An Acad Bras Cienc (2022) 94(1): e20200538 DOI 10.1590/0001-3765202220200538
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ECOSYSTEMS

A new species of *Auriculostoma* (Digenea: Allocreadiidae) in South America: life cycle and phylogenetic relationships

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Abstract: *Auriculostoma* is a genus of digenean (Trematoda) whose adults are parasites of Neotropical freshwater fishes. We describe *Auriculostoma ocloya* n. sp. using morphological and molecular tools, and we elucidated its life cycle, the first known of a species of this genus. The first intermediate host is the bivalve *Pisidium ocloya*, the second intermediate host is the amphipod *Hyalella* sp., and the definite hosts are siluriform fishes. The adult presents a single pair of muscular lobes on either side of the oral sucker with a broad base, stretching from ventrolateral to dorsolateral side, a structure also present in the rest of species of the genus. Nevertheless, the new species differs from all congeners by the combination of several traits, and mainly because on the dorsal side "free" ends of the lobes are absent because they are fused. This is the first study to provide sequence data on larval and adult stages of a species of *Auriculostoma*. Our phylogenetic analysis demonstrated its basal position among species of the genus. Therefore, integrative morphological, molecular, and life cycle data on other South American species of the genus, would contribute to reveal more patterns in the allocreadiid systematics.

Key words: integrative taxonomy approach, Trematoda, parasite, fish, 28S, ITS2 markers.

INTRODUCTION

Scholz et al. (2004) erected the genus *Auriculostoma* to include a new species, *Auriculostoma astyanace*, found in Nicaragua (Central America) and transferred three species formerly described in the genus *Crepidostomum* from South America, *C. platense* Szidat, 1954, *C. macrorchis* Szidat, 1954, and *C. stenopteri* Mañé-Garzón and Gascón, 1973 to the new genus as new combinations. The diagnosis of the genus included the presence of two pairs of muscular oral lobes (one ventrolateral and one prominent dorsolateral). Razo-Mendivil et al. (2014), after studying the oral sucker through the scanning electron microscope (SEM), amended the diagnosis establishing that all species of the genus have a single pair of lobes in the oral sucker.

At present, nine species have been described in this genus (Table I). Four species of *Auriculostoma* from Central America have been described, using an integrative taxonomy approach (i.e., applying morphological and molecular tools); or well were described morphologically and subsequently the sequences of the fragment of ITS2 and 28S from ribosomal RNA (rRNA) (Table I) were obtained, whereas five South American species were described only based on morphological characters.

The life cycle of any of the species of the genus is not yet known. Species belonging to the other related genera (e.g., *Bunodera luciopercae* (Müller, 1776) Lühe, 1909, and *Allocreadium fasciatusi* Kakaji, 1969 use mainly clams of the family Sphaeriidae as first intermediate host and fishes as definitive hosts (Cannon 1971, Madhavi 1978).

Although new helminth species are described annually at an increasing rate, the parallel effort to elucidate life cycles has become disproportionately smaller over time (Blasco-Costa & Poulin 2017). These authors propose further attempts to genetically match adult and juvenile helminth stages in regional faunas, as part of a plea to parasitologists to bring parasite life cycle studies back into mainstream research. A complete characterization of species should include description of all the life stages as an avenue to understand the evolution of the parasites and the comprehension of the way they live in the ecological communities. Therefore, the aim of this work is to describe *Auriculostoma ocloya* n. sp. and elucidate its life cycle in Southern Andes Yungas Forests, Argentina, based on morphological and molecular data.

The ZooBank Life Science Identifier (LSID) of this publication is: urn:lsid:zoobank.org:pub:9541FDBD-F7CC-4476-A667-666139A5E6C5

MATERIALS AND METHODS

Collections and treatment

Samplings were performed in a creek affluent to the Lesser River, Salta, Argentina (24°39'44''S; 65°28'49.07''W), located in Southern Andes Yungas Forests, South America, since October 2017 to March 2018. The Southern Andes Yungas Forests includes mountain slopes between 700 and 1500 m of altitude. It is characterized by a very dense and almost impenetrable mass of vegetation thus is a wet and gloomy environment (Cabrera 1976, Brown et al. 2002). Given the rainfall regime occurs mainly in summer, the volume of rivers fluctuates a lot throughout the year. The creek sampled has a temporary regime with slow water flow, abundant margin vegetation, and clear water, with a high abundance of little catfish (Monasterio de Gonzo 2003).

Individuals of the following animals were collected: fishes of the species *Heptapterus qenqo* Aguilera, Mirande and Azpelicueta, 2010 (Siluriformes, Heptapteridae) (n = 45), *Corydoras micracanthus* Regan, 1912 (Siluriformes, Callichthyidae) (n = 22), and *Psalidodon endy* (Mirande, Aguilera and Azpelicueta, 2006) (Characiformes, Characidae) (n = 37) by using a hand net fishing; clams *Pisidium ocloya* Ituarte, 2005 (Bivalvia, Sphaeriidae) by using a hand net (mesh size of 1 mm) (n = 40), crustaceans *Hyalella* Smith, 1874 (Amphipoda, Hyalellidae) (n = 32), and caddisfly larvae (Trichoptera, Polycentropodidae) (n = 50) by a hand net (mesh size of 2 mm). All specimens were carried alive to the laboratory for a helminthological examination. Fish were dissected after capture, previous anesthesia by exposing a few minutes on ice. The intestines were removed, opened, and examined for adult trematodes under a stereomicroscope. The bivalves were individually placed in 5 ml jars and exposed to light to stimulate cercaria shedding and then dissected under a stereomicroscope to search the intramolluscan stages. Caddisfly larvae and amphipods were dissected under a stereomicroscope to search metacercariae.

All procedures related to the sampling and manipulation of animals were approved by the Secretaría de Medio Ambiente, Ministerio de Ambiente y Producción Sustentable, Gobierno de la provincia de Salta, Argentina (Authorization No. 00340/16) and were according to all the regulations and ethical and legal considerations for the capture and use of animals established by the National Council of Scientific Research and Technical of Argentina.

Table I. Species of *Auriculostoma* associated with their hosts and geographic origin (new species is in bold); 28S and ITS2 sequences are recorded for *Auriculostoma ocloya* n. sp.

