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Molecular, morphological and experimental-infection studies of cercariae of five species in the superfamily Diplostomoidea (Trematoda: Digenea) infecting *Biomphalaria straminea* (Mollusca: Planorbidae) in Brazil

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ABSTRACT

Trematodes belonging to the superfamily Diplostomoidea have complex life cycles involving birds, mammals and reptiles as definitive hosts, and gastropods and different groups of invertebrates and vertebrates as intermediate hosts. Molecular studies of these parasites are numerous, but data from larval stages in molluscs remain scarce, particularly in South America. The present study focused mainly on five morphotypes of longifurcate cercariae found in *Biomphalaria straminea* (Dunker, 1848) from Belo Horizonte, Minas Gerais, Brazil, collected between 2009 and 2017. In each morphotype, nuclear internal transcribed spacer (ITS1-5.8S ITS-2) rDNA and mitochondrial cytochrome *c* oxidase 1 (COI) genes were sequenced. Laboratory-reared fish, *Poecilia reticulata* Peters, 1859 or snails, *Biomphalaria glabrata* (Say, 1818) were exposed to cercariae to obtain metacercariae. The morphology of cercariae, experimentally obtained metacercariae, and phylogenetic analyses led to the identification of three species of Diplostomidae [*Austrodiplostomum compactum* (Lutz, 1928), Crassiphialinae gen. sp. and *Hysteromorpha* sp.] and two species of Strigeidae (*Cotylurus* sp., *Apharyngostrigea* sp.). Previously published sequences allowed species-level identification for only *A. compactum*, although provisional identifications were possible in two cases. First, the COI from cercariae of *Apharyngostrigea* sp. in Brazil matched those of metacercariae from naturally infected *Cnesterodon decemmaculatus* (Jenyns, 1842) in Argentina; although a positive identification is not possible, the material presents morphological similarities with larval stages previously described for *A. simplex*. Secondly, *Cotylurus* sp. resembles *C. lutzi*. Our analysis of previously published COI sequences suggests that *Cotylurus cornutus* (Rudolphi, 1808) has a Holarctic distribution. Both the morphology of experimentally obtained metacercariae and COI sequences indicate that *Hysteromorpha* sp. in Brazil is distinct from congeners in North America [*Hysteromorpha corti* (Hughes, 1929)] and Europe [*Hysteromorpha triloba* (Rudolphi, 1819)].

1. Introduction

Members of the superfamily Diplostomoidea Poirier, 1886 are parasites of mammals, birds and reptiles with complex life cycles, involving molluscs as first intermediate hosts and various vertebrates and invertebrates as second intermediate or paratenic hosts (Niewiadomska, 2002a,2002b; Blasco-Costa and Locke, 2017). Several species have important effects in pisciculture, causing mortality and delayed development, and others occasionally infect humans (Blasco-Costa and Locke, 2017). Over 400 species were recognized by Dubois (1938, 1968, 1970b) and recently Littlewood et al. (2015) reported that 800 species exist in the superfamily. However, knowledge of the life cycles of this group of parasites lags, with larval stages unknown for most species (Blasco-Costa and Locke, 2017).

In South America, too, most diplostomoids are known as adults from vertebrate definitive hosts (about 110 species, compiled by Fernandes and Kohn, 2014; Fernandes et al., 2015), and most life cycles remain unknown. Experimental infections with larvae shed by molluscs have elucidated the life cycle of nine diplostomoids in Argentina, Brazil, and Venezuela (Ostrowski de Núñez and Gil de Pertierra, 2004; Davies and

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Ostrowski de Núñez, 2012; Ritossa et al., 2013; López-Hernández et al., 2018). In contrast, dozens of species of diplostomoids from South America known as larvae from molluscs are assigned to the collective genera *Cercaria* Müller, 1774 and *Furcocercaria* Lamark, 1815, or identified at family or cercarial-type levels (Nasir and Diaz, 1973; Ostrowski de Núñez, 1992; Pinto and Melo, 2013a; Fernández et al., 2016; Fernández and Hamann, 2017). Experimental infections involve considerable logistical challenges, particularly in maintaining the vertebrate and invertebrate hosts of diplostomoids, and this has undoubtedly hindered advances in the knowledge of the life cycles of these parasites.

Molecular tools have improved understanding of systematics and life cycles of trematodes, including the members of the Diplostomoidea, especially diplostomids and strigeids infecting fish and birds in Europe and North America (Locke et al., 2010, 2011, 2015, 2018; Pérez-del Olmo et al., 2014; García-Varela et al., 2015, 2016; Stoyanov et al., 2017; Blasco-Costa and Locke, 2017). Molecular data from diplostomoid cercariae found in gastropod intermediate hosts has lagged behind, but is now beginning to accumulate (Faltýnková et al., 2014; Uhrig et al., 2015; Rosser et al., 2016; Wongsawad et al., 2016; Aksenova et al., 2016; Gordy et al., 2017). However, only recently have DNA sequences from diplostomoids become available in South America, and nearly all data are from adults or metacercariae. These include *Austrodiplostomum compactum* (Lutz, 1928), *Tylodelphys jenynsiae* Szidat, 1969 and *Braunina cordiformis* Wolf, 1903. The only molecular data from South American cercariae of diplostomoids are from *Posthodiplostomum nanum* Dubois, 1937 (López-Hernández et al., 2018). Clearly, further integrative studies of South American diplostomoids are needed, particularly those involving larval stages in snails.

In the present study, five longifurcate cercariae obtained from naturally infected *Biomphalaria straminea* (Dunker, 1848) (Planoribidae) in Brazil were used for morphological, molecular and experimental infection studies. Our data highlight the scarcity of reference sequences for the South American trematode fauna, which remains an obstacle to the molecular elucidation of life cycles of trematodes.

2. Material and methods

2.1. Malacological survey and evaluation of infection with trematodes

Most parasites reported here were obtained from long-term field studies of molluscs in two urban waterbodies in the city of Belo Horizonte, state of Minas Gerais, southeastern Brazil. Cercariae were obtained from snails from the Pampulha reservoir (19° 51' 44.77"S and 43° 58' 29.35"W) collected between 2009 and 2012, and from snails from a lake located at Minas Gerais state Administrative Center (19° 47' 06.20"S and 43° 57' 11.41"W) collected between August 2016 and April 2017. The molluscs were transported to the laboratory, placed individually in 24-well plates with about 3 mL of dechlorinated water, subjected to artificial photostimulation for 2 h, and examined with a stereomicroscope to detect emerged cercariae. A second examination was performed the next day. Molluscs were identified according to Paraense (1975).

Strigeid metacercariae (n = 4) were also collected from four different individual *Cnesterodon decemmaculatus* (Poeciliidae) caught in February, 2014 with a cast net in an urban canal draining into Canal Santiago in the city of La Plata, Buenos Aires, Argentina (34°53'49.2"S, 57°55'44.4"W). The worms were mechanically excysted and placed in 95% ethanol.

2.2. Experimental infection

Longifurcate cercariae obtained from *B. straminea* were used to experimentally infect laboratory-reared fish or molluscs (with approval of Ethics Committee on Animal Use (CEUA-UFMG), protocols 199/2009, 20/2016). The choice of a potential second intermediate host was based on larval morphology. Cercarial morphotypes corresponding to taxa likely to use fish as second intermediate hosts were exposed to groups of 10–20 adult *Poecilia reticulata* Peters, 1859 (Pisces: Poeciliidae), of both sexes, measuring from 2 to 4 cm in total length. Fish were kept with infected molluscs for 24 h in an aquarium containing 1 L of chlorine-free water. Fish were then maintained 7–70 days, fed daily with fish feed, before euthanasia by immersion in benzocaine hydrochloride solution (250 mg/L). The organs of the abdominal cavity (stomach, intestine and liver), gills and fins were transferred to glass slides containing physiological saline solution (0.85% NaCl) and examined under a stereomicroscope for presence of metacercariae.

