

Exploring Neotropical anuran parasites: a morphological, life cycle and phylogenetic study of *Catadiscus marinholutzi* (Trematoda: Diplodiscidae)

Research Article

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Abstract

Amphistome species belonging to the genus *Catadiscus* are poorly studied intestinal trematodes found primarily in Neotropical anurans. Herein, developmental stages of an amphistome species found during herpetological and malacological surveys in a temporary marsh pond from Brazil were subjected to morphological (light and scanning electron microscopy) and molecular analyses. Adult parasites recovered from anurans were identified as *Catadiscus marinholutzi*. Amphistome cercariae found in the planorbid snails *Drepanotrema depressissimum* and *Drepanotrema lucidum* from the same waterbody were used for experimental and molecular studies. Immature parasites, morphologically compatible with members of *Catadiscus*, were experimentally obtained in laboratory-reared tadpoles. Sequencing of a partial region of 28S rDNA gene of both adult and cercariae revealed 100% similarity between these developmental stages, confirming their conspecificity. Phylogenetic analyses were attempted for the first time to reveal the position of a species of *Catadiscus* in the superfamily Paramphistomoidea. *Catadiscus marinholutzi* falls in a virtual polytomy together with other paramphistomoids, which leaves its phylogenetic relationships within the group unclear. Moreover, the high genetic divergence to *Diplodiscus* spp. (10.06–10.84%) cast doubts on the placement of *Catadiscus* within Diplodiscidae. Hence the species composition of the Diplodiscidae should be re-evaluated in further studies using a broader spectrum of related taxa.

Introduction

Knowledge of taxonomy and life cycles of trematodes has advanced over time, both through classical experimental studies (revised by Yamaguti, 1975; Galaktionov and Dobrovolskij, 2003; Bolek *et al.*, 2016; Blasco-Costa and Poulin, 2017) and molecular tools (Blasco-Costa *et al.*, 2016; Assis *et al.*, 2019; Gonchar *et al.*, 2019; Locke *et al.*, 2020). However, studies focused on life cycle of trematodes parasitizing cold-blooded tetrapods, especially amphibians, are comparatively scarce worldwide. This scientific gap is more pronounced in South America, where despite the great diversity of these parasites reported in amphibians, either as definitive or second intermediate hosts (Hamann and González, 2009; Campião *et al.*, 2014; Fernandes and Kohn, 2014; Hamann *et al.*, 2019, 2020a; Queiroz *et al.*, 2019, 2020), most species remain with their life cycle and phylogenetic position unknown. In fact, only one species of trematode that uses anurans as definitive hosts (*Halipegus dubius* Klein, 1905) had its life cycle elucidated by experimental studies in South America (Paraense, 1992; Ostrowski de Núñez and Gil de Pertierra, 2004). Moreover, only recently have trematodes from anurans been studied by molecular means in this continent (Gomes *et al.*, 2013; Queiroz *et al.*, 2019; Hamann *et al.*, 2020b).

Among the poorly known trematodes found in anurans are the members of the superfamily Paramphistomoidea, parasites with a worldwide distribution (Sey, 1991; Jones, 2005a). About four dozen species of amphistomes from two families (Cladorchiidae Fischeoeder, 1901 and Diplodiscidae Cohn, 1904) have been reported in amphibians from different parts of the world (Skryabin, 1949; Yamaguti, 1971; Sey, 1991; Jones, 2005a). Among these, only members of the diplodiscid genus *Catadiscus* Cohn, 1904 (12 species) occur in South America (Sey, 1983, 1991; Campião *et al.*, 2014; Fernandes and Kohn, 2014; Hamann *et al.*, 2014). From an evolutionary point of view, the Neotropical region was considered the centre of early radiation and diversification of the paramphistomoids in amphibians, with subsequent colonization of these hosts in other zoogeographical realms (Sey, 1991). Despite the high diversity of species, the knowledge about the life cycle of these amphibian parasites is restricted to species of the genus *Diplodiscus* Diesing, 1836, *Megalodiscus* Chandler, 1923 and *Opisthodiscus* Cohn, 1904 from Europe, North America and Asia (Sey, 1991). These studies have shown that either a tadpole or adult becomes infected when ingesting encysted metacercariae in food or its own cast-

off skin; an alternative route of infection by swallowing the cercariae was also proposed (Yamaguti, 1975; Grabda-Kazubska, 1980; Bolek and Janovy Jr., 2008; Besprozvannykh *et al.*, 2018). The paucity of knowledge on the life cycle of anuran amphistomes can be due to difficulties in the maintenance of this host under laboratory conditions for experimental infection studies. The scarcity or even absence of molecular information for most species, which would make it easier to link larval stages to their adult counterparts, is a limiting factor as well. In this last aspect, data from no more than six species of trematodes from anurans were included in recent high-level phylogenetic studies involving trematodes or exclusively members of Paramphistomoidea (Olson *et al.*, 2003; Pérez-Ponce de León and Hernández-Mena, 2019; Alves *et al.*, 2020).

In the current study, adult parasites recovered from anurans and amphistome cercariae found in planorbid snails in Brazil were subjected to morphological, experimental and molecular studies. The life cycle of *Catadiscus marinhoi* Freitas and Lent, 1939 was here studied for the first time using this integrative taxonomic approach. Moreover, the first phylogenetic placement of a species of *Catadiscus* within the Paramphistomoidea is attempted and associated implications for the systematics of the group discussed.

Materials and methods

Study area and host sampling

The anurans and snails evaluated in this study were collected in a temporary marsh pond located in a riparian forest of Véstia stream (20°23'43.4"S, 51°23'39.8"W), municipality of Selvíria, State of Mato Grosso do Sul, the Central-West region of Brazil. The pond contains water about 10 months a year, has a maximum depth of 1.6 m, a perimeter of 314 m and a surface area of 5000 m². Adult anurans were collected by the scan searching method (Halliday, 2006), with 1 h of capture effort during 23 samplings between December 2016 and February 2019. The animals were placed individually in plastic bags and transported to the laboratory, where they were measured (snout-vent length) and euthanized [sodium thiopental solution (Thiopentax®, Itapira, SP, Brazil), *via* intraperitoneal]. Tadpoles were collected using a rectangular dip-net (75 cm × 50 cm) and transported to the laboratory. The anurans were subjected to macroscopic inspection, and after this process, viscera were removed, transferred to Petri dishes containing physiological solution (0.9% NaCl) and examined under a stereomicroscope for detecting the presence of parasites. The taxonomic identification of anurans was based on morphological criteria, according to Provet *et al.* (2011).

