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Author for correspondence:
M.S. Queiroz,
E-mail: queiroz51@gmail.com

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Metacercariae of *Heterodiplostomum lanceolatum* (Trematoda: Proterodiplostomidae) found in *Leptodactylus podicipinus* (Anura: Leptodactylidae) from Brazil: a morphological, molecular and ecological study

M.S. Queiroz[1], D. López-Hernández[2], S.A. Locke[3], H.A. Pinto[2] and L.A. Anjos[4]

[1]Universidade Estadual Paulista, Instituto de Biociências de Botucatu – IBB/Unesp, 18618-970, Botucatu, São Paulo, Brazil; [2]Universidade Federal de Mina Gerais – UFMG, Belo Horizonte, Brazil; [3]Department of Biology, University of Puerto Rico at Mayagüez, Call Box 9000, Mayagüez, 00681-9000, Puerto Rico and [4]Departamento de Biologia e Zootecnia – FEIS/UNESP, Universidade Estadual Paulista, Passeio, CEP 15385000 Ilha Solteira, Brazil

Abstract

The trematodes from South American reptiles are poorly known, with only one life cycle completely characterized. We used molecular and morphological methods to characterize diplostomoid metacercariae found in 29 of 86 pointedbelly frogs, *Leptodactylus podicipinus* (Cope, 1862) collected in a marsh pond in Selvíria, in the central-west region of Brazil. The metacercariae were identified as *Heterodiplostomum lanceolatum* Dubois, 1936 (Proterodiplostomidae), a rarely reported species that matures in snakes. In phylogenetic analysis of partial sequences from 28S rDNA, *H. lanceolatum* fell within a polytomy with the proterodiplostomid *Crocodilicola pseudostoma* (molecular divergence of 4.1%) and other members of the superfamily Diplostomoidea. Our collections provide insights into the ecology of this parasite, in that infected frogs were smaller than uninfected frogs, and metacercariae were more numerous in the abdominal cavity and hindlimb muscles than in abdominal muscles, which suggests directions for future research on the transmission and pathology of this proterodiplostomid.

Introduction

South America is home to the second highest number of non-avian reptile species in the world (Uetz *et al.*, 2019), and with 842 species, Brazil ranks third among countries (Costa & Bèrnils, 2018; Uetz *et al.*, 2019). South American reptiles are infected by more than 140 species of digeneans (Fernandes and Kohn, 2014), but the life cycles of these parasites are poorly understood. In fact, studies of the life cycles of trematodes from reptiles are scarce worldwide (Yamaguti, 1975), and in South America, only one is completely elucidated (*Acanthostomum brauni* Garzón & Gil, 1961; Ostrowski de Núñez, 1987). Most trematodes that infect reptiles are adults acquired in trophic interactions with infected second intermediate hosts. These second intermediate hosts likely include larval and adult anurans infected with metacercariae of Opisthogonimidae, Plagiorchiidae, Proterodiplostomidae and Reniferidae (Kehr and Hamann, 2003; Hamann *et al.*, 2006; Schaefer *et al.*, 2006; Hamann and González, 2009; Pinto & Melo, 2012). Although these reports suggest the involvement of amphibians in the transmission of trematodes to reptiles, empirical support for such life cycles is lacking for species in South America.

Members of the family Proterodiplostomidae Dubois, 1936 are flukes that mature exclusively in reptiles, unlike other families of the Diplostomoidea, in which definitive hosts are mainly birds and mammals. The body of adult proterodiplostomids is divided into a flat anterior segment bearing a distinctive, sometimes papillose holdfast organ and a cylindrical or conical posterior segment containing reproductive organs including a paraprostate. The structure of the holdfast organ and the paraprostate are autapomorphies for the Proterodiplostomidae (Niewiadomska, 2002; Hernández-Mena *et al.*, 2017). More than 15 species of this family are reported from reptiles in South America (Fernandes & Kohn, 2014). No complete proterodiplostomid life cycle is known, but fish and amphibians have been implicated as second intermediate host of species of *Crocodilicola*, *Cystodiplostomum*, *Herpetodiplostomum* and *Heterodiplostomum* (Szidat, 1969; Mañé-Garzón & Alonso, 1979; Abdallah *et al.*, 2006; Hamann *et al.*, 2006; Tavares-Dias *et al.*, 2011; Hernández-Mena *et al.*, 2017).