In experiments evaluating molluscs as second intermediate hosts, laboratory-reared *Biomphalaria glabrata* (Say, 1818) were transferred to polystyrene plates containing samples of longifurcate cercariae. At 7 days post-infection (DPI) the molluscs were pressed between glass slides and examined under a stereomicroscope to detect metacercariae.

2.3. Morphological study

Cercariae that emerged from naturally infected molluscs were stained with vital dyes (aqueous solution of 0.05% neutral red or 0.05% Nile blue sulphate) and examined under a light microscope in non-permanent preparations. Samples of each morphotype of longifurcate cercariae or experimentally obtained metacercariae were also killed in water at 70 °C and fixed in 4% formalin. Representative larvae were stained with alum acetocarmine, dehydrated in an ethanol series, clarified in beechwood creosote and mounted in permanent slides with Canada balsam. Measurements of heat-killed and formalin-fixed cercariae and stained and mounted metacercariae were taken using a micrometer eyepiece under a light microscope, and are reported in micrometer (μm) as mean followed by standard deviation and the range in parenthesis. Photographs were taken with a Leica ICC50 HD digital camera mounted to a light microscope. The cell-flame pattern and distribution of sensory hairs were not studied. Parasites were identified to the lowest possible taxonomic category based on the primary literature and the review of larval trematodes by Yamaguti (1975).

2.4. Molecular study

Representative longifurcate cercariae found in *B. straminea* in Belo Horizonte were fixed in 95% ethanol and stored at −20 °C. DNA was extracted using the Wizard® Genomic DNA Purification kit (for aliquots with at least 50 cercariae) or the QIAamp® DNA micro kit (samples containing 3–10 cercariae) following the manufacturer's instructions. The dosage of the obtained DNA was determined in a microvolume spectrophotometer (NanoDrop® ND-1000). The DNA of metacercariae found in naturally infected *C. decemmaculatus* in La Plata was extracted, amplified and sequenced as in Moszczynska et al. (2009).

For the molecular characterization of cercariae and metacercariae from Brazil, two molecular markers were amplified and sequenced: the internal transcribed spacer region (ITS1-5.8S-ITS2) of nuclear rDNA and the mitochondrial cytochrome *c* oxidase subunit I (COI). For the amplification of ITS, we used the primers D1 (F) and D2 (R) of Galazzo et al. (2002). For COI, we used the primers Dice1-F and Dice11-R described by Van Steenkiste et al. (2015) and Plat-diploCOX1F and Plat-diploCOX1R designed by Moszczynska et al. (2009). PCR reactions were performed in a final volume of 25 μl, which included 12.5 μl Platinum Hot Start PCR Master Mix, 1.25 pmol of each primer, and about 50 ng of template DNA. The PCR conditions used were as described by the authors listed above. Amplification products were visualized in 1% agarose gel electrophoresis stained with GelRed® (Biotium, USA). Purification of appropriately sized, single-banded PCR products was performed with polyethylene glycol (PEG) 20% PEG 8000 (Promega, USA) in 2.5 M NaCl solution. Purified DNA was resuspended in 20 μl of ultrapure water, dosed and sequenced in both directions by

capillary electrophoresis in an ABI3730 sequencer using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA) .

Sequences obtained were assembled and edited using the ChromasPro v.2.0.1 (Technelysium Pty Ltd, Australia). For the similarity search we used the Basic Alignment Search Tools (BLAST) program of the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) (National Library of Medicine, USA) and the Barcode of Life Data System (BOLDSYSTEMS) of the Center for Biodiversity Genomics, Canada (http://www.boldsystems.org/). The sequences obtained herein were aligned with similar sequences from the latter public sources in MEGA 7.0 (Kumar et al., 2016). Information related to sequences used in the phylogenetic analyses is presented in Appendix A. The evolutionary models used for the construction of phylogenetic trees were selected with the Bayesian information criterion (BIC) in MEGA 7.0 (TN93 + G + I for COI data and GTR + G for ITS data). Phylogenetic trees were created with Maximum Likelihood (ML) and Bayesian Inference (BI). The nodal support in the ML analysis was measured using the bootstrap test with 1000 replicates. BI analyses were performed with MrBayes v.3.2.6 (Ronquist et al., 2012) using Markov chain Monte Carlo (MCMC) for 1,000,000 generations and sampling every 100 generations. The first 25% of the trees generated were discarded as 'burn-in'. New sequences obtained were deposited in GenBank (accession numbers MH777789-MH777791, MN179319-MN179326, MN179269-MN179277).

3. Results

Five different morphotypes of longifurcate cercariae were found in *B. straminea* during the malacological surveys carried out in Belo Horizonte, Minas Gerais, Brazil. Diplostomoid larvae were found in 103 of 16,235 *B. straminea* collected from the Pampulha Reservoir and in 33 of 100 *B. straminea* from the lake at the state government offices. Measurements of the cercariae obtained in the present study are presented in Table 1. Metacercariae were experimentally obtained for two species and their measures are presented in Tables 2 and 3.

PCR amplifications of the ITS region resulted in bands of approximately 1300 bp. After editing and trimming the aligned sequences, nine 1089-bp fragments were considered for the analyses. Eight COI sequences between 463 bp and 662 bp were considered in the analyses, and trimmed final alignments were 319 bp long. In phylogenetic trees obtained with both markers, sequences of the five morphotypes formed clades with similar taxonomic composition, generally with high support (Figs. 1 and 2). These analyses, along with morphological and experimental infection studies, enabled the identification of the species presented below

3.1. Apharyngostrigea sp. (Strigeidae) (Fig. 3, Tables 1 and 2)

Locality: Pampulha Reservoir, Belo Horizonte, Minas Gerais, Brazil; Canal Santiago, La Plata, Buenos Aires, Argentina

Table 2
Morphometric data from metacercariae (encysted and mechanically excysted) of *Apharyngostrigea* sp. obtained in *Poecilia reticulata* experimentally infected with cercariae emerged from *Biomphalaria straminea* collected at the Pampulha Reservoir, Belo Horizonte, Minas Gerais, Brazil. Abbreviations: DPI: days post-infection; L: length, W: width.

Encysted			
	25 DPI	L	925 ± 201 (614-1203)
		W	737 ± 205 (444-946)
	40 DPI	L	686 ± 33 (653-756)
		W	513 ± 34 (464-567)
	60 DPI	L	709 ± 50 (636-791)
		W	550 ± 76 (430-688)
Excysted	60 DPI		
Body		L	504 ± 47 (416-580)
		W	326 ± 47 (273-409)
Oral sucker		L	56 ± 6 (48-68)
		W	59 ± 7 (43-67)
Ventral sucker		L	83 ± 6 (77-94)
		W	87 ± 8 (77-103)
Tribocytic organ		L	205 ± 17 (184-239)
		W	212 ± 29 (163-253)

Table 3
Morphometric data from excysted metacercariae of *Hysteromorpha* sp. obtained 70 days after the experimental infection of *Poecilia reticulata* with cercariae emerged from *Biomphalaria straminea* collected in Belo Horizonte, Minas Gerais, Brazil. Abbreviations: L: length, W: width.

