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Original paper

First record of *Cotylurus* metacercariae (Trematoda: Strigeidae) in *Biomphalaria straminea* (Planorbidae) from Argentina: morphological and molecular identification

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ABSTRACT. In the study of the biology of trematode species, the knowledge of the larval stages in snail hosts is important to elucidate their complete life cycle. The goal of the present study was to describe a new tetracotyle-type metacercaria found in the freshwater mollusk *Biomphalaria straminea* sampled in a rice field from Corrientes province, Argentina. To this end, 1,768 snails were collected from the cultivated plots and irrigated channels during the flooding periods (from the time of sowing to soon after rice harvesting) between December 2016 and May 2017. We used morphological and molecular analysis to characterize the tetracotyle-type metacercariae. Its morphological traits and the internal transcribed spacers (ITS1 and ITS2 plus 5.8S; ~1200 pb) from nuclear ribosomal DNA (rDNA) were amplified and sequenced. From 1,768 specimens of *B. straminea* screened, 52 were found infected with metacercariae of tetracotyle type (2.9%) that were identified as *Cotylurus* genus. A total of 218 metacercariae were found encysted in the ovotestis or between the mantle and viscera of *B. straminea.* Bioinformatic analysis showed that the metacercarial rDNA sequences shared 94% identity with those of *Cotylurus gallinulae* from Mexico and 100% identity with those of *Cotylurus* sp. from Brazil. In this study, the morphological descriptions are supplemented with the first molecular identification of a metacercaria related to *Cotylurus* parasitizing planorbids from Argentina. Also, our study provides a new morphological description in *B. straminea*, thus broadening the geographical distribution. The life cycle of this *Cotylurus* metacercariae is unknown and there are no reports of adult stages parasitizing waterfowl in Argentina.

Keywords: larval trematodes, Digenea, *Cotylurus*, freshwater snails, agricultural area, phylogeny

Introduction

The life cycle of strigeids involves birds or mammals (definitive hosts) that feed on fish, frogs or snails (second intermediate hosts) [1]. The strigeid *Cotylurus* Szidat, 1928 show metacercariae of tetracotyle type in snails and mature exclusively in birds. This strigeid is mainly characterized by bipartite body consisting of a globular, subglobular or cup-shaped forebody, with lobed holdfast organ and hindbody [1].

Considering South and Central America, a species of the genus *Cotylurus* has been reported in Brazil, Venezuela and Mexico that parasitizes aquatic birds [2–4], in addition the life cycle of a species in Brazil has been described from *Biompalaria glabrata* naturally infected [5]. In Argentina no records of species of this genus in stages adults [6] but are reports of metacercariae in *B. peregrina* [7], *B. tenagophila* and *B. orbignyi* [8].

In the complex life cycle of trematodes, the mollusks represent a "key piece" since they serve as first intermediate hosts in most of the described species [9]. The freshwater snails of genus *Biomphalaria* Preston, 1910, intermediate hosts in the life cycle of some species of *Cotylurus*, inhabit

a wide range of environments; especially isolated, ephemeral, and lentic water bodies from America and Africa [10–12]. Specifically, *Biomphalaria straminea* (Dunker, 1848) is found in diverse environments but prefers shallow, temporary and standing or slow-flowing fresh water bodies, and thus rice fields provide favorable conditions for the development of dense populations of this planorbid snail [13]. In Argentina, this species is restricted to the Northeast and Pampean regions, being extremely frequent in the Río de La Plata basin, namely in the Paraná and Uruguay Rivers [14,15]. In the Northeast region, *B. straminea* has been reported as an intermediate host of 15 species of cercariae, most of them in agricultural environments [16]. However, metacercariae of the family Strigeidae Raillet, 1919 in snails of this region have not yet been studied. Likewise, previous reports on metacercariae of the genus *Cotylurus* in planorbids from Argentina did not include molecular data [8] and morphological characterization of excysted metacercariae [17].

In this context, we aimed to describe morphologically and molecularly a tetracotyle-type metacercaria of *Cotylurus* genus found in the freshwater planorbid *B. straminea* from a rice field of Corrientes, Argentina.