Here, we report naturally occurring metacercariae of *Heterodiplostomum lanceolatum* Dubois, 1936 from the pointedbelly frog, *Leptodactylus podicipinus* Cope, 1862 from the

central-west region of Brazil. We used morphological, molecular and ecological methods to study these parasites, which have not been recorded since the 1980s. Our results provide the first evidence of the involvement of *L. podicipinus* in a proterodiplostomid life cycle and new insights into the epizootiology of this parasite.

Materials and methods

Study area and sample collection

Frogs were collected from a temporary marsh pond located near a riparian forest in Véstia Stream (20°23′43.57″S, 51°23′39.28″W), municipality of Selvíria, state of Mato Grosso do Sul, central-west region of Brazil, between March and December 2017 (four samples) and January and May 2018 (six samples). The pond contains water about ten months of the year, has a maximum depth of 1.6 m, perimeter of 314 m and surface area of 5000 m^2. Mean temperature in 2017 was 25°C with 74% relative humidity (Unesp, 2018). Anurans were collected by a single individual using the scan searching method (Halliday, 2006), with effort of 1 h. The animals were placed individually in plastic bags and transported to the Ecology Laboratory of Parasitism, Unesp, Ilha Solteira. Sampling was conducted under permit SISBIO 58746-4 from the Brazilian Institute of the Environment and Renewable Natural Resources.

Recovery of parasites

In the laboratory, frogs were identified according to Provete *et al.* (2011), sexed and length was measured. After euthanasia with a sodium thiopental solution (Thiopentax®, Itapira, SP, Brazil) administrated intraperitoneally and macroscopic inspection, viscera were removed and transferred to Petri dishes containing physiological solution (0.9% NaCl) and examined under a stereomicroscope. Metacercariae were mechanically freed from cysts, when present, and representatives were either compressed between glass slides and fixed in 10% formalin, fixed in 2.5% glutaraldehyde solution diluted in buffer solution of pH 7.4 for electron microscopy (adapted from Amato *et al.*, 1991) or preserved in 95% ethanol for molecular analysis.

Morphological study

A subsample of metacercariae were stained with haematoxylin, serially dehydrated in ethanol solutions, clarified in methyl salicylate and mounted on permanent slides with Canada Balsam (adapted from Amato *et al.*, 1991). Stained parasites were studied under a Leica DM 2500 optical microscope with differential phase contrast system and photographed with a camera coupled to the microscope. Measurements were made with an ocular micrometre. Details of the excretory system were not evaluated.

For scanning electron microscopy, glutaraldehyde-fixed trematodes were passed through increasingly concentrated acetone, which was later removed in critical point drying in a Leica Microsystems model CPD300. Samples were then metallized on a Quorum model Q150TE and photographed with a Zeiss microscope model EVO-LS15 using SmartSEM (Carl Zeiss Microscopy, Cambridge, UK). The trematodes were identified based on morphology (Dubois, 1936; Travassos *et al.*, 1969, Niewiadomska, 2002, Fernandes & Kohn, 2014). Quantitative descriptors of infection levels followed Bush *et al.* (1997). Samples were deposited in the collection of trematodes of Universidade Federal de Minas Gerais (UFMG-TRE 114) and Coleção Zoológica – ZUFMS (AMP07454–7479).

Molecular analyses

Extraction of DNA from ethanol-fixed metacercariae was performed using the QIAamp DNA micro kit (Qiagen Ltd., Crawley, UK). Partial sequences of the 28S rDNA were obtained by polymerase chain reaction (PCR). We used the forward primer digl2 (5′- AAGCATATCACTAAGCGG -3′) and reverse primer 1500R (5′- GCTATCCTGAGGGAAACTTCg -3′) and PCR conditions described by Tkach *et al.* (2003). Sequencing was performed in both directions by capillary electrophoresis using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc., Foster City, CA), using the same primers used in PCR. The sequences obtained were edited in ChromasPro (Technelysium Pty Ltd, Australia) and the contig was aligned with data from other species of Diplostomoidea available in GenBank (table 1) using MEGA version 7.0 (Kumar *et al.*, 2016). The final, trimmed alignment was 1062 bp in length. The best nucleotide substitution model (GTR + G) was determined based on Bayesian Information Criterion in MEGA 7.0 (Kumar *et al.*, 2016). A species of Clinostomidae (*Clinostomum tataxumui* – MF398321) was selected as the outgroup based on phylogenies published by Olson *et al.* (2003) and Pérez-Ponce de León & Hernández-Mena (2019). Phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian inference (BI) methods. An ML tree was computed in MEGA version 7.0 and nodal support was estimated from 1000 bootstrap pseudoreplicates. The BI analyses were performed in MrBayes version 3.2.6 (Ronquist *et al.*, 2012) using Markov chain Monte Carlo searches on two simultaneous runs of four chains for 1,000,000 generations and sampling every 100 generation. The first 25% of the sampled BI trees were discarded as 'burn-in'. Phylogenetic trees and data files were visualized in FigTree version 1.4.3 (Rambaut, 2016). The new sequence obtained in the present study was deposited in GenBank (accession number MN149353).

