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SANJUAN HERNANDEZ, Claudia de la Caridad; Curini-Galletti, Marco; MONNENS, Marlies; DIEZ GARCIA, Yander & ARTOIS, Tom (2025) Molecular reassessment of the phylogeny of Coelogynoporidae (Platyhelminthes, Proseriata), with the description of two new genera and three new species from Cuba and the Pacific coast of Panama. In: Marine Biodiversity, 55 (4) (Art N° 57).

DOI: 10.1007/s12526-025-01538-9
Handle: http://hdl.handle.net/1942/46656

Molecular reassessment of the phylogeny of Coelogynoporidae (Platyhelminthes, Proseriata), with the description of two new genera and three new species from Cuba and the Pacific coast of Panama

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Short title: Molecular phylogenetic reassessment of Coelogynoporidae

Abstract:

An integrative analysis of the intra-family phylogenetical relationships of Coelogynoporidae Karling, 1966 (Proseriata) was performed using morphological traits and concatenated 18S and 28S rDNA subunits. The molecular data set included 31 specimens of Coelogynoporidae and, as outgroups, two of Calviriidae Martens & Curini-Galletti, 1993, two of Otomesostomidae Midelburg, 1908, and two of Monocelididae Hofsten, 1907, 11 of which were newly sequenced for this research. The genus *Vannuccia* Marcus, 1948 was recovered as paraphyletic, with the species grouping into two distinct clades. These clades are morphologically diagnosable due to the presence or absence, respectively, of accessory stylets within the male copulatory bulb. The species without accessory stylets cluster in a more basal position within the family than those with a stylet. Those with stylets, including the type species *V. martae* Marcus, 1948 and the newly described *V. pacifica* **sp. nov.** and *V. santiaguera* **sp. nov.**, form a monophyletic sister clade to *Prosogynopora riseri* Laumer & Curini-Galletti, 2014.

Therefore, we restrict the genus *Vannuccia* to species with accessory stylets, and erect a new genus, *Anoplovannuccia* **gen. nov.**, for those without stylet, including the new species *A. berovidesi* **sp. nov.** The new species are distinguished from their congeners by the specific morphology of the sclerotised structures of the male copulatory bulb. The two known species of *Invenusta* Sopott-Ehlers, 1976 do not make up a monophyletic grouping in the tree, and the shared morphological features between them are thus mainly based on the absence of characters that may have been lost independently. Consequently, we transfer *I. paracnida* (Karling, 1966) Sopott-Ehlers, 1976 to the new genus *Pseudoinvenusta* **gen. nov.**, now *P. paracnida* **comb. nov.** The findings from our study could provide a foundation for addressing questions about the phylogenetic placement of various Coelogynoporidae species, such as the enigmatic position of *P. riegeri*.

Keywords: systematics, biodiversity, meiofauna, Apingospermata, flatworms

Introduction

The taxon Proseriata comprises small, free-living flatworms, most of which live interstitially in marine sediments (Curini-Galletti 2001). Of the 430 proseriate species described to date (Tyler et al. 2006–2024), most originate from a few well-researched regions, such as the North Sea and the Mediterranean (Schockaert et al. 1989; Curini-Galletti et al. 2020, 2023; Armonies 2023). In contrast, vast areas of the globe have received far less attention, resulting in a possible severe underestimation of the species richness of the taxon. Every new sampling campaign outside comparatively well-known areas has uncovered numerous new species (Willems et al. 2009; Martínez et al. 2019; Jörger et al. 2021; Curini-Galletti et al. 2023). Metabarcoding studies hold promise for addressing this Linnean shortfall (Brito, 2010); however, the number of proseriate sequences in GenBank is exceedingly small compared to the number of environmental OTUs identified by molecular studies (Martínez et al. 2019), resulting in many unassigned sequences.

The phylogenetic system of Proseriata has been the object of numerous studies, and intra-order relationships appear to be assessed (Curini-Galletti et al. 2010; Laumer et al. 2014; Scarpa et al. 2017). On the contrary, intra-family relationships are still far from assessment (Scarpa et al. 2017). In particular, discrepancies are often apparent among morphology-based systems and molecular data (see, e.g. Faubel & Rohde 1998 vs *Monocelis*, and Ax 2008 vs *Otoplana*). Coelogynoporidae Karling, 1966 is one of the families whose in-group relationships are poorly known (Curini-Galletti et al. 2010, Scarpa et al. 2017). Regarding the analysis of Coelogynoporidae, Curini-Galletti et al. (2010) found that the internal support for the monophyly of this taxon is weak, indicating the need for future research with broader sampling. Recently, Scarpa et al. (2017) included sequences from 15 specimens of Coelogynoporidae in the first comprehensive molecular study of Proseriata at the family level. The

phylogenetic trees obtained in that study revealed inconsistencies between the molecular phylogeny and the current taxonomy of the group. For example, the monophyly of the genus *Vannuccia* Marcus, 1948 appears unsupported and, similarly, a specimen of *Coelogynopora* groups with representatives of *Invenusta* Sopott-Ehlers, 1976. Unfortunately, Coelogynoporidae is underrepresented in molecular phylogenetic studies relative to its species richness, resulting in tentative findings.

In this contribution, we take the opportunity to describe three new species that, based on morphological characters, can be assigned to the genus *Vannuccia*, two from Cuba and one from Panama's Pacific coast. Two new species are sequenced (18S and 28S rDNA subunits) and used, together with newly obtained sequences and those available in GenBank, in the most comprehensive molecular phylogenetic analysis of Proseriata.

Material and Methods

Collecting and morphological study

Proseriates were collected in various localities of Cuba and the west coast of Central and Northern America between 2016 and 2023 (Table 1). Live specimens were extracted from the sediment using the $MgCl_2$ decantation method (Schockaert 1996) and studied alive and whole-mounted. Salinity was measured using a handheld refractometer. (HRS 16 KURSS). For a detailed study of the hard structures of the male copulatory organ, a 7% acetic acid solution was added to some of the whole-mounted specimens. After approximately 3 minutes, the acetic acid was carefully removed, and a few drops of orcein acetic acid were added. After another 2 minutes, the coverslip was placed and firmly squeezed. After examination of these specimens, they were preserved using lactophenol. Specimens selected for molecular work were cut in half, and the body's posterior part containing the reproductive organs was fixed as described before. The anterior part of the body was preserved in ethanol (99 %) and stored at -20 ºC. The sclerotised structures were drawn with a camera lucida on a Leica DM 2500 LED microscope, using Nomarski interference contrast. The drawings were improved using the Inkscape 1.1.2 software.