Digenean species	Type host (Order)	Geographic origin	GenBank accession numbers	Reference
Auriculostoma macrorchis (Szidat, 1954) Scholz, Aguirre-Macedo and Choudhury, 2004	*Pachyurus bonariensis* (Perciformes)	Argentina (South America)	-	Szidat (1954), Scholz et al. (2004)
Auriculostoma platense (Szidat, 1954) Scholz, Aguirre-Macedo and Choudhury, 2004	*Iheringichthys labrosus* (Siluriformes) *Pimelodus maculatus* (Siluriformes) *Rhinodoras dorbignyi* (Siluriformes) (type host not assigned)	Argentina (South America) Argentina (South America) Argentina (South America)	-	Szidat (1954), Scholz et al. (2004)
Auriculostoma stenopteri (Mañé-Garzón and Gascón, 1973) Scholz, Aguirre-Macedo and Choudhury, 2004	*Asyphonichthys stenopterus* (Characiformes)	Uruguay (South America)	-	Mañé-Garzón & Gascón (1973), Scholz et al. (2004)
Auriculostoma astyanace Scholz, Aguirre-Macedo and Choudhury, 2004	*Astyanax aeneus* (Characiformes) *Astyanax fasciatus* (Characiformes)	Nicaragua (Central America) Costa Rica (Central America)	HQ833707 KF631422	Curran et al. (2011) Razo-Mendivil et al. (2014)
Auriculostoma diagonale Curran, Tkach and Overstreet 2011	*Stethaprion cf. erythrops* (Characiformes)	Peru (South America)	-	Curran et al. (2011)
Auriculostoma foliaceum Curran, Tkach and Overstreet, 2011	*Bryconops cf. caudomaculatus* (Characiformes)	Peru (South America)	-	Curran et al. (2011)
Auriculostoma totonacapensis Razo-Mendivil, Mendoza-Garfias, Pérez-Ponce de León and Rubio-Godoy, 2014	*Astyanax mexicanus* (Characiformes)	Mexico (Central America)	KF631417-KF631418	Razo-Mendivil et al. (2014)
Auriculostoma lobata Hernández-Mena, Lynggaard, Mendoza-Garfias and Pérez-Ponce de León, 2016	*Brycon guatemalensis* (Characiformes)	Mexico (Central America)	KX954170-KX954174	Hernández-Mena et al. (2016)

Table I. Continuation.

Auriculostoma tica Hernández-Mena, Pinacho-Pinacho, García-Varela, Mendoza-Garfias and Pérez-Ponce de León, 2018	*Gymnotus maculosus* (Gymnotiformes)	Costa Rica (Central America)	MH997001-MH997002	Hernández-Mena et al. (2018)
***Auriculostoma ocloya* n. sp**.	*Heptapterus qenqo* (Siluriformes) *Corydoras micracanthus* (Siluriformes) *Hyalella* sp. (2º IH) *Pisidium ocloya* (1º IH)	Argentina (South America)	MT140284 (ITS2) - MT140287-88(28S) MT140289-90 MT140286 MT140285	**Present study**

IH: intermediate host.

Morphological analysis

All stages were studied *in vivo* using both neutral red and Nile blue stained and unstained specimens. Adults and larvae were killed with hot saline solution and immediately fixed with 10% hot formalin, and then preserved in 70% ethanol; some other specimens were fixed with 100% ethanol to molecular study. Formalin fixed specimens were stained with hydrochloric carmine, dehydrated in an ethanol series, clarified with metylsalicilate, and mounted in Canada balsam to permanent slides. Drawings were made with the aid of a drawing tube attached to a microscope Leica DM 2500; measurements are presented in micrometers (µm) with the minimum and maximum followed by the mean in parentheses. Measurements were taken from whole stained specimens, 10 adults, 10 young rediae (small size, with a prominent pharynx and undifferentiated germ balls), 5 mature rediae (large size, with an inconspicuous pharynx and cercariae differentiated), 10 emerged cercariae, and 5 metacercariae. Measurements of one egg of each adult specimens were obtained, i.e., 10 eggs. Some formalin fixed adult specimens were dehydrated using a graded series of ethanol, critical point dried, and gold coated for observation and microphotograph using a Zeiss Supra 55VP SEM. Specimens of adults, metacercariae, and rediae with cercariae were deposited in the Parasitological Collection of the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia', Buenos Aires, Argentina (MACN-Pa) and in the Parasitological Collection of the Instituto de Biología de Organismos Marinos (IBIOMAR, CCT CONICET-CENPAT), Puerto Madryn, Argentina (CNP-Par).

DNA extraction, PCR amplification and sequencing

DNA was extracted using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma) according to the manufacturer´s instructions. ITS2 and 28S rRNA gene were amplified by Polymerase chain reaction (PCR). The PCRs were performed in a total volume of 50 µl containing 10X buffer (200 mM Tris-HCl pH 8.4 500 mM KCl), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 1 U of Taq polymerase, and 5 µl of genomic DNA. The ITS2 regions were

amplified using as forward primer 5.8S-ITS2: 5'-GCTCGTGTGTCGATGAAGAG-3', situated 114 bp from the 3' end of the 5.8S gene, and as reverse primer 28S-ITS2: 5'-AGGCTTCGGTGCTGGGCT-3', located 34 bp from the 5' end of the conserved region of the 28S rRNA gene. The 28S regions were amplified using as forward primer 28S-28S: 5' GTGAATACCCGCTGAACTTAAGC- 3', situated 16 bp from the 3' end of the conserved region of the 28S rRNA gene, and as reverse primer 28S–28S: 5'-TCTCCTTGGTCCGTGTTTCAA-3', located 868 bp from the 5' end of the conserved region of the DNA. PCR conditions consisted of initial denaturalization at 94 °C for 4 min followed by 30 amplification cycles comprising a denaturalization step of 1 min at 94 °C, annealing at 52 °C (ITS2) and 56 °C (28S rRNA gene) for 1 min, and extension at 72 °C for 1:30 min. Reactions were completed with 4 min at 72 °C followed by cooling to 4 °C. Amplification reactions were carried out in an automated thermal cycler (Applied Biosystems™ 5700 Real-Time PCR Systems). PCR products were fractionated with 1.0% (w/v) agarose gel electrophoresis, stained with Syber safe and visualized with an Image Analyzer Gel. The PCR product genes were sequenced with an ABI Prism 3100 Genetic Analyzer System following the manufacturer's recommendations. The sequences were submitted to the NCBI databases (Table I) and BLAST program was used to compare with closely related sequences. Complete sequences of ITS2 and 28S were aligned using the multiple alignments of DNA-MAN, version 4.03 program. The ITS2 sequences of adults, metacercariae and rediae were compared to elucidate the life cycle.

