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Corresponding author:
L.A.R. Leite;
Email: lar.leite@unesp.br

Morphological and molecular data on *Phyllodistomum* (Digenea: Gorgoderidae) from Brazil, with the description of a new species parasitizing *Hoplias malabaricus* (Bloch, 1794) (Osteichthyes, Erythrinidae)

K.G.A. Dias[1], G. Pérez-Ponce de León[2], R.J. da Silva[1], D.H.M.D. Vieira[1], L.A.R. Leite[1], R.K. de Azevedo[3] and V.D. Abdallah[4]

[1]Departamento de Parasitologia, Universidade Estadual Paulista (UNESP), Instituto de Biociências, Botucatu, São Paulo, Brazil; [2]Escuela Nacional de Estudios Superiores Unidad Mérida, and Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad de México, Mexico; [3]Centro Universitário CESMAC, Maceió, Alagoas, Brazil and [4]Setor de Patologia e Parasitologia, Universidade Federal de Alagoas (UFAL), Instituto de Ciências Biológicas e da Saúde, Maceió, Alagoas, Brazil

Abstract

Phyllodistomum pepirense n. sp. is described from the urinary bladder of *Hoplias malabaricus* (Bloch, 1794), sampled in the Jacaré-Pepira River in São Paulo state, Brazil. The isolates of the new species were recovered as a monophyletic group in the phylogenetic analysis of the 28S rRNA gene, which showed the new species as the sister taxa of *Phyllodistomum virmantasi* Pinacho-Pinacho, Sereno-Uribe, Hernández-Orts, García-Varela & Pérez-Ponce de León, 2021, a species sampled from an eleotrid fish in Southeastern Mexico. The new species differs morphologically from *P. virmantasi* by having a larger body size, slightly lobed testes and ovary, a mostly intercaecal uterus, slightly diverticulated caeca, and vitelline masses irregularly shaped. The new species is also readily distinguished from other species of *Phyllodistomum* Braun, 1899 reported from freshwater fishes in Brazil – namely, *P. rhamdiae* Amato & Amato, 1993 and *P. spatula* Odhner, 1902. The new species is herein described based on morphological characteristics, molecular data from D1–D3 domains of the 28S rRNA gene, host association, and geographical distribution.

Introduction

Trematodes are one of the most diverse groups of parasites found among metazoans (Bray *et al.* 2009). The taxonomic history of the genus *Phyllodistomum* has been controversial and unstable due to the wide intraspecific variation of some species, making their correct identification a challenging task (Petkevičiūtė *et al.* 2014). Currently, this genus is considered one of the most diversified among trematodes because it contains more than 120 species (Pinacho-Pinacho *et al.* 2021). In this context, molecular systematic studies have proven useful to aid in species delineation and to allocate species in their proper taxonomic groupings (Pérez-Ponce de León et al. 2015). *Phyllodistomum* spp. are widely distributed in freshwater and marine environments and are found in the urinary bladder, ureters, intestine, swim bladder, and gall bladder of fishes (Campbell 2008; Mendoza-Garfias & Pérez-Ponce de León 2005).

Among teleosts, characiform fishes are mainly distributed in freshwater environments across the Neotropical region (Baumgartner *et al.* 2012) and possess a highly diverse parasite fauna. *Phyllodistomum* spp. have been reported to infect the urinary bladders of *Hoplias malabaricus* (Bloch, 1794), commonly known as 'traíra' (Oyakawa 2003), in São Francisco and Batalha River basins, Brazil (Costa *et al.* 2015; Gião *et al.* 2020). *Hoplias malabaricus* are carnivorous fish with ambush behavior. They exhibit a wide geographic distribution across the Neotropical biogeographic region; they occur in several hydrographic basins in South America (Oyakawa 2003) and their distribution also extends northwards to Costa Rica in Central America.

In this study, we describe a new species of *Phyllodistomum* from the urinary bladder of *H. malabaricus* in Brazil. The new species description is based on morphological characteristics and other sources of information such as molecular data obtained from the D1–D3 domains of the 28S rRNA gene, host association, and geographical distribution.

Materials and methods

In May 2018 and October 2021, a total of 60 specimens of *H. malabaricus* were collected from the Jacaré-Pepira River, São Paulo state, Brazil. Fish were captured with gill nets of different mesh

sizes placed at different depths. After collection, they were anesthetized with eugenol and then euthanized by spinal section. For parasite collection, all organs were removed, separated in Petri dishes, and examined for trematodes under a stereoscope. Adult trematodes were removed from the urinary bladder and fixed in hot 10% buffered formalin; some specimens were stained with clorhidric carmine and cleared in eugenol. The holotype was mounted in Permount® for morphological study. Morphological analysis and measurements of adult digeneans were made using a microscope with differential interference contrast optics (Leica DMLB 5000, Leica Microsystems). Measurements are given in micrometers (μm). Drawings were made with the aid of a microscope (Leica DMLS, Leiva Microsystems, Wetzlar, Germany) equipped with a drawing tube. Type material was deposited in the Helminthological Collection of the Institute Oswaldo Cruz, Rio de Janeiro, Brazil, with the accession numbers 'CHIOC 000000–000000'.

Some specimens were processed for scanning electron microscopy. These specimens were fixed in 70% ethanol, dehydrated in a graded alcohol series, critical-point dried with carbon dioxide, mounted on aluminum stubs using conductive double-sided tape, coated with gold-palladium, and examined with the use of a FEI Quanta 200 scanning electron microscope.

