Parasitology

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First morphological and molecular identification of the cercaria of *Stomylotrema vicarium* from the endemic apple snail *Pomacea americanista*

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Research Article

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Abstract

The adult fluke *Stomylotrema vicarium* (Stomylotrematidae, Microphalloidea) was described for the first time in *Theristicus caerulescens* in 1901, but the complete life cycle has remained unknown to date. Here, we found a stomylotrematid trematode in the digestive gland of the endemic apple snail *Pomacea americanista*. The digestive gland's tubuloacini were compressed by the trematode larvae placed on connective tissues and haemocoel spaces. Non-virgulate, stylet-bearing cercariae showed three pairs of penetration glands with a body, oral sucker and stylet morphometrically similar to those of stylet-bearing, unencysted young metacercariae of *S. vicarium* found in the aquatic coleopteran *Megadytes glaucus*, and at a lesser extent with cercariae of *S. gratiosus* found in the apple snail *Pomacea maculata*. The larvae molecular phylogeny was inferred using the markers rRNA 28S and ITS1, being these sequences grouped with the sequences of *S. vicarium* obtained from adult flukes. Together, these findings indicate that the life cycle of *S. vicarium* begins in *P. americanista*, thus supporting the hypothesis that the ampullariid snails act as a first intermediate host.

Introduction

Flukes (Platyhelminthes, Trematoda) are parasites that have a complex life cycle, involving several definitive and intermediate hosts. Traditionally, the identification of trematodes has been limited to the morphology of the adult in the definitive host (Jones *et al.*, 2002). However, the continuous and fast growth of molecular databases offers the possibility of advance in the identification of parasite larvae found in different intermediate hosts, overcoming the taxonomic limitations of larval morphology studies or of assays that mimic the parasitic life cycle (Nolan and Cribb, 2004; Blasco-Costa *et al.*, 2016). A molecular approach involving loci with different evolutionary rates (e.g. rRNA 28S gene or ribosomic Intergenic Spacer 1; Schulenburg *et al.*, 1999; Tkach *et al.*, 2000; Olson *et al.*, 2003; Blasco-Costa *et al.*, 2016) has been helpful to identify trematode cryptic species (Miura *et al.*, 2005; Vilas *et al.*, 2005; Razo-Mendivil *et al.*, 2010; Dellagnola *et al.*, 2019*a*). Besides, integrated morphological and molecular approaches with ribosomal markers helped in the identification of fluke mixed larval infections (Dellagnola *et al.*, 2019*a*).

Apple snails (Caenogastropoda, Ampullariidae) are distributed in tropical and subtropical wetlands worldwide (Berthold, 1991; Hayes *et al.*, 2009). The family Ampullariidae includes amphibious species with different degrees of air dependence (Seuffert and Martín, 2009, 2010; Hayes *et al.*, 2015; Rodriguez *et al.*, 2021), i.e. species with presumptively predominant gill respiration that lay gelatinous egg masses underwater and species that strictly depend on aerial lung respiration and lay calcareous eggs out of the water (Hayes *et al.*, 2009).

Pomacea is the most diverse genera of Ampullariidae (Hayes *et al.*, 2015). *Pomacea canaliculata* and *Pomacea maculata* are highly invasive species that affect different agricultural ecosystems of Southeast Asia, Pakistan, Hawaii, North America and Europe causing annual millionaires' economic loss (Rawlings *et al.*, 2007; López-Soriano *et al.*, 2009; Oscoz *et al.*, 2010; Yanygina *et al.*, 2010; Baloch *et al.*, 2012; Hayes *et al.*, 2015; Joshi *et al.*, 2017). On the other hand, *P. americanista* (Ihering 1919) is an endemic snail to the Alto Paraná basin and Iguazú River in the rainforest of Argentina, Paraguay and Brazil, and is found on hard rocky substrates in swiftly flowing waters (Hylton Scott, 1957). Unlike most ampullariids, *P. americanista* is a vulnerable species due to stringent habitat requirements and a narrow geographical range (Martín *et al.*, 2015; Gurovich *et al.*, 2017).

In the context of a broader program dealing with the symbiotic associations of apple snails, a stylet-bearing trematode was found hosted into the connective tissue and haemocoel spaces of the digestive gland in *P. americanista*. The aim of this study was to identify this parasite, plausibly from the suborder Xiphidiata (Plagiorchiida, Digenea), using a morphological and molecular approach. The trematode phylogeny inferred from the gene encoding the large subunit ribosomal RNA (28S rRNA) and the non-coding nuclear region of the ITS1 allowed

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identifying *Stomylotrema vicarium* Braun, 1901 for the first time in an intermediate host; being the stomylotrematid flukes a small group of parasites from which there was a very scarce knowledge about their basic biology and early life cycle.

Material and methods

Collection site and host sampling

During a malacological survey carried out in the Bonito stream, Misiones rainforest, Argentina (27°26′39.60″S, 54°56′12.65″O; Supplementary Fig. 1A) in January 2014 southern summer, specimens similar to the ampullariid *P. americanista* (Ihering, 1919) were collected (Supplementary Fig. 1B and C). Only five individuals (range = 35.1–38.9 mm of shell length and 9.8–34.5 g) of this species were collected for different studies based on their scarcity, scant knowledge about their life history, and restricted geographic distribution (Martín *et al.*, 2015; Gurovich *et al.*, 2017).

