scale bars: A, 20 μm; B, C, 100 μm;

model evaluation approach. The general time-reversible model with gamma distribution and number of invariant sites (GTR + G + I) was the best fit for the data. Ten likelihood replicates and non-parametric bootstrap analyses (100 pseudoreplicates) were performed in GARLI v.0.95 (Zwickl, 2006) under the GTR (6-rate) model (accepted using this version of GARLI) and using default settings and rate matrix specified by jModelTest output.

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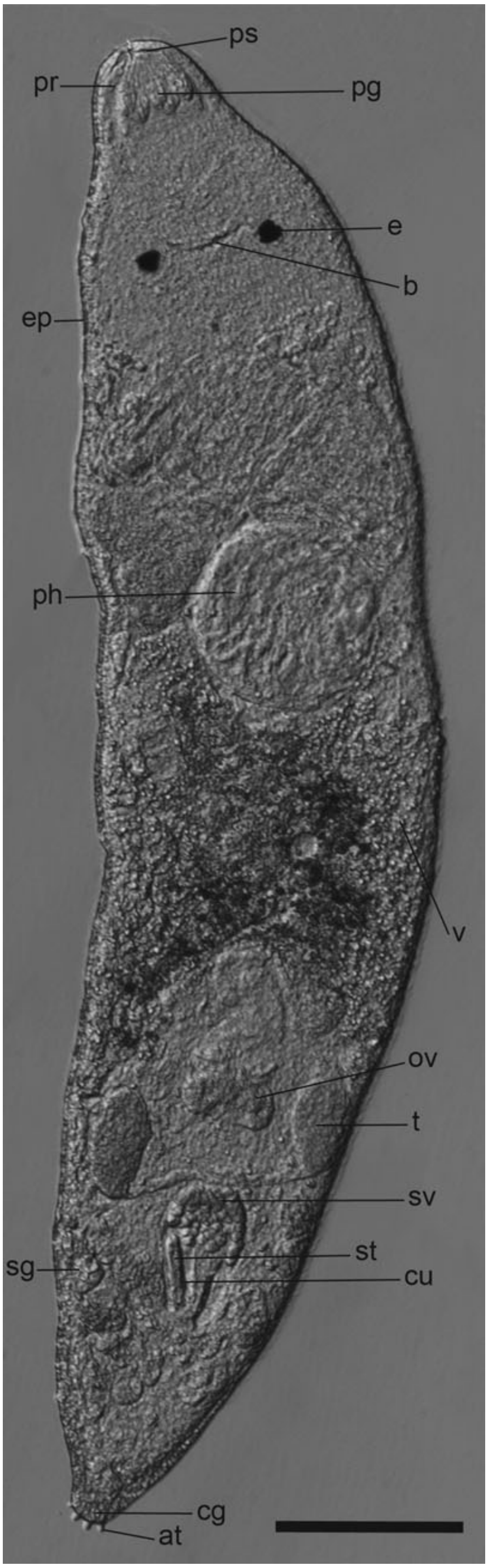

Fig. 4. Dorsal view of extended live anaesthetized *Thinodactylaina tlaoquiahtensis* gen. nov., sp. nov. specimen (squeeze preparation). Abbreviations: at, adhesive toes; b, brain; cg, caudal glands; cu, male cuticular apparatus; e, eye; ep, epidermis; ov, ovary; pg, proboscis glands; ph, pharynx; pr, proboscis; ps, proboscis sheath; sg, shell gland; st, stylet; sv, seminal vesicle; t, testis; v, vitellaria. Scale bar: 50 μm.

RESULTS

Isolation of a previously described species

Five individuals with morphological traits that corresponded with previous descriptions of *Gyratrix hermaphoditus* were discovered and examined, one of which is shown in Figure 1A–C (BBMMI6491-6495). The stylet of our isolate was 70 μm. The total body length was 680 μm; proboscis length was 50 μm (Table 2). Other available material from these isolates includes four separate genomic DNA samples from each of four individuals (BBMMI6496-6499), one of which is represented in the present study, and an 18S rDNA sequence deposited in GenBank under Accession number JN205124.

Molecular phylogenetic analyses

Phylogenetic analyses of the 27-taxon alignment resulted in two main clades (Figure 6): the Schizorhynchia and the Eukalyptorhynchia. The 18S rDNA sequences of *Undicola tofinoensis* sp. nov., *Schizorhinos vancouverensis* sp. nov. and *Linguabana tulai* sp. nov. clustered within the Schizorhynchia; this result was congruent with the fact that all three species possessed the unifying feature of the clade, namely a bifurcated proboscis. *Linguabana tulai* sp. nov. formed the nearest sister lineage to all other members of the Schizorhynchia included in the analysis, albeit with modest statistical support. The Eukalyptorhynchia received robust statistical support (98% bootstrap value) and contained *Thinodactylaina tlaoquiahtensis* sp. nov. and *Rostracilla nuuchahnulthensis* sp. nov. along with the Vancouver Island isolate of *Gyratrix hermaphroditus*.

The relationships within each of the two major clades were poorly resolved. Within the Schizorhynchia, the two karkinorhynchid species *Karkinorhynchus bruneti* and *Cheliplana* cf. *orthocirra* are not monophyletic, nor are the described schizorhynchids *Proschizorhynchus triductibus, Thlyacorhynchus ambronensis and Schizorhynchoides caniculatus*. The only exception was the clade of *Schizochilus* species (98% bootstrap value). Within the Eukalyptorhynchia, all of the putative polycystidids formed a well-supported clade that was the nearest sister group to the cicerinid *Zonorhynchus seminascatus* (97% bootstrap value). The 18S rDNA sequence from the Vancouver Island isolate of *G. hermaphroditus* clustered strongly with the other two *G. hermaphroditus* sequences available in GenBank (100% bootstrap value). The *G. hermaphroditus* clade was most closely related to *Rostracilla nuuchahnulthensis* sp. nov., albeit with modest statistical support (67% bootstrap value). The two new sequences from *Thinodactylaina tlaoquiahtensis* sp. nov. formed a distinct clade that branched from the unresolved polycystidid backbone. With the exception of *R. nuuchahnulthensis*, which is potentially related to *G. hermaphroditus,* phylogenetic pattern was uninformative for informing generic comparisons or assignments.

