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Integrative delimitation of two new, meiobenthic species of flatworms from Panama, constituting two new genera of marine triclads (Platyhelminthes, Tricladida, Maricola)

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Abstract

Although platyhelminths are common members of marine interstitial communities, the number of meiofaunal marine triclads known at present is rather limited. Here, we describe two new monotypic genera of interstitial marine triclads from Panama. In order to shed light on their phylogenetic and taxonomic position, we have followed an integrative approach that includes not only comparative analysis of their morphology but molecular phylogenetic placement with small and large rDNA sequences. Our phylogenies were broadly congruent with recently published molecular phylogenies of the marine triclads. The two new genera appeared not to be closely related, but, nevertheless, do share several morphological features, such as the presence of (1) a secondary gonopore, (2) a glandular region traversed by many septa in a large portion of the penis, (3) a copulatory bursa connected with the secondary gonopore, and (4) two retinal cells per eyecup. However, they do also differ considerably from each other. The following combination of factors made it impossible to assign the two new genera to any of the higher categories within the suborder of the marine triclads: (a) a unique combination of features present in each of the genera that does not fit into any of the current family diagnoses, (b) their present position in the phylogenetic tree, (c) the mismatch between the topologies of the molecular phylogenetic trees and the current taxonomic classification within the suborder. Therefore, the two taxa are classified as genera *incertae sedis*, pending a broader revision of the taxonomy of Maricola.

Key words: marine planarians, Central America, interstitial, integrative taxonomy, phylogeny

Introduction

Since Sluys & Kawakatsu (2005, fig. 31) published their global species richness map of the marine triclads, our knowledge of the pattern of biodiversity of this group of worms has been increased by the description of several new species: two from the Mediterranean Sea (Vila-Farré *et al*. 2010; Delogu & Galletti 2011), seven from China (Yu *et al*. 2013; He *et al*. 2016; Chen *et al*. 2019; Li *et al*. 2019; Li *et al*. 2021), one from the subantarctic region (Volonterio & Brewin 2014), one from northeastern South America (Souza *et al*. 2018), and one, unidentified species from the abyssal slope of the Kuril-Kamchatka Trench in the northwestern Pacific (Kakui & Tsuyuki 2024). Nevertheless, great gaps remain in our knowledge of maricolan biodiversity in northern South America and Central America. Therefore, it was fortunate that one of us (CEL) encountered very small marine triclads while sifting through the sediments of the Wild Cane Reef area in Panama as part of the STRI-Bocas del Toro Meiofauna Taxonomy workshop.

The first interstitial species of marine triclads were described by Holmquist & Karling (1972), viz., *Oregoniplana opisthopora* Holmquist & Karling, 1972 and *Pacifides psammophilus* Holmquist & Karling, 1972, from Oregon and California, respectively. Other, very small interstitial maricolans are *Procerodes* spec. from Tagus River, Portugal (cf. Sluys 1989) and *Miroplana paulula* Sluys, 2005 from brackish water sediments, Coomera River, Queensland,

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Australia (cf. Sluys & Kawakatsu 2005), each being 1.5 mm long in living condition. Thus, the number of meiofaunal marine triclads known at present is rather limited. In this paper, we describe two new species of interstitial marine triclads that turned out to be structurally different from all other maricolans known to date. Although this was evident, the precise assignment of the species in the higher-level taxonomy of the Maricola Hallez, 1892 is complex, while the very small size of the animals prevented the correct observation of certain morphological characters. Moreover, the relationship between the two species is complicated by the presence of several common anatomical structures and by several morphological differences. In order to shed light on their phylogenetic and taxonomic position, we have followed an integrative approach that includes not only the analysis and comparison of their morphology but also a rDNA-based molecular phylogenetic approach.

Materials and methods

Coarse sands and reef rubble were collected manually by snorkel and scientific SCUBA divers as part of the 2010 STRI meiofauna taxonomy training workshop in Bocas del Toro. Sediments were stored in buckets in seawater tanks for several days to concentrate interstitial fauna in the surface layers. Meiofauna were anaesthetized with an isotonic $MgCl_2$ solution and then extracted from sediments through agitation and decantation onto a 63 µm filter.

Animals were extracted from the community in a living state, and the morphology of a few exemplar specimens studied under DIC microscopy using wet whole mount slides, i.e., semi-squashed live whole mounts, applying gentle cover slip pressure to flatten and immobilize animals. Specimens vouchered in this way were rescued from slides and preserved in RNAlater (Ambion) for later nucleic acid extraction. Specimens designated for morphological study were fixed in hot Bouin's fluid adjusted to 9% (w/v) sucrose (Salvenmoser *et al*. 2010). After one day of room temperature fixation, specimens were transferred to 70% ethanol, changing the ethanol several times to leach out residual picric acid, and stored at 4 ^{o}C until histological processing. Whole mounts were prepared using a standard lactophenol fixation and clearing. Histology specimens were embedded in Paraplast (Leica Biosystems) and sectioned at 4 µm intervals, pretreated with 6% $HgCl_2$ and Lugol's iodine, stained in Mallory trichrome, preceded by Mayer's hematoxylin (Presnell & Schreibman 1997), and, subsequently, mounted in Fisher's Permount mounting medium (Fisher Scientific). Glass slides with the histological sections of the material are deposited in the collections of Naturalis Biodiversity Center, Leiden, The Netherlands (RMNH).

Specimens designated for molecular study were extracted, PCR-amplified, and Sanger-sequenced to yield nearly full length 18S and 28S rRNA sequences, following Laumer & Giribet (2014). Sequences for these genes from other Maricola and relevant outgroups were retrieved from NCBI, whose accession numbers are given in Table S1. Sequences were aligned using MAFFT (e-ins-i algorithm) (Katoh & Standley 2013), and concatenated using catsequences (https://zenodo.org/badge/latestdoi/79135872). A maximum likelihood phylogeny was inferred on the supermatrix (length 5,470 bp, of which the first 1900 bp were 18S) using IQ-tree2 v2.2.0.3 (Nguyen *et al*. 2015; Kalyaanamoorthy *et al*. 2017; Hoang *et al*. 2018), executed using options `-T 8 -m MFP+MERGE -B 1000 -p Maricola.partition`, which settled on a single merged partition fit using the GTR+F+I+I+R3 model as optimum. A Bayesian mixture model phylogeny was inferred using the CAT+GTR+G4 model (-cat -gtr) in PhyloBayes-MPI (Lartillot *et al*. 2013), with four chains run between 5355–5377 generations. A posterior consensus tree was generated from two chains with 'bpcomp -x 1000 10', yielding a maxdiff of 0.0394383, indicative of convergence.

Abbreviations used in the figures. b, band; bc, bursal canal; bd, balloon-shaped duct; br, brain; cb, copulatory bursa; cm, circular muscles; cn, canal; cv, cavity; cyg, cyanophil glands; dep, dorsal epidermis; e, eye; ec, eyecup; ed, ejaculatory duct; gd, gonoduct; gid, genito-intestinal duct; gp, gonopore; in, intestine; le, lens; lm, longitudinal muscles; ma, male atrium; mo, mouth opening; ncl, nucleus; od, oviduct; ov, ovary; pb, penis bulb; pg, penial glands; ph, pharynx; pp, penis papilla; sep, septa; sg, shell glands; sgp, secondary gonopore; sp, spermiducal vesicle; spi, spine; sv, seminal vesicle; te, testis; vd, vas deferens; vnc, ventral nerve cord.

Results

Systematic section

Order TRICLADIDA Lang, 1884

Suborder MARICOLA Hallez, 1892

Genus *Minutaplana* Sluys & Vila-Farré, gen. nov. *incertae sedis*

Type species. *Minutapla dimaculosa* Vila-Farré & Laumer, **sp. nov.**

Etymology. The generic name is based on the Latin adjectives *minutus*, small, and *planus*, flat, and alludes to the fact that these flatworms are very small in size. Gender: feminine.

Diagnosis. Marine triclads with: (1) septa in the penis papilla, channelling secretion towards the proximal end of the ejaculatory duct; (2) a secondary gonopore located posteriorly to the primary gonopore; (3) a copulatory bursa that is connected with the secondary gonopore and is located posterior to the male copulatory apparatus and communicates with the exterior through a canal that arises from its posterior wall and opens through the dorsal epidermis near the caudal end of the body; (4) two retinal cells per eyecup; (5) a distinct lens to each eyecup; and (6) numerous nuclei surrounding the ejaculatory duct and the wall of the penis papilla.

Minutaplana dimaculosa Vila-Farré & Laumer, sp. nov.

Material examined. Holotype: RMNH.VER.22264.1, Wild Cane Key/Reef at Bastimentos Island, Bocas del Toro Province, Panamá, 14–18 June 2010, coll. Christopher Laumer, Alejandro Martínez García, Barbara Eder, Sofia Pyataeva, sagittal sections on one slide.

Paratypes: RMNH.VER.22264.2, ibid., sagittal sections on 2 slides; RMNH.VER.22264.3, ibid., sagittal sections on 2 slides; RMNH.VER.22264.4, ibid., horizontal sections on one slide; RMNH.VER.22264.5, ibid., horizontal sections on one slide.

