Parasitology International 64 (2015) 202–210

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Revision of the family Gymnophallidae Odhner, 1905 (Digenea) based on morphological and molecular data

Florencia Cremonte [a,*], Carmen Gilardoni [a], Susana Pina [b], Pedro Rodrigues [b], Cristián Ituarte [c]

[a] Centro Nacional Patagónico (CONICET), Bvard. Brown 2915, U9120ACF Puerto Madryn, Argentina
[b] Laboratory of Fish Pathology and Immunology, ICBAS-Abel Salazar Institute for the Biomedical Science, University of Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
[c] División Zoología Invertebrados, Museo Argentino de Ciencias Naturales, Av. Ángel Gallardo 470, C1405DJR Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 3 November 2014
Received in revised form 28 November 2014
Accepted 4 December 2014
Available online 13 December 2014

Keywords:
Digenean
Gymnophallidae
Molecular phylogeny
Mollusca: Bivalvia

ABSTRACT

This paper reviews the family Gymnophallidae, recognizing as valid seven genera; four within the subfamily Gymnophallinae: *Gymnophallus* Odhner, 1900 (syn. *Meiogymnophallus* Ching, 1965), *Paragymnophallus* Ching, 1973, *Pseudogymnophallus* Hoberg, 1981, and *Bartolius* Cremonte, 2001, and three in the Parvatrematinae: *Parvatrema* Cable, 1953, *Lacunovermis* Ching, 1965, and *Gymnophalloides* Fujita, 1925. Specimens representing one species of each available genus were chosen from those well-described and non controversial species, for which strong morphological information was available, and used for molecular studies (ITS1-5.8S-ITS2-28S rDNA strands were sequenced). The presence or absence of a pars prostatica differentiates between the 2 subfamilies, Gymnophallinae and Parvatrematinae. The characters used to differentiate genera are: location of the ovary (pre-, post- or inter-testicular), size and location of the genital pore (inconspicuous and located at the anterior margin of the ventral sucker, or conspicuous and located at some distance from the anterior margin of ventral sucker), presence of caecal pockets, and presence or absence of ventral pit (a muscular structure which can be either well-developed, similar in size and musculature to the ventral sucker, or be poorly developed). The characters previously used to distinguish among genera that actually should be considered to separate species include: shape of tegument spines (broad, sharp or serrated), presence of lateral projections on the oral sucker (also called papillae or lips), shape of the seminal vesicle (unipartite or bipartite), shape of the prostatic duct (elongate or oval), presence of papillae on the genital pore, shape of the genital atrium (tubular, wide, oval), shape of the vitellaria (follicular in a variable degree, paired or single), shape of the excretory vesicle (V or Y), and extension of uterus (restricted to forebody, at hindbody or extending in both). Additionally, some of these characters may vary with the age of worm. The morphological and molecular information obtained in this study provided strong support for recognizing seven valid genera in the family Gymnophallidae.

1. Introduction

The family Gymnophallidae Odhner, 1905 is a small and homogeneous taxon of marine digeneans [1,2]. Most of its members use mollusks as first intermediate hosts and, with rare exceptions, charadriiform and anseriform birds are their definitive hosts [1,3]. The metacercariae never encyst and are usually parasitic on bivalves, although they have also been reported parasitizing gastropods, brachiopods and polychaetes [1,4,5].

The present state of the taxonomy of the family Gymnophallidae is confusing, despite the attempts made by several authors to clarify it [2,3,6,7]. The family-level unclear taxonomy and status of several genera may be in part the result of both the small size of the specimens (from 300 to 600 μm length) and the difficulties to describe accurately the internal anatomy, imposed by the usually large number of eggs in adults and the massiveness of the accumulation of excretion granules in metacercariae, which strongly makes the observation of anatomical details difficult.

James [8] used the character 'presence or absence of pars prostatica' to distinguish between two subfamilies within Gymnophallidae: the Gymnophallinae, with a pars prostatica present, and the Parvatrematinae, without pars prostatica; in the latter, numerous prostatic cells surround and open directly into the genital atrium. The genera *Parvatrema* (=*Meiogymnophallus*), *Gymnophallus* (=*Paragymnophallus*), *Gymnophalloides* (=*Lacunovermis*) and *Pseudogymnophallus* were proposed by Scholz [2], who tried to stabilize the taxonomy of the family by proposing the suppression of the use of subfamilies (Gymnophallinae and Parvatrematinae) and synonymising a number of genera. A fifth genus, *Bartolius*, was erected by Cremonte [9]. Some authors

* Corresponding author at: Centro Nacional Patagónico (CONICET), Bvard. Brown 2915, U9120ACF Puerto Madryn, Chubut Province, Argentina. Tel.: +54 280 4883184; fax: +54 280 4883543.
E-mail addresses: fcremont@cenpat-conicet.gob.ar (F. Cremonte), gilardoni@cenpat-conicet.gob.ar (C. Gilardoni), smpina@icbas.up.pt (S. Pina), prodrigu@ibmc.up.pt (P. Rodrigues), ituarte@retina.ar (C. Ituarte).

http://dx.doi.org/10.1016/j.parint.2014.12.003
1383-5769/

[e.g., 10–12], continued using gymnophallid generic names disregarding the synonymies proposed by Scholz [2] and recognizing as valid the eight genera, five within the subfamily Gymnophallinae: *Gymnophallus* Odhner, 1900, *Meiogymnophallus* Ching, 1965, *Paragymnophallus* Ching, 1973, *Pseudogymnophallus* Hoberg, 1981, and *Bartolius* Cremonte, 2001, and three in the Parvatrematinae: *Parvatrema* Cable, 1953, *Lacunovermis* Ching, 1965 and *Gymnophalloides* Fujita, 1925. The diagnoses of the genera *Gymnophallus* and *Gymnophalloides* were recently amended [12,13]. Scholz [2] offered a detailed historical account of the family Gymnophallidae, emphasizing the need of reconsideration of the value of some morphological features traditionally used for the definition of genera.

Within the Gymnophallinae, *Gymnophallus* is the genus that includes most of gymnophallid species. The validity of the genus *Meiogymnophallus* was extensively discussed because of its close morphological similarity with *Gymnophallus* [e.g., 14–17]. According to Ching [3] and Lee and Chai [18], both genera differ by the presence or absence of lateral projections on the oral sucker (present in *Meiogymnophallus* and absent in *Gymnophallus*), in the form of the seminal vesicle (bi- or tripartite in *Gymnophallus* an unipartite in *Meiogymnophallus*), and by the presence in *Meiogymnophallus* of papillae in the genital pore. However, there are species described under *Gymnophallus* or *Meiogymnophallus* which show a mix of the mentioned diagnostic features. For example, *Gymnophallus australis* Szidat, 1962 have lateral projections on the oral sucker and a bipartite seminal vesicle [13]; *Meiogymnophallus minutus* (Cobbold, 1859), *Meiogymnophallus jamesoni* Bowers, 1965 and *Gymnophallus rostratus* Bartoli, 1982 do not have lateral projections on the oral sucker and have a unipartite seminal vesicle [15,19,20]. To increase the confusion, in fact, the genus *Meiogymnophallus* was erected by Ching [21] as a new name proposed for the genus *Gymnophalloides* as amended by James [8]. For the exposed reasons, we propose *Meiogymnophallus* as a junior synonym of *Gymnophallus*, and consider the features formerly used to differentiate between genera (i.e., presence of lateral projections on the oral sucker and form of the seminal vesicle) as mere intraspecific variations among species of a single genus.

Paragymnophallus, a genus proposed by Ching [22], is most similar to *Gymnophallus*, only differing in the genital pore, which is conspicuous and located at some distance from the anterior margin of the ventral sucker in the former and inconspicuous and located at the anterior margin of the ventral sucker in the latter. *Pseudogymnophallus* is a monotypic genus and can be differentiated from the other genera by the presence of caecal pockets and the position of the ovary (between testes), a set of characters not present in any other gymnophallid. *Bartolius*, also a monotypic genus, is characterized by the position of the ovary, posterior to testes, a character unique among gymnophallids.

