Zootaxa 2537: 1–18 (2010)
www.mapress.com/zootaxa/

Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

New insights on the phylogenetic relationships of the Proseriata (Platyhelminthes), with proposal of a new genus of the family Coelogynoporidae

MARCO CURINI-GALLETTI[1], BONNIE L. WEBSTER[2], TINE HUYSE[2,3], MARCO CASU[1], ERNEST R. SCHOCKAERT[4], TOM J. ARTOIS[4] & D. TIMOTHY J. LITTLEWOOD[2]

[1] *Dipartimento di Zoologia e Genetica Evoluzionistica, University of Sassari, via Muroni 25, I 07100 Sassari, Italy*
[2] *Parasitic Worms Group, Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK*
[3] *Katholieke Universiteit Leuven, Laboratory of Animal Diversity and Systematics, Ch. Deberiotstraat 32, B-3000 Leuven, Belgium*
[4] *Research group Biodiversity, Phylogeny and Population Studies, Centre for Environmental Sciences, Hasselt University, Campus Diepenbeek, Agoralaan Building D, B-3590 Diepenbeek, Belgium*

Abstract

Proseriata is a diverse and species-rich taxon of Neoophora (Platyhelminthes). The group is divided into two morphologically well-characterised taxa: Lithophora and Unguiphora. Previous molecular analyses, aimed at ascertaining in-group relationships of the Proseriata, were equivocal in supporting the monophyly of the Lithophora, and of one of its families, the Coelogynoporidae. Here we present a new phylogeny of the Proseriata, based on complete ssrDNA and partial (D1-D6) lsrDNA. The analysis includes 36 proseriate species. 13 new sequences from seven species are used, four of which from species of the Coelogynoporidae and one from a new species of the recently established family Calviriidae. Phylogenetic analysis was performed using Bayesian Inference (BI), maximum likelihood (ML) and maximum parsimony (MP). Clades were considered to have high nodal support if BI posterior probability and ML and MP bootstrap percentages were $\geq$90%. The resulting cladogram strongly supports the monophyly of the Lithophora. In addition, the monophyly of the Coelogynoporidae and their inclusion in the Lithophora are corroborated. Within the Lithophora, two sister clades are supported, including Coelogynoporidae+Calviriidae, and Otoplanidae+Archimonocelididae+Monocelididae respectively. In the Coelogynoporidae, there was a poor correspondence between estimates of phylogeny and the monophyly of genera, suggesting the family is in need of systematic revision. Current morphology-based systematic arrangements of Otoplanidae and Monocelididae are not supported by molecular results. The enigmatic taxon *Ciliopharyngiella* (formerly included in the Rhabdocoela) clusters with the Unguiphora, although with low support. One new species of Coelogynoporidae (*Parainvenusta englarorum* n. gen n. sp.) is described and discussed in the appendix.

Key words: phylogeny; taxonomy; ssrDNA; lsrDNA; Unguiphora; Lithophora; *Ciliopharyngiella*

Introduction

The first comprehensive molecular study specifically designed to assess both in-group and out-group phylogenetic relationships of the Proseriata, a species-rich and widespread taxon of Neoophoran flatworms, left two major problems unsolved. In fact, the monophyly of the Proseriata themselves and of one of its major subtaxa, the Lithophora were unsupported by the molecular trees obtained (Littlewood *et al.*, 2000). These results were in stark contrast with most of the morphology-based phylogenies of the group, which instead clearly supported their monophyly (Sopott-Ehlers, 1985; Ehlers, 1985; Ehlers & Sopott-Ehlers, 1990). However, molecular data partially agreed with alternative, morphology based reconstructions, which hypothesized paraphyletic Lithophora (Martens & Schockaert, 1988) and Proseriata (Brüggemann, 1986).

Accepted by W. Sterrer: 11 May 2010; published: 15 Jul. 2010

This latter assumption was based on the similarities in ultrastructure and differentiation of the stylet of the Unguiphora with a taxon, viz. *Ciliopharyngiella intermedia* Ax, 1952, then placed within the Rhabdocoela and more specifically within the Typhloplanoida (see Ehlers, 1972). The position of the taxon *Ciliopharyngiella* Ax, 1952 is debated (see Sopott-Ehlers 2001 for a review), and recent molecular data, although not entirely solving its phylogenetic position, excluded a rhabdocoelan affinity (Willems *et al.*, 2006).

The lack of molecular support for monophyly of either the Lithophora or the Proseriata may have resulted from poor sampling of apparently crucial proseriate taxa. In fact, the non-monophyly of the Lithophora was caused by two and one species respectively of the lithophoran families Coelogynoporidae and Calviiridae, which clustered with the Unguiphora. To add more confusion to the picture, the third coelogynoporid species sequenced, *Cirrifera dumosa* Sopott, 1972, clustered with members of the lithophoran family Monocelididae (Littlewood *et al.*, 2000).

More recent molecular studies supported the monophyly of the Proseriata (Lockyer *et al.*, 2003), and of the Lithophora (Willems *et al.*, 2006). In both instances, however, Proseriata were not the aim of the study, and the number of species considered too limited to offer unequivocal answers, particularly of the 'crucial' taxa (Coelogynoporidae and Calviiridae).

Further studies that encompass a wider sample of Proseriata, and particularly of the two critical families, are needed. The Coelogynoporidae include 9 genera and about 60 species known worldwide, with the majority of species (37) belonging to the single genus *Coelogynopora* Steinböck, 1924 (Tyler *et al.*, 2006; Ax, 2008). In contrast, many of the other coelogynoporid genera have 1–3 species at most. The Calviriidae include at present the genera *Asilomaria* Karling, 1966 and *Calviria* Martens & Curini-Galletti, 1993, with one and three species respectively (Martens & Curini-Galletti, 1993; Schockaert *et al.*, 2009).

Here we present new sequences (nearly complete lsrDNA and complete ssrDNA) from the lithophoran families Coelogynoporidae (four species), Monocelididae (one species), Otoplanidae (one species), and nearly complete lsrDNA for one species of Calviriidae.

Together with sequences available (Littlewood *et al.*, 2000; Litvaitis *et al.*, 1996; Willems *et al.*, 2006), the dataset includes at present 36 proseriate species, and allows the following questions to be addressed: 1) is the Lithophora monophyletic? 2) are the Coelogynoporidae monophyletic? 3) what is the relationship of the Coelogynoporidae and the Calviiridae with respect to the rest of the Proseriata? Furthermore, the new data clarify relationships of the major families of the Proseriata.

Material and methods

The animals were collected from sandy sediment samples and extracted with the $MgCl_2$ decantation technique (Martens, 1984). Specimens were identified alive in slightly squeezed preparations, and then fixed in 95% ethanol and stored at 4°C until DNA extraction. Specimens of the new species (*Parainvenusta englarorum* gen. nov. sp. nov.) were fixed for morphological and karyological study. Whole mounts were made with lactophenol. For microscopical analysis material was fixed in Bouin's fluid, embedded in 60°C Paraplast and cut into serial sagittal sections at 4 µm, stained with Hansen's haematoxylin and eosin-orange and mounted in Eukitt.

The karyotype was determined from acetic-orcein-stained spermatogonial mitoses as described by Curini-Galletti *et al.* (1989). Type material is deposited in the collections of the South Australian Museum (Adelaide, Australia) (SAM) and the Swedish Museum of Natural History (Stockholm, Sweden) (SMNH). Additional material is deposited in the collection of the Zoological Museum of the University of Sassari (Italy) (CZM).

Taxa. Table 1 provides details of which gene fragments were available for each taxon along with GenBank accession numbers, and collection details for newly sequenced taxa. Proseriate sequences available in GenBank were pruned in order to exclude dubious taxonomic attributions, such those retrieved from Japanese specimens identified as '*Otoplana* sp.' (accession D85090). The genus *Otoplana* Du Plessis, 1889 is endemic to the Mediterranean, where it is represented by five species (Lanfranchi & Melai, 2009). The genus, however, has been figured as representative Otoplanidae (see, e.g., De Beauchamp, 1961), and may have been utilised for unrelated intertidal otoplanids. In fact, the diagnostic feature of the genus (i.e. an accessory male pore, see Ax, 1956) can be appreciated only on living, semi-squashed specimens with difficulty, and sections are needed to confirm the identification. Furthermore, since this study included two species of *Cirrifera* Sopott, 1972 determined by us, we decided to exclude the sequences of *Cirrifera dumosa* (accession numbers AJ270154, AJ270169), which did not cluster with the rest of the Coelogynoporidae in a previous analysis (Littlewood *et al.*, 2000). Finally, lsrDNA sequences of *Pseudomonocelis ophiocephala* (Schmidt, 1861) (AY775735 and AY775736) were excluded, as the taxon has been shown to include a complex of cryptic species (Casu & Curini-Galletti, 2006). The sequence of *P. ophiocephala* included in the analysis was obtained from specimens of unambiguous attribution.

Representatives of other turbellarian groups were included in order to root the ingroup correctly: *Stenostomum leucops* Duges, 1828, *Suomina* sp. (Catenulida), *Paromalostomum fusculum* Ax, 1952 (Macrostomida), *Notoplana australis* (Schmarda, 1859) (Polycladida) and *Geocentrophora wagini* Timoshkin, 1984 (Lecithoepitheliata). Finally, the analysis included *Ciliopharyngiella constricta* Martens & Schoackaert, 1981, formerly included in the Rhabdocoela (but see Willems *et al.*, 2006).

