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Intercontinental distributions, phylogenetic position and life cycles of species of *Apharyngostrigea* (Digenea, Diplostomoidea) illuminated with morphological, experimental, molecular and genomic data ☆

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ABSTRACT

When subjected to molecular study, species of digeneans believed to be cosmopolitan are usually found to consist of complexes of species with narrower distributions. We present molecular and morphological evidence of transcontinental distributions in two species of *Apharyngostrigea* Ciurea, 1924, based on samples from Africa and the Americas. Sequences of cytochrome *c* oxidase I and, in some samples, internal transcribed spacer, revealed *Apharyngostrigea pipientis* (Faust, 1918) in Tanzania (first known African record), Argentina, Brazil, USA and Canada. Sequences from *A. pipientis* also match previously published sequences identified as *Apharyngostrigea cornu* (Zeder, 1800) originating in Mexico. Hosts of *A. pipientis* surveyed include definitive hosts from the Afrotropic, Neotropic and Nearctic, as well as first and second intermediate hosts from the Americas, including the type host and type region. In addition, metacercariae of *A. pipientis* were obtained from experimentally infected *Poecilia reticulata*, the first known record of this parasite in a non-amphibian second intermediate host. Variation in cytochrome *c* oxidase I haplotypes in *A. pipientis* is consistent with a long established, wide-ranging species with moderate genetic structure among Nearctic, Neotropic and Afrotropic regions. We attribute this to natural dispersal by birds and find no evidence of anthropogenic introductions of exotic host species. Sequences of CO1 and ITS from adult *Apharyngostrigea simplex* (Johnston, 1904) from *Egretta thula* in Argentina matched published data from cercariae from *Biomphalaria straminea* from Brazil and metacercariae from *Cnesterodon decemmaculatus* in Argentina, consistent with previous morphological and life-cycle studies reporting this parasite—originally described in Australia—in South America. Analyses of the mitochondrial genome and rDNA operon from *A. pipientis* support prior phylogenies based on shorter markers showing the Strigeidae Railliet, 1919 to be polyphyletic.

1. Introduction

Widely distributed species tend to be abundant throughout their ranges (e.g., Borregaard and Rahbek, 2010) and therefore should be among the first encountered in local surveys. However, molecular data rarely support wide distributions in digeneans from aquatic habitats, even those parasites maturing in and dispersed by wide-ranging terrestrial hosts. Freshwater digenean species thought to be cosmopolitan based on morphological records are often revealed by molecular data to consist of complexes of species with narrower distributions. Examples include *Clinostomum complanatum*, *Diplostomum spathaceum*, and *Hysteromorpha triloba*, which were commonly reported from multiple biogeographic regions until DNA sequences showed all three to be limited to

☆ *Note:* DNA sequences reported here are available in the NCBI database under GenBank accession numbers MK510081, MK570088, MT677870, MT679576-86, MT974151 (nucleotide sequences), BioProject PRJNA681586 (Illumina reads).

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the Palearctic (Galazzo et al., 2002; Dzikowski et al., 2004; Locke et al., 2018; López-Hernández et al., 2019). Moreover, species that appear widespread in early molecular studies are sometimes later resolved into distinct lineages from different biogeographic regions (e.g., *Echinostoma trivolvis* and possibly *Echinostoma robustum*, Detwiler et al., 2010; Georgieva et al., 2013, 2014; and see *Hypoderaeum conoideum* in Tantrawatpan and Saijuntha, 2020; Wiroonpan et al., 2021), further underscoring the rarity of truly broad distributions. We are aware of only 18 species of freshwater digeneans in which distributions spanning biogeographic regions are supported with molecular data (Table 1). In contrast, 10 recent molecular surveys collectively recorded 189 freshwater digeneans known only from single biogeographic regions (Supplementary Table S1).