Within Parvatrematinae, *Parvatrema* is distinguished by the absence of a ventral pit, a structure present in the two other genera in the subfamily: *Gymnophalloides* and *Lacunovermis*, which can be differentiated by the size of the genital pore, inconspicuous and located at the anterior margin of the ventral sucker in *Gymnophalloides* and conspicuous and located at some distance from the anterior margin of the ventral sucker in *Lacunovermis*. The generic diagnosis of *Gymnophalloides* was recently amended, incorporating as a diagnostic character the absence of a pars prostatica, for which it was reassigned to the subfamily Parvatrematinae [12].

In the present work, the revised diagnosis of each of the seven genera considered valid and dichotomous key for genera are provided. In addition, molecular information obtained from of well-described species was considered to support the results of the morphological review.

2. Materials and methods

Specimens used for the morphological and molecular studies were obtained from the host and type localities. They were studied alive when possible and/or through stained in toto specimens and histological seriated sections (cases of *G. australis*, *Bartolius pierrei*, *Parvatrema* sp., and *Gymnophalloides nacellae*, and *Lacunovermis macomae*). In other cases, specimens were requested from museum collections and studied under light microscope; in these cases, only morphological study was possible (*Paragymnophallus kinsellai* and *Pseudogymnnophallus alcae*). Specimens of species of the two genera having a ventral pit (*L. macomae* and *G. nacellae*) and one species of a genus with a wide genital pore (*Parvatrema* sp.) were fixed in 10% formaline, postfixed in Bouin's fixative and embedded in resin (Historesin Leica®). Serial transverse and sagittal histological sections (3.5 μm thick) were obtained. In addition, for morphological studies we used adult or infective (completely developed) metacercariae. Moreover, on each diagnosis we stated clearly which character varies with age and how.

Specimens for molecular analysis study were preserved in ethanol 70%. DNA was extracted using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, St. Louis, Missouri) according to the manufacturer's instructions. The ITS1, 5.8S, ITS2 and 28S regions of the rDNA were amplified by PCR. PCRs were performed in a total volume of 50 μl containing 10× buffer (200 mM Tris–HCl pH 8.4, 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. 2 μl of genomic DNA was used as template. The ITS1 regions were amplified 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. The ITS2 regions were amplified using as forward primer 5.8S-ITS2: 5′-GCTCGTGTGTCGATGAAGAG-3′, situated 114 bp from the 3′ end of the 5.8S gene, and as reverse primer 28S-ITS2: 5′-AGGCTTCGGTGCTGGGCT-3′, located 34 bp from the 5′ end of the conserved region of the lsrDNA. The 28S regions were amplified using as forward primer 28S-28S: 5′-GTGAATACCCGCTGAACTTAAGC-3′, situated 16 bp from the 3′ end of the conserved region of the lsrDNA, and as reverse primer 28S–28S: 5′-TCTCCTTGGTCCGTGTTTCAA-3′, located 868 bp from the 5′ end of the conserved region of the lsrDNA. The cycling conditions included an initial denaturation at 94 °C for 5 min followed by 40 cycles of 30 s at 94 °C, 30 s at 54 °C (annealing) for ITS1, 56 °C for ITS2 and 52 °C for 28S 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, USA) and sent for sequencing (Stabvida, Oeiras, Portugal). The complete ITS1, 5.8S, ITS2 and 28S region sequences have been deposited in GenBank (see the accession numbers in Table 1).

Both ITS1-5.8S-ITS2-28S rDNA strands were sequenced and concatenated alignments were performed using MAFFT software [23]. ITS1-5.8S-ITS2 sequences of *Parvatrema duboisi* (AB478508), as well as 2 outgroup species have been retrieved from GenBank for molecular and phylogenetic studies. According to Olson et al. [24], the superfamily Gymnophalloidea, which include the species under analysis, together with the superfamily Bucephaloidea, form the suborder Bucephalata. *Dollfustrema hefeiensis* (EF198238) and *Dollfustrema vaneyi* (EF198216) were selected as outgroup, since these two species belong to the superfamily Bucephaloidea.

Phylogenetic and molecular evolutionary analyses were conducted on the aligned nucleotide sequences of ITS1-5.8S-ITS2-28S and were inferred by both maximum-likelihood (ML) method using MEGA6 [25] and by Bayesian inference (BI) using BEAST v1.8.0 [26]. To determine the evolution model that gave best fit to our dataset, the program jModeltest 2.1.1 [27] was employed, with model selection based on the Akaike information criterion (AIC). Results indicated that the general time reversible model with an estimate of gamma distributed

Table 1
Specimens of the family Gymnophallidae sequenced, host, locality, number of specimens DNA processed (n), collection number of deposited specimens, accession GenBank number, and reference. Abbreviations: CNP-Par: Parasitology Collection of Centro Nacional Patagónico, Puerto Madryn, Argentina; SNU: Seoul National University, Republic of Korea.

Species	Host	Locality	n	Collection #	GenBank	Reference
Gymnophallus australis Szidat, 1962	*Perumytilus purpuratus* (Bivalvia, Mytilidae)	Puerto Deseado (47°45′S, 65°51′W), Argentina	46	CNP-Par 14	KM246854	[13]
Gymnophallus choledochus Odhner, 1900	*Cerastoderma edule* (Bivalvia, Cardiidae)	S. Jacinto Channel (40°30′N, 8°43′W), Aveiro estuary, Portugal	60	CNP-Par 41	KM268112	[33,34]
Gymnophallus minutus (Cobbold, 1859)	*Cerastoderma edule* (Bivalvia, Veneridae)	S. Jacinto Channel (40°30′N, 8°43′W), Aveiro estuary, Portugal	50	CNP-Par 40	KM268111	[15]
Bartolius pierrei Cremonte, 2001	*Darina solenoides* (Bivalvia, Mactridae)	Fracasso Beach (42°25′S, 64°07′W), San Jose Gulf, Argentina	22	CNP-Par 1, 12	KM246855	[9]
Parvatrema sp.	*Tagelus plebeius* (Bivalvia, Psammobiidae)	Mar Chiquita (37°46′S, 57°27′W), Buenos Aires, Argentina	48	CNP-Par 38	KM246856	[35]
Gymnophalloides nacellae Cremonte, Pina, Gilardoni, Rodrigues and Ituarte, 2013	*Nacella* (*Patinigera*) *magellanica* (Gastropoda, Patellidae)	Conejo Island (54°49′S, 68°13′W) Beagle Channel, Argentina	50	CNP-Par 50,51	KM246857	[12]
Gymnophalloides seoi Lee, Chai and Hong, 1993	*Crassostrea gigas* (Bivalvia, Ostreidae)	Shinan-gun in Jeonnam-do Province, Republic of Korea, (34°45′N, 126°08′E)	60	SNU 9225	KM246858	[36]
Lacunovermis macomae (Lebour, 1908)	*Macoma balthica* (Bivalvia, Mactridae)	Tvärminne Zoological Station (59°50′N, 23°15′E), southwestern Finland	55	CNP-Par 37	KM246859	[21,30,32]

among-site rate variation (GTR + G) was the most appropriate. For ML analyses, nodal support was estimated from 500 bootstrap resamplings. The resulting trees were rooted with the outgroup taxon.

