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FISH PARASITOLOGY - ORIGINAL PAPER

Molecular and morphological characterization of the metacercariae of two species of diplostomid trematodes (Platyhelminthes, Digenea) in freshwater fishes of the Batalha River, Brazil

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Abstract

The Diplostomidae include a large group of flatworms with complex life cycles and are frequently found parasitizing the eyes and central nervous system of freshwater fishes. The morphological identification of the metacercariae at species level is not always possible. Thus, molecular tools have become essential to assist in the parasite species determination. This study was aimed at describing two diplostomid metacercariae found in freshwater fish in São Paulo, Brazil, based on morphological characters and in the genetic characterization of COI sequences. Our results showed that the two recognized taxa (*Tylodelphys* sp. and Diplostomidae gen. sp.) appear to be different from the species already described in South America. *Tylodelphys* sp. differs morphologically from *Tylodelphys xenopi*, *T. mashonense*, *T. jenynsiae*, and *T. scheuringi*. The metacercariae of *T. clavata* and *T. conifera* are smaller than *Tylodelphys* sp., while *T. podicipina* is larger than the metacercariae described here. The phylogenetic analysis of COI sequences yielded *Tylodelphys* sp. as the sister species of *Tylodelphys* sp. 4, a species reported from the brain of the eleotrid *Gobiomorus maculatus* in Oaxaca, Mexico. The metacercariae identified as Diplostomidae gen. sp. are morphologically different from the known diplostomid metacercariae and did not match with other diplostomid sequences available. Diplostomidae gen. sp. is recovered as the sister species of *Diplostomum ardeae*. Although the morphological evidence and the COI sequences differentiate the metacercariae found, the absence of adult specimens of both species precludes the specific designation. This is one of the first papers that use an integrative taxonomy approach to describe the species diversity of diplostomid trematodes in Brazil.

Keywords Fish parasites · Flatworms · Trematoda · Cytochrome *c* oxidase · Morphological analysis · Bayesian analysis

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Introduction

Parasites represent a large part of the diversity of known species and are considered to have a high potential for speciation (McCoy 2003); they therefore are among the best candidates for exploring species boundaries with the help of molecular methods (Olson and Tkach 2005). Modern taxonomic practices used in helminths combine morphological data and DNA sequences, allowing the establishment of a link between larval stages and adults in different host species in an ecosystem (Alcántar-Escalera et al. 2013; Chibwana et al. 2013; Blasco-Costa et al. 2017; Presswell and Blasco-Costa, 2019).

Digenetic trematodes of the family Diplostomidae Poirier, 1886 are Platyhelminthes with complex life cycles, widely distributed and with great species diversity. In freshwater fishes, metacercariae of this family can be found encysted in several tissues, but mainly they are unencysted in some habitats such as the eyes, musculature, digestive tract, and central nervous system (Travassos et al. 1969; Niewiadomska 2002). According to Locke et al. (2010), there is a tendency for infection of the cerebral and ocular tissues since those habitats exhibit lower immune response. In cases where infection levels are very high, the metacercariae may cause hemorrhage in the musculature, ocular cataract and lens dislocation, exophthalmia, blindness or cranial distortion causing brain tissue damage (Chappell 1995; Sandland and Goater 2001), and their presence in the host may increase the susceptibility of infected fish to predation (Chappell et al. 1994).

The identification of diplostomid species in all phases of the life cycle is challenging; the identification of the metacercariae at species level is most of the times difficult to achieve; morphological variation according to the host species, size and age, or even according to the density of infection make identification up to species level a difficult task (Graczyk 1991; Niewiadomska and Niewiadomska-Bugaj 1995). The limitations of obtaining the adult parasites from the final hosts (birds) and the difficulty of completing the life cycles experimentally restrict the taxonomic progress in this group. However, recent studies on diplostomids have shown an effective methodological approach. The mitochondrial nicotinamide-adenine dinucleotide dehydrogenase 1 (NAD1) and cytochrome c oxidase subunit 1 (COI) genes, as well as the two internal transcripts of rRNA (ITS1 and ITS2), are the most widely used markers for molecular identification, elucidation of life cycles, and prospecting of cryptic species within the subclass Digenea (Nolan and Cribb 2005; Olson and Tkach 2005; Vilas et al. 2005; Criscione et al. 2005; Pérez-Ponce de León and Nadler 2010). Studies involving the combination of morphological and molecular data in diplostomid metacercariae have been carried out around the world (Chibbwana and Nkwengulila 2010; Chibwana et al. 2013; Otachi et al. 2014; García-Varela et al. 2016a, 2016b; Blasco-Costa et al. 2017); however, these studies have been scarcely performed considering diplostomids in Brazil (e.g., López-Hernández et al. 2018). In Brazil, a large diversity of diplostomids has been documented (Dubois 1968, 1969; Travassos et al. 1969; Fernandes et al. 2015).

The main objective of this study was to identify and characterize the diplostomid metacercariae in 12 species of freshwater fishes belonging to the Characiformes and Siluriformes orders in the Batalha River, State of São Paulo, Brazil, based on the genetic characterization of COI sequences in combination with an analysis of the morphological characters of these organisms. Our study revealed that the two species seem to be different from the species previously described in the region, and likely they will represent new species although their adult forms in fish-eating birds have not been found yet.

Material and methods

Collection and analysis of hosts

Host samples were collected from February 2014 to December 2016 at the Batalha River (21°53′17″ S, 49°13′31″ W), São Paulo State, Brazil. Nylon monofilament gillnets with different mesh types were used for this procedure; nets were placed overnight and removed at dawn. The euthanasia methodologies of the host fish were carried out following the guidelines of the National Council of the Animal Experimentation Control (CONCEA), and the research project was submitted to the Ethical Committee on Animal Use (CEUA) of the Universidade do Sagrado Coração (USC) (authorization no. 3353050417). We examined a total of 323 specimens of fish representing 12 species across four families for diplostomid metacercariae.

Collection of parasites and morphological analysis

The hosts were frozen immediately after collection and defrosted in the laboratory for analysis. They were necropsied and the organs removed, opened, and washed; and the material collected from them was passed through sieves and observed under a stereoscope. The metacercariae were collected, counted, and stored in 70° ethanol for morphological study. For molecular analyses, specimens were stored in absolute ethanol. For morphological analysis, some specimens were also fixed directly in 2.5% glutaraldehyde and sent to the Center for Electronic Microscopy in the Department of Biosciences of Botucatu, Paulista State University "Júlio de Mesquita Filho" (UNESP), to be used in scanning electron microscopy (SEM) preparations (Quanta 200 SEM-FEI

Company). After being removed from the fixative, the specimens were washed in distilled water, then immersed in 0.5% osmium tetroxide solution, washed again in water, and passed through the increasing series of alcoholic dehydration, and then critical point dried and metalized in gold.

For internal morphological analysis, parasites were stained with carmine hydrochloric alcohol and Gomori's trichrome. The stained parasites were diaphanized in creosote of faia or eugenol (clove oil) and permanent slides were mounted in Canada balsam (Eiras et al. 2006). Images of specimens were taken with Motican camera attached to the eyepiece of the optical microscope and then measurements were made with the Motic Images Plus 5.0 software. All measurements are given in micrometers (μm), the mean followed by standard error and range. The ecological parameters of the infections (prevalence, mean intensity, and mean abundance) were calculated according to Bush et al. (1997).