Body	L	822 ± 157 (617–1097)
	W	551 ± 62 (494–686)
Oral sucker	L	57 ± 12 (48–82)
	W	54 ± 13 (41–69)
Ventral sucker	L	37 ± 9 (27–55)
	W	36 ± 11 (21–55)
Pharynx	L	37 ± 7 (34–48)
	W	31 ± 4 (27–34)
Tribocytic organ	L	273 ± 38 (206–315)
	W	133 ± 4 (130–137)

Prevalence of infection in *B. straminea* (Brazil): 8/16,235 (0.049%)
Prevalence of infection in *C. decemmaculatus* (Argentina): 4/44 (9%)

3.1.1. Experimental study

At 16 DPI, free parasites (pre-cystic forms) were found in the abdominal cavity of laboratory-reared *P. reticulata* exposed to cercariae. Encysted metacercariae were recovered at 25, 40 and 60 DPI. Cysts were orally inoculated into mice and young chickens. Immature parasites morphologically similar to excysted metacercariae were recovered in the intestine of mice 24 h after infection, although they were not found after this period. No parasites were found in experimentally infected chickens.

Table 1
Morphometric means ± standard deviations (ranges) in five species of cercariae obtained from *Biomphalaria straminea* in two lakes in Belo Horizonte, Brazil, between 2009 and 2017. Abbreviations: L: length, W: width.

		Apharyngostrigea sp.	*Austrodiplostomum compactum*	*Cotylurus* sp.	Crassiphialinae gen. sp.	*Hysteromorpha* sp.
Body	L	203 ± 16 (177–232)	165 ± 13 (137–206)	172 ± 7 (158–185)	172 ± 4 (165–178)	187 ± 18 (137–206)
	W	81 ± 10 (55–96)	33 ± 3 (27–34)	48 ± 7 (34–62)	31 ± 4 (27–34)	51 ± 11 (34–69)
Tail stem	L	406 ± 26 (355–444)	191 ± 14 (171–206)	193 ± 16 (165–247)	231 ± 18 (165–240)	231 ± 26 (192–274)
	W	158 ± 19 (116–191)	31 ± 4 (27–41)	41 ± 4 (34–48)	33 ± 4 (27–41)	37 ± 6 (27–48)
Furcae	L	184 ± 38 (116–253)	197 ± 9 (178–213)	213 ± 8 (185–226)	203 ± 8 (178–219)	221 ± 15 (205–267)
	W	34 ± 7 (23–58)	15 ± 3 (14–21)	15 ± 3 (14–21)	15 ± 4 (7–27)	16 ± 4 (14–27)
Anterior organ	L	47 ± 3 (42–52)	23 ± 5 (14–34)	32 ± 6 (21–41)	35 ± 3 (33–38)	32 ± 7 (14–41)
	W	29 ± 3 (23–33)	18 ± 4 (14–27)	24 ± 5 (14–34)	20 ± 1 (19–21)	27 ± 5 (21–34)
Ventral sucker	L	19 ± 2 (17–25)	21 ± 5 (14–27)	32 ± 7 (21–43)	—	30 ± 5 (27–41)
	W	18 ± 2 (15–23)	21 ± 6 (14–27)	30 ± 6 (21–43)	—	28 ± 8 (21–41)

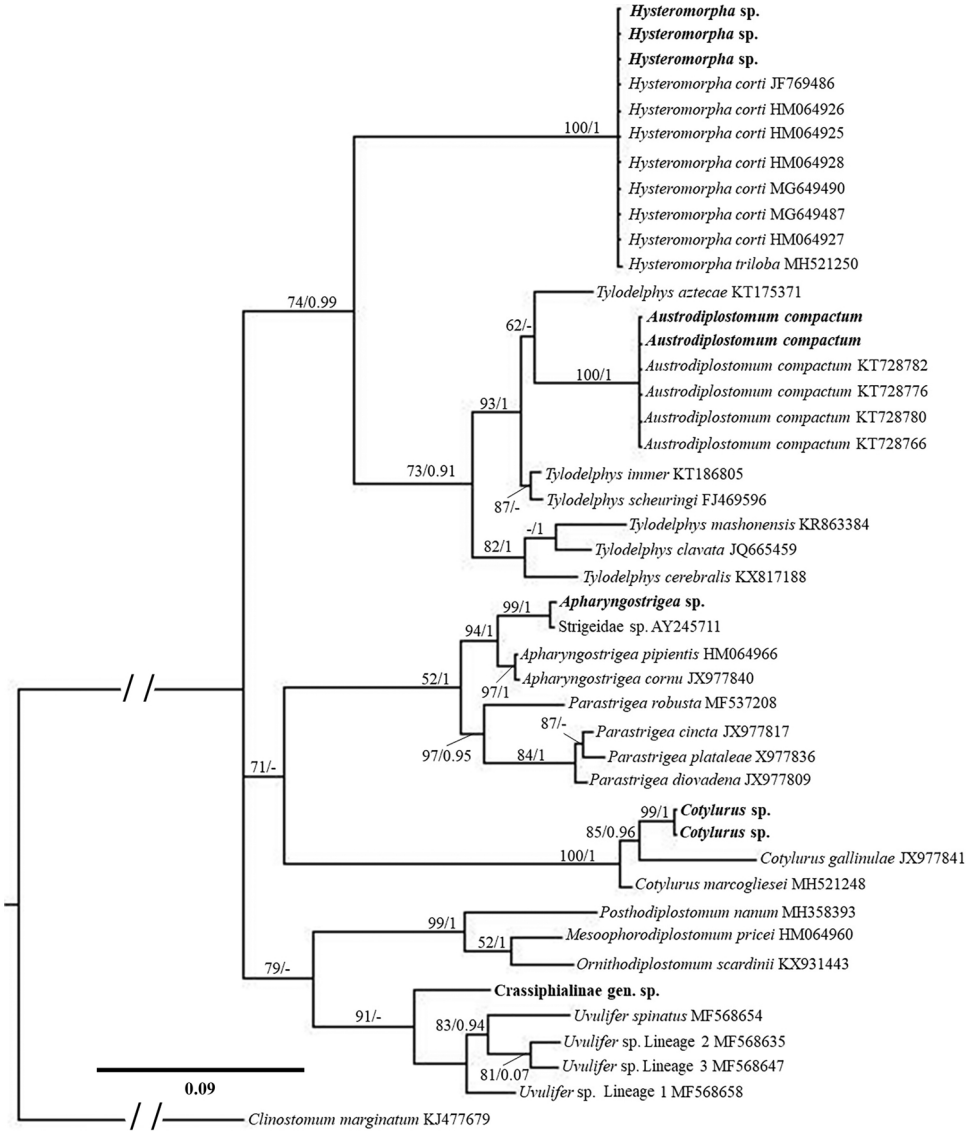

Fig. 1. Phylogenetic relationships between *Apharyngostrigea* sp., *Austrodiplostomum compactum*, *Cotylurus* sp., Crassiphialinae gen. sp. and *Hysteromorpha* sp. collected in the present study (in bold), and including other diplostomoids, inferred from sequences of internal transcribed spacer based on Bayesian Inference (BI) and Maximum Likelihood (ML) analyses. Nodal support is indicated as ML/BI; values < 0.90 (BI) and < 50 (ML) are indicated by a dash.