Distance matrices (p-distance model, i.e. percentage of pairwise character differences with pairwise deletion of gaps) were also calculated with MEGA6. Evolutionary analyses were

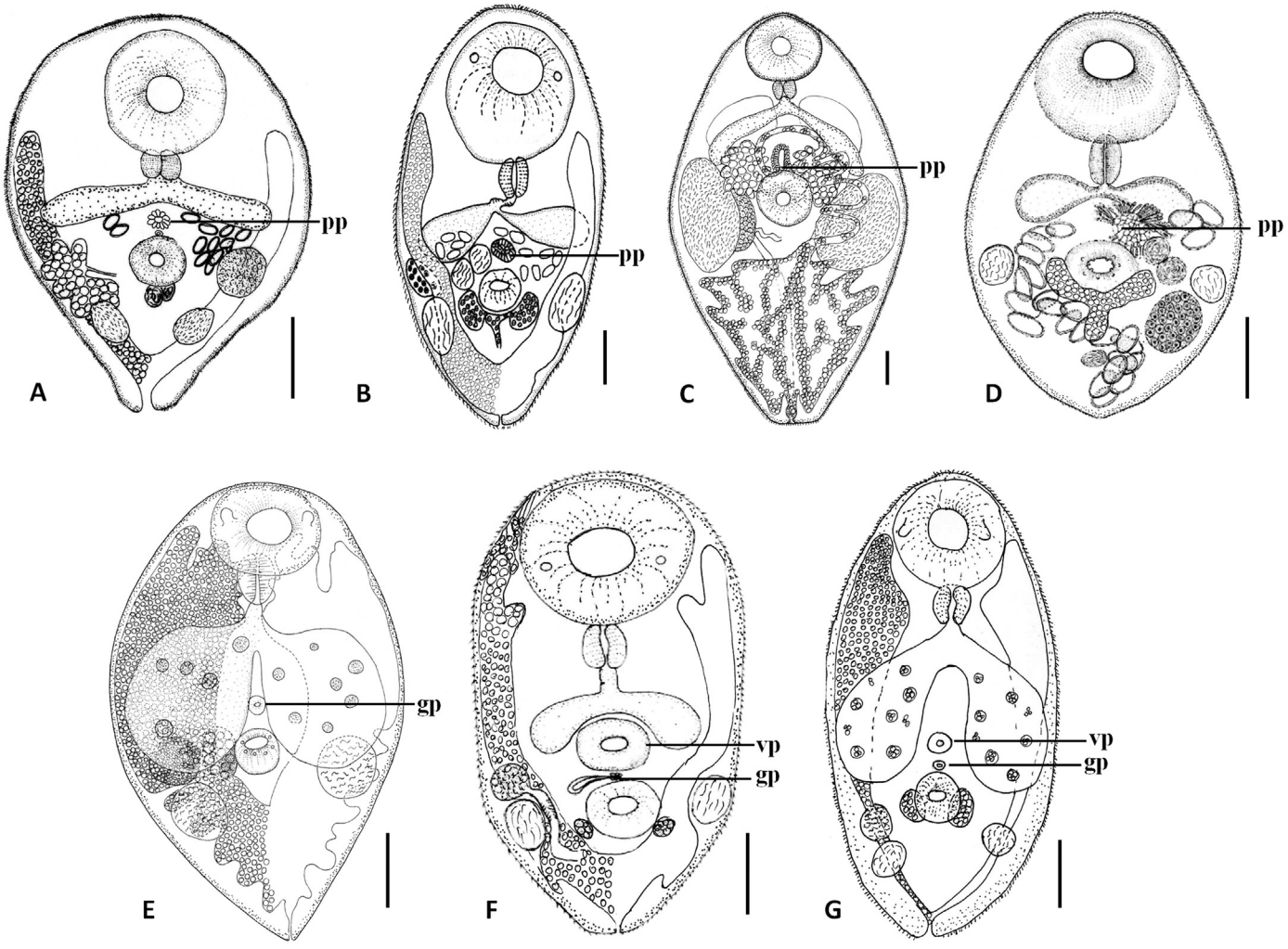

Fig. 1. Schematic drawings of a representative species of each valid genera of Gymnophallidae. A. Adult of *Paragymnophallus kinsellai* Ching, 1995, ventral view (holotype HWML 38347). B. Adult of *Gymnophallus australis* Szidat, 1962, ventral view. C. Adult of *Pseudogymnophallus alcae* Hoberg, 1981, ventral view (paratype USNPC 101575. 0). D. Adult of *Bartolius pierrei* Cremonte, 2001, dorsal view. E. Metacercaria of *Parvatrema* sp., ventral view. F. Metacercaria of *Gymnophalloides nacellae* Cremonte, Pina, Gilardoni, Rodrigues, Chai and Ituarte, 2013, ventral view (cephalic glands of the left side omitted). G. Metacercaria of *Lacunovermis macomae* (Lebour, 1908), ventral view. Abbreviations: gp, genital pore; pp, pars prostatica; vp, ventral pit. Excretory granules represented only on the right side of the excretory vesicle (A, B, E, G). Scale bars: 50 μm.

conducted using the Tajima-Nei model [28] and involved 5 nucleotide sequences with a total of 2322 positions in the final dataset.

3. Results

3.1. Revision of genera diagnoses

Family Gymnophallidae Odhner, 1905
(Figs. 1–4)
Type genus: *Gymnophallus* Odhner, 1900
Diagnosis. Body minute to small, oval to pyriform. Spiny tegument with simple, serrated, or sharply ridged spines covering the entire body surface except for small areas usually around ventral sucker and genital pore. Oral sucker subterminal, larger than ventral sucker, sometimes twice the size of ventral sucker, with or without retractile lateral projections (lips). Prepharynx absent; pharynx well developed; esophagus very short. Intestine bifurcate, caeca widely divergent, short, not reaching to midbody (caeca are larger in metacercariae), with or without dorsal diverticula (also called caecal pockets). Ventral sucker located in middle or posterior third of body. Seminal vesicle unipartite or bipartite. Pars prostatica present or absent (prostatic cells opening into genital atrium). Cirrus and cirrus sac absent. Ejaculatory duct joining the metraterm to form the hermaphrodite duct; genital atrium tubular or oval. Genital pore medial, inconspicuous and located at the anterior margin of ventral sucker, or conspicuous and located at some distance from anterior margin of ventral sucker and sometimes surrounded by muscle fibers or papillae. Ovary smooth, usually pre-testicular, rarely inter-testicular or post-testicular. Two testes, symmetrically or obliquely arranged to each other, located posterolateral to ventral sucker. Laurer′s canal present. Vitellaria paired or single, compact or follicular, located usually at sides or slightly posterior to ventral sucker. Seminal receptacle present or absent. Uterus forming loops in forebody, hindbody or both, usually in the anterior two thirds of body. Eggs small, operculate, embryonated. Excretory vesicle V or Y-shaped, sometimes Y with short stem, with long lateral arms reaching the pharyngeal level, with or without diverticula. Excretory collecting duct short, ciliated; flame cells in doublets, in number of either 16 or 24 in total. Excretory pore terminal. Parasites of gall-bladder, bursa Fabricii or intestine of charadriiform and anseriform birds; occasionally found in mammals (including humans).