DNA extraction, amplification, sequencing of complete ssrDNA and partial (D1-D6) lsrDNA. Specimens were fixed and stored in a minimum of five volumes of 95–100% ethanol. Prior to DNA extraction individual worms were rehydrated in two washes and one 1 h soak in TE (pH 8.0). Total genomic DNA was extracted using DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol. 25 µL amplifications were performed with 3–5 µL of genomic extract (~10 ng) using Ready-To-Go PCR beads (Amersham Pharmacia Biotech) each containing 1.5 U Taq Polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each dNTP and stabilizers including BSA; and 0.4 mM of each PCR primer.

Complete amplification and sequencing of complete nuclear small subunit rRNA genes (ssrDNA) was performed as described previously (Littlewood *et al.*, 1999). Amplification and sequencing of partial nuclear large subunit rRNA gene (lsrDNA) fragments, spanning variable domains D1-D6, followed protocols and primers described in Littlewood *et al.* (2000). Standard 25 µl PCR reactions were performed using PCR Beads (Pharmacia) and the cycling conditions were generally hot start (95°C/5 min) followed by 30 cycles of 94°C/1 min, 50°C/1 min and 72°C /1 min. Amplified products were cleaned directly with Qiaquick PCR purification kit (Qiagen) or were run out on a 1% TAE agarose gel, cut out and purified using Qiaquick gel extraction kit (Qiagen). Sequencing of both strands of each amplicon was carried out on an Applied Biosystems 3730 DNA Analyser, Big Dye version 1.1. Contigs were assembled using Sequencher 4.8 (GeneCodes Corporation).

Alignment. Using the published proseriate alignments of ssrDNA and lsrDNA data presented in Littlewood *et al.* (2000), which was assembled with reference to ribosomal RNA secondary structures, we added newly published sequence data (Willems *et al.*, 2006) and newly sequenced taxa from this study (see Table 1). A concatenated alignment of all available gene fragments (ssrDNA, lsrDNA D3-D6 and lsrDNA D1-D6) was assembled using MacClade 4.08 (Maddison and Maddison, 2005). Ambiguously alignable regions were marked for exclusion in subsequent analyses.

Phylogenetic analyses. The nature of previous studies has resulted in a highly fragmented taxon and gene sampling for the Proseriata; e.g. some taxa are only represented by one gene or partial fragments of lsrDNA, see Table 1. As such, individual gene, or gene fragment, analyses would yield topologies very difficult to compare and contrast in terms of phylogenetic signal. Consequently, all unambiguously alignable data were included in combined analyses.

The dataset was analysed using Bayesian Inference (BI), maximum likelihood (ML) and maximum parsimony (MP). All analyses were run on a four dual-core Opteron-based Unix cluster (http://pug.nhm.ac.uk).

TABLE 1. List of species sampled and sequences used for this study. Accession numbers refer to GenBank codes; accession numbers of new sequences in bold.

Family	Species	collecting details (for newly sequenced taxa only)	ssrDNA	lsrDNA	
				d3d6	d1d6
Catenulida	*Stenostomum leucops* Duges, 1828		AJ012519		AJ270178
	Suomina sp.		AJ012532		AJ270179
Macrostomida	*Paromalostomum fusculum* Ax, 1952				
Polycladida	*Notoplana australis* (Schmarda, 1859)				
Lecithoepitheliata	*Geocentrophora wagini* Timoshkin, 1984				
Incertae sedis	*Ciliopharyngiella constricta* Martens & Schockaert, 1981		AY775754		
Proseriata					
Archimonocelididae	Archimonocelididae gen.nov. sp.nov.		AJ270150		AJ270164
	Archimonocelis crucifera Martens & Curini-Galletti, 1993		AJ270151		AJ270163
	Archimonocelis staresoi Martens & Curini-Galletti, 1993		AJ270152	U40209	AJ270166
	Archimonocelis oostendensis Martens & Schockaert, 1981		AY775732		
Calviriidae	Calviridae gen.nov. sp.nov.	Iceland, Keflavik.	**HM026562**		
	Calviria solaris Martens & Curini-Galletti, 1993		AJ270153		AJ270168
Coelogynoporidae	*Coelogynopora gynocotyla* Steinböck, 1924		AJ243679	U40207	AJ270170
	Coelogynopora axi Sopott, 1972		AY775734		
	Parainvenusta englarorum gen. nov. sp. nov.	Australia, Port Lincoln (SA)	**HM026563**		**HM026556**
	Cirrifera cirrifera Sopott, 1972	France, Roscoff	**HM026564**		**HM026557**
	Cirrifera sopottehlersae Noldt & Jouk, 1988		AY775733		
	Invenusta aestus Sopott-Ehlers, 1976	France, Hendaye	**HM026565**		**HM026558**
	Vannuccia campana Ehlers & Ehlers, 1980	Italy, S. Antioco (Sardinia)	**HM026566**		**HM026559**
Monocelididae	*Archilina israelitica* Curini-Galletti & Martens, 1995			U39927	
	Archiloa rivularis de Beauchamp, 1910		U70077	U40049	
	Duplominona sp. 71862			U41377	
	Duplominona sp. 71863			U41375	
	Minona concharum Curini-Galletti & Cannon, 1996			U42002	
	Minona ileanae Curini-Galletti, 1997			U42000	
	Monocelidinae gen. nov. sp. nov.			U42004	
	Monocelididae gen. nov. sp. nov.	Italy, Valledoria (Sardinia)	**HM026567**		**HM026560**
	Monocelis lineata (Müller, 1774)		U45961	U40203	
	Monocelis longiceps (Duges, 1830)			U40205	
	Monotoplana cf. *diorchis* Meixner, 1938		AJ270159		AJ270174
	Promonotus ponticus Ax, 1959			U40199	

continued next page

TABLE 1. (continued)

Family	Species	collecting details (for newly sequenced taxa only)	ssrDNA	lsrDNA	
				d3d6	d1d6
	Promonotus spinosissimus Martens & Curini-Galletti, 1999			U40201	
	Pseudomonocelis cetinae Meixner, 1943			U40051	
	Pseudomonocelis ophiocephala (Schmidt, 1861)			U40197	
Otoplanidae	*Archotoplana holotricha* Ax, 1956		AJ243676		AJ270165
	Monostichoplana filum (Meixner, 1938)		AJ270158		AJ270173
	Notocaryoplana arctica Steinböck, 1935	England, Plymouth	**HM026568**		**HM026561**
	Otoplanella schulzi (Ax, 1951)			AF022750	
	Paratoplana renatae Ax, 1956		AJ012517		AJ270176
	Xenotoplana acus Ax, Weidemann & Ehlers, 1978		AJ270155		AJ270181
Unguiphora	*Nematoplana* sp.		AJ270160		AJ270175
	Polystyliphora novaehollandiae Curini-Galletti, 1998		AJ270161		AJ270177

Modeltest version 3.7macX (Posada and Crandall, 1998) was used to select a model of evolution using the Akaike Information Criterion. The data were partitioned into two character sets: ssrDNA and lsrDNA in order to select appropriate models. For BI, phylogenetic trees were constructed using MrBayes, version 3.1.2 (Huelsenbeck and Ronquist, 2001). Likelihood settings were set to nst=6, rates=invgamma, ngammacat=4 (equivalent to the GTR+I+G model of evolution, as suggested by Modeltest), estimating parameters separately for each gene. Two MCMCMC runs with 4 chains (temp=0.2) were run for 5 million generations and sampled every 1000 generations. Two million generations were discarded as 'burnin', after plotting log-likelihood values against generation number and checking that log likelihood values had 'plateaued' (usually after 500,000 generations). Nodal support was expressed as posterior probabilities.

For ML, trees were estimated using successive approximation (ML) with PAUP* version 4.0b10 (Swofford, 2002). Model parameters were estimated based on a starting tree determined by neighbor-joining (NJ). A heuristic search was performed implementing the estimated model parameters using nearest-neighbor-interchange (NNI) branch swapping. Model parameters were estimated on the best tree and a heuristic search performed using subtree-pruning-regrafting (SPR) branch swapping. After estimating model parameters, heuristic searches using tree-bisection-reconnection (TBR) branch swapping were performed until the topology remained unchanged; two searches using TBR sufficed. Maximum likelihood bootstrap values for 100 replicates were obtained using Genetic Algorithm for Rapid Likelihood Inference (GARLI) Version 0.951 (Zwickl, 2006) using default settings, except setting 'Genthreshfortopoterm' to 10,000 generations.

For MP, heuristic analyses with tree-bisection-reconnection (TBR) branch-swapping were used with 100 iterations each beginning with random starting trees. MP bootstrap support was assessed with 1000 bootstrap iterations (full TBR heuristic searches).

Clades were considered to have high nodal support if BI posterior probability and ML and MP bootstrap percentages were ≥90%.

Results

Sequence lengths for ssrDNA ranged from 1651–1837 bp, for D3-D6 lsrDNA 826–926 bp and D1-D6 lsrDNA 1641–1729 bp. The concatenated sequence alignment consisted of the following partitions: ssrDNA –

comprising 1979 positions of which 424 were excluded (21.4%), D3-D6 lsrDNA – comprising 986 positions of which 225 were excluded (22.8%), D1-D6 lsrDNA – comprising 1934 positions of which 705 were excluded (36.5%). Of the unambiguously alignable positions the following number (and proportion) of bases were variable per partition: 660 (42.4%) for ssrDNA, 379 (49.8%) for D3-D6 lsrDNA, and 635 (51.7%) for D1-D6 lsrDNA. Under the principle of parsimony these datasets provided 414 (26.6%), 239 (31.4%), and 414 (33.7%) informative characters for ssrDNA, D3-D6 lsrDNA and D1-D6 lsrDNA respectively; percentage values are proportions of unambiguously alignable sites available per partition.

