Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Phylogenetic position of *Gorgoderina parvicava* Travassos, 1922 (Digenea: Gorgoderidae), a parasite of *Leptodactylus labyrinthicus* (Spix, 1824) (Anura: Leptodactylidae) in Brazil

Posição filogenética de *Gorgoderina parvicava* Travassos, 1922 (Digenea: Gorgoderidae), parasita de *Leptodactylus labyrinthicus* (Spix, 1824) (Anura: Leptodactylidae) no Brasil

E. P. Alcantara[a]*, L. P. Úngari[a], M. I. Müller[b], C. Ferreira-Silva[a], E. Emmerich[a], L. H. O'Dwyer[a] and R. J. Silva[a]

[a]Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, Programa de Pós-graduação em Ciências Biológicas (Zoologia), Laboratório de Parasitologia de Animais Silvestres, Botucatu, SP, Brasil

[b]Universidade Federal de São Paulo – UNIFESP, Departamento de Ecologia e Evolucão, Laboratório de Ecologia e Evolução, Unidade José Filippi, Diadema, SP, Brasil

Abstract

During a parasite survey in Brazilian amphibians from São Paulo state, Brazil, *Gorgoderina parvicava* Travassos, 1922 was found in the urinary bladder (11 adult worms) and (five juvenile worms) in the kidneys of the pepper-frog *Leptodactylus labyrinthicus* (Spix, 1824). Parasites were examined by microscopy and 28S rDNA and COI gene were sequenced and analyzed for the molecular study. The phylogenetic reconstructions resulted in identical topologies with highly supported values in the nodes in most clades using Maximum likelihood and Bayesian inference methods and positioned G. parvicava in the subclade formed by species of subfamily Gorgoderinae parasitizing the urinary bladder of amphibians. Molecular phylogenetic data showed that this species is related to other species of *Gorgoderina*. In addition, new molecular data and the analyses of genetic distances provide extra comparative data, which can be applied in further investigations on the taxonomic status and the diversity among *Gorgoderina* spp. and host-parasite relationships.

Keywords: amphibian, helminths, Trematoda, phylogeny.

Resumo

Durante o levantamento de parasitas de anfíbios brasileiros do estado de São Paulo, Brasil, *Gorgoderina parvicava* Travassos, 1922 foi encontrado na bexiga urinária (11 vermes adultos) e nos rins (cinco vermes juvenis) da rã-pimenta *Leptodactylus labyrinthicus* (Spix, 1824). Os parasitas foram examinados por microscopia e os genes 28S rDNA e COI foram sequenciados e analisados para o estudo molecular. As reconstruções filogenéticas resultaram em topologias idênticas com valores suportados nos nós na maioria dos clados, usando métodos de máxima verossimilhança e inferência bayesiana e posicionaram *G. parvicava* no subclado formado por espécies da subfamília Gorgoderinae parasitando a bexiga urinária de anfíbios. Dados filogenéticos moleculares mostraram que esta espécie está relacionada a outras espécies de *Gorgoderina*. Além disso, novos dados moleculares e as análises de distâncias genéticas fornecem dados comparativos extras, que podem ser aplicados em futuras investigações sobre o status taxonômico e a diversidade entre *Gorgoderina* spp. e relações parasita-hospedeiro.

Palavras-chave: anfíbio, helmintos, Trematoda, filogenia.

1. Introduction

The Pepper-frog *Leptodactylus labyrinthicus* (Spix, 1824) has a wide distribution through Brazil, Paraguay, and Bolivia (Heyer, 2005; Santos and Haddad, 2006; Heyer et al. 2021; Frost, 2021). In Brazil, the species occurs mainly near wetlands and has been recorded in open habitats throughout the Cerrado, Caatinga, and central Amazonia (Heyer, 1979; Larson and De Sá, 1998; Carvalho et al., 2013).

Some digenean species were previously reported infecting *L. labyrinthicus* from South America, as follows: *Gorgoderina parvicava* Travassos, 1922, *Choledocystus elegans* (Travassos, 1926) Ruiz, 1949, *Neohaematoloechus neivai* (Travassos and Artigas, 1927) Odening, 1960, *Rauschiella linguatula* (Rudolphi, 1819) Razo-Mendivil, Leon-Regagnon and Pérez-Ponce de Leon,

*e-mail: ednnapaulino@gmail.com
Received: May 5, 2022 – Accepted: November 28, 2022

2006, and *Rauschiella palmipedis* (Lutz, 1928) (Freitas, 1960; Dobbin Junior, 1957; Campião et al., 2014; Kohn and Fernandes, 2014).