Vouchers of the analyzed hosts were deposited in the Nupelia Ichthyology Collection (Nucleus of Limnological Research, Ichthyology and Aquaculture) (NUP), State University of Maringá, municipality of Maringá, Paraná State, and in the Fish Collection of the Laboratory of Biology and Fish Genetics of the Institute of Biosciences of Botucatu (IBB), UNESP, municipality of Botucatu, São Paulo State, Brazil (Voucher numbers IBB 22920, NUP 17245, NUP 17008, IBB 22922, IBB 22916, NUP 17244, NUP 17246, IBB 22921, and IBB 22900). Vouchers of the studied metacercariae were deposited in the Helminthological Collection of the Institute of Biosciences of Botucatu (CHIBB) of UNESP in Botucatu, São Paulo State, Brazil (Voucher numbers CHIBB 542L, CHIBB 543L, CHIBB 544L, CHIBB 545L, CHIBB 546L, CHIBB 547L, CHIBB 548L, CHIBB 549L, CHIBB 550L, CHIBB 551L, CHIBB 552L, and CHIBB 553L).

Molecular data and phylogenetic analyses

DNA extraction of the collected diplostomids was performed with the DNeasy Blood & Tissue Kit (Qiagen, Germany). The COI gene was amplified using a PCR protocol with the primer pair MplatCOX1dF (5′-TGTAAAACGACGGCCAGTTT WCITTRGATCATAAG-3′) and MplatCOX1dR (5′-CAGG AAACAGCTATGACTGAAAYAAYAIIGGATCICCACC-3′) (Moszczynska et al. 2009). Amplifications were performed on Bio-Rad Mycycler thermal cycler (Bio-Rad Laboratories Pty Ltd., Gladesville, Australia), with initial denaturation at 94 °C for 1 min, followed by 5 cycles at 94 °C for 40 s, 45 °C for annealing for 40 s, and 72 °C for 1 min. Then, 35 cycles were performed at 94 °C for 40 s, 51 °C for annealing temperature for 40 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. Conventional PCR amplifications were performed in 25 μl of reactions containing 5 μl of the extracted DNA and 1 μl of each PCR primer using Ready-to-Go PCR beads (PureTaq™ Ready-to-Go beads, GE Healthcare, Chicago, USA). The solution consisted of stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of puReTaq DNA polymerase and reaction buffer. The bead was reconstituted to a final volume of 25 μl and the concentration of each dNTP was 200 μM in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, and 1.5 mM MgCl 2. After checking for the presence of the expected DNA amplicons on a 1% agarose gel in TBE buffer, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen®, CA, USA). Automated sequencing was performed directly on PCR products purified from samples using the BigDye Ready-Cycle Sequencing Reaction Kit v.3.1 (Applied Biosystems, Foster City, CA, USA) for sequencing cycles. Samples were sequenced on an Applied Biosystems ABI 3500 DNA genetic analyzer.

For phylogenetic analysis, sequences were assembled in Sequencher™ v. 5.2.4 (Gene Codes, Ann Arbor, MI) software and to confirm sequence identities, they were analyzed in BLAST (http://blast.ncbi.nlm.nih.gov). The partial sequences obtained from the COI gene were aligned with sequences of other diplostomids (Table 1). Sequences were aligned using Geneious version 7.1.3 (Kearse et al. 2012) with Muscle algorithm (Edgar 2004). The presence of stop codons and indels were checked in Geneious 7.1.3 (Kearse et al. 2012). To evaluate the occurrence of substitution saturation, we estimated the Iss index using DAMBE 5 software (Xia 2013). *Apatemon* sp. (KM212028) (Kuhn et al. 2015) and *Australapatemon mclaughlini* Gordy et al. 2017 (KY587406) (Gordy et al. 2017) were used as outgroups for rooting the trees.

The JModelTest 2.1.1 program (Posada 2008) was used to select the most appropriate evolutionary model for the data set for Bayesian inference (BI) and maximum likelihood (ML) analysis. BI was performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) and ML was run using PhyML (Guindon and Gascuel 2003), with the nucleotide substitution model GTR + I + G. Markov chain Monte Carlo (MCMC) chains were made for 10 million trees generated. The burn-in has been set to 25%. The maximum likelihood supports were determined by the bootstrap, 100 replicates. Phylogenetic trees were visualized and edited in FigTree v1.4 (Rambaut 2012). In order to calculate the genetic divergence, an alignment containing only sequences of adult diplostomids possessing pseudosuckers (*Austrodiplostomum*, *Diplostomum*, *Hysteromorpha*, and *Tylodelphys*) was used to produce a distance matrix. The genetic divergence between the sequences was calculated using the Kimura 2-parameter distance model (Kimura 1980) in MEGA6 (Tamura et al. 2013).

Table 1 Species of Diplostomidae and Strigeidae presented in the molecular phylogenetic analyses with details of host, locality, and GenBank accession numbers

Parasite species	Host	Locality	GenBank reference number	Reference
Strigeidae				
Apatemon sp.	*Gasteroteus aculeatus*	Norway	KM212028	Kuhn et al. 2015
Australapatemon mclaughlini		Canada	KY587406	Gordy et al. 2017
Diplostomidae				
Austrodiplostomum ostrowskiae		USA	KR271028	Locke et al. 2015
Austrodiplostomum ostrowskiae			MF124271	Blasco-Costa and Locke 2017
Diplostomidae gen. sp. (haplotype 1 and 2)	*Hypostomus regani, H. strigaticeps, H. hermanni, H. iheringii, H. ancistroides, H. albopunctatus, H. paulinus, Loricaria prolixa, L. piracicabae Rhamdia quelen Hoplosternum littorale*	Brazil		Present study
Diplostomum ardeae	*Ardea herodias*	Canada	KR271033	Locke et al. 2015
Diplostomum baeri	*Perca flavescens*	USA	MF142178	Ubels et al. 2018
Diplostomum huronense	*Ambloplites rupestris, Catostomus commersonii Notemigonuscrysoleucas, Notropis stramineus/volucellus, Perca flavescens, Pimephales notatus, Larus argentatus, Larus delawarensis*	Canada	KR271068	Locke et al. 2015
Diplostomum indistinctum	Lymnaeidae gen. sp., *Cephalorhynchus commersonii, Neogobius melanostomus, Notemigonus crysoleucas, Larus* sp.	Canada	KR271076	Locke et al. 2015
Diplostomum mergi	*Radix auricularia*	Canada	KY271543	Locke et al. 2015
Hysteromorpha triloba	*Notropis hudsonius, Notemigonus crysoleucas, Ictalurus nebulosus, Catostomus commersonii*	Canada	JF769473	Locke et al. 2011
Hysteromorpha triloba	*Notropis hudsonius, Notemigonus crysoleucas, Ictalurus nebulosus, Catostomus commersonii*	Canada	JF769467	Locke et al. 2011
Neodiplostomum americanum		Canada	JF904537	Locke et al. 2011
Tylodelphys aztecae	*Podilymbus podiceps*	Mexico	KT175316	Garcia-Varela et al. 2016b
Tylodelphys clavata		China	KY271544	Dang 2016
Tylodelphys clavata			KR271480	Locke et al. 2015
Tylodelphys excavata	*Planorbarius corneus*	Czech Republic	KC685344	Chibwana et al. 2013
Tylodelphys immer	*Coregonus clupeaformis, Notropis hudsonius, Perca flavescens, Salvelinus fontinalis, Gavia immer*	Canada	KR271493	Locke et al. 2015
Tylodelphys jenynsiae	*Cnesterodon decemmaculatus*	Argentina	KR271496	Locke et al. 2015
Tylodelphys mashonensis	*Clarias gariepinus*	Tanzania	KR863382	Chibwana et al. 2013
Tylodelphys scheuringi	*Ambloplites rupestris*	Canada	HM064915	Locke et al. 2010
Tylodelphys scheuringi		Canada	FJ477223	Moszczynska et al. 2009
Tylodelphys sp. (haplotype 1 and 2)	*Hoplias malabaricus*	Brazil		Present study
Tylodelphys sp.	*Gobiomorphus cotidianus*	New Zealand	KU588148	Blasco-Costa et al. 2017
Tylodelphys sp.	*Gobiomorphus cotidianus*	New Zealand	KU588149	Blasco-Costa et al. 2017
Tylodelphys sp. 1	*Clarias gariepinus*	Tanzania	KC685355	Chibwana et al. 2013
Tylodelphys sp. 2	*Clarias gariepinus*	Tanzania	KC685358	Chibwana et al. 2013
Tylodelphys sp. 2	*Micropterus salmoides, Tilapia zillii, Oreochromis leucostictus, Barbus paludinosus, Cyprinus carpio*	Kenya	KF809494	Otachi et al. 2014
Tylodelphys sp. 3	*Lepomis microlophus*	USA	KR271515	Locke et al. 2015
Tylodelphys sp. 4	*Gobiomorus maculatus*	Mexico	KR271518	Locke et al. 2015
Tylodelphys sp. 5	*Dormitator latifrons, Galaxias maculatus*	Mexico	KR271521	Locke et al. 2015