3.1.2. Morphological characterization

Cercaria (Fig. 3A, B): Body oval, covered by small spines. Oral sucker trapezoid, ventral sucker equatorial with crown of small spines. Prepharynx very small, pharynx oval, small. Esophagus short, bifurcating midway between pharynx and ventral sucker. Intestinal cecum septate, bifurcating in middle of forebody and ending in postacetabular region, with yellow-orange material. Six pairs of penetration glands in two clusters antero-lateral to ventral sucker. Tail stem bulbous, wider than body, lacking caudal bodies. Body and tail with brownish granules. Genital primordium irregular in posterior of body. In resting position, body retracted and furcae extended approximately 90° to tail stem.

Metacercaria (Fig. 3C, Table 2): Body of experimentally obtained specimens enclosed in oval, bilayered cyst. Cyst wall 200–300 µm thick, comprising an inner, resistant, thin, transparent layer and outer, fragile, opaque, whitish, thick layer. At 60 DPI, mechanically excysted metacercariae piriform, with suckers and tribocytic organs evident. Body of two metacercariae from naturally-infected *C. decemmaculatus* in Argentina, which genetically matched cercariae of *Apharyngostrigea* sp. (see below; one para- and one syngenophore *sensu* Pleijel et al., 2008) similar, 375–382 in total length, 251–265 wide; oral sucker 50–62 × 56–74, and ventral sucker 66–71 × 84–102.

3.1.3. Remarks

The cercaria of *Apharyngostrigea* sp. is morphologically and metrically similar to that of *Apharyngostrigea simplex* (Johnston, 1904), a species also reported in *B. straminea* from Argentina (Ostrowski de Núñez, 1989). *Cercaria inflaticauda*, described from *Biomphalaria pfeifferi* (Krauss, 1848) in the Democratic Republic of Congo (Fain, 1953), has similar measurements, but differs from *Apharyngostrigea* sp. in the presence of non-pigmented eyespots. In South America, larvae with similar morphology to that here reportedwere found in *Biomphalaria tenagophila* (d'Orbigny, 1835) from Florianópolis, state of Santa Catarina (Espíndola et al., 1992), Niterói, state of Rio de Janeiro (Boaventura et al., 2002), in *Biomphalaria peregrina* (Orbigny, 1835) from the Paraná River (Dias et al., 2002), and in *B. tenagophila* and *Biomphalaria occidentalis* Paraense, 1981 from Argentina (Fernández et al., 2016). These records from South America may all represent the same species, but ascertaining this requires additional study.

The metacercariae here obtained experimentally in *P. reticulata* and

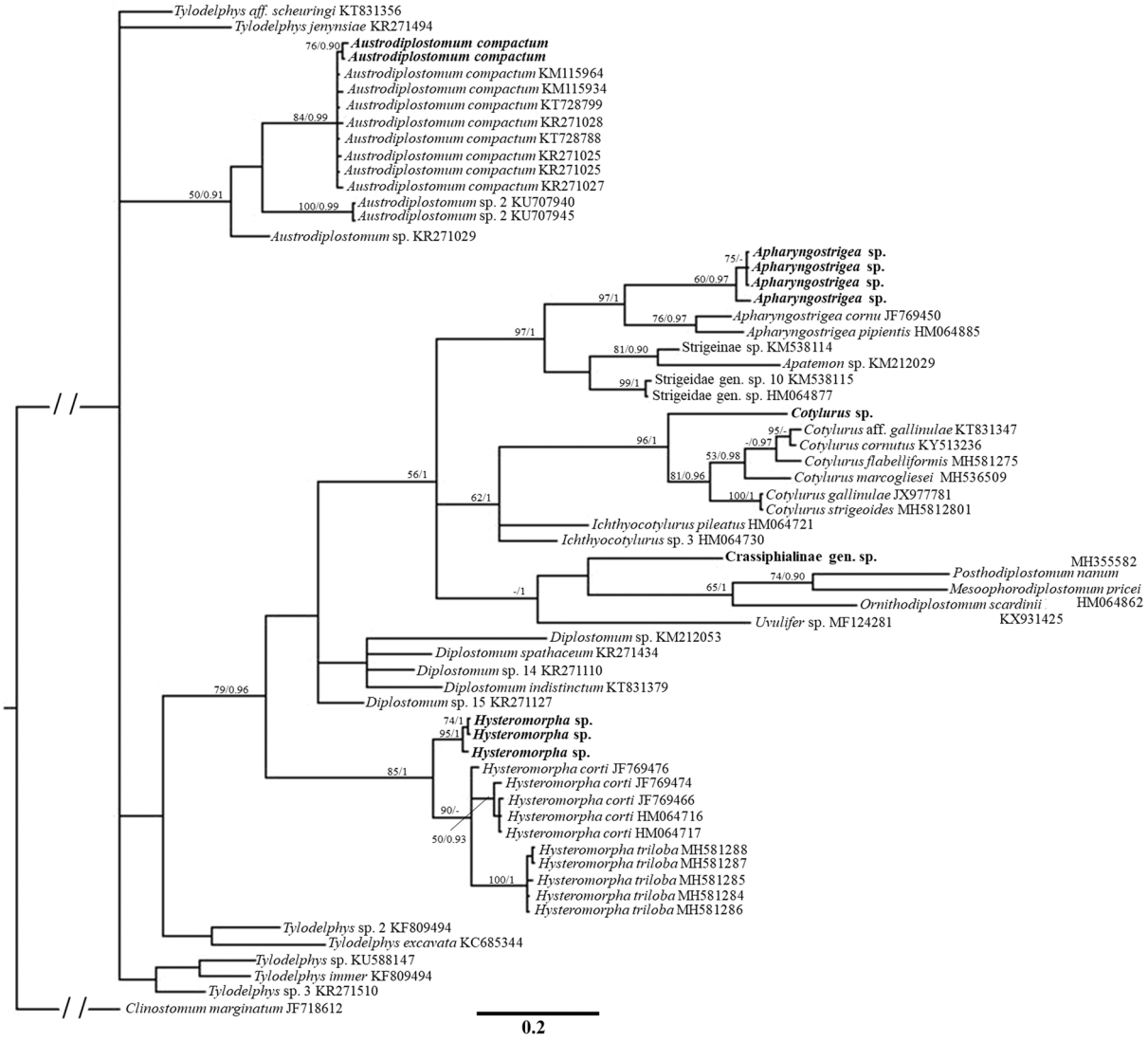

Fig. 2. Phylogenetic relationship between *Apharyngostrigea* sp., *Austrodiplostomum compactum*, *Cotylurus* sp., Crassiphialinae gen. sp. and *Hysteromorpha* sp. collected in the present study (in bold) and among other diplostomoids inferred from sequences of partial cytochrome c oxidase sequences based on Bayesian Inference (BI) and Maximum Likelihood (ML) analyses. Nodal support is indicated as ML/BI; values < 0.90 (BI) and < 50 (ML) are indicated by a dash.