A long-term malacological survey was carried out monthly between May 2017 and February 2019 (29 field expeditions with a duration of 1 h). The snails were also collected using a rectangular dip-net, placed in a plastic sack and transported to the laboratory for the parasitological analyses. In the laboratory, the collected snails were placed individually in polystyrene microplates and subjected to artificial photostimulation for 2 h followed by exam using a stereomicroscope. A new exam was performed after an overnight period (Fernandez *et al.*, 2008). Subsamples of the snails collected were used for taxonomic identification, which was based on morphological traits, according to Paraense and Deslandes (1956, 1957) and PAHO (1968).

Experimental infection tests

For the current study, a morphotype of amphistome cercariae found in *Drepanotrema lucidum* (Pfeiffer, 1839) was selected to experimental infection study. Aiming to obtain parasite-free tadpoles, we sampled anuran eggs from the pond at the beginning of

the rainy season of 2018. Eggs of two anuran species were collected: *Physalaemus cuvieri* Fitzinger, 1826 (Leptodactylidae) and *Trachycephalus typhonius* (Linnaeus, 1758) (Hylidae). The laboratory-reared tadpoles were kept in rectangular plastic trays (60 cm × 35 cm × 15 cm) with 3 L of filtered water and aquarium pumps. They were fed daily with fish feed. In the first experiment, we evaluated the formation of metacercariae on tadpole skin. For this, tadpoles of *T. typhonius* (Gosner stage 26–28) ($n = 3$) and *P. cuvieri* (Gosner stage 27–38) ($n = 3$) were placed individually in wells of a 6-well polystyrene plate containing 10 mL chlorine-free water and at least ten cercariae. The plate was examined under a stereomicroscope for the presence of encysted metacercariae on tadpole or at the bottom of the well after 2, 4, 6, 12 and 24 h exposure to the cercariae. We tried to experimentally infect tadpoles of *T. typhonius* ($n = 6$) to obtain adult parasites. The laboratory-reared anurans were placed with one infected snail in a 1 L aquarium for 80 h. After the exposure, the tadpoles were necropsied in 2 days interval for the recovery of trematodes.

Morphological study

Samples of adult amphistomes from different species of anuran hosts naturally infected were slightly compressed between glass slide and coverslip and fixed in formalin, or killed in hot water (70°C) and fixed in 2.5% glutaraldehyde solution diluted in a buffer solution of pH 7.4. Samples of worms 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). The cercariae that emerged from the snail hosts were initially studied alive in non-permanent preparations stained with vital dyes (0.05% neutral red or Nile blue sulphate). For morphometric analyses, they were killed in hot water (70°C), fixed in 2.5% glutaraldehyde solution diluted in a buffer solution of pH 7.4. Two naturally infected snails were dissected to recover rediae.

The developmental stages were studied under a Leica DM 2500 optical microscope with a differential phase contrast system and photographed with a camera coupled to the microscope. Measurements were taken with the aid of Leica software. Morphometric data are presented in micrometres as the mean followed by the standard deviation and range in parentheses. For scanning electron microscopy (SEM), some heat-killed (70°C) and glutaraldehyde-fixed trematodes were passed through increasingly concentrated acetone, which was later removed by critical point drying in a Leica Microsystems model CPD300. Samples were then sputter-coated with gold for 3 min (film thickness: 5 nm) on a Quorum model Q150TE and photographed with a Zeiss microscope model EVO-LS15 using SmartSEM. The taxonomic identification of adult trematodes was based on morphological traits according to different authors (Travassos *et al.*, 1969; Sey, 1983, 1991; Jones, 2005a, 2005b). The quantitative descriptors of parasitism followed Bush *et al.* (1997). Voucher specimens were deposited in the collection of trematodes of UFMG (UFMG-TRE 119).

Molecular analyses

Samples of adult parasites recovered from naturally infected *Leptodactylus podicipinus* (Cope, 1862) (Leptodactylidae) and cercariae emerged from *Drepanotrema depressissimum* (Moricand, 1839) (Planorbidae) were fixed in ethanol for molecular analyses. The DNA was extracted using the QIAamp DNA micro kit (Qiagen Ltd., Crawley, UK) and quantified using a microvolume spectrophotometer (NanoDrop Lite; Thermo Fisher Scientific). A partial region of the 28S rDNA gene (~1200 bp) was amplified using the primers (dig12 and 1500R) and polymerase chain reaction (PCR) conditions described by Tkach *et al.* (2003). PCR

reactions were performed in a final volume of 25 μ L, which included 12.5 μ L Platinum Hot Start PCR Master Mix, 1.25 pmol of each primer and about 50 ng of template DNA. Amplification products were visualized in 1% agarose gel electrophoresis stained with UniSafe Dye 20.000 \times (Uniscience) and purified with polyethylene glycol 20% (PEG 8000, Promega, USA). The sequencing was done in two directions by capillary electrophoresis using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Inc., Foster City, CA) in an ABI3730 sequencer, with the same primers used in PCR.

Contiguous sequences were assembled in ChromasPro (Technelysium Pty Ltd., Australia) and aligned with 44 sequences available for paramphistomoids in the GenBank (Table S1) using default parameters of MAFFT (Katoh and Standley, 2013) implemented in the Guidance2 web server (<http://guidance.tau.ac.il/>; Sela et al., 2015). Unreliable positions in the alignment were identified and removed using the Gblock web server (<http://phylogeny.lirmm.fr/>; Dereeper et al., 2008) with less stringent settings. Uncorrected *P*-distances were calculated using MEGA 7.0 (Kumar et al., 2016). Phylogenetic reconstructions were performed with the Bayesian inference (BI) and maximum likelihood (ML) criteria, based on the GTR + F + I + G₄ model, predicted as the best estimator by the small sample size corrected Akaike information criterion in ModelFinder (Kalyaanamoorthy et al., 2017) within IQ-TREE (Nguyen et al., 2015). Microscaphid and mesometrid digeneans were used as outgroups (see Table S1) based on previous studies (Olson et al., 2003; Pérez-Ponce de León and Hernández-Mena, 2019).

The ML trees were generated via IQ-TREE and nodal supports were estimated with 10 000 UFBoot iterations (Minh et al., 2013). BI analyses were conducted in MrBayes ver. 3.2 (Ronquist et al., 2012) running two independent MC3 runs of four chains (one cold, three heated) for 10 million generations, sampling tree topologies every 1000th generation, with the first 25% of samples discarded as burn-in; all above-mentioned analyses were run on the computational resource CIPRES (Miller et al., 2010). Tracer v.1.6 (Rambaut et al., 2014) was used to check the convergence and mixing of different parameters and to confirm that the effective sample size (ESS) of each parameter was adequate to provide reasonable estimates of the variance in model parameters (i.e. ESS values >200). FigTree ver. 1.4.2 (Rambaut, 2012) was used for tree visualization and Adobe® Photoshop® CS5 (Adobe Inc., USA) for additional editing. Newly generated sequences were deposited in the GenBank database (accession number MW618662 and MW618663).