Tissue sampling and histological procedures for light microscopy

Snails collected were submersed in an ice/water bath (~4°C for 15 min) for inducing relaxation and minimizing pain before careful shell cracking. Samples for light microscopy were obtained from sacrificed individuals by cutting 2–3 mm thick slices of the digestive gland with a razor blade from the gland surface, close to the kidney boundary. Tissues were then fixed in 4% paraformaldehyde in a buffered solution (43 mM NaCl, 1.8 mM KCl, 10.0 mM HEPES and 30 mM EDTA; pH 7.6; Cueto *et al.*, 2015) for 24 h at 20°C and kept in 70% ethanol. These were subsequently dehydrated in increasing concentrations of alcohol, embedded in a resin-paraffin mixture (Histoplast®, Argentina) and sectioned (5 μm). Sections were stained with haematoxylin-eosin and a trichrome stain (Alcian Blue, Nuclear Fast Red and eosin) as reported previously (Dellagnola *et al.*, 2017). Digestive epithelial cells, intracellular symbiotic corpuscles and storage cells were morphologically identified as reported previously for Neotropical (Castro-Vazquez *et al.*, 2002; Vega *et al.*, 2005, 2007; Giraud-Billoud *et al.*, 2008; Dellagnola *et al.*, 2019*b*) and Indomalayan apple snails (Meenakshi, 1955; Takebayashi, 2013).

Trematode larvae were also obtained from the digestive gland by the osmotic lysis of glandular cells, followed by sequential sedimentations (Dellagnola *et al.*, 2019*a*). This fraction, enriched in trematode parasites, was used for morphometric and molecular (see next section) determinations. The micrographs were taken with a Nikon Eclipse 80i microscope, fitted with a Nikon DS-Fi1-U3 digital camera, using either Nomarski or bright field Differential Interference Contrast microscopy. The morphometry of the larval morphological features was made with ImageJ 1.51j8 (Rasband, 2012).

DNA extraction, PCR and sequencing

The isolated trematodes were washed and then centrifuged at 2300 ***g*** (5 min). The DNA was extracted using the CTAB method (Porebski *et al.*, 1997). Total DNA was suspended in sterile ultrapure water (Biodynamics) overnight at 4°C and then incubated for 20 min at 65°C for removing DNase activity. The DNA was quantified using a 260/280 absorbance ratio. The whole region of the large ribosomal RNA subunit (28S rRNA gene) and the complete ITS1 non-coding nuclear region (placed between the end of the 18S rRNA gene and the beginning of the 5.8S rRNA gene) were amplified by PCR and then sequenced for phylogenetic studies (Schulenburg *et al.*, 1999; Van Steenkiste *et al.*, 2015). The primers and the reaction conditions (cycles of denaturation, annealing and extension) used for each PCR are shown in Tables 1 and 2, respectively. All PCRs were performed in a total volume of 20 μL, containing 10 ng of total DNA, 0.2 μM primers, 0.8 mM nucleotides mix and 1 U of recombinant Taq polymerase (Invitrogen). The reactions were performed in a Mastercycler Gradient thermal cycler (Eppendorf). The size of both PCR amplicons was confirmed by 0.9% low-melting-point agarose gel electrophoresis. Amplicons were then purified according to the manufacturer's protocol of the PuriPrep-GP Kit (Inbio Highway®, Argentina) and sequenced by the dideoxynucleotide method at the Instituto de Biotecnología (INTA-Castelar, Argentina) and Macrogen Inc. (Korea). The electropherograms were analysed, assembled and corrected using Chromas 2.6.2 (Technelysium Pty Ltd., Tewantin Qld, Australia). Both sequences were deposited in GenBank® (accession numbers MW480895 for 28S rRNA and MW481318 for ITS1).

Table 1. Oligonucleotide primers

Amplified region	Specific primer set	Reference
rRNA 28S	LSU-5f: 5′-TAGGTCGACCCGCTGAAYTTAAGCA-3′ LSU-1500r: 5′-GCTATCCTGAGGGAAACTTCG-3′	Tkach *et al.* (2003)
ITS1	S20T2f: 5′-GGTAAGTGCAAGTCATAAGC-3′ 5.8s1: 5′-GCTGCGCTCTTCATCGACA-3′	Bartoli *et al.* (2000)

Trematoda phylogenetic relationships

A preliminary similarity analysis among the trematodes sequences obtained and those deposited in the GenBank® database was done using the BLASTn search (Zhang *et al.*, 2000). Two rRNA 28S gene sequences (KY982863 and MF155659) were found in the BLASTn analysis with an identity up to 95%. These sequences showed different coverage (KY982863 = 95%; MF155659 = 86%); thus, two independent rRNA 28S phylogenetic trees were run with the rest of the rRNA 28S sequences. Besides, sequences of the ITS1 region showed a percentage of coverage lesser than 95% and an identity lesser than 95% in the BLASTn analysis; thus, a unique tree was run.

Trematoda homologous sequences of both loci (rRNA 28S and ITS1) were downloaded, aligned (Clustal Omega; Sievers *et al.*, 2011) and ordered in an iterative process. The alignments were edited and trimmed to the shortest sequence length using MegaX (Kumar *et al.*, 2018). The final alignments were made with MAFFT version 7 using the iterative refinement method G-INS-I (Katoh *et al.*, 2017). Parameters and nucleotide substitution models were calculated with Smart Model Selection (Lefort *et al.*, 2017) using AIC likelihood-based statistical criteria. Final Maximum Likelihood (ML) trees were inferred using PhyML 3.0 (Guindon *et al.*, 2010). For both rRNA 28S and ITS1 trees, the General Time Reversible model (γ distributed with invariant sites) was predicted as the best estimator by the Akaike information criterion.