Ecological analysis

Bartlett homogeneity and Shapiro–Wilk normality tests were applied to verify the premises necessary for parametric tests. We used Student's *t*-test to determine whether frog size (snout vent length, SVL) differs in infected and uninfected anurans, and simple linear regression to test for a relationship between SVL and intensity of infection. Because data did not meet the assumptions for parametric tests, we used Kruskal–Wallis tests to determine if infection intensity differs in three different sites (cavity, musculature and hindlimb muscles). The foregoing statistical tests were performed with Bioestat 5.30 (Ayres *et al.*, 2007).

Results

We found 172 proterodiplostomid metacercariae encysted in the abdominal and hindlimb muscle or free in the body cavity of 29 out 86 (32.6%) *L. podicipinus* (mean size 18 mm, range 10–25 mm) collected in 2017 and 2018 from a temporary pond in Selvíria, Mato Grosso do Sul, Brazil. Metacercariae were found free in the abdominal cavity as well as encysted in the limb and abdominal musculature in one host (3.4%). Metacercariae occurred in both limbs and free in the cavity in 11 hosts

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Table 1. Morphometric data of metacercariae of *Heterodiplostomum lanceolatum* found in *Leptodactylus podicipinus* from Brazil (mean in micrometres followed by standard deviation and range between parenthesis) and *H. lanceolatum* (metacercariae and adults) and *H. helicopsis* (adults) found in snakes in South America (maximum and minimum measurements in micrometres).

		Heterodiplostomum lanceolatum					*Heterodiplostomum helicopsis*
Reference		This study	Mañé-Garzón & Alonso (1979)	Dubois (1936)	Ruiz & Rangel (1954)	Lunaschi & Sutton (1985)	Mañé-Garzón & Alonso (1976)
Locality		Brazil	Uruguay	Brazil	Brazil	Argentina	Uruguay
Developmental stage		Metacercaria	Metacercaria	Adult	Adult	Adult	Adult
Host		*Leptodactylus podicipinus*	*L. ocellatus*	*Coluber* sp.	*Xenodon guentheri*	*Helicops carinicaudus*	*H. carinicaudus*
Body total	L	4050 ± 1486 (1241–6036)	5810–7100	4950–6460	8051–9551	8016–10464	3940–4640
	W	700 ± 205 (248–879)		550–720	1342–1421	1104–1512	520–730
Forebody	L	2243 ± 969 (711–3225)	3010–3950	2850–3960	3973–4657	4512–5664	2059–2925
	W	678 ± 208 (248–879)	760–1460	550–720	1342–1421	1104–1512	
Hindbody	L	1807 ± 785 (532–2811)	2730–3950	2100–2550	4078–5052	3456–4800	1732–2044
	W	639 ± 179 (211–715)	600–1160	400–570	789–921	720–946	
Oral sucker	L	32 ± 9 (23–45)	40–70	31–48	92–111	60–68	
	W	41 ± 9 (27–51)		42–51		64–88	36–57
Ventral sucker	L	84 ± 31 (20–148)	130–180	98–115	296–320	191–229	
	W	115 ± 36 (35–173)	160–260	100–120		241–249	112–176
Pharynx	L	46 ± 6 (37–51)	60–110	38–50	80–104	80–88	
	W	40 ± 5 (27–46)		39–48		51–61	46–68
Tribocytic organ	L	773 ± 328 (198–1211)	1010–1240	1200–1650	1526–1710	1488–2241	653–1235
	W	130 ± 38 (91–192)	370–420	270–340	473–552		156–241
Ovary	L	75 ± 29 (42–119)	100–130		216	120–183	
	W	100 ± 29 (51–146)	100–130	120–150		144–166	86–140
Anterior testis	L	130 ± 65 (62–211)	160–220	180–207		204–264	100–213
	W	182 ± 72 (83–281)	140–230	180–248		216–332	113–241
Posterior testis	L	147 ± 66 (51–228)	180–240	180–255			142–255
	W	220 ± 84 (71–315)	160–270	190–239			128–270
Muscular sac	L	182 ± 53 (95–258)	180–310	170–190	442–563	380–498	185–284
	W	168 ± 68 (85–335)		100–145	253–352	199–252	113–200
Eggs	L			81–91	135–178		128
	W			43–61	74–104		42

L, length; W, width.