Identification of the specimens was based on the diagnostic features referenced in existing literature (Marcus 1948; Tajika 1977; Ax and Sopott-Ehlers 1979; Ehlers and Ehlers 1980; Curini-Galletti 2014). Measurements were taken along the central axis of the structures. The position of structures is expressed in percentages of the total body length (distance from the anterior tip of the body). The holotypes are deposited in the Museum of Nature Hamburg –Zoology (ZMH). The remaining materials, including paratypes, are deposited in the reference collection of Hasselt University (HU).

90 **DNA extraction, PCR amplification and sequencing**

91 The total DNA was extracted using a salting-out protocol for low-input tissues (C. Laumer, pers.comm.). Each
92 specimen was treated with 195 µl TNES buffer and 5 µl proteinase K (Invitrogen) at 55 °C for ± 30 minutes.
93 Subsequently, 65 µl 5M NaCl and 290 µl 96 % EtOH were added to the lysate, using 1.5 µl yeast tRNA
94 (Invitrogen) as a coprecipitant, and the result was stored for at least 1h at -20 °C. Purification was performed
95 through two EtOH (70 %) wash steps. DNA was eluted overnight at 4 °C in 100 µl of 0.1X TE buffer with 0.02
96 % Tween™ 20 (Thermofisher).

97 The PCRs for 18S and 28S rDNA regions were carried out using the primers 18S: A (forward) GCG AAT GGC
98 TCATTA AAT CAG and B (reverse) CTTGTT ACG ACT TTT ACT TCC (Littlewood & Olson, 2001); and
99 28S: LSU5 (forward) TAG GTC GAC CCG CTG AAY TTA AGC A, and LSUD6-3 (reverse) GGA ACC CTT
100 CTC CAC TTC AGT C (Littlewood *et al.*, 2000). PCRs were carried out for a total volume of 25 µl using
101 PuReTaq Ready-To-Go PCR beads (GE Healthcare), which included 2.5 µl of each primer (2 µM), 1 µl of DNA,
102 and 19 µl of purified water. PCR reactions were carried out on a Bio-Rad PCR T-100 Touch thermal cycler
103 programmed as follows: 1 cycle of 2 min at 94 °C, 40 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min and 30
104 s at 72 °C, 5 min at 72 °C and a final cooling at 4 °C were carried out. The PCR products were verified on a 1.4
105 % agarose gel, stained with GelRed™, posteriorly, were commercially purified and sequenced by Macrogen
106 Europe B.V. (Amsterdam) under BigDye™ terminator cycling conditions on an ABI3730XL DNA Sequencer.

107 **Molecular phylogenetic analysis**

108 The molecular data set included 31 specimens of Coelogynoporidae and, as the outgroup, two of Calviriidae
109 Martens & Curini-Galletti, 1993, two of Otomesostomidae Midelburg, 1908, and two of Monocelididae Hofsten,
110 1907. Forty-nine sequences were mined from GenBank, and 22 were newly sequenced for this study. The
111 accession numbers of the analysed material are listed in Table 2. The new sequences were assembled in
112 Geneious v11.1.5 (Kearse et al. 2012). The consensus sequences were subsequently submitted to a BLAST
113 search (Altschul et al. 1990) on the NCBI website (ncbi.nlm.nih.gov) to check for signs of contamination. Partial
114 18S and 28S rDNA sequences were aligned separately using the online version of MAFFT v7 (Katoh & Standley
115 2013; Katoh et al. 2019), specifying the Q-INS-i algorithm. The resulting alignment was processed on the
116 Gblocks v0.91b server (Castresana 2000) to eliminate ambiguously aligned regions, selecting options for a less
117 stringent pruning. After alignment, 1,444-pb-long sequences for 18S and 1,494-pb-long for 28S were obtained.
118 The alignments were concatenated in Geneious, and the initial partition scheme defining gene boundaries was

constructed manually. The concatenated alignment and partition files were input into the ModelFinder tool on IQ-TREE webserver (Trifinopoulos et al. 2016) to calculate the best-fitting evolutionary models. The evolutionary model was evaluated using Akaike information criterion (AIC). The best-fitting model was TVMe+I+G4 for 18S and GTR+F+I+G4 for 28S.

The Maximum Likelihood (ML) analysis was conducted on the IQ-TREE server (Nguyen et al. 2015). Branch support was assessed by SH-aLRT branch tests (Guindon et al. 2010) and ultrafast bootstrapping (UFBoot) (Hoang et al. 2018), both with 1,000 replicates. Bayesian Inference (BI) was conducted on the CIPRES Science Gateway (Miller et al. 2010). The Metropolis-coupled Markov Chain Monte Carlo (MC^3) implemented in MrBayes v3.2.6 (Ronquist et al. 2012) was used. The best-fitting models were implemented as previously specified by ModelFinder. Two independent runs of 10,000,000 generations each were implemented, and sampling was carried out every 1,000. The initial 25 % of samples were discarded as burn-in, and the rest were combined to generate a 50 % majority rule consensus tree. Convergence was confirmed by the average standard deviation of split frequencies (ASDSF) values below 0.01 and the potential scale reduction factor (PSRF) close to 1. Posterior probabilities (pp) were used as support values. The phylogenetic trees obtained by both analyses (ML and BI) were visualised and rooted in FigTree v1.4.4 (Rambaut 2018). The clades with low support were collapsed when the results of the three performed tests fell below their respective thresholds (SH-aLRT < 80, UFboot < 95, pp < 0.95).

Abbreviations used in the figures

ao, accessory organ; **as**, accessory stylet; **b**, bursa; **br**, brain; **cb**, copulatory bulb, **cg**, common genital pore; **ci**, cirrus; **g**, gut; **ov**, ovary; **pa**, paracnida; **pb**, prostate body cell's; **ph**, pharynx; **pg**, prostate glands; **pv**, prostate vesicle; **sp**, spines; **st**, statocyst; **sv**, seminal vesicle; **t**, testis; b**c**, bursal canal; **vi**, vitellarium.

Results

Molecular phylogeny of Coelogynoporidae

The BI and ML methods produced identical topologies (Fig. 1). A tree before the nomenclatural actualisation is represented in Figure 1a, while in Figure 1b, the new taxonomy is used. This section follows Figure 1a to describe the molecular phylogeny results. Coelogynoporidae was recovered monophyletic and contains two main sister clades. The first clade (Clade 1 in Fig. 1a) contains *Parainvenusta englarorum* Curini-Galletti, 2010 + *Invenusta paracnida* (Karling, 1966) Tajika, 1981, and the second one encompasses the remaining family representatives (pp = 0.99; SH-aLRT = 92.3; UF-boot = 93) (Fig. 1a).