For the large 28S ML tree, the estimated proportion of invariable sites was 0.339; the number of discrete γ categories was 4, and the γ shape parameter was 0.843. This tree was constructed using 103 taxa with 1221 positions in the last alignment dataset. For the short 28S ML tree, the estimated proportion of invariable sites was 0.332; the number of discrete γ categories was 4, and the γ shape parameter was 0.844. This tree was constructed using 104 taxa with 1068 positions in the last alignment dataset. For the ITS1 tree, the estimated proportion of invariable sites was 0.130; the number of discrete γ categories was 4, and the γ shape parameter

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Table 2. PCR conditions for each genic region amplified

Amplified region	PCR step	Cycles	Temperature (°C)	Time
rRNA 28S	Initial denaturalization	1	94	5 min
	Denaturalization	35	94	45 s
	Annealing	35	62	30 s
	Extension	35	72	2 min
	Final extension	1	72	10 min
ITS1	Initial denaturalization	1	95	3 min
	Denaturalization	35	95	30 s
	Annealing	35	55	30 s
	Extension	35	72	2 min
	Final extension	1	72	7 min

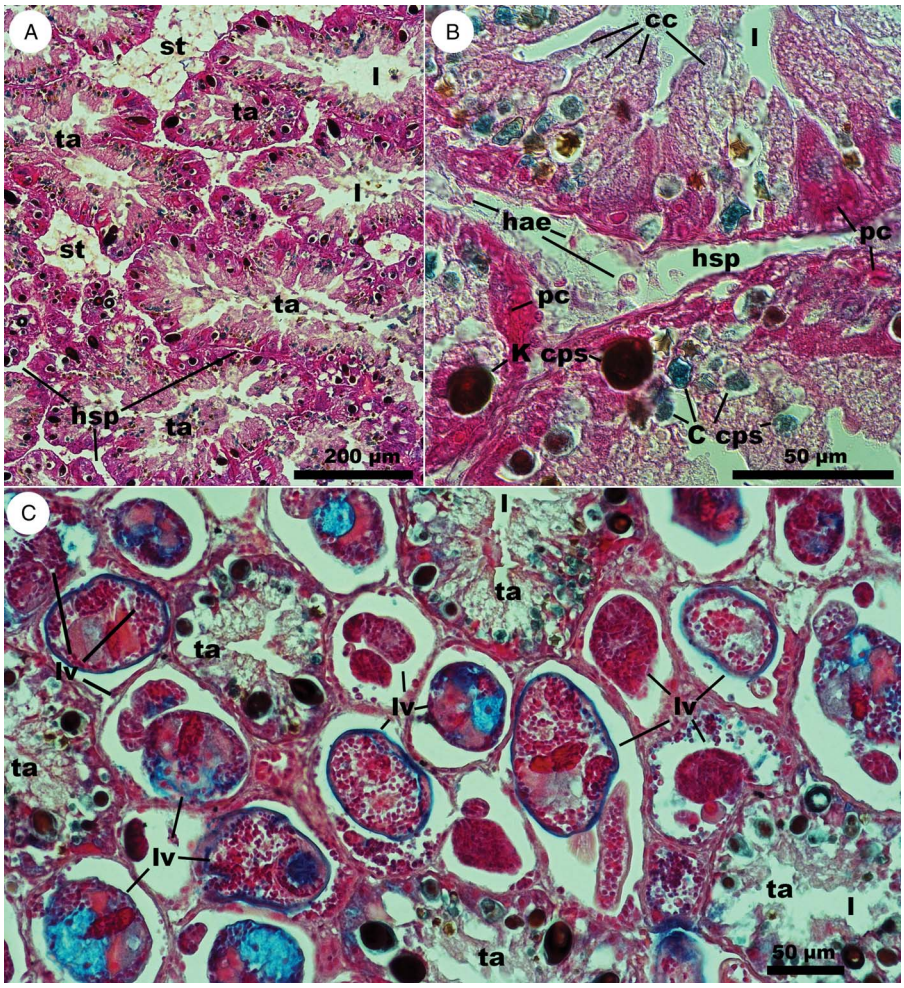

Fig. 1. Histology of the digestive gland of *P. americanista*. (A) Trichrome stain section of the digestive gland from a non-parasitized host, showing a typical tubuloacinar structure of the digestive gland of the ampullariid snails. (B) DIC micrograph of a trichrome stain section showing the haemocoel spaces, the columnar and pyramidal cells, and the symbiotic C and K corpuscles that form the digestive epithelium. (C) Trichrome stain section of the digestive gland from a parasitized host, showing a large number of sporocyst carrying cercariae in different stages of development; which occupied the haemocoel spaces and connective tissue between tubuloacini. cc, columnar cells; C cps, C corpuscles; hae, haemocytes; hsp, haemocoel space; K cps, K corpuscles; l, (tubuloacinar) lumen; lv, (trematode) larvae; pc, pyramidal cells; ta, tubuloacinus; st, storage tissue.

was 1.147. This tree was constructed using 53 taxa with 1822 positions in the last alignment dataset.

A bootstrap analysis (100 replicates) was performed by each phylogenetic hypothesis. Sequences of the Schistosomatidae (Diplostomida) were used as the outgroup in all trees.

Results

The digestive gland of P. americanista

Figure 1 shows a non-parasitized digestive gland of *P. americanista*. This organ was mainly composed of elongated, irregular alveoli. The interstice is made up of narrow haemocoel spaces and vessels surrounded by storage tissue (Fig. 1A). Two digestive epithelial cells (pyramidal and columnar) lined an alveolar lumen of irregular width (Fig. 1B). Numerous symbiotic corpuscles 'C' and 'K' were found in the basal third of the epithelial cells. The symbiotic K corpuscles, black/brownish in colour and with an oval shape, were located in the acidophilic cytosol of pyramidal cells. Symbiotic C corpuscles were housed in vesicles of columnar cells and they reacted positively to Alcian Blue. Furthermore, cercariae-carrying sporocysts occupied huge interstitial spaces in the infected specimen of *P. americanista* (Fig. 1C). Secretions and the external epithelium of these larvae showed alcianophily.