Table 1 indicates the taxonomic spread and gene partitions over which there were missing data. The proportion of missing data varied according to data partition. When considering only unambiguously alignable sites in the alignment, the proportion of missing data (expressed as the number of base positions missing per total number of base positions x100) was as follows: 22.3% for ssrDNA, 36.4% for D1-D2 lsrDNA, 6.8% for D3-D6 lsrDNA and 18.1% considering lsrDNA as a whole (D1-D6).

Rather than provide phylogenetic estimates for each data partition, which would result in omitting key proseriate taxa, we chose to analyse the complete concatenated alignment, without ambiguously alignable positions. Various authors have shown that even highly incomplete sequences can be safely included in phylogenetic analyses in order to place the taxa they represent accurately (e.g. see Wiens & Moen, 2008) and references therein for a general review, and evidence of the robustness of Bayesian phylogenetics to missing data.

All three methods of phylogenetic analysis employed yielded very similar estimates of proseriate interrelationships, with only minor differences in tree topology amongst some of the coelogynoporid species. Figure 1 shows the ML solution with nodal support indicated from each of the ML and MP bootstrapping, and BI posterior probabilities.

The ML tree had a -ln likelihood score of 22914.9 and was identical in its estimate of proseriate interrelationships to the BI solution (-ln = 22617.2); the BI solution differed slightly from the MP and ML solution in placing the macrostomid turbellarian as the sister group to the proseriates. MP analysis resulted in 240 equally parsimonious trees with length = 4160, CI = 0.487 and RI = 0.505; the strict consensus of these trees yielded a topology entirely congruent with the likelihood model-based solutions.

Nodal support amongst solutions estimated by each of the phylogenetic methods was reasonably high throughout the trees (summarised on the ML tree in Figure 1).

All analyses resolved Proseriata + *Ciliopharyngiella* as a monophyletic assemblage. Indeed, *Ciliopharyngiella* clustered within the Unguiphora, though with low support. In contrast to previous analyses (Littlewood *et al.*, 2000), the monophyly of Lithophora, as well as of the major lithophoran families, including the Coelogynoporidae, was well supported. Sister-group relationships of Lithophora with Unguiphora + *Ciliopharyngiella* was likewise well supported. Within Lithophora, two sister clades were supported, including Coelogynoporidae + Calviriidae, and Otoplanidae + Archimonocelididae + Monocelididae, respectively.

Within most of the families, the number of genera and species sequenced is still too limited to allow inferences on intra-family relationships. However, the results obtained for the Coelogynoporidae deserve consideration, as particularly problematic, with instances of poor nodal support and topological differences between different phylogenetic estimates. In particular, the placement of *Coelogynopora axi* Sopott, 1972, *Vannuccia campana* Ehlers & Ehlers, 1980 and of the two species of *Cirrifera* differed between BI, ML and MP; Fig. 1 only shows the ML solution but the low nodal support in this clade highlights the conflict between different phylogenetic estimates. For all analyses, the genus *Cirrifera* was consistently resolved as paraphyletic. BI resolved *Coelogynopora axi* as nested within *Cirrifera* species, and ML (Fig. 1) shows *C. axi* as sister to *C. cirrifera* Sopott, 1972. All topologies relating these taxa were represented amongst the equally parsimonious trees found with MP. Furthermore, the genus *Coelogynopora* was resolved as paraphyletic in all phylogenetic estimates.

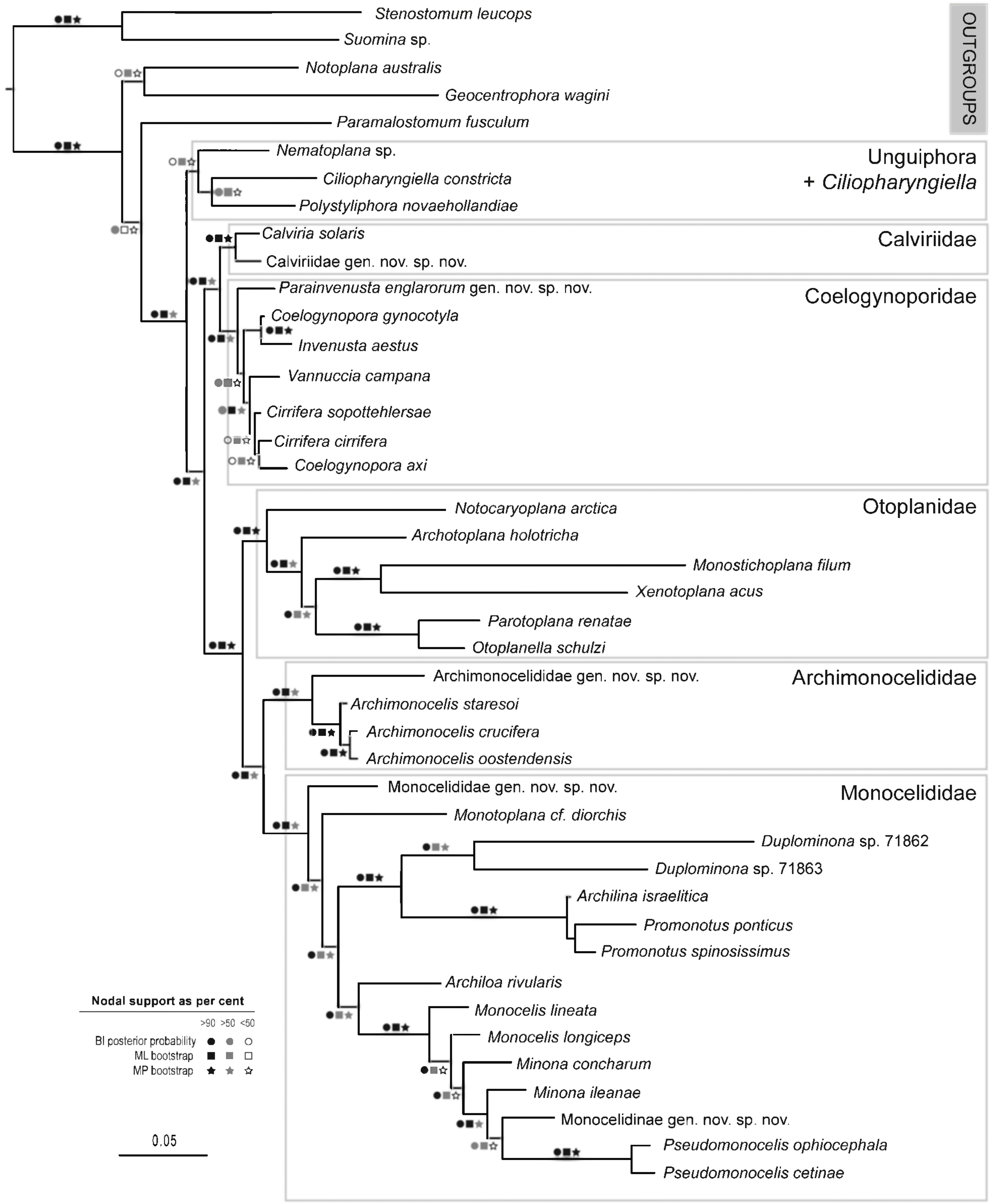

FIGURE 1. Maximum-likelihood estimate of the interrelationships of the Proseriata based on analysis of combined ssrDNA and lsrDNA data partitions (see Table 1). Major taxon names are indicated. Nodal support is indicated for BI (posterior probabilities), ML (bootstrap, n = 100) and MP (bootstrap, n=1000) as a percentage. The branch length scale is the number of substitutions per site.

Discussion

With respects to previous analyses (Littlewood *et al.*, 2000; Willems *et al.*, 2006), the widening of both taxonomic and molecular sampling gave an unambiguous tree with most basal nodes highly supported, which offers precious insights on the in-group relationships of the Proseriata:

Monophyly of Lithophora

Sopott-Ehlers (1985) divided the Proseriata into two suborders: the Unguiphora, which include taxa with, *i.a.*, pigment in the mantle cells of rhabdomeric receptors, and without a statocyst, and the Lithophora, which include taxa without pigment in the mantle cells and with a statocyst. An alternative hypothesis was proposed by Martens & Schockaert (1988), who assumed that the presence of the statocyst is the plesiomorphic condition for the Proseriata, secondarily lost in the Unguiphora. In this scenario, the family Monocelididae was assumed as the sister taxon to the rest of the Proseriata, the Paramonocelidida, characterised by the presence of a brain-capsula and polyploidy-derived karyotype (see Martens *et al.*, 1989).