In the second main clade, *Vannuccia* appears polyphyletic. *Vannuccia campana* Ehlers & Ehlers, 1980 and *Vannuccia rotundouncinata* Ax & Sopott-Ehlers, 1979 (Clade 2 in Fig. 1a) are the sisters of the rest of Coelogynoporidae (pp = 0.99; SH-aLRT = 91.3; UF-boot = 79). *Coelogynopora* Steinböck, 1924 is polyphyletic. *C. axi* Sopott, 1972, *C. tenuis* Meixner, 1938, and *C. gynocotyla* Steinböck, 1924 (Clade 3 in Fig. 1a) form an unresolved trichotomy (pp = 1; SH-aLRT = 99.6; UF-boot = 100) and are the sisters of a major group that encompasses clade 4 and 5 representatives (pp = 0.78; SH-aLRT = 84.2; UF-boot = 45). Clades 4 and 5 are sisters (pp = 0.77; SH-aLRT = 88.1; UF-boot = 50) (Fig. 1a).

In clade 4 (Fig. 1a), *Cirrifera* spp. Sopott, 1972 and the grouping of *C. bidentata* + *I. aestus* and *C. gynocotyla* A and B are sisters (pp = 0.99; SH-aLRT = 88.6; UF-boot = 76). The genus *Cirrifera* is monophyletic and *C. sopottehlersae* Noldt & Jouk, 1988 is sister of *Cirrifera* (pp = 0.97; SH-aLRT = 84.5; UF-boot = 81). *C. dumosa* Sopott, 1972 and *C. cirrifera* Sopott, 1972 are sister species (pp = 1; SH-aLRT = 92.3; UF-boot = 95). Meanwhile, *C. bidentata* is the sister species to *Invenusta* and *C. gynocotyla* A and B (pp = 0.99; SH-aLRT = 96.2; UF-boot = 93).

In clade 5 (Fig. 1a) *Vannuccia* sp. "Australia" is the sister of *Prosogynopora riseri* Laumer & Curini-Galletti, 2014 + other *Vannuccia* species (pp = 1; SH-aLRT = 100; UF-boot = 99). *P. riseri* is well-supported as the sister of *V. martae* Marcus, 1948 (type species of *Vannuccia*) + two new species (pp = 1; SH-aLRT = 99.1; UF-boot = 100). The relationships between *V. martae* and *Vannuccia* **sp. nov. 2** and *Vannuccia* **sp. nov. 1** are unresolved (Fig. 1B).

Furthermore, the results exposed the existence of rampant polyphyletism in some former genera such as *Invenusta*, *Vannuccia*, and *Coelogynopora* (Fig. 1a). Of particular interest for the descriptions of the new species, at first all identified as species of *Vannuccia* based on morphology, is the polyphyly of the genus, which made it necessary to introduce a new genus in the following taxonomic section (see also below for its justification), and to re-define the genus *Vannuccia* accordingly.

Taxonomy

Proseriata Meixner, 1938

Lithophora Steinbock, 1925

Apingospermata Laumer, Giribet, Curini-Galletti, 2014

Coelogynoporidae Karling, 1966

176 ***Vannuccia* Marcus, 1948**

177 **Emended diagnosis (after Curini-Galletti, 2014).** Species of Coelogynoporidae with duplex-type copulatory
178 organs, including at least part of the proximally-placed seminal and prostate vesicles. A spiny cirrus and
179 accessory organ(s) provided distally with stylet(s) and connected basally to the copulatory organ is present. The
180 cirrus opens posteriorly into the common genital atrium, provided with a common gonopore. The bursa is
181 connected to a long bursal canal, opening anteriorly into the common atrium.

182

183 **Type species.** *Vannuccia martae* Marcus, 1948, by original designation.

184 **Other species.** *Vannuccia glandulifera* (Faubel & Rohde, 1998) Curini-Galletti, 2014, *V. hastata* Ax & Ax,
185 1974, *V. pacifica* **sp. nov.**, *V. santiaguera* **sp. nov.**, *V. tripapillosa* Tajika, 1977, and *V. umbilica* Sopott, 1972.

186 ***Vannuccia pacifica* sp. nov.**

187 (Figs. 2b, e, f; 3a-d)

188 http://zoobank.org/urn:lsid:zoobank.org:act:xxx

189 **Material. Panama:** Observations on live animals. Two whole mounts from the intertidal zone, in the coarse
190 sand of Isla Taboga (February 25, 2016), Restinga Beach (Type locality), one of which is designed as a holotype
191 (ZMH VXXX PN-101) and the other one cut in half; the posterior part with the reproductive organs whole
192 mounted designed paratype, anterior part and used for molecular analysis (HU No. XXX PN-102). One whole
193 mount from coarse sand of the intertidal zone of Achotines Bay, designated as reference material and used in
194 molecular analysis (HU XXX Voucher A).

195 **Etymology.** The species is named after the region where it was found, the Panama's Pacific coast.

196 **Diagnosis.** Species of *Vannuccia* whose cirrus presents a distal cluster of 6–8 large spines, 19–32 µm long,
197 hook-shaped and with flap-like apophyses and 6–7 smaller spines, 6–10 µm long. A cluster of 10–15 proximal
198 spines, 4–6 µm long, is present, separated from the previous spines by an unarmed area. The accessory stylet is
199 40–48 µm long, with a poorly sclerotised, paddle-like basis and a straight, narrow-tubular distal part, with a
200 sharply pointed distal opening.

201 **Description.** Live specimens up to 5 mm long (Fig. 2b). The epidermis is entirely ciliated, with a few sensory
202 bristles in the anterior margin. Posterior end with few adhesive glands. Numerous paracnida are scattered along

the body, appearing as shiny rods in live specimens, and small rhabdoid glands are also present. The gut (Fig. 2b: g) extends above the encapsulated brain (Fig. 2b: br) and the statocyst (Fig. 2b: st) into a narrow, barely noticeable precerebral gut. A short and collar-shaped pharynx (Fig. 2b: ph) is located in the posterior third of the body.

Male genital system. With 15–30 testes (Fig. 2b: t) arranged into a single row anterior to the pharynx. The copulatory organ (Fig. 2e) is provided with a thick outer muscular lining. It contains a single seminal vesicle (Fig. 2 b, e; 3a: sv), a long prostate vesicle (Fig. 2b, e: pv), an eversible spiny cirrus (Fig. 2b, e: ci), and an accessory organ armed with a stylet (Fig. 2b, e: as).

