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# A NEW SPECIES OF GYMNOPHALLID (DIGENEA) AND AN AMENDED DIAGNOSIS OF THE GENUS *GYMNOPHALLOIDES* FUJITA, 1925

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ABSTRACT: Morphological and molecular evidence suggest that specimens formerly described as *Lacunovermis* sp. from *Nacella* (*Patinigera*) spp. (Patellogastropoda: Patellidae) belong to a new species of *Gymnophalloides* Fujita, 1925. Based on the new information, they are identified as *Gymnophalloides nacellae* n. sp. The new species differs from *Gymnophalloides tokiensis*, *Gymnophalloides seoi*, and *Gymnophalloides heardi* mainly through the presence of a group of papillae located on the ventral surface between oral and ventral suckers. A detailed morphological study revealed the lack of pars prostatica, a character previously reported in *G. seoi*, which is why it was formerly placed in the Gymnophallinae. Molecular information proved that *G. nacellae* is close to *G. seoi*, being nestled together with *Parvatrema* representatives. This molecular information, along with the absence of pars prostatica, allows these 2 genera to be placed in Parvatrematinae. An amended diagnosis of *Gymnophalloides* is provided. Histological sections of mantle epithelium of the limpet show metacercariae attached by their oral and ventral suckers in a similar manner to *G. seoi* in its host, the oyster *Crassostrea gigas*. Tissue reaction includes cells of outer mantle epithelium being stretched by sucker attachment, hemocyte infiltration of connective tissue between mantle epitheliums, and abnormal calcareous deposition on the inner surface of the shell.

Gymnophallidae Odhner, 1905 is a small and homogeneous group of marine digeneans (Scholz, 2002). Most members use molluscs as first intermediate hosts and, with rare exceptions, charadriiform and anseriform birds as definitive hosts (Bartoli, 1974). The metacercariae never encyst and are usually parasitic in bivalves, but they have also been reported parasitizing gastropods, brachiopods, and polychaetes (Bartoli, 1974; Ching, 1995b).

Despite the many studies undertaken since the first description of a gymnophallid species by Odhner in 1900, including the revisions by James (1964), Ching (1995a), and Scholz (2002), the statement by Stunkard and Uzmann (1958) regarding the many taxonomic uncertainties within the family remain valid: "... the situation is chaotic and one of utter confusion." Contributing to this confusing status are the small size of the specimens and difficulties in accurately describing the internal anatomy, in part due to the large number of eggs in gravid adults and the massive numbers of excretion granules that accumulate in metacercariae which, in turn, prevent adequate internal observation. Gymnophallids should be studied alive including, when possible, histological serial sections (Cremonte et al., 2008).

Presently, 8 gymnophallid genera are recognized, with 6 distributed to the Gymnophallinae (characterized by the presence of a pars prostatica): *Gymnophallus* Odhner, 1900, *Gymnophalloides* Fujita, 1925, *Meiogymnophallus* Ching, 1965, *Paragymnophallus* Ching, 1973, *Pseudogymnophallus* Hoberg, 1981, and *Bartolius* Cremonte, 2001; and 2 in the subfamily Parvatrematinae (without a pars prostatica): *Parvatrema* Cable, 1953 and *Lacunovermis* Ching, 1965 (James, 1964; Cremonte, 2001; Cremonte et al., 2008).

Two genera, *Gymnophalloides* and *Lacunovermis*, have a conspicuous accessory sucker referred to as the ventral pit. Despite both genera sharing the presence of a ventral pit, they are included in different subfamilies according to the record of the presence or absence of pars prostatic. Ching (1972) examined histological sections of metacercariae of *Gymnophalloides tokiensis* from the same host as Fujita (1925), reporting a "pars prostatica well developed." However, the structure they illustrate is an ejaculatory duct with some cells (supposed to be near the genital atrium). The pars prostatica is easily discernible (see fig. 8 in Cremonte [2001], fig. 3 in Chai et al. [2007], and fig. 6 in Cremonte [2008]). Consequently, *Gymnophalloides* was formerly placed in the Gymnophallinae, characterized by the presence of a pars prostatica (James, 1964). We considered that the presence of pars prostatic in the *Gymnophalloides* species was erroneously reported and, thus, that *Gymnophalloides* belongs to Parvatrematinae.

*Gymnophalloides tokiensis* Fujita, 1925, the type species of the genus, was described by Fujita (1925) from metacercariae in the Japanese oyster from Tokyo Bay, Japan. It was redescribed by Ching (1972), who examined histological sections of metacercariae from the same host and locality. *Gymnophalloides seoi* Lee, Chai, and Hong, 1993 was first described from worms recovered after the antihelminthic treatment of a human patient in Korea. It probably corresponds to *G. tokiensis* (Lee et al., 1993; Lee and Chai, 2001). The third species in the genus is *G. heardi* Ching, 1995, described from marsh rice rats, *Oryzomys palustris* Harlan, in Florida (Ching, 1995b). Martorelli and Morriconi (1998) assigned metacercariae found between the mantle and shell of the limpets *Nacella* (*Patinigera*) *magellanica* (Gmelin, 1791) and *Nacella* (*Patinigera*) *deaurata* (Gmelin, 1791) from Beagle Channel (southern Patagonia) to *Lacunovermis* Ching, 1965.

In the present paper, we describe metacercariae from *N. magellanica* and *N. deaurata* as a new species, and include it in *Gymnophalloides*. New information provided from ITS1 rDNA sequence data obtained from specimens of the new species and from *G. seoi*, along with morphological characters, shows that the new species is nested with *Parvatrema* spp. (Parvatrematinae). In addition, an amended diagnosis of *Gymnophalloides* and a description of the host tissue reaction are provided herein.