Materials and Methods

Study area and sampling procedure

The study site was an agricultural area with cultivated rice plots connected or associated with the Paraná River basin, located at approximately 85 km South of Corrientes city, in Corrientes province, Argentina (27°40'N 58°48'W). Snails (n=1768) were collected manually from the edge of the cultivated plots and irrigated channels during the flooding periods, from the time of sowing to soon after the harvest, between December 2016 and May 2017. The collection of snails was performed by two people in the morning for 1.5 hours, using simple mesh nets, locally named "copos" (25 cm frame diameter). The studied channels (n=2) were approximately 2 m wide and 250 m long. Snails were transported to the laboratory and dissected to search for metacercariae infections.

Morphological analyses

Cysts were isolated from the host, counted, and its diameter measured using an ocular micrometer. Metacercariae were studied alive and drawings were made using a Leica microscope DM 2500 with the aid of a camera lucida (Leica Microsystems, Germany). Measurements given in the description of metacercariae are based on heat-killed, formalin-fixed specimens and expressed in micrometers (μm), with the range followed by the mean ± SD in parentheses. Metacercariae fixed in hot 4% formalin were preserved in vials with 70% ethanol and deposited at the Helminthological Collection of the Centro de Ecología Aplicada del Litoral (CECOAL), Corrientes, Argentina.

Photographs were taken with a Leica DFC 295 (Leica Microsystems, Germany) camera mounted on a Leica DM2500 microscope. Specimens to be studied by scanning electron microscopy (SEM) were dehydrated in an ethanol series, dried using the critical point technique, coated with gold-palladium, and examined under a Jeol 5800 LV Scanning Electron Microscope (Joel, Tokio, Japon). The overall prevalence was calculated following [18].

Molecular analysis

DNA extraction followed the protocol of PureLink genomic DNA mini kit (ThermoFisher, Carlsbad, CA, USA). The internal transcribed spacers ITS1 and ITS2 plus 5.8S region from nuclear ribosomal DNA (~1200 pb) were amplified by PCR, using the same primers (BD1 5'-GTCGTAACAAGGTTTCCGTA-3′ and BD2 5'-ATCTAGACCGGACTA GGCTGTG-3') and conditions published by [4]. Each PCR reaction contained 1× buffer, 2.5 mM total $MgCl_2$, 0.2 mM each dNTPs, 10 μM each primer, Q solution 1X, 0.02 U/μl Qiagen Taq DNA polymerase enzyme (Qiagen, Hilden, Germany), and 15 ng of DNA. The mix was placed in a Takara thermocycler (Takara-Bio, Shiga, Japan) under the following conditions: initial denaturation at 94ºC for 1 min, and subsequently 35 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and extension at 72ºC for 1.5 min, and a final extension phase at 72ºC for 10 min. The PCR products were visualized in 1% agarose gels stained with ethidium bromide, then precipitated with 70% ethanol and quantified using a Spectrophotometer NanoVue Plus (Biochrom, a division of Harvard Bioscience, Inc., USA). The amplicons were sequenced using a BigDye terminator cycle sequencing kit, version 1.1 (Applied Biosystems, Foster City, CA), purified by FastGene Dye Terminator Removal kit (Nippon Genetics Co., Ltd, Tokyo, Japan) and analysed on