SYSTEMATICS

RHABDOCOELA Meixner, 1925
KALYPTORHYNCHIA Graff, 1905
EUKALYPTORHYNCHIA Meixner, 1928
Thinodactylaina tlaoquiahtensis gen. nov., sp. nov.

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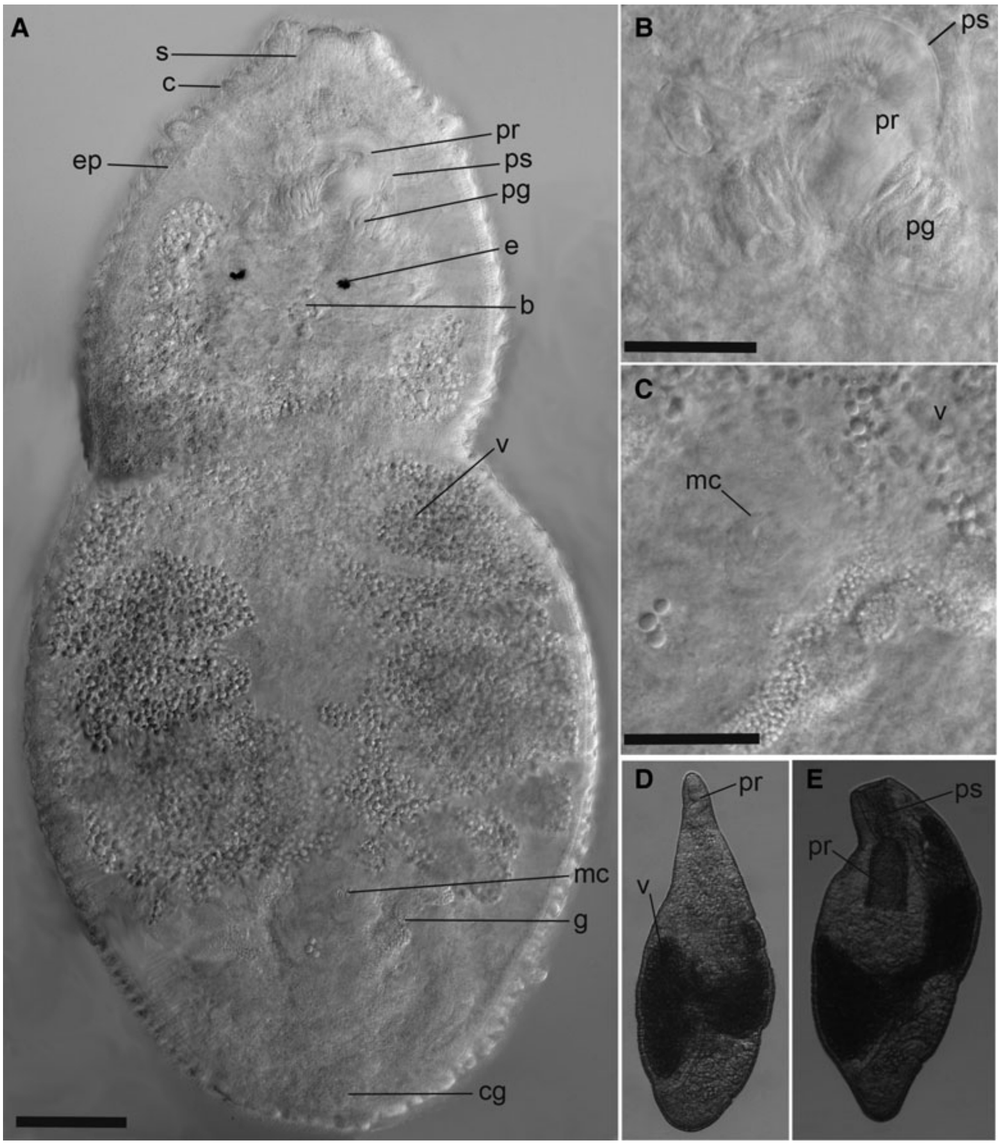

Fig. 5. (A–C) Dorsal view of a gluteraldehyde-preserved *Rostracilla nuuchahnulthensis* gen. nov., sp. nov. specimen (squeeze preparation); (D, E) dorsal view of a live anaesthetized specimen (squeeze preparation); (A) full body; (B) head region, showing details of the proboscis and proboscis glands; (C) posterior region showing the male copulatory organ from (A); (D) proboscis extended in live specimen; (E) proboscis of live specimen retracted into the proboscis sheath. Abbreviations: b, brain; c, cilia; cg, caudal glands; e, eye; ep, epidermis; g, germarium; mc, male copulatory organ; pg, proboscis glands; pr, proboscis; ps, proboscis sheath; s, sphincter; v, vitellaria. Scale bars: A, 50 μm; B, C, 50 μm).

type material

A gluteraldehyde-fixed and preserved specimen (holotype, BBMMI6504); specimen in Figure 4A (iconotype); genomic DNA from one individual (BBMMI6505).

type locality

Long Beach near Tofino on the western coast of Vancouver Island (49°03′20.42″N 125°43′40.16″W) at low tide in a small sand pool in a section of beach somewhat less wave-exposed than for that of *Schizorhynchus tofinoensis* sp. nov. Coll. R.J.R. on 10 September 2009.

other locality

Specimen from GenBank Accession number JN205123: low tide fine sand sample taken from Pachena Beach on the western coast of Vancouver Island (48°47.551′N 125°06.974′W; near Bamfield, BC). Coll. R.J.R. on 26 June 2009.

etymology

Genus name is from the Greek word *thinos* (beach, shore) and the feminized version of the Greek *daktylos* (toe) in reference to the adhesive toes of the type species, which allow the animal to stick to sand grains. This species is named in honour of the First Nations of Tla-o-qui-aht, whose traditional lands extend to the Long Beach area where *Thinodactylaina tlaoquiahtensis* sp. nov. was found, and who are part of the Nuu-chah-nulth First Nations of the western coast of Vancouver Island. This species helps educate the public in this region about a particular place, its culture, and the unseen biodiversity adapted to living there.