## MATERIALS AND METHODS

### Morphology and histopathology

From December 2005 to August 2010, specimens of *N.* (*P.*) *magellanica* and *N.* (*P.*) *deaurata* (Patellogastropoda: Patellidae) were collected from rocky shores of Conejo Island (54°49′S, 68°13′W), Beagle Channel, and Puerto Deseado (47°45′S, 65°51′W), Argentina. Limpets were collected during low tide and maintained in aquaria with aerated seawater until

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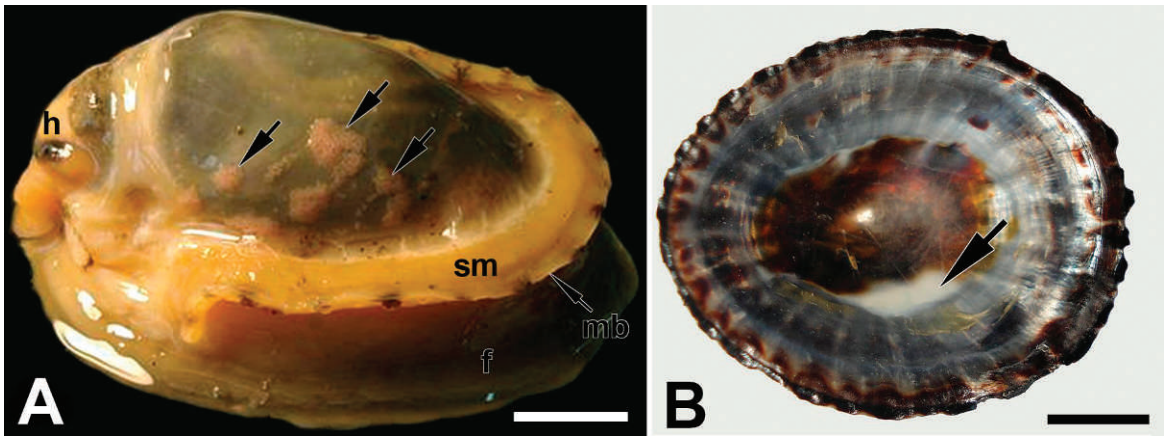


FIGURE 1. (**A**) *Nacella* (*Patinigera*) *magellanica* parasitized by *Gymnophalloides nacellae* metacercaria. A specimen without shell showing clusters of metacercariae (arrows). (**B**) Inner shell surface showing abnormal calcareous deposition just above the site where larvae were located (arrow). Abbreviations: f, foot; h, head; mb, mantle border; sm, shell muscle. Scale bars = 10 mm.

examination. Metacercariae were studied alive after staining with neutral red and Nile blue. They were incubated in vitro at 39–40 C in small Petri dishes with physiological solution or with NCTC 109 medium with, and without, antibiotics. Development of metacercariae was observed at different time intervals over 30 hr. Three chicks were also exposed to 60 metacercariae each and euthanized and necropsied at 24-hr intervals, but sexually mature adult stages could not be obtained.

Metacercariae were killed with hot physiological solution and fixed in 10% formalin, stored in 70% ethanol, stained with Semichons's acetocarmine, cleared in methyl salicylate, and mounted in Canada balsam. For histology, portions of the mantle with clusters of metacercariae attached, and 35 larvae free from the mantle, were fixed in Bouin's fluid, dehydrated in ethanol series, and embedded in Historesin Leica® (Leica, Nussloch, Germany). Serial sections at a thickness of 3.5 μm were obtained and stained with Gill's hematoxylin and eosin. Drawings were made with the aid of a camera lucida and photographs taken with a digital camera attached to a light microscope. Some metacercariae were fixed in 4% glutaraldehyde in sodium cacodylate buffer (0.1 M; pH 7.4), dehydrated in an ethanol series, dried by rinsing for a few minutes in hexamethyldisilazane, gold coated, and observed using a JEOL JSM 6360 LV scanning electron microscope (SEM) (JEOL, Tokyo, Japan). Dimensions, measured on mounted specimens, are given in micrometers with the mean value followed by the range in parentheses (10 specimens measured unless otherwise indicated). The sucker-ratio was calculated as: oral sucker length:ventral sucker length. The forebody was measured from the anterior extremity to the anterior margin of the ventral sucker. In August 2009 and February 2010, a total of 186 specimens of both limpet species was collected at Puerto Deseado, necropsied, and the metacercariae counted; prevalence and intensity were calculated according to Bush et al. (1997). For comparative purposes, specimens of *G. tokiensis* and *G. seoi* housed at Meguro Parasitological Museum of Tokyo, Tokyo, Japan were studied.