Phylogenetic analysis

Sequences of the 28S rRNA gene of the new species (2 adults from *Heptapterus qenqo*, 2 adults from *Corydoras micracanthus*) were aligned, using ClustalW implemented by MEGA 7.0. program (Kumar et al. 2016), with those of the congeneric species and sequences of other species of allocreadiids and callodistomids (used as outgroup) recently analyzed by Hernández-Mena et al. (2018). Additionally, sequences of other species of allocreadiids were included, i.e., *Creptotrema funduli* Mueller, 1934, *Allocreadium lobatum* Wallin, 1909, and *Crepidostomum cornutum* (Osborn, 1903) Stafford, 1904. Phylogenetic and molecular evolutionary analyses were inferred by both Bayesian inferences (BI) using MrBayes v.3.2 (Ronquist et al. 2012) and Maximum likelihood (ML) using RAxML v.8.2 software's (Stamatakis 2014). To determine the evolutionary model that gave the best fit to our dataset, the jModeltest 2.1.1 program (Darriba et al. 2012) was employed, with model selection based on the Akaike information criterion (AIC). Results indicated that the general time reversible model with an estimate of gamma distributed among-site rate variation (GTR+G+I) was the most appropriate. BI analysis was carried out running four independent Markov Chain Monte Carlo for 10 million generations and sampling tree topologies every 1000 generations (printfreq = 1000, samplefreq = 1000, diagnfreq = 1000). For ML analyses, nodal support was estimated from 1000 bootstrap re-samplings. The phylogenetic tree obtained was drawn with the FigTree v.1.4.4 software. Genetic distances among taxa were estimated using uncorrected p distances (p-distances) with the Mega v.7 program.

RESULTS

We found the adult stage of a new species of *Auriculostoma* in the intestine of *Heptapterus qenqo* and *Corydoras micracanthus*; *Psalidodon endy* was not infected with this trematode. The bivalve specimens were parasitized with

rediae containing cercarial stages, which were occupying the gonad and digestive gland. Only amphipods were found infected with metacercariae; caddisfly larvae were uninfected.

Superfamily Allocreadioidea Looss, 1902

Family Allocreadiidae Looss, 1902

Genus *Auriculostoma* Scholz, Aguirre-Macedo and Choudhury, 2004

***Auriculostoma ocloya* n. sp.**

ZooBank Life Science Identifier (LSID) - urn:lsid:zoobank.org:act:41C47287-E076-4979-B370-F1CF3CD6E6E5

Descriptions

Adult (Figs. 1, 2)

Body elongated, tapered anteriorly and posteriorly, 854–1522 (1224) in length, 216–393 (296) in width. Smooth tegument (Fig. 2a). Remnants of eyespots present, scattered, located between pharynx and genital pore (Fig. 1a). Oral sucker subterminal, with subtriangular oral aperture (Fig. 2a), and one pair of muscular ventral lobes whose broad base stretches dorsolaterally (Figs. 2b, 2d), and appear to be fused because there are not "free" ends present (Fig. 2b); 117-176 (147) in length, 113–193 (157) in width, with dome-like papillae arranged in four rows: 4 apical, 4 pre-anterior, 6 anterior (Figs. 2b, 2c), 5 in the outer surface (Fig. 2c). Ventral sucker 122–193 (165) in length by 113–208 (165) in width. Prepharynx absent; pharynx, 49–74 (64) in length, 56–86 (74) in width; esophagus curved, 49–73 (62). Cecal bifurcation between one third to one half of distance between oral sucker and ventral sucker; ceca ending between posterior testis and posterior end of body. Testes oval, entire, oblique, contiguous in posterior half of body. Anterior testis 110–174 (142) in length by 98–147 (127) in width. Posterior testis 115–152 (132) in length by 98–149 (110) in width. Cirrus sac ovoid 147–269 (194) in length, containing internal seminal vesicle, pars prostatica, and unspined looping cirrus opening into genital atrium (Fig. 1c). Genital pore located at level of cecal bifurcation. Ovary pretesticular, median, rounded, immediately posterior to or overlapping ventral sucker, 95–154 (134) in length by 95–184 (124) in width. Seminal receptacle sac located immediately posterior to ovary, Laurer's canal present (both seen only in live specimens after eggs released) (Fig. 1b). Mehlis' gland not observed. Vitelline follicles numerous, lateral, extra and intracecal, extending from oral sucker to posterior end of body, confluent in post-testicular region. Uterus coiling between posterior testis and ovary, containing about 30 eggs. Eggs collapsed, deformed in permanent mounts; 44–61 (47) in length by 24–37 (29) in width, apparently not embryonated into live specimens, (Fig. d). Excretory vesicle tubular, extending to anterior testis.

Redia (Figs. 3a, b)

Groups of rediae in each infected clam include individuals of different states of development. Young immature rediae measured 137–237 (184) in length by 31–55 (42) in width, with pharynx 25–32 (30) in length by 16–32 (24) in width; and slender gut. Mature rediae translucid and motile, measured 500–700 (583) in length by 100–200 (133) in width, possess muscular pharynx, 25–37 (31) in length by 18–31 (28) in width; gut (not easily visible in fully mature individuals), and contained l up to 4 developing cercariae and germinal balls.