For the molecular study, some specimens were fixed in 100% ethanol. Total genomic DNA was extracted from whole worms using the Qiagen Dneasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Fragments of the D1–D3 domains of the 28S rRNA were amplified by polymerase chain reaction (PCR) with primers dig12 (5'-AAGCATATCACTAAGCGG-3') (Tkach *et al.* 2000) and reverse LSU1500R (5'-GCTATCCTGAGGGAAACTTCG-3') (Tkach *et al.* 2003). Amplification was performed in a Bio-Rad Mycycler (Bio-Rad Laboratories Pty Ltd., Gladesville, Australia) with initial denaturation at 94°C for 3 min, followed by 45 cycles of 94°C for 45 s, 54°C for 45 s, 72°C for 1:30 min and a final extension at 72°C for 10 min. PCR reactions were performed in 25 μl reactions containing 2 μl of extracted DNA and 1 μl of each PCR primer using PCR Ready-to-Go beads (Pure TaqTMReady-to-GoTM beads, GE Healthcare, Chicago, USA). The solution consisted of stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ± 2.5 units of puReTaq DNA polymerase, and reaction buffer. Each bead was reconstituted to a final volume of 25 μl. PCR products were analyzed by electrophoresis on 1% agarose gel stained with GelRed and visualized under UV light. The products of the PCR reaction for the 28S rRNA gene were purified and then sequenced with primers dig12 (5'-AAGCATATCACTAAGCGG-3') (Tkach *et al.* 2000) and reverse LSU1500R (5'- 90 GCTATCCTGAGGGAAACTTCG-3') (Tkach *et al.* 2003) with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied) and precipitation reaction by Ethanol/EDTA/Sodium Acetate, according to the protocol suggested by the manufacturer. Automatic sequencing by capillary electrophoresis was performed on the ABI3730xl DNA Analyzer (Applied Biosystems). PCR results were purified with the Qiagen purification kit before sequencing. The obtained partial sequences were assembled and edited using Sequencher 4.8 software (Gene Codes Corporation) to obtain consensus sequences. The consensus sequences were aligned with partial sequences of 28 genetically similar species obtained from GenBank using the ClustalW algorithm (Larkin *et al.* 2007) and standard settings in Geneious 7.1.3 software (Kearse *et al.* 2012).

The phylogenetic analysis of the 28S rRNA gene included the newly sequenced individuals plus 36 sequences downloaded from Genbank (Table 1). We used sequences of four species of allocreadiids (i.e., *Allocreadium lobatum* Wallin, 1909; *Creptotrematina batalhensis* Dias and Abdallah, 2020; *Wallinia brasiliensis* Dias and Abdallah, 2018; and *W. caririensis* Silva and Yamada, 2020) as outgroups. Species of this family were used because they are the sister taxon of gorgoderids (Choudhury *et al.* 2017). Sequences were aligned using the MUSCLE software (Edgar 2004) implemented in the Geneious Server Database (version 7.1.3), using default settings (Kearse *et al.* 2012). The alignment was trimmed to the shortest sequence, and the homologous regions were aligned. The substitution saturation index was estimated using DAMBE5 (Xia 2013), and the number of base substitutions per site between sequences was calculated. Phylogenetic analyses were done using maximum likelihood in RaxML version 8 (Stamatakis 2014) using the Kimura 2-parameter model of substitution. Standard error estimates were obtained using the bootstrap procedure (1,000 replicates). The model parameter and bootstrap value (1,000 repetitions) were also estimated using the RaxML program, which was performed through an online computer site CIPRES (Miller *et al.* 2010). Figtree ver 1.1.2 was used to visualize phylogenetic trees.

Results

Family Gorgoderidae Looss, 1901

Genus *Phyllodistomum* Braun, 1899

Phyllodistomum pepirense Dias, Pérez-Ponce de León, Silva and Abdallah n. sp. (Figures 1–4)

Description (based on eight whole-mounted adult specimens): Body spatulate, 902–3363 (4235 ± 449) long, distinctly divided in forebody and hindbody. Tegument wrinkled. Forebody elongated, neck-like, 1673–2110 (1862 ± 167) long, 657–1180 (838 ± 162) wide, 40–52% (44%) of total body length, possessing ventrally six pairs of dome-like papillae (Figure 4B). Hindbody foliate, widest at testes level, 1607–2942 (2372 ± 383) long, 2211–3076 (2532 ± 274) wide, with numerous randomly distributed tegumental papillae. Oral sucker terminal, 438–602 (502 ± 62) long, 414–577 (491 ± 64) wide, with five pairs of papillae on outer border and one pair on inner anterior border (Figure 4C). Mouth opening subventrally. Ventral sucker pre-equatorial, smaller than oral sucker, 302–379 (343 ± 27) long, 340–398 (357 ± 21) wide, with four papillae on the inner surface (Figure 4D). Oral sucker/ventral sucker length/width ratios 1:0.55–1:0.82 (1:0.69), 1:0.61–1:0.84 (1:0.74), respectively. Prepharynx and pharynx absent. Esophagus 226–307 (264 ± 32) long. Caeca long, wide, extending laterally to almost reach posterior, slightly diverticulated, 488–662 (588 ± 74) from posterior end of body.

Testes two, ellipsoid, in middle region of body, slightly lobed, post-ovarian, intercaecal, and slightly oblique (Figures 1–3). Right testis, 253–488 (328 ± 73) long, 202–326 (248 ± 47) wide; left testis, 277–498 (341 ± 74) long, 175–291 (240 ± 35) wide. Seminal vesicle sac-like, 189–256 (210 ± 21) long, 9–124 (101 ± 11) wide. Genital pore median, intercaecal, between intestinal bifurcation and ventral sucker, 819–1353 (1068 ± 170) from the anterior extremity.

Ovary lobed, pre-testicular, dextral, 159–290 (206 ± 44) long, 19–283 (214 ± 50) wide. Mehlis' gland median, weakly developed, slightly anterior to vitelline follicles. Laurer's canal not observed. Uterus with few loops; loops exceptionally extracecal. Metraterm weakly muscular, dorsal to seminal vesicle, opening into genital pore. Vitelline glands composed of two opposing masses containing three to five follicles, located between ovary and testes; right mass 137–308 (205 ± 63) long, 87–171 (113 ± 27) wide; left mass 103–285 (167 ± 57) long, 65–183 (124 ± 39) wide. Eggs ovoid, 25–35 (29 ± 2)

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Table 1. Sequences used in phylogenetic analyses of 28S rDNA gene: Parasite species, hosts, locality, GenBank accession number, and references