Results

Molecular characterization

Two types of metacercariae were found, one resembling those of *Tylodelphys* and one resembling those of *Diplostomum*. The COI sequences of the *Tylodelphys* specimens from Brazil (649- and 658-bp long) were identical. The newly generated sequences were aligned with 27 diplostomids retrieved from the GenBank database, in addition to other two members of Diplostomidae obtained in this study (Table 1). The Iss index indicated no saturation in the transitions or

transversions; Iss.c values were greater than the Iss values. The final alignment for phylogenetic analysis was trimmed to 474 bp and ML and BI analyses were performed. The metacercariae of *Tylodephys* sp. herein characterized morphologically represent a separate lineage and is nested in a clade consisting of *Tylodelphys* sp. 4 (GenBank KR271518) from *Gobiomorus maculatus* (Günther, 1859) in Oaxaca, Mexico, and *T. jenynsiae* (GenBank KR271027) from *Cnesterodon decemmaculatus* (Jenyns, 1842) in Argentina. The data obtained, based on K2P distance matrix (Supplementary Table 1), showed the lowest nucleotide interspecific distance of 9.2% with respect to *Tylodelphys* sp. 4 (Oaxaca, Mexico), 9.4% with *Tylodelphys* sp. 6 (Mississippi, USA) and 11.2% with *T. jenynsiae*. These high genetic divergence values clearly show that the metacercariae from Brazil constitute a separate species, although it cannot be ruled out that it does not belong to one of the five valid species occurring in South America.

The COI sequences of Diplostomidae gen. sp. were 625–642 bp long and were different in five nucleotidic sites, with 1.2% of intraspecific divergence (Supplementary Table 1). Haplotypes of Diplostomidae gen. sp. were aligned with 27 diplostomids retrieved from GenBank database plus two specimens of *Tylodelphys* sp. that are also characterized morphologically in this study (Table 1). The phylogenetic tree (Fig. 1) shows that the metacercariae of the diplostomid herein characterized are nested as the sister group of *Diplostomum ardeae* Dubois, 1969 a parasite of the Great blue heron, *Ardea herodias* Linnaeus, 1758 in Canada, a relationship with strong nodal support (0.99 and 91 for posterior probabilities and bootstrap, respectively). Interestingly, these clades did not form a monophyletic assemblage along with species of *Diplostomum*; actually, our phylogenetic tree showed that *Hysteromorpha triloba* (Rudolphi, 1819) and *Neodiplostomum americanum* were sister taxa of most species of *Diplostomum*, and the newly generated sequences along with *D. ardeae* were the sister group of *Tylodelphys* spp. and *Austrodiplostomum* spp., although these relationships were supported by low bootstrap support values (see Fig. 1). The COI genetic divergence between the newly generated sequences and *D. ardeae*, based on K2P distance matrix (Supplementary Table 1), varied from 6.9 to 7.6%, although genetic divergence values with *Hysteromorpha* and with species of *Diplostomum* was higher, 12.9–13.5% and 13.4–13.8%, respectively in average. Our molecular data allow us to speculate that *D. ardeae* and the sequences from Brazilian freshwater fish represent a separate genus that requires further description and name, and that the specimens from Brazil represent a different species from the one described from Canada.

The metacercariae of Diplostomidae were found in all fish species analyzed, and 4828 specimens were counted. The diplostomids obtained from the fish of the order Siluriformes were exclusively found in the lens. Instead, the metacercariae found in *H. malabaricus* infected the vitreous humor of the eyes and the visceral cavity of their hosts (Table 2).

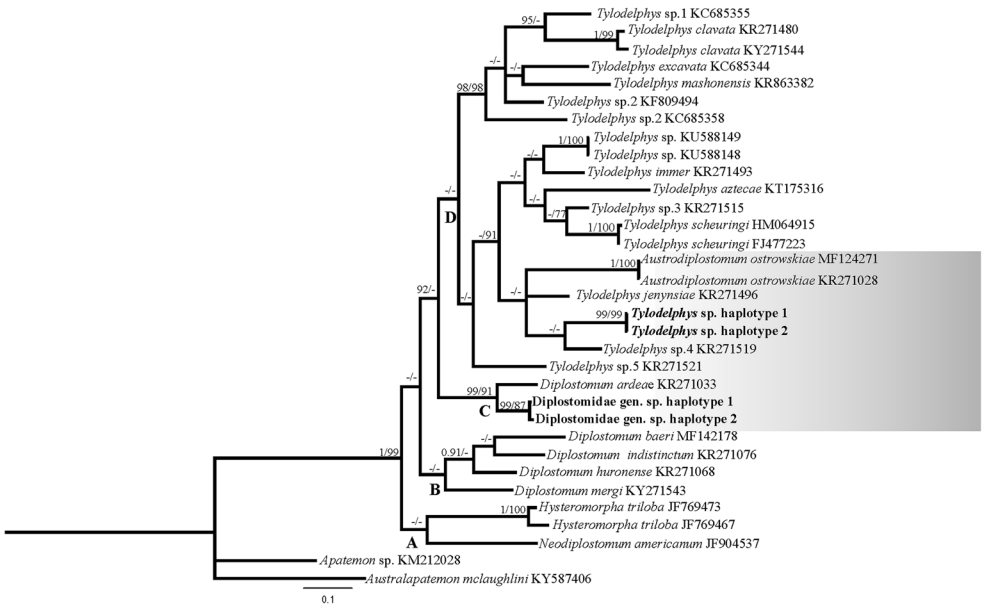

Fig. 1 Bayesian and maximum likelihood phylogram reconstructed based on cytochrome C oxidase (COI) sequences of Diplostomidae. Newly sequences species are in bold. Main clades are denoted in bold as A–D. Numbers besides names are GenBank acession numbers. Nodal support values are given above branches (posterior probabilities < 0.90 and bootstrap < 70% are not shown). Gray square indicates the phylogenentic position of the putative new species. The Strigeids *Apatemon* spp. and *Australapatemon mclaughlini* were used as outgroup. The scale bar indicates the number of substitutions per site