Gorgoderina Looss, 1902 comprises 57 described species, widely distributed around the world that are parasites of the urinary bladder of several anuran and caudata species (Mata-López et al., 2005; Kohn and Fernandes, 2014; Bursey et al., 2014; Velázquez-Urrieta and Pérez-Ponce de León, 2021). In Brazil, seven *Gorgoderina* species have been reported. Despite the large amount of information available on *Gorgoderina* spp., there are no molecular studies for these species in Brazil.

Studies have demonstrated that molecular analysis is an important tool for the identification and phylogenetic analyses of trematodes (Gomes et al., 2013; Müller et al., 2021; Queiroz et al., 2021; Franceschini et al., 2021). Here, we provide the morphological and the first molecular assessment of *G. parvicava* from *L. labyrinthicus* in Brazil using the mitochondrial gene cytochrome c oxidase I (COI) and the large subunit of the 28S ribosomal gene.

2. Materials and Methods

One adult male specimen of *L. labyrinthicus*, snout-vent length (SLV) 145 mm and weight (W) 245 g, was collected on January 14, 2020, at the Carandá Farm (21°52'30.28"S, 48°13'50.78"W), municipality of Araraquara, São Paulo state, Brazil. During necropsy, digeneans were found in the urinary bladder and kidneys.

The parasites found were fixed in alcohol-formalin-acetic acid solution under the light pressure of a coverslip for 10 min and transferred to 70% alcohol for further processing. Three fresh specimens were added to 96% ethanol for molecular analyses. Digeneans were stained with carmine, cleared with eugenol, and analyzed in a computerized system for image analysis (V3 Leica Application Suite, Leica Microsystems, Wetzlar, Germany) in a microscope with differential interference contrast. Voucher specimens were deposited in the Helminthological Collection of the Instituto de Biociências, Universidade Estadual Paulista (UNESP), municipality of Botucatu, São Paulo State, Brazil, under #CHIBB 9200. Access to the genetic data was authorized by the Brazilian Ministry of Environment (Sisgen A6199F3). The frog host was deposited at the Herpetological Collection of the Universidade Federal do Ceará, municipality of Fortaleza, Ceará State, Brazil, under #CHUFC- A10088.

The morphological analysis was based on five whole-mounted adult specimens. Measurements are presented as range and are expressed in micrometers, except in the indicated cases. The diagnosis of *Gorgoderina* follows Bray et al. (2008).

Genomic DNA was extracted from a portion of three specimens using a DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, with a final volume of 30 µl. DNA fragments were amplified using a partial 28S rDNA gene containing D1/D3 divergent domains and cytochrome c oxidase subunit 1 (COI). The primers and cycling conditions used to amplify and sequence the partial 28S ribosomal DNA (rDNA) (D1–D3) followed Mendoza-Palmero et al. (2015) and for the mtDNA, COI was amplified using the primers (JB3 and JB4) and polymerase chain reaction (PCR) conditions described by Morgan and Blair (1998). The PCR reactions were performed using 3 µl of DNA extract, 1 µl of each primer, 7.5 µl of ultrapure water (Sigma-Aldrich, United Kingdom), and 12.5 µl Master Mix MyFiTM Mix Bioline®, with a final volume of 25 µl

PCR amplicons (3 µl) were visualized on GelRed (Biotium, Fremont, CA, USA) and purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Sequencing reactions were performed directly on the purified PCR products, with the same PCR primes, using a BigDye v3.1 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and sequences were run on an ABI 3500 DNA genetic analyzer (Applied Biosystems).

Contiguous sequences from each molecular marker were assembled and edited in Sequencher v. 5.2.4 (Gene Codes, Ann Arbor, MI). Sequences of partial 28S rDNA and COI were aligned with sequences of *Gorgoderina* spp. and *Phyllodistomum* spp. downloaded from GenBank for comparative purposes. Sequences of *Glypthelmins tuxtlasensis* Razo-Mendivil, León-Règagnon and Pérez-Ponce de León 2004 was used as an outgroup of COI analysis and *Degeneria halosaur* (Bell 1887) Campbell 1977 was used as an outgroup of 28S rDNA.