Parasite	Host	Locality	GenBank	Reference
1-*Allocreadium lobatum*	*Semotilus corporalis* (Fish)	United States	EF032693	Curran *et al*. 2006
2-*Creptotrematina batalhensis*	*Astyanax lacustris* (Fish)	Brazil	MT512642	Dias *et al*. 2020
3-*Wallinia brasiliensis*	*Astyanax lacustris* (Fish)	Brazil	MH520995	Dias *et al*. 2018
4-*Wallinia caririensis*	*Astyanax bimaculatus* (Fish)	Brazil	MW024899	Silva *et al*. 2020
5-*Phyllodistomum vaili*	*Mulloidichthys vanicolensis* (Fish)	Australia	KF013187	Cutmore *et al*. 2013
6-*Phyllodistomum hyporhamphi*	*Hyporhamphus australis* (Fish)	Australia	KF013190	Cutmore *et al*. 2013
7-*Phyllodistomum* sp.	*Epibulus insidiator* (Fish)	French Polynesia	KF013179	Cutmore *et al*. 2013
8-*Phyllodistomum* sp.	*Cephalopholis boenak* (Fish)	Australia	KF013175	Cutmore *et al*. 2013
9-*Gorgoderidae* sp.	*Lioconcha castrenses* (Bivalve mollusc)	Australia	KF013193	Cutmore *et al*. 2013
10-*Phyllodistomum hoggettae*	*Plectropomus leopardos* (Fish)	Australia	KF013191	Cutmore *et al*. 2013
11-*Phyllodistomum cribbi*	*Zoogoneticus quitzeoensis* (Fish)	Mexico	KT376720	Pérez-Ponce de León *et al*. 2015
12-*Phyllodistomum wallacei*	*Xenotaenia resolanae* (Fish)	Mexico	KT376715	Pérez-Ponce de León *et al*. 2015
13-*Phyllodistomum inecoli*	*Heterandria bimaculata* (Fish)	Mexico	KC760199	Razo-Mendivil *et al*. 2013
14-*Phyllodistomum spinopapillatum*	*Profundulus balsanus* (Fish)	Mexico	KM659388	Pérez-Ponce de León *et al*. 2015
15-*Phyllodistomum folium*	*Gasterosteus aculeatus* (Fish)	Lithuania	AY277707	Petkeviciute *et al*. 2004
16-*Phyllodistomum lacustri*	*Ictalurus punctatus* (Fish)	Mexico	HQ325019	Rosas-Valdez *et al*. 2011
17-*Phyllodistomum staffordi*	*Ameiurus melas* (Fish)	Mexico	HQ325028	Rosas-Valdez *et al*. 2011
18-*Gorgodera cygnoides*	Amphibian	Ukraine	AF151938	Tkach *et al*. 2000
19-*Gorgoderina* sp.	*Rana* sp. (Amphibian)	Mexico	HQ325007	Rosas-Valdez *et al*. 2011
20-*Phyllodistomum magnificum*	*Tandanus tandanus* (Fish)	Australia	KF013186	Cutmore *et al*. 2013
21-*Phyllodistomum brevicecum*	*Umbra limi* (Fish)	Canada	KC760207	Razo-Mendivil *et al*. 2013
22-*Xystretrum solidum*	*Sphoeroides testudineus* (Fish)	United States	KF013188	Cutmore *et al*. 2013
23-*Xystretrum* sp.	*Sufflamen fraenatum* (Fish)	Australia	KF013176	Cutmore *et al*. 2013
24-*Pseudophyllodistomum johnstoni*	*Macrobrachium australiense* (Decapode)	Australia	KF013182	Cutmore *et al*. 2013
25-*Phyllodistomum macrocotyle*	*Dreissena polymorpha* (Bivalve mollusc)	Lithuania	AF533015	Stunzenas *et al*. 2004
26-*Phyllodistomum* cf. *symmetrorchis*	*Clarias gariepinus* (Fish)	Kenya	KF013171	Cutmore *et al*. 2013
27-*Phyllodistomum centropomi*	*Centropomus parallelus* (Fish)	Mexico	KM659384	Pérez-Ponce de León *et al*. 2015
28-*Phyllodistomum virmantasi*	Eleotridae	Mexico	MW804317	Pinacho-Pinacho *et al*. 2021
29-*Phyllodistomum pepirense* n. sp.	*Hoplias malabaricus*	Brazil	*	Present study
30-*Phyllodistomum pepirense* n. sp.	*Hoplias malabaricus*	Brazil	*	Present study
31-*Nagmia floridensis*	*Dasyatis sabina* (Stingray)	United States	EF032691	Curran *et al*. 2006
32-*Nagmia* sp.	*Stegostoma fasciatum* (Shark)	Australia	KF013192	Cutmore *et al*. 2013
33-*Plesiochorus* sp.	*Caretta caretta* (Turtle)	United States	KF013180	Cutmore *et al*. 2013
34-*Anaporrhutum* sp.	*Chiloscyllium punctatum* (Fish)	Australia	KF013184	Cutmore *et al*. 2013
35-*Staphylorchis cymatodes*	*Chiloscyllium punctatum* (Fish)	Australia	HM486318	Cutmore *et al*. 2010
36-*Dicrocoelium* sp.	*Ovis aries* (Sheep)	Spain	AY222261	Olson *et al*. 2003
37-*Brachylecithum lobatum*	*Corvus corone* (Crow)	Czech Republic	AY222260	Olson *et al*. 2003
38-*Degeneria halosauri*	*Halosauropsis macrochir* (Fish)	NE Atlantic Ocean	AY222257	Olson *et al*. 2003
39-*Paracreptotrematina limi*	*Umbra limi* (Fish)	United States	HQ833706	Curran *et al*. 2011
40-*Encyclometra colubrimurorum*	Frog	Ukraine	AF184254	Tkach *et al*. 2001

*GenBank Access number will be added after acceptance of the manuscript.

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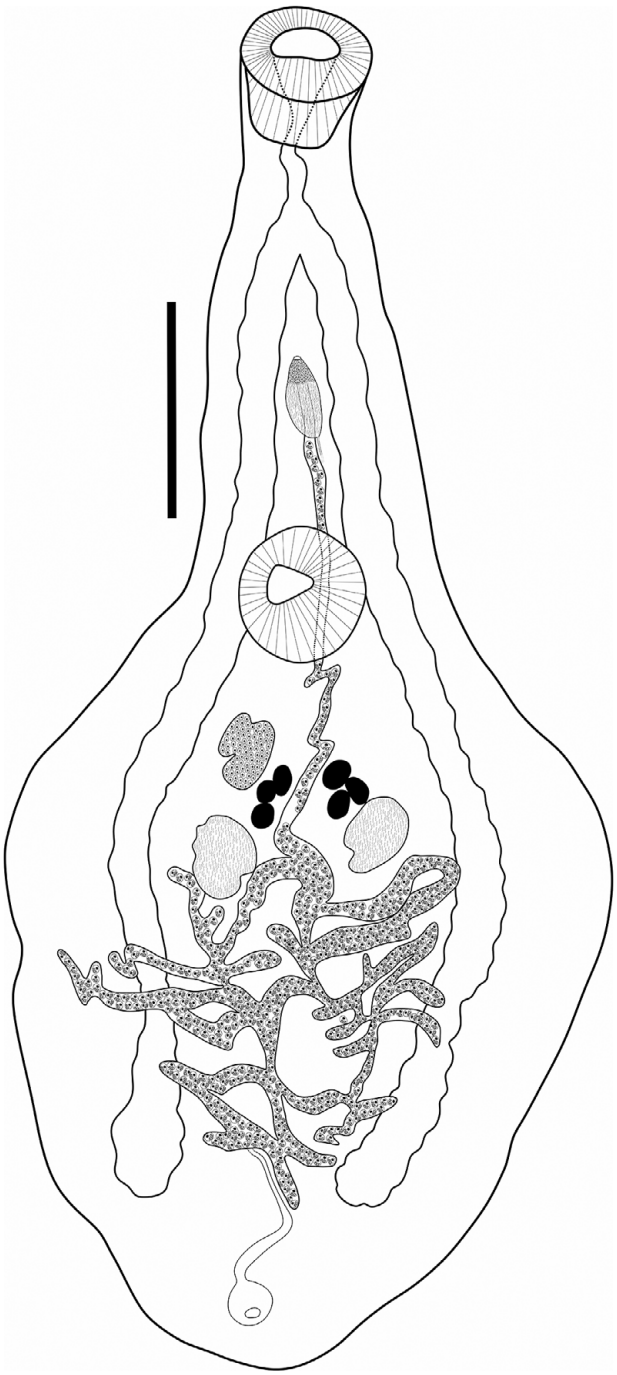