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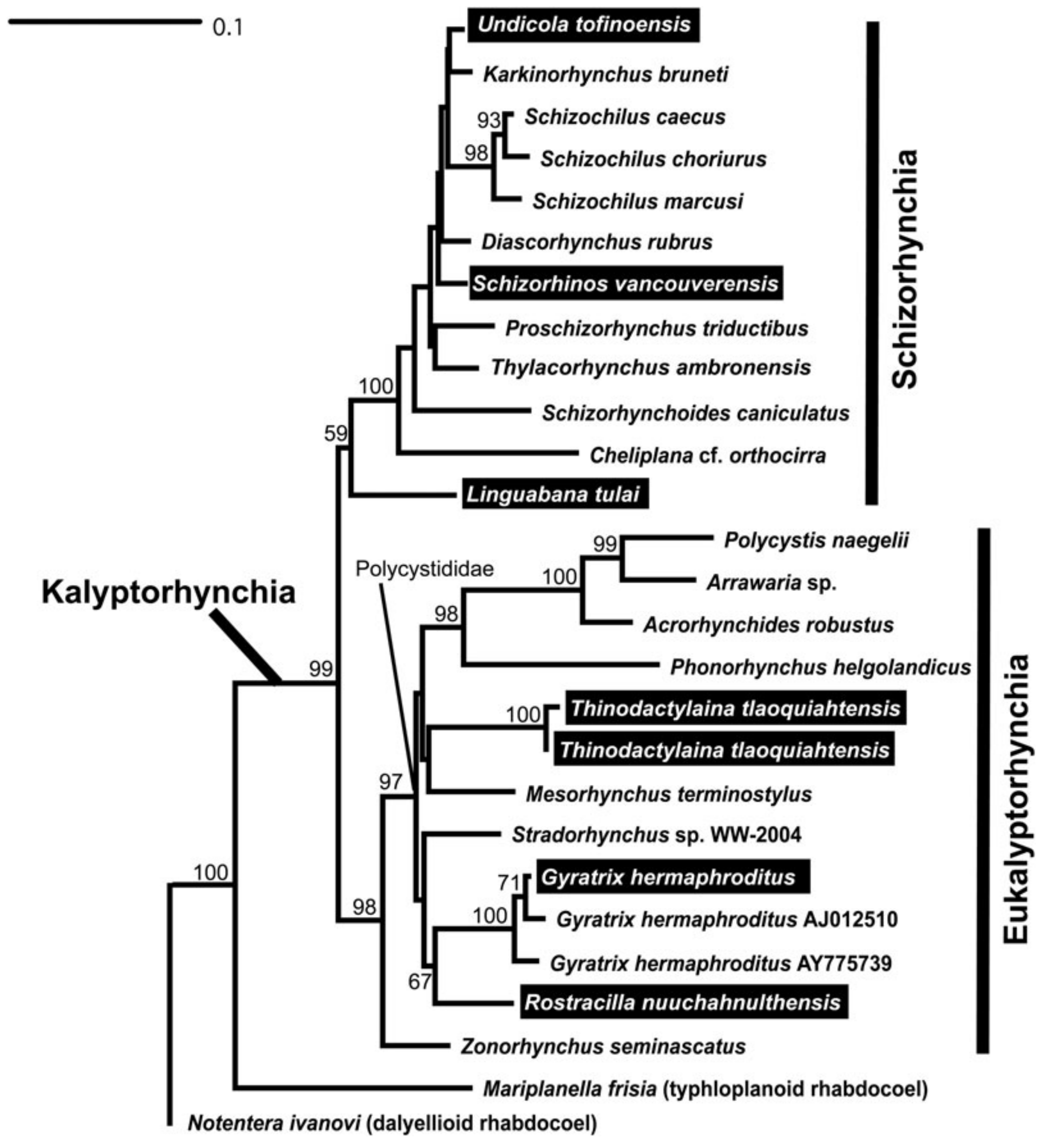

Fig. 6. Maximum likelihood tree (GARLI; Zwickl, 2006, ln $L = -10003.42044$) inferred from 18S rDNA sequences from 27 species of kalyptorhynchs (1653 sites); the positions of a new isolate of *Gyratrix hermaphroditus* and five new kalyptorhynch species are outlined in black. Numbers represent bootstrap values greater than 50.

DESCRIPTION

Head is blunt with an indistinct, sheathed, unbranched proboscis without hooks (Figure 4). There are no whiskers. Prominent proboscis glands are clustered into an anterior snout-like region of the head. A curved brain region is clearly visible between two large eyespots. A large round muscular pharynx is present midway down the body. Diffuse paired vitellaria are present on either side of the body below the pharynx. There are two distinct and kidney-shaped testes below the vitellaria, that each have a duct that meets in the male stylet region. The tail is pointed and bears four adhesive toes. Body is mostly brownish in colour. Extended body length is 550 μm and retracted body length is 340 μm. Proboscis length is 15 μm and stylet length is 20 μm.