Table 2 Quantitative descriptors of the Diplostomidae metacercariae collected in different neotropical fish species of the Tietê-Batalha basin, São Paulo, Brazil (*N*, number of analyzed fish; *P%*, prevalence expressed as percentage; *MA*, mean abundance; *SE*, standard error; *MI*, mean intensity)

Host species	*N*	P%	MA ± SE	MI ± SE
Callichthyidae				
Hoplosternum littorale	53	86.8	7.5 ± 1.2	8.6 ± 1.3
Erythrinidae				
Hoplias malabaricus	41	68.3	32.5 ± 7.1	47.5 ± 9.1
Heptapteridae				
Rhamdia quelen	10	20	4.1 ± 2.7	20.5 ± 1.5
Loricariidae				
Hypostomus regani	60	95	17.6 ± 1.8	18.5 ± 1.7
Hypostomus strigaticeps	30	93.3	14.8 ± 2.4	15.8 ± 2.4
Hypostomus hermanni	31	96.7	21.3 ± 2.1	22 ± 2.1
Hypostomus iheringii	17	100	27.1 ± 2.8	27.1 ± 2.8
Hypostomus ancistroides	25	80	6.4 ± 1.6	8.1 ± 1.8
Hypostomus albopunctatus	11	81.8	5.3 ± 2.4	6.4 ± 2.9
Hypostomus paulinus	3	33.3	3.3 ± 3.3	10
Loricaria prolixa	40	85	7.5 ± 1.4	8.8 ± 1.5
Loricaria piracicabae	2	50	5 ± 5	10

Morphologically, the metacercariae of *H. malabaricus* belong to the genus *Tylodelphys*. In the present study, we present the detailed description of this diplostomid.

Phylum Platyhelminthes Gegenbaur, 1859

Class Trematoda Rudolphi, 1808

Subclass Digenea Carus, 1863

Order Diplostomida Olson, Cribb, Tkach, Bray & Littlewood, 2003

Family Diplostomidae Poirier, 1886

Tylodelphys sp.

Host: *Hoplias malabaricus* (Bloch, 1794) (Characiformes, Erythrinidae)

Locality: Batalha River, Tietê-Batalha drainage basin, SP, Brazil (21°53′17″ S, 49°13′31″ W).

Site of infection: vitreous humor (eyes) and visceral cavity

Description: based on 41 specimens stained and measured

Non-encysted metacercariae; body linguiform; body undivided; anterior end rounded; posterior end conical non-segmented body. Oral sucker sub-terminal. Pseudosuckers (lappets) and pre-pharynx absent. Muscular pharynx oval; esophagus short; intestinal ceca extend to the posterior part of body up to the posterior border of holdfast organ; V-shaped excretory vesicle; excretory pore terminal. Ventral sucker round to oval, located at middle portion of body and anterior to the holdfast organ. Holdfast organ oval, strongly muscular, with a longitudinal slit. Primordia of gonads posterior to the holdfast organ, forming a cell mass located at the end of the ceca branches. Small calcareous corpuscles distributed in two rows along the body (Figs. 2a and 3). All the measures, ratios, and distances between structures are given in Table 3.

Remarks: Morphologically, our specimens correspond with the diagnosis of *Tylodelphys* provided by Niewadomska (2002) in that they have a bipartite body and a non-trilobed anterior end. The metacercariae of *Tylodelphys* have been previously found in the brain of *H. malabaricus* in Laguna Salta La Vieja, el Chaco Province, Argentina (Szidat 1969). In the same study, Szidat (1969) described *Tylodelphys jenynsiae* based only on the metacercaria. However, since the metacercariae were found in only one analyzed specimen of *H. malabaricus*, this author preferred not to name the species (see Szidat 1969).

Thirty species of *Tylodelphys* are described worldwide. The metacercariae of 18 of these species are known, and eight of these were described and named based only on the morphological characters of their metacercariae (Blasco-Costa et al. 2017), without the description of the adult individuals. According to García-Varela et al. (2016b) and Blasco-Costa et al. (2017), species whose description would have been made only based on the morphological characters of metacercariae should be considered *incertae sedis*, since the link between these forms and potential species already described from adults cannot be established only through morphological data. However, some species described in the past based only on the morphology of their metacercariae were validated after molecular analysis.

In South America, five congeneric species are considered valid: *Tylodelphys adulta* Lunaschi & Drago, 2004, *Tylodelphys brevis* Drago & Lunaschi, 2008, *Tylodelphys elongata* (Lutz, 1928) Dubois, 1936, and *Tylodelphys americana* (Dubois, 1936), whose descriptions are based on adults obtained in fish-eating birds in Argentina, Brazil, and Venezuela; and *T. jenynsiae*, a parasite of the visceral cavity of fish; only the metacercarial stage is known, and it has been characterized both morphologically and molecularly (Fernandes et al. 2015, Blasco-Costa et al. 2017). The species characterized in the present study differs from the metacercariae of *Tylodelphys aztecae* Garcia-Varela, Sereno-Uribe, Pinacho-Pinacho, Hernández-Cruz & Pérez-Ponce de León, 2015, a species recently described from the intestine of the podicipedid *Podilymbus podiceps* (Linnaeus, 1758) in Mexico (García-Varela et al. 2016b), because it presents a larger size of most morphometric characters; in addition, the metacercariae of *T. aztecae* possess calcareous corpuscules forming at least six rows along the body, whereas in the present study, *Tylodelphys* sp. presents only two rows of calcareous corpuscles; in addition, *T. aztecae* is found in the body cavity of an endemic fish species across central Mexico, *Goodea atripinnis* Jordan, 1880.