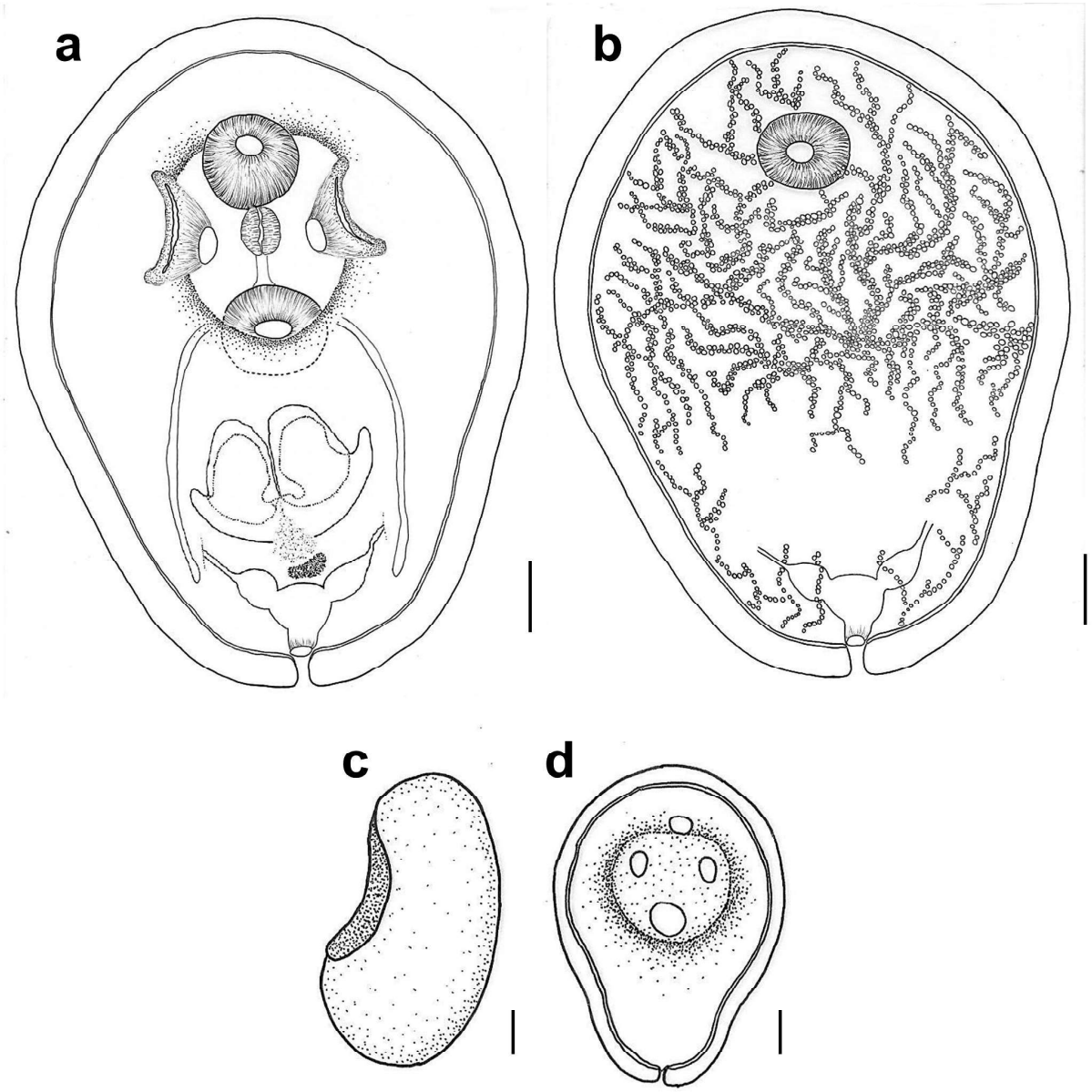

Figure 1. Metacercaria of *Cotylurus* sp. (a) larva encysted, ventral view; (b) larva encysted showing calcareous concretions of excretory vessels, ventral view; (c) cyst, lateral view; (d) cyst, ventral view. Scale bars= 50 μm

an Applied Biosystems Hitachi 3130 Genetic Analyzer automated sequencer, employing the primers BD1 and BD2. The sequences obtained were assembled and edited on Chromas Lite V 2.6.5 (https://technelysium.com.au/wp/chromas/). Data were analysed on MEGA X [19]. A Blast-n was carried out in GenBank for comparison with available sequences from other species. Sequences related to *Cotylurus* sp. from Hernández-Mena et al. [4] were downloaded from GenBank, aligned and analysed with a nucleotide substitution model. The Maximum Likelihood method was used to obtain a phylogenetic tree with 1000 bootstrap replications. Sequences have been deposited on GenBank (MT276343).

Results

From 1,768 specimens of *Biomphalaria straminea* screened, 52 were found infected with metacercariae of tetracotyle type (2.9%). A total of 218 metacercariae were found encysted in ovotestis or between the mantle and viscera.

***Cotylurus* sp. (Figs. 1, 2)**

Specimen deposited: accession number CECOAL (17032920)

Cyst (measurements based on ten cysts): tetracotyle cyst oval to pear-shaped, 340–470 (391.0±41.2) long by 210–350 (299.0±39.8) wide, cyst wall composed by two layers: an outer hyaline non-cellular layer, 15–30 (20.7±4.5) thick, and a tough inner layer, 3–6 (4.1±1.2) thick. Larvae not easily released; once layers were ruptured they adhered to the cuticle of the parasite and cannot be removed without injuring them. Ventral surface with four external openings in the widest portion of the body: the top opening leading directly to the oral sucker, the side openings leading to the pseudosuckers, and the lower opening leading anteriorly to the ventral sucker and immediately behind to the holdfast organ. There is an aperture in the cyst adjacent to the excretory pore; refractive granules of the excretory bladder may be forced out through this canal.