Two datasets of DNA sequences were constructed and analyzed. Alignment I comprised the partial 28S gene dataset of *Gorgoderina* spp. and representative species of gorgoderids. This molecular marker was used because it is the molecular marker with the largest number of sequenced species, thus allowing us to test the position of our species in the family. The alignment II comprised the mitochondrial COI gene dataset of *Gorgoderina* spp.. Although there are not many *Gorgoderina* sequences, this marker provides information on intragenotypic differences.

Newly obtained sequences were aligned using the MUSCLE (Edgar, 2004) implemented in Geneious version 11.1.4 (Kearse et al., 2012). To evaluate the occurrence of substitution saturation, the Iss index was estimated in DAMBE 6 (Xia, 2013). The best-fit model for nucleotide substitution was determined by the Akaike information criterion in jModelTest program (Posada, 2008). Phylogenetic analyses were performed using Bayesian inference and maximum likelihood (ML) using MrBayes and RaxML analysis was carried out using the computational resource CIPRES (Miller et al., 2010). The COI sequences were assessed using the default parameters of the Muscle algorithm (Edgar, 2004) implemented in Geneious 7.1.3 (Kearse et al., 2012). Stop codons and indels were also checked in Geneious 7.1.3 (Kearse et al., 2012), translation frame 1, invertebrate mitochondrial. The substitution saturation index was estimated in DAMBE 5 (Xia, 2013) to calculate its occurrence.

The Bayesian Bayesian inference for both analyses was run with the nucleotide substitution model GTR+I+G. To search with the Markov chain Monte Carlo method, chains were run with 10,000,000 generations, saving one tree every 1,000 generations. On the burn-in, the first 25% of generations were discarded, and the consensus trees were estimated using the remaining trees. Bayesian posterior probabilities (BPP) cutoff was considered > 90%. The branch support for ML was determined by

performing 1,000 bootstrap replicates. The Bootstrap cutoff was considered > 75%. The trees were visualized in FigTree v1.3.1 (Rambaut, 2009). Genetic divergence was calculated using the Kimura 2-parameter model in MEGA7.0.20 software (Kimura, 1980; Tamura et al., 2013).

3. Results

Sixteen digeneans were found, 11 adults in the urinary bladder and 5 juveniles in the kidneys of *L. labyrinthicus*. Based on the observed morphological features and morphometric analyses, the digenean was identified as *G. parvicava* (Figures 1 and 2; Table 1).

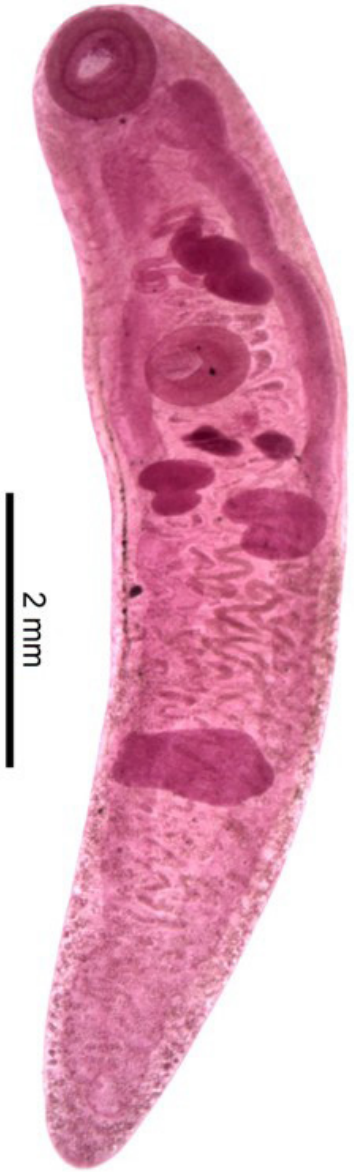

Figure 1. *Gorgoderina parvicava* Travassos, 1922 (Gorgoderidae) parasite of *Leptodactylus labyrinthicus* (Spix, 1824) (Leptodactylidae) from Carandá Farm, municipality of Araraquara, São Paulo state, Brazil. Ventral view.