The cirrus presents a cluster of 6–8 larger spines distally (Fig 2e, f, 3a, d: sp 3), measuring 19–32 µm long ($\bar{x}$ = 26 µm; n = 22). The distal part of the spines is hook-shaped, with a proportionally very long and recurved distal tip ending into an obtuse point. The spines are provided with a submedian, flap-like apophyse. The apophysis is 4–6 µm long ($\bar{x}$ = 5 µm; n = 14) and 6–9 µm wide ($\bar{x}$ = 8 µm; n = 14). The area below the apophysis is 5–7 µm wide ($\bar{x}$ = 6 µm; n = 7) and appears poorly sclerotised and, in some cases, hardly noticeable. *In vivo*, these spines appear neatly arranged in three rows, with the two largest placed most distally. This regular arrangement is lost in squeezed whole mounts. Anterior to the previously mentioned cluster of spines, 6–7 smaller spines (Fig 2e, f: sp 2) are present, 6–10 µm long ($\bar{x}$ = 8 µm; n = 11) and 1–2 µm wide at their bases. Each spine has a large, flap-like submedian apophysis and a slightly recurved, sharp distal tip. The apophysis is flap-like, 2–3 µm long ($\bar{x}$ = 2 µm; n = 7) and 2 µm wide ($\bar{x}$ = 2 µm; n = 4). A cluster of 10–15 spines (Fig. 2e, f; 3b: sp 1) is present in the most proximal part of the cirrus, separated from the previous spines by a 40–50 µm long, unarmed section. These thin and slender spines are 4–6 µm long ($\bar{x}$ = 5 µm; n = 14), slightly curved with an acute distal tip, and 0.7–0.9 µm wide at their base, below the apophysis ($\bar{x}$ = 0.8 µm; n = 6). The apophysis is flap-like, 1 µm long ($\bar{x}$ = 1 µm; n = 5), and 0.6–0.8 µm wide ($\bar{x}$ = 0.7 µm; n = 5). These spines are barely noticeable in living specimens and only observable in squeezed whole mounts.

The accessory stylet (Fig. 2e, f; 3c: as) is connected to an accessory prostate gland. The stylet is 40–48 µm long ($\bar{x}$ = 43 µm; n = 3). Its paddle-like basis is poorly sclerotised and 11–14 µm wide ($\bar{x}$ = 13 µm; n = 3). The distal part is straight, narrow-tubular, measuring about half the length of the whole stylet, with a sharply pointed distal opening. The cirrus opens into the common genital atrium, opening to the outside through the common genital pore (Fig. 2b, e: cg).

Female genital system. The vitellaria (Fig. 2b: vi) are located on both sides of the body and extend posterior to the pharynx. The ovaries (Fig. 2b: ov) are positioned anterior to the pharynx. A small, round bursa (Fig. 2b: b) is present caudally, lined by a high epithelium; it is connected to a long, strongly muscular bursal canal (Fig. 2b, 3a: bc), opening anteriorly into the common atrium.

***Vannuccia santiaguera* sp. nov.**

(Fig. 2a, c, d; Fig. 3 e, f)

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Vannuccia sp. 1 in Diez et al. (2023)

Material. Cuba: Observations on live animals. Seven specimens from Sardinero (Type locality), Santiago de Cuba (May 11, 2020), two used in the molecular analysis (DJ-35, DJ-33) and five whole mounted, one of which is designated holotype (ZMH VXXX J-31) and four paratypes (HU No. XXX–XXX J-29, J-32, J-34, H-44); collected in the sublittoral, 0.5 m deep, from the *Thalassia testudinum* K. D. Koenig grassland slope, fine-grained sand with abundant organic matter (salinity: 35 ‰). One whole mount from Cayo Damas, Guamá, Santiago de Cuba (October 21, 2020) (HU XXX J-55); collected in the intertidal (salinity: 34 ‰). One whole mount from Siboney (HU XXX P-15), Santiago de Cuba (June 5, 2017); from the intertidal muddy-sandy bottom, fine-grained sand with abundant organic matter (salinity: 35 ‰).

Etymology. The species is named after the region where it was found, Santiago de Cuba.

Diagnosis. Species of *Vannuccia* whose cirrus is provided with about 30 spines arranged into three longitudinal rows. The spines are hook-shaped, 10–13 µm long, slightly larger and straighter towards the distalmost part, with a rounded apophysis. The accessory stylet is 34-36 µm long, with an ovoid, paddle-like basis and a long tubular distal part.

Description. Live specimens measure about 5 mm long (Fig. 2a, 3e). The statocyst (Fig. 2a; 3e: st) is 200 µm far from the anterior margin of the body. The brain (Figs. 2a; 3e: br) is encapsulated and situated just behind the statocyst. Rod-shaped paracnida (Figs. 3e: pa) are present throughout the body. The collar-shaped pharynx (Figs. 2a; 3e: ph) is located in the posterior third of the body. The intestine (Fig. 2a: g) extends along most of the length of the body; a pre-cerebral intestinal diverticulum was observed above the statocyst.

Male genital system. With about 20 testes (Figs. 2a; 3e: t), arranged in a row and extending over the medium body line anterior to the pharynx. The male duplex-type copulatory bulb (Fig. 3e: cb) is placed at 80 %. The seminal vesicle (Fig 2a, c: sv) is intrabulbar and lies proximally into the male copulatory bulb. It is connected to a prostate vesicle (Fig. 2a, c: pv) lined with glandular tissue, whose cell bodies are extrabulbar (Fig. 2a, c: pb). The muscular cirrus (192 µm long in the holotype) (Figs. 2a, c: ci; 3f) is provided with 25-30 spines (Figs. 2c, d: sp; 3f), arranged into three longitudinal rows. The spines are hook-shaped, 10–13 µm long ($\bar{x}$ = 12 µm; n = 48) and 2– 3 µm ($\bar{x}$ = 2 µm; n = 48) of maximum width at their bases below the rounded apophysis, 3–5 µm long ($\bar{x}$ = 4 µm; n = 12). The cirrus spines are similar in shape, slightly larger and straighter towards the distalmost part of the cirrus.

An accessory muscular organ, provided with a stylet connected to a glandular vesicle (Figs. 2c, d; 3f: as), is present. The stylet is proximally paddle-like and progressively becoming tubular distally and measures 34–36 µm in length ($\bar{x}$ = 35 µm; n = 5) and 13–17 µm at its widest, poorly sclerotised proximal part ($\bar{x}$ = 15 µm; n = 5). The distal section of the tube has a rounded opening and is 2 µm in diameter (($\bar{x}$ = 2 µm; n = 5). The male bulb opens proximally into the common genital atrium (Fig. 2c: cg).