### DNA extraction, PCR amplification, and sequencing

Specimens of *G. nacellae* from *Nacella* (*Patinigera*) *magellanica* from Conejo Island, Beagle Channel, Argentina, specimens of *G. seoi* from *Crassostrea gigas* (Ostreidae) from Shinan-gun in Jeonranam-do (Province), the Republic of Korea, and specimens of *Parvatrema* sp. from *Tagelus plebeius* (Psammobiidae) from Mar Chiquita (37°46′S, 57°27′W), Argentina described by Vázquez et al. (2006) were fixed in ethanol 70%. DNA from 60, 50, and 48 metacercariae of *G. seoi*, the new species of *Gymnophalloides*, and *Parvatrema* sp., respectively, was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, Missouri) according to the manufacturer's instructions. The ITS1 region of the rDNA was amplified by PCR using as forward primer 18S-ITS1: 5′-CCGTCGCTACTACCGATTGAA-3′, situated 141 bp from the 3′ end of the conserved region of the ssrDNA, and as reverse primer 5.8S-ITS1: 5′-CGCAATGTGCGTTCAAGATGTC-3′, located 95 bp from the 5′ end of the 5.8S gene. PCRs were performed in a total volume of 50 μl containing 1× buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.4 μM of each primer, and 1 U of platinum *Taq* polymerase. Two microliters of genomic DNA were used as template. The cycling conditions included an initial denaturation at 94 C for 5 min followed by 40 cycles of 30 sec at 94 C, 30 sec at 54 C (annealing), and 2 min at 72 C, with a final extension step of 10 min at 72 C. Amplified PCR products were electrophoretically separated in a 1% (w/v) agarose gel stained with ethidium bromide. Negative controls for the PCR were always run to control for contamination. Relevant bands were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, California), cloned into pGEM-T Easy vectors propagated in JM109 High Efficiency Competent Cells (Promega, Madison, Wisconsin), and sent for sequencing (Stabvida, Oeiras, Portugal). The complete ITS1 sequences, including partial sections of the 18S and 5.8S, have been deposited in GenBank and have accession numbers JN381024, JN381025, and JN381026.

### Phylogenetic analyses

Both ITS1 rDNA strands were sequenced and alignments were performed using Multalin software (available at http://bioinfo.genotoul.fr/multalin/multalin.html). ITS1 sequences of *Parvatrema duboisi* (AB478508), as well as 2 outgroup species, were obtained from GenBank for molecular and phylogenetic studies. According to Olson et al. (2003), the superfamily Gymnophalloidea, which includes the species under analysis (*Gymnophalloides seoi*, the new species, and *Parvatrema* spp.), together with the superfamily Bucephaloidea, form the suborder Bucephalata. *Dollfustrema hefeiensis* (EF198238) and *Dollfustrema vaneyi* (Tseng, 1930) (EF198216) were selected as outgroups because these 2 species belong to the Bucephaloidea (no other ITS1 sequence for this group is available in GenBank). Phylogenetic analyses were conducted on the aligned nucleotide sequences of ITS1 using MEGA 4.0 (Tamura et al., 2007). The neighbor-joining (NJ) method of Saitou and Nei (1987) was performed using the program's default settings. The reliability of internal branches in the NJ tree was assessed using bootstrap analysis with 1,000 replicates. The resulting network was rooted with the outgroup taxa.

## DESCRIPTION

### *Gymnophalloides nacellae* n. sp.

Syn. *Lacunovermis* sp. sensu Martorelli and Morriconi, 1998
(Figs. 1–5)

*Diagnosis (metacercariae [Figs. 2–4]):* Body oval with rounded anterior end and slightly pointed posterior end, 338 (301–380) long × 183 (142–215) maximum wide. Spines arranged transversely, covering entire body surface, except in region from pharynx to ventral sucker (Fig. 4). Forebody 202 (165–255) long.

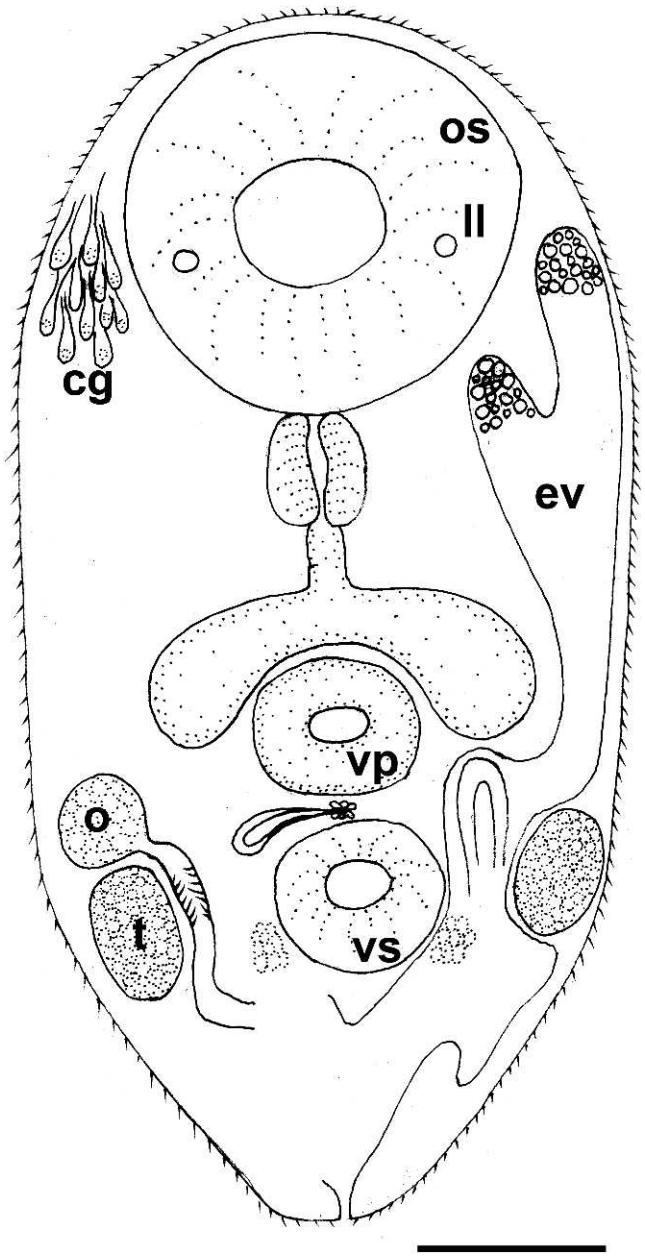


FIGURE 2. Schematic drawing of *Gymnophalloides nacellae* n. sp., ventral view (excretory granules and cells not drawn, only left part of excretory vesicle and right cephalic glands drawn). Abbreviations: cg, cephalic glands; ev, excretory vesicle; ll, lateral lip; o, ovary; os, oral sucker; t, testis; vp, ventral pit; vs, ventral sucker. Scale bar = 50 μm.