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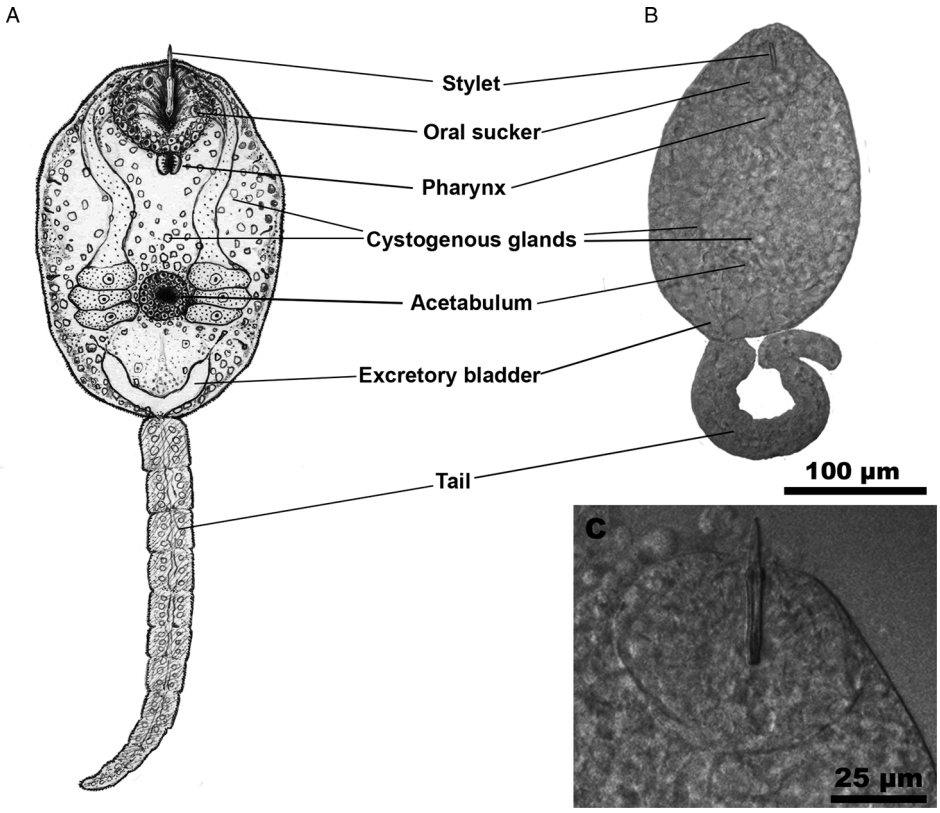

Fig. 2. Cercaria isolated from the digestive gland of *P. americanista*. (A) Scale drawing of a representative cercaria with their anatomical references. (B) Unstained xiphidiocercaria. (C) Detail of the oral sucker showing a well-developed, sclerotized stylet with a thickened base and pointed anterior end that exceeds the anterior body margin.

Both the location and size of the trematode appear to compress the tubuloacini and reduce their acinar lumen.

Morphology and morphometry of the trematode larvae

Stained sections of the digestive gland and isolated parasites were used for morphological and morphometric analysis, respectively. The xiphidiocercaria showed a conspicuous, sclerotized stylet in the oral sucker (Fig. 2A–C) which has a thickened base pointed anterior end that can exceed the anterior body margin, 36 μm (range = 25–43, $n = 6$) long and 5 μm (range = 3–7, $n = 5$) wide at the base (Fig. 2C). The body was ovoid, 213 μm long (range = 182–241, $n = 17$) and 147 μm wide (range = 132–164, $n = 17$) with a mean long/wide relationship of 1.5 (1.3–1.6, $n = 16$). The body had abundant cystogenic glands. The tail was 245 μm long (range = 195–300, $n = 9$) and 26 μm wide (range = 24–29, $n = 9$); fin-folds were not observed. The tail was larger than the body (T/B), 1.19 (range = 1.0–1.3, $n = 6$). Oral sucker was rounded, sub-terminal, 56 μm (range = 46–66, $n = 19$) long and 59 μm (range = 44–69, $n = 19$) wide. The digestive system was not observed possibly due to the presence of numerous cystogenic cells.

Abundant, Nuclear Fast Red-stained, cystogenic unicellular glands were found along the parasite's body (Fig. 3A–C). The parasite showed three pairs of large, eosinophilic penetration glands (Fig. 3A–D) at the mid-body, arranged laterally to the acetabulum. The first and second pair of penetration glands showed cells with finely granular cytoplasmic content (Fig. 3A, C and D); the third pair showed hyalinized contents (Fig. 3D). Ducts of the penetration glands opened on both sides of the stylet (Fig. 3A and B). No virgula organ and genital primordium were observed. Cells of the acetabulum appear basophilic in haematoxylin-eosin preparations (Fig. 3A) with red nuclei in trichrome preparations (Fig. 4C and D). The formula of the excretory system could not be recognized. The V-shaped excretory bladder was associated with the central duct of the tail (Fig. 3D–F). Tiny spines covered the body and tail surfaces. These spines were parallel to the tail's main axis (Fig. 3E and G).

PCR and sequence similarities

The PCR assay showed amplicons about 1300 and 700 bp (Supplementary Fig. 2) using, respectively, the primer sets LSU-5f and LSU-1500r (for rRNA 28S) and S20T2f and 5.8s1 (for ITS1) and total DNA from the digestive gland of an infected individual of *P. americanista*.

The size and coverage of the rRNA 28S gene of microphalloidean trematodes were conserved and had few internal gaps. Interestingly, the rRNA 28S of the xiphidiocercaria of *P. americanista* (Supplementary Fig. 3A) showed a per cent of identity major than 95% with sequences of *S. vicarium* (Stomylotrematidae) reported in two vertebrate hosts (a bird, KY982863; and a mammal, MF155659). On the other hand, the comparative analysis of the ITS1 of microphalloidean trematodes showed a less conserved locus than the rRNA 28S gene. The coverage and size (~700–1000 bp) of the ITS1 region were variable. The ITS1 of the xiphidiocercaria of *P. americanista* (Supplementary Fig. 3B) and those of the order Plagiorchiida showed a similarity of <92%.