Figure 1. *Phyllodistomum pepirense* n. sp. holotype: Whole mount specimen collected from the urinary bladder of *Hoplias malabaricus* from Jacaré-Pepira River, municipality of Ibitinga, São Paulo state, Brazil. Ventral view. Scale bar 500 μm.

long, 18–25 (23 ± 1.5) wide. Excretory vesicle I-shaped; excretory pore subterminal.

Taxonomic summary

Type-host: *Hoplias malabaricus* (Bloch, 1794) (Osteichthyes, Erythrinidae)

Type-locality: Jacaré-Pepira River, municipality of Ibitinga (21°53'30.5"S; 48°48'33.0"W)

Infection site: Urinary bladder

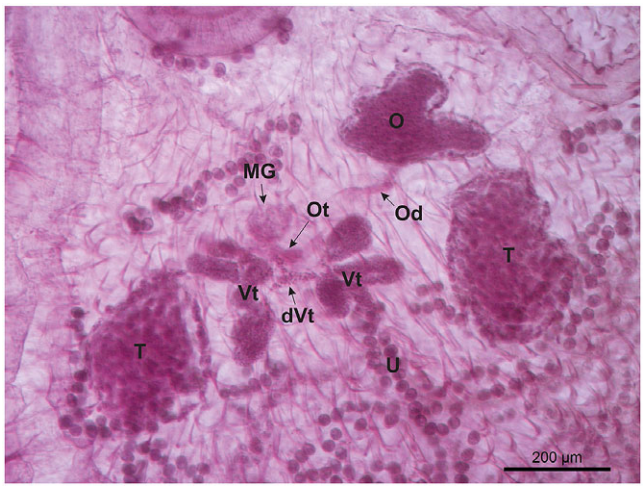

Figure 2. Detail of the post-acetabular region of the holotype of *Phyllodistomum pepirense* n. sp. highlighting the ovary (O), oviduct (Od), Mehlis' gland (MG), Ootype (Ot), Vitelline follicles (Vt), Vitelline ducts (dVt), Uterus (U), and Testes (T). Carmine staining, dorsal view.

Prevalence: 35%

Mean abundance: 0.42 digeneans per examined host

Etymology: The specific epithet *pepirense* refers to the name of the river (Jacaré-Pepira River) where the parasite was discovered.

Remarks

Phyllodistomum pepirense n. sp. possesses the characteristic morphological features that place it in its genus (Campbell 2008; Bray 2009). Five species of *Phyllodistomum* have been reported from Brazil, but only two currently valid species were described from freshwater fishes – namely, *P. rhamdiae* Amato & Amato, 1993, a parasite of *Rhamdia quelen* (Quoy & Gaimard, 1824), and *P. spatula* Odhner, 1902 from *Colossoma macropomum* (Cuvier, 1818); *Pimelodella laticeps* Eigenmann, 1917; and *Rhamdia sapo* (Valenciennes, 1836) in Argentina (see Kohn *et al.* 2007). The new species differs from *P. rhamdiae* by having slightly lobed testes, a hindbody with irregular margins, and the uterus intercaecal and extracaecal, occupying most of the hindbody. The species *P. spatula* was first recorded in Brazil by Fernandes (1984) in the Ceará state from *C. macropomum* and differs morphologically from the new species in body size, caeca width, shape of vitelline masses, and the distribution of the uterus occupying most of the hindbody. The record of *Phyllodistomum* sp. from *H. malabaricus* in the Jacaré-Pepira River in São Paulo state, Brazil, by Leite *et al.* (2021) most likely corresponds with the new species we describe herein.

Several species of *Phyllodistomum* have also been described further north in the Neotropical region. One of them, *P. centropomid*, was described from the urinary bladder of *Centropomus paralellus* Poey, 1860 in Veracruz, Mexico (Mendoza-Garfias & Pérez-Ponce de León 2005). The new species is distinguished morphologically from *P. centropomi* because the body length of *Phyllodistomum pepirense* n. sp. is larger [3360 to 4900 (4230) vs 1796 to 2610 (2200)] in *P. centropomi.* The new species lacks the three or four slight undulations on the lateral surface of the hindbody, which possesses muscular indentations. The vitellarium in the new species is composed of two groups of three to five follicles; in *P. centropomi*, vitellarium comprises two compact oval masses. At last, the uterus in *P. pepirense* n. sp. possesses few loops, mostly intercaecal and partially caecal, and in *P. centropomid*, the