Results

Herpetological and malacological surveys

In the herpetological study, 296 specimens of adult anurans of 17 species were collected and evaluated for trematode infections. From these, 76 specimens (24.9%) belonging to five species [*Boana raniceps* (Cope, 1862), *Pseudis platensis* Gallardo, 1961 – both Hylidae; *Leptodactylus macrosternum* Miranda-Ribeiro, 1926, *L. podicipinus*, *Pseudopaludicola* sp., – all Leptodactylidae] were found harbouring amphistomes in the large intestine. Basic descriptive ecological parameters of the infections are shown in Table 1. The following species of adult anurans were not found infected with amphistome trematodes: *Dendropsophus elianeae* (Napoli and Caramaschi, 2000) (*n* = 7), *Dendropsophus minutus* (Peters, 1872) (*n* = 22), *Dendropsophus nanus* (Boulenger, 1889) (*n* = 44), *Scinax fuscovarius* (Lutz, 1925) (*n* = 6), *Scinax nasicus* (Cope, 1862) (*n* = 12), *T. typhonius* (*n* = 2) – all Hylidae; *Leptodactylus mystaceus* (Spix, 1824) (*n* = 2), *L. mystacinus* (Burmeister, 1861) (*n* = 1), *P. cuvieri* (*n* = 2), *Physalaemus nattereri*

Table 1. Ecological parameters for *Catadiscus marinholutzi* recovered in five anuran species sampled in Selviria, Central-Western Brazil, from December 2016 to February 2019

Anuran species	Prevalence of infection (%)	Mean intensity of infection (range)
<i>Boana raniceps</i>	5/27 (19.2)	1.80 ± 0.43 (1–4)
<i>Leptodactylus macrosternum</i>	3/12 (25)	17.33 ± 3.08 (3–43)
<i>Leptodactylus podicipinus</i>	55/106 (51.88)	5.23 ± 0.27 (1–21)
<i>Pseudopaludicola</i> sp.	3/17 (17.6)	2.66 ± 0.40 (2–4)
<i>Pseudis platensis</i>	10/20 (50)	10.10 ± 0.89 (1–29)

(Steindachner, 1863) (*n* = 8) – all Leptodactylidae; *Dermatonotus muelleri* (Boettger, 1885) (*n* = 6) – Microhylidae; *Pithecopus azureus* (Cope, 1862) (*n* = 6) – Phyllomedusidae. Additionally, 261 specimens of tadpoles belonging to 11 species [*B. raniceps* (*n* = 49); *D. minutus* (*n* = 4), *D. nanus* (*n* = 67), *D. muelleri* (*n* = 50), *L. podicipinus* (*n* = 20), *P. platensis* (*n* = 8); *P. cuvieri* (*n* = 6), *P. nattereri* (*n* = 14), *S. fuscovarius* (*n* = 18), *S. nasicus* (*n* = 6) and *T. typhonius* (*n* = 19)] were examined; however, none of them was found harbouring encysted metacercariae on skin or trematodes in the intestine.

In the malacological study, a same morphotype of amphistome cercaria was found in 3/420 (0.7%) of the specimens of *D. depressissimum*, and 6/2236 (0.3%) of *D. lucidum* (Pfeiffer, 1839) (Planorbidae) collected in the current study. Specimens of *Biomphalaria occidentalis* (Planorbidae) (*n* = 1013), *Pomacea* sp. (Ampullariidae) (*n* = 66) and *Physa marmorata* Guilding, 1828 (Physidae) (*n* = 360) were also collected and examined for infection with larval trematodes, but no amphistome cercariae were found in these species.

Experimental infection study

During the experimental infection study carried out with the amphistome cercariae found in snails, larval encystment on tadpole skin was not observed. However, live cercariae and encysted metacercariae were found at the bottom of the container in all intervals. After 24 h of exposure, only metacercariae were observed. The keeping of tadpoles of *T. typhonius* in the same aquarium containing one naturally infected snail resulted in the infection of one of the six exposed specimens (precise pathway of infection not observed). In total, 14 immature worms were recovered in the intestine of this tadpole 8 days after the exposure.

Morphological study and taxonomic identification

Morphological examination of adult parasites found in anurans enabled us to identify these amphistomes as *Catadiscus marinholutzi*. Members of *Catadiscus* are typified by having the lumen of acetabulum constricted in two regions (Jones, 2005b) and may be distinguished from each other by several morphological traits (e.g. caeca length, arrangement and extent of vitelline follicles, pharynx/acetabulum and acetabulum/body ratios) (Sey, 1991). *Catadiscus marinholutzi* can be readily distinguished from most of its congeners by the presence of vitelline follicles confluent anteriorly. An exception is *Catadiscus propinquus* Freitas and Dobbin Jr., 1956, of which *C. marinholutzi* differs by the presence of larger vitelline follicles and pharynx/acetabulum ratio (Sey, 1991). In all infected host species, we observed the presence of ovigerous worms at different stages of maturation, i.e. juvenile as well as fully mature (gravid) specimens with eggs in the uterus and, as previously reported for *C. propinquus* by Hamann (2004). Comparative morphometric data, based on fully mature adult

Table 2. Morphometric data of *Catadiscus marinhoi* found in anurans from Brazil and of the most similar species, *Catadiscus propinquus*

Reference	<i>C. marinhoi</i>		<i>C. propinquus</i>
	Current study	Freitas and Lent (1939)	Freitas and Dobbin Jr (1956)
Host	<i>Leptodactylus podicipinus</i>	<i>Leptodactylus latrans</i>	<i>Lithobates palmipes</i>
N	9	8	27
Body	L	1363 ± 281 (1138–2052)	1610–2490
	W	753 ± 153 (560–1086)	1050–1290
Pharynx	L	70 ± 14 (53–95)	80–100
	W	97 ± 17 (76–120)	–
At pharyngeal sacs level	L	149 ± 27 (117–190)	200–220
	W	133 ± 23 (109–180)	180–220
Appendages	L	80 ± 15 (60–102)	–
Oesophagus	L	162 ± 30 (117–196)	280–330
Oesophageal bulb	L	83 ± 12 (70–101)	150–180
	W	56 ± 9 (46–75)	60–100
Acetabulum	L	462 ± 40 (370–500)	700–900
	W	380 ± 35 (315–420)	530–980
Pharynx/acetabulum		3.3 ± 0.55 (2.6–4)	3.44*
Testis	L	131 ± 32 (92–167)	280–530
	W	214 ± 31 (181–259)	330–550
Ovary	L	99 ± 16 (84–127)	100–170
	W	111 ± 24 (78–143)	120–150
Eggs	L	99 ± 12 (73–110)	113–126
	W	56 ± 7 (42–63)	59–71
Vitelline follicles	L	95 ± 12 (83–120)	82–163*
	W	130 ± 20 (104–170)	82–112*

L, length, W, width.