Etymology. The specific epithet is derived from the Greek *dis*, twice, and the Latin adjective *maculosus*, spotted, and alludes to the two pigmented spots on the head of the animal.

Ecology and distribution. The species is known only from the type locality (9.350650° N, 82.172417° W) at the Wild Cane Key in the Caribbean Sea, in the interstitial sediments of a coarse sand area at a depth of 3 m.

Diagnosis. As for the genus.

Description. In elongated state, living sexually mature specimens up to 1.7 mm in length and 0.5 mm in width (Fig. 1). The preserved holotype specimen was 1.3 mm long, as determined from histological sections. Body broadly oval-shaped, with rounded hind end and with the greatest width of the body at about the posterior third of the animal. Anterior to the eyes the body shows a narrowing, after which the body margins slightly diverge to form a triangular-shaped front end with a small, rounded protuberance at its mid-point. The two eyes are far removed from the frontal margin and lie close together at a distance of 1/5-1/6th of the width of the head. Dorsal and ventral body surfaces are hyaline, except for two brown spots at the level of the narrowing of the head. Histologically, the pigment granules of these spots resemble those of the eyecup, albeit being more irregular in shape.

The eyes (eyecup diameter 18–21 µm in histological sections) contain two retinal cells, and are placed dorsally, just below the body wall musculature and above the brain. The eye has an oval lens (10–14 µm diameter in sections) situated in the opening of the pigment cup (Fig. 2A, B). The so-called “Substanzinseln” are present in the brain (cf. Sluys 1989).

In front of the brain of the holotype there is a cavity (maximum diameter 138 µm) lined with a brown structure that contains a brown glandular secretion, probably originating from the parenchyma dorsally to the cavity (Fig. 2C). The same cavity is also present in other specimens, e.g. RMNH.VER.22264.4 and RMNH.VER.22264.3, either in front or behind the brain.

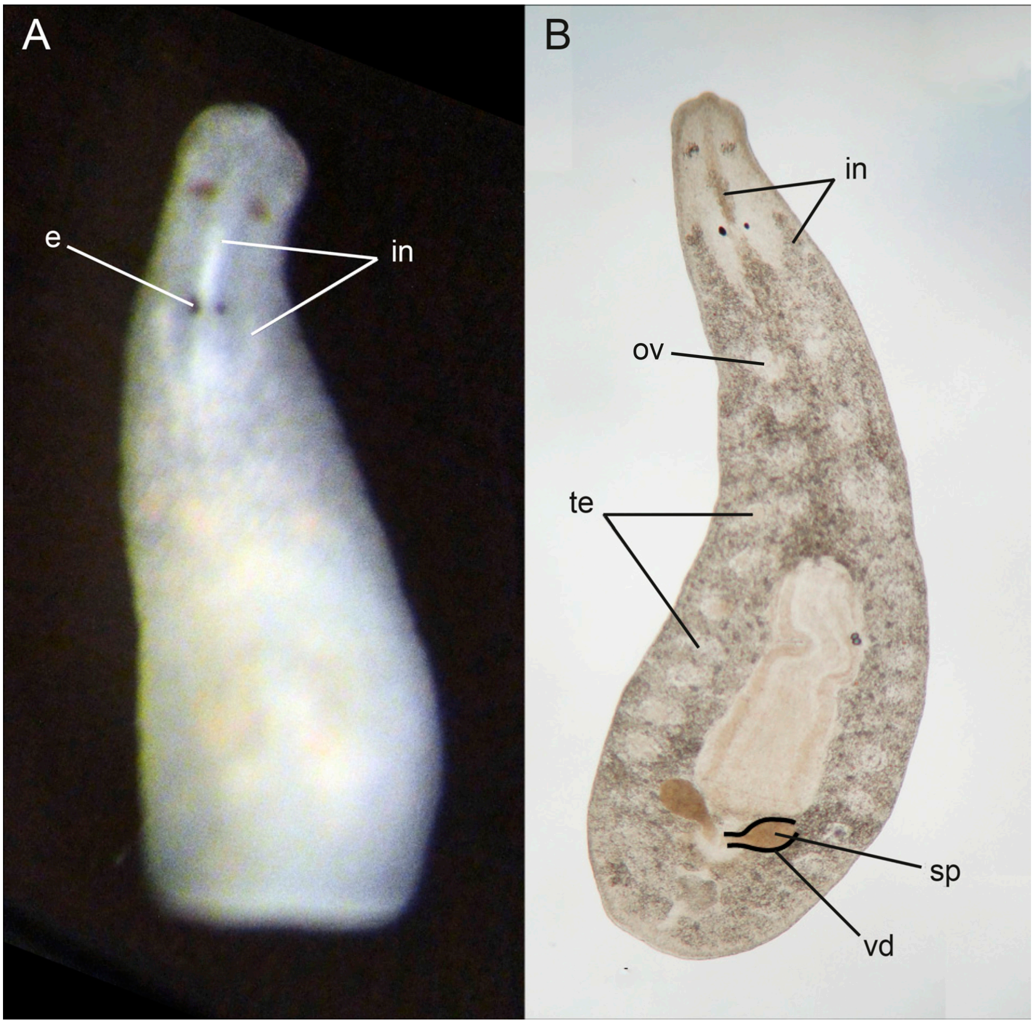

FIGURE 1. *Minutaplana dimaculosa*. (A, B). Photographs of live specimens; anterior to the top. Scale bars not available.

The anterior branch of the intestine extends anterior to the eyes and reaches almost the level of the pigment spots. The cylindrical pharynx lies approximately in the middle of the body and measures between 1/3 and 1/2 of the body length. The outer epithelium of the pharynx is ciliated, except at its proximal section (first third of the pharynx not ciliated in specimens RMNH.VER.22264.3 and RMNH.VER.22264.4), and is underlain by a layer of longitudinal muscles, followed by a layer of circular muscles. Very thick outer layer of circular muscles present underneath the inner pharynx epithelium, followed by an inner layer of longitudinal muscles fibres. The mouth lies at the posterior end of the pharyngeal pocket, close to the hind wall of the pharyngeal pouch. In specimen RMNH.VER.22264.2 the mouth is situated just behind the penis papilla and is connected to the pharyngeal pouch through an irregularly-shaped canal.

The ventral epidermis of the body is underlain by a layer of circular muscles, followed by a layer of longitudinal muscle. The longitudinal layer becomes very thick at the anterior end of the body (thickness of the layers at the anterior end: circular layer, 4 µm; longitudinal, 9 µm) (Fig. 2B). A zone of ventral adhesive papilla is present.

The large and ventrally located testes extend posteriorly in two rows from a short distance behind the ovaries to the level of the copulatory apparatus; the follicles occupy about 3/5th of the dorsoventral diameter, extending dorsally beyond the midline of the body.

At the posterior end of the pharyngeal pouch, the very narrow vasa deferentia widen to form very large, sac-shaped spermiducal vesicles that contain sperm. These spermiducal vesicles occupy approximately two-thirds of the dorsoventral diameter of the body. After penetrating the penis bulb, the vasa deferentia bend ventrally and open separately into the ejaculatory duct inside the penis papilla (Figs 3; 4). The ejaculatory duct runs centrally through the penis papilla and widens just before the tip to form an intrapenial cavity. The ejaculatory duct is lined with a thin