The first molecular study of the Proseriata (Littlewood *et al.*, 2000) did not fully support either hypothesis. In fact, whereas the monophyly of the Lithophora was unsupported, this was due to members of the lithophoran families Coelogynoporidae and Calviriidae which clustered with the Unguiphora, while the Monocelididae clustered with the rest of the lithophoran taxa. More recently, in contrast, the monophyly of Lithophora was retrieved in a large-scale molecular study of the phylogeny of the Rhabdocoela, which included ssrDNA sequences of 15 proseriate species (Willems *et al.*, 2006). Results of the present, much larger study also support the monophyly of the Lithophora, and fully agree with the scenario depicted by Sopott-Ehlers (1985). Characters supporting the Paramonocelidida (Martens & Schockaert, 1988) should therefore be carefully reconsidered as possibly homoplasious. The Monocelididae, in fact, include many minute, interstitial taxa, and the absence of the brain-capsula in the group may be due to secondary reduction, induced by their non-burrower life-style (see Curini-Galletti, 2001). Furthermore, alternative hypotheses of karyotype evolution in the Proseriata (i.e. a secondary reduction of chromosome numbers occurring in the Monocelididae) should be investigated.

The Coelogynoporidae

Characterisation of the Coelogynoporidae on morphological grounds has always been problematical. In particular, its distinction from the Otoplanidae has been a matter of contention, as stressed by Karling (1966, p. 493), who stated that "there is no single characteristic, which alone separates these taxa". Characters shared by Coelogynoporidae and Otoplanidae include the monogonoporid condition (i.e. the presence of a single, common, genital opening), which has been considered as a synapomorphy for the two families (Martens & Schockart, 1988). The first molecular results did not support the monophyly of Coelogynoporidae + Otoplanidae, and led to consider the alternative hypothesis, i.e that the monogonoporid condition in the two groups is a homoplasious character (Littlewood *et al.*, 2000; Curini-Galletti 2001). The retrieved monophyly of the Coelogynoporidae in present study, the presence of the digonoporid condition (i.e. the separation of male and female pores) in the sister taxon Calviriidae, as well as in the Unguiphora and in several other lithophoran taxa (Meidiamidae, Archimonocelididae, and Monocelididae), and the lack of sister-taxon relationships between Coelogynoporidae and Otoplanidae, further suggest that the monogonoporid condition has been attained independently in the two latter taxa. However, a diagnosis of the family Coelogynoporidae remains problematic, and should include a combination of characters (monogonoporid condition, entirely ciliated body, distal (if any) fusion of oviducts, and absence of cephalic sensory groove) which, taken singly, are not unique to the taxon.

The placement of *Cirrifera sopottehlersae* Noldt & Jouk, 1988 and *C. cirrifera* in the molecular trees obtained in this study suggests that the puzzling result of a previous molecular study (Littlewood *et al.*, 2000) is due to the misidentification of the specimen sequenced, attributed to *C. dumosa*, whose placement among the Monocelididae, as the sister taxon of *Archiloa rivularis* de Beauchamp, 1910, made no morphological sense. In fact, the cirrus of *C. dumosa* and that of the species of *Archiloa* de Beauchamp, 1910 consists of numerous, minute spines, and may be confused in observations on unsqueezed specimens (cf Ax, 1954; Sopott, 1972). This case further corroborates the proposal that, whenever possible, sequences deposited in GenBank should be accompanied by references of voucher material and/or pictures of the specimens analysed (Pleijel *et al.*, 2008) that may be used to validate the identification.

Even with the removal of *C. dumosa*, correspondence of molecular clusters with coelogynoporid genera, as delimited at present, is far from satisfying. In most instances, however, the internal support of the clusters is poor, and further research, which includes a broader taxonomic sampling, is needed. The sister group relationship among *Coelogynopora gynocotyla* Steinböck, 1924 and *Invenusta aestus* Sopott-Ehlers, 1976 is one of the few well-supported nodes, and suggests that the large genus *Coelogynopora* may include unrelated taxa and needs a serious work of taxonomic refinement.

The Calviriidae

The group has a short but troubled taxonomic history. The first species described, *Asilomaria ampullata*, was included in the Monocelididae (Karling, 1966). Later, Martens & Curini-Galletti (1993) placed *A. ampullata*, together with three species of the newly described genus *Calviria*, in the Calviriinae, as a subfamily of the Archimonocelidae. Neither hypothesis was supported by the first molecular studies of the Proseriata, which rather showed a relationship of *Calviria solaris* Martens & Curini-Galletti, 1993 with part of the Coelogynoporidae (Littlewood *et al.*, 2000; Willems *et al.*, 2006). Curini-Galletti (2001) thus suggested a removal of the Calviriinae from the Archimonocelididae, and a placement of the group as a subfamily of the Coelogynoporidae. Recently, the taxon was given family rank (Schockaert *et al.*, 2009).

Present analysis placed *C. solaris* and Calviriidae gen. nov. sp. nov. from Iceland (Schockaert *et al.*, in prep) as the sister taxon of the Coelogynoporidae; the clade Calviriidae + Coelogynoporidae is the sister taxon of the rest of the Lithophora.

This relationship is of particular interest. Both in the Coelogynoporidae and the Calviriidae, in fact, as well as in the Unguiphora (the sister taxon of the Lithophora), a common female duct, when present, is very short, and results from the distal fusion of the oviducts. A similar condition is found in *Ciliopharyngiella,* where the two oviducts fuse just before their opening into the common atrium (Ax, 1952a). In contrast, in the Archimonocelidae, Monocelididae, in most of the Otoplanidae, and in the newly established Meidiamidae, for which no sequences are available at present, a noticeably long female duct, which results from a fusion of the oviducts in front of the copulatory organ, is present. The presence of a long female duct is thus assumed to be a synapomorphy for these taxa. In this group, a terminal fusion of the oviduct is present only in very few Otoplanidae, and the distribution of the character appears rather bizarre. At least within the genus *Parotoplana* Meixner, 1938, congeneric species present different conditions (see Ax, 1956, p. 718, fig. 150; Lanfranchi, 1978: p. 250, fig. 3), and there is the suspicion that the genito-intestinal connection may have been misinterpreted as one of the oviducts (Delogu & Curini-Galletti, unpublished data).

The Otoplanidae

In the most recent taxonomic arrangement of the Otoplanidae (Ax, 2008), the family was subdivided into the Euotoplanida Ax, 2008, which includes the subfamilies Otoplaninae Hallez, 1910; Bulbotoplaninae Ax, 1956; Parotoplaninae Ax, 1956; Cirroplaninae Miller & Faubel, 2003; and the taxon "*Archotoplana*" Ax, 1956, which lacks the apomorphic feature of the ciliation restricted to a ventral sole found in the Euotoplanida (Ax, 2008; Miller & Faubel, 2003).

At present, a molecular scrutiny of this scheme, including representatives of the various subfamilies, is lacking. Indeed, the molecular sampling of species of Otoplanidae is still very poor, relative to the diversity of the taxon, which includes well over 100 species (Ax, 2008; Delogu & Curini-Galletti, 2009; Tyler *et al.*, 2006).

As incomplete as it may be, our molecular tree offers a solution in conflict with current taxonomy. In particular, *Archotoplana holotricha* Ax, 1956 does not emerge as the sister taxon of the rest of the Otoplanidae, thus supporting neither the basal position of *Archotoplana* nor the monophyly of the Euotoplanida. Furthermore, the monophyly of at least one subfamily of the Euotoplanida, the Otoplaninae (which includes the genera *Otoplanella* Ax, 1956, *Monostichoplana* Ax, 1956, *Notocaryoplana* Steinböck, 1935, and *Xenotoplana* Ax, Weidemann & Ehlers, 1978, for which sequences are available), is clearly not supported by molecular results.

The Archimonocelididae

With the recent removal of the genera *Calviria* and *Meidiama* Marcus, 1946, the scope of the Archimonocelididae has been considerably reduced, and, presently, it only includes the genus *Archimonocelis* Meixner, 1938 (Schockaert *et al.*, 2009; Martens & Curini-Galletti, 1993). There are, however, numerous, still not formally described taxa from Australia, such as Archimonocelididae gen. nov. sp. nov. used in the present analysis, which reveal a far greater diversity of the Archimonocelididae than published data alone may suggest. At present, the morphological characters used to define the family (Schockaert *et al.*, 2009) appear to adequately represent this taxon, which is supported by the molecular tree.

The Monocelididae

Not surprisingly, this large and diverse family, the most species-rich in the Proseriata (Tyler *et al.*, 2006), has been the subject of several studies aimed to assess intra-group phylogenetic relationships. The earliest attempt to produce a systematic arrangement by Karling (1978), resulted in the division of the Monocelididae in two subfamilies, based on the presence (Mononinae) or absence (Monocelidinae) of the accessory prostatoid organ, a muscular-glandular structure armed with a stylet. Litvaitis *et al.* (1996), considering molecular, morphological and karyological data, limited the scope of the Monocelidinae to genera provided with a simplex-type copulatory bulb, and placed the remaining genera of the Monocelididae, with duplex-type copulatory bulb, in the Duplomonocelidinae, a possibly paraphyletic taxon (see in the appendix for a definition of simplex and duplex-type copulatory organs). In this scheme, the presence of the prostatoid organ was considered to be a plesiomorphic feature for the Monocelididae. Later, based on molecular analyses, taxa ascribed to the family Monotoplanidae (= Globuliphorinae) were moved to the Monocelididae Duplomonocelidinae (Littlewood *et al.*, 2000; Curini-Galletti, 2001).

Faubel & Rohde (1998), considering morphology alone, added two new subfamilies to Karling's scheme (1966): the Archiloinae and the Cannoninae.