COMPARISONS WITH OTHER SPECIES

Body length is less than 1/3 of the body length of *Polycystis naegelii* Kölliker, 1845. *Thinodactylaina* sp. nov. can be differentiated from other polycystidids on the basis of its distinctive 18S rDNA sequence. *Thinodactylaina tlaoquiahtensis* sp. nov. is also distinct from *Gyratrix hermaphroditus* in that it has a smaller, unarmed proboscis, different stylet morphology, and adhesive toes. *Thinodactylaina tlaoquiahtensis* sp. nov. is distinct from *R. nuuchahnulthensis* sp. nov. on the basis of its much smaller proboscis, more anterior position of its proboscis glands, and larger stylet.

DISTRIBUTION AND ECOLOGY

Fine sand, exposed but low-impact sand pools (i.e. deeper pockets of water subjected to irregular influx of new water, but not quite a tide pool) and/or fine sand exposed to waves.

DNA SEQUENCE

An 18S rDNA sequence was deposited in GenBank (JN205122).

Table 1. Species included in the analyses and their GenBank Accession numbers.

Species	GenBank	Locality	Reference
Acrorhynchides robustus (Karling, 1931)	AY775737	Germany	Willems *et al.*, 2006a
Arrawaria sp.	AJ243677	Not published	Littlewood *et al.*, 1999
Cheliplana cf. *orthocirra* Ax, 1959	AJ012507	Not published	Littlewood *et al.*, 1999
Diascorhynchus rubrus Boaden, 1963	AJ012508	Not published	Littlewood *et al.*, 1999
Gyratrix hermaphroditus Ehrenberg, 1831	AJ012510	Not published	Littlewood *et al.*, 1999
Gyratrix hermaphroditus Ehrenberg, 1831	AY775739	Sweden	Willems *et al.*, 2006a
Gyratrix hermaphroditus Ehrenberg, 1831	JN205124	Vancouver Island	Rundell & Leander
Karkinorhynchus bruneti Schilke, 1970b	AY775740	Germany	Willems *et al.*, 2006a
Linguabana tulai gen. nov., sp. nov.	JN205121	Vancouver Island	Rundell & Leander
Mariplanella frisia Ax & Heller, 1970	AJ012514	Not published	Littlewood *et al.*, 1999
Mesorhynchus terminostylus Karling, 1956	AY775741	Sweden	Willems *et al.*, 2006a
Notentera ivanovi Joffe, Selivanova & Kornakova, 1997	AJ287546	Not published	Littlewood & Olson, 2001
Phonorhynchus helgolandicus (Metschnikoff, 1865)	AY775742	Sweden	Willems *et al.*, 2006a
Polycystis naegelii Kölliker, 1845	AY775743	Greece	Willems *et al.*, 2006a
Proschizorhynchus triductibus Schilke, 1970b	AY775744	Belgium	Willems *et al.*, 2006a
Rostracilla nuuchahnulthensis gen. nov., sp. nov.	JN205125	Vancouver Island	Rundell & Leander
Schizochilus caecus L'Hardy, 1963	AY775745	Germany	Willems *et al.*, 2006a
Schizochilus choriurus Boaden, 1963	AY775746	Belgium	Willems *et al.*, 2006a
Schizochilus marcusi Boaden, 1963	AY775747	Belgium	Willems *et al.*, 2006a
Schizorhinos vancouverensis gen. nov., sp. nov.	JN205120	Vancouver	Rundell & Leander
Schizorhynchoides caniculatus L'Hardy, 1963	AY775748	Germany	Willems *et al.*, 2006a
Stradorhynchus sp. WW-2004	AY775738	Australia	Willems *et al.*, 2006a
Thinodactylaina tlaoquiahtensis gen. nov., sp. nov.	JN205122	Vancouver Island	Rundell & Leander
Thlyacorhynchus ambronensis Schilke, 1970b	AY775749	France	Willems *et al.*, 2006a
Undicola tofinoensis gen nov., sp. nov.	JN205119	Vancouver Island	Rundell & Leander
Zonorhynchus seminascatus Karling, 1956	AY775750	Germany	Willems *et al.*, 2006a

Rostracilla nuuchahnulthensis gen. nov., sp. nov.

TYPE MATERIAL

A gluteraldehyde-fixed and preserved specimen (holotype, BBMMI6506; Figure 5A–C); specimen pictured in Figure 5D, E (iconotype); genomic DNA from one individual (BBMMI6507).

TYPE LOCALITY

Long Beach near Tofino on the western coast of Vancouver Island (49°03′20.42″N 125°43′40.16″W) at low tide in wave-exposed section of beach. Coll. R.J.R. on 10 September 2009.

ETYMOLOGY

Genus name is from the Latin word *rostrum* (snout) and the feminized version of the Latin *cillo* (put in motion) in reference to the prominent and expressive proboscis of the type species. The species is named in honour of the lands of the Nuu-chah-nulth people, a group of fifteen related First Nations of the western coast of Vancouver Island. The Nuu-chah-nulth people were traditionally known for their sophisticated hunting techniques (exemplified by the eversible proboscis of *Rostracilla nuuchahnulthensis* sp. nov.), which involved the capture of animals such as salmon, halibut, seals and sometimes whales. This species helps educate the public in this region about a particular place, its culture, and the unseen biodiversity adapted to living there.

DESCRIPTION

Head region is distinct and bears a heavily ciliated proboscis sheath opening, posterior to which the proboscis can be curved and substantially retracted (Figure 5A, B, D, E). Large proboscis glands are present at the base of the proboscis, posterior to which are the eyespots. The pharynx is obscured by the paired vitellaria, which appear as a vest of brown granules on either side of the body. The male cuticular region is minute and crown-shaped (Figure 5A, C). The tail region is blunt. Body is mostly brownish in colour. Total body length is 490 μm, proboscis length is 60 μm, and male copulatory organ length is 10 μm.