Paragymnophallus Ching, 1973
(Fig. 1A)
Type species: *Paragymnophallus odhneri* Ching, 1973.
Diagnosis. Body small, oval to pyriform or fusiform, spinose. Oral sucker subterminal, large, without lateral projections. Prepharynx absent, pharynx well developed. Caeca short, without dorsal diverticula. Ventral pit absent. Ventral sucker smaller than oral sucker, located in middle or posterior third of body. Seminal vesicle bipartite. Pars prostatica present. Genital pore conspicuous and located at some distance from the anterior margin of ventral sucker. Ovary pre-testicular. Testes symmetrical or diagonal in hindbody. Vitellaria single, follicular, located posterolateral to ventral sucker. Seminal receptacle present or absent. Uterus in fore- and/or hindbody. Excretory vesicle Y-shaped, with long stem, reaching pharyngeal level.
Other species: *P. kinsellai* Ching, 1995.
Gymnophallus Odhner, 1900
(Figs. 1B, 2A, 3A)
Syn. *Meiogymnophallus* Ching, 1965.
Type species: *Gymnophallus deliciosus* (Olsson, 1893).
Diagnosis. Body small, oval, spinose. Oral sucker subterminal, large, with or without lateral projections. Prepharynx absent, pharynx well developed. Caeca short, without dorsal diverticula. Ventral pit absent. Ventral sucker smaller than oral sucker, located in middle or posterior third of body. Seminal vesicle unipartite or bipartite. Pars prostatica present. Genital pore inconspicuous and located at the anterior margin of ventral sucker. Ovary pre-testicular. Testes symmetrical or diagonal in hindbody. Vitellaria usually paired, sometimes single, compact or follicular, located close to ventral sucker. Seminal receptacle present or absent. Uterus in fore-, mid- and/or hindbody. Excretory vesicle Y or V-shaped, reaching pharyngeal to oral sucker level.
Other species: *Gymnophallus minutus* (Cobbold, 1859) (syn. *Gymnophallus cambrensis* Cole, 1938, *Gymnophallus margaritarum* (Dubois, 1901)); *Gymnophallus somateriae* (Levinsen, 1881) (syn: *Gymnophallus gibberosus* Loos-Frank, 1971); *Gymnophallus bursicola* Odhner, 1900 (syn. *Gymnophallus bilis* Brinkmann, 1956); *Gymnophallus margaritae* (Lebour, 1907); *Gymnophallus glandosa* (Lebour, 1908); *Gymnophallus oedemiae* Jameson and Nicoll, 1913; *Gymnophallus micropharyngeus* (Lüehe, 1898); *G. deliciosus* (Olsson, 1893); *Gymnophallus choledochus* Odhner, 1900 (syn. *Gymnophallus*

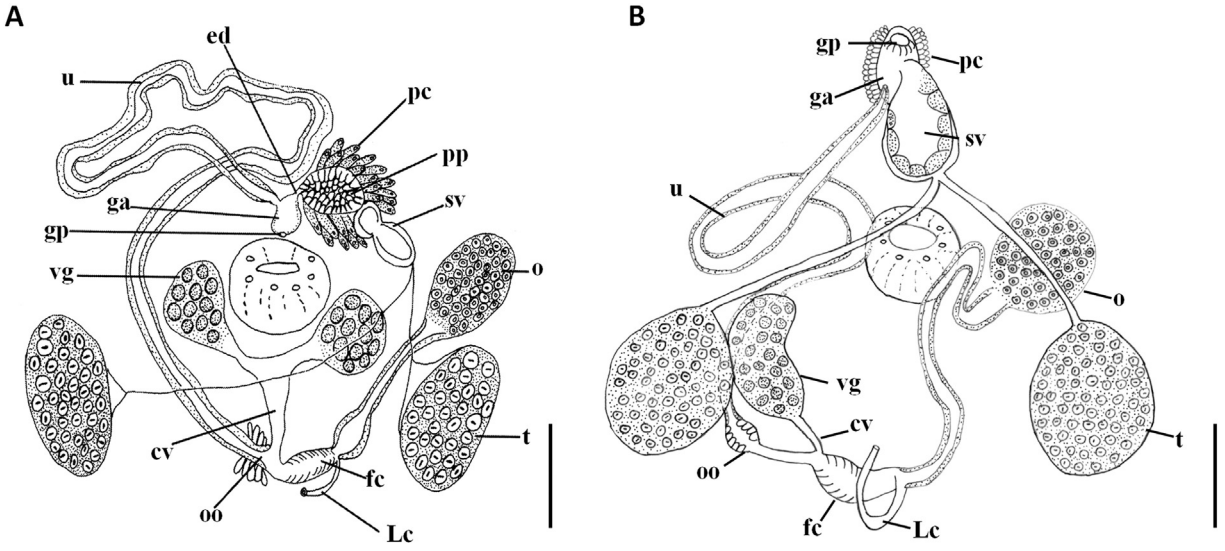

Fig. 2. Schematic drawing of the genital system of infective metacercariae as an example of the character used to distinguish between the subfamilies Gymnophallinae and Parvatrematinae. A. *Gymnophallus australis* Szidat, 1962, pars prostatica present, ventral view. B. *Parvatrema* sp., prostatic cells open into genital atrium, ventral view. Abbreviations: cv, common vitelline duct; ed, ejaculatory duct; fc, fertilization chamber; ga, genital atrium; gp, genital pore; Lc, Laurer's canal; o, ovary; oo, oötype; pc, prostatic cells; pp, pars prostatica; sv, seminal vesicle; t, testis; u, uterus; vg, vitelline gland. Scale bars: 50 μm.

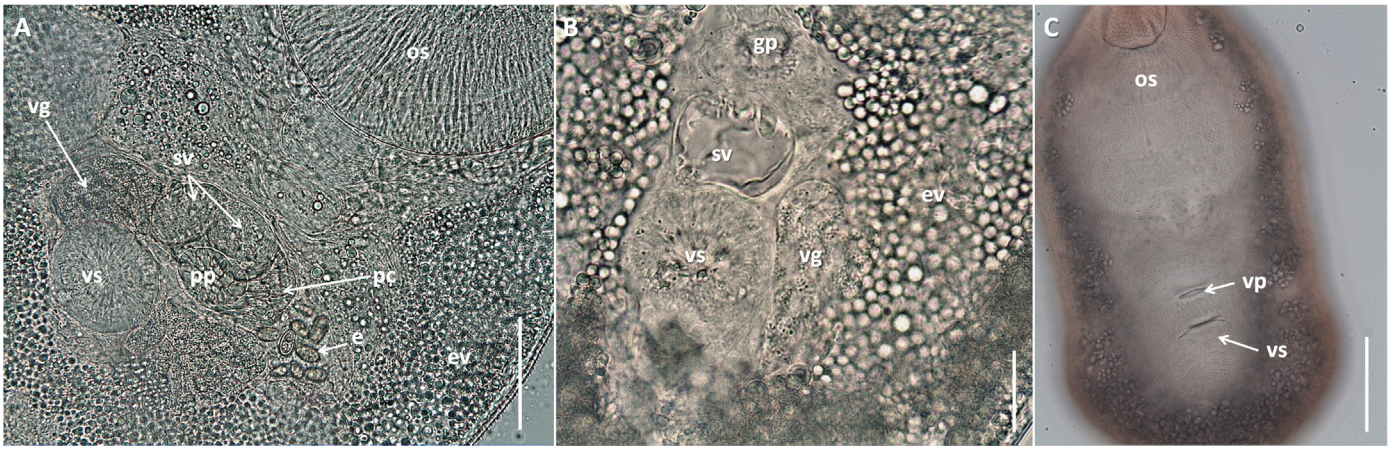

Fig. 3. Photographs of living gymnophallid specimens showing examples of diagnostic characters. A. Young adult of *Gymnophallus australis* Szidat, 1962, pars prostatica, ventral view. B. Metacercaria of *Parvatrema* sp., genital pore conspicuous, dorsal view. C. Metacercaria of *Gymnophalloides nacellae* Cremonte, Pina, Gilardoni, Rodrigues, Chai and Ituarte, 2013, ventral pit, ventral view. Abbreviations: e, egg; ev, excretory vesicle; gp, genital pore; os, oral sucker; pp, pars prostatica; sv, seminal vesicle (bipartite in A and unipartite in B); vg, vitelline gland; vs, ventral sucker; vp, ventral pit. Scale bars: 50 μm (A, C), 20 μm (B).

fulbrighti (Hutton, 1952)); *G. bursicola* Odhner, 1900; *Gymnophallus dapsilis* Nicoll, 1907; *Gymnophallus strigatus* Lebour, 1908; *Gymnophallus ovoplenum* James and Nicoll, 1913; *Gymnophallus affinis* Jameson and Nicoll, 1913 (syn.: *Gymnophallus multigemulus* (Ching, 1965)); *Gymnophallus tapetis* (Fujita in Dollfus, 1925); *Gymnophallus macrostomus* Yamaguti, 1939; *Gymnophallus perligena* Palombi, 1940; *Gymnophallus obscurus* Ching, 1960; *Gymnophallus nereicola* Rebecq and Prévot, 1962; *G. australis* Szidat, 1962; *Gymnophallus minor* Ryschikov, 1963; *Gymnophallus skrjabini* Ryschikov, 1963; *Gymnophallus ceratostomus* Zimbaljuk and Leonov, 1963; *Gymnophallus jamesoni* (Bowers, 1965); *Gymnophallus fossarum* Bartoli, 1965; *Gymnophallus charadrii* Kulachkova, 1966; *G. rostratus* Bartoli, 1982; *Gymnophallus rebecqui* Bartoli, 1983; *Gymnophallus japonicus* Rybakov, 1984; *Gymnophallus sinovaculae* (Chai, Han, Choi, Kim, Guk, Shin and Lee, 2007).