These views of the taxonomy of the group were summarised by Tyler *et al.* (2006): the Monocelididae are split into six subfamilies, listed below. For each of them, the genera for which species have been sequenced are indicated between brackets:

- Archiloinae Faubel & Rohde, 1998 (*Archilina* Ax, 1959; *Archiloa*)
- Cannoninae Faubel & Rohde, 1998 (*Pseudomonocelis* Meixner, 1943)
- Globuliphorinae Westblad, 1952 (*Monotoplana* Meixner, 1938)
- Mononinae Karling, 1978 (*Minona* Marcus, 1946; *Duplominona* Karling, 1966)
- Monocelidinae Midelburg, 1908 (*Monocelis* Ehrenberg, 1831)
- Promonotinae Marcus, 1949 (*Promonotus* Beklemischev, 1927).

Our molecular analysis confirmed that the genera *Monocelis*, *Minona*, and *Pseudomonocelis,* all with a simplex-type copulatory bulb, form a monophyletic clade, thus supporting the Monocelidinae sensu Litvaitis *et al.*, 1996. The result emphasises the phylogenetic relevance of the characters "bulb of the simplex-type" and of the *Monocelis*-like karyotype (Curini-Galletti & Martens, 1990) . Both characters are also present in the undescribed species Monocelidinae gen. nov. sp.nov. Due to the remote position in the molecular tree of species of the genera *Minona* and *Duplominona*, the subfamily Mononinae is clearly not supported. The monophyly of the Cannoninae, which include genera with simplex (*Pseudomonocelis, Acanthopseudomonocelis* Curini-Galletti & Cannon, 1995) and duplex-type copulatory bulb (*Cannona* Faubel & Rohde, 1998) is unlikely, but we have no molecular-sequence data on any species of *Cannona* so this part of the phylogenetic tree remains unsettled. Furthermore, molecular data do not support the subfamily Archiloinae, as species of *Archiloa* and *Archilina* do not form a monophyletic clade.

Indeed, apart from the monophyletic Monocelidinae, the rest of the Monocelididae forms a rather heterogenous assemblage of taxa with duplex-type cirrus, corresponding to the paraphyletic Duplomonocelidinae (Litvaitis *et al.*, 1996). More data are clearly needed before a satisfactory systematic arrangement of the family can be proposed.

The position of *Ciliopharyngiella*

In our analysis, *C. constricta* clustered within the Unguiphora, although with poor support (Fig. 1). Albeit scarcely probative, this result may be taken as indicative of a close relationships of *Ciliopharyngiella* with the Proseriata. A similar result was obtained by Willems *et al.* (2006), who included a large sample of Rhabdocoela. The similarities of *Ciliopharyngiella* with Proseriata, based on general topography of female genital structures and brain morphology, were already remarked by the author of the taxon (Ax, 1952a), who ascribed it to the Proseriata, although with some hesitancy. Later, the taxon was removed from the Proseriata for the presence of a bulbous-type pharynx rosulatus (Proseriata have a plicatus-type pharynx) and the structure of the male system, and assigned to the Typhloplanoida (Ehlers 1972). *Ciliopharyngiella* has received considerable phylogenetic attention, since it was assumed as the earliest derived taxon of the Rhabdocoela (Ehlers 1972; Ax, 1995). Affinities of the taxon on morphological grounds are, however, still a matter of contention. The pharynx rosulatus of *Ciliopharyngiella* has a unique morphology among the Rhabdocoela (Ax, 1995), and some of its supposed autapomorphies (such as the presence of ciliation both internally and externally) are indeed shared with the Proseriata. Claimed synapomorphies with Rhadocoela and Prolecithophora (see Sopott-Ehlers 2001) are not unequivocal, such as the lack of true lamellated rhabdites, a character assumed to be shared with representatives of the Prolecithophora, but lacking in the Proseriata as well (Sopott-Ehlers, 1985). Indeed, some of the observed characters on which rhabdocoelan affinities were based, ‘might be a convergence’ (Sopott-Ehlers, 2001, p. 163).

Furthermore, there are reasonable suspicions that some of the supposed affinities with the Rhabdocoela, and of the very numerous autapomorphies of the taxon (Sopott-Ehlers, 2001), are biased by the overall poor knowledge of the Unguiphora. Bruggemans (1986) pointed at synapomorphies among *Ciliopharyngiella* and Unguiphora in the development and ultrastructure of the stylet. Unfortunately, the genus *Ciliopharyngiella* is still poorly known. It includes at present two species: the type species, *C. intermedia* and *C. constricta*, which is the only one sequenced. At least one more species has been found in the Mediterranean, but the material was inadequate for species description (MC-G unpubl. data). At this moment, the poor knowledge and limited molecular sampling of both Unguiphora and *Ciliopharyngiella* do not allow further phylogenetic inferences.

Conclusion

The molecular database available provided solutions to most of the open questions, concerning the intra-group relationships of the Proseriata:

- the monophyly of Lithophora and of Coelogynoporidae is supported;
- the sister-group relationships of Coelogynoporidae and Calviriidae is revealed; as well as their sister-group relationships with a clade including the rest of the Lithophora (Otoplanidae+Archimonocelididae+Monocelididae).

These results highlight the effects of the widening of taxon and molecular samplings, together with the pruning from the database of, at best, potentially misidentified species.

However, there are still open issues that need to be addressed in future research:

- present definitions and delimitations of coelogynoporid genera are far from satisfying, and should be assessed on larger molecular and denser taxonomic samples;
- the two families Otoplanidae and Monocelididae are both very large and diverse, and their intra-group relationships still have to be elucidated in detail;

- two families - Otomesostomidae and the newly established family Meidiamidae (Schockaert *et al.*, 2009) - have yet to be assessed for molecular-sequence data;
- the position of *Ciliopharyngiella* and its possible inclusion within the Proseriata need to be thoroughly assessed.

Acknowledgements

Samplings in Iceland were made possible thanks to a grant allowed by the Sangerdi Marine Centre, Iceland. Dr. Giulia Ceccherelli kindly provided the sample from Plymouth, where *Notocaryoplana arctica* was found. Two unknown referees significantly improved the first version of the manuscript.

References

Ax, P. (1951) Die Turbellarien des Eulitorals der Kieler Bucht. *Zoologische Jahrbücher (Systematik, Ökologie und Geographie der Tiere)*, 80, 277–378.

Ax, P. (1952a) *Ciliopharyngiella intermedia* nov. gen. nov. spec. Repräsentant einer neuen Turbellarien-Familie des marinen Mesopsammon. *Zoologische Jahrbücher (Systematik, Ökologie und Geographie der Tiere)*, 81, 286–312.

Ax, P. (1952b) Neue psammobionte Turbellaria Macrostomida aus der Verwandtschaft von *Paromalostomum*. *Zoologischer Anzeiger (Jena)*, 149, 99–107.

Ax, P. (1954) Zwei neue Monocelididae (Turbellaria, Proseriata) aus dem Eulitoral der Nord- und Ostsee. *Kieler Meeresforschungen*, 10, 229–242.

Ax, P. (1956) Monographie der Otoplanidae (Turbellaria). Morphologie und Systematik. *Abhandlungen der Matematisch-naturwissenschaftlichen Klasse 1. Akademie der Wissenschaften und der Literatur Mainz*, 13, 499–796.

Ax, P. (1959) Zur Systematik, Ökologie und Tiergeographie der Turbellarienfauna in den ponto-kaspischen Brackwassermeeren. *Zoologische Jahrbücher (Systematik, Ökologie und Geographie der Tiere)*, 87, 43–184.

Ax, P. (1995) *Multicellular Animals: A New Approach to the Phylogenetic Order in Nature. Vol. I.* Springer Verlag, Berlin-Heidelberg-New York, 225 pp.

Ax, P. (2008) Plathelminthes aus Brackgewässern der Nordhalbkugel. *Abhandlungen der Mathematisch-naturwissenschaftlichen Klasse 1. Akademie der Wissenschaften und der Literatur Mainz*, 1, 1–696.

Ax, P., Wiedemann, E. & Ehlers, B. (1978) Zur Morphologie sublitoraler Otoplanidae (Turbellaria, Proseriata) von Helgoland und Neapel. *Zoomorphologie*, 90, 113–133.

Beklemischev, W.N. (1927) Über die Turbellarienfauna des Aralsees. *Zoologische Jahrbücher. Abteilung für Systematik, Geographie und Biologie*, 54, 87–138.

Brüggeman, J. (1986) Ultrastructural investigations on the differentiation of genital hard structures in free-living platyhelminthes and their phylogenetic significance. *Hydrobiologia*, 132, 151–156.

Casu, M. & Curini-Galletti, M. (2006) Genetic evidence for the existence of cryptic species in the mesopsammic flatworm *Pseudomonocelis ophiocephala* (Rhabditophora: Proseriata). *Biological Journal of the Linnean Society*, 87, 553–576.

Curini-Galletti, M. (1997) Contribution to the knowledge of the Proseriata (Platyhelminthes: Seriata) from the Gulf of Elat (Red Sea): Genus *Minona* Marcus, 1946. *Israel Journal of Zoology*, 43, 121–131.

Curini-Galletti, M. (1998) The genus *Polystyliphora* Ax, 1958 (Platyhelminthes: Proseriata) in eastern Australia. Journal of Natural History, 32, 473–499.

Curini-Galletti, M. (2001) The Proseriata. *In*: Littlewood, D.T.J., Bray, R.A. (Eds) *Interrelationships of the Platyhelminthes*. Taylor and Francis: London and New York, pp. 41–48.

Curini-Galletti, M. & Cannon, L.R.G. (1995) Contribution to the knowledge of the Proseriata (Platyhelminthes: Seriata) from eastern Australia. II. Genera *Pseudomonocelis* Meixner, 1943 and *Acanthopseudomonocelis* n. g. *Contributions to Zoology*, 65, 271–280.