COMPARISONS WITH OTHER SPECIES

The most distinctive features of *R. nuuchahnulthensis* sp. nov. are its highly retractable proboscis and its tiny crown-shaped male cuticular region, which was unique among the specimens examined in this study. It can be differentiated from other polycystidids on the basis of its distinctive 18S rDNA sequence. In our phylogenetic analysis (Figure 6), *R. nuuchahnulthensis* is most closely related to *Gyratrix hermaphroditus*, the type species for *Gyratrix* Ehrenberg 1831. However, the genital anatomy of *R. nuuchahnulthensis* differs from this species in clearly lacking a long and prominent stylet.

DISTRIBUTION AND ECOLOGY

Fine sand at wave-exposed site. Only known from type locality.

Table 2. Key features of kalyptorhynch flatworms from the Pacific North-west. Clade E = the Eukalyptorhynchia; Clade S = the Schizorhynchia. All localities are in British Columbia, Canada.

Species	Clade	Locality	Habitat	Body length (μm)	Proboscis length (μm)	Length of stylet/male copulatory apparatus (μm)	Shape of posterior end	Species	Whiskers	Size and position of testes	Size and position of male cuticular apparatus	Organization of vitellaria
Linguabana tulai gen. nov., sp. nov.	S	Long Beach, Vancouver Island	Intertidal, fine sand, low impact waves	800	70	7	Blunt	*Linguabana tulai* gen. nov., sp. nov.	Absent	Large, triangular, anterior to vitellaria	Small, posterior to vitellaria	Paired, serially repeated, diffuse clusters of granules
Schizorhinos vancouverensis gen. nov., sp. nov.	S	Stanley Park, English Bay, Vancouver	Intertidal, course sand, low impact waves	1400	200	Unknown	Pointed	*Schizorhinos vancouverensis* gen. nov., sp. nov.	Present	Obscured	Obscured	Unpaired, widespread, dark, granular
Undicola tofinoensis gen. nov., sp. nov.	S	Long Beach, Vancouver Island	Intertidal, fine sand, high impact waves	930	150	70	Blunt	*Undicola tofinoensis* gen. nov., sp. nov.	Present	Small, posterior to vitellaria	Ovoid, Posterior to vitellaria	Unpaired, tiny clustered granules
Gyratrix hermaphroditus	E	Trevor Channel, Barkley Sound, Vancouver Island	15–10 m depth, shell hash	680	50	70	Blunt	*Gyratrix hermaphroditus*	Absent	Obscured	Large, posterior to vitellaria	Unpaired, diffuse granules
Rostracilla nuuchahnulthensis gen. nov., sp. nov.	E	Long Beach, Vancouver Island	Intertidal, fine sand, high impact waves	490	60	10	Blunt	*Rostracilla nuuchahnulthensis* gen. nov., sp. nov.	Absent	Obscured	Small, crown-shaped, Posterior to vitellaria	Paired, granular
Thinodactylaina tlaoquiahtensis gen. nov., sp. nov.	E	Long Beach Beach, Pachena Beach, Vancouver Island	Intertidal, fine sand, low and high impact waves	550	15	20	Pointed with 4 adhesive toes	*Thinodactylaina tlaoquiahtensis* gen. nov., sp. nov.	Absent	Two, kidney-shaped, posterior to vitellaria	Large, posterior to vitellaria	Paired, diffuse

DNA SEQUENCE
An 18S rDNA sequence was deposited in GenBank (JN205125).

SCHIZORHYNCHIA Meixner, 1928
Family SCHIZORHYNCHIDAE Graff, 1905
Undicola tofinoensis gen. nov., sp. nov.

TYPE MATERIAL
Specimen pictured in Figure 2A fixed and preserved in gluteraldehyde (BBMMI6508); genomic DNA from one individual (BBMMI6500).

TYPE LOCALITY
Long Beach near Tofino on the western coast of Vancouver Island (49°03′20.42″N 125°43′40.16″W) at low tide in wave-exposed section of beach. Coll. R.J.R. on 10 September 2009.

ETYMOLOGY
Genus name is from the Latin word *undicola* (one that lives in waves), in reference to the type species' habitat in a wave-swept section of beach. The species name comes from the town nearest the species' locality, an exposed wave and wind-swept spot on the western coast of Vancouver Island, a region popular with surfers. Tofino was named in honour of cartographer Admiral Vicente Tofiño.

DESCRIPTION
Head region is elongate with a sheathed bifurcated proboscis without hooks (Figure 2A). Whiskers (elongate cilia) are present at the anterior tip of the head. Posterior to the proboscis there are two prominent eye spots. There is a prominent muscular pharynx approximately halfway down the body. Posterior to the pharynx is the vitellarium, which appears as hundreds of tiny, sometimes clustered, granules. The ovoid region of the stylet apparatus is the most prominent feature of the tail region. There is a single ovary. The body terminates in a blunt, heavily ciliated tail. Body is mostly brownish in colour. Total body length is 930 μm, proboscis length is 150 μm, and stylet length is 70 μm.

COMPARISONS WITH OTHER SPECIES
This species is distinguished from the other Long Beach, Vancouver Island schizorhynch described here (*Linguabana tulai* sp. nov.) based on its longer body, its longer proboscis (which is twice as long as that of *L. tulai* sp. nov.) and its enormous stylet, which is ten times longer than the stylet of *L. tulai* sp. nov. *Undicola tofinoensis* sp. nov. has much smaller and less distinct testes than *L. tulai* sp. nov. *Undicola tofinoensis* sp. nov. has a shorter body length and proboscis length than *Schizorhinos vancouverensis* sp. nov., the species from English Bay (Vancouver), and has a 18S DNA sequence distinct from both of the schizorhynch species described here. *Undicola tofinoensis* sp. nov. has a shorter body length than most of the other schizorhynch species in Figure 6, and also lacks proboscis hooks, unlike *Karkinorhynchus bruneti* Schilke, 1970b. The placement of the testes in *Schizorhynchus tofinoensis* sp. nov. is also different from that of *K. bruneti*, whose testes are near the mid-body pharynx (Schilke, 1970b).