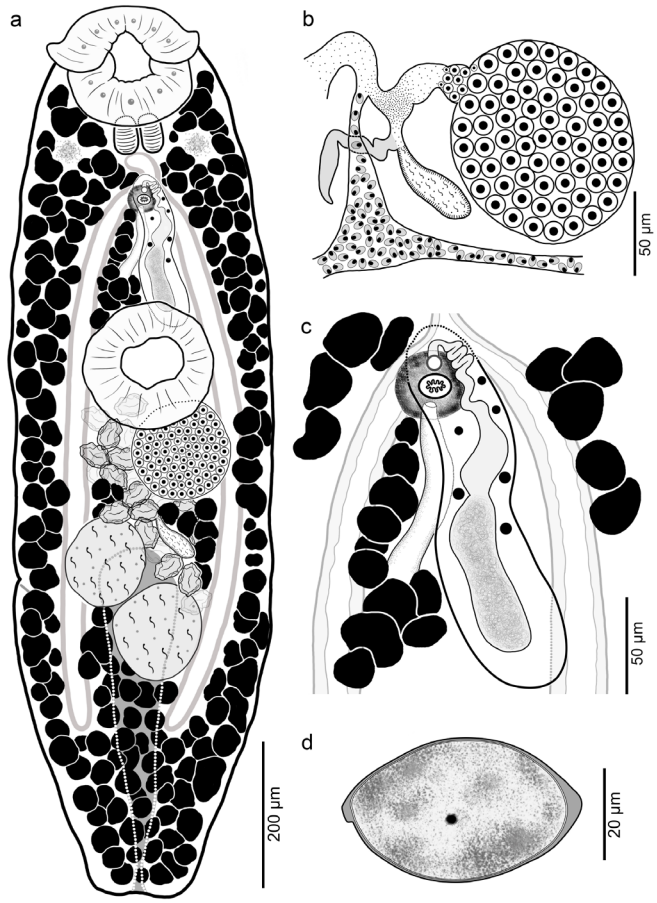

Figure 1. ***Auriculostoma ocloya*** **n. sp. a) Adult, ventral view. b) Terminal genitalia, ventral view. c) Cirrus sac and genital atrium. d) Egg.**

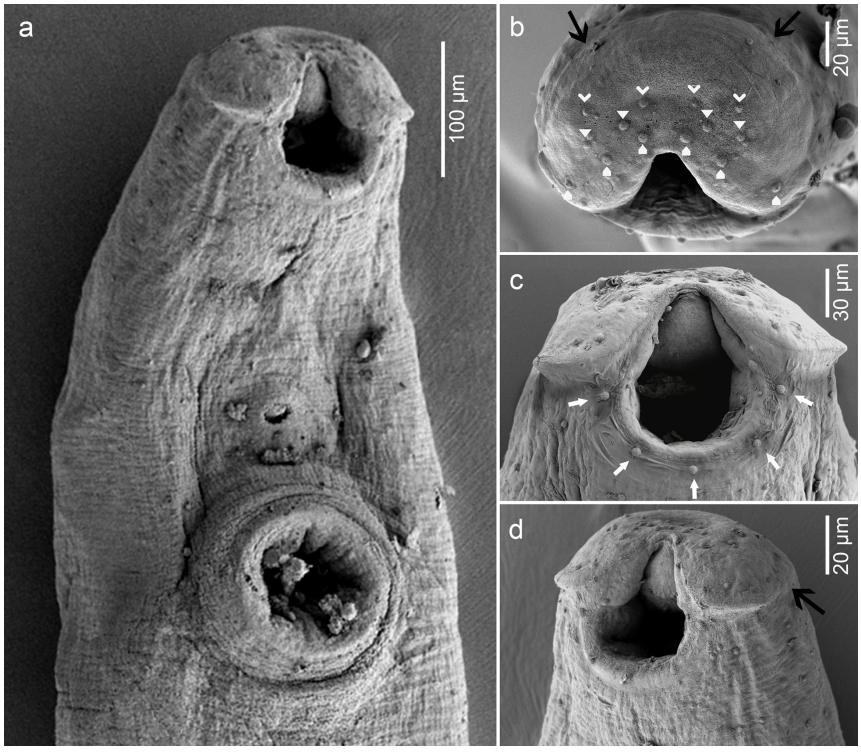

Figure 2. ***Auriculostoma ocloya*** **n. sp. SEM photographs. a) Anterior end with oral sucker bearing muscular lobe on either side, genital atrium and ventral sucker. b) Pair of ventral muscular lobes with a broad base stretches and fuses on the dorsal side (black arrow); papillae arranged in three rows: 6 anterior (pentagon), 4 pre-anterior (triangle), and 4 apical (arrowhead). c) Distribution of the five dome-like papillae over outer surface of oral sucker (arrows). d) Lateral view of muscular lobes stretches to dorsolateral side (black arrow).**