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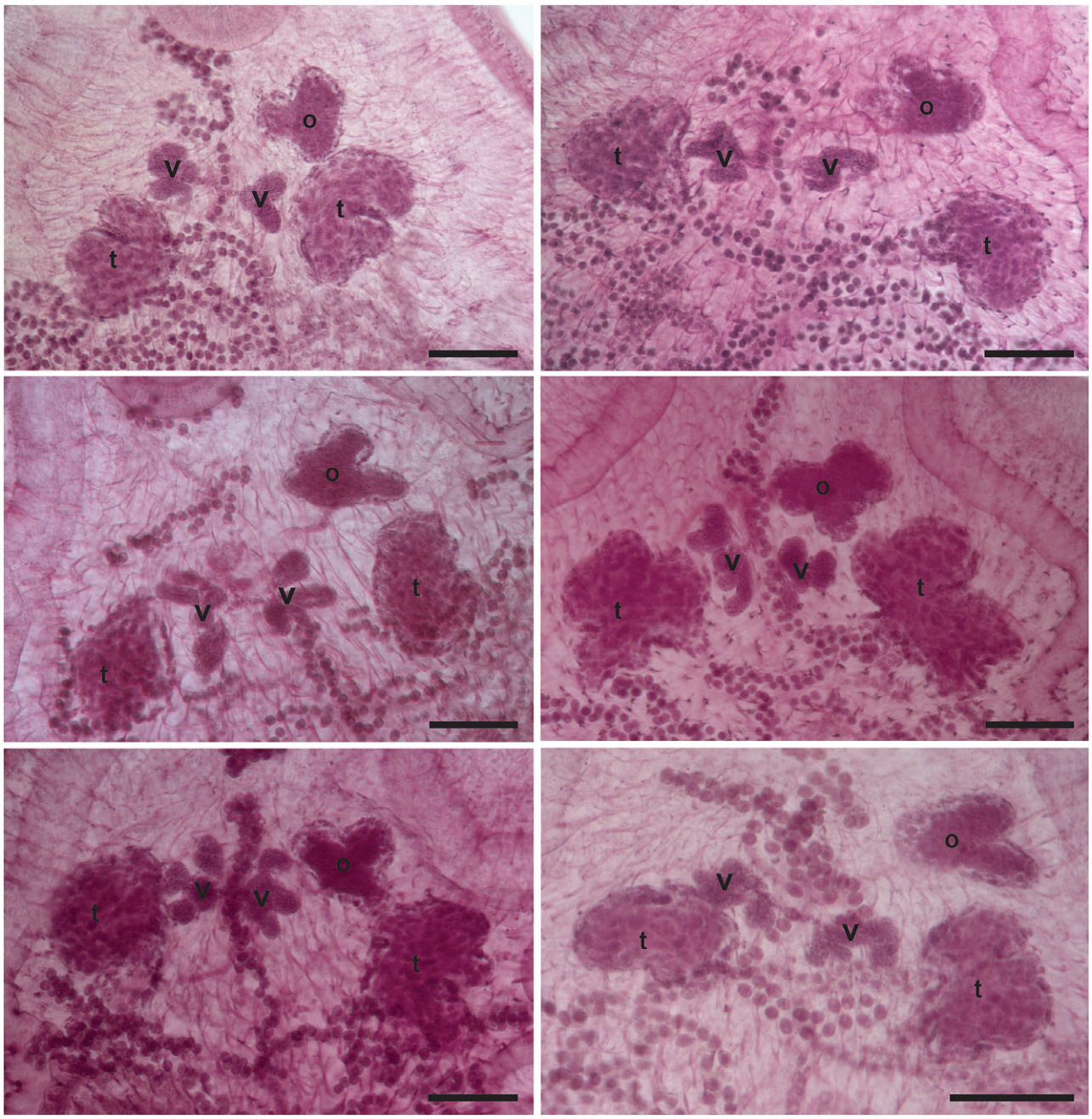

Figure 3. Detail of the post-acetabular region of some paratypes of *Phyllodistomum pepirense* n. sp. highlighting the ovary (o), Vitelline follicles (v), and Testes (t). Carmine staining, dorsal view. Scale bar 200 μm.

uterus occupies most of the hindbody and extends into the extra- and inter-caecal area.

More recently, Pinacho-Pinacho *et al.* (2021) described five additional new species from Mexico and Central America through an integrative taxonomy approach: *P virmantasi*, Pinacho-Pinacho, Sereno-Uribe, Hernández-Orts, García-Varela & Pérez-Ponce de León, 2021; *P. romualdae* Pinacho-Pinacho, Sereno-Uribe, Hernández-Orts, García-Varela & Pérez-Ponce de León, 2021; *P. isabelae* Pinacho-Pinacho, Sereno-Uribe, Hernández-Orts, García-Varela & Pérez-Ponce de León, 2021; *P. scotti* Pinacho-Pinacho, Sereno-Uribe, Hernández-Orts, García-Varela & Pérez-Ponce de León, 2021; and *P. simonae* Pinacho-Pinacho, Sereno-Uribe, Hernández-Orts, García-Varela & Pérez-Ponce de León, 2021. *Phyllodistomum virmantasi* was described from the urinary bladders of *Gobiomorus dormitor* Lacepède, 1800 and *Eleotris* sp. (Eleotridae). Our specimens more closely resemble one of them, *P. virmantasi*; however, the new species can be differentiated by having a larger body size [3362–4902 (4234) vs 1898–3497 (2480)]. Furthermore, *P. pepirense* n. sp. possesses a larger oral sucker bearing ten papillae on the outer surface and two papillae on the inner surface, whereas *P. virmantasi* possesses 12 papillae on the outer surface and four on the inner surface. In addition, *Phyllodistomum pepirense* n. sp. differs in size, form, and position of the ovary (i.e., lobed, slightly dextral, and located a short distance from the ventral sucker, whereas in *P. virmantasi*, the ovary is subspherical, smooth, and almost contiguous with the ventral sucker). The