(37.9%), in both the limbs and the abdominal musculature in two (6.8%) and only in limb musculature in six hosts (20.7%). Nine hosts (31.0%) had only free metacercariae in the abdominal cavity. Metacercariae were recovered from frogs collected in six of ten sampling events. Prevalence of infection ranged from 25% (1/4 frogs infected in March 2018) to 100% (9/9 in January 2018). As described in the following, the worms were identified as *H. lanceolatum*.

Systematics

Superfamily: Diplostomoidea Poirier, 1886
Family: Proterodiplostomidae Dubois, 1936
Genus: *Heterodiplostomum* Dubois, 1936

Heterodiplostomum lanceolatum **Dubois, 1936 (metacercariae)** (figs 1–3, table 1)

Description

General. Based on 16 excysted metacercariae. Body bi-segmented, with hindbody connected dorsally with forebody. Forebody flattened, spatulate. Oral sucker subterminal, pharynx globular, larger than oral sucker. Oesophagus very small, bifurcating in anterior region of forebody into long intestinal caeca extending laterally into posterior region of hindbody, terminating anterior to copulatory bursa. Ventral sucker oval-shaped, located in middle region of forebody, anterior to holdfast organ. Holdfast organ prominent, elongate, located in posterior portion of forebody. Hindbody cylindrical, smaller than forebody, containing well-developed sexual organs in most specimens. Ovary oval, subspherical, located anteriorly to testes. Two subspherical testes, tandem, transversely elongate, entire. Copulatory bursa ovoid, eversible paraprostate duct inside a muscular sac similar to a cirrus-sac. Paraprostate tubular with ejaculatory duct and uterus each opening separately into copulatory bursa. Excretory pore terminal. Genital pore opening dorsally in hindbody. In some specimens, primordial vitelline glands surrounding holdfast organ and extending in two rows to ovary region (fig. 1).

Remarks

Despite marked variation in size (total length 1.23–6.06 mm) and development, metacercariae of *H. lanceolatum* examined here varied little in body shape, position of oral and ventral suckers, shape and disposition of holdfast and reproductive organs, the proportions among these organs and among organs relative to body size (fig. 3). Some metacercariae were found free in the host's abdominal cavity, but their morphology was similar to encysted forms. These specimens may have been pre-cystic stages or they may have been accidentally excysted during necropsy.

The morphology and measures of the parasites obtained here are similar to those of metacercariae identified as *H. lanceolatum* found in *Leptodactylus ocellatus* in Uruguay by Mañé-Garzón and Alonso (1979). These authors also found metacercariae encysted in the abdominal muscle and limbs. Unlike us, they did not report free metacercariae in the corporal cavity, which may be due the small number of animals necropsied (three hosts).

Several metacercariae we obtained from *L. podicipinus* were advanced in development and morphometrically similar to the type specimens (adults) of *H. lanceolatum* described by Dubois (1936) from *Coluber* sp. collected in Brazil, especially in the