Pseudogymnophallus Hoberg, 1981

(Fig. 1C)

Type and only species: *P. alcae* Hoberg, 1981.

Diagnosis. Body small, oval to pyriform, spinose. Oral sucker subterminal, large, without lateral projections. Prepharynx absent, pharynx well developed. Caeca long, extending into posterior third of body; arch of each cecum with dorsal diverticula. Ventral pit absent. Ventral sucker smaller than oral sucker, located in middle third of body. Seminal vesicle slightly bipartite. Pars prostatica present. Genital pore inconspicuous and located at the anterior margin of ventral sucker. Ovary, submedian, inter-testicular. Testes symmetrical or diagonal in hindbody. Vitelllaria paired, located anterolateral to ventral sucker. Seminal receptacle absent. Uterus in hindbody. Excretory vesicle Y-shaped, reaching pharyngeal level.

Bartolius Cremonte, 2001

(Fig. 1D)

Type and only species: *B. pierrei* Cremonte, 2001.

Diagnosis. Body small, oval, spinose. Oral sucker subterminal, large without lateral projections. Prepharynx absent, pharynx well developed. Caeca short, without dorsal diverticula. Ventral pit absent. Ventral sucker about half size of oral sucker, located in middle third of body. Seminal vesicle bipartite. Pars prostatica present. Genital pore inconspicuous and located at the anterior margin of ventral sucker. Ovary post-testicular. Testes symmetrical or diagonal in hindbody. Vitellaria paired, compact, located close to

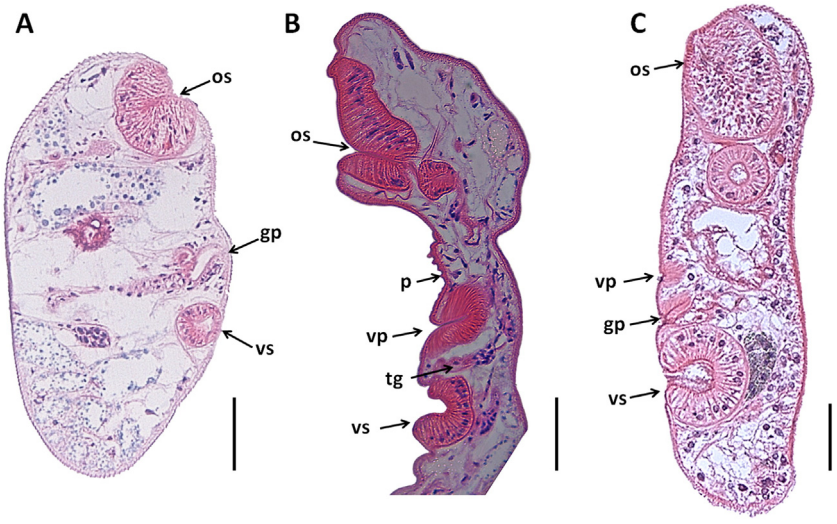

Fig. 4. Histological sections of species representing the 3 genera the subfamily Parvatrematinae. A. Metacercaria of *Parvatrema* sp. showing a conspicuous genital pore located anterior at certain distance of the anterior edge of the ventral sucker and absence of ventral pit. B. Metacercaria of *Gymnophalloides nacellae* Cremonte, Pina, Gilardoni, Rodrigues, Chai and Ituarte, 2013, showing an inconspicuous genital pore located at the edge of the ventral sucker and a well developed ventral pit. C. Metacercaria of *Lacunovermis macomae* (Lebour, 1908), showing a conspicuous genital pore located anterior at certain distance of the anterior edge of the ventral sucker and surrounded by fiber muscle and a poor developed ventral pit. Abbreviations: gp, genital pore; os, oral sucker; p, papillae; tg, terminal genitalia; vs, ventral sucker; vp, ventral pit. Scale bars: 50 μm.

ventral sucker. Seminal receptacle present. Uterus in fore- and hindbody. Excretory vesicle V-shaped, reaching pharyngeal to oral sucker level.

Parvatrema Cable, 1953

(Figs. 1E, 2B, 3B, 4A)

Type species: *Parvatrema borinqueñae* Cable, 1953.

Diagnosis. Body small, oval, spinose. Oral sucker subterminal, large, usually with lateral projections. Prepharynx absent, pharynx well developed. Caeca short, without dorsal diverticula. Ventral pit absent. Ventral sucker in middle to posterior third of body. Seminal vesicle usually unipartite, sometimes bipartite. Pars prostatica absent, prostatic cells opening into genital atrium. Genital pore conspicuous, wide, and located at some distance from the anterior margin of ventral sucker. Ovary post-testicular. Testes symmetrical or diagonal in hindbody. Vitellaria usually single, sometimes paired, follicular or compact, located close to ventral sucker. Seminal receptacle present or absent. Uterus in fore- and hindbody. Excretory vesicle V or Y-shaped, reaching pharyngeal to oral sucker level.

Other species: *P. duboisi* (Dollfus, 1923) (syn. *Gymnophallus perla* (Sinitzin, 1911); *G. bursicola* Odhner, 1900; *Parvatrema timondavidi* Bartoli, 1963; *Parvatrema lintoni* (Linton, 1928) (syn. *Distomum* Linton, 1928); *P. ovoplenum* (Jameson and Nicoll, 1913); *Parvatrema borealis* Stunkard and Uzmann, 1958*; Parvatrema donacis* Hopkins, 1958; *Parvatrema homoeotecnum* James, 1964; *Parvatrema isostoma* Belopolskaja, 1966; *Parvatrema rebunense* Shimazu, 1975; *Parvatrema bushi* Ching, 1995; *Parvatrema polymesoda* Ching, 1995; *Parvatrema chaii* Sohn, Na, Ryang, Ching and Lee, 2007; *Parvatrema margaritense* (Ching, 1982).

Gymnophalloides Fujita, 1925

(Figs. 1F, 3C, 4B)

Type species: *Gymnophalloides tokiensis* Fujita, 1925.

Diagnosis. Body small, oval to pyriform, spinose. Oral sucker subterminal, large, with lateral projections. Prepharynx absent, pharynx well developed. Caeca short, without dorsal diverticula. Ventral pit present, well developed, similar in size to ventral sucker. Ventral sucker smaller than oral sucker, located in posterior third of body. Seminal vesicle unipartite or bipartite. Pars prostatica absent, prostatic cells opening into genital atrium. Genital pore inconspicuous and located at the anterior margin of ventral sucker. Ovary pre-testicular. Testes symmetrical or diagonal in hindbody. Vitellaria single or paired, follicular or compact, located close to ventral sucker. Seminal receptacle present or absent. Uterus in forebody. Excretory vesicle V or Y-shaped, reaching pharyngeal to oral sucker level.

Other species: *Gymnophalloides seoi* Lee, Chai and Hong, 1993; *Gymnophalloides heardi* Ching, 1995; *G. nacellae* Cremonte, Pina, Gilardoni, Rodrigues and Ituarte, 2013.

Lacunovermis Ching, 1965

(Figs. 1G, 4C)

Type and only species: *L. macomae* (Lebour, 1908) (syn. *Lacunovermis conspicuus* (Ching, 1965) and *Lacunovermis macroporus* (Jameson and Nicoll, 1913)).