When intercontinental ranges in freshwater digeneans have molecular support, mechanisms responsible for these unusually wide distributions are sometimes unclear. New molecular records showing a wide distribution might indicate that a parasite (i) has long been present, but overlooked, in parts of its range, or (ii) has recently expanded its geographic distribution. One way to distinguish among scenarios i and ii is to compare genetic diversity of parasite populations (e.g., Morgan et al., 2005), with the expectation that recently established populations will show a small subset of the genetic diversity of a source population. Hosts are also relevant. Finding a newly recorded parasite in a native host suggests long local history (i), while discovering it in an exotic host may indicate co-introduction (ii). However, the dispersal abilities of hosts affect the strength of these inferences. Among freshwater

Table 1
Digeneans with life cycles associated with freshwater habitats in which distributions spanning biogeographic regions have been demonstrated with molecular data. The countries where sequenced specimens originate are listed with the life stage sequenced in parenthesis (C, cercaria; M, metacercaria; A, adult) and markers studied (CO1, cytochrome *c* oxidase 1; ND, NADH dehydrogenase; ITS, internal transcribed spacer; mt, mitochondrial).

	Neotropic	Nearctic	Palearctic	Afrotropic	Indo-Malay	Australasia	Molecular marker	Source
Trichobilharzia querquedulae	Argentina (A)	Canada (A), USA (A)		South Africa (A)		New Zealand (A)	CO1, ND4, ITS	Ebbs et al., 2016
Schistosoma mansoni	Brazil (C, A), Venezuela (A), Guadeloupe (A), Puerto Rico (A)		Egypt (C, A)	11 sub-Saharan African countries (C, A), Madagascar (C)			2530 bp mt DNA	Morgan et al., 2005
Clinostomum heluans	Brazil (M), Bolivia (M)	Mexico (A)					CO1	Briosio-Aguilar et al., 2018
Austrodiplostomum compactum (as *A. ostrowskiae*)	Brazil (M)	USA (M)					CO1	Locke et al., 2015
Diplostomum sp. 14			People's Republic of China (M), Iraq (M)	South Africa (M)			CO1	Hoogendoorn et al., 2020
Diplostomum sp. 16			Iraq (M)	South Africa (M)			CO1	Hoogendoorn et al., 2020
Posthodiplostomum centrarchi[a]		Canada (M)	Spain (A), Portugal (M), Bulgaria (M), Solavakia (M), Czech Republic (M)				CO1, ITS	Kvach et al., 2017; Stoyanov et al., 2017
Posthodiplostomum sp. 8[a]		Canada (M)	Portugal (M)				ITS	Kvach et al., 2017
Cotylurus cornutus		Canada (C)	Norway (C)				CO1	López-Hernández et al., 2019
Echinostoma caproni			Egypt	Madagascar, Cameroon			CO1, ND1	Morgan and Blair, 1998a
Echinostoma paraensei	Brazil					Australia (C)	ND1	Morgan and Blair, 1998b
Echinostoma miyagawai			Poland (A), Czech Republic (A, C), Germany, Spain (A)			Australia (M)	ND1	Georgieva et al., 2014
Fasciola hepatica	Peru (A), Uruguay		Spain, Italy, Ireland, Iran. China			Australia	ITS1, ND1	Ichikawa-Seki et al., 2016
Fasciola gigantica			Egypt, Japan	Nigeria (A), Zambia	Vietnam, Thailand, Indonesia		ND1, CO1, pepck and pold RFLP	Ichikawa-Seki et al., 2017
Centrocestus formosanus[a]	Peru (C)				Vietnam, Thailand		28S	Pulido-Murillo et al., 2018
Haplorchis pumilio[a]	Brazil (C, M [b], A [b]), Peru (C), Mexico (M)				Thailand (M)		CO1, ITS2, 28S	Lopes et al., 2020; Martínez-Aquino et al., 2019
Philophthalmus gralli[a]	Peru (A), Costa Rica (C)				Bangladesh (A)		ITS1, ITS2, CO1, ND1	Biswas et al., 2021
Drepanocephalus spathans	Brazil (C), Mexico (A)	Canada (A), USA (C, A), Mexico (A)					CO1, ND1, ITS	Hernández-Cruz et al., 2018

[a] Wide range associated with introduction of intermediate host.
[b] Experimental infection.

digeneans, intermediate hosts often have more strictly aquatic life cycles and lower dispersal ability, and may therefore constrain parasite distributions to a greater extent than definitive hosts. For example, the wide distributions of at least five of the 18 species in Table 1 are associated with introductions of intermediate hosts into new biogeographic regions, and Ebbs et al. (2016) discuss the possible role of another invasive intermediate host, *Physa acuta*, in maintaining the global distribution of *Trichobilharzia querquedulae*.