Asterisks indicate data calculated from figures presented in the cited references.

specimens obtained from *L. podicipinus*, are shown in Table 2. Measurements obtained from selected mature worms from other four anuran host are presented in Table S2, which are also compatible with *C. marinhoi*. Overall, the morphology of immature worms obtained experimentally in tadpoles matches that of the adult forms. The link between cercariae and adults is also supported using molecular data (100% similarity in the 28S rDNA). The morphometric data obtained for larval stages are shown in Table 3 and compared with morphologically similar species reported in South America.

Taxonomic summary

Catadiscus marinhoi Freitas and Lent, 1939 (Figs 1–4; Tables 2 and 3).

Type-host and locality: *Leptodactylus latrans* (Steffen, 1815), Aquidauana, State of Mato Grosso do Sul, Brazil.

Definitive hosts: *Leptodactylus macrosternum* (New Host Record – NHR), *Leptodactylus podicipinus*, *Pseudopaludicola* sp. (NHR) (Anura: Leptodactylidae), *Boana raniceps* (NHR), *Pseudis platensis* (NHR) (Anura: Hylidae).

First intermediate hosts: *Drepanotrema lucidum* and *Drepanotrema depressissimum* (Mollusca: Planorbidae).

Locality: Selvíria (New Locality Record), State of Mato Grosso do Sul, Brazil.

Representative DNA sequences (partial 28S rDNA): MW618663 from *D. lucidum* (snail host) and MW618662 from *L. podicipinus* (anuran host).

Morphological description

Adults (Figs 1 and 2, Table 1): Body piriform with smooth tegument (Figs 1A, B and 2A). Mouth terminal with undulated edges surrounding oral aperture (Fig. 2A, B). Pharynx muscular, with two evident pharyngeal sacs (Fig. 1C). Oesophagus long, with a muscular bulb. Two small caeca reaching up to equatorial region. Genital pore small, pre-equatorial, opening at caeca bifurcation level, with a prominent sucker-like structure (genital papillae) surrounding the genital pore (Figs 1A, D and 2A, C). Cirrus sac globose. Acetabulum ventral and subterminal (Figs 1A, B and 2A); large, with a transversal constriction (Figs 1A, B and 2A, D). Testis single, oval, smooth, intercaecal, median or submedian (Fig. 1A and B). Ovary rounded or oval, sometimes elongated, testicular or post-testicular (Fig. 1A and B). Vitellaria with large follicles, larger than eggs, lying between the acetabulum and caecal bifurcation, confluent anteriorly (Fig. 1A and E). Uterus sinuous, dorsal, overlapping the acetabulum zone, extending to bifurcal region. Eggs oval, operculated, smaller than vitelline follicles.

Cercariae (Figs 3 and 4A–C, Table 3): Body ovoid with black pigmentation, except in mouth and pharynx region (Fig. 4A). Two pigmented, square-shaped eyespots in the anterior body,

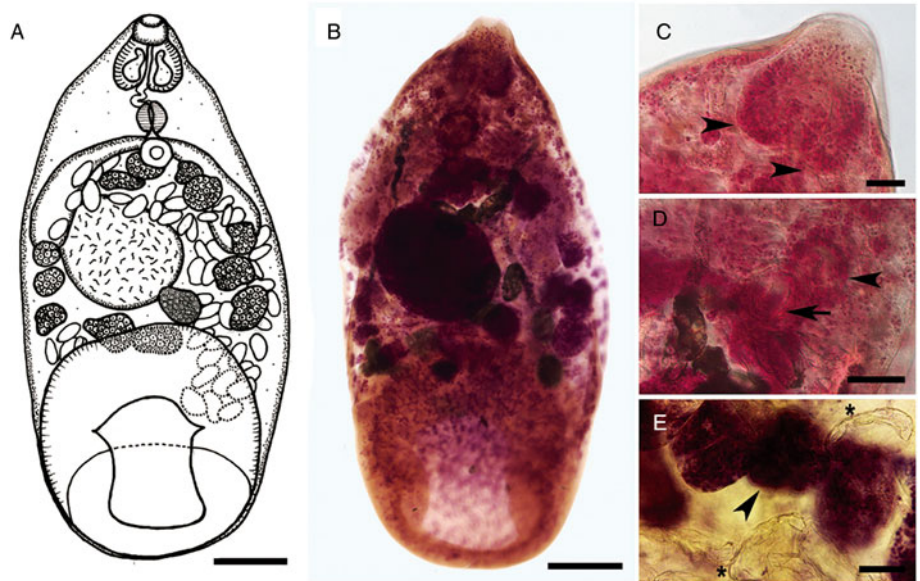


Fig. 1. *Catadiscus marinholutzi* adult recovered from *Leptodactylus podicipinus* from Brazil: (A) Line drawing of a mature worm. (B) Body in ventral view of a haematoxylin-stained worm. (C) Details of pharynx with primary sacs (arrowhead). (D) Details of oesophageal bulb (arrowhead) and genital pore (arrow). (E) Details of the relative size of vitelline follicles (arrowhead) and eggs (asterisks). Scale bars: A, B = 200 μ m; C, E = 50 μ m, D = 100 μ m.

Table 3. Morphometric data of cercariae, metacercariae and rediae of *Catadiscus marinholutzi* found in *Drepanotrema* spp. from Brazil

	<i>C. marinholutzi</i>		<i>Catadiscus uruguayensis?</i>	<i>Paramphistomum</i> sp.?
	Reference	Current study	Ostrowski de Núñez (1979)	Veitenheimer-Mendes and Almeida-Caon (1989)
Host		<i>Drepanotrema depressissimum</i> <i>Drepanotrema lucidum</i>	<i>Drepanotrema kermatoides</i>	<i>D. kermatoides</i>
		Brazil	Argentina	Brazil
<i>Cercariae</i>				
Body	L	276 \pm 25 (218–318)	212–297	315–540
	W	183 \pm 13 (156–204)	233–265	252–342
Pharynx	L	53 \pm 14 (42–96)	43–54	50–81
	W	66 \pm 12 (50–101)	46–59	50–81
Acetabulum	L	89 \pm 11 (75–126)	68–92	80–135
	W	113 \pm 9 (71–128)	86–100	80–135
Tail	L	588 \pm 41 (520–660)	551–710	495–630
	W	60 \pm 4 (54–69)	63–74	54–108
<i>Metacercariae</i>	L	218 \pm 11 (232–181)	200	225–234
	W	180 \pm 12 (159–202)	200	225–234
<i>Rediae</i>				
Body	L	802 \pm 120 (583–1066)	–	431–666
	W	212 \pm 28 (162–265)	–	100–180
Pharynx	L	77 \pm 8 (64–91)	60	36–45
	W	82 \pm 6 (73–96)	50	36–45

L, length; W, width.