DISTRIBUTION AND ECOLOGY
Fine sand at wave-exposed site. Species is only known from type locality.

DNA SEQUENCE
An 18S rDNA sequence was deposited in GenBank (JN205119).

Linguabana tulai gen. nov., sp. nov.

TYPE MATERIAL
Specimen pictured in Figure 2B. Genomic DNA from two specimens (i.e. two separate extractions (BBMMI6509–6510), one of which we used here).

TYPE LOCALITY
Long Beach near Tofino on the western coast of Vancouver Island (49°03′20.42″N. 125°43′40.16″W) at low tide in a small sand pool in a section of beach somewhat less wave-exposed than for that of *Undicola tofinoensis* sp. nov. Coll. by R.J.R. on 10 September 2009.

ETYMOLOGY
Genus name is from the Latin word *lingua* (tongue) and the Anglo-Saxon *bana* (slayer, that which hurts or destroys) in reference to the type species' two-tongued proboscis that is used to capture microscopic prey. The new species is dedicated to the Tula Foundation, which generously funded the work herein and has championed the basic discovery and exploration pursuits of the Centre for Microbial Diversity and Evolution at the University of British Columbia.

DESCRIPTION
Head region is elongate with a sheathed bifurcated proboscis without hooks (Figure 2B). There are no whiskers. Posterior to the proboscis there are two prominent eye spots. Posterior to the head region are two large, light-coloured, triangular-shaped testes, which are slightly larger than the round muscular pharynx, which is just posterior to the second testis. Posterior to the pharynx is an elongate region of vitellaria, which appear as serially-repeated diffuse clusters of granules on either side of the body. The small stylet region is posterior to the vitellaria. The single ovary is close to the end of the tail. The tail is slightly rounded. Body is mostly brownish in colour. Total body length is 800 μm, proboscis length is 70 μm, and stylet length is 7 μm.

COMPARISONS WITH OTHER SPECIES
Differences between this species and *U. tofinoensis* sp. nov. are described above. The most remarkable features of this species are its anteriorly positioned large triangular testes. The position of the testes is similar to that of *Karkinorhynchus bruneti,* however the shape and size are different. The long, pronounced serial repetition of granular vitellaria was also unique among Vancouver Island kalyptorhynchs, which made this species a striking schizorhynch. Its 18S rDNA sequences also distinguishes it from all other kalyptorhynchs discussed here (Figure 6)

distribution and ecology

Fine sand, exposed but low-impact sand pools (deeper pockets of water subjected to irregular influx of new water, but not quite a tide pool). Only known from type locality.

dna sequence

An 18S rDNA sequence was deposited in GenBank (JN205121).

Schizorhinos vancouverensis gen. nov., sp. nov.

type material

A gluteraldehyde and osmium tetroxide-fixed specimen preserved in 70% ethanol, catalogue number BBMMI6501 (holotype); three specimens pictured in Figure 3A–C (iconotypes); genomic DNA from two specimens (i.e. two separate extractions (BBMMI6502–6503), one of which we used here).

type locality

High tide 3 m from shore at the beach near Stanley Park on English Bay (Vancouver; 48°17′18.48″N 123°08′37.78″W). Coll. by R.J.R. on 19 August 2009.

etymology

Genus name is from the Greek word *schizo* (cleave, split) and the feminine Greek *rhinos* (snout) in reference to the split proboscis characteristic of the Schizorhynchia. The species is named for the type locality, which is in the city of Vancouver (named for Captain George Vancouver, who explored the city's Burrard inlet in 1792).

description

Head region is elongate with a very long sheathed bifurcated proboscis without hooks (Figure 3A, B). Whiskers (elongate cilia) are present at the anterior tip of the head. Posterior to the proboscis there are two prominent eyespots. There is a prominent muscular pharynx approximately halfway down the body. Posterior to the pharynx is the vitellarium, which is a dark, granular area that obscures most of the other reproductive organs. The body terminates in a slightly pointed tail that includes prominent caudal glands. Body is mostly brownish in colour. Extended body length is 1400 μm and retracted body length is 1200 μm. Proboscis length is 200 μm.

comparisons with other species

This species' locality is distinct from the other kalyptorhynchs described here; this species was never found on Long Beach (Vancouver Island). Perhaps this species prefers the slightly lower salinities present in English Bay. *Schizorhinos vancouverensis* sp. nov. has the longest proboscis of any kalyptorhynch currently known from the Vancouver or Vancouver Island area (this study). It is also clearly distinct from other kalyptorhynchs based on its 18S rDNA sequence.

distribution and ecology

Course sand, exposed to waves but low wave activity. Only known from type locality.

dna sequence

An 18S rDNA sequence was deposited in GenBank (JN205120).

DISCUSSION

Comparative morphology of the new isolates and other North American species

We erected new binomials for the five new species described here because they do not conform to existing genera in the literature (starting with Seth Tyler's database: turbellaria.umaine.edu) and do not cluster closely with existing 18S rDNA sequences. All five of the new kalyptorhynch isolates had an eversible proboscis, which was either prominent and bifurcated (Schizorhynchia: *Undicola tofinoensis* sp. nov., *Linguabana tulai* sp. nov., *Schizorhinos vancouverensis* sp. nov.; Figures 2A, B & 3), diminutive and unbranched (Eukalyptorhynchia: *Thinodactylaina tlaoquiahtensis* sp. nov.; Figure 4) or prominent and unbranched (Eukalyptorhynchia: *Rostracilla nuuchahnulthensis* sp. nov.; Figure 5). The new isolates also differed in their body length, proboscis length and appearance, presence of 'whiskers' (e.g. elongated cilia), shape of the posterior end and reproductive morphology (e.g. stylet length, appearance of the vitellarium, and relative positions of the testes and male cuticular apparatus) (Table 2). All of the isolates were capable of attachment and rapid release to and from sand grains using their posterior body region and associated caudal glands.