Metacercaria (measurements based on ten encysted metacercariae): larva tetracotyliform, 370–415 (395.0±21.6) in total length. Forebody and hind body difficult to differentiate. No spines on tegument. Subterminal oral sucker, 45–70 (57.9±8.8)×55–75 (65.4±7.0); glandulo-muscular pseudosuckers on the posterolateral edges of the oral sucker, fan-shaped slits extending dorsolaterally from their relatively small openings on the ventral surface. Muscular pharynx, 30–40 (35.0±7.1)×28–33 (30.5± 3.5); pharynx to oral sucker length ratio 1:1.1–2.3. Esophagus very short; ceca extending laterally to almost the posterior end of the body. Ventral sucker, 40–55 (48.0±5.1)×50–67 (55.9±8.0). Suckers' width ratio 1:0.9–1.5. Holdfast organ lobes immediately behind ventral sucker, not projecting from opening, a pair of lobes distinguishable on the anterior face of the holdfast organ, and an additional pair situated on the posterior face. Proteolytic gland difficult to see. A small single mass of genital primordia is present in the posterior portion of the body. Complex excretory vessels with small spherical calcareous concretions throughout the body, mostly in the forebody.

Remarks

The metacercariae of *Cotylurus flabelliformis* and *Cotylurus* sp. both have a cyst with a hyaline wall, external openings of the oral and lateral suckers and the cavity of the holdfast organ in the

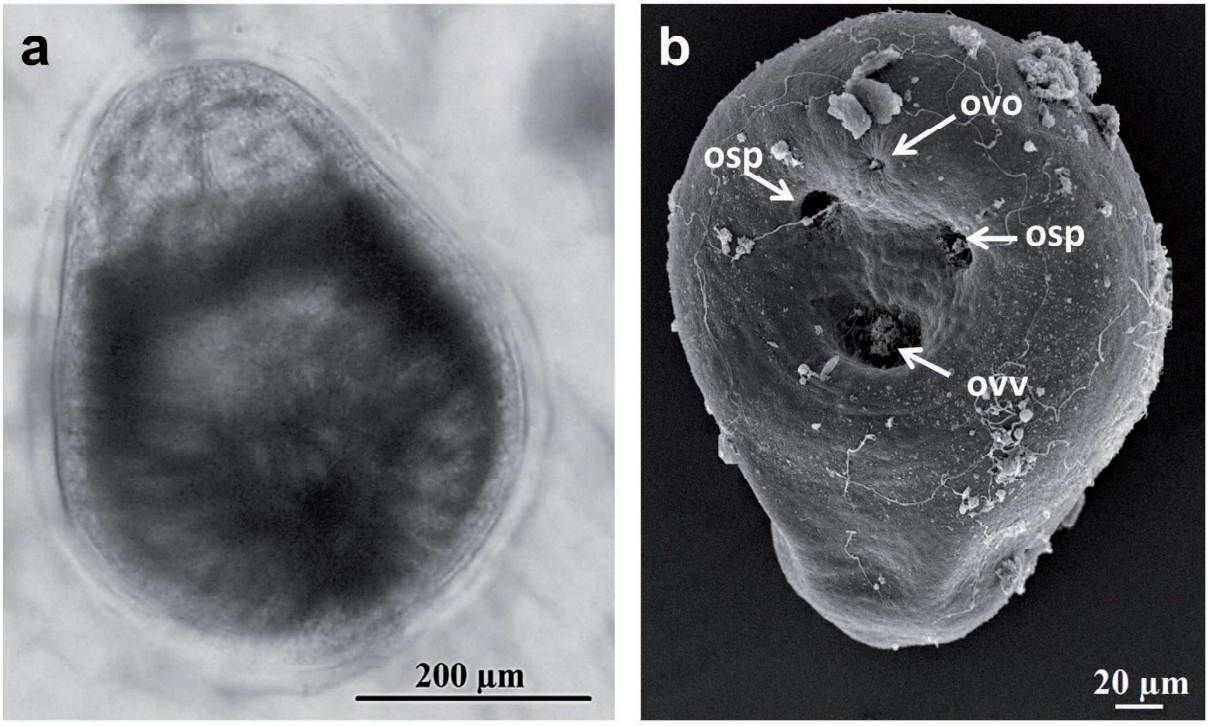

Figure 2. Light micrographs and scanning electron micrograph of *Cotylurus* sp. metacercaria. (a) cyst, note the calcareous concretions of excretory vessels throughout the body; (b) cyst, ventral view. Abbreviations: ovo, opening of oral sucker; ovv, opening of ventral sucker; ops, opening of pseudosuckers