Data reported for amphistome cercariae found in *D. kermatoides* in South America by other authors are shown for comparison.

between pharyngeal sacs and oesophageal bulb (Figs 3 and 4B). Abundant cystogenous cells in two shapes, with granular contents in lateral-body and with rod-shaped contents in the mid-body (Figs 3 and 4B). Mouth terminal with undulated border, followed by a muscular pharynx with two pharyngeal sacs longer and wider than pharynx. Oesophagus long, ending with a muscular bulb. Two relatively short caeca, posteriorly enlarged, running laterally up to the mid-body, distant from the acetabulum. Acetabulum large, subterminal, constricted in the middle region (Figs 3 and

4A, C). Excretory system constituted by an oval excretory vesicle, pre-acetabular, two main collecting ducts, with numerous small granules inside, united above excretory vesicle, running sinuously up to posteriormost pharyngeal region, surpassing the eyespots. Medial anastomosis of main collecting ducts absent. Additional data of the excretory system were not observed because of the intense pigmentation of the body. Tail long, more than twice the body length, cylindrical, progressively tapered, presenting a long caudal excretory duct slightly enlarged towards tip.

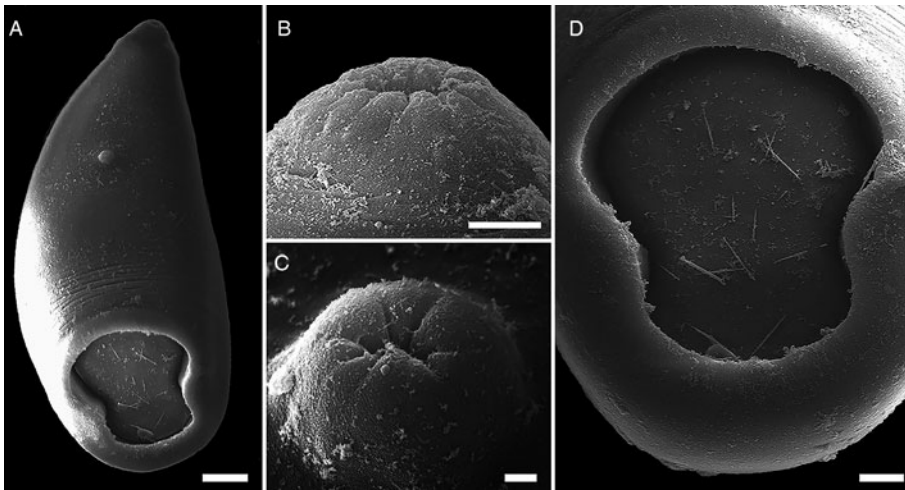


Fig. 2. SEMs of *Catadiscus marinholutzi* from *Leptodactylus podicipinus*. (A) Body in ventral view showing the genital pore. (B) Oral opening surrounded by undulated edges. (C) Details of the genital pore bearing a genital papillae. (D) Details of the median transversal constriction of acetabulum. Scale bars: A = 50 μ m, B = 10 μ m, C = 2 μ m, D = 20 μ m.

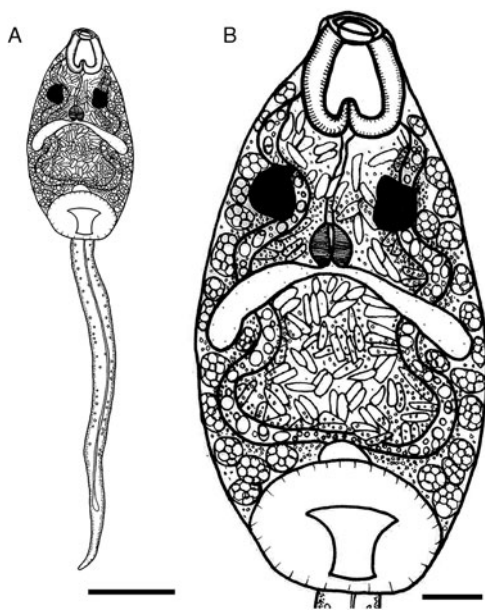


Fig. 3. Line drawing of cercariae of *Catadiscus marinholutzi* found in *Drepanotrema lucidum*. (A) Whole view of cercaria. (B) Details of cercarial body. Scale bars: A = 100 μ m, B = 25 μ m.

Metacercariae (Fig. 4D): Cysts spherical, cystic wall thin.

Rediae (Fig. 4E and F): Body transparent, elongated. Pharynx subterminal. Caecum short, sac-like, with brown granules inside. Several germinal balls (8–10) at different stages of development scattered in the body.

Remarks

The morphology and measurements of adults of *C. marinholutzi* reported in this study are compatible with those described by Freitas and Lent (1939). Although full range of variation for most morphometric data is higher in our specimens than those reported in the original description, the mean body length and width of our individuals are slightly smaller than the interval reported by Freitas and Lent (1939). These differences may come from different fixation and mounting processes, comparison of worms at different stages of the ontogenic development, or it indicates a high morphometric variability within the species. Such variability, which may be host-induced (e.g. host size, age and geographical distribution), cannot be overlooked in species delineation of *Catadiscus* based on morphological features alone.

Few cercariae of *C. marinholutzi* reported here in *Drepanotrema* spp. emerged from the snails on the morning and encysted on the bottom of the container. They belong to the Diplocotylea group of amphistome cercariae, characterized mainly by the presence of pharyngeal sacs and absence of medial anastomosis in the main collecting ducts (Sewell, 1922; Dawes, 1968; Schell, 1985). The presence of a transverse constriction in the acetabulum, a trait known for the adult stages of the genus *Catadiscus*, is reported here in the cercariae. Considering amphistome cercariae reported in *Drepanonetrema* spp. from South America, those described as the probable larval stage of *Catadiscus uruguayensis* Freitas and Lent, 1939 by Ostrowski de Núñez (1979) presents measurements similar to the morphometric data gathered from *C. marinholutzi*. However, some morphological differences are noticeable, especially concerning the position of the caeca (long, extending up to the acetabulum), extension of main ducts of the excretory system and position of the excretory vesicle (dorsal to acetabulum), absence of an oesophageal bulb and especially the non-constricted acetabulum. The amphistome cercariae reported from *Drepanotrema kermatoides* (Orbigny, 1835) in Brazil by Veitenheimer-Mendes and Almeida-Caon (1989) is significantly longer and wider than the cercariae of *C. marinholutzi* and possess medial anastomosis in the main excretory ducts, eyespots with lenses and an acetabulum without transverse constrictions. Despite unequivocal experimental evidence is still required to determine the route of infection, the specimen of *T. typhoni* became infected probably by the ingestion of encysted metacercariae present in the food. Anyway, the results obtained reveal that the ingestion of its own shed skin is not a mandatory event, given the recuperation of parasites occurs before metamorphosis.