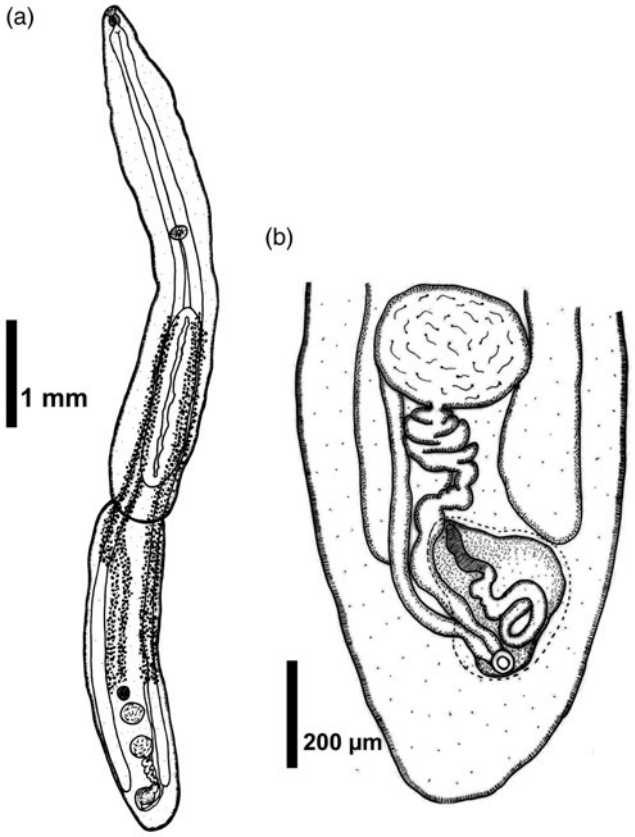

Fig. 1. Metacercariae of *Heterodiplostomum lanceolatum*. (a) Whole view of a parasite in advanced stage of development. Note the presence of vitellaria extending to anterior margin of the holdfast organ, characteristic of this species. (b) Details of terminal genitalia.

total length, fore- and hindbody widths, and the dimensions of suckers, holdfast organ, sexual organs and copulatory bursa. The only differences with the adult types of *H. lanceolatum* are in the maturity of the sexual organs and absence of eggs. Ruiz and Rangel (1954) reported large adults of *H. lanceolatum* from *Xenodon coluber* (total worm length 8.05–9.39 mm vs. 4.95–6.46 mm in Dubois (1936), cf. up to 6.04 mm in our specimens) and attributed this to differences in specimen preservation. Metacercariae we collected differed from adult stages of *Heterodiplostomum helicopsis* Mañé-Garzón & Alonso 1976, the only other species of *Heterodiplostomum*, in the extent of the intestinal caeca and vitelline glands. In *H. helicopsis*, the intestinal caeca extend to the distal extremity of the hindbody, surpassing the region of the copulatory bursa, and the vitellaria extend anteriorly to the bifurcation of oesophagus. In metacercariae of *H. lanceolatum*, the caecae terminate anterior to copulatory bursa and the vitellaria do not surpass the holdfast organ. Scanning electron microscopy revealed a smooth, non-papillose surface on the holdfast organ and the genital and excretory pores dorsal and terminal in the posterior hindbody (fig. 2).

We consider that the morphology of the metacercariae, particularly those advanced in development and with morphology similar to that described for adults, amply supports our identification of *H. lanceolatum*. Mañé-Garzón and Alonso (1979) identified metacercariae of *H. lanceolatum* based on similar reasoning. Future molecular studies of adult parasites recovered in naturally infected snakes will be helpful for unequivocal linkage of the developmental stages of *H. lanceolatum*. Experimental infections

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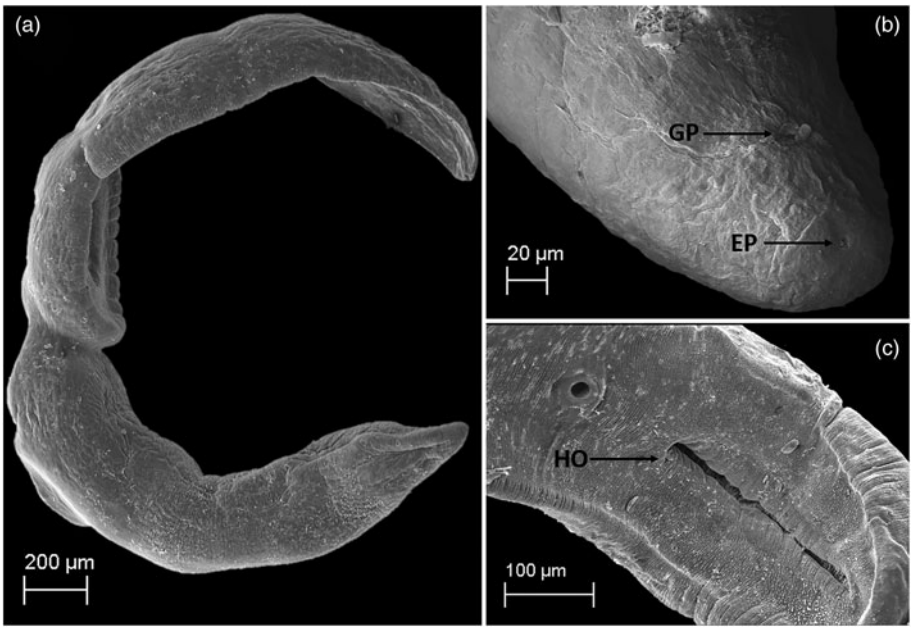

Fig. 2. Scanning electron microscopy of metacercariae of *Heterodiplostomum lanceolatum* found in *Leptodactylus podicipinus* from Brazil: (a) lateral view; (b) dorsal view of hindbody extremity, showing excretory (EP) and genital (GP) pores; (c) holdfast organ (HO) lacking papillae.