Table 1. The list of taxa used in this study, definitive host species, collection locality of material, and GenBank accession numbers

Classification/Species	GenBank accession number	Host species (Order)	Locality, country	Reference
Diplostomida, Clinostomidae				
Clinostomum marginatum (bio-material DNA2075OOAX)[a]	KJ477679	*Ardea alba* (Pelecaniformes)	Middle-America	20
Diplostomida, Diplostomidae				
Tylodelphys aztecae (isolate DNA1902)	KT175371	*Goodea atripinnis* (Cyprinodontiformes)	Tlahuac, Southern Mexico City	21
Tylodelphys cerebralis (voucher HS-TR/2016/04)	KX817188	*Channa punctata* (Anabantiformes)	Meerut (U.P.), India	22
Tylodelphys clavata (isolate CL91)	JQ665459	*Coregonus lavaretus* (Salmonidae)	Lake Constance, Germany	23
Tylodelphys immer (voucher T.RH.Sf.RBI.5.2)	KT186805	*Coregonus clupeaformis* (Salmonidae) *Notropis hudsonius* (Cypriniformes) *Perca flavescens* (Perciformes) *Salvelinus fontinalis* (Salmoniformes) *Gavia immer* (Gaviiformes)	Quebec, Canada	24
Tylodelphys mashonensis (voucher LkV_E2Dm1.1)	KR863384	*Clarias gariepinus* (Siluriformes)	Lake Victoria, Tanzania	25
Tylodelphys scheuringi (isolate T.LH.S.R.1.1)	FJ469596	*Ambloplites rupestris* (Perciformes)*	Nova Scotia, Canada	26
Diplostomida, Strigeidae (Tribe: Cotylurini)				
Cardiocephaloides medioconiger (isolate DNA593)	JX977842	*Larus* sp. (Charadriiformes)	Campeche, Mexico	4
Cardiocephaloides medioconiger (isolate DNA594)	JX977843	*Larus* sp. (Charadriiformes)	Campeche, Mexico	4
Cardiocephaloides sp. (MGV-2013 isolate DNA181)	JX977844	*Larus occidentalis* (Charadriiformes)	Baja California Sur, Mexico	4
Cotylurus gallinulae (isolate DNA596)	JX977841	*Aythya affinis* (Anseriformes)	Sonora, Mexico	4
Cotylurus marcogliesei (isolate S.IN.Lc.MTL.2.5)	MH521248	*Lophodytes cucullatus* (Anseriformes)	Quebec, Canada	27
Cotylurus sp. (HAP-BH-Cidade Administrativa-2)	MN179272	*Biomphalaria straminea* (Basommatophora)	Minas Gerais, Brazil	17
Cotylurus sp. (HAP-BH-Cidade Administrativa-1)	MN179271	*Biomphalaria straminea* (Basommatophora)	Minas Gerais, Brazil	17
Cotylurus sp. (CECOAL)	MT276343	*Biomphalaria straminea* (Basommatophora)	Corrientes, Argentina	[b]
Cotylurus syrius (voucher 3LF-2712)	MF628093	*Cygnus olor* (Anseriformes)	Czech Republic	28
Cotylurus syrius (voucher 3LF-3907)	MF628099	*Cygnus olor* (Anseriformes)	Czech Republic	28
Cotylurus syrius (voucher 3LF-2710)	MF628091	*Cygnus olor* (Anseriformes)	Czech Republic	28
Ichthyocotylurus pileatus	AJ301886	*Perca fluviatilis* (Perciformes)	Finland	29
Diplostomida, Strigeidae (Tribe: Strigeini)				
Australapatemon burti (isolate DNA138)	JX977787	*Anas diazi* (Anseriformes)	Estado de México	4
Australapatemon burti (isolate DNA180)	JX977785	*Anas americana* (Anseriformes)	Baja California Sur, Mexico	4
Australapatemon burti (isolate DNA182)	JX977786	*Anas cyanoptera* (Anseriformes)	Estado de México	4
Apharyngostrigea comu (isolate DNA568)	JX977839	*Nyctycorax nyctycorax* (Pelecaniformes)	Sinaloa, Mexico	4
Apharyngostrigea comu (isolate DNA1006)	JX977840	*Nyctanassa violacea* (Pelecaniformes)	Veracruz, Mexico	4
Apharyngostrigea comu (isolate DNA1008)	JX977838	*Butoroides virescens* (Pelecaniformes)	Veracruz, Mexico	4
Parastrigea cincta (isolate DNA445)	JX977820	*Eudocimus albus* (Pelecaniformes)	Nayarit, Mexico	4
Parastrigea cincta (isolate DNA706)	JX977816	*Eudocimus albus* (Pelecaniformes)	Sinaloa, Mexico	4
Parastrigea cincta (isolate DNA707)	JX977818	*Eudocimus albus* (Pelecaniformes)	Sinaloa, Mexico	4
Parastrigea diovadena (isolate DNA443)	JX977803	*Eudocimus albus* (Pelecaniformes)	Tamaulipas, Mexico	4
Parastrigea diovadena (isolate DNA926)	JX977811	*Eudocimus albus* (Pelecaniformes)	Veracruz, Mexico	4
Parastrigea diovadena (isolate DNA802)	JX977798	*Eudocimus albus* (Pelecaniformes)	Veracruz, Mexico	4
Parastrigea plataleae n. sp. (isolate DNA858)	JX977826	*Platalea ajaja* (Pelecaniformes)	Guerrero, Mexico	4
Parastrigea plataleae n. sp. (isolate DNA859)	JX977825	*Platalea ajaja* (Pelecaniformes)	Guerrero, Mexico	4
Parastrigea plataleae n. sp. (isolate DNA861)	JX977834	*Platalea ajaja* (Pelecaniformes)	Oaxaca, Mexico	4
Parastrigea plataleae n. sp. (isolate DNA1041)	JX977836	*Platalea ajaja* (Pelecaniformes)	Sinaloa, Mexico	4