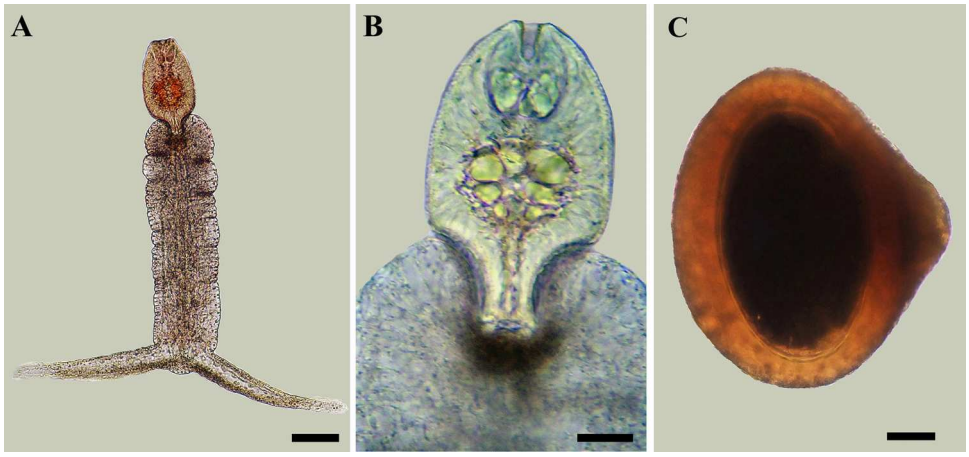

Fig. 3. *Apharyngostrigea* sp.: (A) Cercaria from naturally infected *Biomphalaria straminea*. (B) Detail of cercarial body. (C) Metacercaria from body cavity of experimentally infected *Poecilia reticulata*. Scale bars: 100 µm (A, C), 25 µm (B).

in naturally infected *C. decemmaculatus* are similar to those reported also in *P. reticulata* from Belo Horizonte by Pinto and Melo (2012) and to *A. simplex* obtained experimentally in *C. decemmaculatus* in Argentina by Ostrowski de Núñez (1989). Encysted parasites were longer in specimens obtained 25 DPI than 40 and 60 DPI (One-way ANOVA, $F = 15.42$; $p < 0.0001$), suggesting size reduction in the first days after encystment and stable size after 40 DPI.

3.1.4. Molecular study

Phylogenetic analysis of the ITS dataset resulted in a phylogram where sequences of cercariae of *Apharyngostrigea* sp. from Brazil formed a clade with those of *Apharyngostrigea pipientis* (Faust, 1918) from Canada and *Apharyngostrigea cornu* (Zeder, 1800) from Mexico, diverging by 2.3% from both species (Fig. 1). The ITS of *Apharyngostrigea* sp. differs by 0.3% from Strigeidae gen. sp. in Israel, which suggests they are congeneric species. Phylogenetic analyses showed a close relationship between members of *Apharyngostrigea* and *Parastrigea*, represented by *Parastrigea diovadena* Dubois and Macko, 1972, *Parastrigea cincta* (Brandes, 1888), *Parastrigea plataleae* Hernández-Mena, García-Prieto and García-Varela, 2014 from Mexico, and *Parastrigea robusta* Szidat, 1928 from Germany, with intergeneric ITS divergence from 5.1 to 15.6%.

In phylogenetic analysis of COI sequences (Fig. 2), *Apharyngostrigea* sp. fell within a well-supported clade with congeners. Sequences of COI from cercariae from *B. straminea* in Brazil and metacercariae from *C. decemmaculatus* in Argentina varied by 0–1.9%, indicating conspecificity. The COI of *Apharyngostrigea* sp. differs by 11.3%–12.2% from North American isolates of *A. cornu* and *A. pipientis*. The topology of phylogenetic trees also reveals that *Apatemon* sp. is closely related to *Apharyngostrigea* spp., with intergeneric divergences of 8.8–15.7%.

3.2. Austrodiplostomum compactum (Table 1)

Locality: Lagoa da Pampulha, Belo Horizonte, Minas Gerais, Brazil.
Prevalence of infection: 71/16,235 (0.44%).

3.2.1. Remarks

Cercariae of *A. compactum.* identified by experimental infections, were described by Pinto and Melo (2013b). The ethanol-fixed cercariae we used for molecular study are consistent with those data, and with those reported for *A. compactum* from *B. straminea* in Argentina (Ostrowski de Núñez, 1982) and from *Biomphalaria havanensis* (Pfeiffer, 1839) from the USA (Rosser et al., 2016).

3.2.2. Molecular study

The ITS sequences of cercariae here studied and metacercariae of *A. compactum* obained from naturally infected *Oreochromis niloticus* by Pinto et al. (2014) were identical to those of *A. compactum* (=*A. ostrowskiae*, see Ostrowski de Núñez, 2017) from the USA. The COI sequences we obtained from *A. compactum* differed by 0.3%–1.3% from those of isolates of this species from the USA, Mexico, Brazil and Peru. All these isolates formed a clade with significant support.

3.3. Cotylurus sp. (Strigeidae) (Fig. 4, Table 1)

Locality: Lake at Minas Gerais state Administrative Center, Belo Horizonte, Minas Gerais, Brazil.

Prevalence of infection of cercariae in *B. straminea*: 2/21 (9.5%).

Prevalence of infection of metacercariae in *B. straminea*: 30/79 (38%).

3.3.1. Experimental infection

No metacercariae were found in laboratory-bred *B. glabrata* seven days after exposure to cercariae of *Cotylurus* sp.

3.3.2. Morphological characterization

Cercariae (Fig. 4A, B; Table 1): Body elongated; anterior end rounded with small spines, posterior end truncated. Anterior organ subterminal, longer than wide. Ventral sucker in posterior half of body. Prepharynx small, pharynx muscular, slightly elongated. Esophagus short, bifurcating anterior to ventral sucker. Intestinal cecum relatively short, extending approximately to posterior margin of ventral sucker. Four pairs of penetration glands in posterior half of body, post-acetabular. Eyespots non-pigmented, anterior to ventral sucker. Tail stem shorter than furcae, with 5 pairs of caudal bodies. Resting cercarial body retracted with furcae extending approximately 90° to tail stem.

Metacercariae (Fig. 4C): *Tetracotyle*-type, piriform, 302 ± 17 (279–329) by 255 ± 12 (237–277). Cyst wall hyaline, 21 ± 2 (17–25) thick. Excysted metacercariae not examined morphologically.

3.3.3. Remarks

The cercariae of *Cotylurus* sp. are similar to *Cercaria caratinguensis*, which Ruiz (1953) described from *B. glabrata* in Minas Gerais, Brazil. Since then, several authors have recorded similar larvae in different species of molluscs in Brazil (Milward de Andrade and Campos, 1969; Souza et al., 1998; Carvalho et al., 2001; Moraes et al., 2009; Eduardo et al., 2012; Ohlweiler et al., 2013), Argentina (Ostrowski de Núñez, 1972; Martorelli et al., 2013; Fernández and Hamann, 2017) and Venezuela (Nasir, 1979). Basch (1969) described *Cotylurus lutzi* from experimental infections from naturally infected *B. straminea* from Minas Gerais, Brazil. The cercariae and metacercariae of *Cotylurus* sp. in the present study are morphologically similar to *C. lutzi*, and are from the same geographic area and first intermediate host as *C. lutzi*, but morphological study of adults is necessary for a positive identification.

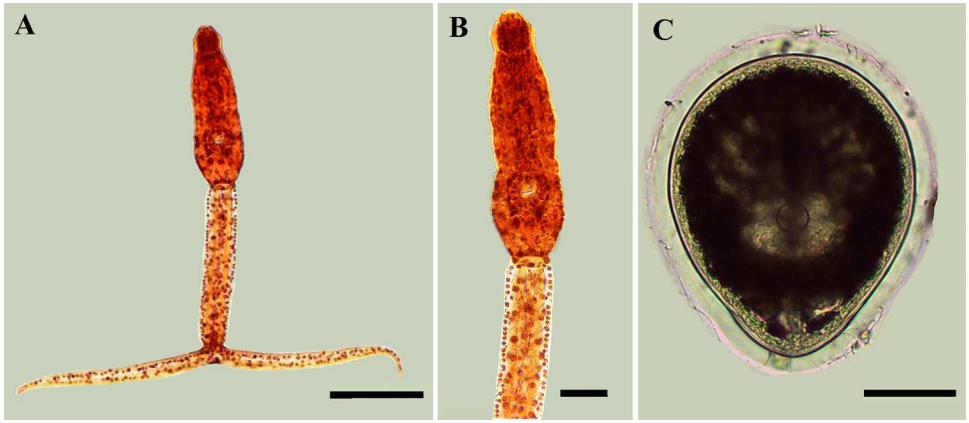

Fig. 4. *Cotylurus* sp.: (A) Cercaria from naturally infected *Biomphalaria straminea.* (B) Detail of cercarial body. (C) Metacercaria from naturally infected *Biomphalaria straminea*. Scale bars: 100 µm (A, C), 25 µm (B).