Oral sucker 96 (85–102) long × 102 (91–112) wide, with circle of 16 papillae surrounding mouth and 18 scattered around oral sucker; lateral lips present, 2 in number, eversible (Fig. 4B–E). Oral sucker retractible (Fig. 4A). Cephalic glands present, 22 in number. Ventral pit 41 (30–50) long × 52 (30–66) wide. Papillae present between oral sucker and ventral pit, approx. 30 in number (Fig. 4A–D, F). Ventral sucker 38 (30–46) long × 47 (42–52) wide, with outer circle of 6 papillae (Fig. 4 F). Sucker-ratio 1:0.46 (0.41–0.53). Pharynx ovoid, 31 (27–38) long × 29 (26–30) wide. Esophagus 20 (10–29) long. Caeca sacciform, filled with granular material, 53 (36–73) long × 31 (23–47) wide, reach to mid-ventral pit region. Testes ovoid, located at level of ventral sucker; testis posterior to ovary may be slightly diagonal. Left testis 46 (30–60) long × 31 (23–47) wide; right testis 43 (28–53) long × 30 (24–42) wide. Seminal vesicle unipartite. Pars prostatica absent; prostatic cells opening into genital atrium. Genital pore small, inconspicuous, located close to anterior margin of ventral sucker (Fig. 2). Ovary rounded, 29 (23–36) long × 21 (16–28) wide, located anterior to right testis. Oviduct short, originating dorsal to ovary. Fertilization chamber ciliated. Uterus ascends toward genital atrium, forming long loop anterior to ventral pit. Vitelline gland paired, formed by about 8 follicles each, oval, close to ventral sucker (not evident in metacercaria). Flame cell formula 2 [(2+2) + (2+2)] = 16. Excretory vesicle Y-shaped, with very short stem and 2 long ventral arms extending and bifurcating at oral sucker level; dorsally formed by multiple branched arms (Fig. 3A), filled with spherical excretory granules. Metacercariae always in clusters, pink colored when alive, attached to outer mantle epithelium by oral or oral and ventral suckers (Figs. 1, 5).

Pre-adult specimens obtained after 24–30 hr of in vitro culturing of metacercariae showed active spermatozoa in their testes and in the seminal vesicle. Vitellaria became evident.

## Taxonomic summary

*Type host: Nacella* (*Patinigera*) *magellanica* (Gmelin, 1791) (Patellogastropoda: Patellidae).

*Other host: Nacella* (*Patinigera*) *deaurata* (Gmelin, 1791) (Patellogastropoda: Patellidae).

*Site of infection:* Extrapallial space (between mantle and shell) (Fig. 1).

*Prevalence, range, and mean intensities:* 92.5%, 1–177 (33).

*Etymology:* Specific name refers to the molluscan host.

*Type locality:* Puerto Deseado (47°45′S, 65°51′W), Santa Cruz Province, Argentina.

*Other locality:* Conejo Island (54°49′S, 68°13′W), Beagle Channel, Tierra del Fuego Province, Argentina.

*Specimens deposited:* Stained whole-mounts of metacercariae were deposited at the Parasitology collection, Museo Argentino de Ciencias Naturales, Buenos Aires, Argentina (holotype and 3 paratypes MACN-Pa 532/1, 532/2), Centro Nacional Patagónico, Puerto Madryn, Argentina (Paratypes CNP-Par 50), U.S. National Parasite Collection, Beltsville, Maryland, U.S.A. (Paratypes USNPC 105642).

## Remarks

*Gymnophalloides nacellae* n. sp. differs from the other species in the genus, i.e., *G. tokiensis*, *G. seoi*, and *G. heardi*, in having a group of papillae located between the ventral pit and ventral sucker. In addition, the new species is distinguished from *G. heardi* by the shape of vitellaria, i.e., a single lobe in *G. heardi* (Ching, 1995b) and paired follicular structures in *G. nacellae* n. sp. Moreover, *G. tokiensis* and *G. seoi* have bipartite seminal vesicles (Ching, 1972; Lee et al., 1993), while the seminal vesicle in *G. nacellae* n. sp. is unipartite.

Due to an erroneous morphological interpretation of a structure reported as a "wide genital pore," actually corresponding to a body fold, Martorelli and Morriconi (1998) considered the species studied here as belonging to *Lacunovermis*. The misinterpretation originated in the fact that they studied fixed and contracted specimens.