The three new schizorhynch isolates (*Undicola tofinoensis* sp. nov., *Linguabana tulai* sp. nov., *Schizorhinos vancouverensis* sp. nov.) did not possess cirri, which distinguished them from the described North American Pacific coast species *Cheliplana californica* Karling, 1989 and *C. elkhornica* Karling, 1989, *Schizochilus hoxholdi* Karling, 1989 and *Proschizorhynchella inflata* Karling, 1989. The three new isolates did possess eyes, which distinguished them from the eyeless *Proschizorhynchella schilkei* Karling, 1989 and *Paraschizorhynchoides glandulis* Karling, 1989. The relatively large size (2500 μm) and the presence of a tongue-like process on the stylet distinguished *Proschizorhynchella linguata* Karling, 1989 from these three new isolates.

The three new schizorhynch species also differ from three western Atlantic species, *Parathylacorhynchus reidi* Dean, 1980, *Proschizorhynchus nahantensis* Doe, 1974 and *Proschizorhynchus papillatus* Doe, 1974. *Parathylacorhyuchus reidi*, *Proschizorhynchus nahantensis* and *P. papillatus* are opaque and over 2100 μm in body length; *P. reidi* also has three distinctive bands of holdfasts (Dean 1980). The three new schizorhynch isolates lacked these traits, were brownish in colour, and were much smaller (≤1400 μm). *Undicola tofinoensis* sp. nov. differed from *Proschizorhynchus nahantensis* and *P. papillatus* by possessing testes that were posterior to the pharynx. *Linguabana tulai* sp. nov. had testes positioned anterior to the pharynx and has a much smaller copulatory organ than previously described species of schizorhynchs. The proboscis length of all three new species is shorter (≤200 μm) than in *P. nahantensis* and *P. papillatus.*

The two new eukalyptorhynch isolates (*Thinodactylaina tlaoquiahtensis* sp. nov. and *Rostracilla nuuchahnulthensis* sp. nov.) lacked cirri, which distinguished them from the North American Pacific coast species *Cicerina bicirrata* Karling, 1989, *C. triangularis* Karling 1989, *Duplacrorhynchus minor* Schockaert & Karling, 1970, *D. major* Schockaert & Karling, 1970 and *Yaquinaia microrhynchus* Schockaert & Karling, 1970. The two new eukalyptorhynch isolates also possessed eyes, which distinguishes them from the eyeless North American Pacific coast species *Placorhynchus pacificus* Karling, 1989 and *Uncinorhynchus pacificus* Karling, 1989. *Gyratrix proaviformis* Karling & Schockaert, 1977 is yellowish in colour and twice the size of the two eukalyptorhynch isolates we describe here. *Paraustrorhynchus pacificus* Karling & Schockaert, 1977 has a much larger (140 µm) cuticular organ than the two eukalyptorhynch isolates we describe here. Neither of the two new eukalyptorhynch isolates had a dark blue mantle that is distinctive of *Alcha evelinae* Marcus, 1949.

The two new eukalyptorhynch isolates also differ from the North Atlantic species *Cystiplana rubra* Dean, 1977 and the very large (4000 µm) *Crassicollum musculare* Dean, 1977 by lacking longitudinal red stripes and by being much smaller (≤550 µm) than these North Atlantic species. The North Atlantic species *Gnathorhynchus riseri* Karling, 1995 possesses proboscis hooks that are absent in the isolates we describe here; *Placorhynchus doei* Karling, 1995 has testes anterior to the pharynx with a different stylet structure than in the isolates we describe here.

Kalyptorhynch evolution

The molecular phylogenetic analyses suggest that the Schizorhynchia and Eukalyptorhynchia are monophyletic groups within the Kalyptorhynchia (Figure 6). Thus the distinction between an unbranched proboscis and a bifurcated proboscis may be phylogenetically informative and, if so, this distinction occurred relatively early in the evolution of kalyptorhynch rhabdocoels. Presumably, the difference between unbranched and bifurcated proboscises reflects different feeding strategies for different prey items. Research on the functional morphology of schizorhynchoid vs eukalyptorhynchoid proboscises in relation to the food preferences within each kalyptorhynch clade will be necessary to better understand the biological significance of these proboscis types. More extensive sampling within Kalyptorhynchia is needed to better resolve basal relationships. And as with other rhabdocoel lineages, the relationships within the Schizorhynchia and the Eukalyptorhynchia are still unclear, likely because of poor taxon sampling (Willems *et al.*, 2006a). This poor resolution limits our ability to draw compelling inferences about the evolution of morphological features within the subclades.

However, the meiofaunal species described here are clearly new, based not only on molecular phylogenetic evidence, but also on morphological features, such as presence or absence of adhesive toes, proboscis length, male cuticular apparatus differences, stylet length and position of the testes (Table 2). Many putatively closely related schizorhynch species also possess differences in major organ systems. For example, *Schizochilus marcusi* adults have 18–24 testes, whereas many other schizorhynchids have only two or four testes. Although some have reported that testes number can be variable in filiform meiofaunal taxa, or fluctuate with age (Karling, 1989). *Schizochilus choriurus caecus* Boaden, 1963 (presumably referable to the species recorded in GenBank as *Schizochilus caecus*) has eyes, but *Schizochilus choriurus* Boaden, 1963 reportedly does not. *Schizochilus marcusi* also lacks eyes, and possesses a sheathed cirrus (a male reproductive structure) that other *Schizochilus* species lack. These distinctions among species have probably led to the proliferation of genera.