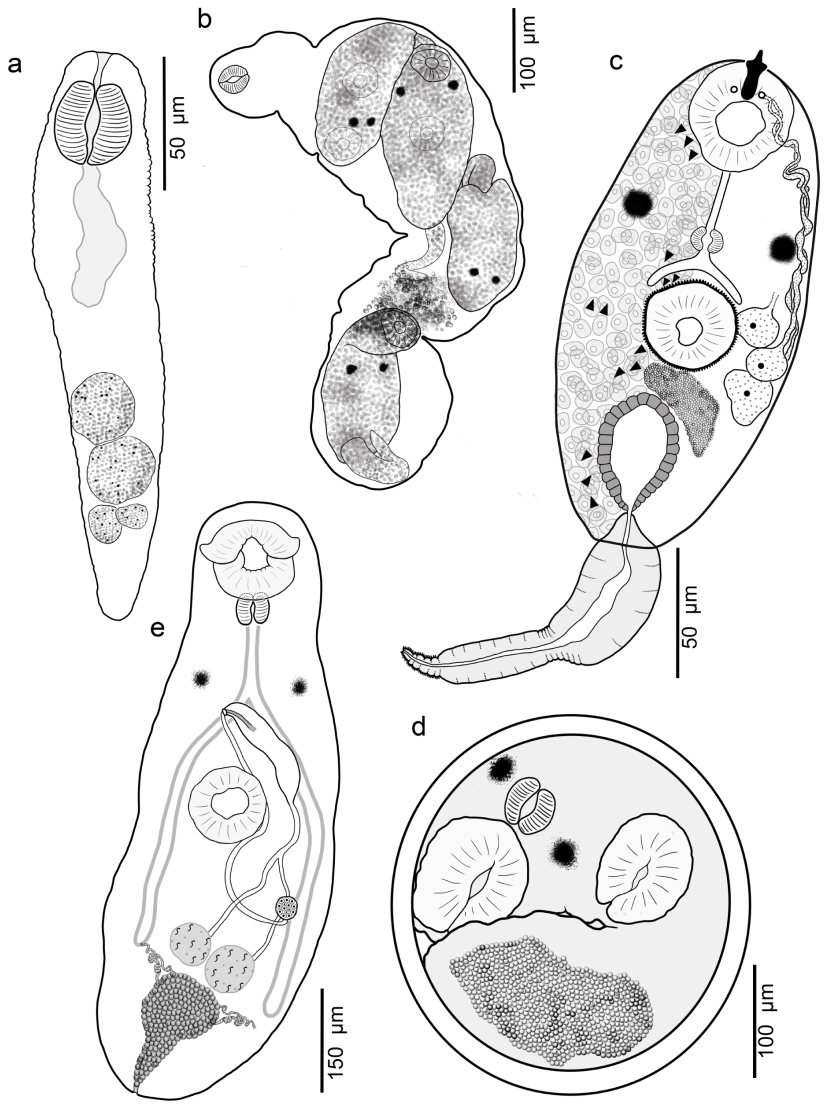

Figure 3. Line drawings of the larval stages of *Auriculostoma ocloya* n. sp. a) Immature redia. b) Mature redia. c) Cercaria, ventral view, flame cells and cystogenous glands of the left side are omitted, penetration glands of the right side are omitted. d) Encysted metacercaria. e) Excysted metacercaria, ventral view.

Cercaria (Fig. 3c)

Ophtalmoxiphidiocercaria. Body oval, elongated, 157–265 (195) in length by 59–98 (77) in width. Forebody 56–88 (72) in length. Smooth tegument. Pigmented eyespots located anteriorly to pharynx. Oral sucker subterminal, 29–59 (41) in length by 29–59 (43) in width. Digestive system with long prepharynx, musculous pharynx, 9–12 (10) in length by 10–17 (14) in width; short esophagus; cecal bifurcation anterior to ventral sucker, ceca not extending posterior to the anterior border of ventral sucker. Ventral sucker 29–49 (40) in length by 29–59 (42) in width, with scalloped outer margin. Stylet fairly robust with anterior shoulders and tapered spine, 17–24 (21) in length. Cystogenous glands filling all body. Three penetration glands at either side of the ventral sucker; ducts passing anteriorly and opening dorsally, at either side of stylet. Genital primordium immediately posterior to ventral sucker. Excretory system formed by 28 flame cells, 14 at each side of body. Flame cell formula appeared to be 2[(3 + 3) + (2 + 3 + 3)]. Excretory bladder surrounded by cells arranged in a single layer; lumen continues with median caudal canal and variable in shape. Tail 100–245 (186) in length, without finfolds.

Metacercaria (Figs. 3d, e)

Cyst spherical, surrounded by thin, double layered cyst wall, 255–393 (343) of diameter (Fig. 3d). Body 393–883 (574) in length by 88–344 (190) in width. Oral sucker subterminal 86–157 (117) in length by 73–147 (109) in width. Pharynx, 37–55 (47) in length by 36–59 (45) in width. Eyespots present. Esophagus long. Ceca bifurcating anterior to ventral sucker, extending up to end of testes. Ventral sucker equatorial, 100–132 (110) in length by 61–130 (86) in width. Excretory bladder filled with refractive granules. Genital primordia well developed, resemble genital organs of adult worms (Fig. 3e).

Taxonomic summary

Type host: *Heptapterus qenqo* Aguilera, Mirande and Azpelicueta, 2010 (Teleostei, Heptapteridae).

Other host: *Corydoras micracanthus* Regan, 1912 (Teleostei, Callichthyidae).

Type locality: a creek affluent to the Lesser River, Salta, Argentina (24°39´43.56´´S; 65°28´49.079´´W).

First intermediate host: *Pisidium ocloya* Ituarte, 2005 (Bivalvia, Sphaeriidae).

Second intermediate host: *Hyalella* sp. Smith 1874 (Crustacea, Hyalellidae).

Site of infection: redia in gonad and digestive gland, metacercaria in thoracic segments between gills, and adult in intestine.

Prevalence: adult, 71% in the type host (32 fish infected of 45 fish examined), and 9% in *C. micracanthus* (2 fish infected of 22 fish examined); redia, 72% (29 clams infected of 40 clams examined); and metacercaria, 34% (11 amphipods infected of 32 amphipods examined).

Mean intensity (range): adult, 5.2 (1–15) in the type host; metacercaria, 2 (1–8).

Etymology: The epithet 'ocloya' refers to the specific name of the first intermediate host; 'ocloya' is referring to an indigenous tribe in the north of Argentina.

Gen Bank accession numbers: rediae (ITS2, MT140285), metacercariae (ITS2, MT140286), adults from *H. qenqo* (28S, MT140287, MT140288; ITS2, MT140284), adults from *C. micracanthus* (28S, MT140289, MT140290).

Deposited specimens: Holotype MACN-Pa 712 (adult). Paratypes MACN-Pa 713 (2 adults), MACN-Pa 714 (cercariae), MACN-Pa 715 (metacercaria); 1 paratype CNP-Par 193 (1 adult).