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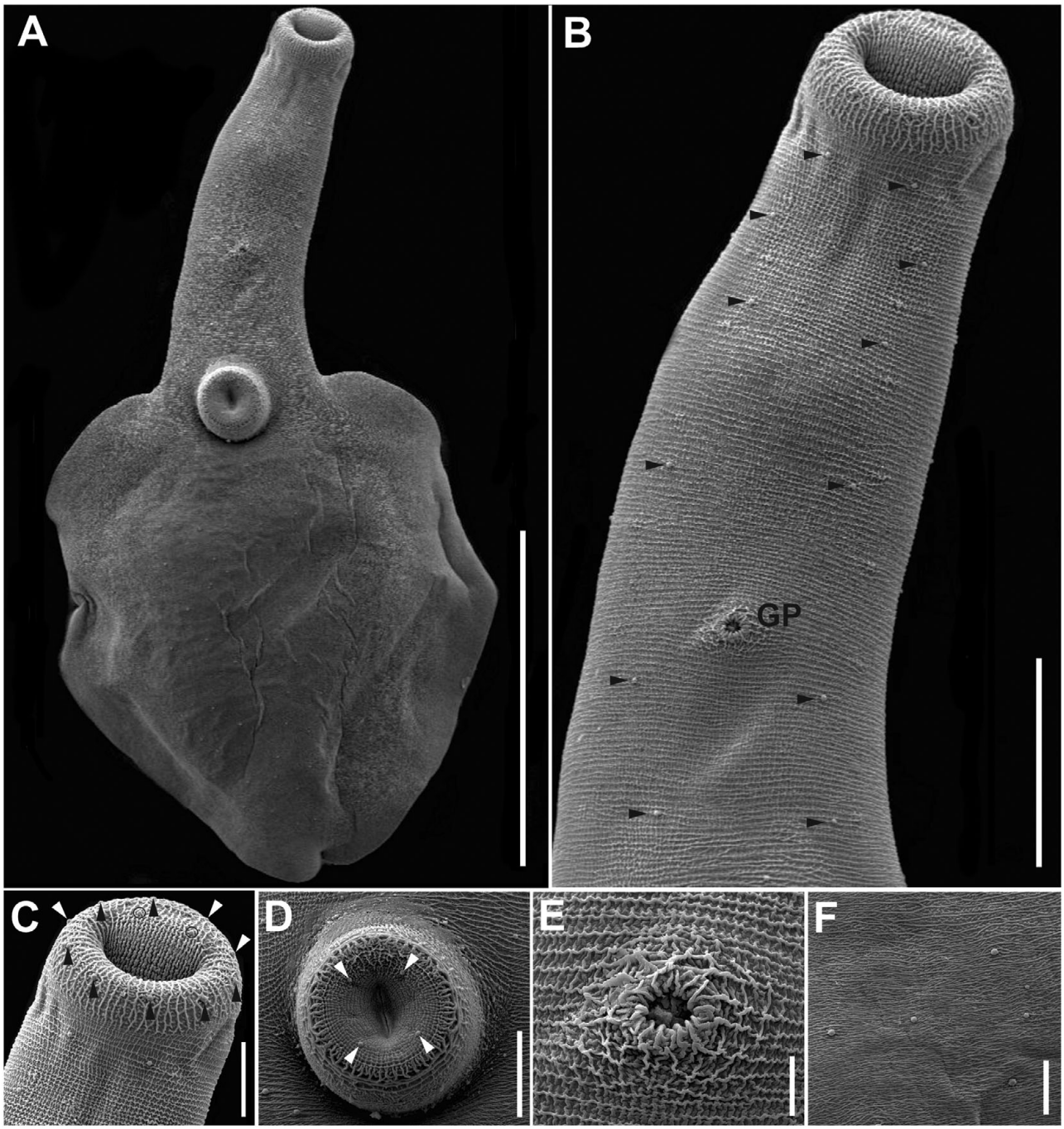

Figure 4. Scanning electron micrographs of *Phyllodistomum pepirense* n. sp. A) Total view, scale 1 mm; B) detail of the forebody with six pairs of dome-like papillae (black head arrows), scale 250 μm; see the genital pore (GP); C) detail of the oral sucker, highlighting the presence of 10 papillae on the outer surface (white and black head arrows) and two on the inner surface (black circle), scale 100 μm; D) detail of the ventral sucker, highlighting the presence of four papillae on the inner surface (white head arrows), scale 100 μm; E) detail of the genital pore, scale 25 μm; and F) detail of some papillae of the hindbody, scale bar 50 μm.

vitellarium in the new species is composed of two groups of three to five follicles, whereas in *P. virmantasi*, the vitellarium consists of two masses that are spherical to slightly elongate. Finally, *P. virmantasi* has an extensively coiled uterus that is inter- and extracaecal and occupies most of the hindbody, whereas in *P. pepirense* n. sp., the uterus possesses few loops, which are mostly intercaecal.

An additional record of *Phyllodistomum* sp. was reported by Choudhury *et al.* (2017) from a closely related species of host, *Hoplias microlepis* (Günther, 1864), in Panama. However, the single specimen reported by these authors was not characterized morphologically or molecularly, hindering a comparison with the new species we describe herein. Considering the host and geographical location of this species of *Phyllodistomum*, we hypothesize that it represents the same species; however, this needs to be corroborated by sampling more specimens from Panama and characterizing the species morphologically and molecularly.

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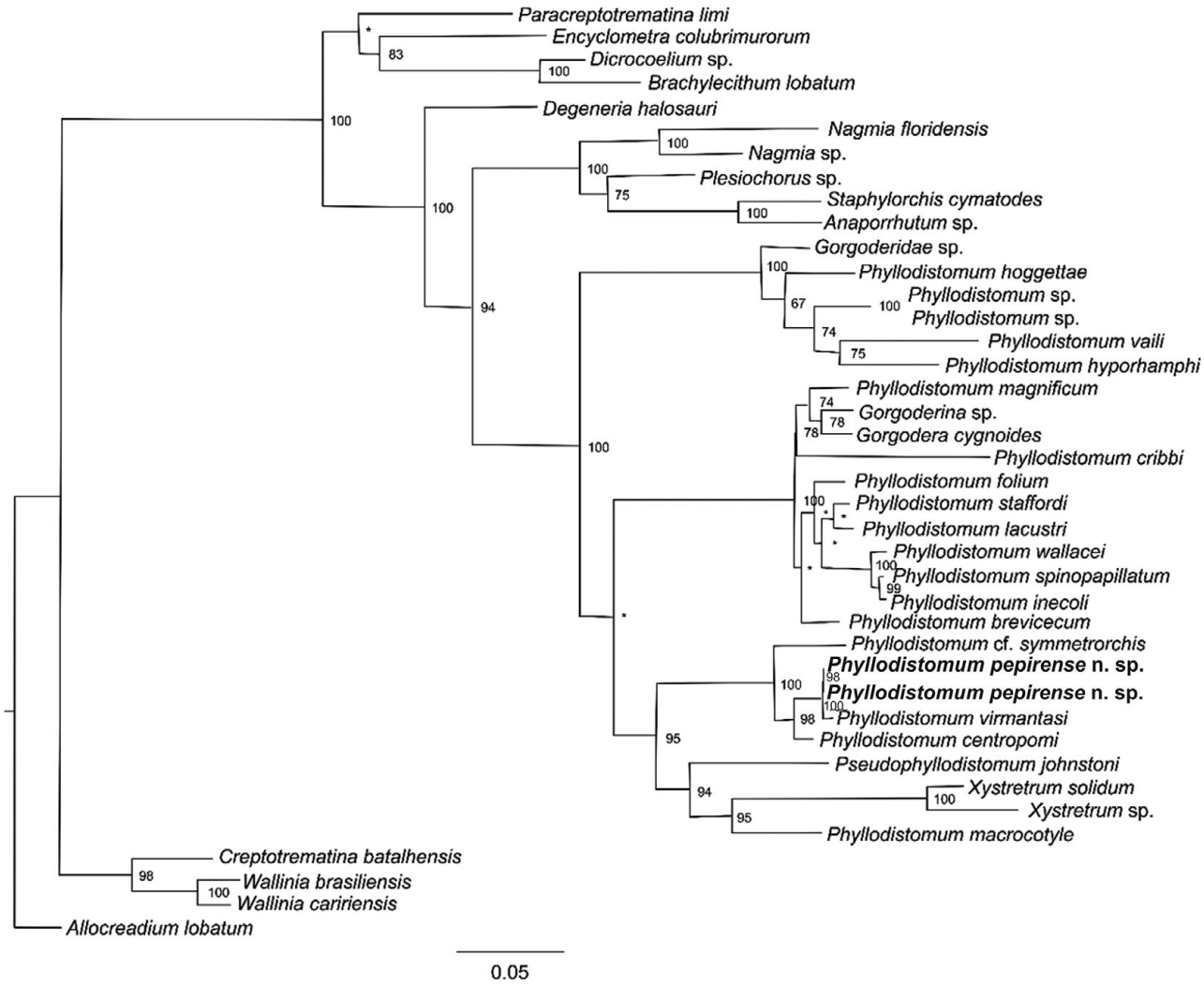