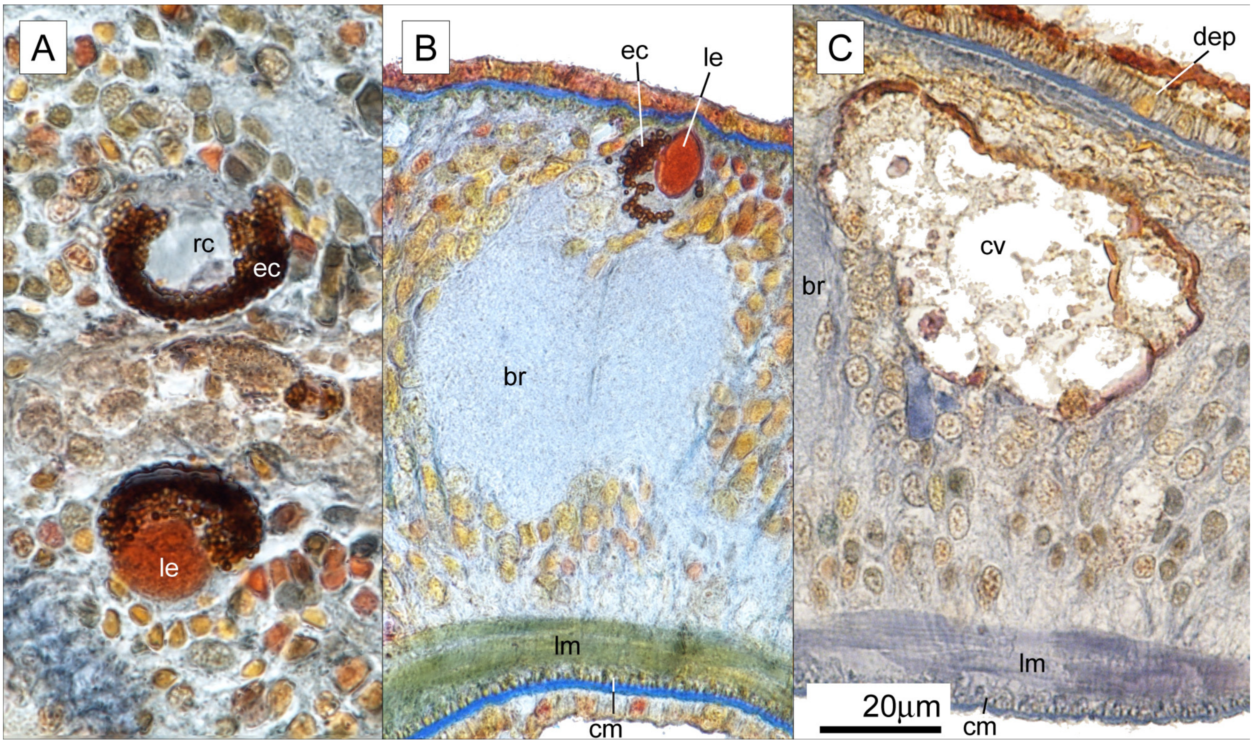

FIGURE 2. *Minutaplana dimaculosa*. Photomicrographs. (A) Paratype RMNH.VER.22264.5, horizontal section through the two eyecups; (B) Paratype RMNH.VER.22264.4, sagittal section through the brain and an eyecup; dorsal body surface to the top; (C) Holotype RMNH.VER.22264.1, sagittal section through the brain and the cavity; dorsal body surface to the top.

nucleated epithelium. Surrounding the ejaculatory duct and the walls of the penis papilla are numerous, densely red-staining nuclei, particularly near the tip of papilla, where they mix with the circular muscles underneath the lining epithelium (Fig. 4A). The mesenchyme of the penis papilla receives an abundant cyanophilic secretion from the parenchyma around the atrium and houses septa that converge towards the dorsal wall of the intrapenial cavity (Figs 3; 4A). The rounded penis bulb is formed by longitudinal muscles intermingled with a few circular muscle fibres. The narrow male atrium is lined with a thin epithelium that is underlain by a layer of circular muscle fibres, followed by a layer of longitudinal fibres. The gonopore lies approximately under the tip of the penis papilla.

The ovaries occupy about 1/3rd of the dorsoventral diameter of the body. They are located at the base of the posterior end of the brain, lying over the ventral nerve cords. There are no traces of oviducts in any of the specimens examined. Only the central section of the short and narrow bursal canal is visible and receives the openings of the shell glands all along it (Fig. 3).

The connection between the bursal canal and the atrium and between the bursal canal and the copulatory bursa is only faintly evident (Figs 3; 4A). The big and oval-shaped copulatory bursa is located just behind the atrium and is lined with a tall epithelium that is covered with a thin layer of muscle fibres. The bursa opens to the exterior through a canal, lined with a nucleated epithelium, that arises from its posterior wall and runs parallel to the ventral body surface before it bends dorsally to open through the dorsal epidermis near the end of the body, thus forming a secondary gonopore (Figs 3; 4B). This canal is surrounded by a thin muscle layer, while its diameter decreases progressively towards its distal end.

Genus *Pusillaplana* Sluys & Laumer, gen. nov. *incertae sedis*

Type species. *Pusillaplana rubella* Sluys, **sp. nov.**

Etymology. The generic name is based on the Latin adjectives *pusillus*, very little, and *planus*, flat, and alludes to the fact that these flatworms are very small in size. Gender: feminine.

Diagnosis. Marine triclads with: (1) septa in the penis papilla, channelling secretion towards the proximal end of the ejaculatory duct; (2) a secondary gonopore located posteriorly to the primary gonopore; (3) a copulatory bursa that is connected with the ventrally located secondary gonopore and is located posterior to the male copulatory apparatus; (4) two retinal cells per eyecup; (5) absence of an eye lens; (6) a far posterior location of the ovaries, the gonads being situated directly in front of the male copulatory apparatus; (7) an oviducal loop posterior to the gonoduct and copulatory bursa; (8) oviducts that communicate with the copulatory bursa through balloon-shaped sections; (9) a genito-intestinal duct connecting the oviducal loop with the gut; and (10) a ring of distinct, sclerotic spines on the tip of the penis papilla.

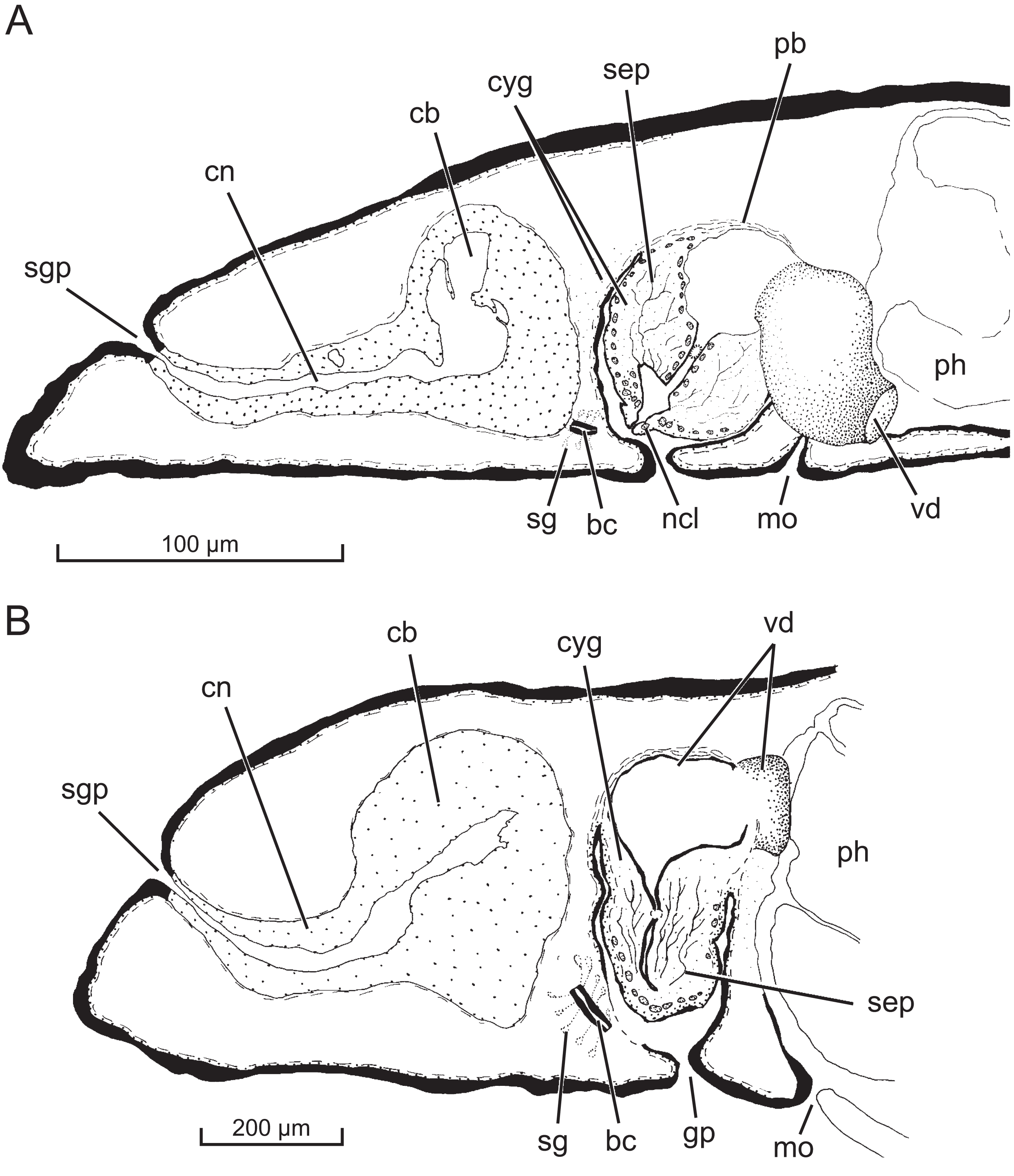

FIGURE 3. *Minutaplana dimaculosa*. Sagittal reconstruction of the copulatory apparatus of (A) holotype RMNH.VER.22264.1 and (B) paratype RMNH.VER.22264.3; anterior to the right.