Remarks:

The specimens of *Auriculostoma ocloya* n. sp. agree to the diagnosis of *Auriculostoma* (Razo-Mendivil et al. 2014) by having a single pair of muscular lobes on either side of the oral sucker with a broad base stretching from ventrolateral to dorsolateral; however, *A. ocloya* n. sp. can be readily distinguished because the "free" ends of lobes on the dorsal side (see Razo-Mendivil et al. 2014, Hernández-Mena et al. 2018) are not present in our specimens because appear to be fused (Fig. 2b). In addition, it differs from the other congeners by the combination of the following characters: small size, vitelline follicles extending from oral sucker and small cirrus sac. However, the new species most resembles *Auriculostoma platense* because the specimens have the cirrus sac not reaching beyond the posterior margin of the ventral sucker, but they differ in that *A. platense* has a large and pointed posterior "free" end of the lobes on the oral sucker whilst *A. ocloya* n. sp. has less developed the muscular lobes on the dorsal side on the oral sucker. Moreover, the new species has vitelline follicles confluent in the postesticular region, whereas those of *A. platense* are not confluent; both species can also be distinguished by the egg size (smaller in the new species, 47µm versus 75 µm in length). *Auriculostoma ocloya* n. sp. differs from its congeners *A. macrorchis*, *A. foliaceum*, and *A. stenopteri* by having oblique entire testes rather than irregular testes in

tandem (Table II). Additionally, these mentioned three species have the uterus located anterior to the testes (versus the uterus reaching the testicular level in the new species). In *A. ocloya* n. sp. and *A. totonacapensis* the genital pore is located at the level of the cecal bifurcation and the testes are oblique, although in some specimens of *A. totonacapensis* the testes seem to be in tandem (Razo-Mendivil et al. 2014). The last mentioned species differs from the new species in the extension of the vitelline follicles, which reach the esophagus (versus reaching the oral sucker in the new species). In addition, the excretory vesicle extends up at the posterior margin of the posterior testis (versus anterior testis in the new species). *Auriculostoma tica* can be distinguished from *A. ocloya* by having a bean-shaped ovary (versus entire and rounded in the new species). *Auriculostoma diagonale* and the type species of the genus, *A. astyanace,* have a long cirrus sac, clearly differing from the new species, in which the cirrus sac is not surpassing the ventral sucker. Furthermore, in *A. astyanace,* the anterior extension of the vitelline follicles reaches the intestinal bifurcation and in *A. diagonale* reaches the pharynx level, whereas vitelline follicles reach the oral sucker in the new species. In *A. lobata,* the ventral sucker is pre-equatorial, the testes are multilobulated and separate, whilst in the new species the ventral sucker is equatorial, and the testes are entire and contiguous.

The body surface of *A. ocloya* n. sp. possesses 16 dome-like papillae on the oral sucker surface, which are arranged in four rows (only seen at SEM) (Fig. 2b). *Auriculostoma ocloya* n. sp., *A. lobata*, and *A. totonacapensis* possess six anterior dome-like papillae on the oral sucker, whilst *A. tica* possesses eight anterior dome-like papillae. Moreover, in the new species, the inner surface dome-like papillae are absent, and the pre-anterior dome-like papillae are adding. The papillae on the ventral sucker were not observed in *A. ocloya* n. sp. (Fig. 2a).

Molecular analyses

The sequences of the amplified ITS2 fragment of adults of *A. ocloya* n. sp. from *H. qenqo* and *C. micracanthus* were 281 bp long. The sequences encoding the ITS2 region of the rediae from *P. ocloya* and metacercariae from *Hyalella* sp. (Table I) were found identical to the adult sequence.

Sequences of the 28S rRNA gene obtained from the two adults of *H. quenqo* presented 944 bp (isolate 1), 938 bp (isolated 2), and from the two adults of *C. micracanthus* 948 bp (isolated 3) and 873 bp (isolated 4). The alignment of these four sequences resulted in 758 bp and they were identical. ML and BI analyses based on the 28S rRNA gene resulted in trees with the same topology (Fig. 4). Both analyses revealed the genus *Auriculostoma* monophyletic, but without strong support (bootstrap ML: 86%; posterior probability BI: 1). *Auriculostoma ocloya* n. sp. is found as a sister to all other sequenced members of *Auriculostoma* (*A. astyanace*, *A. lobata*, *A. tica*, and *A. totonacapensis*).

The genetic distance between *A. ocloya* n. sp. and *A. lobata* reached 1.61%, meanwhile between the species here described and *A. astyanace* and *A. tica* was 1.74 and 2.41% respectively. Moreover, *A. totonacapensis* and the new species reached 3.22%. Intergeneric genetic divergence between species of *Auriculostoma* and *Wallinia* values ranged from 2.95 to 6.84%, between the genera *Auriculostoma* and *Creptotrematina*, values varied between 4.16 to 4.96% and between *Auriculostoma* and *Creptotrema* genetic distance was relatively high, varying from 5.09 to 6.43%.

Table II. Comparison of *Auriculostoma* species (new species is in bold); measurements are presented in micrometers, given range and mean in parenthesis.

Digenean species	Body length	Body width	Oral sucker lobes on the dorsal side	Ventral sucker (VS) position	Vitelline follicles anterior extension	Testes position	Genital pore	Number of eggs	Egg size (length by width)
Auriculostoma macrorchis[a]	1500	500	Feebly developed	First third of body	Oral sucker	Tandem	Posterior to cecal bifurcation	More than 20 [b]	55 29
Auriculostoma platense[a]	800	200	Well developed. (triangle shape "free"end)	Second third of body	Esophagus	Oblique	Anterior margin of VS	4 – 5	75 39
Auriculostoma stenopteri	750–1320	216–308	Feebly devoloped	First third of body	Oral sucker	Tandem	Anterior to cecal bifurcation	2 – 10	68–76 28–40
Auriculostoma astyanace	1900–2900 (2900)	400–488 (488)	Well developed. (triangle shape "free"end)	First third of body	Cecal bifurcation	Tandem	Between VS and cecal bifurcation	40 – 60	55–59 34–41
Auriculostoma diagonale	1207–1600	352–474	Well developed	First third of body	Pharynx	Oblique	Between VS and cecal bifurcation	-	54–57 28–32
Auriculostoma foliaceum	1993	450	Well developed	First third of body	Cecal bifurcation	Tandem	Between VS and cecal bifurcation	About 20	58–63 28–29
Auriculostoma totonacapensis	1028–2003 (1402)	287–568 (398)	Well developed[c] (rounded shape "free" end)	First third of body	Cecal bifurcation	Oblique	At cecal bifurcation	14 [b]	48–65 (54) 30–41 (34)
Auriculostoma lobata	2539–3010 (2735)	502–639 (581)	Well developed[c] (triangle shape "free" end)	First third of body	Cecal bifurcation	Tandem	Between VS and cecal bifurcation	More than 20 [b]	45–58 (52) 23–35 (27)
Auriculostoma tica	1551–2118 (1778)	424–568 (478)	Feebly developed[c] (rounded "free" end)	First third of body	Genital pore	Oblique	At mid-level of esophagus	More than 20 [b]	41–70 (54) 23-39 (29)
***Auriculostoma ocloya* n. sp.**	854–1522 (1224)	216–393 (296)	**Without "free" end[c]**	Second third of body	Oral sucker	Oblique	At cecal bifurcation	About 20	44–61 (47) 24–37 (29)

[a] specimens with a measure for each structure.
[b] represents value not provided in the original descriptions; these were calculated from the drawings for comparative purpose only.
[c] character observed through SEM microscopy.