Female genital system. The vitellaria (Figs. 2a; 3e: vi) extend along both body sides from behind the brain to the anterior part of the pharynx. The two ovaries (Figs. 2a; 3e: ov) are anterior to the pharynx. The female atrial organs open into the common gonopore, posterior to the male system. The bursa (Fig. 2a: b) is weakly muscular, located subcaudally in the body, and filled with sperm. It opens into the more muscular bursal canal (Fig. 2a: bc). Abundant gland cells open at the connection of the female duct with the common genital pore. The common gonopore opens at 90%.

***Anoplovannuccia* gen. nov.**

(Figs. 2g, h, 4e, f)

Etymology. The genus name is coined on *Vannuccia*, with the prefix anoplo- (from Greek: unarmed).

Diagnosis. Coelogynoporidae with the characters as *Vannuccia*, but without accessory organ(s) with stylet(s).

Type species. *Anoplovannuccia campana* (Ehlers & Ehlers, 1980) **comb. nov.**, here designated.

Other species. *Anoplovannuccia berovidesi* **sp. nov.**, *A. rotundouncinata* (Ax & Sopott-Ehlers, 1979) **comb. nov.**, *A. talea* (Marcus, 1954) **comb. nov.**, and *A. tripapillosa* (Tajika, 1977) **comb. nov.**

***Anoplovannuccia berovidesi* sp. nov.**

(Figs. 2g, h; 4a, b, c)

http://zoobank.org/urn:lsid:zoobank.org:act:xxx

Vannuccia sp. 2 in Diez et al. (2023)

Material. Cuba: Siboney (Type locality), Santiago de Cuba (March 30, 2022); sublittoral, 2 m deep, fine-grained, *Halimeda*-rich sand; salinity: 34 ‰. Observations on one live animal, later cut in half. The posterior part contains the reproductive organs whole mounted (holotype, ZMH VXXX), the anterior part stored in alcohol for molecular

Etymology. Species named after the late Prof. Dr. Vicente Berovides Álvarez (Havana University, Cuba). Prof. Berovides dedicated his life to the study and conservation of Cuban biodiversity. At the time of his death, he held the title of Emeritus Professor of Havana University, where he contributed to the education of hundreds of bachelor and postgraduate biologists.

Diagnosis. Species of *Anoplovannuccia*, whose cirrus is armed with 40 spines arranged in six longitudinal rows. The spines increase in size distally, from 4 µm to 7 µm. The smaller, proximal spines have curved, hook-like distal tips and large, rounded, flap-like apophyses. Distalmost, larger spines are straighter, featuring a small and curved distal tip with short apophyses.

Description. Live specimen about 5 mm long. The epidermis is entirely ciliated, with numerous paracnida (Fig. 4B: pa) and small rod-shaped rhabdoid glands. The brain is encapsulated and situated immediately behind the statocysts. The pharynx (Fig 4a, b: ph) is collar-shaped at 80 % of the body length.

Male genital system. With about 20 testes (Fig. 4a) anterior to the pharynx, extending along the body in a row in its middle line. The copulatory bulb (Fig. 4a, b: cb) is immediately behind the pharynx. The seminal (Fig. 2g: sv) and the prostate (Fig. 2g: pv) vesicles are intrabulbar. The cirrus (Fig. 2g; 4c: ci) is armed with 40 spines (Fig. 2g, h: sp; 4c), arranged in six longitudinal rows and increasing in size distally from the 4 µm to 7 µm long. The smaller, proximal spines have recurved, hook-like distal tips and large, rounded, flap-like apophyses, nearly orthogonal to the central axis of the stylet. The spines become progressively larger and straighter distally, and their apophyses end point downwards and gradually become more elongated and larger, to 3 µm. Distalmost spines are nearly straight, with a tiny, recurve distal tip. Their apophyses are short, roughly rectangular, and almost parallel to the spine's central axis (Fig. 2h). The cirrus opens into a common genital pore through the common genital atrium.

Female genital system. The vitellaria extend laterally along the body in two rows, from behind the brain and to in front of the pharynx. The ovaries (Fig. 4a: ov) are anterior to the pharynx and posterior to the testes. The bursa is present in the posterior end of the body and is connected to the common genital atrium through the bursal canal.

Discussion

Members of the family Coelogynoporidae are typical components of high-energy, mesopsammic communities in cold-water habitats of both hemispheres (Sopott 1972; Ax 2008; Curini-Galletti et al. 2010; Volonterio and Ponce De León 2021). Their presence elsewhere is limited: only a few species are known from the Mediterranean, where they were considered boreal relics (Sopott-Ehlers 1976; Jouk et al. 2019). Besides a questionable assignation to the genus *Carenscoilia* Sopott, 1972 of specimens found in the Galapagos Islands (Ax & Ax 1974), the genus *Vannuccia* is the only notable exception to the cold-water distribution of the family: its members are, in fact, widespread in temperate to tropical areas (Marcus 1948, 1954; Sopott 1972; Ax & Ax 1974; Tajika 1977; Ax & Sopott-Ehlers 1979; Ehlers & Ehlers 1980; Faubel & Rohde 1998; Curini-Galletti 2014, Jouk et al. 2019).

The genus *Vannuccia* was introduced by Marcus (1948) for a new species of Coelogynoporidae, *V. martae*, from the southeast coast of Brazil. The new species was considered distinct from all known members of the family at that time, included in the single genus *Coelogynopora* Steinbock, 1924, because of the presence of insunk epithelial nuclei and a single seminal vesicle situated anteriorly to the gonopore (Marcus 1948). Later Marcus (1954) added a second Brazilian species to the genus, *V. talea* Marcus, 1954: a much larger species with more numerous and differently shaped spines in its copulatory organ. Six additional species of the genus *Vannuccia* were later described from the Canary Islands (Ehlers & Ehlers 1980), the Galapagos (Ax & Ax 1974), eastern Australia (Faubel & Rohde 1998), Japan (Tajika 1977, 1981), the northwest coast of U.S.A. (Ax & Sopott-Ehlers 1979), and the North Sea (Sopott 1972). Members of *Vannuccia* are easily distinguishable from other coelogynoporids by the presence of a spiny, eversible cirrus: most other coelogynoporids possess a penis papilla provided with spines and/or stylet(s) (Ax & Sopott-Ehlers 1979). The only other coelogynoporid genera with a duplex-type, eversible cirrus are *Cirrifera* Sopott, 1972 and *Parainvenusta* Curini-Galletti, 2010. However, *Cirrifera* is characterised by one or two long, seminal vesicle(s) located posteriorly to the common gonopore and oriented forwards, an extra-bulbar prostate vesicle, and the absence of a bursa. In contrast, in members of *Vannuccia*, the seminal and prostate vesicles are intrabulbar and oriented backwards; the prostate vesicle is contained, at least partially, within the copulatory bulb, and a bursa is present. *Macroatrium setosum* Riser, 1981, the morphology of which is not known in detail, also appears to have a cirrus: its seminal vesicles