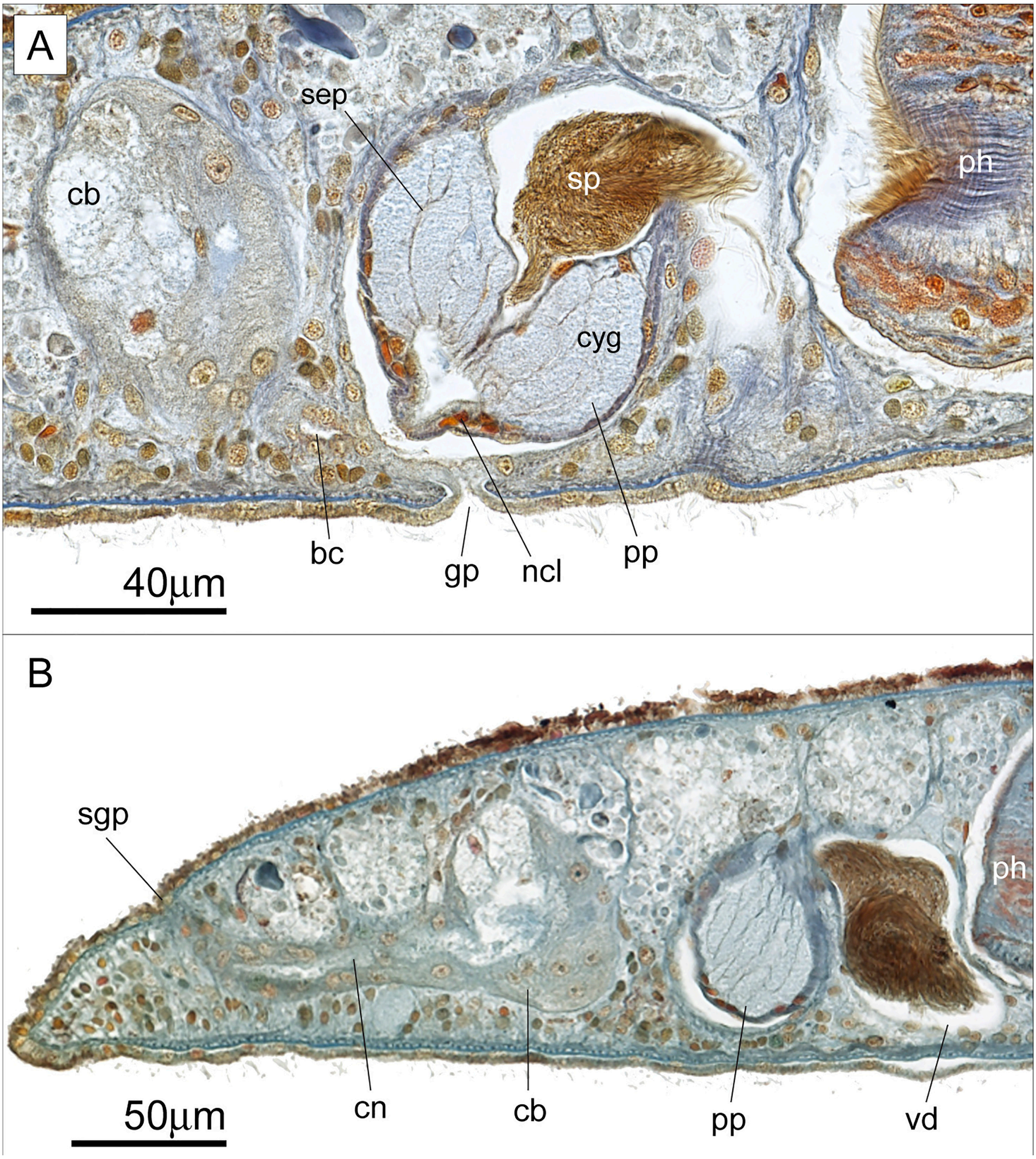

FIGURE 4. *Minutaplana dimaculosa*. Photomicrographs of sagittal sections of the copulatory apparatus of holotype RMNH. VER.22264.1; anterior to the right. (A) Detail of the male copulatory apparatus; (B) Section through the entire copulatory apparatus, showing the position of the penis papilla, copulatory bursa, and the opening of the secondary gonopore.

Pusillaplana rubella Sluys, sp. nov.

Material examined. Holotype: RMNH.VER.22265.1, east of Wild Cane Key, Bocas del Toro, Panama, 9.35169444° N, 82.16200000° W, 14–18 June 2010, in sediment, coll. Daniel Gouge, Ashleigh Smythe, Katrine Worsaae, sagittal sections on 1 slide.

Paratypes: RMNH.VER.22265.2, ibid., sagittal sections on 1 slide; RMNH.VER.22265.3, ibid., horizontal sections on 1 slide; RMNH.VER.22265.4, ibid., whole mount on 1 slide; RMNH.VER.22265.5, ibid., whole mount on 1 slide.

Other material: RMNH.VER.22266.1, sagittal sections on 1 slide.

Etymology. The specific epithet is derived from the Latin *rubellus*, red, and alludes to the red pigmentation of the animals.

Ecology and distribution. The animals were part of a highly diverse interstitial community collected from medium coarse sand on Wild Cane Reef in 1–1.5 m wide channels between coral heads at a depth of up to 6.4 m.

Diagnosis. As for the genus.

Description. In extended condition, living specimens up to 0.8 mm in length and 0.3 mm in width. The preserved holotype specimen was 0.62 mm long, as determined from histological sections. Head rounded and tail obtusely pointed. Dorsal surface provided with reddish pigment, which is arranged in two bands that run from the tail to shortly behind the eyes and converge to a single branch that extends between the eyes forwards to the frontal margin (Fig. 5). In living specimens, the pigment is more diffusely arranged in the middle part of the body, thus forming a broad pigmented area (Fig. 5A), while in one of the paratype whole mounts (RMNH.VER.22265.5) the red pigment is here more distinctly arranged as two bands (Fig. 5B). The ventral surface is pale and provided with a zone of adhesive papillae.

The eyes are positioned on top of the brain (Fig. 6A) and consist of a pigment cup housing two retinal cells; an eye lens is absent.

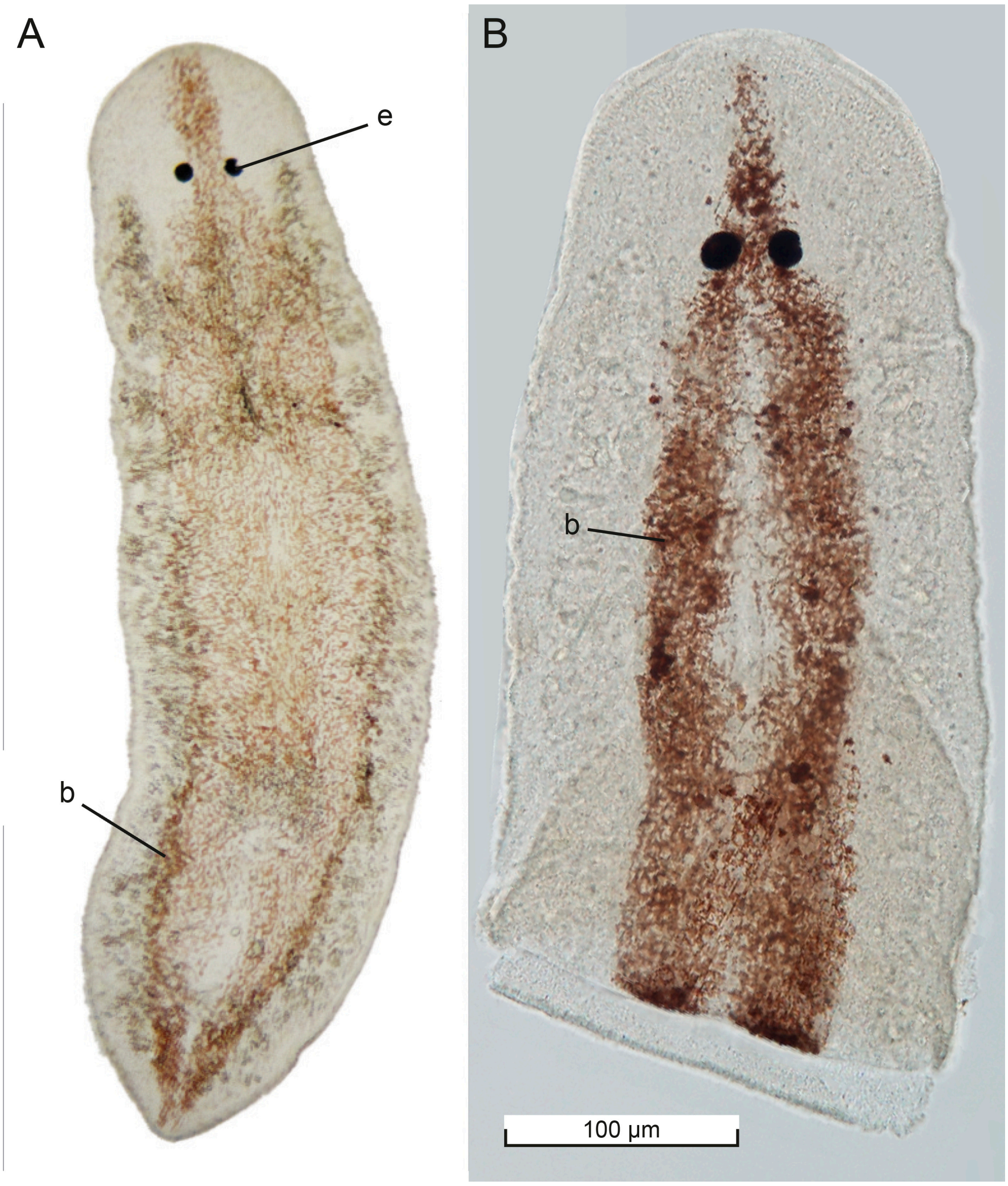

FIGURE 5. *Pusillaplana rubella*. External features of (A) live specimen (scale bar not available) and (B) specimen RMNH.VER.22265.5, whole mount; the tail is folded forwards; anterior to the top