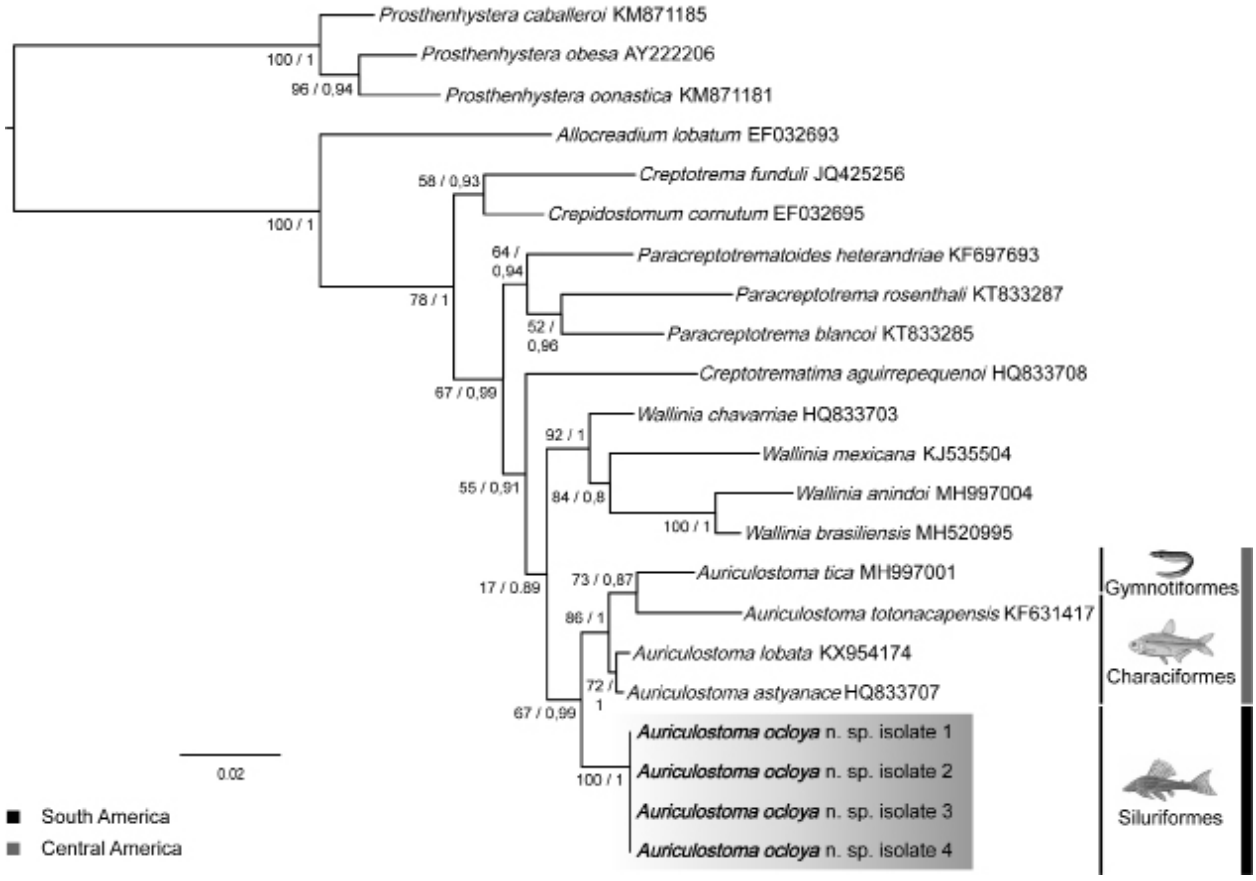

Figure 4. Phylogenetic tree based on Bayesian inference and Maximum likelihood analyses of the 28S dataset showing the relationship between the new species and the other species of *Auriculostoma*. Order of host and geographical origin of each species of the *Auriculostoma* are indicated. Values above the branches represent Bayesian inference posterior probabilities (PP) followed by the Maximum likelihood bootstrap support (BS).

DISCUSSION

We describe a new species of allocreadiid digenean of the genus *Auriculostoma* parasitizing fishes from South America. For the first time, we elucidated the complete life cycle for a species of this genus, given a complete characterization of the new species.

Morphological study of the specimens here studied allowed us to determine the presence of a single pair of muscular lobes on either side of the oral sucker; a similar condition is observed in *Creptotrema*. However, we have been observed by SEM that the muscular lobes on either side of the oral sucker have a broad base, stretching from ventrolateral to dorsolateral, as is present in the rest of the species of the genus *Auriculostoma* (Razo-Mendivil et al. 2014, Hernández-Mena et al. 2018). Nevertheless, on the dorsal side, the "free" ends of the lobes are not present in our specimens because their appear to be fused (Fig. 2b). In this sense, we agree with Razo-Mendivil et al. (2014) in that morphological study solely based on light microscopy seems not to be useful to clearly distinguish the morphology of the muscular lobes; therefore, the use of SEM is helpful as it can provide some additional character. Furthermore, the new species differ of the species of *Creptotrema* by the absence of a prepharynx, seminal receptacle inconspicuous, and Laurer's canal present.