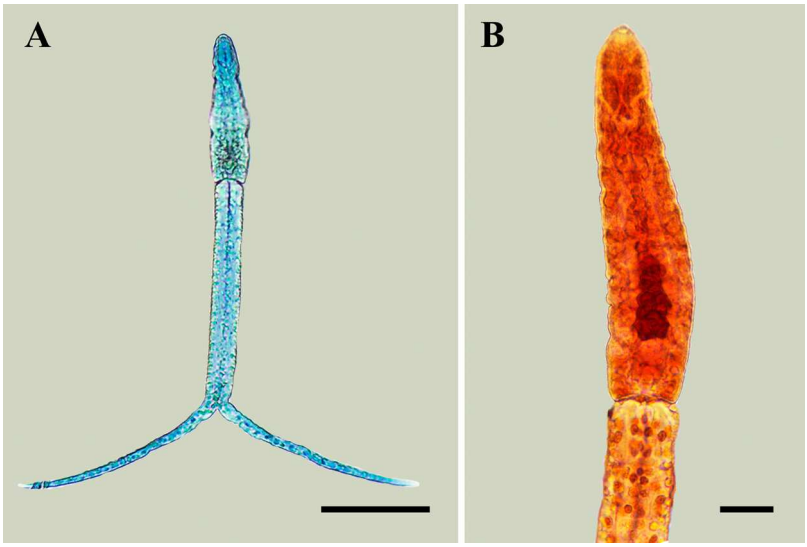

Fig. 5. Crassiphialinae gen. sp.: (A) Cercariae from naturally infected *Biomphalaria straminea*. (B) Detail of cercarial body. Scale bars: 50 µm (A), 25 µm (B).

3.3.4. Molecular study

Phylogenetic analysis of ITS (Fig. 1) supported the morphological identification of *Cotylurus* sp., and sequences obtained from cercariae and metacercariae were identical. The ITS sequences fell in the same clade as *Cotylurus strigeoides* Dubois, 1958 (=*C. gallinulae,* see Locke et al., 2018) from Mexico, and *Cotylurus marcogliesei* Locke et al., 2018 from Canada, with divergences of 5.2% and 4.4%, respectively. The ITS of *Cotylurus* sp. varied by 14.2–16.8%, 14.2–16.4% and 13.7–15.6% in relation to *Apharyngostrigea* spp., *Uvulifer* spp. and *Parastrigea* spp., respectively.

Analyses of COI revealed that *Cotylurus* sp. belongs to a clade containing congeners (Fig. 2), from which the COI of *Cotylurus* sp. differed by 8.8–12.5%. A sequence identified as *Cotylurus* aff. *gallinulae* in western Canada grouped with *C. cornutus* from Norway, with a difference of 1.9%, suggesting that these isolates are conspecific. Members of the *Cotylurus* clade differed from *Apatemon* sp. by 19.1%, from *Apharyngostrigea* spp. by 16.0–21.6%, and from *Ichthyocotylurus* spp. by 14.4–16.3%.

3.4. Crassiphialinae gen. sp. (Diplostomidae) (Fig. 5, Table 1)

Locality: Lake at Minas Gerais state Administrative Center, Belo Horizonte, Minas Gerais, Brazil.

Prevalence of infection: 2/21 (9.5%).

3.4.1. Experimental study

Attempts to infect *P. reticulata* and *B. glabrata* with cercariae of Crassiphialinae gen. sp. did not yield metacercariae.

3.4.2. Morphological characterization

Cercariae (Fig. 5): Body elongated with width similar to caudal stem. Anterior organ subterminal, ventral sucker absent. Prepharynx present, pharynx muscular. Esophagus long. Intestinal caeca not observed. Two pairs of penetration glands in posterior half of body. Caudal stem without caudal bodies. Body curved ventrally at resting position with tail stem straight and furcae at 45° in relation to tail stem.

3.4.3. Remarks

The cercariae here preliminarily identified as Crassiphialinae gen. sp. differ from all cercariae of Diplostomoidea in which the life cycle has been elucidated, especially because the ventral sucker and eyespots are lacking. A larva with similar morphology and measurements was found in *B. tenagophila* and described as Furcocercaria Nº1 in Uruguay (Martorelli et al., 2013).

3.4.4. Molecular study

In the phylogenetic analyses of the ITS dataset, Crassiphialinae gen. sp. fell within a clade with species of the genus *Uvulifer* (Fig. 1). The Brazilian isolates diverged by 4.3–5.2% from *Uvulifer*¸ which suggests it belongs to a distinct genus, given that ITS varies by 1.6–3.7% within *Uvulifer*. COI sequences of Crassiphialinae gen. sp. (Fig. 2) formed a clade with other members of the Crassiphialinae, and divergences ranged from 16% (*Uvulifer* sp.) to 19.7% (*Mesoophorodiplostomum pricei*).

3.5. Hysteromorpha sp. (Diplostomidae)

Localities: Pampulha Reservoir and lake at Minas Gerais state Administrative Center, Belo Horizonte, Minas Gerais, Brazil

Prevalence of infection: 24/16,235 (0.15%) in Pampulha Reservoir, and 1/21 (5%) in the lake at Minas Gerais state Administrative Center.

3.5.1. Experimental study

Five specimens of *P. reticulata* were exposed to cercariae of *Hysteromorpha* sp. Two fish were negative at 7 DPI and eight free metacercariae were found in the musculature of one of three fish at 70 DPI.

3.5.2. Morphological characterization

Cercariae (Fig. 6 A, B): Body elongate. Anterior organ oval. Ventral sucker in posterior half of body. Prepharynx small, pharynx muscular. Esophagus bifurcating near middle of body, anterior to ventral sucker; caeca slightly sinuous, confined to anterior part of body. Two pairs of penetration glands, one pair pre-acetabular and one para-acetabular. Caudal stem without caudal bodies, furcae long. In resting position, cercarial body ventrally curved, with tail stem straight and furcae at approximately 45°.

Metacercariae (Fig. 6C): Body piriform, divided in two parts. Forebody oval, hindbody conical. Pseudosuckers lateral to oral sucker. Pharynx small, ventral sucker well developed, near bifurcation of intestinal caeca. Tribocytic organ well developed, longitudinally elongated, in middle of body. Intestinal caeca prominent anterior to ventral sucker and extending to posterior of hindbody. Genital organs not well differentiated.