* Lab host

[a] Outgroup

[b] This study

cyst, an opening in the cyst wall adjacent to the excretory pore through which refractive granules of the reserve bladder come out, and scattered clumps of refractile granules throughout the body. However, *C. flabelliformis* has a composed cyst which extends laterally to the almost posterior end of the body of a single wall and the body with spinose cuticle. Both *Cotylurus brevis* and *Cotylurus* sp. have a cyst wall with two layers (an outer gelatinous hyaline layer and a tough opaque inner layer) and an opening in the cyst wall adjacent to the excretory pore through which refractive granules of the reserve bladder come out. However, *C. brevis* differs in having a larger cyst (446–540/400–500 vs. 340–470/ 210–350) and a wider outer layer (39–48 vs. 15–30); further, the cyst of *Cotylurus* sp. has a perforated anterior end. Finally, *Cotylurus lutzi* and *Cotylurus* sp. either encyst in the ovotestis of the snail host or remain free in its mantle, have a cyst with oval to pear-shaped, body without spines, no forebody and differentiated hind body, ventral surface leading to four openings arranged in the form of a cross in the widest portion of the body, which lead to the oral sucker, pseudosuckers and ventral sucker and holdfast organ respectively, pseudosuckers with fan-shaped slits, and similar number and arrangement of lobes of holdfast organ (one pair anterior face and one pair posterior face of holdfast organ). In contrast to *Cotylurus* sp., *C. lutzi* has a delicate and thinner hyaline cyst wall (3 vs. 15–30) without layers and openings at its end, smaller body length (220–240 vs. 370–415), and