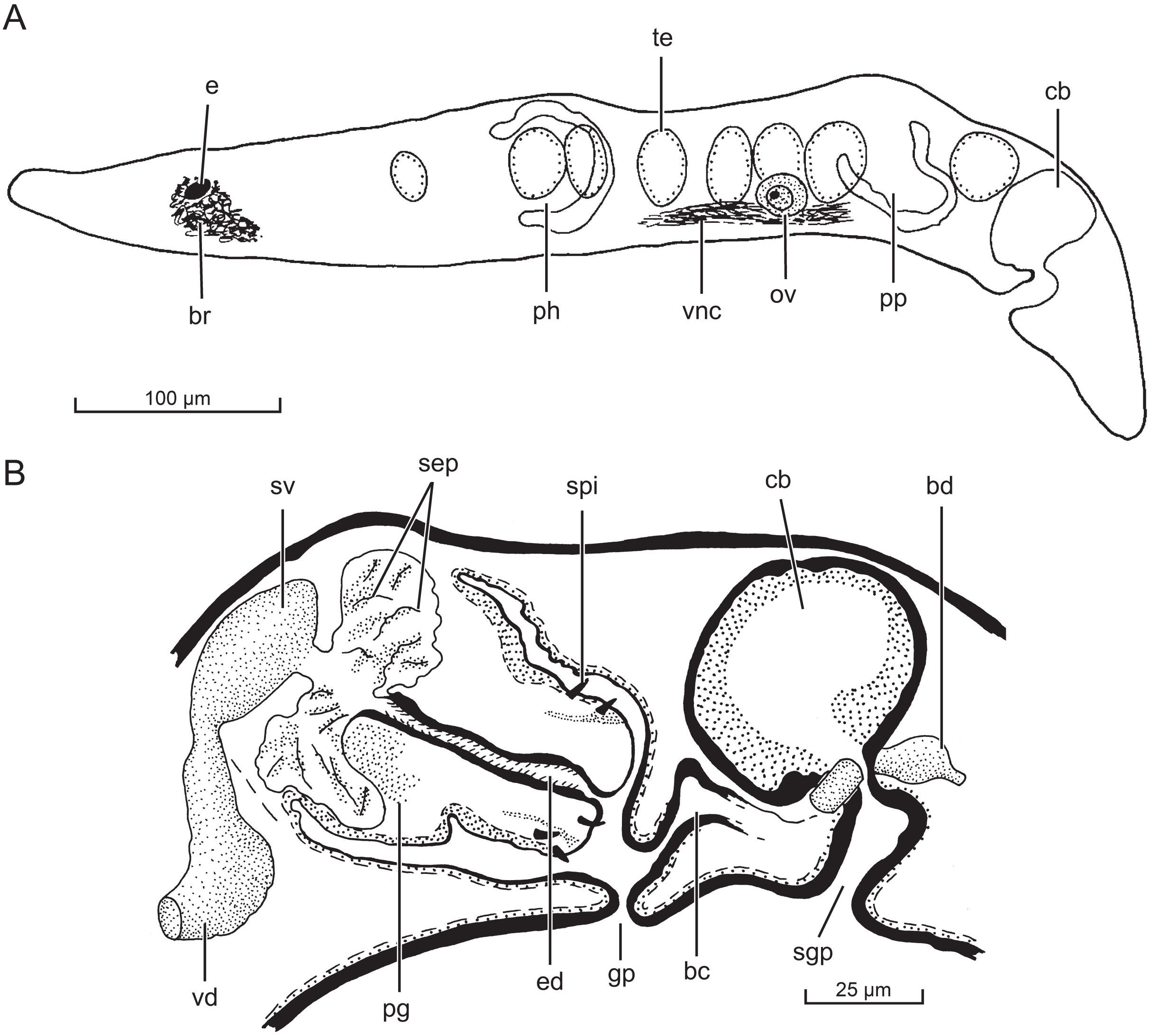

FIGURE 6. *Pusillaplana rubella*. (A) Holotype: RMNH.VER.22265.1, sagittal reconstruction of the positions of ovary, testes, eye, pharynx, and copulatory apparatus on one side of the body; anterior to the left; (B) holotype RMNH.VER.22265.1, sagittal reconstruction of the copulatory apparatus; anterior to the left.

The comparatively very short pharynx is fully situated in the anterior portion of the body and measures about 1/13th of the body length in preserved specimens (Figs 6A; 8). The pharyngeal musculature is of the usual, planariid type. The mouth opening is situated at the posterior end of the pharyngeal pocket.

The testes are located in the middle of the body, i.e., the follicles cannot be considered to be situated closer to the ventral or to the dorsal body surface. The testis follicles extend posteriorly from about half-way between the brain and the root of the pharynx to a position between the penis papilla and the copulatory bursa; there may be no more than about 8 follicles on either side of the body.

Concerning the sperm ducts, we could only distinguish a single broad duct that penetrates the penis bulb and opens into an egg-shaped intrabulbar seminal vesicle (Fig. 6B). Probably, this single duct represents a common vas deferens, resulting from the fusion of two sperm ducts outside of the male copulatory apparatus. However, separate sperm ducts or their point of fusion could not be traced in the histological sections.

The seminal vesicle communicates via a small opening with the proximal section of the penis that is traversed by many septa and thus forms a kind of mesh, which receives the abundant, erythrophilic secretion of penis glands (Figs 6B; 9). These septa converge towards the proximal end of the ejaculatory duct, which runs straight towards the blunt tip of the penis papilla; the duct is lined with a ciliated, probably infranucleated, epithelium. The penis papilla occupies almost the entire male atrium, which opens to the exterior via a separate gonopore (Figs 6B; 7A; 8).

The penis papilla is basically cone-shaped, with a blunt tip. The papilla is divided into a broad, conical basal section that is separated from a more narrow and tubular distal part by a slight constriction at about halfway the length of the penis papilla. The papilla is covered with a very thin epithelium, which is underlain by a well-developed layer of circular muscles, followed by a thin layer of longitudinal muscle fibres.

The tip of the penis papilla carries a ring of distinct, sclerotic spines. These spines are most clearly evident in slightly squeezed, live specimens, and thus, it was determined that there are about 20 spines encircling the tip of the penis (Fig. 7B). Each spine measures about 7.4 µm in length.

The small ovaries are situated directly in front of the male atrium and the penis papilla, immediately dorsally to the ventral nerve cords (Fig. 6A). Each ovary consists of one or two, big oocytes.

A conspicuous structure of the female copulatory apparatus is formed by a copulatory bursa that lies immediately behind the male atrium and opens ventrally to the exterior through its own, separate gonopore (Figs 6A, B; 7A; 8). The ball-shaped bursa is lined by a nucleated epithelium, while a large section of its lumen is filled with a coarse-grained, yellowish substance of unknown nature or origin. The bursa communicates via a very narrow opening with a well-developed gonoduct, leading to the gonopore. The histological sections suggest the presence of a connecting duct or bursal canal between the male atrium and the copulatory bursa or gonoduct. Unfortunately, only the section of this connecting duct opening into the male atrium is well-developed, whereas its connection with the female apparatus remains obscure.

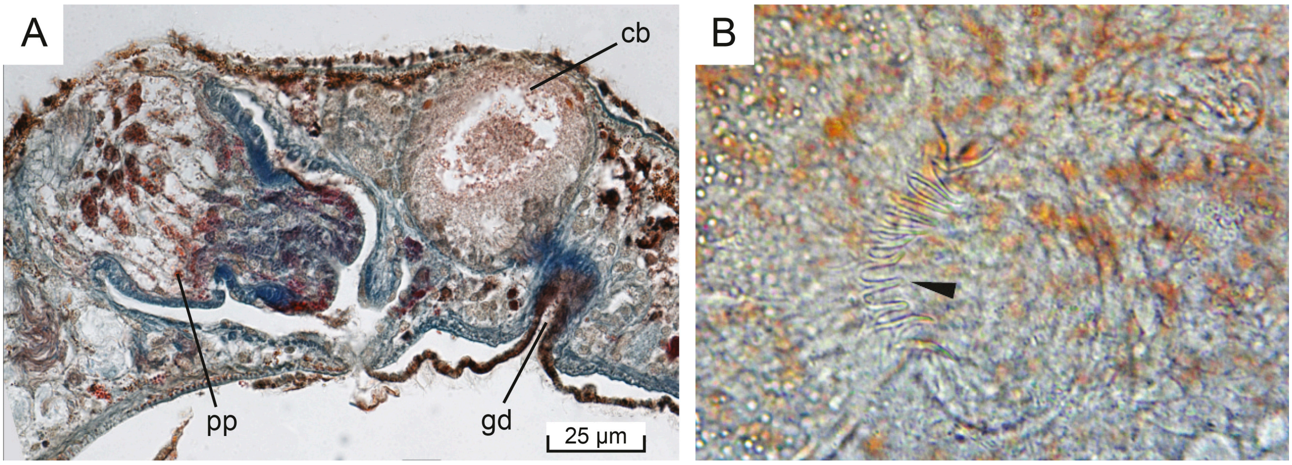

FIGURE 7. *Pusillaplana rubella*. (A) Holotype RMNH.VER.22265.1, photomicrograph of sagittal section of the copulatory apparatus; anterior to the left; (B) video still of ring of sclerotic spines on the penis papilla, indicated by arrowhead; scale bar not available; anterior to the left.