Species of the genus *Apharyngostrigea* Ciurea, 1927 are well suited for testing wide distributions based on morphological taxonomy, with pre-molecular records of several species indicating transcontinental ranges spanning biogeographic regions. Members of this genus mature mainly in ardeids (Ukoli, 1967; Dubois, 1968), highly mobile avian hosts (Kushlan and Hancock, 2005) that make broad distributions plausible. Known life cycles involve freshwater intermediate hosts, namely planorbid snails from which emerge distinctive furcocercous cercariae forming metacercariae in fish or amphibians (Olivier, 1940; Yamaguti, 1975; Ostrowski de Núñez, 1989). Mitochondrial (mt) DNA has been sequenced in a limited number of samples in the Americas (Locke et al., 2011; Hernández-Mena et al., 2014; López-Hernández et al., 2019), but available sequences from samples in other regions are from slowly evolving nuclear rDNA markers (Tkach et al., 2001; Olson et al., 2003; Kim et al., 2020). This situation has effectively prevented molecular assessment of the geographic distribution of species of *Apharyngostrigea*.

Here we describe molecular and morphological data from two species of *Apharyngostrigea* variously sampled from intermediate and definitive hosts from the Americas and Africa. *Apharyngostrigea pipientis* (Faust, 1918) was recovered in Africa and throughout the American continents. Data also support prior reports of the Australian species *Apharyngostrigea simplex* (Johnston, 1904) in South America. Whole mt and complete nuclear rDNA sequences also provide insights on the phylogenetic position of the genus *Apharyngostrigea*.

2. Materials and methods

2.1. Specimen collection and morphological study

Snails, amphibians and birds infected with *A. pipientis* or *A. simplex* were collected in Tanzania, Argentina, Brazil, Canada and the United States (Table 2). *Apharynogstrigea simplex* (n = 110 worms) were taken from the intestine of an *Egretta thula* shot in June of 2017 in Argentina with authorization from the Ministerio de Desarrollo Agrario de la provincia de Buenos Aires (Disposición 95/14).

Apharyngostrigea pipientis were found in the intestines of salvaged carcasses of *Botaurus lentiginosus* (≈50 worms) and *Nycticorax nycticorax* (three worms) in 2012 at Le Nichoir avian rehabilitation centre in Hudson, Quebec, Canada. A single specimen of *A. pipientis* was obtained from a nestling of *Ardea herodias* that died during a nesting survey on Ile aux Herons, offshore from Montreal, Canada, in 2006. Four adult *A. pipientis* were collected from *Ardea alba* at Marcela Farm near El Colorado, Formosa Province, Argentina, in 2016. In the Mwanza Gulf of Lake Victoria, Tanzania, five adult *A. pipientis* were sequenced from two of 12 infected (of 33 examined) *Ardea cinerea* (intensities from 1-29 worms) and six adult worms were sequenced from four of five infected *Ardea alba* (of 21 examined) (intensities 1–15) in 2012 (see below for sequencing methods). A metacercaria of *A. pipientis* was obtained in 2009 from *Rana pipiens* in North Dakota. Cercariae of *A. pipientis* were obtained from *Biomphalaria straminea* and *Biomphalaria tenagophila* collected during long term malacological studies carried out in urban lakes from Belo Horizonte, and Contagem, state of Minas Gerais, Brazil. Snails were placed individually in 24-well plates with approximately 3 mL of dechlorinated water, subjected to artificial photostimulation for 2 h, and examined with a stereomicroscope to detect infection. Additionally, samples of cercariae from *B. straminea* from the Pampulha Reservoir, Belo Horizonte, were used to experimentally infect laboratory-reared *Poecilia reticulata*, with the approval of the Ethics Committee on Animal Use of the Universidade Federal de Minas Gerais, Brazil (protocol 199/2009). For molecular study, we used two samples of cercariae obtained in 2019.