The *G. parvicava* specimens found in *L. labyrinthicus* presented the following morphology: Elongated body with tapered ends. Smooth tegument, without spines. Oral sucker subterminal. Acetabulum cylindrical to ovoid, slightly smaller than oral sucker. Pharynx absent. Esophagus short and frequently sinuous. Intestinal caeca robust extending until almost the body end. Excretory vesical I-shaped. Excretory pore terminal. Genital pore located after cecal bifurcation, at the middle distance between oral sucker and acetabulum, and slightly dislocated to the right side. Seminal vesicle placed just anteriorly to acetabulum, sinuous, enlarged at basal portion, and thin at distal portion, where prostate composed of few and large cells are distributed. Testes mainly intercaecal, with irregular contours. Anterior testis dislocated to left side,

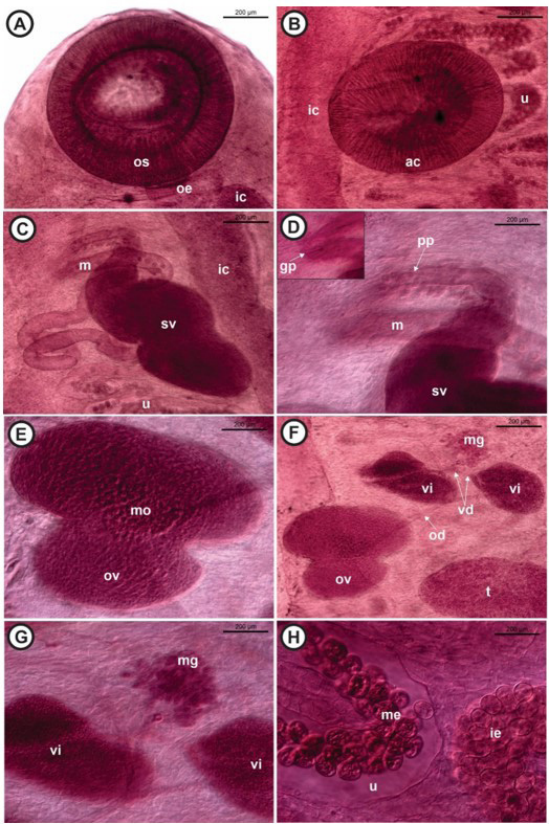

Figure 2. *Gorgoderina parvicava Travassos,* 1922 (Gorgoderidae) parasite of *Leptodactylus labyrinthicus* (Spix, 1824) (Leptodactylidae) from Carandá Farm, municipality of Araraquara, São Paulo state, Brazil. A) Detail of the oral sucker, oesophagus, and intestinal caeca; B) Acetabulum; C) Detail of the region of seminal vesicle and metraterm; D) Detail of the terminal genitalia and metraterm, highlighting the genital pore (left above corner); E) Ovary; F) Region of the ovary highlighting the Mehlis' gland and vitelline follicles; G) Detail of the Mehlis' gland; H) Eggs in the uterus, highlighting part of the descending loop of the uterus with immature eggs and an ascending part with mature eggs. Legend: ac – acetabulum, gp – genital pore, ic – intestinal caeca, ig – immature eggs, m – metraterm, me – mature eggs, mg – Mehlis' gland, mo – mature oocytes, od – ovary duct, oe – oesophagus, os – oral sucker, ov – ovary, pp – pars prostatica, sv – seminal vesicle, t – testis, u – uterus, vd – vitelline ducts, vi – vitelline follicles.

Table 1. Morphometry of *Gorgoderina parvicava*. Measurements are in micrometers, except in the indicated cases.

Host	Present study	Travassos (1922)	Fernandes (1958)	Suriano (1965)
	Leptodactylus labyrinthicus	***Leptodactylus latrans***	***Leptodactylus pentadactylus***	***Leptodactylus latrans***
Body length (mm)	8-12.3	6-11	11-14	3-9.7
Body width (mm)	1.4-1.7	1-2	1.7-2	0.6-1.5
Oral sucker diameter	742.3–861.4	470-700	770-970	500-900
Ventral sucker diameter	574.3–746.8	310-560	730-930	250-500
Distance oral sucker to ventral sucker	1,638.3–2,194			
Esophagus length	101.1–439.7	170-470		
Distance of intestinal caeca to the posterior end	395.8–871.4			
Anterior testis (length x width)	414.9–867.2 x 528.5–1,030	520-870 x 310-710	830-1,370	250-600 x 400-1,000
Posterior testis (length x width)	610.3–1,170x 485–760.8	610-1,000 x 310-780	1,370	280-510 x 300-1,000
Seminal vesicle length	759.3–968.7	780		
Ovary (length x width)	347.7–588.3 x 307.2–457.2	450-640 x 260-430	530-670	280-500 x 250-800
Eggs (length x width)	22.8–44.8 x 18.9–23.9	39-42 x 28	25-33 x 21	11-27 x 10-20