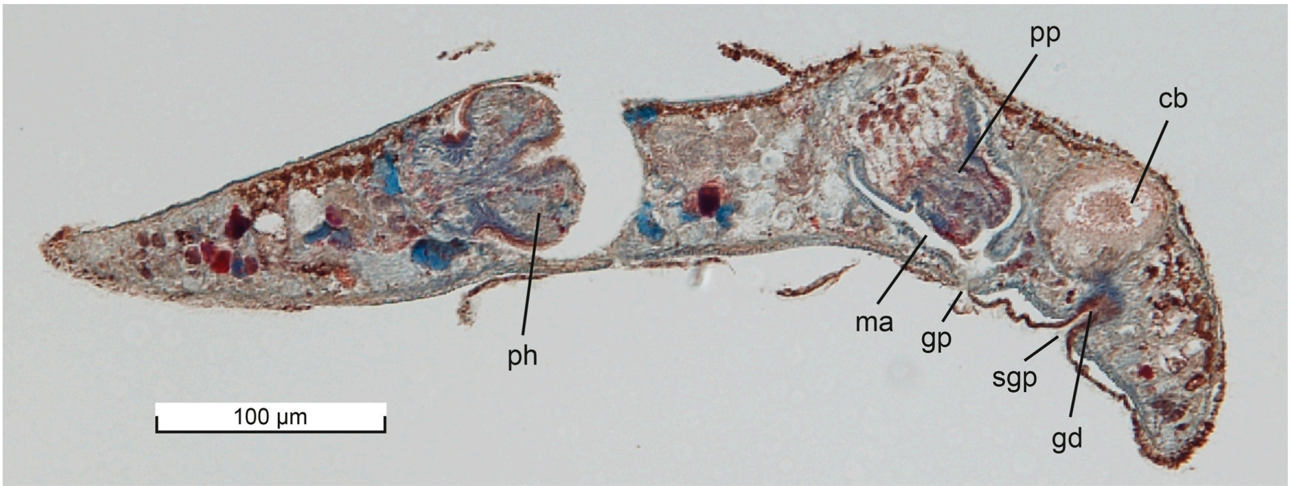

FIGURE 8. *Pusillaplana rubella*. Photomicrograph of sagittal section of the entire holotype RMNH.VER.22265.1, showing the pharynx, penis papilla, copulatory bursa, and the ventral openings of the gonoduct and the male atrium.

The course of the oviducts and their communication with the female copulatory apparatus is complex. At the level of the gonoduct connecting the bursa with the secondary gonopore, each oviduct throws off a very short and narrow duct that quickly expands to form a balloon-shaped duct. The latter communicates with the copulatory bursa precisely at the point of the narrow connection between bursa and gonoduct (Figs 6B; 10A). The openings of these balloon-shaped sections of the oviduct into the bursa are symmetrical and situated close together, resulting in the fact that these sections actually also communicate with each other (Fig. 10A, B).

After having thrown off the small connecting ducts to the balloon-shaped sections, the oviducts continue their course backwards and join behind the gonoduct, thus forming a loop (Fig. 10B). The oviducts, including the balloon-shaped sections, are lined with a nucleated and ciliated epithelium.

From the oviducal loop arises a narrow duct, containing sperm, that runs dorsally and ends in close proximity of a gut branch. Although no open connection between duct and gut was observed, this is most likely a genito-intestinal duct (Fig. 10A, B).

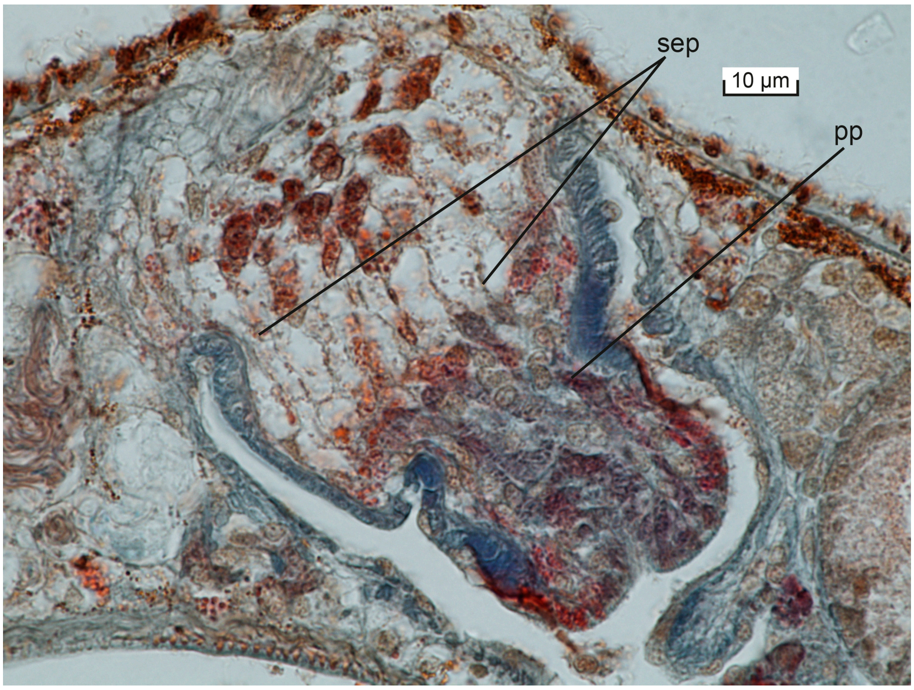

FIGURE 9. *Pusillaplana rubella*. Photomicrograph of a sagittal section of the penis papilla of holotype RMNH.VER.22265.1, showing the septa; anterior to the left.

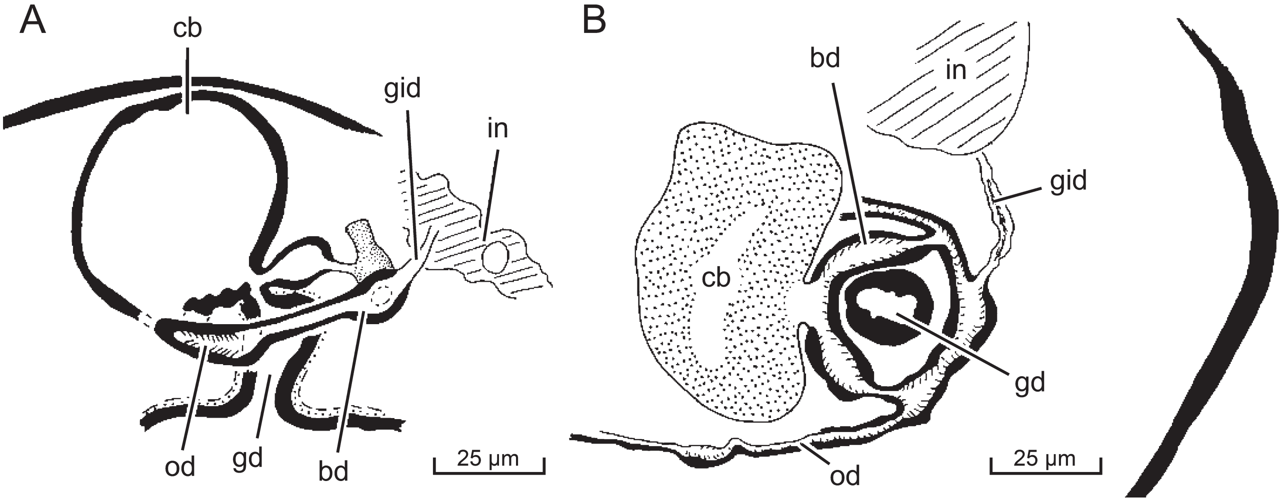

FIGURE 10. *Pusillaplana rubella.* (A) Holotype RMNH.VER.22265.1, sagittal reconstruction, showing the connections between oviducts, copulatory bursa, and intestine; posterior to the right; (B) paratype RMNH.VER.22265.3, horizontal reconstruction, showing the course and connections between oviducts, copulatory bursa, and intestine; posterior to the right.