Figure 5. Phylogenetic tree based on Maximum Likelihood analysis of partial sequences of the 28S nuclear rDNA gene. Bootstrap support values with an asterisk representing values not supported by the analyses (<70%). GenBank accession numbers are provided in Table 1. Branch length scale bar indicates the number of substitutions per site. *Allocreadim lobatum*, *Creptotrematina batalhensis*, *Wallinia caririensis,* and *Wallinia brasiliensis* were used as outgroup.

Phylogenetic analysis

Two adult specimens of *Phyllodistomum pepirense* n. sp. were successfully sequenced. The alignment of 28S rDNA sequences included 34 gorgoderid species, and four allocreadids were used as outgroup. The final alignment was 841 bp long. Maximum likelihood phylogenetic trees yielded *Phyllodistyomum pepirense* n. sp. as the sister taxon of *P. virmantasi* and these two, together, as the sister group of *P. centropomid.* Both species distributed farther north in the Neotropical region parasitizing distantly related species of hosts (Figure 5). These relationships are well-supported by high bootstrap values. The genetic divergence between the species-pair *Phyllodistomum pepirense* n. sp. and *P. virmantasi* was 1%, whereas the divergence between these two species and *P. centropomi* varied from 2% to 3% (Table 2).

Discussion

The genus *Phyllodistomum* is one of the genera with the largest species richness of Trematoda, parasitizing both freshwater and marine fishes, and also amphibians, and being recorded in different regions of the world (Cribb *et al.* 2002). In North America, there are approximately 43 species described (Pérez-Ponce de León *et al.* 2007; Pinacho *et al.* 2021). Conversely, in South America, the genus is species-poor, with only five species reported (Kohn *et al.* 2007). Of these, three species of *Phyllodistomum* were described in Brazil. The first described species of the genus was *P. mugilis* from *M. platanus* in the Baia de Guanabara, Rio de Janeiro state (Knoff & Amato 1992), and later, the species *P. rhamdiae* was described from *R. quelen* in the Guandu River, Rio de Janeiro state (Amato & Amato 1993). This latter species has also been recorded in *H. malabaricus* in the Batalha River, São Paulo state (Gião *et al.* 2020), although considering host association, it is more likely that the report may correspond with the new species we describe in this study. One species, *P. spatula*, seems to be widely distributed in Brazilian fishes and was first recorded in Ceará, Brazil, by Fernandes (1984) from *C. macropomum* (Cuvier, 1818). Later, it was reported infecting *H. malabaricus* and *H. intermedius* from the São Francisco River from Minas Gerais state (Costa *et al.* 2015) and *Acestrorhynchus falcirostris* Cuvier, 1819 from the municipality of Manus, Amazonas state (Fernandes *et al.* 2017). We also believe that at least the records by Costa *et al.* (2015) may correspond to the

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Table 2. Percentage (%) of Kimura-2-Parameters genetic divergence of 28S rRNA among Gorgoderidae species downloaded from Genbank and *Phyllodistomum* n. sp. Species of Allocreadiidae (1–4) were used as outgroup.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
1- *Allocreadium lobatum*																																							
2- *Creptotrematina batalhensis*	11																																						
3- *Wallinia brasiliensis*	13	8																																					
4- *Wallinia caririensis*	12	8	3																																				
5- *Phyllodistomum vaili*	37	33	32	30																																			
6- *Phyllodistomum hyporhamphi*	33	33	32	30	11																																		
7- *Phyllodistomum* sp.	35	33	32	31	11	10																																	
8- *Phyllodistomum* sp.	37	36	34	33	12	10	3																																
9- *Gorgoderidae* sp.	34	32	30	30	11	10	9	8																															
10- *Phyllodistomum hoggettae*	34	32	31	32	12	9	8	8	6																														
11- *Phyllodistomum cribbi*	34	34	34	31	29	27	26	28	26	26																													
12- *Phyllodistomum wallacei*	30	30	31	30	24	24	22	24	22	21	13																												
13- *Phyllodistomum inecoli*	30	30	31	30	24	24	22	24	22	22	12	1																											
14- *Phyllodistomum spinopapillatum*	30	31	32	30	24	24	22	24	22	22	12	1	0																										
15- *Phyllodistomum folium*	30	30	31	30	24	23	22	24	21	21	10	5	4	4																									
16- *Phyllodistomum lacustri*	30	31	31	30	25	23	22	24	22	21	12	4	4	4	3																								
17- *Phyllodistomum staffordi*	29	30	30	28	24	22	21	23	21	20	11	4	4	4	3	2																							
18- *Gorgodera cygnoides*	30	30	30	28	25	24	23	24	21	21	10	7	6	6	4	5	5																						
19- *Gorgoderina* sp.	29	30	31	30	27	25	23	25	23	21	10	7	7	7	5	6	5	3																					
20- *Phyllodistomum magnificum*	30	31	32	30	26	23	22	24	23	20	11	7	6	6	5	5	5	4	4																				
21- *Phyllodistomum brevicecum*	30	30	30	29	25	24	24	25	22	22	11	5	5	5	3	4	4	5	5	4																			
22- *Xystretrum solidum*	36	37	37	35	30	27	27	30	26	27	30	25	25	25	25	24	23	25	25	26	25																		
23- *Xystretrum* sp.	37	37	37	36	31	30	29	31	28	30	32	29	28	28	28	27	26	27	26	28	27	6																	
24- *Pseudophyllodistomum johnstoni*	31	31	34	32	24	23	23	23	20	20	24	20	20	20	18	18	17	19	19	20	18	18	19																
25- *Phyllodistomum macrocotyle*	32	32	33	32	22	21	22	23	21	21	21	18	18	18	17	16	16	18	18	18	17	15	18	13															
26- *Phyllodistomum* cf. *symmetrorchis*	33	33	35	33	25	25	25	26	22	21	24	20	21	20	19	18	18	19	20	19	19	23	25	16	14														