Diagnosis. Body small, oval to pyriform, spinose. Oral sucker large, with lateral projections. Prepharynx absent, pharynx well developed. Caeca short, without dorsal diverticula. Ventral pit present, poorly developed, represented by a slight invagination of the ventral body surface, smaller that ventral sucker. Ventral sucker smaller than oral sucker, located at middle to posterior third of body. Seminal vesicle unipartite. Pars prostatica absent, prostatic cells opening into genital atrium. Genital pore conspicuous and located at some distance to anterior margin of ventral sucker, usually with papillae. Ovary pre-testicular. Testes symmetrical or diagonal in hindbody. Vitellaria paired, compact, close to ventral sucker. Seminal receptacle present or absent. Uterus in fore- and/or hindbody. Excretory vesicle Y or V-shaped, reaching the pharyngeal level.

4. Taxonomic keys

Key to subfamilies

1. Prostatic cells arranged into a pars prostatica……..…… ……**Gymnophallinae**

1′. Pars prostatica absent (prostatic cells opening into genital atrium) ……. **Parvatrematinae**

Key to genera of Gymnophallinae

1. Ovary pre-testicular…….…… ……...…….. 2

1′. Ovary inter-testicular or post-testicular ……..…… ……...……..3

2. Genital pore conspicuous and located at some distance anterior to ventral sucker……..…… ……..…….. *Paragymnophallus*

2′. Genital pore inconspicuous and located at the anterior margin of ventral sucker..…….…… ……. *Gymnophallus*

3. Ovary inter-testicular, caeca with diverticula ……..…..... *Pseudogymnophallus*

3′. Ovary post-testicular…… ……. *Bartolius*

Key to genera of Parvatrematinae

1. Ventral pit present……..…… ……. *Parvatrema*

1′. Ventral pit absent…… ……...2

2. Ventral pit well developed (similar in size than ventral sucker), genital pore inconspicuous and located at the anterior margin of ventral sucker..……...……. *Gymnophalloides*

2′. Ventral pit poorly developed (as an invagination of the ventral surface, smaller in size than the ventral sucker), genital pore conspicuous and located at some distance from the anterior margin of ventral sucker …..……...…..……..…….. *Lacunovermis*

5. Molecular phylogenetic analyses

For the majority of gymnophallid species, the ITS1-5.8S-ITS2-28S nucleotide sequences obtained gave single products such as 2210 bp for *G. australis* and *G. minutus*, 2137 bp for *Parvatrema* sp., 2141 for *G. nacellae*, 2171 bp for *G. choledochus*, 2199 for *G. seoi* and 2157 for *L. macomae*. In the particular case of *B. pierrei*, the 28S region could not be sequenced and the ITS1-5.8S-ITS2 sequence gave a single product of 1257 bp. For *P. duboisi* and the outgroup species *D. hefeiensis* and *D. vaneyi*, only the ITS1-5.8S-ITS2 sequences were available in GenBank, from which single products of 1394 bp and 1452 bp were respectively retrieved.

Maximum-likelihood (ML) and Bayesian Inference (BI) analyses resulted in trees with the same topology (Fig. 5). Moreover, both analyses revealed the presence of two clades (posterior probability BI: 0.97; bootstrap ML: 61%) corresponding with the two subfamilies: Gymnophallinae, including *G. australis*, *G. minutus* and *G. choledochus*; and Parvatrematinae, including *G. nacellae*, *G. seoi*, *Parvatrema* sp., *P. duboisi* and *L. macomae*. Furthermore, phylogenetic trees support the synonymy between the genera *Gymnophallus* and *Meiogymnophallus*, since *G. australis* and *G. minutus* (syn. *M. minutus*) species formed a highly supported clade (pp BI: 1.00; bootstrap ML: 100%), both also grouping with *G. choledochus* (pp BI: 1.00; bootstrap ML: 84%).

It is noteworthy that *L. macomae* clustered with *Parvatrema* spp. (*Parvatrema* sp. and *P. duboisi*), forming a strongly supported clade both in BI and ML analyses (Fig. 5). Moreover, the sequence of *L. macomae* differed from that of *P. duboisi* and *Parvatrema* sp. by 0.16%, a value much lower when compared to 0.22–0.25% divergence in relation to sequences of *Gymnophalloides* spp. (Table 3).

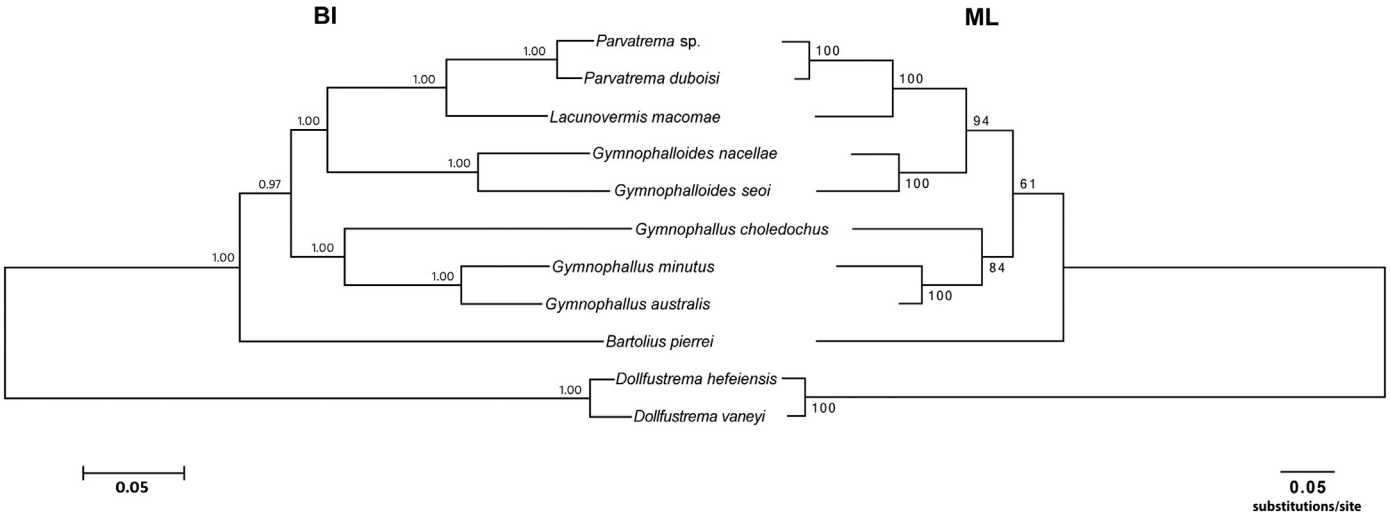

Fig. 5. Phylogenetic trees resulting from the Bayesian Inference (BI, left) and maximum-likelihood (ML, right) analyses using 18S-ITS1-5.8S-ITS2-28S sequences of species of Gymnophallidae (at least one available species from each genus). BI nodal support is indicated as posterior probabilities and ML nodal numbers represent bootstrap values (%, n = 500 replicates). Scale bar indicates substitutions/site.

6. Discussion

This study intends to clarify the taxonomy of the Gymnophallidae family by reviewing previous classifications, reevaluating some morphological characters and incorporating molecular information. Both morphological and molecular data revealed the presence of two subfamilies: Gymnophallinae, including *G. australis*, *G. minutus*, and *G. choledochus*; and Parvatrematinae, including *G. nacellae*, *G. seoi*, *Parvatrema* sp., *P. duboisi*, and *L. macomae*.

The species *B. pierrei* appear as a separate clade, probably reflecting the strong morphological difference (ovary post-testicular), a character unique among gymnophallids. However, we consider the molecular data as not strong enough to warrant erecting a new subfamily.