Phylogenetic analyses

The two identical, newly generated 28S sequences from cercariae and adults of *C. marinholutzi* were 1219 bp long. The alignment of 46 sequences comprised of 1431 positions, of which 453 were excluded, resulting in a final alignment of 978 positions with 278 parsimony-informative sites. The phylogenetic trees resulting from the BI and ML analyses were identical in topology, except for the relationship among representatives of *Diplodiscus* (see ML tree depicted in Fig. S1). The ingroup formed a virtual polytomy composed of five clades equally related to each other: (i) amphistome parasites of warm-blooded terrestrial hosts (collapsed clade), including representatives of the Zygotocylidae, Gastrodiscidae, Paramphistomidae, Gastrothylacidae and Olveriiidae; (ii) cladorchids (Cladorchiidae) that have colonized aquatic and terrestrial cold-blooded hosts - except for *Solenorchis travassosi* Hilmy, 1949, known from the marine mammal *Dugong*

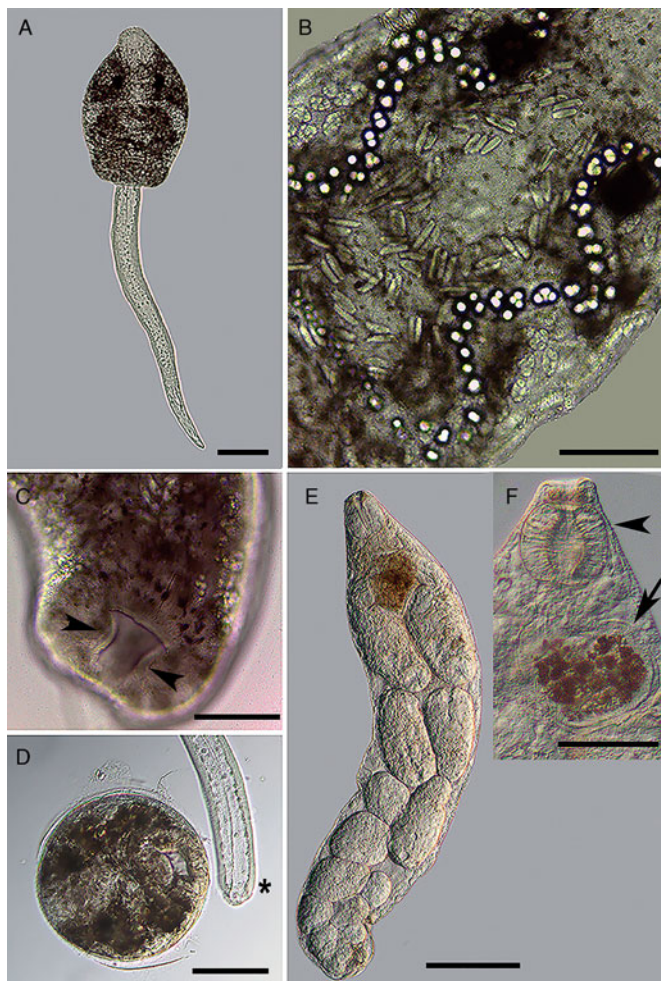


Fig. 4. Larval stages of *Catadiscus marinholutzi* found in *Drepanotrema lucidum*. (A) Whole-mount of the amphistome cercaria, ventral view. (B) Details of cercarial body. (C) Details of the constriction in the acetabulum of cercaria (arrowhead). (D) Recently encysted metacercaria and detail of the detachment of the tail from the body (asterisk). (E) Whole-mount of redia. (F) Details of anterior region of redia, evidencing the pharynx (arrowhead) and caecum (arrow). Scale bars: A, D, F = 100 μm ; B, C = 50 μm . E = 200 μm .

dugon (Müller, 1776) – from unrelated hosts and geographical regions; (iii) fish cladorchids from the Neotropical region; (iv) members of *Diplodiscus*, type genus of the Diplodiscidae from the Palaearctic region and (v) isolates of *C. marinholutzi* (also Diplodiscidae) from its intermediate and final hosts (Fig. 5).

Even though *C. marinholutzi* appeared as an independent lineage within the Paramphistomoidea, its phylogenetic relationships remain unclear. In addition, there is no evidence for the monophyly of the Diplodiscidae considering the branch support of the group formed by *C. marinholutzi* and *Diplodiscus* spp. (32% UFbootstrap and 0.58 posterior probability support values) and the relatively high genetic divergence verified between them (ranging from 10.06 to 10.84%; 65–70 nt differences). The species of *Megalodiscus*, found primarily in amphibians and once included within Diplodiscidae, grouped in a moderately supported clade with other cladorchids from unrelated hosts and zoogeographical realms, while the remaining representatives of Cladorchidae from Neotropical fishes formed a well-supported clade; genetic divergence between *C. marinholutzi* and *Megalodiscus* spp. ranged from 12.48 to 13.47% (80–87 nt differences).

Pairwise comparisons within well-supported monophyletic assemblages revealed lower intergeneric divergences in the Gastrodiscidae (1.08%; 7 nt differences), Zygocotylidae (1.70%; 11 nt differences), and Neotropical cladorchids (1.39–6.19%;

9–40 nt differences) than that exhibited between the diplodiscids *Catadiscus* and *Diplodiscus* (see above).

Discussion

The results obtained here for *C. marinholutzi* from anuran and snail hosts bring new insights into the biology and taxonomy of the genus *Catadiscus*, formed by poorly studied trematodes found primarily infecting amphibians from Neotropics. Using an integrative taxonomic approach for studying *C. marinholutzi*, we successfully linked cercariae found in snails and adults recovered from amphibians. Moreover, this study represents the first attempt to include a species of *Catadiscus* into a phylogenetic context. Our results suggest that a taxonomic reorganization may be necessary to accommodate species of this genus.