Fig. 3. Metacercariae of *Heterodiplostomum lanceolatum* found in *Leptodactylus podicipinus* from Brazil at different stages of development.

to obtain adults is a less viable method of pursuing this linkage, as the snake definitive hosts of *H. lanceolatum* present significant challenges for permitting and maintenance in laboratory settings.

Taxonomic summary

Type host. Leptodactylus podicipinus Cope, 1862.

Site of infection. Abdominal and hindlimb muscles (encysted) and abdominal cavity (unencysted).

Prevalence of infection. 33.7% (29/86).

Mean intensity of infection. 5.9 ± 6.5.

Mean abundance of infection. 2.0 ± 4.7.

Molecular characterization and genetic comparison

The 1146-bp 28S sequence of *H. lanceolatum* from *L. podicipinus* did not match any others deposited in GenBank. The most similar sequences were from *Crocodilicola pseudostoma* Willemoes-Suhm, 1870, the only proterodiplostomid with comparable data in GenBank, from which three 28S sequences diverge by at least 4.1% from our data. The 28S sequence of *H. lanceolatum* differed by 5.3–6.5% from those of the Diplostomidae, by 5.4–6.7% from the Strigeidae, by 13.0% from the Brauninidae and by 13.9% from the Cyathocotylidae. In phylogenetic analysis of a 1062-bp alignment, *H. lanceolatum* fell within a strongly supported clade containing the proterodiplostomid *C. pseudostoma* and a clade of Strigeidae + Diplostomidae, but the relationship among these three lineages was not resolved (fig. 4).

Ecological analysis

Infected frogs (SVL = 16.2 ± 3.7) were smaller than uninfected frogs (18.5 ± 3.2, $P < 0.005$), but SVL was unrelated to intensity of infection ($R^2 = 0.018$, $P > 0.05$). Infection intensity was greater in the hindlimb muscles (4.95 ± 4.05) and in the body cavity (2.57 ± 2.08) than in the abdominal muscles (range 2–10, $n = 3$) ($P = 0.0002$). Infected frogs were collected only in part of the pond (prevalence in north-west margin = 49%, prevalence in south-east margin = 0%).