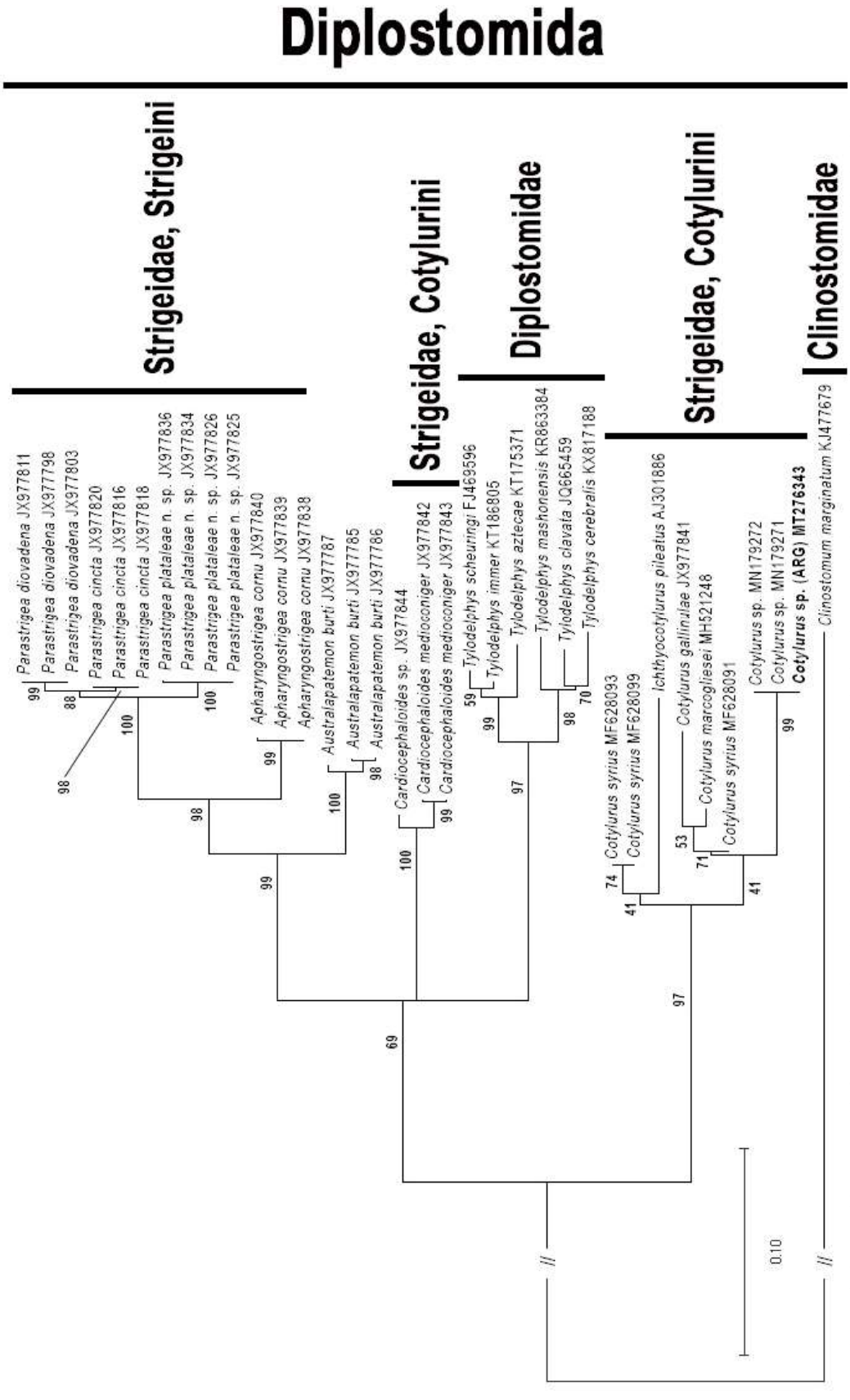

Figure 3. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model [30]. The tree with the highest log likelihood (-7484.04) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (Bootrstap values – 1000 replications). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.0894)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 21.68% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 35 nucleotide sequences. There were a total of 1365 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [13]

smaller diameter of oral sucker (30 vs. 55–75) and ventral sucker (32 vs. 50–67).

In Brazil, López-Hernández et al. [17] found metacercariae of *Cotylurus* sp. in *B. straminea*, cyst have a similar size (279–329/237–277 vs. 340–470/210–350) and a similar outer layer (17–25 vs. 15–30). The morphological characterization of excysted metacercariae were not examined.