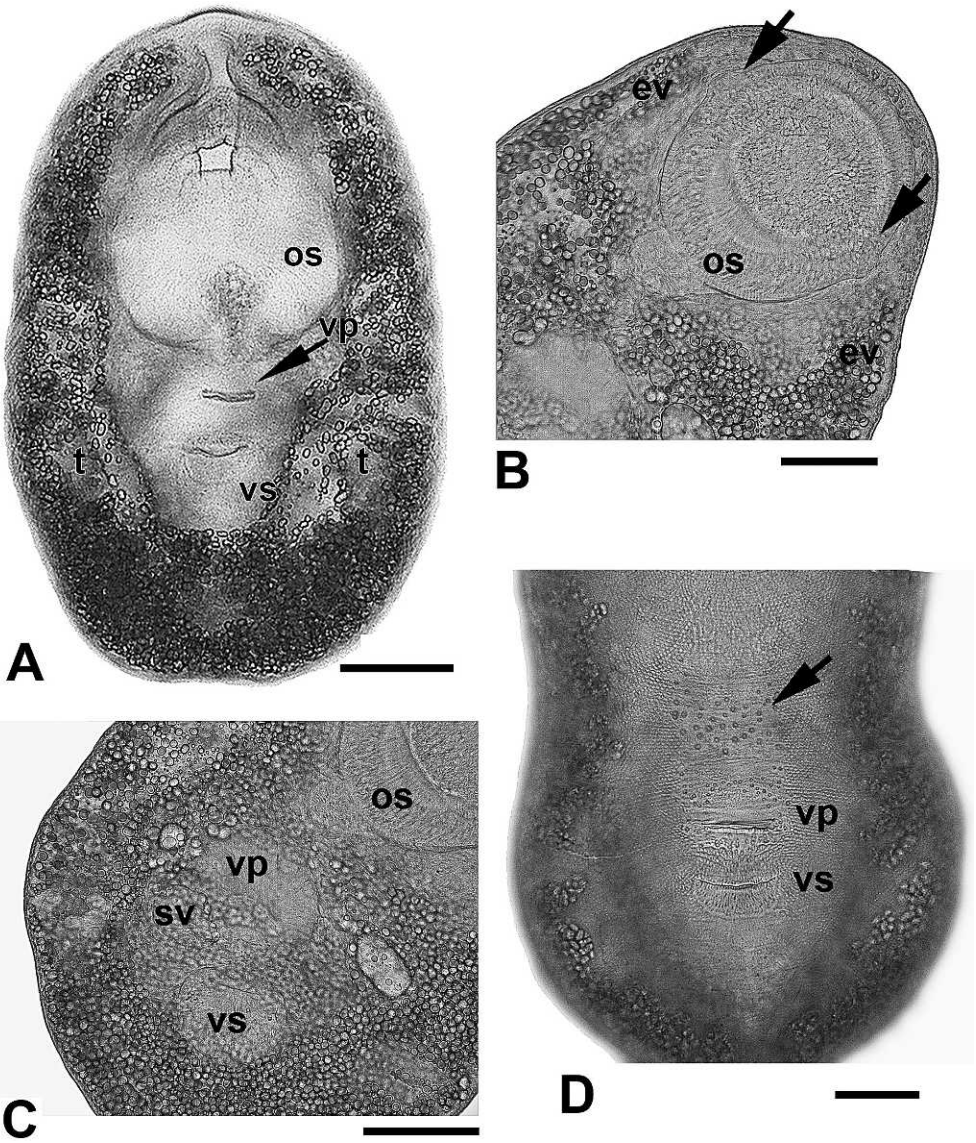


FIGURE 3. Photomicrograph of live metacercariae of *Gymnophalloides nacellae* n. sp. (**A**) Specimen with retracted oral sucker, ventral view. (**B**) Anterior end showing oral sucker with eversible lateral lips (arrows) and tips of bifurcating branches of excretory vesicle. (**C**) Detail of ventral sucker, ventral pit, and seminal vesicle. (**D**) Ventral surface of mid-posterior body showing papillae between oral and ventral suckers (arrow). Abbreviations: ev, excretory vesicle; os, oral sucker; vp, ventral pit; vs, ventral sucker; t, testis; sv, seminal vesicle. Scale bars: A, C, D = 40 μm; B = 30 μm.

The information coming from morphology (the lack of pars prostatica) and molecular studies of *G. nacellae* n. sp. reported in the present paper reveal that this species is closely related to *G. seoi*. Moreover, both species are associated with those representatives of the genus *Parvatrema*, supporting their inclusion in the Parvatrematinae. Thus, the 'ventral pit' must be the diagnostic character to distinguish between *Gymnophalloides* and *Parvatrema*.

## Histopathology

The metacercariae of *G. nacellae* n. sp. occur in small clusters attached to the outer mantle epithelium in the extrapallial space of the gastropod host (Fig. 1A). Virtually all extrapallial space may be potentially occupied by the larvae, although larvae usually occur in groups along the extrapallial space immediately above the shell muscle (Fig. 1).

Metacercariae were observed most-commonly attached by means of the oral sucker; however, in some cases, histological evidence indicated attachment by both the oral and ventral sucker (Fig. 5D). The attachment position varied from vertical to parallel with respect to the mantle surface. The ventral pit was never involved in attachment (Fig. 5C–E).

The outer mantle epithelium is formed by tall columnar cells (about 25 μm in height) with centrally elongated nuclei; the inner mantle epithelium consists of cubical cells (about 6–7 μm in diameter) with rounded nuclei. Between outer and inner epithelia, a layer of connective tissue (about 60 μm thick) is present, with fibrous elements and bundles of muscle fibers of variable