Curini-Galletti, M. & Cannon, L.R.G. (1996) The genus *Minona* (Platyhelminthes, Seriata) in Eastern Australia. *Zoologica Scripta*, 25, 193–202.

Curini-Galletti, M., Puccinelli, I. & Martens, P.M. (1989) Karyometrical analysis of ten species of the subfamily Monocelidinae (Proseriata, Platyhelminthes) with remarks on the karyological evolution of the Monocelididae. *Genetica*, 78, 169–178.

Curini-Galletti, M. & Martens, P.M. (1990) Karyological and Ecological Evolution of the Monocelididae

(Plathelminthes Proseriata). *Marine Ecology, Pubblicazioni della Stazione Zoologica di Napoli,* 11, 255–261.

Curini-Galletti, M. & Martens, P.M. (1995) *Archilina israelitica* n. sp. (Platyhelminthes Proseriata) from the eastern Mediterranean. *Bollettino di Zoologia*, 62, 267–271.

De Beauchamp, P. (1910) *Archiloa rivularis* n.g. n. sp. Turbellarié Alloeocoele d'eau douce. *Bulletin de la Société Zoologique de France,* 35, 211–219.

De Beauchamp, P. (1961) Classe des Turbellariés. *In*: Grassé, P.P. (Ed.), *Traité de Zoologie IV (I)*, Masson, Paris, pp. 35–212

Delogu, V. & Curini-Galletti, M. (2009) Otoplanidae (Platyhelminthes, Proseriata) from the northern Adriatic Sea. *Meiofauna Marina*, 17, 133–144.

Duges, A. (1828) Recherches sur l'organisation et les moeurs des planariees. *Annales des sciences naturelles*, 15, 139–182.

Duges, A. (1830) Aperçu de quelques observations nouvelles sur les Planaires et plusieurs genres voisins. *Annales des Sciences naturelles*, 21, 72–91.

Du Plessis, G. (1889) Note sur 'l'*Otoplana intermedia*'. *Zoologischer Anzeiger*, Leipzig , 12, 339–342.

Ehlers, U. (1972) Systematisch-phylogenetische Untersuchungen an der Familie Solenopharyngidae (Turbellaria, Neorhabdocoela). *Mikrofauna Meeresboden*, 11, 1–78.

Ehlers, U. (1985) *Das phylogenetische System der Plathelminthes*. G. Fischer Ed., Stuttgart/New York, 317 pp.

Ehlers, U. & Ehlers, B. (1980) Zur Systematik und geographischen Verbreitung interstitieller Turbellarien der Kanarischen Inseln. *Mikrofauna Meeresbodens*, 80, 573–593.

Ehlers, U. & Sopott-Ehlers, B. (1990) Organization of statocysts in the Otoplanidae (Plathelminthes): An Ultrastructural analysis with implications for the phylogeny of the Proseriata. *Zoomorphology*, 109, 309–318.

Faubel, A. & Rhode, K. (1998) Sandy beach meiofauna of eastern Australia (Southern Queensland and New South Wales). IV. Proseriata, Plathelminthes. *Mitteilungen des hamburgischen zoologischen Museums und Instituts*, 95, 7–28.

Hallez, P. (1910) Un nouveau type d'alloiocoele (*Bothriomolus constrictus* n. g., n. sp.). *Archives de Zoologie experimentale et generale*, 3, 611–664.

Huelsenbeck, J.P. & Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17, 754–755.

Jouk, P.E.H., Martens, E.E. & Schockaert, E.R. (2007) *Cirrifera genitoductus* sp.n. (Platyhelminthes, Proseriata, Coelogynoporidae) from the Belgian coast, with observations on its ultrastructure and its significance for the taxonomy of the Coelogynoporidae. *Belgian Journal of Zoology*, 137, 223–230.

Karling, T.G. (1956) Morphologisch-histologische Untersuchungen an den männlichen Atrialorganen der Kalyptorhynchia (Turbellaria). *Arkiv för Zoologi*, 9, 187–279.

Karling, T.G. (1966) Marine Turbellaria from the Pacific Coast of North America IV. Coelogynoporidae and Monocelididae. *Arkiv för Zoologi*, 18, 493–528.

Karling, T.G. (1978) Anatomy and systematics of marine Turbellaria from Bermuda. *Zoologica Scripta*, 7, 225–248.

Lanfranchi, A. (1978) Morphology and taxonomy of two new otoplanids (Turbellaria, Proseriata) from the Ligurian Sea. *Zoologica Scripta*, 7, 249–254.

Lanfranchi, A. & Melai, M. (2009) A new marine flatworm (Plathelminthes: Rhabditophora: Otoplanidae) from the Ligurian coast. *Journal of the Marine Biological Association of the United Kingdom,* Published online by Cambridge University Press. doi:10.1017/S0025315409990427

Littlewood, D.T.J., Rohde, K. & Clough, K.A. (1999) The interrelationships of all major groups of Platyhelminthes: phylogenetic evidence from morphology and molecules. *Biological Journal of the Linnean Society*, 66, 75–114.

Littlewood, D.T.J., Curini-Galletti, M. & Herniou, E.A. (2000) The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. *Molecular Phylogenetics and Evolution,* 16, 449–466.

Litvaitis, M.K., Curini-Galletti, M., Martens, P.M. & Kocher, T.D. (1996) A reappraisal of the systematics of the Monocelididae (Platyhelminthes, Proseriata) – Inferences for rDNA sequences. *Molecular Phylogenetics and Evolution*, 6, 150–156.

Lockyer, A.E., Olson, P.D. & Littlewood, D.T.J. (2003) Utility of complete large and small rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. *Biological Journal of the Linnean Society,* 78, 155–171.

Maddison, W.P. & Maddison, D.R. (2005) *MacClade: analysis of phylogeny and character evolution.* Sinauer Associates, Sunderland, Massachusetts

Marcus, E. (1946) Sobre Turbellaria brasileiros. *Boletim da Faculdade de Filosofia, Ciências e Letras da Universidade de São Paulo, série Zoologia,* 11, 1–254.

Marcus E. (1948) Turbellaria do Brasil. *Boletim da Faculdade de Filosofia, Ciências e Letras da Universidade de São Paulo, série Zoologia,* 13, 111–243.

Marcus E. (1949) Turbellaria Brasileiros (7). *Boletim da Faculdade de Filosofia, Ciências e Letras da Universidade de São Paulo, série Zoologia* 14, 7–156.

Martens, P.M. (1984) Comparison of three different extraction methods for Turbellaria. *Marine Ecology Progress Series*, 14, 229–234.

Martens, P.M. & Curini-Galletti, M. (1993) Taxonomy and phylogeny of the Archimonocelididae Meixner, 1938 (Platyhelminthes, Proseriata). *Bijdragen tot de Dierkunde*, 63, 65–102.

Martens, P.M. & Curini-Galletti, M. (1999) Revision of *Promonotus* Beklemischev, 1927 (Platyhelminthes: Proseriata) with description of two new species from the Mediterranean. *Hydrobiologia,* 412, 131–142.

Martens, P.M., Curini-Galletti, M. & van Oostveldt, P. (1989) Polyploidy in Proseriata (Platyhelminthes) and its phylogentical implications. *Evolution*, 43, 900–907.

Martens, P.M. & Schockaert, E.R. (1981) Sand dwelling Turbellaria from the Netherlands Delta area. *Hydrobiologia*, 84, 113–127.

Martens, P.M. & Schockaert, E.R. (1988) Phylogeny of the digonoporid Proseriata. *Fortschritte der Zoologie/Progress in Zoologie*, 36, 399–403.

Meixner, J. (1938) Turbellaria (Strudelwuermer). I. Die *Tierwelt der Nord- und Ostsee*. 33 (IVb), 1–146.

Meixner, J. (1943) Über die Umbildung einer Turbellarienart nach Einwanderung aus dem Meere ins Süßwasser. *Internationale Revue der gesamten Hydrobiologie und Hydrographie*, 43, 458–465.

Midelburg, A. (1908) Zur Kenntnis der Monocelididae. *Zeitschrift für Wissenschaftliche Zoologie*, 88, 81–108.

Miller, W. & Faubel, A. (2003) New species of the suborder Proseriata (Platyhelminthes: Turbellaria) from the eastern Australian intertidal zone, with ecological comments. *Mitteilungen des hamburgischen zoologischen Museums und Instituts*, 100, 27–57.

Müller, O.F. (1774) Vermium terrestrium et fluviatilium, seu animalium infusoriorum, helminthicorum et testaceorum, non marinorum, succincta historia. Vol. primi pars altera. *Havniae et Lipsiae*. 4, 52–72.

Noldt, U. & Jouk, P. (1988) *Cirrifera sopottehlersae*, a new species of Coelogynoporidae (Plathelminthes, Proseriata) from marine sediments of the Belgian and German North Sea coast. *Stygologia*, 4, 242–248.

Pleijel, F., Jondelius, U., Norlinder, E., Nygren, A., Oxelman, B., Schander, C., Sundberg, P. & Thollesson, M. (2008) Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. *Molecular Phylogenetics and Evolution*, 48, 369–371.

Posada, D. & Crandall, K.A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics,* 14, 817–818.

Riser, N. (1981) New England Coelogynoporidae. *Hydrobiologia*, 84, 139–145.