Phylogenetic reconstruction based on the rRNA 28s gene and the ITS1 region

Figure 4 shows the phylogenetic position of the trematode larval stages from *P. americanista*. The large rRNA 28S ML tree (Fig. 4A), based on 103 trematode sequences and 1221 homologous positions in the final alignment dataset, showed strong monophyletic support (bootstrap value = bv = 100%) from the order Plagiorchiida La Rue, 1957. It included three suborders: (1) Echinostomata (92%) represented by the families Echinochasmidae (100%), Psilostomidae (91%), Hismanthlidae (100%), Fasciolidae (100%) and Echinostomatidae (68%); (2) Opisthorchiata (100%) represented by the families Opisthorchiidae

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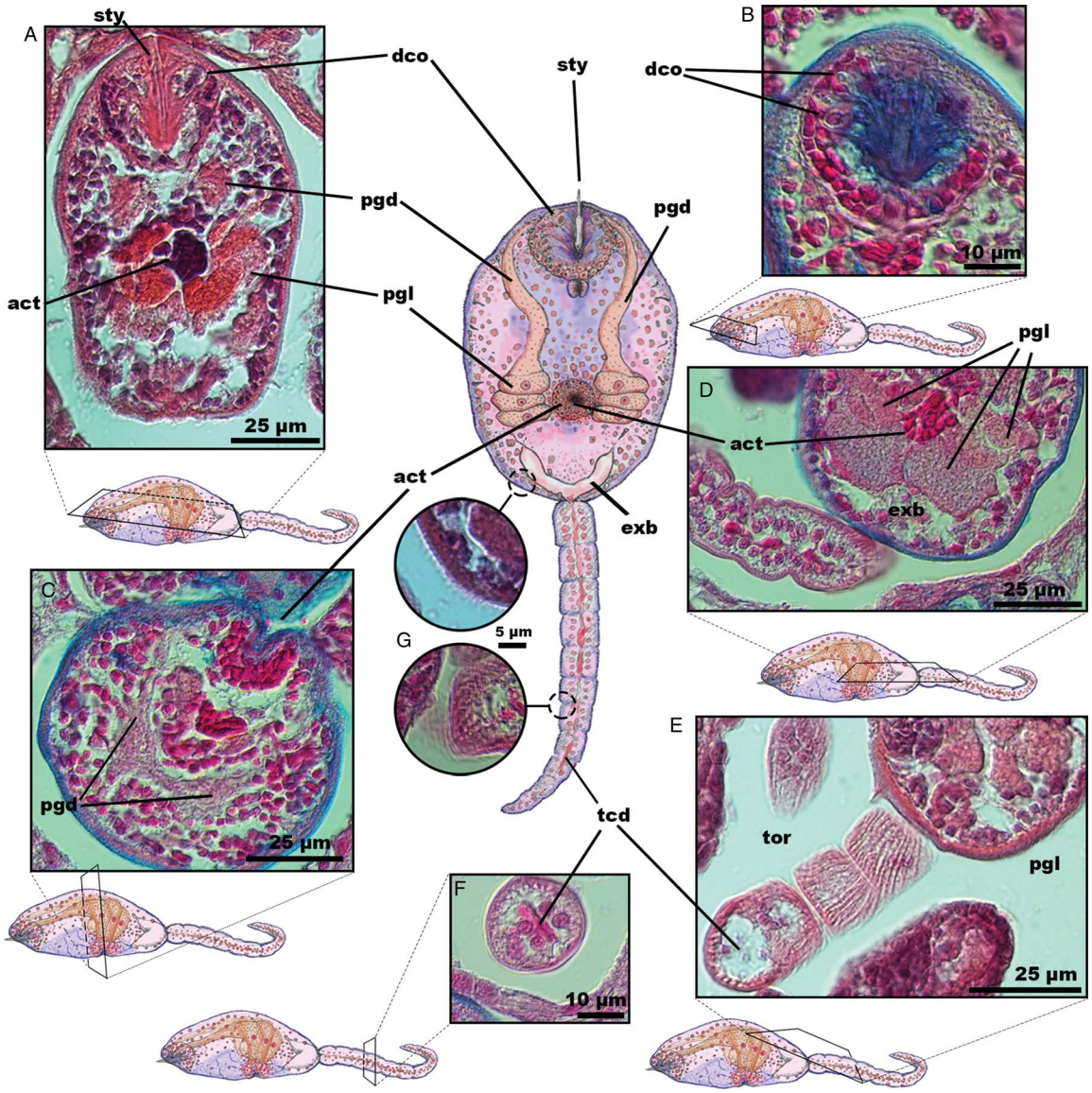

Fig. 3. Anatomy of the cercaria from *P. americanista*. (A and E) Haematoxylin-eosin stained intramolluscan cercariae. (B–D and F) Trichrome stained intramolluscan cercariae. (G) Spines around the parasite's body and tail. act, acetabulum; dco, duct opening; exb, excretory bladder; pgd, penetration gland ducts; pgl, penetration glands; sty, stylet; tcd, tail's central duct; tor, tail ornamentation.

(100%) and Heterophyidae (99%); and (3) Xiphidiata (72%). This latter clade is a monophyletic group of trematodes in which the cercarial stage carries a stylet in their oral sucker. In Xiphidiata, the superfamily Microphalloidea (100%) was grouped with five monophyletic families: Omphalometridae (100%), Haematoloechidae (98%), Telorchiidae (94%), Plagiorchiidae (100%) and Choanocotylidae (67%). The superfamily Microphalloidea was composed of the families Pleurogenidae (54%), Collyriclidae (a single sequence, JQ231122), Prosthogonimidae (99%), Microphallidae (82%), Stomylotrematidae (100%), Phaneropsolidae (100%), Lechithodendriidae (100%) and *Cercaria nigrospora* (MK259981). The families Phaneropsolidae (sequences KY982862, KJ700422 and MH532430) and Stomylotrematidae were sister taxa (74 and 72% in Fig. 4A and B, respectively). Sequences (KY982863 and MF155659) of adults of *S. vicarium* were strongly grouped with the trematode larvae found in *P. americanista* (Fig. 4A and B), with a genetic distance <0.02 and 0.05%, respectively.