just behind the ovary. Posterior testis postequatorial, transversally to the body. Ovary smaller than testes, placed between anterior testis and vitelline follicles, with irregular contours. Vitelline follicles composed of two follicles, compacts, and with regular contour. Mehlis gland located in middle position, anterior e near vitelline follicles. Ovary duct and short vitelline ducts pass to the Mehlis' gland. Canal de Laurer not oberverd. Uterus distributed from Mehlis' gland to posterior end of body, numerous loops, inter and extracaecal. Descending loops filled with immature eggs and ascending loops filled with mature eggs (Figures 1 and 2; Table 1).

The genomic DNA was extracted from three adult worms and five new sequences were obtained; three partial sequences of the 28S rDNA gene (1,527 bp, 1,558 bp, and 1,537 bp) (Genbank acess: OP965546, OP965561, OP966658) and two of the COI mtDNA (446 bp and 405 bp) (Genbank acess: OP965562, OP965563). DNA sequences were generated and two alignments were built. The first one included 28S rDNA sequences of *G. parvicava* and other gorgoderid trematodes to test their phylogenetic position within the family (Figure 3). The 28S rDNA final alignments included 36 sequences of Gorgoderoidean digeneans available in the Genbank. Sequences from all individuals were trimmed to match the shortest consensus sequence. The final 28S rDNA alignment consisted of 745 bp long. Genetic divergence among specimens of *G. parvicava* was 0.1% (Supplementary Material, Table S1), indicating conspecificity. The genetic distance of sequences of *G. parvicava* varied 9.4% from *Gorgoderina africana* Meskal, 1970 parasite of *Hyperolius viridiflavus* (Duméril and Bibron, 1841) (Hyperoliidae), 7% from *Gorgoderina rosamondae* Velázquez-Urrieta and Pérez-Ponce de León 2021 parasite of *Lithobates berlandieri* (Baird, 1859) (Ranidae) and 8.9%-9.1% from *Gorgoderina* sp. (MW684072-73) parasite of *Lithobates vaillanti* (Brocchi, 1877) (Supplementary Material, Table S1).

The 28S Bayesian inference (BI) and maximum likelihood (ML) method resulted in identical topologies with highly supported values in the nodes in most clades (Figure 3). The 28S tree shows that only *Pseudophyllodistomum* Cribb, 1987and *Xystretrum* Linton, 1910 form natural groups. The phylogenetic analysis yielded two main clades labeled as A and B; clade A grouped two species of *Phyllodistomum* parasite of fishes. The main clade B is divided into two subclades B1 and B2 (Figure 3). The subclade B1 high PP (posterior probability) and bootstrap support for the clade comprising species of *Phyllodistomum* Braun, 1899, *Pseudophyllodistomum*, and *Xystretrum* species. Subclade B2 presented well-supported nodes and was subdivided into clades B2.1 and B2.2. The B2.1 presented low support and is formed by *Phyllodistomum* species and the subclade B2.2 presented low support and is formed by *Gorgoderina* and *Gorgodera* species (Figure 3), plus two species of *Phyllodistomum*. *Gorgoderina parvicava* appears in the subclade formed by species of subfamily Gorgoderinae parasitizing the urinary bladder of amphibians. The genus is not monophyletic since *Gorgodera* is nested within the genus.

The two newly generated COI sequences have shown 0.3% of genetic divergence among them (Supplementary Material, Table S2). Both phylogenetic analyses (ML and BI) revealed similar phylogenetic patterns; *G. parvicava* clustered with *Gorgoderina* spp. from Mexican frogs