3.5.3. Remarks

The cercariae obtained in the present study show similarity in

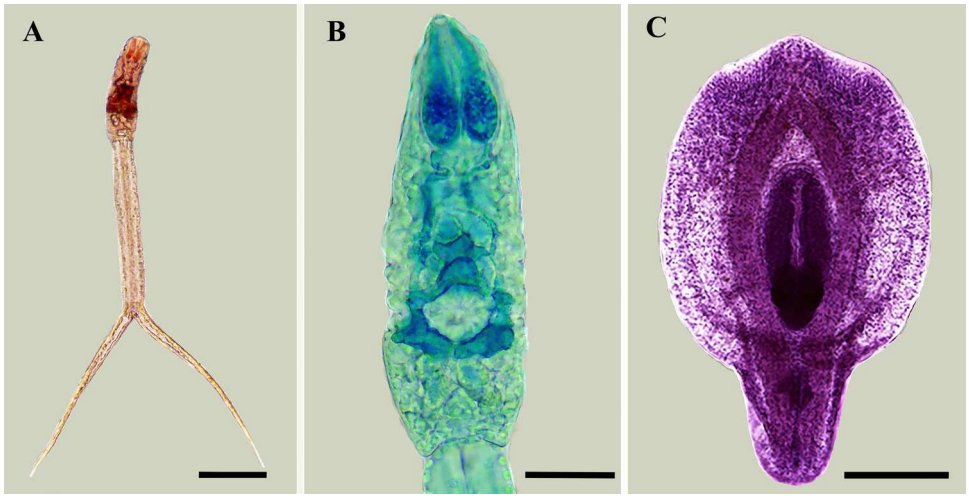

Fig. 6. *Hysteromorpha* sp. (A) Cercariae from naturally infected *Biomphalaria straminea*. (B) Detail of cercarial body. (C) Metacercaria from body cavity of experimentally infected *Poecilia reticulata*. Scale bars: 100 μm (A), 25 μm (B), 200 μm (C).

morphology and morphometrics to the larvae of *Hysteromorpha corti* (Hughes, 1929) described as *H. triloba*, by Hugghins (1954) from experimentally infected *Gyraulus deflectus* (Gould, 1840) (= *G. hirsutus*). A larva found in *Biomphalaria kuhniana* (Clessin, 1883) from Venezuela and described as *Cercaria patoica* by Nasir and Diaz (1973) also presents morphology and measures similar to our cercariae of *Hysteromorpha* sp. In Brazil, cercariae identified as *H. triloba* were reported in *B. peregrina* collected in the Paraná River (Souza et al., 2008). *Furcocercaria* sp. XIX, which was described from *B. straminea* in Argentina (Fernández and Hamann, 2017), differs from *Hysteromorpha* sp. mainly by the disposition of penetration glands and by the presence of caudal bodies in the tail stem.

The metacercariae here obtained experimentally in fish are similar but not identical to those of *H. corti*, a North American species recently distinguished from European *H. triloba* based on phylogenetic analysis of COI and ITS and metacercarial morphology (Locke et al., 2018). The metacercariae of *Hysteromorpha* sp. have a smaller ventral sucker than both *H. corti* [76 ± 4 (73–83) (Hughes, 1929), 50 (47–54) (Sereno-Uribe et al., 2019), and 60 ± 6 (56–68) (Locke et al., 2018) and *H. triloba* [60–82 (70 ± 8) (Locke et al., 2018)]. The tribocytic organ in *Hysteromorpha* sp. is also longer than in *H. corti* (136–195 in Sereno-Uribe et al., 2019 and Locke et al., 2018) and the European *H. triloba* [160–229 (193 ± 27), Locke et al., 2018)].

3.5.4. Molecular study

Analysis of ITS corroborated the identification of *Hysteromorpha* sp. Our isolate forms a clade together with *H. triloba* from Italy and *H. corti* from Canada and Mexico. There were three variable positions in the aligned ITS sequences 1017–1261 in length, i.e., 0.1–0.3% variation, and no mutations distinguished *Hysteromorpha* sp. from *H. corti*, whereas one transition distinguished *H. triloba* from the other two species (Fig. 1).

Phylogenetic analysis of COI sequences also grouped the isolates of *Hysteromorpha* spp. (Fig. 2). Within *Hysteromorpha*, isolates from North America, Italy and those from Brazil fell within two distinct subclades. COI varied among the Brazilian isolates (0.0–0.9%) less than in the North American *H. corti* (3.8–4.7%) and similarly to *H. triloba* (0.3–0.8%). The molecular divergences between *Hysteromorpha* sp. and *H. triloba* from Italy and *H. corti* from North America were 8.5% and 6.6–8.5%, respectively, similar to that between *H. corti* and *H. triloba* (6.6–7.5%). Differences between species of the genera *Diplostomum* and *Hysteromorpha* were 12.5–17.2%.

4. Discussion

The morphological, molecular and experimental-infection studies of five different morphotypes of longifurcate cercariae found in *B. straminea* from Brazil led to several new geographical and host records for the Diplostomoidea. In only one case, *A. compactum*, did previously published data allow molecular identification to species. To identify or describe the four cercariae here identified at generic or subfamilial levels, data from adults and from further molecular or experimental-infection studies are needed.

Species of the genus *Apharyngostrigea* Ciurea, 1924 parasitize birds, mainly Ardeidae. In Brazil, three species, all with life cycle unknown, have been reported in ardeids: *Apharyngostrigea brasiliana* (Szidat, 1928), *Apharyngostrigea cornu* (Zeder, 1800), and *Apharyngostrigea multiovata* (Pérez-Vigueras, 1944) (Travassos et al., 1969; Dubois, 1970a; Arruda et al., 2001; Noronha et al., 2009; Fernandes et al., 2015). Both ITS and COI confirm a close relationship between *Apharyngostrigea* sp. from Brazil with North American isolates of *A. cornu* and *A. pipientis*. Interestingly, ITS from *Apharyngostrigea* sp. from Brazil grouped with an unidentified strigeid from *Egretta garzetta* (Linnaeus, 1766) from Israel (Dzikowski et al., 2004), and varied from the latter by just 0.3%, which suggests the Israeli isolate also belongs to *Apharyngostrigea*. The COI sequences from cercariae of *Apharyngostrigea* sp. indicate they are conspecific with metacercariae from naturally infected *C. decemmaculatus* from Argentina. This, together with our experimental infections of another poeciliid as second intermediate host, suggests the species is *A. simplex*, given that Ostrowski de Núñez (1989) infected *C. decemmaculatus* with similar cercariae collected in the same region we collected the sequenced metacercariae.

Since Lutz (1928) described *A. compactum* from the Neotropical cormorant *Nannopterum brasilianus* (Gmelin, 1789) in Venezuela, this parasite has been reported in the same host elsewhere in South America (Ostrowski de Núñez, 1968, 1982; Drago et al., 2011; Monteiro et al., 2017) and in *Nannopterum auritus* (Lesson, 1831) in North America (Flowers et al., 2004; Rosser et al., 2016). In some reports, this species was identified as *Austrodiplostomum ostrowskiae* Dronen, 2009, a species recently synonymized with *A. compactum* (Ostrowski de Núñez, 2017). The life cycle of *A. compactum* involves planorbids and fish as first and second intermediate hosts, respectively (Ostrowski de Núñez, 1982). Since the mid-1990s, *A. compactum* has often been reported in fish in the Americas (Kohn et al., 1995; Overstreet and Curran, 2004; Galaviz-Silva et al., 2013; Ramos et al., 2013; Pinto et al., 2014).