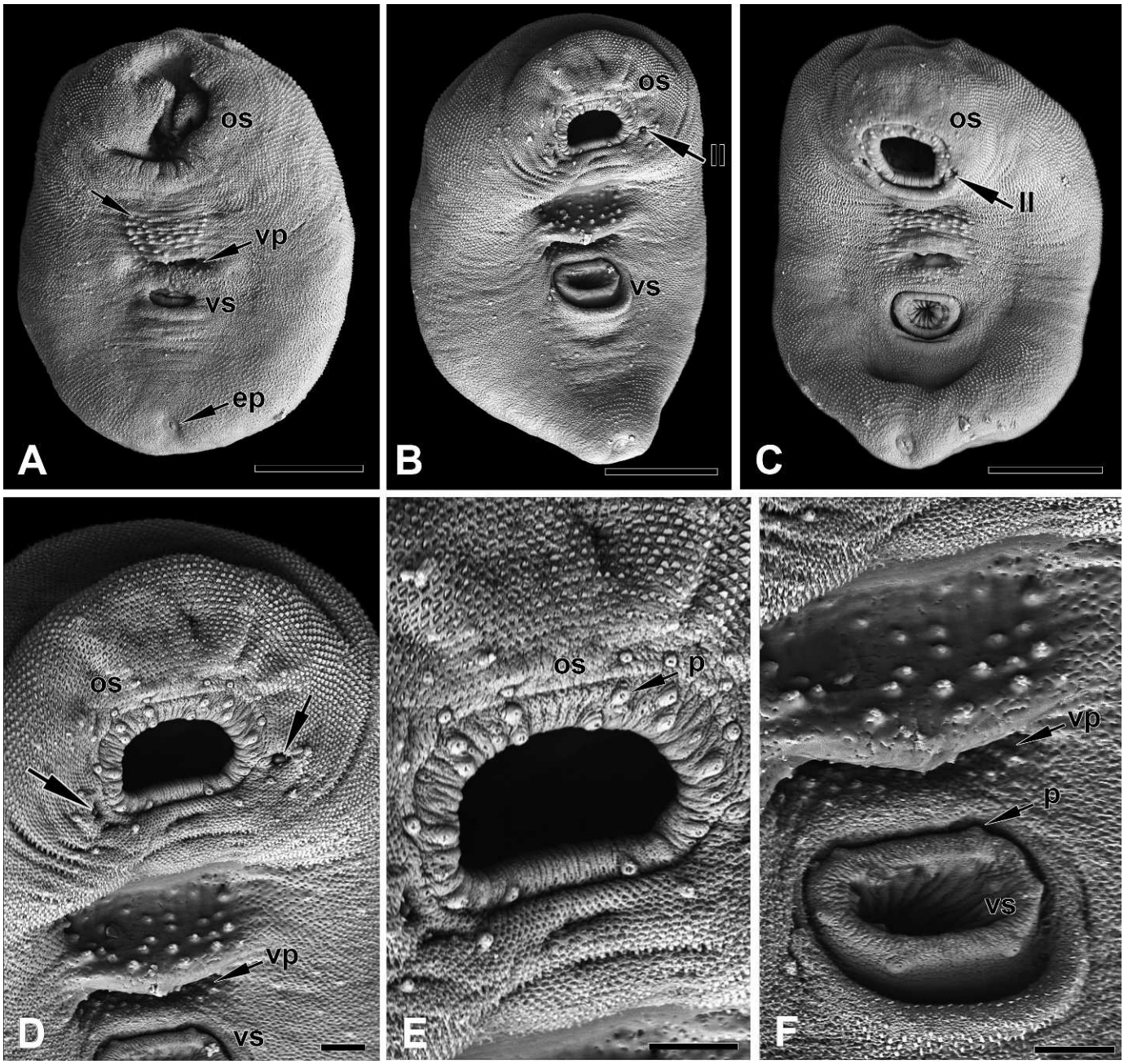


FIGURE 4. SEM photomicrographs of *Gymnophalloides nacellae* n. sp. (**A**) Specimen with retracted suckers, ventral view. (**B–C**) Specimens with relaxed suckers, ventral views showing lateral lips at side of oral sucker and ventral pit. (**D**) Oral sucker with papillae and eversible lateral lips (arrows). (**E**) Detail of D showing the spatial distribution pattern of papillae. (**F**) Detail of ventral sucker with a circle of six papillae. Abbreviations: ep, excretory pore, os, oral sucker; p, papillae; ll, lateral lip; vp, ventral pit; vs, ventral sucker. Scale bars: A–C = 50 μm; D–F = 10 μm.

development according to the zone considered (Fig. 5B). The site of the outer mantle epithelium at the point of attachment of the metacercariae is mechanically altered by the vacuum force produced by the sucker. The shape and size of the cells at the point of attachment of each larva were morphologically altered; the cytoplasm and nuclei became slender and taller, forming a sort of mammilla that corresponds to the concavity of the metacercaria oral sucker (Fig. 5C–E).

The attachment of the parasite primarily affects the outer lining epithelium of the mantle. However, the adjacent connective tissue was also affected; some of the fibrous elements showed a moderate departure from the normal structure accompanying the distortion of epithelial cells (Fig. 5C, E). There was a consistent presence of a moderate number of hemocytes just beneath the attachment of larvae in the spaces among cells, and fibers of the connective tissue were also evident (Fig. 5E).