343 are posterior to the gonopore, and no prostate vesicle was described (Riser 1981). *Parinvenusta englarorum*
344 Curini-Galletti, 2010, from Tasmania, has a complex, convoluted cirrus, not provided with spines, and the
345 seminal vesicles are posterior to the copulatory bulb and lack a prostate vesicle (Curini-Galletti et al. 2010).
346 Faubel and Rhode (1998) introduced the genus *Stilivannuccia* Faubel & Rhode, 1998 for species previously
347 attributed to *Vannuccia* and characterised by the presence of accessory stylet(s) connected to thick muscular
348 fibres and capable of moving independently from the cirrus itself. The study of numerous specimens of *V.*
349 *martae* (type species of the genus *Vannuccia*) from southeast Brazil, however, revealed the presence of three of
350 such accessory stylets attached by long, mobile muscle bundles to the cirrus basis (Curini Galletti 2014). Marcus
351 (1948) did not describe these muscles; the stylets are mentioned in the original species description but assumed
352 to be part of the cirrus. The presence of accessory stylets in the type species of the genus *Vannuccia*
353 automatically rendered *Stilivannuccia* a junior synonym of *Vannuccia* (Curini Galletti 2014).
354 However, our phylogenetic reconstructions show that the presence/absence of accessory stylets indeed has
355 phylogenetic significance: species assigned to the genus *Vannuccia*, as re-defined by Curini-Galletti (2014),
356 appear polyphyletic and placed in different clades according to the presence/absence of accessory stylets. The
357 first clade includes species without accessory stylets (*Anoplovannuccia campana* **comb. nov**. and
358 *Anoplovannuccia rotundouncinata* **comb. nov**.) (Fig. 1b). The second clade is phylogenetically distant from the
359 first, grouping the type species *V. martae* with the newly introduced species *V. pacifica* **sp. nov.** and *V.*
360 *santiaguera* **sp. nov.** (Fig. 1b), all sharing the presence of accessory stylets. Even though morphological features
361 such as a duplex type copulatory bulb, spiny and eversible cirrus, the position of seminal and prostate vesicles,
362 and the presence of the bursa are shared by the members of the two clades, the presence of accessories stylets
363 appears to be a taxonomically decisive morphological character. These results raised the necessity of the erection
364 of a new genus to accommodate species that, unlike the type species of *Vannuccia*, *V. martae*, do not possess
365 accessory stylets – paradoxically, in an opposite process to that of Faubel and Rhode (1998). We introduced
366 *Anoplovannuccia* **gen. nov.** to accommodate these species and propose an official emendation of *Vannuccia* to
367 include the presence of accessory stylets in its diagnostic criteria.
368 It is worth noting that the clade including the accessory stylets-bearers *Vannuccia* species and *Prosogynopora*
369 *riegeri*, also includes "*Vannuccia*" sp. Australia (Fig. 1a) (Coelogynoporidae indet. B in Fig. 1b). This
370 undescribed species shares the presence of accessory stylets with its congeners but possesses a separate patch of
371 atrial spines surrounding the common genital pore (Curini-Galletti; unpublished results). The numerous
372 Australian members of *Vannuccia* s.l. will be the subject of a separate contribution (Curini-Galletti in prep).

Species justification

Both stylet-bearing species described here, *V. santiaguera* **sp. nov.** from Cuba and *V. pacifica* **sp. nov.** from Panama's Pacific coast, have a single accessory stylet. The morphology of the cirrus spines can distinguish these two species. In *V. santiaguera* **sp. nov.** the spines are uniform in shape and size. Conversely, in *V. pacifica* **sp. nov.**, the distalmost spines are notably larger, ranging between 19–32 µm. In addition, a distinct patch of small, proximal spines, separated from the larger distal spines by a spine-free tract of cirrus, is present in this species.

The three other species of *Vannuccia* (*V. martae* from Brazil, *V. umbilica* from the North Sea, and *V. glandulifera* from eastern Australia) all have two to three accessory stylets. The distalmost spines of the cirrus of *V. martae* are 21–30 µm long, distinctly larger than in the other species (18 µm long in *V. glandulifera* and 20 µm long in *V. umbilica*), and comparable to *V. pacifica* **sp. nov.**, but its cirrus is uniformly spiny, without any tract devoid of spines. The cirrus spines of *V. umbilica* have a characteristic shape (Sopott 1972), with a disk-shaped basis, and its accessory stylets are accompanied by 3–4 spines, absent in other species of the genus. The spines of *V. glandulifera* increase progressively in size distally, reaching a length of 18 µm, and are thus much smaller than the distal spines of *V. pacifica* **sp. nov.** In addition, the spination of its cirrus does not show any bare patch.

The only known species of *Vannuccia* with a single accessory stylet, and thus comparable to our new species, is *V. hastata*, from the Galapagos. It is distinct from *V. santiaguera* for the presence of marked differences in size among the cirrus spines, ranging from 32 µm to 4 µm, and for the presence of a bare patch devoid of spines. The shape of the stylet of *V. hastata* is most similar to that of *V. pacifica* **sp. nov.**, but it is much larger (85 µm vs. 40–48 µm, respectively). Similarly to the new species, *V. hastata* has a distal group of larger spines, separated from the proximal, smaller spines by a short tract devoid of spines. However, the distal group of spines of *V. hastata* includes five spines, whereas there are 6–8 large and 6–7 small spines in *P. pacifica* **sp. nov.** The proximal group of spines is similar in both species and consists of 9–11 small spines (5–8 µm long) in *V. hastata* and 10–15 (4–6 µm long) in *V. pacifica* **sp. nov.** The shape of the large spines differs between the two previously mentioned species. Notably, the apophyses are nearly basal in *V. hastata* but submedian in *V. pacifica* **sp. nov.** The presence of two separate groups of spines in both species from the Pacific Ocean are unique features among representatives of *Vannuccia* and suggest a close relationship between the two species.