We proposed *Meiogymnophallus* as a junior synonym of *Gymnophallus* based on their close morphological features and molecular data from the studied gene sequences. *Meiogymnophallus* was created by Ching [21], with *M. multigemmulus* as the type species. Bowers et al. [17] placed *Gymnophallus rebequi* Bartoli, 1983 within the genus *Meiogymnophallus* based on "the location of the adult parasite in the gut of the final host, the nature of genitalia and flame-cell formula". In this asseveration, there are no clear or specific characters to justify the separation of both genera. Other authors based a similar opinion in the presence of lateral projections in the oral sucker; however, there are species described in both genera which present a combination of the mentioned features [13,15,19,20].

Based on the presence of a ventral pit in *Gymnophalloides* and *Lacunovermis*, which is absent in other gymnophallid genera, Yamaguti [29] and Scholz [2] considered the two genera as synonyms. Ching [3] refused this synonymy, reaffirming that the primary difference between the two genera is in the shape and location of the genital pore. Indeed, molecular data support the opinion that *Gymnophalloides* and *Lacunovermis* are different. This fact leads us to re-examine the so called "ventral pit" in both genera as well as its significance as a diagnostic character. In *Lacunovermis* (with only one species, *L. macomae*, recognized as valid), the ventral pit was illustrated in detail (Figs. 1a, b, 5a, d) by Pekkarinen [30] and described as "an invagination of the ventral surface", "a transverse slit with a narrow lumen smaller in size than the ventral sucker" [30,31]. It was corroborated by our examinations of stained whole mounted specimens and histological sections from newly collected specimens. In our opinion, it should be considered a "poorly developed ventral pit", in comparison with the "well developed ventral pit" present in *Gymnophalloides*, similar in size and musculature development to the ventral sucker (see Lee and Chai [18] and Cremonte et al. [12]). Evidence from histological sections of metacercariae of *G. seoi* and *G. nacellae* attached to the mantle host suggest that the ventral pit would not function as an accessory sucker (Figs. 4B, C). Molecular data showed that *L. macomae* nestled with *Parvatrema* spp., not with *G. nacellae*, a topology which originated in the fact that the size of the genital pore would be a morphological character stronger than the presence of a ventral pit.

In this revision, we clarified which characters should be used to differentiate genera (Table 2) and which should be regarded as diagnostic at species level within a genus. The presence of lateral projections in the oral sucker, the seminal vesicle form (uni- or bipartite), and the vitellaria shape (single or paired, compact or follicular) appears to be variable characters among gymnophallid species. In addition, regarding the shape of the vitellaria, sometimes there is no clear difference between the follicular and compact arrangements currently observed in digeneans; the vitellaria may appear as more or less follicular, and it can be arranged in either one (single) or two lobes (paired) at sides of the ventral sucker. The shape of the excretory vesicle (Y- or V-shaped), is a confusing character because the stem of a Y-shaped vesicle

Table 2
Genera of the family Gymnophallidae Odhner, 1900. In the cases in which the genital pore is inconspicuous, it is located at the anterior margin of the ventral sucker; and when the genital pore is conspicuous it is wide, sometimes surrounded by muscular fibers or papillae and it is located at some distance from the anterior margin of ventral sucker.

Character/genus	*Gymnophallus* Odhner, 1900 (syn. *Meiogymnophallus* Ching, 1965)	*Paragymnophallus* Ching, 1973	*Pseudogymnophallus* Hoberg, 1981	*Bartolius* Cremonte, 2001	*Parvatrema* Cable, 1953	*Gymnophalloides* Fujita, 1925	*Lacunovermis* Ching, 1965
Pars prostatica	Present	Present	Present	Present	Absent	Absent	Absent
Ventral pit	Absent	Absent	Absent	Absent	Absent	Present	Present
Ovary position	Pre-testicular	Pre-testicular	Inter-testicular	Post-testicular	Pre-testicular	Pre-testicular	Pre-testicular
Caecal pockets	Absent	Absent	Present	Absent	Absent	Absent	Absent
Genital pore	Inconspicuous	Conspicuous	Inconspicuous	Inconspicuous	Conspicuous	Inconspicuous	Conspicuous

Table 3
Pairwise nucleotide sequence comparisons between *Lacunovermis macomae*, *Parvatrema* spp. and *Gymnophalloides* spp. calculated as percentage of nucleotide differences (gaps treated as missing data) for the aligned ITS1-5.8S-ITS2-28S sequences (n = 2322 bp).

No.		1 *L. macomae*	2 *P. duboisi*	3 *Parvatrema* sp.	4 *G. nacellae*	5 *G. seoi*
1	*L. macomae*					
2	*P. duboisi*	0.16				
3	*Parvatrema* sp.	0.16	0.042			
4	*G. nacellae*	0.22	0.33	0.24		
5	*G. seoi*	0.25	0.37	0.26	0.11	

may be extremely short in some cases, resembling a V-shaped one; for this, it should be regarded as a character to differentiate species.

Frequently, it is possible to study gymnophallids from metacercariae obtained from their molluscan host because they have all genital organs developed (including the uterus) and are more easily obtained than adults from birds. For that reason, it is necessary considering that some structures can vary with the developmental stage (young or infective metacercaria) and with the age of the adult worms. For example, metacercaria are usually bigger than the adult stage; caeca are bigger and full of material in metacercaria and smaller and empty in the adult, spines are usually sharp in cercaria and change to serrated in adult, oral sucker grows from cercaria to adult stage but ventral sucker remains of similar size (then, the oral-ventral suckers ratio change); also, the form of the vitellaria has been reported as varying with the age of the specimens. The extent of the uterus (i.e. in the fore- or hindbody) also varies with the age of the specimens (in young adults, the first eggs appear in the terminal part of the uterus). Data about the development from metacercaria to adult given above are mainly from *L. macomae* [30,31], *B. pierrei* [9], and *G. australis* [13].

In summary, the character presence or absence of a pars prostatica is used to separate two subfamilies: Gymnophallinae (with pars prostatica) and Parvatrematinae (without pars prostatica). The main unambiguous characters proposed here as useful for distinguishing among the seven gymnophallid genera considered valid, are the position of the ovary (pre-, post- or inter-testicular), the presence or absence of a ventral pit and its degree of development, the size and location of the genital pore (inconspicuous and located at the margin of ventral sucker or conspicuous and located at some distance from the ventral sucker) and the presence or absence of caecal diverticula (also called caecal pockets).

Gymnophallus (=*Meiogymnophallus*) is the more specious genus with about 27 valid species, followed by *Parvatrema*, with about 14 valid species, and the remaining only have two or four (*Paragymnophallus* and *Gymnophalloides*, respectively) or one (*Pseudogymnophallus*, *Bartolius* and *Lacunovermis*) described species. Gymnophallids should be studied "in vivo", when possible on metacercarial stage cultivating to adult stage and/or through histological serial sections. It would be desirable that from now, the new species be described with detailed morphology and providing molecular information from DNA sequences, contributing to clarify the taxonomy at family and subfamily levels.

Acknowledgments

The authors express their gratitude to Dr. Takashi Iwaki, curator at Meguro Parasitological Museum, Tokyo, to Dr. Scott Gardner, curator of the Parasitological Collection, of The Harold W. Manter Laboratory of Parasitology (HWML) for the loan of specimens, and Dr. Eric Hoberg, curator of the National Parasite Collection, USA, (USNPC), and to Dr. Risto Vainola (Finnish Museum of Natural History, University of Helsinki) for collecting and sending specimens of the bivalve *Macoma balthica*. Special thanks are owed to Pierre Bartoli (Centre d'Océanologie de Marseille, France) for his marvelous work on gymnophallids and for his warm encouragement to undertake this revision. FC, CG and CI are members of CONICET. The present study was funded by ANPCyT (PICT2010-0730 and PICT 2013-1702). We are also indebted to FCT — Fundação para a Ciência e a Tecnologia, Ministério da Ciência e do Ensino Superior, Portugal for the grant SFRH/BPD/81377/2011 to S. Pina and Erasmus Mundus Fundation for the grant under Eurotango program to C. Gilardoni.