The ITS sequences from our samples of *A. compactum* in *B. straminea* were identical to isolates of this species (some identified as *A.*

ostrowskiae) from the USA. The COI sequences of cercariae of *A. compactum* from Brazil grouped with isolates from fish and birds in the USA, Mexico and Peru (O'Hear et al., 2014; Locke et al., 2015; Rosser et al., 2016; García-Varela et al., 2016). The COI variation among these isolates (0.3–1.3%) indicates conspecificity and, given the spatial scale of the sampling, low intraspecific variability. Sequences from two unidentified *Austrodiplostomum* spp. known only from metacercariae in North American fish (Locke et al., 2015; Rosser et al., 2016) differed by 9.7% and 10% from *A. compactum* sequenced in Brazil. Similar variation (10%) between these two North American isolates suggests the existence of at least three species of this genus in the Americas. Molecular data are needed from the type species of the genus, *Austrodiplostomum mordax* Szidat and Nani, 1951, to determine the status of *A. compactum* and the two unnamed North American species of *Austrodiplostomum* recorded by Locke et al. (2015) and Rosser et al. (2016).

Members of the genus *Cotylurus* are intestinal parasites in birds, using aquatic molluscs as both first and the second intermediate hosts (Yamaguti, 1975; Niewiadomska, 2002b). In South America, three species have been reported, *Cotylurus cornutus* (Rudolphi, 1808), *Cotylurus gallinulae* (Lutz,1928) and *C.lutzi* (Fernandes et al., 2015), among which the life cycle is known only for the last species. Cercariae of *Cotylurus* sp. from *B. straminea* collected in the present study did not infect laboratory-raised *B. glabrata*, even if molecular data confirmed naturally-occurring infections of *Cotylurus* sp. metacercariae in the closely related *B. straminea*. Previous reports have established that *B. glabrata* is a competent host for metacercariae of *Cotylurus*, both in natural (Ruiz, 1953) and experimental infections (Basch, 1969). *Biomphalaria glabrata* may be an unsuitable host for *Cotylurus* sp., but other factors may also have prevented infection in our study, such as age of molluscs, post-emergence temporal window of cercarial infectivity, and cercarial dosage.

Molecular data have recently been published from several members of the genus *Cotylurus* (Gordy et al., 2016; Hernández-Mena et al., 2017; Soldánová et al., 2017; Locke et al., 2018). The COI sequences from *Cotylurus* sp. from the present study differed by 11–12.9% from *C. strigeoides* from Mexico and Canada, *C. cornutus* from Norway, and *C. marcogliesei* from Canada, which is comparable to interspecific differences between congeners in other strigeid genera (7.5–15.6%) (Blasco-Costa et al., 2016a,b). The ITS sequences we obtained differed by 5.1% from *C. strigeoides* (=*C. gallinulae*) from *Aythya affinis* (Eyton, 1838) in Northern Mexico (Hernández-Mena et al., 2017; Locke et al., 2018), indicating that *Cotylurus* sp. is congeneric (but not conspecific) with the latter. We noted that the CO1 sequence of strigeid cercariae from *Stagnicola elodes* (Say, 1821) from western Canada (Gordy et al., 2016) differs by only 1.9% from and sporocysts of *C. cornutus* from *Radix balthica* (Linnaeus, 1758) from Norway (Soldánová et al., 2017). This strongly suggests these isolates are conspecific. *Cotylurus cornutus* was described in Europe and has been recorded throughout the Holarctic (Dubois, 1968), but without molecular support until now. The ability of *C. cornutus* to persist on different continents can be attributed to the relatively wide spectrum of lymnaeids that may serve as first intermediate hosts (see *Radix* and *Stagnicola* in Correa et al., 2010) and the mobility of its definitive hosts, which include Charadriidae and Anatidae (Dubois, 1968).

Cercariae of Crassiphialinae gen. sp. are here reported for the first time in Brazil. Experimental-infections with these larvae did not yield metacercariae for further identification of this parasite, nor did analysis of ITS or COI yield matches at species or genus levels. However, analysis of COI indicates Crassiphialinae gen. sp. from Brazil are closely related to *Uvulifer* spp., while ITS divergences of at least 10% were obtained between Crassiphialinae gen. sp. and other diplostomoid sequences. On this basis, we believe the second intermediate host of Crassiphialinae gen. sp. is probably a fish, as in other species of *Uvulifer* and other members of this subfamily, even if experimental infection of *P. reticulata* was unsuccessful. The material from Brazil may belong to a crassiphialinid genus that has not yet been sequenced, because cercariae lacked non-pigmented eyespots, which are present in *Uvulifer* (Yamaguti, 1975; Faltýnková et al., 2007). The absence of ventral sucker seems to be a typical feature of cercariae of Crassiphialinae.

In South America, adults of species of the genus *Hysteromorpha* Lutz (1931) are reported mainly in cormorants. The type species, *Hysteromorpha triloba* (Rudolphi, 1819), was described in Europe and until recently considered cosmopolitan (Dubois, 1938, 1953, 1970a,b, Caballero and Diaz-Ungria, 1958; Drago et al., 2011). However, isolates of *Hysteromorpha* from Europe and North America differ in COI and ITS, and in the morphology of metacercariae, leading to the resurgence of the North American species as *H. corti* (Locke et al., 2018). The ITS sequences of *Hysteromorpha* sp. from Brazil were identical to those of *H. corti*. However, the COI of *Hysteromorpha* sp. from Brazil diverges by 6.6–7.5% from *H. corti*, and by 8.2–8.5% from *H. triloba*, with the Brazilian isolates forming a distinct clade in the phylogenetic analysis, suggesting that they are distinct species. Noting the distinct species of *Hysteromorpha* in Europe and North America, Locke et al. (2018) suggested that the South American isolates may also prove to be a different species. Our results support this prediction, and cast doubt on the pan-American distribution of a single *Hysteromorpha* species (e.g., Sereno-Uribe et al., 2019).

One plausible factor constraining the distribution of species of *Hysteromorpha*, which are undoubtedly widely dispersed by cormorants, is the planorbid first intermediate host. *Hysteromopha corti* is known from *Gyraulus deflectus* (Say, 1824) (limited to North America) and *Hystermorpha* sp. occurs in *B. straminea* (limited to South America, Paraense, 2001). Although both are planorbids, these species are not closely related (Jørgensen et al., 2004), which also provides further evidence that *Hystermorpha* sp. and *H. corti* are distinct species. Thus, our results suggest the existence of at least three species of *Hysteromorpha* among the sequences evaluated, one in each region (North America, Europe and South America). Data from the adult form of *Hysteromorpha* sp. will advance the taxonomic status of this species.

Molecular data from diplostomoids are rapidly accumulating (Blasco-Costa and Locke, 2017), yet with two commonly used markers we achieved only one identification to species, and in one case even genus could not be determined. This reflects a lack of molecular data from diplostomoids in the Neotropical region, where parasite species discovery effort lags behind host species discovery effort (Jorge and Poulin, 2018). Blasco-Costa and Poulin (2017) called for renewed efforts to experimentally elucidate helminth life cycles, but many difficulties are inherent to this approach. In the diplostomoids, developmental stages and species vary widely in host specificity, and even the phylum of suitable hosts for experimental assays may be unclear. In this sense, molecular studies may provide the most efficient means of accelerating the elucidation of life cycles in regions with poorly characterized diversity. Such studies seem necessary for the specific identification of some of the cercariae of Diplostomoidea here evaluated. Indeed, our identifications relied heavily on existing sequence data from Europe and North America. The identifications made, although often provisional, allowed inferences about probable hosts and life-cycle patterns not apparent from experimental infections. Such information may be useful in approaches related to wildlife management, conservation and for the identification and surveillance of species of potential importance in aquaculture.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.actatropica.2019.105082.

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