(*Continued*)

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Table 2. (*Continued*)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
27- *Phyllodistomum centropomi*	31	31	33	32	23	24	25	26	20	21	24	19	19	19	18	18	16	18	19	19	18	22	23	14	13	5													
28- *Phyllodistomum virmantasi*	31	32	33	33	24	25	25	26	21	21	23	20	20	20	19	19	17	18	19	20	19	23	24	14	14	6	**3**												
29- ***Phyllodistomum pepirense* n. sp.**	32	33	34	33	25	25	25	26	21	22	24	21	21	21	20	20	18	19	20	21	20	23	24	14	14	5	2	**1**											
30- ***Phyllodistomum pepirense* n. sp.**	33	34	36	35	25	26	26	27	22	22	25	21	21	21	20	20	18	19	21	21	20	24	25	14	15	6	3	**1**	0										
31- *Nagmia floridensis*	33	35	33	32	29	27	29	30	27	27	30	26	26	26	27	27	25	26	27	27	26	31	30	27	27	29	28	28	28	29									
32- *Nagmia* sp.	29	31	30	29	27	25	27	28	25	27	26	28	24	24	24	24	24	23	23	25	26	24	28	30	24	26	28	27	27	28	11								
33- *Plesiochorus* sp.	27	31	30	28	26	26	25	26	24	23	28	23	23	23	24	23	22	23	23	24	23	25	28	23	23	24	23	22	23	23	14	13							
34- *Anaporrhutum* sp.	28	31	31	30	32	32	31	33	29	29	32	27	28	28	28	28	26	27	28	27	27	31	34	28	29	31	29	29	30	31	20	19	13						
35- *Staphylorchis cymatodes*	29	32	32	31	33	31	32	33	30	30	34	28	28	28	29	30	28	28	29	30	28	34	37	29	30	30	27	27	28	29	21	17	13	8					
36- *Dicrocoelium* sp.	24	26	27	25	34	29	32	33	31	30	31	29	30	30	28	30	30	30	30	30	29	37	38	30	34	30	29	30	31	32	29	27	28	31	31				
37- *Brachylecithum lobatum*	27	29	28	26	36	33	33	34	34	33	34	32	33	33	31	34	33	33	33	35	32	41	40	31	34	32	31	31	32	33	30	29	30	36	36	7			
38- *Degeneria halosauri*	24	26	25	23	25	25	25	27	23	24	24	22	22	22	22	22	21	22	22	24	21	28	30	22	22	23	21	22	22	23	20	17	16	19	20	20	22		
39- *Paracreptotrematina limi*	22	27	26	23	29	31	30	32	28	30	30	26	27	27	26	26	26	26	27	27	27	32	33	28	28	28	25	26	27	28	24	22	21	26	27	17	19	16	
40- *Encyclometra colubrimurorum*	22	23	25	23	32	28	28	29	26	27	31	28	28	28	27	28	28	28	27	27	28	33	36	29	29	29	27	27	28	29	25	25	25	26	28	17	20	19	15

species described here. Still, this requires further verification by analysing the morphology of the specimens in more detail and, preferentially, by obtaining sequence data from specimens sampled in the same locality. Our study increased the diversity of species within the genus *Phyllodistomum* in South America, although the current concept of the genus is controversial, and several studies have demonstrated that the genus needs revision because it seems to be paraphyletic (Cutmore *et al.* 2013; Petkevičiūtė *et al.* 2020; Pinacho-Pinacho *et al.* 2021).

Molecular tools have proven very useful for species delimitation within *Phyllodistomum* in combination with the use of other characteristics such as morphology (including scanning electron microscopy to describe the type, number, and arrangements of papillae), geographical distribution, and host association. In some cases, preparation and fixation of the parasite have led to controversial species identification because of the influence of this procedure on the morphological traits of individuals (Bakke 1988). Also, there seems to be a pattern of host specificity among species of *Phyllodistomum*, although many species are not yet sequenced, and reports of some species infecting certain groups of hosts require further verification, in addition to the potential to find cryptic species complexes as in the case of *P. lacustri* in catfishes of North America (Rosas-Valdez *et al.* 2011). The finding of the new species as a parasite of the erythrinid *H. malabaricus* in South America raises an interesting hypothesis about the distribution of this gorgoderid along with its hosts. *Phyllodistomum* sp. was reported from another erythrinid, *Hoplias microlepis*, from the Rio Chagres in the Soberania National Park, Panama (Choudhury *et al.* 2017). This erythrinid, along with *H. malabaricus*, reaches its northernmost distribution range in Costa Rica, Central America. It seems plausible to postulate that the specimens from Panama will more likely represent the new species.

Finally, the number of *Phyllodistomum* spp. is still increasing as authors approach the description of new congeneric species worldwide through an integrative taxonomy approach (Petkevičiūtė *et al.* 2020; Pinacho-Pinacho *et al.* 2021). Once a taxonomic review of the genus is conducted as more species of the genus are sequenced, the classification scheme for the group will be modified to determine monophyletic groupings corresponding with the generic rank, and then the diversity of all the genera of gorgoderids will be known.

Ethical standard. The fish collection was authorized through the Sistema de Autorização e Informação da Biodiversidade (SISBIO) under #40998-2. All animal procedures were performed in full compliance with the Ethics Committee for Animal Experimentation (CEUA #3353050417) of the Universidade Estadual Paulista (São Paulo State University - UNESP).

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Competing interest. No potential conflict of interest was reported by the authors.

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