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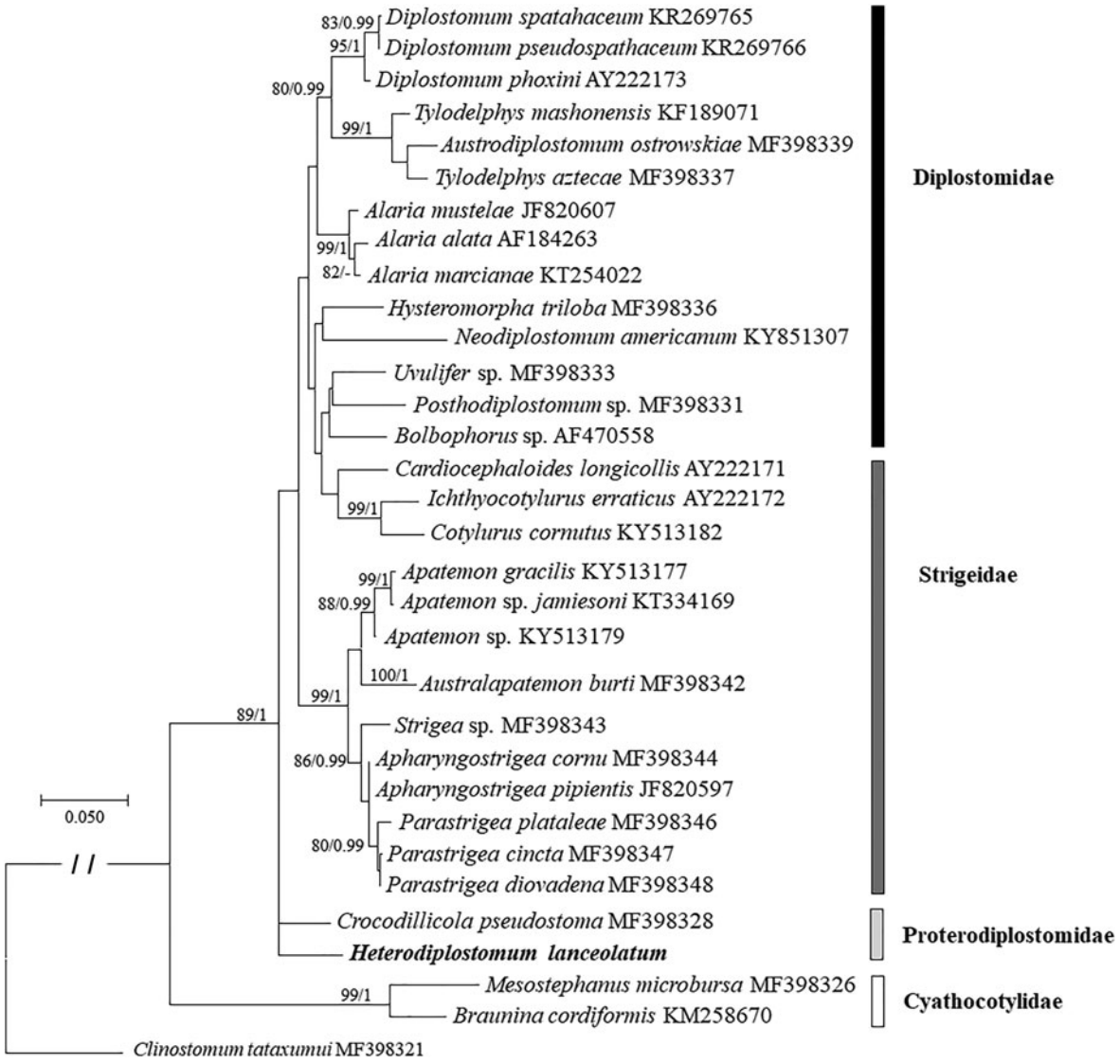

Fig. 4. Phylogenetic relationships of *Heterodiplostomum lanceolatum* (in bold) with selected members of the superfamily Diplostomoidea as inferred from analysis of partial sequences of 28S rDNA with the GTR + G model analysed by Bayesian Inference (BI) and Maximum Likelihood (ML) methods. Nodal support is indicated as ML/BI; values <0.95 (BI) and <70 (ML) are not shown.

Discussion

Like other members of the subfamily Ophiodiplostominae Dubois, 1936, species of the genus *Heterodiplostomum* mature exclusively in snakes and possess a large, non-papillose holdfast organ and vitellaria in both the fore- and hindbody (Niewiadomska, 2002). In *Heterodiplostomum*, the holdfast organ occupies about 40% of the length of the forebody and the vitellaria extend in two rows from the anterior holdfast organ to the testes, and the ejaculatory duct and uterus open separately inside the copulatory bursa, without a hermaphroditic duct (Niewiadomska, 2002). These characteristics are described only for reproductively active adults from naturally infected snakes (Dubois, 1936; Ruiz & Rangel, 1954; Lunaschi & Sutton, 1985), but we observed similar features in metacercariae in *L. podicipinus*, which possess remarkably developed reproductive organs. Adults identified as *H. lanceolatum* by Ruiz & Rangel (1954) and Lunaschi & Sutton (1985) are much larger than the type specimens described by Dubois (1936), which are similar in size to the metacercariae we encountered. A previous report of metacercariae of *Heterodiplostomum* sp. in *Leptodactylus chaquensis* in Argentina (Hamann *et al.*, 2006) did not include morphological data, whereas our identification of *H. lanceolatum* is based on light and scanning electron microscopy, as well as molecular phylogenetic analysis. The advanced development of the gonads in metacercariae of *H. lanceolatum* suggests the production of eggs probably starts shortly after infection of the snake definitive host. Progenetic metacercariae have also been reported in another proterodiplostomid, *C. pseudostoma* (Willemoes-Suhm, 1870), which infects fish and matures in crocodilians (Pérez-Ponce de León *et al.*, 1992; Guidelli *et al.*, 2003).