The species identified in the current study as *C. marinholutzi* was originally described by Freitas and Lent (1939) from anurans identified as *Leptodactylus ocellatus* (L.) and *Leptodactylus caliginosus* Girard, 1853 [both currently considered synonyms to *Leptodactylus latrans* (Steffen, 1815)] collected in Salobra and Aquidauana (district of Camisão), localities from State of Mato Grosso Sul, Brazil, not so far from our sampling area (about 600 km). Later, additional reports of the infection of leptodactylids (*L. latrans* and *L. podicipinus*) with this amphistome were registered in different localities in Brazil (Travassos, 1940; Goldberg et al., 2009; Graça et al., 2017). The species was also reported in *Rhinella fernandezae* (Gallardo, 1957) from Argentina (Hamann et al., 2013), greatly expanding its distribution. Out of South America, the species was found in colubrid snakes [*Coniophanes bipunctatus* (Günther, 1858), *Coniophanes imperialis* (Baird and Girard, 1859), *Coniophanes quinquevittatus* (Duméril, Bibron and Duméril, 1854), *Leptodeira septentrionalis* (Kennicott, 1859) and *Thamnophis* sp.] from Mexico (Thatcher, 1964; Sey, 1991; Paredes-León et al., 2008). In the current study, four new definitive hosts (*L. macrosternum*, *Pseudopaludicola* sp., *B. raniceps* and *P. platensis*) are reported for *C. marinholutzi*, including the first record in hylid anurans. Environments that present seasonal floods, i.e. water expansion and retraction in annual cycles, such as the Pantanal wetlands and our sampling area, promote ecological opportunities via ecological fitting (Janzen, 1985) for new host–parasite associations (see D’Bastiani et al., 2020 and references therein). This process may have boosted the encounters of *C. marinholutzi* with a diverse array of aquatic and semiaquatic anuran hosts. Regardless of environmental conditions, these findings are not unexpected given that *Catadiscus* species are known to have broad host specificity. Besides amphibians and non-avian reptiles, adult forms of *Catadiscus* spp. have been found in the golden apple snail *Pomacea canaliculata* (Ampullariidae) and the annual fishes *Austrolebias* spp. (Rivulidae) in Argentina and Uruguay, respectively (Hamann, 1992; Vettorazzi et al., 2020).

The morphometric data obtained here are compatible with the description of *C. marinholutzi*. Also, first SEM images of a *Catadiscus* species demonstrated more clearly the presence of a constriction in the acetabulum of *C. marinholutzi*, a typical trait of the genus *Catadiscus* but described as being relatively poorly developed in this species (Freitas and Lent, 1939). The presence of a small genitalium bearing a prominent genital papillae is evidenced for the first time in species of *Catadiscus* by the aid of SEM. Histological sections of this structure are broadly used to characterize different groups of paramphistomoids (see Sey, 1991), but likely due to their small size, diplodiscid taxa have never been evaluated by means of this approach. Therefore, further comparative studies of the terminal genitalium among members of the Diplodiscidae may be useful to support or refute the current classification scheme of the family. Morphologically, *C. marinholutzi* most closely resembles *C. propinquus*. Both

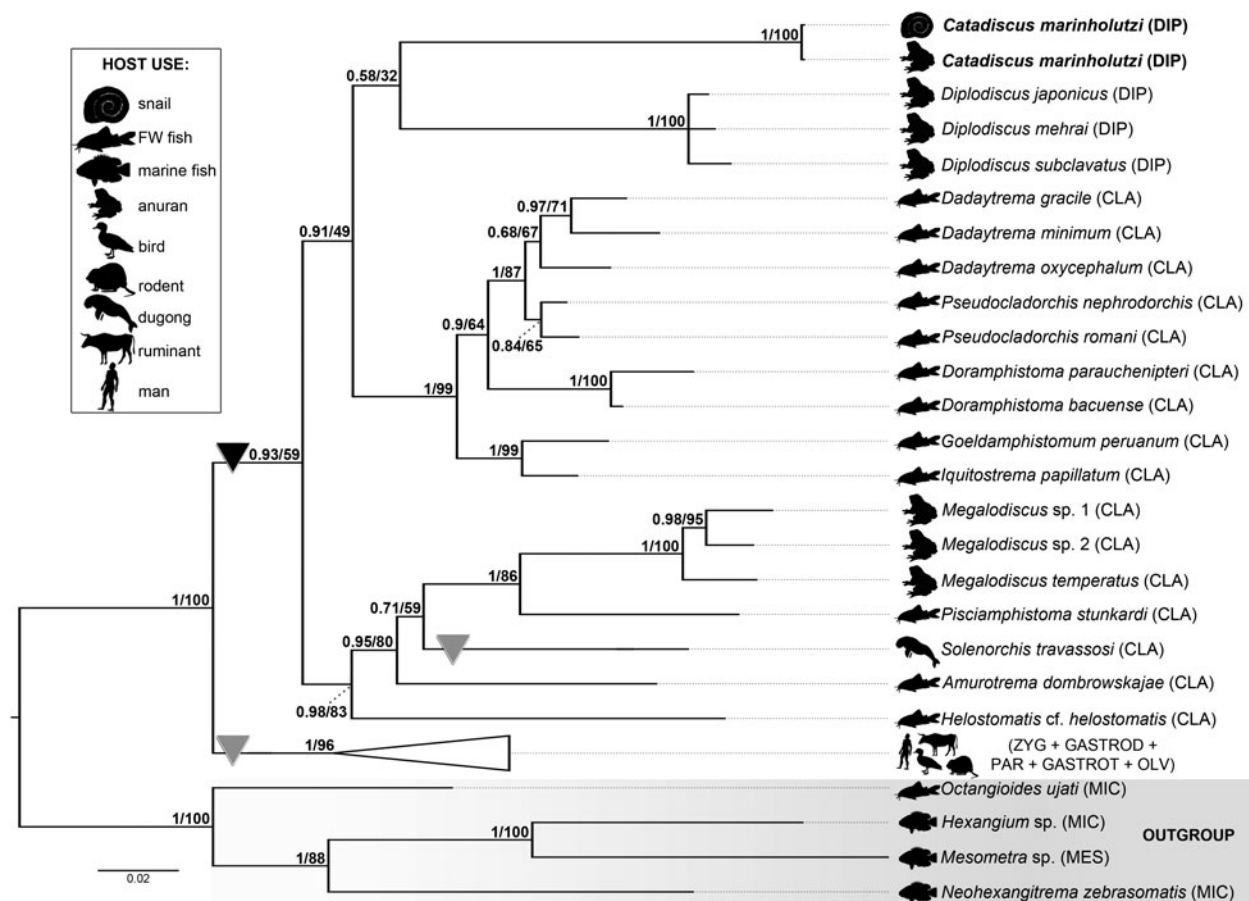


Fig. 5. BI phylogram based on partial 28S sequences of *Catadiscus marinhoi* and selected members of the Paramphistomoidea. BI posterior probabilities and ML UFbootstrap support values are shown above nodes. Newly generated sequences are highlighted in bold. Parasitism in cold- and warm-blooded hosts are indicated by a black and a grey triangle, respectively. Branch length scale bar indicates the number of substitutions per site. CLA, Cladorchiidae; DIP, Diplodiscidae; GASTROD, Gastrodiscidae; GASTROT, Gastrothylacidae; MES, Mesometridae; MIC, Microsophidiidae; OLV, Olveriidae; PAR, Paramphistomidae; ZYG, Zygocotylidae.