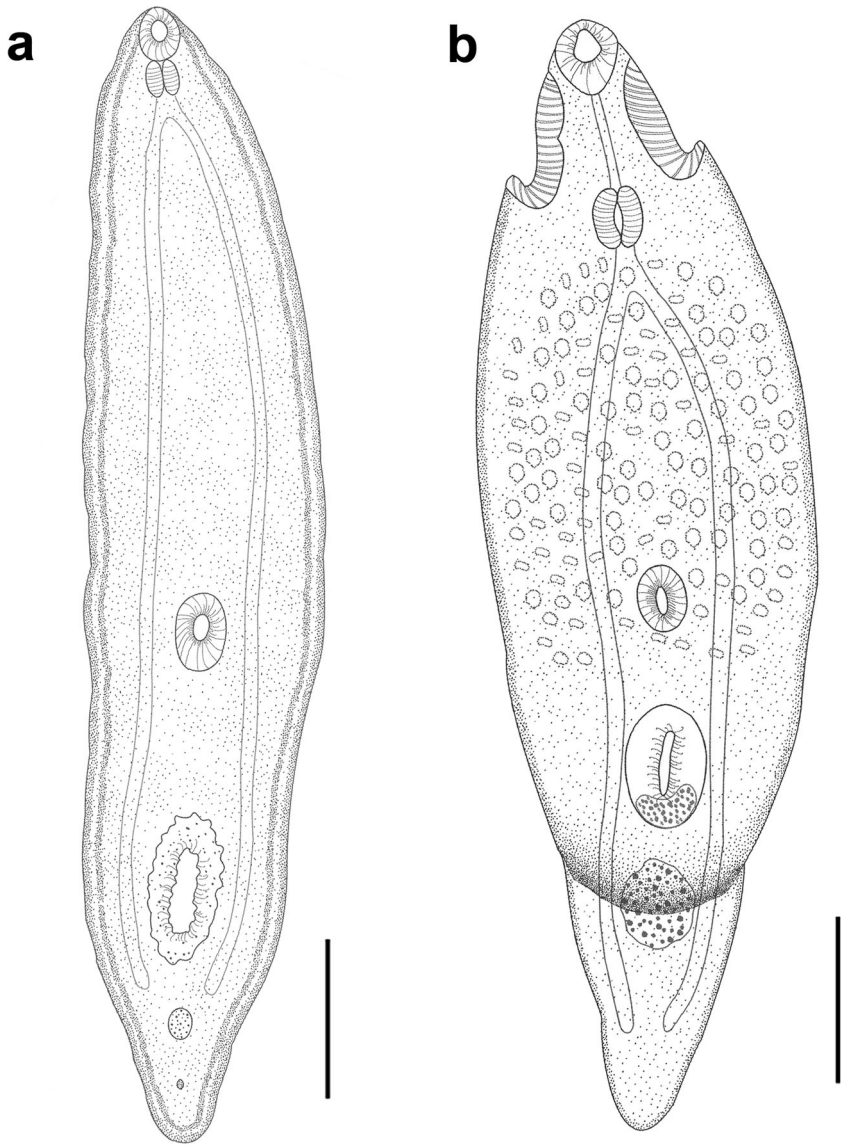

Fig. 2 Diplostomidae metacercariae from fish collected in the Batalha River, Tietê-Batalha basin, State of São Paulo, Brazil. **a** *Tylodelphys* sp., found in the vitreous humor (eyes) and visceral cavity of *Hoplias malabaricus* (Characiformes, Erythrinidae). **b** Diplostomidae gen. sp., found on the lens of Siluriformes fish (scale = 100 μm)

The metacercariae we characterize differs further from the metacercariae of other *Tylodelphys* species; *Tylodelphys xenopi* (Nigrelli & Maraventano, 1944) has an anterior segment of the body bearing conspicuous pseudosuckers and concentrated corpuscles in the anterior end of the body (King and Van As,1997). The metacercariae of *Tylodelphys mashonense* Beverley-Burton, 1963 also shows a bi-segmentation of the body (which seems to disappear in adult individuals), and the posterior segment is longer (Moema et al. 2013; Chibwana et al. 2015). *T. jenynsiae* and *Tylodelphys scheuringi* (Hughes, 1929) present a fold on the ventral surface, resembling a body segmentation (Hughes 1929; Szidat 1969). The specimens of *Tylodelphys clavata* (von Nordmann, 1832) and *Tylodelphys conifera* (Mehlis, 1846) are smaller than in *Tylodelphys* sp. from Brazil, while *Tylodelphys podicipina* (Kozicka & Niewiadomska, 1960) is larger than the metacercariae described here.

Diplostomidae gen. sp.

Hosts: *Hypostomus regani* (Iheringi, 1905), *H. strigaticeps* (Regan, 1908), *H. hermanni* (Ihering, 1905), *H. iheringii* (Regan, 1908), *H. ancistroides* (Ihering, 1911), *H. albopunctatus* (Regan, 1908), *H. paulinus* (Regan, 1905), *Loricaria prolixa* (Isbrücker & Nijssen, 1978), *L. piracicabae* (Ihering, 1907) (Siluriformes, Loricariidae); *Rhamdia quelen* (Quoy & Gaimard, 1824) (Siluriformes, Heptapteridae); *Hoplosternum littorale* (Hancock, 1828) (Siluriformes, Callichthyidae)

Locality: Batalha River, Tietê-Batalha drainage basin, SP, Brazil (21°53′17″ S, 49°13′31″ W)

Site of infection: crystalline lens (eyes)

Description: based on 39 specimens stained and measured

Non-encysted metacercariae occurring between the layers of the lens. Living metacercariae highly mobile, stretching and