Anoplovannuccia berovidesi **sp. nov.** from Cuba should be compared with the species now included in *Anoplovannuccia* **gen. nov.** The spiny area of the new species is much shorter, and the number of spines (40) is lower than that of the other species. The morphology of the spines is also unique: they are hook-shaped

proximally with a large, flap-like median apophysis, and they gradually become straighter distally, featuring a narrower and more basal apophysis (Fig 2h). *Anoplovannuccia campana* **comb. nov.** has six rows of claw-shaped spines, without apophysis, measuring 5–12 µm (Ehlers & Ehlers 1980) and *A. talea* **comb. nov.** has more than 100 spines of 11 µm long within the cirrus. Distally, the cirrus of *A. talea* **comb. nov.** is provided with a girdle of six, 44-µm-long spines. *Anoplovannuccia rotundouncinata* **comb. nov.** likewise has about 100 cirrus spines with a unique morphology: their bases are spherical, and the apophysis is lacking.

In *Anoplovannuccia tripapillosa* **comb. nov.** from Japan, the copulatory bulb has an intrabulbar seminal vesicle and a short main cirrus (120 µm long), provided with 1-µm-long spines. The main cirrus is connected at its basis to two muscular accessory cirri armed with numerous tiny spines similar to those of the prostate cirrus (Tajika 1977). Ax and Sopott-Ehlers (1979) described specimens from the west coast of the U.S.A. (Puget Sound) that differed from the Japanese specimens due to the presence of three accessory cirri instead of two, as *V. tripapillosa americana* Ax & Sopott-Ehlers, 1979.

The new species here introduced display large differences in the morphology of the hard structures within the male copulatory organ, enabling their distinction from each other and other species within the genus. The distribution, number, size, and shape of the cirrus spines show considerable variation among the presently known and newly proposed species. Additionally, the number of accessory stylets in species of *Vannuccia* ranges from one to three. The morphological features previously discussed validate the status of *V. pacifica* **sp. nov.**, *V. santiaguera* **sp. nov.**, and *Anoplovannuccia berovidesi* **sp. nov.** as new species.

Systematic implications and nomenclatural acts

The newly presented phylogenetic tree raises questions about the current systematics of the family Coelogynoporidae. Besides the genus *Vannuccia*, two other genera appear polyphyletic and need to be discussed:

Genus *Invenusta* Sopott-Ehlers, 1976

Two species of *Invenusta* are presently known: *I. aestus* (type species) from the eastern Atlantic (Canary Islands and south-west Atlantic coast of Europe) (Sopott-Ehlers 1976) and *I. paracnida* (Karling, 1966) Sopott-Ehlers, 1976 from the western U.S.A. (Karling 1966). The two species share several features, including the absence of sclerotised structures in the copulatory organ, the absence of a bursa, and the presence of unusually long paracnida, that were assumed to be synapomorphies for the two species by Sopott-Ehlers (1976). The absence of any sclerotised structures is indeed a rare character in Coelogynoporidae, only found in *Coelogynopora gynocotyla* (but see below), *Pseudovannuccia hirutai* Tajika, 1981, and *Parainvenusta englarorum* Curini-

433 Galletti, 2010, and, given at least the position of the former and the latter species in our trees (Fig. 1A), it is
434 likely to be the result of independent losses rather than synapomorphy. A bursa is lacking in representatives of
435 *Cirrifera*, *Carenscoilia*, and *Pseudovannuccia* Tajika, 1981 and may have been similarly lost independently.
436 Paracnida differ in morphology between species of *Invenusta*: the much shorter paracnida of *I. aestus* have the
437 eversible part provided with barbs, while they are granular basally and extremely long and convoluted in *I.*
438 *paracnida* (Sopott-Ehlers 1981, Curini-Galletti et al. 2010). Furthermore, the pharynx is short and vertically
439 oriented, and the copulatory organ is anteriorly oriented in *I. aestus*, whereas the pharynx is tubular and
440 horizontal, and the copulatory organ is vertical in *I. paracnida* (Karling 1966; Sopott-Ehlers 1981). The shared
441 characters of *Invenusta* species are thus mainly based on the absence of characters that may have happened
442 independently, and the monophyly of the genus has already been questioned on morphological grounds (Curini-
443 Galletti et al. 2010). One of the main morphological differences between the two species is the presence of a
444 characteristically long and winding genito-intestinal duct in *I. paracnida*, often filled with sperm (Karling 1966;
445 Curini-Galletti pers. obs.), that may act as a temporary bursa. No comparable structure is present in *I. aestus*. Our
446 molecular results further support the unrelatedness of the two species, as they are remotely placed in the trees.
447 Instead, *I. paracnida* is placed in the same clade as *Parainvenusta englarorum* (Fig. 1a). Both species share
448 similar, extremely long paracnida; the Tasmanian *P. englarorum*, however, has a duplex-type, unarmed cirrus
449 extremely long and convolute within the double-walled copulatory bulb; it also possesses a true copulatory bursa
450 (Curini-Galletti et al. 2010).
451 *Invenusta paracnida* thus needs to be removed from the genus *Invenusta*, and a new genus needs to be erected to
452 accommodate this species (see Fig. 1b). To this aim, we introduced a new genus-level taxon:
453 *Pseudoinvenusta* **gen. nov.**
454 **Etymology.** Genus coined on *Invenusta*, with the prefix pseudo- (from Greek: 'false')
455 **Diagnosis.** Coelogynoporidae with long, convoluted paracnida, simplex-type copulatory organ, unarmed penis
456 papilla, and short, ovoid seminal vesicles. Without a prostate vesicle. With a common genital atrium and a
457 single, common genital opening. Without a true bursa, but with a long genito-intestinal connection.
458 **Type species.** *Pseudoinvenusta paracnida* (Karling, 1966) **comb. nov.**, here designated.
459
460 **Genus *Coelogynopora* Steinböck, 1924**
461 The polyphyletism of this genus in our molecular tree (Fig. 1a) is exclusively due to specimens of
462 *Coelogynopora gynocotyla* (labelled A and B in Fig. 1a). While the North Atlantic specimen (labelled C in Fig.

1a) clusters with other *Coelogynopora* species, the two Mediterranean specimens attributed to the species cluster with *Invenusta aestus*. This suggests a possible misidentification of these specimens by Scarpa et al. (2017), who deposited these sequences. *Coelogynopora gynocotyla* is the only member of the genus without hard parts (Jouk et al. 2019), and a superficial attribution to this taxon of *Coelogynopora*-like specimens without stylets is thus possible. Mediterranean specimens are small, and the presence/absence of a bursa, the key character discriminating among members of *Coelogynopora* and *Invenusta*, may have been obscured by the long seminal vesicles of these specimens, and by the possible presence of a large genito-intestinal duct (Curini-Galletti pers. obs.). Therefore, a more thorough morphological study of the Mediterranean specimens should be undertaken to assess their status. For the moment, these should be reported as "Coelogynoporidae indet." (see Fig. 1b). This is not the only case of sequences wrongly assigned to members of Coelogynoporidae deposited in Genbank: the specimens sequenced by Littlewood et al. (2000) and identified as *Cirrifera dumosa* Sopott, 1972 turned out to belong to a member of the genus *Archiloa* de Beauchamp, 1910 (Monocelididae) (Curini-Galletti et al. 2010). Indeed, the cirrus of both species, though distantly related, present similar, numerous minute spines and may be confused in superficial observations on living, unsqueezed specimens (*cf.* Ax 1954; Sopott 1972).