Phylogenetic analysis

The molecular phylogenies inferred using the IQ-tree partitioned model and the PhyloBayes mixture model show nearly identical topologies and branch lengths (Supplementary Figure S1; Fig. 11). *Pusillaplana rubella* is firmly recovered with full support in both trees as the sister taxon of a clade composed of *Pentacoelum kazukolinda* (Kawakatsu & Mitchell, 1984), *Pacifides quadriauriculatum* Ma & Wang, 2021, *Sabussowia dioica* (Claparède, 1863), and *Cercyra hastata* Schmidt, 1861. In contrast, the position of *Minutaplana dimaculosa* is less certain: in the Bayesian CAT+GTR tree (Fig. 11) it falls as the sister-group to a maximally supported large clade including the aforementioned species, plus our representatives of the genera *Oregoniplana* Holmquist & Karling, 1972, *Palombiella* Westblad, 1951, *Nerpa* Marcus, 1948, *Paucumara* Sluys, 1989, *Ectoplana* Kaburaki, 1917, and *Sluysia* Leal-Zanchet, Souza & Riutort, 2018. Instead, in the ML tree (Supplementary Figure S1), *Minutaplana dimaculosa* is recovered as the sister-group of *Sabussowia ronaldi*, albeit with negligible bootstrap and aLRT support values; this pair is then strongly supported as the sister-group to the aforementioned large clade. Hence our data do not decisively resolve the trichotomy between this large clade, *Minutaplana dimaculosa*, and *Sabussowia ronaldi* Delogu & Curini-Galletti, 2011, despite new full-length sequences from both 18S and 28S rDNAs being provided for the latter in this study. Other remarkable results of this analysis include the positioning of an unfortunately unvouchered, green-pigmented maricolan from mangroves in the vicinity of Bocas del Toro ("Maricola sp. undetermined") as sister-group to *Nerpa fistulata* Wang & Chen, 2019. The two sequences from specimens of *Bdelloura propinqua* Wheeler, 1894 were recovered as a sister-group, albeit with only a short branch-length separation, to *Bdelloura candida* (Girard, 1850). Sequences of two newly discovered, still-undescribed deep-sea specimens assigned to Maricola were included ("Maricola sp. KK–2023") for the sake of completeness, but appear distantly related to *Minutaplana dimaculosa* and *Pusillaplana rubella*, instead being positioned (Supplementary Figure S1) as the sister-group to a large clade constituting all descendants of the common ancestor of *Sabussowia ronaldi* and *Oregoniplana geniculata*, similar to their position in Kakui & Tsuyuki (2024). It is noteworthy that the CAT+GTR Bayesian tree (Fig. 11) instead recovers these taxa in a 4-way polytomy with *Uteriporus* and *Bdelloura*.

Discussion

Comparative evaluation. Our phylogenetic results are broadly congruent with recently published molecular phylogenies of the Maricola (Yang *et al.* 2018, Li *et al.* 2019, Benítez-Álvarez *et al.* 2020, Li *et al.* 2021, Vásquez-Doorman *et al.* 2022). Characteristic of all of these studies, including our present one, is that the topologies of their phylogenetic trees do not coincide with the current taxonomic classification within this suborder (cf. Sluys 1989, Sluys *et al.* 2009). This becomes clear when currently known categorical ranks, for example, taxonomic families, are specified for the species included in the molecular phylogenetic analysis. Then, it is immediately apparent that particular families are not restricted to one portion of the tree, as one would expect, but that these recur at various positions (Supplementary Figure S1; Fig. 11). This formed for us one more reason to refrain from assigning *Minutaplana* and *Pusillaplana* to one of the current taxonomic families and, therefore, to consider these to be genera *incertae sedis*. Although we would have preferred to follow a conservative approach to taxonomy and thus not to increase the number of monotypic genera, especially in a group already so rich in these as Maricola, the results of the phylogenetic analysis undisputedly showed that *M. dimaculosa* and *P. rubella* are not closely related.

Nonetheless, *M. dimaculosa* and *P. rubella* do share a good number of morphological features, viz., the presence of (1) a secondary gonopore, (2) a glandular region traversed by many septa in a large portion of the penis, (3) a copulatory bursa connected with the secondary gonopore, and (4) two retinal cells per eyecup. Some of these characters are known also from other maricolans or from other triclads.

For example, secondary caudal or ventral gonopores posterior to the primary gonopore have been described for the following marine triclads: *Oregoniplana pantherina* Sluys, 1989, *O. opisthopora* Holmquist & Karling, 1972, *O. geniculata* Li & Wang, 2019, *Uteriporus vulgaris* Bergendal, 1890, *Meixnerides armatus* Westblad, 1952, *Nerpa evelinae* Marcus, 1948, *Tiddles evelinae* Marcus, 1963, and *Sluysia triapertura* Leal-Zanchet & Souza, 2018. However, all of these taxa are otherwise structurally very much different from the two new species from Panama (cf. Sluys 1989; Souza *et al.* 2018). The gross morphology of the genus *Oregoniplana* Holmquist & Karling, 1972 seems to come closest to that of *Minutaplana* and *Pusillaplana*. However, *Oregoniplana* lacks a clear bursa (although some specimens of *O. opisthopora* may exhibit an ill-defined sac-shaped vesicle or bursa—cf. Sluys 1989), while

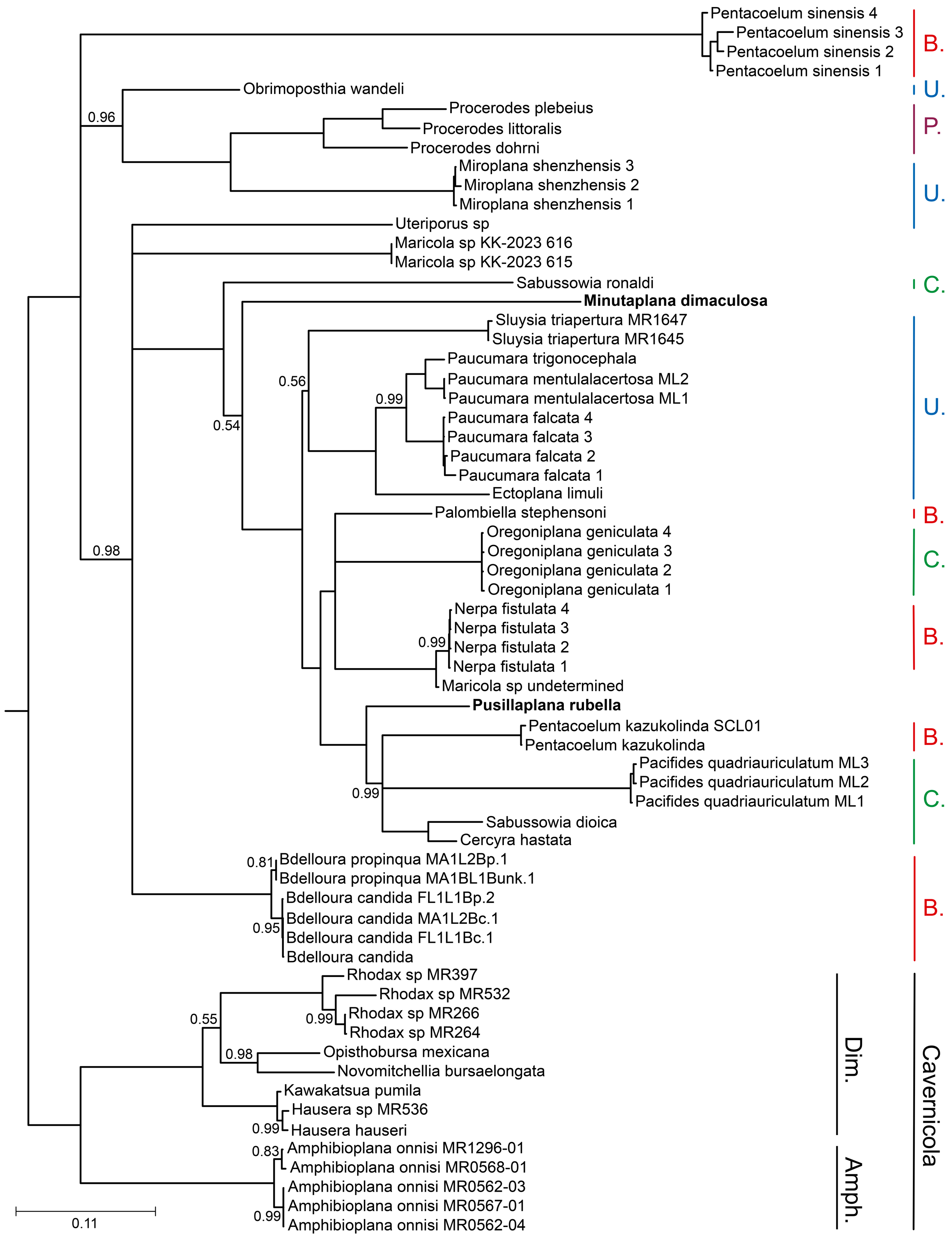

FIGURE 11. Majority rule consensus Bayesian phylogram from two independent MCMC chains using the CAT+GTR mixture model, based on the concatenated 18S and 28S rRNA data matrix. Nodes receiving maximum posterior probability are left unlabelled. Scale bar is in units of inferred substitutions per site. Abbreviations: Amph., Amphioplanidae; B., Bdellouridae; C., Cercyridae; Dim., Dimarcusidae; P., Procerodidae; U., Uteriporidae.

its ovaries are accompanied by stromatic sacs from which the oviducts arise, and the number of testes is reduced to a pair of very large follicles. All of this differs very much from the situation in *Minutaplana* and *Pusillaplana*.