Two species of *Heterodiplostomum* are known, both restricted to South America. *Heterodiplostomum lanceolatum*, the only species reported in Brazil, was described by Dubois (1936) from a snake identified as *Coluber* sp. collected by Natterer and deposited at Helminthological Collection of the Natural History Museum of

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Vienna. Later, *H. lanceolatum* was recorded in *Mastigodryas bifossatus* and *Xenodon guentheri* from Brazil (Ruiz & Rangel, 1954; Fernandes & Kohn, 2014), in *M. bifossatus*, *Hydrodynastes gigas* and *Liophis poecilogyrus reticulatus* in Paraguay (Dubois, 1986, 1988), *Bothrops alternata*, *Helicops infrataeniatus*, *Helicops leopardinus* and *Hydrodynastes gigas* in Argentina (Lunaschi & Sutton, 1985; Lunaschi & Drago, 2010). *Heterodiplostomum helicopsis* Mañé-Garzón & Alonso 1976 was described from *Helicops carinicaudus* from Uruguay.

The only other published DNA sequences of proterodiplostomids are from *C. pseudostoma* (Hernández-Mena *et al*., 2017), a member of the subfamily Polycotylinae Monticelli, 1888, which mature in chelonians and crocodilians. In phylogenetic analysis, *C. pseudostoma* was as an early divergent member of a clade containing the Diplostomidae and Strigeidae (Hernández-Mena *et al*., 2017), which is consistent with our analysis of 28S from *H. lanceolatum* (fig. 4). However, the relationship between this clade, *H. lanceolatum* and *C. pseudostoma* was unresolved, which was unexpected given that both proterodiplostomids are parasites of reptiles and share the unusual morphological characters unique to the family (Niewiadomska, 2002). Considering that *C. pseudostoma* and *H. lanceolatum* belong to different subfamilies, we expect new clades to emerge among the proterodiplostomids as more members of this family are sequenced. However, it remains to be seen whether commonly sequenced nuclear ribosomal and mitochondrial markers will provide sufficient resolution at suprageneric levels (Locke *et al*., 2018).

Given the logistical difficulties of maintaining the reptilian hosts of proterodiplostomids in the laboratory, molecular data will be critical to understanding life cycles in this enigmatic group. Despite the high diversity of species of Proterodiplostomidae from South America, metacercariae of only four genera are known, all from fish and amphibians (*Cystodiplostomum*, *Crocodilicola*, *Heterodiplostomum*, *Herpetodiplostomum*) (Szidat, 1969; Mañé-Garzón & Alonso, 1979; Hamann *et al*., 2006; Ferrari-Hoeinghaus *et al*., 2007; Tavares-Dias *et al*., 2011; Hernández-Mena *et al*., 2017). While metacercariae of *Heterodiplostomum* sp. were reported in *L. chaquensis* in Argentina (Hamann *et al*., 2006), and of *H. lanceolatum* in *L. ocellatus* (L.) in Uruguay (Mañé-Garzón and Alonso, 1979), ours is the first record of metacercariae of *Heterodiplostomum* infecting anurans in Brazil.

Future molecular connections among proterodiplostomid developmental stages can test what seems to emerge from the fragmentary information currently available. In known life cycles, proterodiplostomids that mature in crocodiles encyst as metacercariae in fish, while those maturing in snakes encyst in anurans, reflecting trophic relationships in the transmission of larval forms to definitive hosts. Molecular data will also be critical for illuminating the first intermediate hosts of proterodiplostomids, which are entirely unknown (Blasco-Costa & Locke, 2017) and which may include any of the dozens of distinct, unidentified diplostomoid cercariae known in South America (Pinto & Melo, 2013; Fernandez & Hamann, 2017; López-Hernández *et al*., 2018).

Almost half the frogs collected in the present study were infected with *H. lanceolatum*. The smaller size of infected frogs could reflect high transmission rates, such that smaller frogs have greater risks of both infection and snake predation. The smaller size of infected frogs may also be of pathogenic origin, as these the worms were substantial in size compared to their host. Infection intensity was concentrated in the hindlimb muscles (4.95 ± 4.05) and in the abdominal cavity (2.57 ± 2.08). Infected frogs were collected only in one margin of the pond, which we suspect to be related to differences in aquatic vegetation that provides habitat for definitive or first intermediate hosts, but verifying this will require additional study.

We have provided the first DNA sequence of a proterodiplostomid in Brazil and a new morphological data from a larval stage of *H. lanceolatum*. These data will contribute to better understanding of the life cycles, diversity and evolutionary relationships in the poorly studied Proterodiplostomidae, and are of value in a geographic region where molecular sampling has lagged. The variations in infection levels and host size we observed also suggest directions for future research on the transmission and pathology of this parasite.

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