Schmarda, L.K. (1859) Neue wirbellose Thiere beobachtet und gesammelt auf einer Reise um die Erde 1853 bis 1857. Erster Band. Turbellarien, Rotatorien und Anneliden. W. Engelmann, Leipzig ed., 1–164.

Schmidt (1861) Untersuchungen ueber Turbellarien von Corfu und Cephalonia, nebst Nachträgen zu früheren Arbeiten. *Zeitschrift für Wissenschaftliche Zoologie,* 11, 1–32.

Schockaert, E.R., Curini-Galletti, M., De Ridder, W., Volonterio, O. & Artois, T. (2009) A new family of lithophoran Proseriata (Platyhelminthes), with the description of seven new species from the Indo-Pacific and South America, and the proposal of three new genera. *Zoological Journal of the Linnean Society,* 155, 759–773.

Sopott, B. (1972) Systematik und Ökologie vor Proseriaten (Turbellaria) der deutschen Nord-seeküste. *Mikrofauna Meeresboden*, 13, 1–72.

Sopott-Ehlers, B. (1976) Interstitielle Macrostomida und Proseriata (Turbellaria) von der französischen Atlantikküste und den Kanarischen Inseln. *Mikrofauna Meeresboden*, 60, 539–571.

Sopott-Ehlers, B. (1985) The phylogenetic relationships within the Seriata (Platyhelminthes). *In* : Conway Morris, S., George, D.J., Gibson, R. & Platt, H.M. (Eds), *The origin and relationships of lower invertebrates*. Oxford University Press, Oxford, pp. 159–167.

Sopott-Ehlers, B. (2001) *Ciliopharyngiella intermedia* (Platyhelminthes : Neoophora) – enigmatic as ever? Ultrastructural features and phylogenetic implications. *Belgian Journal of Zoology,* 131 (suppl. 1), 159–165.

Steinböck, O. (1924) Untersuchungen über die Geschlechtstrakt-Darmverbindung bei Turbellarien nebst einem Beitrag zur Morphologie des Tricladendarmes. *Zeitschrift für Morphologie und Ökologie der Tiere*, 2, 461–504.

Steinböck, O. (1935) Turbellarien aus Ostgrönland. *Videnskabelige Meddelelser Dansk Naturhistorisk Forening*, 98, 235–241.

Swofford, D.L. (2002) *PAUP* 4.0: Phylogenetic Analysis Using Parsimony (*and other methods)*. Sinauer Associates, Sunderland, MA.

Tajika, K.-I. (1981) Marine Turbellarien aus Hokkaido, Japan V. Coelogynoporidae (Proseriata). *Journal of the Faculty of Science, Hokkaido University, Series VI, Zoology,* 22, 451–473.

Timoshkin, O. (1984) New species of the genus *Geocentrophora* (Turbellaria, Prorhynchidae) from Lake Baikal. *Zoologichesky Zhurnal,* 63, 1125–1136.

Tyler, S., Schilling, S., Hooge, M. & Bush, L.F. (2006) *Turbellarian taxonomic database. Version 1.5.* Available from http:// turbellaria.umaine.edu

Westblad, E. (1952) Some new "Alloeocoels" (Turbellaria) from the Scandinavian west coast. *Univ Bergen Arbok, Naturvet rekke*, 7, 1–27.

Wiens, J.J. & Moen, D.S. (2008) Missing data and the accuracy of Bayesian phylogenetics. *Journal of Systematics and*

Evolution, 46, 307–314.

Willems, W.R., Wallberg, A., Jondelius, U., Littlewood, D.T.J., Backeljau, T., Schockaert, E.R. & Artois, T.J. (2006) Filling a gap in the phylogeny of flatworms: relationships within the Rhabdocoela (Platyhelminthes), inferred fron 18S ribosomal DNA sequences. *Zoologica Scripta,* 35, 1–17.

Zwickl, D.J. (2006) *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. The University of Texas, Austin.* Available from: www.bio.utexas.edu/faculty/antisense/garli/Garli.html

Appendix

Fam. Coelogynoporidae Karling, 1966

Genus *Parainvenusta* Curini-Galletti gen. nov.

Diagnosis: Coelogynoporidae with long paracnida surrounding the genital pore and in the tail. Copulatory organ of the duplex type, containing a convoluted unarmed cirrus. With bursa, and long, muscular genito-intestinal connection.

Etymology: The genus name is coined after the genus *Invenusta* Sopott-Ehlers, 1976, and reflects the superficial similarity between the two taxa.

Parainvenusta englarorum Curini-Galletti sp. nov.

(Fig. 2)

Holotype: One specimen sagittally-sectioned (SAM AHC29915)

Type locality: Port Lincoln, South Australia. Sleaford Bay, Wreck beach (34°51'36.71"S; 135°43'41.19"E), fine sand, Jan. 2001

Additional material: one specimen sagittally sectioned (paratype: SMNH type-7950); four specimens sagittally sectioned (CZM 181-183); two specimens frontally-sectioned (CZM 184, 185); four whole mounts (CZM 186-189); two karyological slides (CZM 190, 191.). Sleaford Bay, Lone Pine beach (34°52'01.45"S; 135°42'44.98"E), medium-fine sand, Jan. 2001: three specimens sagittally-sectioned (CZM 192-194).

Etymology: the species is dedicated to Giuseppe and Saturna Englaro, for their long and strenuous struggle for the respect and actuation of the wills of their daughter Eluana (1970–2009), in irreversible vegetative state for 17 years, giving an exemplary lesson of laicity, love, and faith in legality.

Description. Animals slender; fixed mounts up to 6 mm long. Anterior end with few sensory bristles. Body entirely ciliated; cilia about 3 µm long. Epithelium with insunk nuclei. With numerous adhesive papillae in the tail; a few are scattered along the length of the body.

Different types of glands are present at the periphery of the body:

i) small, intensely stained eosinophylic glands (g_1 in Fig. 2 C), about 5 µm long, with the main body below the basal lamina, and whose necks pierce the overlaying epithelium;

ii) numerous ovoid rhabdoids (Figs 2A–C: r), 15–20 µm long, particularly dense in the caudal region;

iii) long paracnida, containing an eversible tube, distinctly granular at its proximal end. Two types of paracnida, differing in position and morphology, can be recognized. A few (6–10), very large (up to 60 µm long) and nearly straight, are placed radially around the genital area, and in most specimens form a rosette around the genital opening (Figs 2A, B: p_1). Others (20 – 30 µm long), more numerous and more irregularly coiled, are distributed mostly in the caudal region (Figs 2A, B: p_2), where they are intermingled with rhabdoid glands.

The brain is encapsulated, and abuts anteriorly the statocyst, which is about 20 µm across. The two neural cords originating ventrally from the brain are easily appreciable in sections of the anterior part of the body.

Subepithelial musculature thin, formed by an external, circular and an inner longitudinal layer. Numerous dorso-ventral fibres are present, regularly arranged along the length of body. The gut extends from the cephalic area to the tail region.

Pharynx in the posterior 3rd of body. It is tubular, up to 500 µm long, and directed horizontally. It is provided with a thin layer of external longitudinal musculature and an inner, thicker, circular component. The pharynx is ciliated (cilia 2.5–3 µm long) apart from the distal tip, where long pharyngeal glands, which extend considerably anterior to the pharynx, discharge. With a short (20–30 µm long) oesophagous, lined with unciliated, nucleated epithelium.

Pharyngeal pocket lined with unciliated, nucleated epithelium; mouth ciliated.

Male genital system. With few testes (20–30), arranged in two irregular rows in front of the ovaries. The testes are placed laterally, abutting the vitellaria, and often intermingled with them.

Copulatory organ consisting of a globular bulb, connected to two seminal vesicles. The seminal vesicles are lined by a thin, flat epithelium, and extend laterally and posteriorly from the bulb, into which they enter dorsally. The bulb is 45–50 µm across, lined externally by a flat, intranucleated epithelium. Below the epithelium, a strong muscular lining, 10–12 µm thick, is present. It consists of relatively few and very large muscle cells, arranged into an external longitudinal and an inner, thicker, circular layer. The bulb is posteriorly oriented, and is connected to the body by a relatively small, ventral area, crossed by muscular fibres which are connected to the body musculature. The bulb contains a cirrus, which originates from the fusion inside the bulb of the distalmost portions of the two seminal vesicles. No prostatic vesicle was observed, nor cell bodies that could be identified as prostatic glands. The cirrus is unarmed, long and very convoluted, so that its length is impossible to measure with any degree of confidence in sections. It is lined internally, at least in parts, by a basal lamina, without overlying epithelium, and it is surrounded by a thin coating of muscular cells, more noticeable distally. The cirrus opens ventrally into the atrium. The rest of the bulb appears filled with somewhat loose parenchymatic cells, with ‘empty’ spaces among cells.

Female genital system. Vitellaria at both sides of the body, in front of the pharynx. Two ovaries in front of the pharynx.

Oviducts ciliated; they run posteriorly, and open independently at the two sides of a small bursa. This is nearly spherical, with a lumen about 10 µm across, and, in most specimens, contains sperm. The bursa is lined by an unciliated cuboidal epithelium, and opens anteriorly into the dorsal wall of the atrium through a short, ciliated bursal canal, 10–15 µm long, which receives the female glands. Posteriorly, the bursa is connected to a long (50–60 µm in sections), S-shaped duct, surrounded by very strong circular muscles. The muscle ring closest to the bursa is the strongest, and may act as a sphincter. This duct is lined by an unciliated, nucleated epithelium, and opens into the posterior-most portion of the gut, thus forming a genito-intestinal connection. In most specimens, the posterior gut appears filled with sperm, suggesting the existence of a distinct resorbing bursa in this part of the intestine.