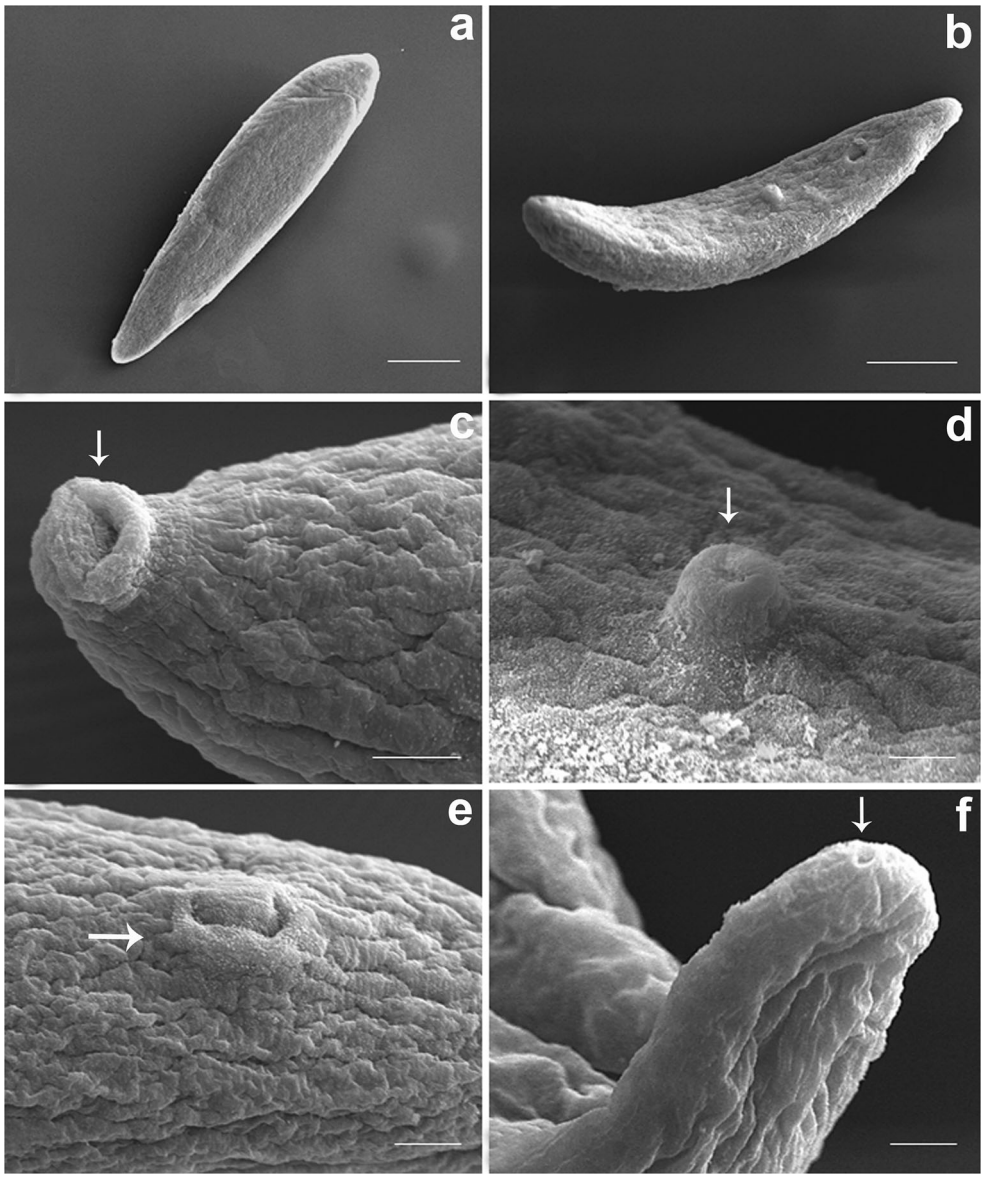

Fig. 3 Scanning electron microscopy (SEM) images of the *Tylodelphys* sp. metacercariae (Platyhelminthes, Digenea), parasite of *Hoplias malabaricus* (Characiformes, Erythrinidae) from the Batalha River, Tietê-Batalha basin, State of São Paulo, Brazil. **a** Dorsal view. **b** Ventral view. **c** Previous region with highlight to the oral sucker. **d** Median region, with prominence for ventral sucker. **e** Middle-posterior region, with the opening of the holdfast organ **f** Posterior region with excretory pore opening (structures highlighted with white arrow) (**a**, **b** = 100 μm scale; **c**, **d**, **e**, **f** = 10 μm scale)

contracting their bodies. Foliate and elongated body even when compressed; bi-segmented; forebody larger than hindbody; short hindbody, conical. Oral sucker subterminal; two lateral accessory pseudosuckers; prepharynx present; pharynx oval; esophagus short; intestinal caeca extending posteriorly beyond the region of primordia of gonads. Ventral sucker round to oval, at midline of body, anterior to holdfast organ; holdfast organ rounded to oval in some specimens, with longitudinal slit, and sometimes protruding. Visible primordia of gonads located posteriorly to holdfast organ, in the form of a cell mass. Incipient vitelline follicles along the body, from the posterior end of the pharynx to the posterior end of ventral sucker (Figs. 2b and 4). All the measures, distances, and ratios are given in Table 3.

Remarks: This diplostomid metacercaria seems to possess some morphological characteristics apparently not present in other groups. Our specimens are similar to species currently allocated into genera considered valid within the family Diplostomidae occurring in the Neotropical region, such as *Austrodiplostomum*, *Tylodelphys*, *Sphincterodiplostomum* (Dubois, 1936); *Hysteromorpha*, *Dolichorchis* (Dubois, 1961); and *Diplostomum* because they all possess a pair of pseudosuckers lateral to the oral sucker. However, the metacercariae of the species we characterize herein differ from *Hysteromorpha* species in that they are encysted on the surface of the intermediate hosts, as it may occur in *H. triloba* causing the black spot disease (see Illan et al. 2013). In addition, *Hysteromorpha*

Table 3 Morphometric data of the metacercariae of *Tylodelphys* sp. parasites of *Hoplias malabaricus* (Characiformes, Erythrinidae), and of the metacercariae of Diplostomidae gen. sp. parasites of Siluriformes fish of the Tietê-Batalha basin, State of São Paulo, Brazil (*W*, width; *L*, length; *SE*, standard error)

	Diplostomidae gen. sp. ($n = 39$)	*Tylodelphys* sp. ($n = 41$)
	Mean ± SE (range)	**Mean ± SE (range)**
Body (W)	176.8 ± 29.4 (86.5–224.7)	150.6 ± 25.5 (90.5–195.1)
Body (L)	654.4 ± 88.6 (543.7–1017.4)	683.8 ± 78.5 (551.4–1033.1)
Forebody (W)	177.0 ± 29.3 (92.7–230)	–
Forebody (L)	535.2 ± 72.6 (417.3–810.2)	–
Hindbody (W)	99.7 ± 20.5 (39.2–133.7)	–
Hindbody (L)	120.4 ± 23.8 (87–203.1)	–
Oral sucker (W)	36.1 ± 7.7 (11.7–48)	32.4 ± 10.3 (14.5–55.5)
Oral sucker (L)	37.8 ± 11.0 (13.2–59.6)	33.1 ± 9.4 (10.5–54.2)
Ventral sucker (W)	41.3 ± 11.7 (11.9–65.2)	10.1 (16.9–52.6)
Ventral sucker (L)	42.8 ± 10.6 (20.7–76.3)	36.2 ± 9.9 (20.8–66.1)
Pseudosuckers (W)	17.7 ± 4.8 (7.7–28.9)	–
Pseudosuckers (L)	68.8 ± 10.1 (55.3–104.6)	–
Holdfast organ (W)	59.6 ± 11.8 (24.9–94.9)	46.8 ± 7.4 (29.7–64.7)
Holdfast organ (L)	65.3 ± 10.7 (44.1–86.7)	93.4 ± 16.8 (71.2–144.8)
Pharynx (W)	22.3 ± 4.7 (14–30.4)	30.2 ± 7.3 (23.5–36.8)
Pharynx (L)	26.8 ± 4.8 (17.7–35.3)	24.7 ± 2.2 (23–27.7)
Distance ventral sucker–anterior end	417.3 ± 67.3 (303.5–653.9)	418.7 ± 6.1 (309.9–483.3)
Distance ventral sucker–holdfast organ	139 ± 23.7 (101.3–206.9)	206.5 ± 3.8 (141.8–254.9)
Hindbody (W)/forebody (W)	0.6 ± 0.1 (0.4–0.7)	–
Hindbody (L)/forebody (L)	0.2 ± 0.1 (0.2–0.3)	–
Body (W)/pseudosuckers (W)	10.5 ± 2.3 (6.8–17.7)	–
Body (L)/pseudosuckers (L))	9.6 ± 0.9 (6.8–11.3)	–
Pseudosuckers (W)/oral sucker (W)	0.5 ± 0.1 (0.3–0.8)	–
Pseudosuckers (L)/oral sucker (L)	2.0 ± 0.9 (1.0–4.8)	–
Pseudosuckers (W)/pharynx (W)	0.8 ± 0.2 (0.4–1.4)	–
Pseudosuckers (L)/pharynx (L)	2.5 ± 0.5 (1.8–4.1)	–
Body (W)/holdfast organ (W)	3.0 ± 0.6 (1.9–4.7)	3.3 ± 0.1 (1.7–53)
Body (L)/holdfast organ (C)	10.1 ± 1.8 (7.1–15.7)	7.5 ± 0.2 (6.2–10.0)
Forebody (W)/holdfast organ (W)	3.0 ± 0.6 (1.9–4.7)	–
Forebody (L)/holdfast organ (L)	8.3 ± 1.5 (5.9–12.3)	–
Oral sucker (W)/pharynx (W)	1.8 ± 0.5 (1.2–3.3)	1.3 ± 0.1 (0.8–1.9)
Oral sucker (L)/pharynx (C)	1.6 ± 0.4 (0.8–2.3)	1.6 ± 0.05 (1.3–1.9)

has no prepharynx, and the anterior end is trilobate, wider anteriorly and attenuated posteriorly, whereas the metacercaria described in this study has tapered anterior and posterior ends (Travassos et al. 1969; Niewiadomska 2002).