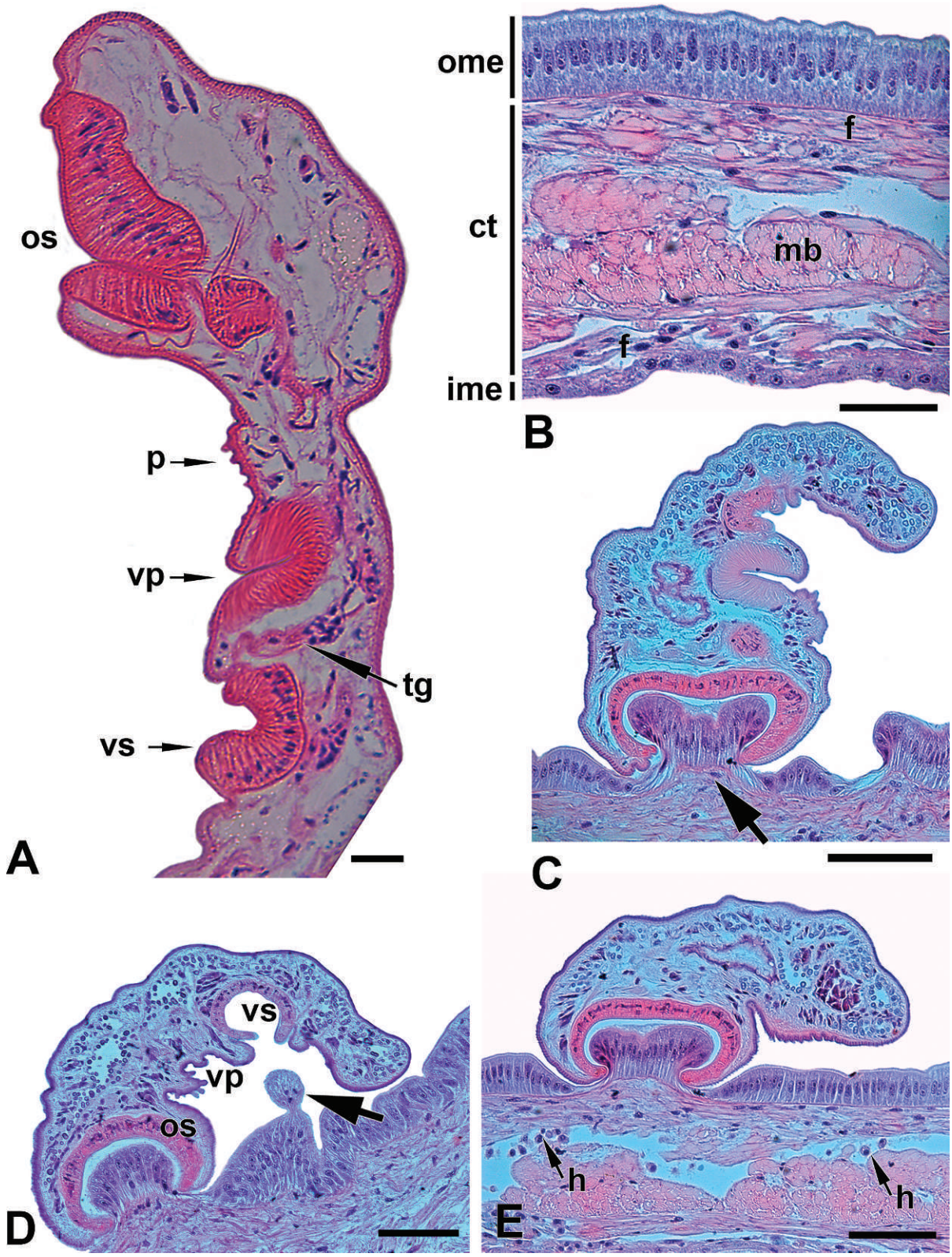


FIGURE 5. Histological sections of metacercariae of *Gymnophalloides nacellae* n. sp. (**A**) Sagittal section of anterior part of body. (**B**) Transverse section showing the normal histology of the mantle. (**C**) Larva attached perpendicular to the outer mantle epithelium; mechanical distortion of the mantle epithelium and underneath connective tissue (arrow) is noticeable. (**D**) Larva attached by oral and ventral (the latter dislodged) suckers; note the persistent injury of outer epithelium (arrow) determined by attachment of ventral sucker. (**E**) Larva attached parallel to the mantle surface. Abbreviations: ct, connective tissue; f, fibers of connective tissue; ime, inner mantle epithelium; mb, muscle bundles; ome, outer mantle epithelium; os, oral sucker; p, papillae; vp, ventral pit; tg, terminal genitalia; vs, ventral sucker; h, hemocytes. Scale bars: A = 20 μm; B = 25 μm; C–E = 50 μm.

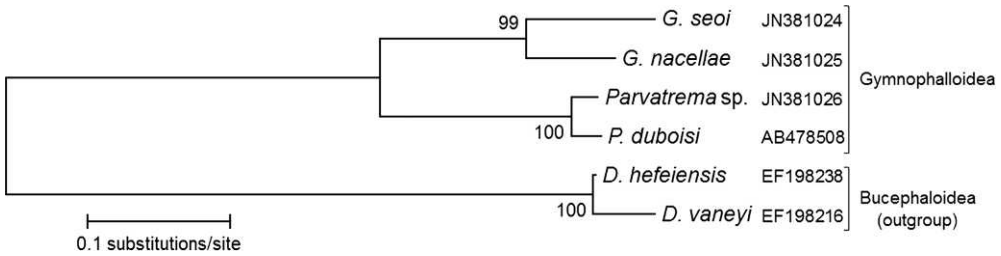


FIGURE 6. Clustering diagram depicting sequence similarity relationship among *Gymnophalloides seoi*, *Gymnophalloides nacellae* n. sp., *Parvatrema* sp., *Parvatrema duboisi*, *Dollfustrema hefeiensis*, and *Dollfustrema vaneyi* species, inferred from ITS1 rDNA. The tree was constructed using the neighbor-joining (NJ) method of Saitou and Nei (1987), with pair-wise deletion of gaps. Numbers at nodes represent bootstrap values (%, n = 1,000 replicates).

### Molecular phylogenetic analysis

PCR amplification of the rDNA from the metacercariae of *G. seoi*, *G. nacellae* n. sp., and *Parvatrema* sp. produce a product of 1,020 bp, 957 bp, and 937 bp long, respectively. After alignment of PCR products sequences, putative 18S and 5.8S regions were identified through comparison with identical regions of other digeneans, and the sequences encoding for the ITS1 region were, correspondingly, 741 bp, 694 bp, and 699 bp long. The comparison by BLASTn of the novel ITS1 sequences revealed the existence of a high similarity sequence, deposited in GenBank, encoding for the same region and belonging to *Parvatrema duboisi*.