species possess vitelline follicles confluent anteriorly (Sey, 1991); however, they can be distinguished by the pharynx/acetabulum ratio (higher than 1:3 in *C. marinhoi* vs lower than 1:3 in *C. propinquus*). Moreover, the presence of larger vitelline follicles (larger than eggs) is clearly the main differential feature between *C. marinhoi* and *C. propinquus*. In fact, in *C. propinquus*, the vitelline follicles are smaller [28–56 $\mu\text{m} \times 28$ –42 μm , as obtained from line drawings published by different authors (Travassos *et al.*, 1969; Hamann, 2004)] than those in *C. marinhoi* studied here (71–142 $\mu\text{m} \times 63$ –170 μm) or calculated from the drawings in the original description (82–163 $\mu\text{m} \times 82$ –112 μm).

In the samples of *C. marinhoi* evaluated in this study it was verified the presence of worms at different stages of maturation, including ovigerous juveniles and mature worms. Similar results were also found in *C. propinquus* from Argentina (Hamann, 2004). We consider that the presence of worms at different stages of maturation can contribute to the overestimation of the *Catadiscus* diversity as some species were described based on few apparently not complete mature specimens [e.g. *Catadiscus pygmaeus* (Lutz, 1928)]. Moreover, it is not possible to know if juvenile ovigerous worms were considered in species descriptions. Thus, we recommend that comparative morphometric studies should be based on fully developed parasites. Further studies to evaluate the impact of the differences in parasite maturation, fixation techniques as well as host-induced phenotypic plasticity are also required. That being said, to overcome any obstacle for the reliable identification of *Catadiscus* spp., the combining of different sources of information (e.g. morphology and DNA) are required.

In the current study, the link between larval and adult stages of *C. marinhoi* was based on experimental and molecular approaches. Both larval stages (cercariae and metacercariae) have a constricted acetabulum, as observed in their adult counterparts. Interestingly, in other genera of the Paramphistomoidea, including in some species of Diplodiscidae, the shape of acetabulum and arrangement of intestinal caeca in adults and cercariae are similar (Yamaguti, 1975; Besprozvanynkh *et al.*, 2018). Such similarities between larvae and adults are thought to be common among 'primitive' trematodes (Galaktionov and Dobrovolskij, 2003). Despite the necessary caution related to identification of larval stages, it is possible that the study of cercariae can support the identification at least at the generic level of amphistomes from anurans.

Regarding the first intermediate host, species of the South American genus *Drepanotrema* (Planorbidae) are confirmed to harbour larval stages of a species of *Catadiscus*. Ostrowski de Núñez (1979) described the putative cercariae of *C. uruguayensis* based on herpetological and malacological findings recovered in a single pond in Uruguay. The cercariae of *C. marinhoi* described in our study are larger than those identified as *C. uruguayensis*. It is important to note that both line drawing and description of cercariae of *C. uruguayensis* by Ostrowski de Núñez (1979) do not show the acetabulum medially constricted, a feature easily observed in the described adults of *C. uruguayensis*. Given the lack of experimental or molecular confirmation, cercariae suggested by Ostrowski de Núñez (1979) as being *C. uruguayensis* may belong to another amphistome group, which deserves new studies.

Most species of *Diplodiscus* with known life cycles have planorbids of the subfamily Planorbinae as intermediate hosts. The finding of *Drepanotrema* spp. as host of *C. marinholutzi* is an additional evidence of the involvement of this host group in the transmission of amphistomes from anurans. Aspects related to specificity to the first intermediate host require more in-depth studies on paramphistomoids, especially in Diplodiscidae, since populations of the same species were reported infecting phylogenetically distant planorbid genera (Sey, 1991). In the case of *C. marinholutzi*, it appears to be specific to species of *Drepanotrema*, given the other planorbid examined (1013 specimens of *Biomphalaria occidentalis*) were not found infected with this parasite. Regarding the infection of the definitive host, it probably occurs by the ingestion of encysted metacercariae present in food, as already elucidated to several other paramphistomoids, including amphibian parasites (Yamaguti, 1975). However, new studies are required to test the possibility of infection by ingestion of cercariae, which is a real possibility considering anuran tadpoles, as verified to *Diplodiscus* (Besprozvannykh, *et al.*, 2018).

The phylogenetic reconstructions based on 28S rDNA sequence data revealed that representatives of *Catadiscus* and *Diplodiscus* (type genus of Diplodiscidae) did not cluster in a well-supported clade. Hence the monophyly of the family Diplodiscidae was not supported in our molecular phylogeny. In addition to that, unusually high intergeneric divergence among these two diplodiscid taxa, much higher than in other monophyletic assemblages recovered from our analyses, provides further evidence for the non-monophyly of the group that will likely require a reevaluation of species composition and the proposal of a new family for species of *Catadiscus*. Nevertheless, there are no sequences to species of other genera currently included in the family Diplodiscidae [*Progonimodiscus* Vercammen-Grandjean, 1960 (in frogs from Africa), *Dermatemytrema* Price, 1937 (in turtles from North America), *Pseudodiplodiscus* Manter, 1962 (in fish from South America), and *Australodiscus* Sey, 1983 (in frogs from Oceania – Australia)] (Sey, 1991; Jones, 2005b).

Morphologically, members currently included in Diplodiscidae can be separated into two groups: with (*Progonimodiscus*, *Australodiscus* and *Diplodiscus*) and without (*Catadiscus*, *Dermatemytrema* and *Pseudodiplodiscus*) central peduncle or sucker in acetabulum (Jones, 2005b); in the latter case all species were described from the Americas. Acetabulum morphology may be phylogenetic informative and therefore useful for higher-level classification of these amphistomes from the New World, but, as above-mentioned, the relative taxonomic value of such characters should also be assessed in further phylogenetic studies. Even though Yamaguti (1971, p. 393) proposed the subfamily Catadiscinae to include amphistomes from amphibians and reptiles having a constriction in the acetabulum, this taxon was not considered in any subsequent revision (Sey, 1983, 1991, Jones, 2005b). Whether further studies confirm the distinctive molecular signature of *Catadiscus* species as suggest by the 28S rDNA gene for *C. marinholutzi*, we might consider elevating the Catadiscinae *sensu* Yamaguti (1971) to the familial level in order to accommodate all members of this genus.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182021000330>

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Conflict of interest. None.

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