The species of diplostomid we characterize does not belong also to the genus *Austrodiplostomum* because their species lacks ventral sucker; from species in the genus *Tylodelphys* our specimens differ because they possess an indistinctly bipartite body, and usually inconspicuous pseudosuckers; further, the metacercariae of species of *Sphincterodiplostomum* show a trilobate anterior end and are distinctly bi-segmented, lack prepharynx and possess a median globular sphincter; the metacercariae of species of *Dolichorchis* also lack prepharynx, and possess a distinctly bi-segmented body (Travassos et al. 1969; Niewiadomska 2002).

The metacercariae of *Diplostomum* species morphologically resemble those of Diplostomidae gen. sp. herein characterized because both are distinctly bi-segmented and possess conspicuous pseudosuckers; however, the ratio between the anterior and posterior segments of Diplostomidae gen. sp. is larger than that observed in *Diplostomum* spp. In addition, vitelline follicles in *Diplostomum* spp. metacercariae extends from the middle to the end of the anterior segment (Travassos et al. 1969; Niewiadomska 2002), while in Diplostomidae gen. sp., follicles are restricted to the area between the posterior border of the pharynx and posterior border of the ventral sucker.

Discussion

Phylogenetic position of the two diplostomid species and taxonomic implications

This study generated four novel COI sequences of diplostomids, two belonging to *Tylodelphys* sp. and two belonging to Diplostomidae gen. sp. from Brazil. The phylogenetic analyses yielded identical topologies for BI and Ml analyses with five main clades, labeled as A–D (Fig. 1). The clade A is composed by *Hysteromorpha triloba* (for sequences obtained from birds in the Nearctic region, although the species was originally described from South America) (Lutz 1931) and *Neodiplostomum americanum* (Locke et al. 2011); clade B is formed by *Diplostomum* species distributed worldwide as a monophyletic lineage; clade C includes the newly generated sequences of Diplostomidae gen. sp., and *D. ardeae* which occurs in the Nearctic region. These relationships show that they form an independent genetic lineage for which a new genus needs to be erected, as previously suggested by Locke et al. (2015). The clade D is composed by *Tylodelphys* species distributed worldwide, the species from this study, and *Austrodiplostomum ostrowskiae* from USA (Table 1, Fig. 1).

The species from Brazil herein characterized belong into that new genus and seems to represent a new species, considering the high values of COI divergence levels (6.9–7.6%, see supplementary Table 1). The two species exhibit an allopatric distribution with "*Diplostomum*" *ardeae* occurring in Canada,

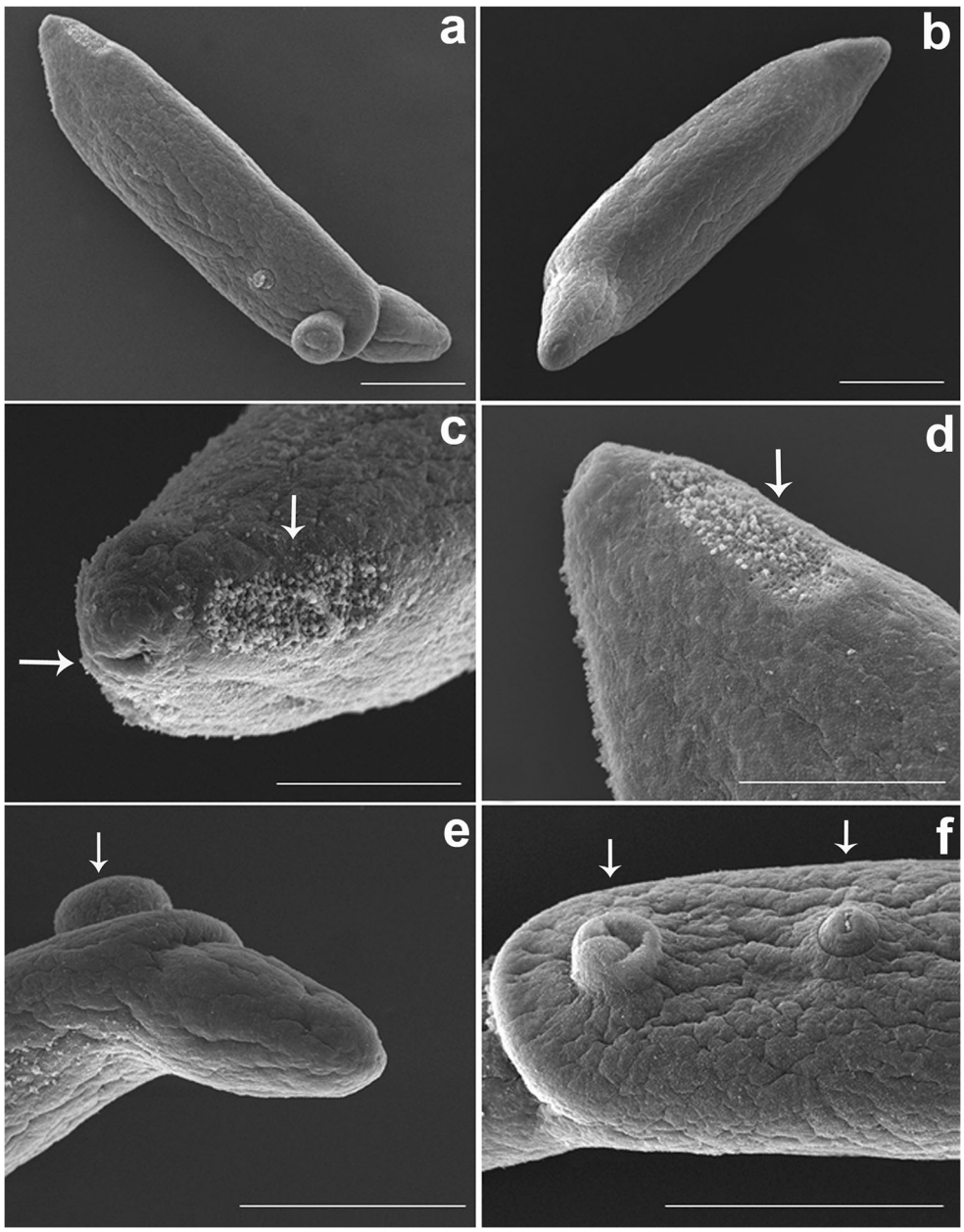

Fig. 4 Scanning electron microscopy (SEM) images of the metacercariae of Diplostomidae gen. sp. (Platyhelminthes, Digenea), parasite of crystalline lens of Siluriformes fish collected in the Batalha River, Tietê-Batalha basin, State of São Paulo, Brazil. **a** Ventral view. **b** Dorsal view. **c** Previous region with the opening of the oral sucker and the pseudosuckers. **d** Lateral pseudosuckers. **e** Lateral view of the posterior region with the holdfast organ above (ventral face), demonstrating that there is no separation of the forebody and hinbody. **f** Holdfast organ and ventral sucker (structures highlighted with white arrow) (**a**, **b**, **e**, **f** = 100 μm scale; **c**, **d** = 50 μm scale)