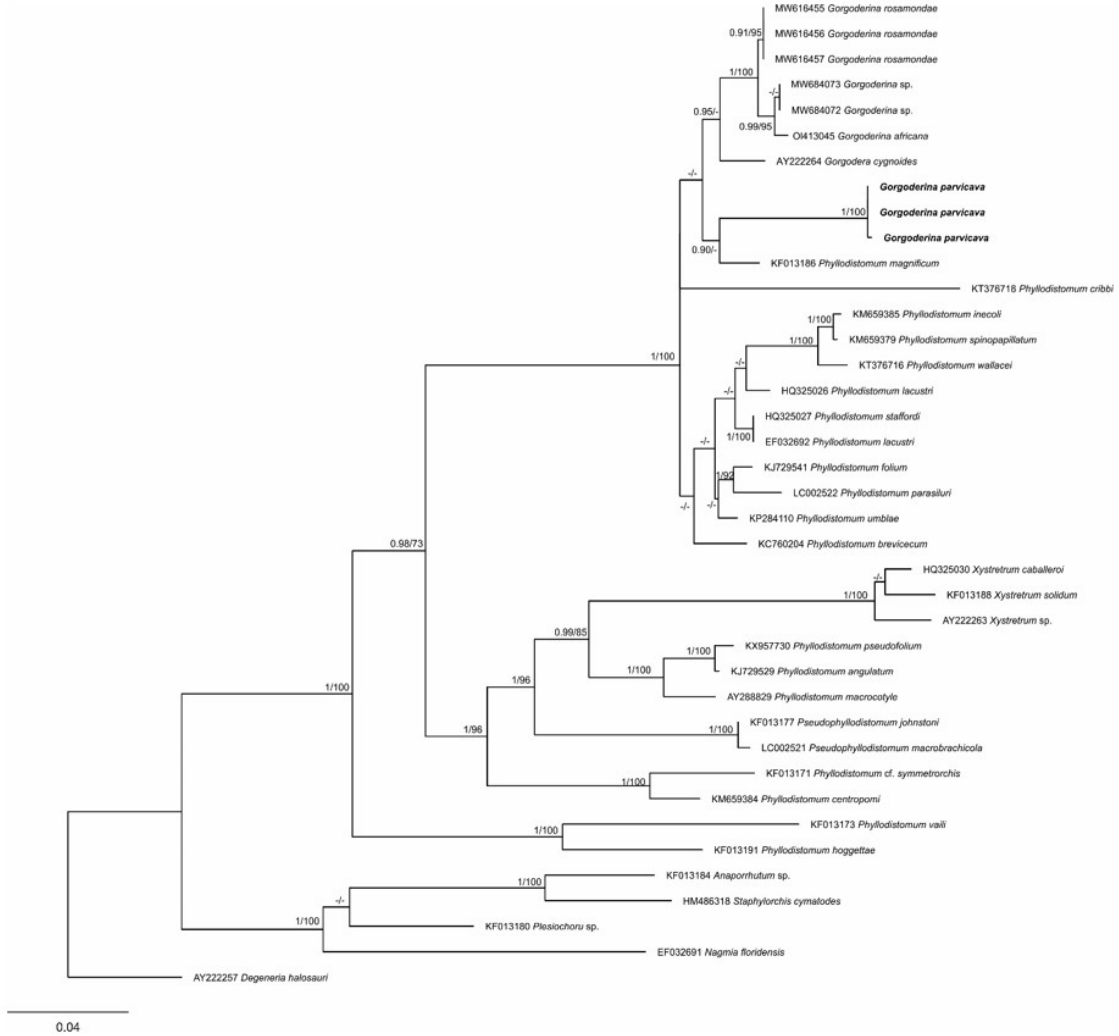

Figure 3. Maximum Likelihood topology based on partial 28S ribosomal DNA sequences of gorgoderid trematodes. GenBank accession numbers are indicated next to species names. Numbers above nodes represent supported nodes by posterior probabilities for Bayesian Bayesian inference and bootstrap for maximum likelihood analyses respectively (posterior probabilities > 0.90 and bootstrap scores > 70). Branch length scale bar indicates the number of substitutions per site.

(Figure 4). The COI pairwise distance between *G. parvicava* and *G. rosamondae* has shown a genetic divergence of 25% and 26% between *G. parvicava* and *Gorgoderina* sp.. Regarding the *G. rosamondae* and *Gorgoderina* sp., the divergence reported was 9% (Supplementary Material, Table S2).

4. Discussion

Gorgoderina parvicava here reported had its morphology compared to other studies with this digenean species (Travassos, 1922; Fernandes, 1958; Suriano, 1965) (Table 1) and its identification was confirmed, except for Suriano (1965), who included a young specimen in its analysis, incorrectly expanding the morphological variation known for the species.