Apart from the marine triclads mentioned above, secondary gonopores occur also in two members of the suborder Cavernicola Sluys, 1990, viz., *Opisthobursa mexicana* Benazzi, 1972 and *O. josephinae* Benazzi, 1975. But, again, a suite of other features indicates great structural differences between *Opisthobursa* Benazzi, 1972 on the one hand and *Minutaplana* and *Pusillaplana* on the other hand (cf. Sluys 1990), suggesting that presence of a secondary gonopore in these genera only represents superficially similar and non-homologous characters. This notion is corroborated by the separate phylogenetic and taxonomic position of the Cavernicola (Supplementary Figure S1; Fig. 11).

The shape of the body of *M. dimaculosa* is very similar to that of *Paucumara trigononcephala* (Ijima & Kaburaki, 1916) (living specimens up to 4 mm long). Particularly, the head is similar in shape and also the position of the eyes, at a considerable distance from the anterior margin. Nevertheless, the pigmentation pattern is more complex in *P. trigonocephala* as compared with *M. dimaculosa*, in which body pigmentation is reduced to two brown spots between the eyes and the tip of the head.

Although the presence of clear anatomical similarities initially suggested that *M. dimaculosa* and *P. rubella* might be taxonomically closely related, which notion was falsified by the molecular results, they do also differ considerably from each other. Notable differences concern (1) overall body size (*P. rubella* being considerably smaller than *M. dimaculosa*), (2) the unusual, far posterior location of the ovaries in *P. rubella*, (3) the equally unusual oviducal loop and communications in *P. rubella*, (4) presence of penial spines in *P. rubella*, (5) presence of an eye lens in *M. dimaculosa*, (6) the precise position of the secondary gonopore (caudo-dorsal in *M. dimaculosa* and postero-ventral in *P. rubella*), (7) presence of a genito-intestinal duct in *P. rubella,* (8) presence of a short and anteriorly located pharynx in *P. rubella*. The presence of a common vas deferens in *P. rubella* would be an additional and important difference between both species, in case the presence of this feature would be established beyond doubt.

In most of the marine triclads the ovaries are positioned directly behind the brain, whereas in only a few species they are located much more posteriorly in the body. However, the far posterior location of the ovaries in *P. rubella* is unprecedented in marine triclads and is only approached by the genus *Puiteca* du Bois-Reymond Marcus, 1955, in which the ovaries occur directly posterior to the pharyngeal pouch (cf. Sluys 1989).

Sclerotic penial spines, in one form or the other, are present in several species of marine triclads: *Stummeria margina* (Hallez, 1906), *Pacifides psammophilus* Holmquist & Karling, 1972, *P. gladiatoris* Sluys, 1989, *Puiteca rigida* du Bois-Reymond Marcus, 1955, *Meixnerides armatus* Westblad, 1952, *Jugatovaria spinosa* Sluys & Ball, 1989, *J. polynesiana* Sluys & Cannon, 1989, *Nesion arcticum* Hyman, 1956, *Miroplana trifasciata* Kato, 1931. However, in most of these species the spines are located within the ejaculatory duct and in none are they arranged in an annular zone on the outside of the penis papilla (cf. Sluys 1989).

Within the marine triclads presence of an eye lens is distributed somewhat haphazardly across the higher taxa, such as families and superfamilies. Nevertheless, most instances are found within the Superfamily Bdellouroidea Diesing, 1862. There are only three cases of convergent evolution of eye lenses outside of this Superfamily, viz., *Procerodes lacteus* Ijima & Kaburaki, 1916, *Stummeria marginata* (Hallez, 1906), and *Sabussowia ronaldi* Delogue & Curini Galletti, 2011 (cf. Sluys 1989; Delogue & Curini Galletti 2011).

However, some other features suggest affinity with another superfamily, the Cercyroidea Böhmig, 1906. For this superfamily three defining features were mentioned by Sluys (1989), viz., a long extrabulbar common vas deferens, septa around the ejaculatory duct, and ovaries located at a distance behind the brain. The extrabulbar vas deferens may be present in *P. rubella*, albeit there is no certainty about this (see above), but it is certainly absent in *M. dimaculosa*. Several, but not all, species of the Cercyroidea have septa around the ejaculatory duct, channelling the secretion of penial glands. Perhaps these structures are homologous with the penial septa in *Minutaplana* and *Pusillaplana*. The septa in *Minutaplana* and *Pusillaplana* also resemble the penial septa in the maricolan genus *Obrimoposthia* Sluys & Ball, 1989, which belongs to the Bdellouroidea.

With respect to the ovaries, *P. rubella* certainly matches the posterior position of the gonads mentioned in the diagnosis of the Cercyroidea, but *M. dimaculosa* does not conform to the definition in that its female gonads are placed directly behind the brain. Thus, it appears that with respect to the current classification of the Maricola (cf. Sluys, 1989; Sluys *et al*. 2009), the genus *Minutaplana* exhibits a mosaic of features preventing its placement at any of the higher categorical ranks.

Equally, or even more difficult is its placement within one of the currently known families. Such has happened before during the taxonomic history of the Maricola, when new and aberrant species and genera induced workers to coin new family names. However, such names seldom fared well and generally unnecessarily complicated classifications. Therefore, Sluys (1989, p.74) argued that "… one should refrain from introducing new families in the taxonomic description of … new species and/or genera. New families should only be created within the scope of comprehensive revisionary studies of the marine triclads." In this paper we have followed this advice and consider *Minutaplana* and *Pusillaplana* to be genera *incertae sedis*.

With respect to the usual size and position of the pharynx in *M. dimaculosa* and the short and anteriorly located pharynx of *P. rubella*, we do here only wish to point out that the latter condition is unusual in marine triclads. Apart from *P. rubella*, it is known only from two species of *Jugatovaria* Sluys & Ball, 1989 (cf. Sluys & Cannon 1989) and three species of *Pacifides* Holmquist & Karling, 1972 (cf. Sluys 1989; Li *et al.* 2021).

Other sequences. New rRNA sequences from specimens whose vouchers have been lost were included in this study in hopes they may be of small utility. In particular, we included sequences from two specimens from near the town of Orleans, Cape Cod, Massachusetts, identified as corresponding to *Bdelloura propinqua*. This determination was made on the basis of external morphology, the body size being notably smaller from *B. candida* collected in the same locality, and the posterior showing no constriction demarcating the caudal disc, but rather a continuous taper to a small, squared tail. The small but definite and well-supported divergence between these species visible in the molecular phylogenies (Supplementary Figure S1; Fig. 11), corresponding to 9 substitutions in the sequenced 1270 bp segment of 18S rRNA, is clear evidence for the status of these specimens as separate species from *B. candida.* This is noteworthy as literature reports on *B. propinqua* are scarce, presumably because its shares many similarities with its sister-species, *B. candida* (cf. Sluys 1989).

Sequences of an undetermined specimen ("Maricola sp. undetermined" in Table S1,Supplementary Figure S1, and Fig. 11) displaying remarkable dorsal green stripes, collected by CEL from mangrove forests near Bocas del Toro, Panama, were also included. Unfortunately, both photomicrographs and physical vouchers from this species have been lost, but the molecular phylogenies place this specimen as sister to *Nerpa fistulata,* which has a transparent body (Chen *et al*. 2019). As the only other member of this genus, *N. evelinae* Marcus 1948*,* is described as a colourless species, we remark that these data provisionally suggest the existence of a new species within or closely related to *Nerpa* is yet to be characterized from the Central American Caribbean coast.

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Additional information

Conflict of interest

The authors declared that there are no conflicts of interest.

Ethical statement

The specimens analysed in this study were collected under permits issued to Jon Norenberg for all collections done in the STRI Bocas del Toro Meiofauna Diversity and Taxonomy workshop by Mario Quirós, Director General, Ecargado, Autoridad de los Recursos Acuáticos de Panama: Resolución DGOMI-PICFC-No. 40 de 31 Octubre de 2011.

Author contributions

Specimens were collected by CEL, who also prepared the histological sections and performed the molecular analyses. MVF and RS examined the preparations and made the taxonomic descriptions. RS developed the taxonomic evaluation of the available data and wrote the first draft of the manuscript. All authors contributed to, read, and approved the final version of the manuscript.

Data availability

All of the data related to the findings of this study are available in the main text and/or can be accessed through information provided in the text.

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Supplementary Materials. The following supporting information can be downloaded at the DOI landing page of this paper:

Table S1. GenBank accession numbers for the taxa and sequences of 18S and 28S used in the present study. New taxa and sequences documented in this study appear in bold.

Figure S1. Maximum likelihood phylogram from the IQtree partitioned analysis, using the concatenated 18S and 28S rRNA data matrix, with ultra-fast bootstrap support values (1000 replicates using the -bnni correction) plotted. Nodes receiving full bootstrap support are left unlabelled. Scale bar is in units of inferred substitutions per site. Abbreviations: Amph., Amphioplanidae; B., Bdellouridae; C., Cercyridae; Dim., Dimarcusidae; P., Procerodidae; U., Uteriporidae.