and the species herein characterized occurring in Brazil. This distribution pattern might be consistent with migration patterns of the bird definitive hosts. Other species of diplostomids are widely distributed across the Americas. *Austrodiplostomum compactum* (Lutz, 1928) (Dubois, 1970) *Hysteromorpha triloba* (Rudolphi, 1819) are distributed from North America southwards to Brazil, in South America (Ostrowski de Nuñez 2017; Sereno-Uribe et al. 2018, 2019). Likewise, this wide distribution pattern is also found in some other trematodes allocated in different families, but having birds as definitive hosts, e.g., the echinostomatid *Drepanocephalus spathans* (Dietz, 1909), and the clinostomid *Clinostomum heluans* Braun, 1899 (Hernández-Cruz et al. 2017; Briosio-Aguilar et al. 2018) (Table 1; Fig. 1).

The integrative taxonomy approach followed in our study allowed us to suggest that the metacercariae of *Tylodephys* sp. represent a separate species, although sampling adult forms from fish-eating birds will be necessary to test this hypothesis. Excepting for *T. jenynsie* for which COI sequences are available (Locke et al. 2015), at least other four valid species of *Tylodelphys* are found in South America (Fernandes et al. 2015). A molecular link must be established between the metacercariae that we found and the adults, to discriminate the possibility that the larval forms do not correspond with the adults of species validated on morphological grounds. In diplostomids, species can be clearly distinguished from each other by COI sequences (Locke et al. 2010).

Our results also provide support for the recognition of a new genus of Diplostomidae as previously suggested by Locke et al. (2015) through a phylogenetic analysis. It was suggested that *D. ardeae* could have been erroneously classified as a member of *Diplostomum*. Based on the current phylogenetic analysis, together with the morphological data, we can suggest that *D. ardeae* together with the unidentified Diplostomidae metacercariae characterized in the present work are part of a distinct genus not yet described. The importance of analyzing molecular data to confirm the morphological differentiation and to discriminate the diplostomid metacercariae found in fish is now well known (Locke et al. 2015; García-Varela et al. 2016a, 2016b; Blasco-Costa et al. 2017; Soldanova et al. 2017; Sereno-Uribe et al. 2018). Morphologically, the adults of *D. ardeae* originally described by Dubois (1969) from *A. herodias* in the USA, are very similar to the metacercariae of Diplostomidae gen. sp. that we characterize in this study even though the description of *D. ardeae* is based on adult individuals obtained in their definitive hosts (Dubois 1969). In particular, some characteristics such as foliate and linguiform body, pointing abruptly to the anterior extremity, two conspicuous pseudosuckers, long prepharynx, and oval pharynx, and the shape and location of the ventral sucker and holdfast organ are similar between *D. ardeae* and the metacercariae we characterize herein. However, the formal description of Diplostomidae gen. sp. of the present study is pending a further analysis of their life cycle that involves the finding of adult forms in the definitive hosts in the same geographic area, since the adult individual is morphologically unknown, and a link needs to be established between the two stages of the life cycle to corroborate conspecificity. This emphasizes the need for more taxonomic research on this trematode group. One possibility would be to obtain larvae and adults through experimental infections, completing all stages of the life cycle and allowing the morphological characterization of each phase to be integrated into a formal species description.

Host-specificity patterns

Diplostomidae metacercariae exhibit in many cases a low host specificity with respect to their second intermediate hosts. For instance, *Austrodiplostomum compactum* (Lutz, 1928) has been recorded in at least 38 fish species across Brazil (Vital et al. 2016). Low levels of host specificity have been associated with the habitat where these diplostomids ocurr, mainly in the lens and eye of their hosts. Locke et al. (2010) suggested that lower immune response in the lens might increase the possibilities of the metacercariae to develop, allowing species to infect a greater variety of hosts than those that inhabit other tissues. The lens capsule also forms an additional physical barrier to cell-mediated immune responses that are important in defense mechanisms against metacercariae and other helminths (Shariff et al. 1980; Kreider et al. 2007). Parasites that infect the lens or other organs are subject to different adaptive responses of the immune system (Karvonen et al. 2005), but the selection forces in tissues outside the lens are more striking than lens inside, since innate immune responses of the host will occur during the time of parasite migration through the host body (around 24 h). This implies that speciation rates are higher in species with metacercariae residing outside the host lens (Locke et al. 2015). Our study with the metacercariae of *Tylodelphys* sp. further illustrates a case of this type of specificity. This metacercaria only occur in the vitreous humor of the eyes and visceral cavity of the *H. malabaricus* analyzed. The same pattern was observed by Szidat (1969).

Regarding Diplostomidae gen. sp. in the present study, all the analyzed hosts species of the order Siluriformes were infected by these metacercariae, demonstrating this parasite might be specific at ordinal level of the host, but it is not host specific at species level. This low specificity results in the increase of available habitats for these parasites, as well as an increase in the possibilities of encounter and the number of routes for the definitive hosts (Locke et al. 2010, 2015).

In studies with Diplostomidae metacercariae where the interaction between molecular characterization and morphological variation evaluation occurred, it was observed that the number of morphospecies was higher than the number of genetically identified lines in their samples, demonstrating that

the identification of the species based only on morphological characters is not always sufficient for a specific baseline (Blasco-Costa et al. 2017). Furthermore, missing adult forms from their definitive hosts prevents a proper morphological description; currently, the genetic library contains mainly unidentified provisional species, many of which were distinguished primarily or only by DNA sequence analysis (see Locke et al. 2015). This is the reason of why an integrative taxonomy approach remains the basis to accomplish the proper species identification (Nadler and Pérez-Ponce de León 2011; García-Varela et al. 2016a). In the present study, we were able to conciliate both the morphological and molecular data, with the delimitation of only two morphotypes/species.

Our study and the paper by López-Hernández et al. (2018) are the first to use an integrative taxonomy approach to shed some light on the diversity of diplostomid trematodes in South America. However, more studies are needed to characterize molecularly the high diversity of this parasitic group of freshwater fishes across Brazil, and ideally, this should be coupled with finding adult forms in their definitive hosts and establishing a link between larval forms and adults.

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Compliance with ethical standards

The euthanasia methodologies of the host fish were carried out following the guidelines of the National Council of the Animal Experimentation Control (CONCEA), and the research project was submitted to the Ethical Committee on Animal Use (CEUA) of the Universidade do Sagrado Coração (USC) (authorization no. 3353050417).

Conflict of interest The authors declare that they have no conflict of interest.

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