Figure 4C shows the ITS1 ML phylogenetic tree. There were no representatives of Stomylotrematidae in the database. Both trematode sequences found in apple snails, MW481318 from *P. americanista* (this work) and MH532426 from *Asolene platae* (Dellagnola *et al.*, 2019*a*) were grouped together (62%). Both sequences were sisters with the sequence JQ231122 (*Collyriclum faba*, Collyriclidae, 66%). Also, these sequences were grouped with a sequence (HQ650133) belonging to Microphallidae (74%). Pleurogenidae, Prosthogonimidae or Lecithodendriidae sequences were not found in the public DNA databases. The whole monophyly of all trematode families was conserved, being the exception of the paraphyletic Heterophyidae.

Discussion

The complete life cycle of *S. vicarium* (Stomylotrematidae, Xiphidiata) remained incomplete for more than 100 years, from the original description of the adult parasite by Braun in 1901. Here, a morphological, morphometric and molecular study allowed us to identify for the first time the larvae of *S. vicarium* living in the digestive gland of the ampullariid *P. americanista*.

Phylogeny

Both ML trees placed the xiphidiocercaria of *P. americanista* inside the superfamily Microphalloidea Ward 1901, a monophyletic group of stylet-bearing trematodes from the suborder

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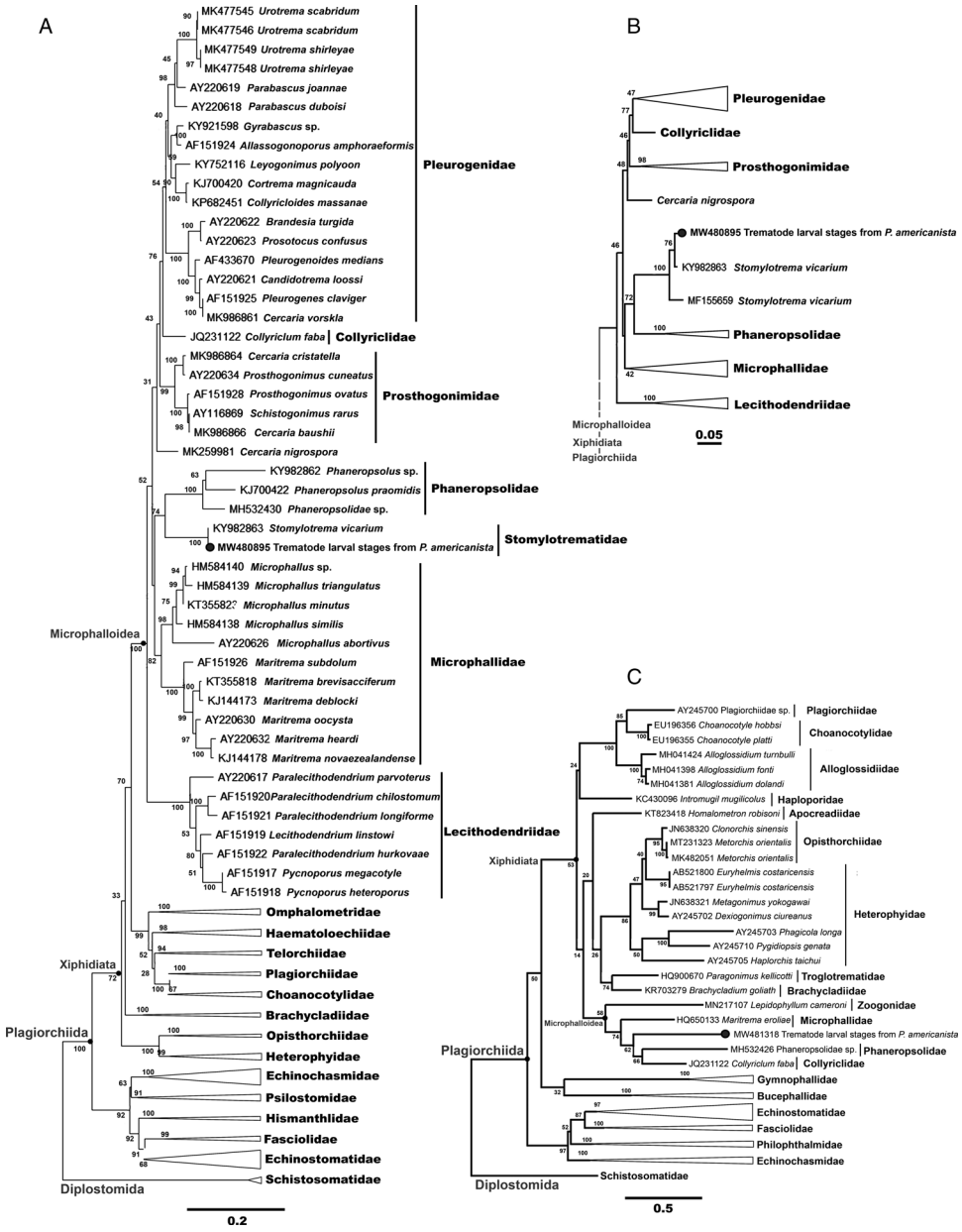

Fig. 4. Phylogeny of the trematode larval stages of *P. americanista*. (A) Large rRNA 28S ML tree based on 103 trematode taxa and 1221 homologous positions. The families of trematodes are compressed, except for those sequences of the superfamily Microphalloidea. (B) Short rRNA 28S ML tree based on 104 trematode taxa with 1068 homologous positions. The families of trematodes are compressed, being the exception of those sequences of Stomylotrematidae. For simplicity, only the Microphalloidea subtree is shown. (C) Complete ITS1 ML tree based on 53 taxa with 1822 homologous positions. The families of trematodes are compressed, being the exception of those sequences of the order Plagiorchiida. In the three trees, sequences of Schistosomatidae (Diplostomida) were used as outgroup. The bootstrap values are shown in each node. The black bars represent the number of substitutions per site.