Systematic confusion persists within the kalyptorhynchs. It is unclear which morphological features beyond general proboscis structure (i.e. bifurcated or not) are phylogenetically informative, and therefore systematics of the group may be more pragmatically resolved with molecular phylogenetic data. Within the eukalyptorhynch family Polycystididae in particular, species often possess significant morphological differences from the type genus *Polycystis* (Schockaert & Karling, 1970), leading to the proliferation of genera (i.e. 47 polycystidid genera, according to Cannon, 1986). For example, *Mesorhynchus terminostylus* was originally placed within Polycystididae, but differences in the proboscis ultrastructure of this species compared with other polycystidids led some authors to doubt this placement (DeVocht, 1991). However, the phylogenetic tree inferred from our data does place *M. terminostylus* within the Polycystididae.

The best-known polycystidid, *Gyratrix hermaphroditus,* is considered either a variable cosmopolitan species or a complex of cryptic species (Heitkamp, 1978; Puccinelli & Curini-Galletti, 1987; Curini-Galletti & Puccinelli, 1990, 1994, 1998; Therriault & Kolasa, 1999; Artois & Tessens, 2008). Morphological data are important for helping to resolve this question. The stylet length of our isolate from Vancouver Island (70 µm) is much shorter than the stylet lengths for *G. hermaphroditus* reported from Zanzibar, Seychelles, Kenya, Indonesia and Réunion (Artois & Tessens, 2008). For example, our isolate's stylet is half the length of the longest stylet observed on individuals from Réunion. However, the stylet length of our *G. hermaphroditus* is very close in size to individuals from the Hawaiian Islands and coastal California, which were noted to have the smallest stylet sizes ever recorded for *G. hermaphroditus* (Karling & Schockaert, 1977). No DNA sequence data are available for these individuals, so molecular phylogenetic comparisons cannot be made. Such comparisons are important for determining whether *G. hermaphroditus* is truly cosmopolitan, or a complex of cryptic species (e.g. the latter of which was the case with a meiofaunal sea slug; Jörger *et al.*, 2012).

Each of the new isolates had an unarmed proboscis (lacking hooks, spines or ridges), which is characteristic of the Schizorhynchidae. But because no morphological features justified alliance with a particular schizorhynchid genus, and molecular systematics was equivocal (i.e. our isolates did not cluster with any of the putative schizorhynchid species *Schizochilus* spp., *Proschizorhynchus triductibus, Thylacorhynchus ambronensis* or *Schizorhynchoides caniculatus*), we have established unique binomials for the new isolates. The same was the case for our new eukalyptorhynch isolates. Although both new eukalyptorhynch species fall within the Polycystididae in the molecular phylogenetic tree, neither phylogenetic nor morphological data brings them within existing genera; thus, new binomials were established. Taxonomy within the Polycystididae is rather outdated (Willems *et al.*, 2006b), but may eventually be improved upon using molecular approaches to understand phylogenetic relationships within this large family.

Rostracilla nuuchahnulthensis sp. nov. is the nearest sister lineage to the *G. hermaphroditus* clade (67% bootstrap), yet there are major differences in the male copulatory apparatus between *R. nuuchahnulthensis* sp. nov. and *G. hermaphroditus*. *Rostracilla nuuchahnulthensis* sp. nov. has a very small

https://doi.org/10.1017/S0025315413001471 Published online by Cambridge University Press

(10 μm) male copulatory organ that is superficially similar in appearance to the schizorhynch *Cheliplana setosa* (Karling, 1983a). In contrast, the stylet of our *G. hermaphroditus* specimen is larger and spear-like.

Differences in prey capture mode might also be important in the evolution of kalyptorhynch species. *Undicola tofinoensis* sp. nov., *Linguabana tulai* sp. nov., *Thinodactylaina tlaoquiahtensis* sp. nov. and *Rostracilla nuuchahnulthensis* sp. nov. were all collected at low tide from relatively exposed fine sand at Long Beach near Tofino on the western coast of Vancouver Island. The proboscis length of schizorhynch species *L. tulai* sp. nov. is half that of *U. tofinoensis* sp. nov.; moreover, eukalyptorhynch species *T. tlaoquiahtensis* sp. nov. and *R. nuuchahnulthensis* sp. nov. both have shorter proboscises than the schizorhynch species, and the proboscis of *T. tlaoquiahtensis* sp. nov. is much shorter than species *R. nuuchahnulthensis* sp. nov. These differences in proboscis length suggest differences in prey preference that would allow all of these predators to co-occur in the same environment. Differences in body size among all of the Tofino kalyptorhynch species, also suggest consumption of different-sized prey (MacArthur, 1972). All of these species also differ in the length of their stylets and in the arrangement of the associated copulatory apparatus; these differences likely reflect reproductive isolation in the same habitat and ensure successful copulation between conspecifics.

In conclusion, kalyptorhynchs are important components of meiofaunal ecosystems that offer opportunities for addressing questions relating to the evolution of novel reproductive and feeding structures. DNA sequences used in combination with improved sampling are essential for estimating the overall biodiversity of kalyptorhynchs and for delimiting the boundaries between different species within the group and other microscopic animals that co-occur in the same habitat.

ACKNOWLEDGEMENTS

We thank the staff of the Bamfield Marine Sciences Centre (Bamfield, BC, Canada) for hosting our field research, and S. Sparmann and K. Wakeman for laboratory and translation assistance. We also thank the anonymous referees for their thoughtful comments.

FINANCIAL SUPPORT

This work was supported by the Tula Foundation (Centre for Microbial Diversity and Evolution) and the Canadian Institute for Advanced Research, Programme in Integrated Microbial Diversity.

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Correspondence should be addressed to:
R.J. Rundell
Department of Environmental and Forest Biology, State University of New York, College of Environmental Science and Forestry, Syracuse, New York 13210, USA
email: rundell@esf.edu