The geographical distribution of this parasite in the Neotropics is wide, and many anurans host were previously reported parasitized with *G. parvicava*, as follows: *Leptodactylus chaquensis* Cei, 1950, *L. labyrinthicus*, *L. latrans* (Steffen, 1815) (= *L. ocellatus*), *L. pentadactylus* (Laurenti, 1768), *R. crucifer* (Wied, 1821), *Rhinella diptycha* (Cope, 1862), *R. icterica* (Spix, 1824), *R. marina* (Linnaeus, 1758), and *Pseudis paradoxa* (Linnaeus, 1758) from Brazil; *L. chaquensis*, *L. latrans,* and *Rhinella fernandezae* (Gallardo, 1957) from Argentina; *Atelopus bomolochos* Peters, 1973, *Leptodactylus rhodonotus* (Günther, 1869), *Rhinella limensis* (Werner, 1901), *Telmatobius culeus* (Garman, 1876), *Telmatobius jelskii* (Peters, 1873), *Telmatobius macrostomus* (Peters, 1873), *Telmatobius peruvianus* Wiegmann, 1834, and *Telmatobius* sp. from Peru; *Rhinella diptycha* from Paraguay; *L. latrans* from Uruguay; and *Pseudis paradoxa* and *Lithobates palmipes* (Spix, 1824) from Venezuela (Campião et al., 2014; Kohn

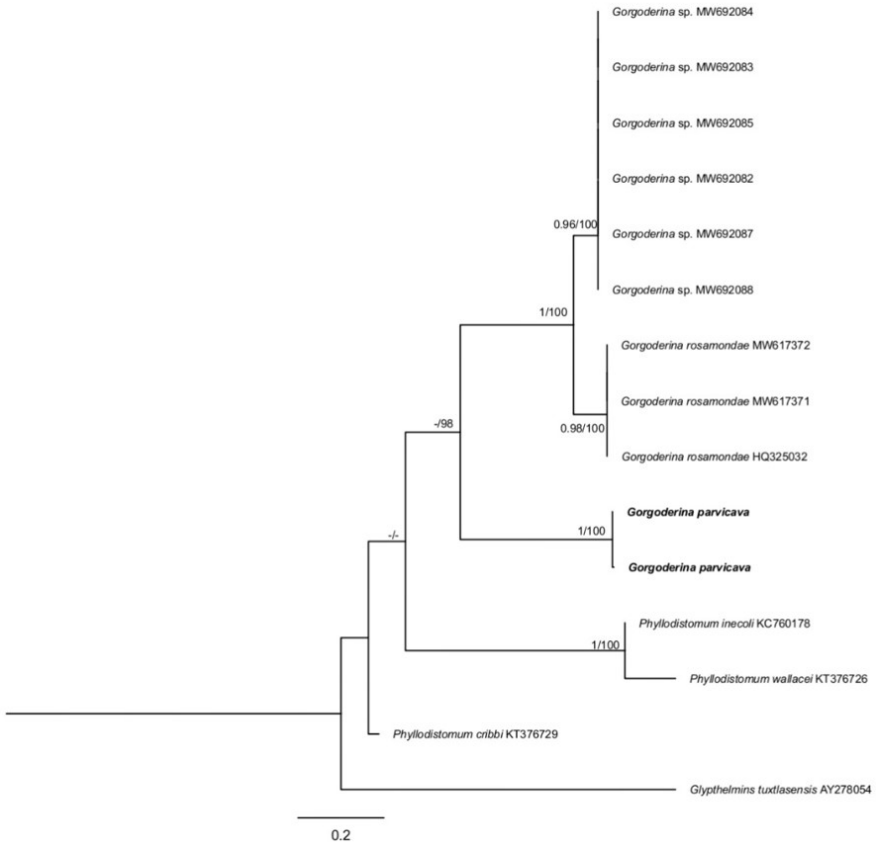

Figure 4. Maximum Likelihood topology based on COI sequences of *Gorgoderina*, showing the phylogenetic position of the adults of *Gorgoderina parvicava* from Carandá Farm, municipality of Araraquara, São Paulo State, Brazil. Numbers above nodes represent supported nodes by posterior probabilities for Bayesian Bayesian inference and bootstrap for maximum likelihood analyses respectively (posterior probabilities > 0.90 and bootstrap scores > 70). Branch length scale bar indicates the number of substitutions per site.

and Fernandes, 2014; Toledo et al., 2018). Infection with *G. parvicava* in *L. labyrinthicus* has also been reported (Travassos, 1922; Fernandes, 1958; Suriano, 1965). Besides the wide distribution and the number of hosts associated with this digenean, few works provided morphometric data of the species and none of them carried out molecular approaches for *G. parvicava*.