Xiphidiata. The rRNA 28S trees supported the monophyletic status of Stomylotrematidae as an independent clade from Pleurogenidae, as was hypothesized by Kanarek *et al.* (2017). Phaneropsolidae was the sister clade of Stomylotrematidae, which also supports their status as an independent family (Kanarek *et al.*, 2014, 2017; Bell *et al.*, 2018; Dellagnola *et al.*, 2019*a*), while Lecithodendriidae remained as a basal group inside of Microphalloidea (Tkach *et al.*, 2003; Kanarek *et al.*, 2014; Dellagnola *et al.*, 2019*a*). The rRNA 28S trees grouped sequences of *S. vicarium*; i.e. those from larvae found in the apple snail *P. americanista* (this study) and from adults reported in the bird *Sclerurus mexicanus* (KY982863) and in the opossum *Philander opossum* (MF155659). Sequences from *S. vicarium* showed a low genetic distance (0.02–0.05%). These findings indicate that *P. americanista* is an early intermediate host of larvae stages *S. vicarium*.

The ITS1 tree showed conserved high-rank phylogenetic relationships. Collyriclidae was a clade derived from Microphalloidea, but this phylogenetic hypothesis should be interpreted with caution since none of the ITS1 sequences of the *insertae sedis C. nigrospora*, Prosthogonimidae and Pleurogenidae have been deposited in the molecular database. The sequence similarity between the xiphidiocercaria from *P. americanista* and those from the order Plagiorchiida could indicate the limited availability of sequences in the ITS1 database.

Morphological traits

The cercaria of *S. vicarium* from *P. americanista* showed three penetration glands, a morphological feature that appears to be a synapomorphy in Stomylotrematidae (Pinto *et al.*, 2015, this paper). The number of pairs of penetration glands would be

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Table 3. Hosts and remarks of the *Stomylotrema vicarium* life cycle

Stage	Host	Locality and country	Morphotype	Methodology of determination	Reference
Second (cercaria)	Aquatic snails				
	Caenogastropoda, Ampullariidae				
	Pomacea americanista	Misiones, Argentina	Xiphidiocercaria	Morphology and molecular ID; rRNA 28S sequence MW480895 and ITS1 sequence MW481318	This study
Third (metacercaria)	Aquatic insects				
	Coleoptera, Dytiscidae				
	Megadytes glaucus	Buenos Aires, Argentina	Unencysted and encysted metacercaria	Morphology	Ostrowski de Núñez (1978)
	Hemiptera, Belostomatidae				
	Belostoma dilatatum	Rio Grande do Sul, Brazil	Encysted metacercaria	Morphology	Amato and Amato (2006)
Four (adult)	Birds				
	Accipitriformes, Accipitridae				
	Busarellus nigricollis	Formosa, Argentina	Adult worm	Morphology	Lunaschi *et al.* (2007)
	Buteogallus meridionalis				
	Charadriiformes, Charadriidae				
	Vanellus chilensis	Experimental infection	Adult worm	Morphology	Ostrowski de Núñez (1978)
	Charadriiformes, Laridae				
	Larus dominicanus	Argentina	Adult worm	Morphology	Szidat (1964)
	Galliformes, Phasianidae				
	Gallus	Experimental infection	Adult worm	Morphology	Ostrowski de Núñez (1978)
	Meleagris gallopavo	Florida, USA	Adult worm	Morphology	Hon *et al.* (1978)
	Gruiformes, Gruidae				
	Antigone canadensis	Florida, USA	Adult worm	Morphology	Forrester *et al.* (1975)
	Pelecaniformes, Ardeidae				
	Egretta caerulea	Maria la Gorda, Cuba	Adult worm	Morphology	Macko *et al.* (1999)
	Nyctanassa violacea	Louisiana, USA	Adult worm	Morphology	Lumsden and Zischke (1963)
	Pelecaniformes, Threskiornithidae				
	Theristicus caerulescens	Brazil	Adult worm	Morphology	Braun (1901)
	Eudocimus albus	Florida, USA	Adult worm	Morphology	Bush and Forrester (1976)
	Paseriformes, Furnariidae				
	Sclerurus mexicanus	Cordillera Azul National Park, Peru	Adult worm	Morphology and molecular ID; rRNA 28S sequence (KY982863)	Kanarek *et al.* (2017)
	Podicipediformes, Podicipedidae				
	Podiceps dominicus	Guanahacabibes Lake Reservation, Cuba	Adult worm	Morphology	Macko *et al.* (1999)
	Strigiformes, Strigidae				
	Bubo virginianus	Florida, USA	Adult worm	Morphology	Kinsella *et al.* (2001)
	Mammals				
	Didelphimorphia, Didelphidae				
	Philander opossum	Chiapas, Mexico	Adult worm	Morphology and molecular ID; rRNA 28S sequence (MF155659)	Ramírez-Cañas *et al.* (2019)

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Table 4. Morphometric comparison between early larvae from stomylotrematid trematodes [mean (μm), range (μm; brackets) and number of samples]