Phylogenetic analyses were conducted on the aligned sequences of ITS1 rDNA using the NJ method. The resulting tree (Fig. 6) revealed that *G. nacellae* n. sp. described in this study branch grouped closely with *G. seoi*, and this relationship is strongly supported by high bootstrap values (99%). As expected, *Parvatrema* sp. and *P. duboisi* are also closely related. Moreover, species of *Gymnophalloides* grouped with species of *Parvatrema*, with the exclusion of outgroups, suggesting that members of the 2 genera belong to the same higher taxon; this grouping also received high bootstrap support.

## AMENDED DESCRIPTION

### *Gymnophalloides* Fujita, 1925

*Diagnosis:* Gymnophallidae Odhner, 1900. Parvatrematinae James, 1964. Body small, oval to pyriform, spinose, with ventral pit located anterior to ventral sucker. Oral sucker sub-terminal, large, with lateral projection at each side, larger than ventral sucker. Ventral sucker in posterior part of body. Prepharynx absent; pharynx present; esophagus short. Ceca saccate, dilated, very short, never passing midbody. Testes paired, symmetrical, in posterior half of body; seminal vesicle pretesticular, voluminous, uni- or bipartite; pars prostatica absent, prostatic cells opening into genital atrium. Genital pore median, small, immediately anterior to ventral sucker. Ovary ovoid, dextral, pretesticular. Vitellarium single, thickly lobed organ, or vitellaria paired, close to ventral sucker. Uterus filling available space in anterior and middle third of body. Eggs small, elliptical, and thin-shelled. Excretory vesicle with long arms. Intestinal parasites of humans, rodents, and birds.

### Taxonomic summary

*Type species: Gymnophalloides tokiensis* Fujita, 1925 (based on metacercaria).

*Other species: G. seoi* Lee, Chai and Hong, 1993; *G. heardi* Ching, 1995; and *G. nacellae* n.sp.

## DISCUSSION

The generic diagnosis of *Gymnophalloides* is here amended to incorporate as a diagnostic character the absence of a pars prostatica, i.e., prostatic cells opening directly into the genital atrium. The character 'presence or absence of pars prostatica' was used by James (1964) to distinguish between 2 subfamilies within Gymnophallidae, i.e., Gymnophallinae, with a pars prostatica, and the Parvatrematinae, lacking this structure. In the latter subfamily, numerous cells surround, and open directly into, the genital atrium.

Based on the presence of a ventral pit in *Gymnophalloides* and *Lacunovermis*, which is absent in other gymnophallid genera, Yamaguti (1971) and Scholz (2002) considered the 2 genera as synonymous. Ching (1995b) refused this synonymy, insisting that the primary difference between the 2 genera is in the shape and location of the genital pore. Considering that the genital pore frequently opens in a "pocket" or in a body fold, it may appear as a "wide" genital pore. It remains to be demonstrated whether size of the genital pore is a valid character to separate *Lacunovermis* from *Gymnophalloides*. At present, the only valid species of *Lacunovermis* is *Lacunovermis macomae* (Lebour, 1908), which occurs in the Baltic Sea and the northern Pacific Ocean (Pekkarinen, 1984; Pekkarinen and Ching, 1994). Molecular evidence from specimens of the only valid species of *Lacunovermis*, i.e., *L. macomae*, is required to confirm the validity of the genus. When Yamaguti (1971) and Ching (1972) discussed the synonymy of *Gymnophalloides* and *Lacunovermis*, the presence or absence of pars prostatica was not considered although, according to James (1964), this character was important enough to place each genus in distinct subfamilies.

Based on the site of infection and the tissue damage caused by *G. nacellae* n. sp., there is a remarkably great similarity with *G. seoi*. Whereas the 2 species infect very different hosts, i.e., the new species is in a gastropod and *G. seoi* occurs in a bivalve, the mode of attachment to the mantle and the tissue reaction elicited are identical (Jong-Yil Chai, unpubl. obs.). Metacercariae of both species attach to the host mantle mainly by the oral sucker. In

addition, abnormal calcareous deposits were observed on the inner surface of the valve just above the site where larvae are located.

Histology of the areas of the mantle affected by the attachment of larvae showed that the outer epithelium is not altered; neither hyperplasia nor metaplasia was observed, but a significant mechanical stretching of epithelial cells determined by the vacuum force of the sucker of metacercariae was noticeable. The absence of hyperplasia or metaplasia of the outer mantle epithelium may serve as evidence that the larval infection is innocuous. However, a moderate hemocyte infiltration was observed in the connective tissue between outer and inner mantle epitheliums beneath the location where the sucker of the larvae are in contact with the mantle. The infiltration of hemocytes in the connective tissue affected by the infection signals a tissue reaction elicited as a defense mechanism or tissue response to an injury. Furthermore, after dislodging the larvae from their attachment in the mantle, a pear-shaped protrusion corresponding to the site of contact with the oral or ventral sucker involved in the attachment remained; this fact indicates that the alteration of the mantle epithelium is, to a certain degree, not transitory.

The information coming from morphology (the lack of pars prostatica) and molecular studies of *G. nacellae* n. sp. reported in the present paper reveal that this species is closely related to *G. seoi*. Moreover, both species are associated with those representatives of the genus *Parvatrema*, supporting their inclusion in the subfamily Parvatrematinae. Thus, 'ventral pit' must be the diagnostic character to distinguish between *Gymnophalloides* and *Parvatrema*.

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