Velázquez-Urrieta and Pérez-Ponce de León (2021) divided *Gorgoderina* into two main groups according to ventral/oral sucker ratio: (1) the ventral sucker is less than twice the size of the oral sucker; and (2) the ventral sucker is more than twice the size of the oral sucker. Considering this criterium, *G. parvicava* should be included in group 1 while the other *Gorgoderina* spp. used in the 28S phylogeny belong to group 2. This morphological distinction could explain the position of these species in two clades and the supposed paraphilia. Besides the morphology of *G. parvicava* being well known, this is the first sequenced species of group 1 and also in South America. The description of new sequences of *Gorgoderina* spp. from South America can change this scenario. Future studies will be necessary to prove the paraphyly of *Gorgoderina*.

The characteristics commonly used for species delimitation of *Gorgoderina* such as the body length, caeca length, location and shape of the ovary, testes, and vitelline follicles are variable among individuals of the same species (Bravo-Hollis, 1948). For the species delimitation, a set of tools would be needed: analysis of scanning electron microscopy, life cycle, and molecular data (Krull, 1935; Rankin, 1939; Mata-López and León-Règagnon, 2005; Bolek et al., 2009; Mata-López et al., 2005). Nonetheless, molecular data for congeneric species is very limited and few species were studied for molecular biology: *Gorgoderina africana* (18S and 28S genes) from Central Africa, *Gorgoderina rosamondae* (28S, ITS, and COI) and *Gorgoderina* sp. (28S, ITS and COI) from Mexico, *Gorgoderina attenuata* (Stafford, 1902) Stafford, 1905 (ITS), and *Gorgoderina simplex* (Looss, 1899) (ITS) from USA.

According to the present results in the 28S phylogenetic analyses, some nodes were strongly supported. However, the clade with the *Gorgoderina* spp. sequences were poorly supported (Figure 3). This may result from limitations of the molecular ribosomal marker (28S), which may not be suitable for the analysis of interrelationships of major lineages of Gorgoderidae. Sequences of the 28S rRNA gene were used to test the phylogenetic position of *G. parvicava* within the family Gorgoderidae, confirming the paraphyletic nature of the *Phyllodistomum* spp. (Velázquez-Urrieta and

Pérez-Ponce de León, 2021), and suggesting the paraphyly of the genus *Gorgoderina.*

The high genetic divergence between *G. parvicava* and the other *Gorgoderina* spp. and the position of the new sequence in the tree suggest a further revision of the genus. Although not supported by ML, Bayesian inference is supported in the clade of the sequence with *Phyllodistomum magnificum* Cribb 1987 (Figure 3).

The phylogenetic analysis using COI mtDNA has shown the three species (*Gorgoderina rosamondae*, *Gorgoderina* sp., and *G. parvicava)* comprising different subclades and *G. parvicava* appears as a sister group to *G. rosamondae* and *Gorgoderina* sp. That result is expected because both *G. rosamondae* and *Gorgoderina* sp. are from the same locality (Los Tuxtlas, Mexico).

Here we provide new 28s and mtDNA COI sequences of morphologically identified *G. parvicava* along with phylogenetic analyses This study gives preliminary information that should be accessed in future studies to help unravel phylogenetic relationships in Gorgoderidae and the genus *Gorgoderina* in the Neotropical region, contributing to the knowledge of digeneans worldwide.

Acknowledgements

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for providing a research fellowship for EPA (141322/2018-7), CFS (140871/2017-9), and R.J.S. (311635-2021-0) and to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for LHO (2018/09623-4), LPU (2018/ 00475-9), RJS (2019/25223-9) and M.I.M (2017/16546-3).

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Supplementary Material

Supplementary material accompanies this paper.

Table S1. The matrix shows the pairwise distance estimated for the 28SrDNA within species of gorgoderid trematodes retrieved from GenBank and the species identified (in bold) in this study.

Table S2. The shaded matrix (upper) shows the similarity percentage (%) in the nucleotide sequences, and the unshaded matrix (lower) shows the pairwise distance estimated for the COI within species of gorgoderid trematodes retrieved from GenBank and the species identified (in bold) in this study.

This material is available as part of the online article from https://doi.org/10.1590/1519-6984.263745