Species	*Stomylotrema vicarium*		*Stomylotrema gratiosus*
Larval stage and host	Xiphidiocercaria of *P. americanista* (this study)	Young, stylet-bearing metacercariae of *M. glaucus* (Ostrowski de Núñez, 1978)	Xiphidiocercaria of *P. maculata* (Pinto *et al.*, 2015)
	Body		
Long	213 (182–241), 17	(150–270), 4	170 (157–177), 20
Wide	147 (64–132), 17	(101–192), 4	90 (82–102), 20
L/W	1.5 (1.3–1.6), 16	Not reported	Not reported
	Tail		
Long	245 (195–300), 9	Not reported	216 (147–272), 20
Wide	26 (24–29), 9	Not reported	28 (27–32), 20
	Oral sucker		
Long	56 (46–66), 19	(57–75), 4	57 (53–62), 20
Wide	59 (44–69), 19	(50–71), 4	50 (46–55), 20
	Stylet		
Long	36 (25–43), 6	(38–42), 4	45 (43–53), 20
Wide	5 (3–7), 6	(4–8), 4	Not reported
	Other morphological features		
Tail/body ratio	1.19 (1.0–1.3), 6	Not reported	Not reported
Pairs of penetration glands	3	Not reported	3
Virgula organ	Absent	Not reported	Absent
Excretory bladder	V-shaped	V-shaped	Not reported

variable in the basal clades of Microphalloidea (3 in Stomylotrematidae; 2 or 4 in Microphallidae; 3 in *C. nigrospora*; 2, 3 or 4 in Lecithodendriidae or Phaneropsolidae). On the other hand, the presence of four pairs of penetration glands could be a synapomorphy in the derived clades Collyriclidae, Pleurogenidae and Prosthogonimidae (Heneberg *et al.*, 2015; Kudlai *et al.*, 2015; Dellagnola *et al.*, 2019*a*; Shchenkov *et al.*, 2020).

The virgula organ does not follow a consistent pattern into Microphalloidea. It has been hypothesized that the virgula is a synapomorphy from 'lecithodendriid-like' flukes, formally Lecithodendriidae, Gyrabascidae, Phaneropsolidae and Leyogonimidae (Lotz and Font, 2008). However, some cercariae of these taxa have no virgula (Kudlai *et al.*, 2015; Dellagnola *et al.*, 2019*a*; Shchenkov *et al.*, 2020). Furthermore, the shape and size of this organ are highly variable (Shchenkov *et al.*, 2020) indicating that this organ would have evolved independently several times.

Life cycle

The complete life cycle of *S. vicarium* is hypothesized in Table 3. The miracidium hatches from an egg released in the feces of Neotropical birds (Ostrowski de Núñez, 1978; Macko *et al.*, 1999; Lunaschi *et al.*, 2007, Kanarek *et al.*, 2017). After that, it swims up to find an individual of the apple snail (as *P. americanista*) and forms sporocysts-containing cercariae (Fig. 1). The apple snail *P. canaliculata* has also been hypothesized as the first intermediate host of *S. vicarium* (Ostrowski de Núñez, 1978), although it has not been displayed. So, a non-virgulate xiphidiocercaria (Figs 1–3) is released from the snail digestive gland and crosses through the intestine toward the aquatic environment. This xiphidiocercaria was similar in morphometry to the unencysted, stylet-bearing young metacercariae of *S. vicarium* reported in the visceral cavity of the dytiscid *Megadytes glaucus* (Coleoptera, Dytiscidae) (Ostrowski de Núñez, 1978). Also, metacercaria develops inside of the coelomic cavity of the aquatic insects *M. glaucus* (Coleoptera, Dytiscidae) (Ostrowski de Núñez, 1978) and *Belostoma dilatatum* (Hemiptera, Belostomatidae) (Amato and Amato, 2006). The parasite's biological cycle is completed when these insects are eaten by several Neotropical birds, and then flukes develop in an adult individual. Recently, adults of *S. vicarium* have been identified in the mammal *P. opossum* (Ramírez-Cañas *et al.*, 2019) which indicates that the definitive host range could be broader than described so far.

Stomylotrematid flukes and apple snails

To date, two species of the congeneric stomylotrematid flukes *S. gratiosus* and *S. vicarium* have been associated with the apple snails *P. maculata* (Pinto *et al.*, 2015) and *P. americanista* (this work), respectively. The xiphidiocercaria of *S. gratiosus* was similar to the non-virgulate *Cercaria peculiaristylata* from *Pomacea glauca* (Nasir and Acuña, 1966) and *Cercaria marisa* from the ampullariid *Marisa cornuarietis* of Venezuela (Nasir and Díaz, 1968). Nevertheless, the xiphidiocercariae of *S. gratiosus* and *S. vicarium* may be differentiated by the morphometry of the body, tail and stylet (Table 4); however, this finding must be confirmed by molecular studies. Other xiphidiocercariae have been reported in apple snails (Damborenea *et al.*, 2006; Dellagnola *et al.*, 2019*a*) but the adult remains unknown.

Results presented here suggest two hypotheses that should be explored deeply: (1) Neotropical ampullariids act as reservoirs

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of different trematodes of the suborder Xiphidiata, (2) a first symbiotic association event occurred between an ancestor stomylotrematid trematode and ampullariid freshwater snail, which resulted in different species of flukes that were co-evolved with the species of the genus *Pomacea*. Further and integrated ecological, morphological and molecular studies must be done to clarify these hypotheses.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S003118202100158X

Data. Raw data are available at Figshare: Dellagnola, Federico; Campoy-Diaz, Alejandra, Vega, Israel (2021): 'First morphological and molecular identification of the cercaria of *Stomylotrema vicarium* from the endemic apple snail *Pomacea americanista*'. Figshare. Dataset. https://figshare.com/articles/dataset/Morphology_and_molecular_phylogeny_of_Stomylotrema_vicarium_in_a_first_intermediate_host/14510655.

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Author contribution. Federico A. Dellagnola: conceptualization, morphometric and molecular *in silico* analyses, writing – review and editing, graphical design, elaboration, and funding acquisition. Alejandra D. Campoy-Diaz: writing – review and editing. Israel A. Vega: conceptualization, formal analysis, resources, writing – review and editing, and funding acquisition.

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Ethical standards. Not applicable.

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