In Salta, Argentina, Davies [8] described the metacercariae of *Cotylurus* sp. from *Biomphalaria orbignyi* and *B. tenagophila*. These are similar to the metacercaria described here in having a pear-shaped cyst, without differentiated forebody and hind body, pseudosuckers in the same location (latero-posterior to the oral sucker), and similar size of the oral sucker (59×64 vs. 57.9×65.4). However, they have a smaller cyst (285–354/216–275 in *B. orbignyi* and 246–304/233–236 in *B. tenagophila* vs. 340–470/210–350), a larger ventral sucker (64×69 vs. 48.0×55.9), and one pair of lobes in the holdfast organ.

Molecular analysis

The nuclear rDNA sequences of the analyzed specimens were aligned with others obtained by blast in GenBank and from other publications (Tab. 1). A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 1.0894)). The variation-rate model allowed for some sites to be evolutionarily invariable ([+I], 21.68% sites). The phylogenetic tree obtained with the maximum likelihood method supports the morphological identification of *Cotylurus* sp. and grouped the studied specimen close to *Cotylurus gallinulae* in the Cotylurini family branch. It is observed the paraphyletic state of the Strigeidae branch, this might be due to the difference in the length of the different sequences. When only the ITS2 region was considered in the analysis, this situation was resolved and the group is shown as monophyletic (data not shown). The sequences from the tetracotyle-type cercaria from Corrientes showed a 100% identity with sequences from *Cotylurus* sp. from Brazil [17] and 94% identity with sequences from *C. gallinulae* from Mexico [4]. Altogether, the 6% divergence between their sequences and the high bootstrap value of the branch (99%) suggests that the specimens found in Corrientes and Brazil belong to a different species (Fig. 3).

Discussion

The larva studied here is morphologically similar to the metacercariae of the genus *Cotylurus* that are tetracotyle-type and encyst in snails and leeches [1] Three species of this genus have cercariae that encyst in mollusks: *C. flabelliformis*, *C. brevis*, and *C. lutzi* [31], all with comparable characteristics to the *Cotylurus* sp. described in this study. On the other hand, in South and Central America, reports about the genus *Cotylurus* include descriptions of the life cycle of *C. lutzi* in Brazil [5], adult stages of *Cotylurus gallinulae gallinulae* (Lutz, 1928) parasitizing *Gallinula chloropus* (Linnaeus, 1758) in Brazil and Venezuela, and *Aythya affinis* Eyton, 1838 in Mexico [2–4]. To date, no reports of adult stages of *Cotylurus* species parasitizing aquatic birds have been published in Argentina. The available information describes metacercariae parasitizing *B. straminea* in Brazil [17], *B. peregrina* in the Patagonian region [7], *B. tenagophila* and *B. orbignyi* in Salta province [8], only the latter including a description of the larva. However, it is known that adult stages of *Cotylurus* spp. parasitize aquatic birds from the families Anatidae, Charadriidae, and Scolopacidae in the Holarctic, Neotropical, and Oriental regions [1]. According to Lesterhuis [32], these three families comprise the largest number of species of aquatic birds that live in rice fields in Northeastern Argentina. Thus, many of these bird species might act as definitive hosts in the life cycle of the trematodes whose larvae we described here.

Our results of morphological and molecular analyses confirm that the metacercariae found in Corrientes, Argentina belong to the *Cotylurus* genus. Besides, the result of ITS sequence revealed a strong association with *Cotylurus* metacercariae analyzed in Brazil [17]. They sharing the molecular morphology and we are in the same geographic basin, this also adds to the evidence. Future incorporation of molecular studies of the partial COI region of adult stages will be sequenced to corroborate the taxonomy of metacercariae in our region. Moreover, the 6% divergence found when comparing its rDNA sequences with those of *C. gallinulae* from Mexico suggests that *Cotylurus* of Corrientes belongs to a different species.

Regarding the metacercariae infection, the 2.9% prevalence of *Cotylurus* sp. in Corrientes is closer to that detected in Salta (1.41% in *B. tenagophila* and 2.12% in *B. orbignyi*), whereas the prevalence of

Cotylurus sp. from the Patagonian region (23–27.7%) and Brazil (38%) are higher [7,8,17]. The fact that the metacercariae were found parasitizing *B. straminea* represents the first report of these snails acting as intermediate hosts of *Cotylurus* genus in Argentina.

In conclusion, we have provided the first molecular analysis of *Cotylurus* metacercariae in Argentina, which will help in the identification of the different life stages. Also, a new morphological description of this tetracotyle metacercariae that infects new host snails.

Acknowledgements

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