References

[1] Bartoli P. Recherches sur les Gymnophallidae F. N. Morozov, 1955 (Digenea) parasites d'oiseaux des côtes de Camargue: systématique, biologie et ecologie. Thèse Marseille: Université d'Aix-Marseille; 1974.
[2] Scholz T. Family Gymnophallidae Odhner, 1905. In: Gibson DI, Jones A, Bray RA, editors. Keys to the Trematoda, Vol. 1. Wallingford: CAB International; 2002. p. 245–51.
[3] Ching HL. Evaluation of characters of the family Gymnophallidae Morozov, 1955. Can J Fish Aquat Sci 1995;52(Suppl. 1):78–83.
[4] Ching HL. Four new gymnophallid digeneans from rice rats, willets, and mollusks in Florida. J Parasitol 1995;81(6):924–8.
[5] Cremonte F. Enfermedades de moluscos bivalvos de interés comercial causadas por metazoos. In: Figueras A, Novoa B, editors. Enfermedades de moluscos bivalvos de interés en Acuicultura. Spain: Observatorio Español de Acuicultura; 2011. p. 331–85.
[6] Cable RM. The life cycle of *Parvatrema borinqueñae* gen. et. sp. nov. (Trematoda: Digenea) and the systematic position of the subfamily Gymnophallinae. J Parasitol 1953;39:408–21.
[7] Stunkard HW, Uzmann JR. Studies on digenetic trematodes of the genera *Gymnophallus* and *Parvatrema*. Biol Bull 1958;115:276–302.
[8] James BL. The life cycle of *Parvatrema homoeotecnum* sp. nov. (Trematoda: Digenea) and a review of the family Gymnophallidae Morozov, 1955. Parasitology 1964;54:1–41.
[9] Cremonte F. *Bartolius pierrei* n. g., n. sp. (Digenea: Gymnophallidae) from the Península Valdés, Argentina. Syst Parasitol 2001;49:139–47.
[10] Chai JY, Han ET, Choi D, Seo M, Kim JL, Guk SM, et al. A new gymnophallid trematode from the intestine of mice infected with metacercariae from the razor clam *Sinovaculata constricta*. J Parasitol 2007;93:132–7.
[11] Fermer J, Culloty SC, Kelly TC, O'Riordan RM. Intrapopulation distribution of *Meiogymnophallus minutus* (Digenea, Gymnophallidae) infections in its first and second intermediate host. Parasitol Res 2009;105:1231–8.
[12] Cremonte F, Pina S, Gilardoni C, Rodrigues P, Chai J-Y, Ituarte C. *Gymnophalloides nacellae* n. sp. (Digenea: Gymnophallidae), and an amended diagnosis of the genus *Gymnophalloides* Fujita, 1925. J Parasitol 2013;99:85–92.
[13] Cremonte F, Vázquez N, Ituarte C. The development of *Gymnophallus australis* Szidat, 1962 (Digenea: Gymnophallidae) from the Patagonian coast (Argentina) from metacercaria to adult, with an amended diagnosis of *Gymnophallus* Odhner, 1905. Syst Parasitol 2008;69:23–31.
[14] Bartoli P. Développement expérimental d'une métacercaire nouvelle de la famille des Gymnophallidae Morozov, 1955 (Trematoda: Digenea), parasite de lamellibranches marins. Bull Soc Zool de France 1965;90:571–80.
[15] Bowers EA, James BL. Studies on the morphology, ecology and life-cycle of *Meiogymnophallus minutus* (Cobbold, 1859) comb. nov. (Trematoda: Gymnophallidae). Parasitology 1967;57:281–300.
[16] Bowers EA, Bartoli P, James BL. A demonstration of allopatric sibling species within the Gymnophallidae (Digenea). Syst Parasitol 1990;17:143–52.
[17] Bowers EA, Bartoli P, Russell-Pinto F, James BL. The metacercariae of sibling species of *Meiogymnophallus*, including *M. rebecqui* comb. nov. (Digenea: Gymnophallidae), and their effects on closely related *Cerastoderma* host species (Mollusca: Bivalvia). Parasitol Res 1996;82:505–10.
[18] Lee SH, Chai JY. A review of *Gymnophalloides seoi* (Digenea: Gymnophallidae) and human infections in the Republic of Korea. Korean J Parasitol 2001;39:85–118.
[19] Bowers EA. A description of *Meiogymnophallus jamesoni* sp. nov. (Trematoda: Gymnophallidae) from the intestine of the common scoter, *Melanitta nigra* L. Ann Mag Nat Hist 1965;8:277–83 Series 13,.
[20] Bartoli P. *Gymnophallus rostratus* n. sp. (Trematoda: Gymnophallidae) parasite de Lamellibranches marins de Camargue (France). Vie marine 1982;4:51–8.
[21] Ching HL. Life Cycles of *Lacunovermis conspicuus* n. gen., n. sp. and *Meiogymnophallus multigemmulus* n. gen., n. sp. (Gymnophallidae: Trematoda) from *Macoma inconspicua* and Diving Ducks from Vancouver, Canada. Proceedings of the Helminthological Society of Washington, 32; 1965. p. 53–63.
[22] Ching HL. *Paragymnophallus odhneri* gen. n., sp. n. (Trematoda: Gymnophallidae) for *Gymnophallus somateriae* sensu Odhner (1900, 1905). Can J Zool 1973;51:807–10.
[23] MAFFT software. available at http://www.ebi.ac.uk/Tools/msa/mafft/.
[24] Olson PD, Cribb TH, Tkach VV, Bray RA, Littlewood DTJ. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). Int J Parasitol 2003;33:733–55.
[25] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. Mega6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725–9.
[26] Drummond AJ, Rambaut A, Suchard MA. BEAST v1.8.0. Bayesian evolutionary analysis sampling trees. Department of Computer Science. University of Auckland; 2002–2013.
[27] Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods 2012;9:772.
[28] Tajima F, Nei M. Estimation of evolutionary distance between nucleotide sequences. Mol Biol Evol 1984;1:269–85.
[29] Yamaguti S. Synopsis of digenetic trematodes of vertebrates. Digenea of Bird. Tokyo: Keigaku Publishing Co; 1971.
[30] Pekkarinen M. Anatomy, histology and maturing of the metacercaria of *Lacunovermis macomae* (Trematoda: Gymnophallidae) from brackish-water

Macoma balthica (Southwestern Finland, Baltic Sea). Ann Zoologici Fennici 1984;21: 481–98.
[31] Pekkarinen M, Ching HL. Comparisons of gymnophallid digeneans from North Pacific and Baltic clams, *Macoma balthica* (Bivalvia). J Parasitol 1994;80:630–6.
[32] Pekkarinen M. Development of the cercaria of *Lacunovermis macomae* (Trematoda: Gymnophallidae) to the metacercaria in brackish-water *Macoma balthica* (Bivalvia). Ann Zoologici Fennici 1986;23:237–50.
[33] Russell-Pinto F. Alguns aspectos do parasitismo de *Cerastoderma edule* (Bivalvia) por trematodes digénios. Açoreana 1989;7:115–24.
[34] Russell-Pinto F, Gonçalves JF, Bowers E. Digenean larvae parasitizing *Cerastoderma edule* (Bivalvia) and *Nassarius reticulatus* (Gastropoda) from Ria de Aveiro, Portugal. J Parasitol 2006;92:319–32.
[35] Vázquez N, Ituarte C, Navone GT, Cremonte F. Parasites of the stout razor clam *Tagelus plebeius* (Psammobiidae) from the southwestern Atlantic Ocean. J Shellfish Res 2006;25(3):877–86.
[36] Lee SH, Chai JY, Hong ST. *Gymnophalloides seoi* n. sp. (Digenea: Gymnophallidae), the first report of human infection by a gymnophallid. J Parasitol 1993;79(5):677–80.