The common atrium is narrow, lined with an unciliated epithelium. The genital pore is surrounded by a sphincter.

Karyotype. The only plate suitable for study had n=5, with Chrom I isobrachial, and the rest of chromosome pairs heterobrachial. The smallest chromosome pair was about half the size of Chrom I.

Discussion. The combination of characters found in the new species is unique, and, although clearly attributable to the Coelogynoporidae for a combination of characters (see discussion above), could not be fit into any extant genus. Indeed, albeit biased as this may be by incomplete taxonomic sample of coeloegynoporid genera, the species occupies a basal, isolated position in the molecular tree (Figure 1).

As recalled by the etymology, *Parainvenusta englarorum* gen. nov. sp. nov. superficially resembles species of *Invenusta*, which possess similar strikingly long paracnida, and have an unarmed copulatory organ. Two species of *Invenusta* are known: *I. aestus* Sopott-Ehlers, 1976 (type species) from the eastern Atlantic (Canary Islands and French Atlantic coast) and *I. paracnida* (Karling, 1966) from western U.S.A. (Bodega Bay, California, and S. Juan islands, Washington). Both species have long paracnida, spread all over the body, and a copulatory organ provided with a muscular penis papilla, with the two seminal vesicles external to the bulb. Both lack a bursa, and the oviducts enter a ciliated diverticulum of the atrium. However, the two species appear to differ to an extent that their congenerity may be questioned. Among the main differences, the paracnida of *I. aestus* have the eversible part provided with barbs, while they are granular basally (and thus similar to the new species) in *I. paracnida*. The pharynx is short and oriented vertically, and the copulatory organ is anteriorly oriented, in *I. aestus*, while the pharynx is tubular and horizontal and the copulatory organ vertical in *I. paracnida*. Furthermore, in *I. paracnida* a long, convoluted genito-intestinal duct is present, while this is absent in *I. aestus* (Karling, 1966, Sopott-Ehlers, 1976). The structure of the female genital system in *I. paracnida* – consisting of a diverticulum of the atrium which receives the oviducts, and a long, winding genito-intestinal connection - is indeed comparable to that of the new species. However, in *P. englarorum*, the ciliated duct, here interpreted as a bursal canal, leads to a bursa which receives the outlets of the oviducts, and is histologically different from the rest of the female system.

Furthermore, the copulatory organ is of the simplex type in *Invenusta*, *i.e.* it is formed by a single muscular wall which surrounds the last portion of the ejaculatory duct (Karling, 1956; Litvaitis *et al.*, 1996). In contrast, in *P. englarorum* the cirrus bulb contains a (presumably) eversible cirrus, surrounded by a fluid matrix. The presence of two muscular walls, an outer and inner one, this latter corresponding to the last portion of the ejaculatory duct, fits the definition of duplex-type copulatory bulb (Karling, 1956).

Finally, the position of *P. englarorum* and *I. aestus* in the molecular tree (Fig. 1) does not support a close phylogenetic relationship among the two taxa.

Within the Coelogynoporidae, a duplex–type copulatory organ is present in the genera *Cirrifera* Sopott, 1972, *Vannuccia* Marcus, 1948, and *Stilivannuccia* Faubel & Rohde, 1998. In these taxa, however, the cirrus is provided with numerous spines; and accessory stylets may be present. In the genus *Cirrifera*, the seminal vesicle is entirely extracapsular, and a prostatic vesicle is present. Furthermore, species of *Cirrifera* have a vertically oriented pharynx, and lack a bursa and true paracnida (Jouk *et al.*, 2007). In *Vannuccia* and *Stilivannuccia*, the seminal vesicle is anterior to the copulatory bulb, an intra-bulb prostatic vesicle is generally present, as well as a long female duct leading to the bursa.

A cirrus is also present in *Macroatrium setosum* Riser, 1981 from N.W. Atlantic (New England); although its morphology is not known in details, it is spiny, broad, and cup-shaped, and thus very different from that of the new species. *M. setosum* lacks paracnida, and is provided with a comparatively very large atrium and a seminal bursa roughly of the same size as the atrium (Riser, 1981).

Similarly to *Parainvenusta englarorum, Pseudovannuccia hirutai* (Tajika, 1981) is characterized by an unarmed copulatory organ. However, in this species the copulatory organ is of the simplex type (Faubel & Rohde, 1998), with a highly developed, strongly muscular penis papilla. Furthermore, the seminal vesicle is entirely within the copulatory bulb and anterior to the gonopore (Tajika, 1981).

Given the morphological distinctiveness of the new species, its inclusion in any of the genera above would render that genus exceedingly heterogenous; and a new genus is thus proposed for it.

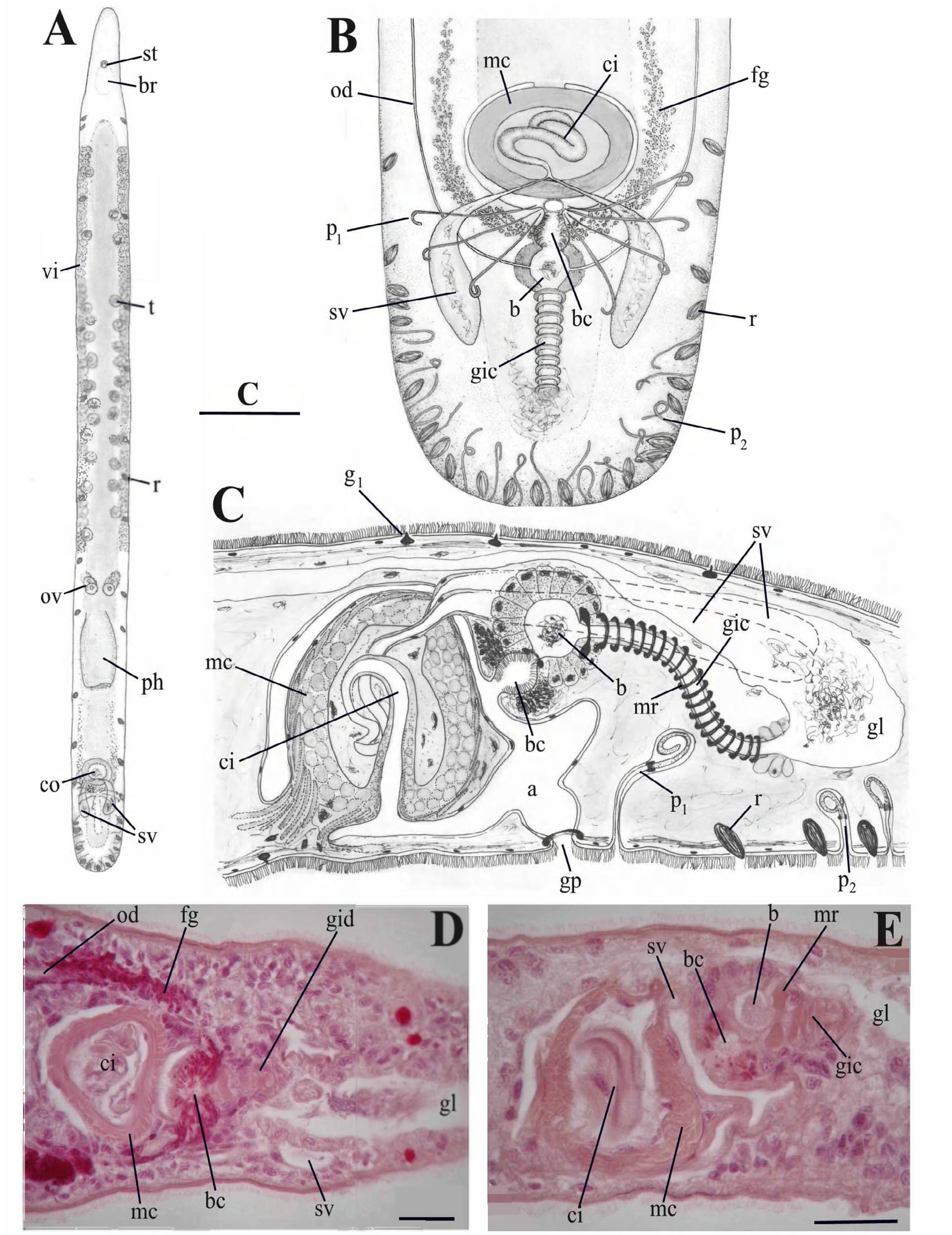

FIGURE 2. *Parainvenusta englarorum* **nov. sp. nov. gen.** A: general appearance; B: Postpharyngeal genital organs, based on observations on living squeezed specimens and frontal sections; C: sagittal reconstruction of the postpharyngeal genital organs; D,E: genital area from frontal (D) and sagittal (E) sections. Scale bar : 25 µm . Abbreviations: a: atrium; b: bursa; bc: bursal canal; br: brain; ci: cirrus; co: copulatory organ; fg: female glands; g_1: gland; gic: genito-intestinal connection; gl: gut lumen; gp: genital pore; mc: muscular lining of the cirrus bulb; mr: muscle ring; od: oviduct; ov: ovary; p_1: paracnida (genital area); p_2: paracnida (caudal area); ph: pharynx; r: rhabdoid gland; st: statocyst; sv: seminal vesicle; t: testis; vi: vitellaria.