In the phylogenetic tree, based on sequences of the 28S rRNA gene, *A. ocloya* n. sp. appears within a clade containing other species of the genus *Auriculostoma.* Even though the support of individual lineages represented by samples of individual species is poor, a geographical/host group pattern can be distinguished. A single South American species, *A. ocloya* n. sp., from catfishes is a sister taxon to all Central American taxa from fishes of the order Characiformes and Gymnotiformes (Fig. 4). This means that although *Creptotrema* and *Auriculostoma* share morphological characters, our phylogenetic analysis shows that *Creptotrema* is most closely related to *Crepidostomum* (Fig. 4). The only species of *Creptotrema* that has been sequenced to date is *C. funduli,* a species of North America. Manter (1962) after revision of the type specimen of this species in New York, USA, concluded that the presence of the oral lobes was doubtful so he classified *C. funduli* as an Opecoelidae. Despite were doubts, Curran (2008) observed the type material for *C. funduli*, and confirmed that this species is an allocreadiid belonging to the genus *Creptotrema*. In addition, he recorded this species in a new definitive host in Mississipi, USA, and subsequently sequenced it (Curran 2012). We agree with Caira & Bogéa (2005), who highlighted that circumscription of the allocreadiid genera is controversial because many of them are defined by their possession of a unique combination of states of non - unique characters, such as oral sucker lobes, the posterior extent of the uterus and anterior extent of the vitelline follicles. This situation increases when these morphological characters are not easy to distinguish.

The use of ITS2 markers made it possible to confirm that the life cycle of *A. ocloya* n. sp. involves three hosts. In the molluscan first intermediate host, *Pisidium ocloya*, the cercariae develop inside the rediae; then they emerge and infect amphipods (*Hyalella* sp.) and when they are preyed by the fishes *Heptapterus qenqo* and *Corydoras micracanthus,* the metacercariae develop into the adult form. Therefore, we found as first intermediate host a sphaeriid clam; which is commonly used amongst species of the Allocreadiidae (see Wootton 1957, Anderson et al. 1965). The prevalence of *A. ocloya* n. sp. in the bivalve was unexpectedly high (about 70%). Considering that this clam harbor rediae in its gonads and digestive gland, which result in castration of the host, it is supposed that the cost of being infected will be high to the host population. Coexistence, even with high-prevalence infections is likely due to a compensatory reproductive mechanism to ensure the reproduction of the host population. For example, infection of *Pisidium amnicum* with *Bunodera luciopercae* (Müller, 1776) (Allocreadiidae) has been shown to occur after reproduction of the clam (Rantanen et al. 1998). This might be the situation in the clam population here studied.

The second intermediate host of the allocreadiid species is generally an insect larva such as larvae of mayflies or chironomids, caddisfly larvae, copepods or crayfishes (Caira & Bogéa 2005). In this study, we found that *A. ocloya* n. sp. uses amphipods (*Hyalella* sp.) as a second intermediate host, seemingly failing to invade caddisfly larvae, which shares the aquatic area with *P. ocloya*.

The adults of *A. ocloya* n. sp. were found in *Heptaterus qenqo* and *Corydoras micracanthus*, but not in *Psalidodon endy*. It is not surprising within this genus that two different families of fishes serve as definitive hosts of a species. For example, *A. platense* was recorded from *Rhinodoras dorbignyi* (Siluriformes: Doradidae) and *Pimelodus maculatus* (Siluriformes: Pimelodidae) by Szidat (1954). Even *A. platense* was found parasitizing fishes belonging to

a different order, *Rhamphichthys rostratus* (Gymnotiformes: Rhamphichthyidae) according Ostrowski de Núñez et al. (2017). A similar situation occurs with *A. macrorchis*, which was reported from *Pachyurus bonariensis* (Scienidae) and then from *Auchenipterus* spp. (Auchenipteridae), *Luciopimelodus pati* (Pimelodidae), and *Rhinodoras dorbignyi* (Doradidae) (Ostrowski de Núñez et al. 2017). Typically, the specificity of the developmental stages infecting the second and definitive hosts may be less strict than those infecting the first host in digeneans (Jousson & Bartoli 2001). That specificity in the definitive host is mainly associated with host ecology, such as feeding habits (Adamson & Caira 1994, Jousson et al. 2000). In our study, both fish parasitized species are siluriform and share certain feeding habits and lifestyles; they live on the bottom of water bodies, with abundant aquatic vegetation, while *P. endy,* which was found free of parasites, lives in pelagic habitats (Monasterio de Gonzo 2003).

The poor knowledge of South American species of *Auriculostoma* and the morphological diagnosis based on oral sucker lobes increase the problems in the unresolved systematics of Allocreadiidae. Therefore, the genetic database should be substantially improved. In this sense, a complete characterization of our new species based on an integrative taxonomic approach represents a contribution to revealing more patterns in allocreadiids systematics.

Acknowledgments

The authors would like to thank Cristián Ituarte for the identification of bivalves; Margarita Ostrowski de Núñez, Nathalia Arredondo (both from Argentina), and Janine Caira (from USA) to provide access to databases on parasitic helminths, Sebastián Quinteros and Francisco Sylvester are acknowledged for their constructive suggestions on the manuscript. Thanks are also due to Lorena Ailán Choke, Néstor Huertas, Javier Cepéda, Facundo Leguizamón, and Daniel Paredes for their assistance in the fieldwork, and to the Secretaría de Medio Ambiente del Gobierno de la Provincia de Salta for allowing us to collect the aquatic organisms. This work was financed in part by Consejo de Investigación from Universidad Nacional de Salta (grant number A 2193/3, A 2481/0 CI-Davies and B 259/2018 CI-Cristóbal), Salta, República Argentina.

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How to cite

LIQUIN F, GILARDONI C, CREMONTE F, SARAVIA J, CRISTÓBAL HA & DAVIES D. 2022. A new species of *Auriculostoma* (Digenea: Allocreadiidae) in South America: life cycle and phylogenetic relationships. An Acad Bras Cienc 94: e20200538. DOI 10.1590/0001-3765202220200538.

Manuscript received on April 13, 2020;
accepted for publication on September 29, 2020

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FLORENCIA LIQUIN examined hosts, found, identified parasites, described them and wrote the manuscript. JOSÉ SARAVIA and HÉCTOR CRISTÓBAL sequenced trematodes and constructed phylogenetic trees. CARMEN GILARDONI contributed to morphological and molecular processing of parasites and writing of the manuscript. FLORENCIA CREMONTE contributed to the analysis of data, writing, structuring the text and revision of the final manuscript. DORA DAVIES designed the project, examined hosts, contributed to the analysis and interpretation of data and revision of the final manuscript submission.