The case of *Prosogynopora riegeri*

The species has a problematic phylogenetic position, as different strategies of alignment variously suggested a sister-group relationship with (or within) Coelogynoporidae, with Calviriidae, or with a clade composed of all other Lithophora (Laumer et al. 2014). In our analysis, based on a broader species dataset, *P. riegeri* nested within Coelogynoporidae into a clade consisting of *Vannuccia* species and "Coelogynoporidae indet. C Australia". Consistent with our findings, Scarpa et al. (2017) identified, but not discussed, a sister species relationship between *P. riegeri* and *Vannuccia martae*. Its position is nonetheless morphologically puzzling, as the only character shared with the accessory stylet-bearing member of the clade is the presence of a cirrus. *Prosogynopora riegeri* has numerous autapomorphies; indeed, the opening of the cirrus at the very caudal end of the animal and the female pore separate from the male pore and opening immediately behind the pharynx are unique features not only for Coelogynoporidae but even for all proseriates (Laumer et al. 2014).

Conclusions

Our findings reveal the non-monophyly of several established genera, underscoring the necessity for a rigorous review of the current system of Coelogynoporidae. This paper, introducing the genera *Anoplovannuccia* **gen.**

nov. and *Pseudoinvenusta* **gen. nov.**, contributed to resolving discrepancies between molecular and morphological systematics. Furthermore, our molecular phylogenetic analyses showed that accessory stylet is a key morphological trait with significant phylogenetic implications within the family and offered new insights into the systematic position of *Prosogynopora riegeri*.

However, our study represents the initial step toward developing a natural and comprehensive systematics of the Coelogynoporidae. The limited number of species sequenced thus far (18 out of 67 described species, the majority of which belong to the genus *Coelogynopora*) and the absence of representatives from the genera *Macroatrium* Riser, 1981, *Pseudovannuccia* (Tajika, 1981) Faubel & Rohde, 1998, and *Ezona* Tajika, 1980 in our dataset highlights the need for further research. Continued efforts in sequencing and analysing additional species will be essential to achieving a more complete understanding of the evolutionary relationships within this family.

Acknowledgements

Mrs. Ria Vanderspikken is thanked for her invaluable administrative support in the lab and her help in organising the collecting campaigns. The research that led to the results presented in this publication was carried out with infrastructure funded by EMBRC Belgium - FWO project GOH3817N.

Statements:

Funding:

This research was partially financed by the project 'VLIR P1. Valorisation of environmental scientific services for climate stress mitigation in eastern of Cuba' (Universidad de Oriente, Cuba—VLIR, Belgium). CS is supported by BOF-Hasselt University under grant BOF21BL07. YLD is supported by a Georg Forster Research Fellowship (Alexander von Humboldt Foundation, Germany, grant number 3.2 - CUB - 1226121 - GF-P). MCG received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, "Dalla ricerca all'impresa" INVESTIMENTO 1.4 – D.D. 1034 17/06/2022, CN00000033)

Conflict of interest:

- The authors have no financial or non-financial conflicts of interest to declare that are relevant to the content of the manuscript.

Ethical approval:

- No animal testing was performed during this study.

Sampling and field studies:

- No specific permissions for field sampling were necessary because these animals are unregulated. The study is compliant with CBD and Nagoya protocols.

Data availability:

Author Contribution Statement:

- Author Contribution: Conceptualisation and Methodology, CS, MC-G, MM, YLD and TA; Field collecting and Formal Analysis, CS and YLD; Resources and Funding Acquisition, YLD and TA; Original Draft Preparation, Review and Editing, CS, MC-G, MM, YLD and TA. All the authors read and approved the manuscript.

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602 **Table 1.** Sampled localities.

603 **Table 2.** Accession numbers of sequences refer to GenBank codes (* new sequences).

604 **Figure 1.** Majority-rule consensus tree from the Bayesian Inference (BI) and Maximum Likelihood (ML) of the
605 concatenated 18S + 28S rDNA dataset of Proseriate. Without nomenclatural changes (**a**), after introducing
606 taxonomic changes (**b**). Branches with support values below the thresholds in the legend of the three analyses
607 were collapsed. Symbols above branches represent posterior probabilities (⋆⋆⋆ 0.98 ≤ pp < 100; ⋆⋆: 0.95 ≤ pp <
608 0.98; ⋆: pp < 0.95). Symbols below branches indicate SH-aLRT (●●●: 85 ≤ SH-aLRT < 100; ●●: 80 ≤ SH-
609 aLRT < 85; ●: SH-aLRT < 80) and ultrafast bootstrap values (■■■: 95 ≤ UFboot < 100; ■■: 90 ≤ UFboot < 95;
610 ■: UFboot < 90) from the maximum likelihood analysis. Branches without symbols have pp = 1, SH-aLRT =
611 100, and UFboot = 100. Taxa from which new sequences were obtained for this study are highlighted in bold.

612 **Figure 2.** Body organisation (live animal) of *V. santiaguera* **sp. nov.** (**a**) and *V. pacifica* **sp. nov.** (**b**). Male
613 copulatory bulb of *V. santiaguera* **sp. nov.** (**c**), *V. pacifica* **sp. nov.** (**e**), and *A. berovidesi* **sp. nov.** (**g**).
614 Sclerotised structures of the male copulatory bulb of *V. santiaguera* **sp. nov.** (**d**), *V. santiaguera* **sp. nov.** (**f**), *V.*
615 *berovidesi* **sp. nov.** (**h**). All drawings represent the morphology of the holotypes of each species.

616 **Figure 3.** *V. pacifica* **sp. nov.** atrial organs, live animal (**a**); cirrus spines (**b**, **d**); male copulatory bulb of the
617 holotype (**c**). *V. santiaguera* **sp. nov.** body organisation (**e**), male copulatory bulb (**f**).

618 **Figure 4.** *Anoplovannuccia berovidesi* **sp. nov.** body organisation (**a**, **b**); cirrus's spines (**c**) of holotype.