Specimens were preserved in 70% or 95% ethanol (after manual excystment, in the case of the metacercaria). Adults of *A. simplex* were stained with a 1:6 dilution in 96% ethanol of hydrochloric carmine, dehydrated, cleared in xylene, and mounted in Canada balsam. Measurements are given in micrometres (μm) unless otherwise stated, as the range followed by mean in parentheses. Adults of *A. simplex* were not drawn as Ostrowski de Núñez (1989) provided detailed morphological accounts of all developmental stages. An adult of *A. pipientis* from *A. alba* in Argentina was drawn with the aid of a camera lucida. Vouchers of Tanzanian material were not kept, but specimens were photographed before DNA extraction from whole worms.

Cercariae from *B. straminea* were stained with vital dyes (aqueous solution of 0.05% neutral red or 0.05% Nile blue sulfate) and examined under a light microscope in non-permanent preparations. Subsamples of cercariae were killed in water at 70 °C and fixed in 4% formalin. Measurements of fixed cercariae and experimentally obtained metacercariae were taken using a micrometer eyepiece under a light microscope. Photographs of these larval stages were taken with a Leica ICC50 HD digital camera mounted to a light microscope. Flame-cell patterns and sensory hair distributions were not studied.

2.2. Molecular study

DNA was extracted from one adult *A. simplex* using 100 μl of a 5% Chelex suspension in deionized water and 2 μl of proteinase K, an overnight incubation at 56 °C, then at 90 °C 8 min and centrifuged at 13,148*g* for 10 min. One μl of the supernatant was used as a template for PCR with primers BD1F-BD2 R (Luton et al., 1992) and Dice 1F, Dice 11 R and Dice 14 R (Van Steenkiste et al., 2015). PCR products obtained with Master Mix (Productos Bio-Lógicos, Argentina) were visualized in 1% agarose gel electrophoresis stained with ethidium bromide and sequenced by Macrogen Inc. (South Korea). Extraction, amplification and sequencing of DNA from adults and metacercariae of *A. pipientis* was conducted at the Canadian Centre for DNA Barcoding (Canada) using the primers and protocols of Moszczynska et al. (2009) or Van Steenkiste et al. (2015) for CO1. DNA was extracted from cercariae of *A. pipientis* using the QIAamp DNA micro kit following the manufacturer's instructions. The dosage of the obtained DNA was determined in a microvolume spectrophotometer (NanoDrop® ND-1000). The PCR also used the primers and conditions described by Van Steenkiste et al. (2015), and purification of amplicons and sequencing were as described in López-Hernández et al. (2019).

Contigs of the foregoing sequences were edited and aligned with Geneious (Kearse et al., 2012) and using MEGA X (Kumar et al., 2018). One specimen of *A. pipientis* from the aforementioned salvaged *B. lentiginosus* carcass was used for Illumina sequencing, after DNA extraction with a Qiagen DNEasy blood and tissue kit (GmbH, Germany), following the manufacturer's protocol with two 200 μL elutions yielding 164.8 ng of DNA (Qubit dsDNA assay). This DNA was shotgun sequenced in one tenth of a lane on an Illumina HiSeq 4000 and 150-bp paired-end libraries were built with Nextera adapters at Genewiz (NJ, USA). Illumina reads were assembled into an rDNA operon using as an initial scaffold a consensus from an alignment of sequences from *Cardiocephaloides medio-*

Table 2
Origins and GenBank accession numbers of DNA sequences generated or analyzed in the present study. In *Apharyngostrigea pipientis*, unique haplotypes of partial cytochrome *c* oxidase 1 (CO1) are assigned identifiers H1-H9.

Species	Host	Locality	GenBank						Source[b]
			CO1 (haplotypes)	ITS	Mitochondrial genome	rDNA operon	18S	28S	
Apharyngostrigea pipientis									
	Ardea alba egretta	El Colorado, Formosa, Argentina	MT943785-6 (H1, H2)						1
	A. alba	Mwanza, Lake Victoria, Tanzania	MT943772-6, MT943778 (H2, H4, H4, H4, H4, H8)						1
(as *A. cornu*)	*A. alba*	Pánuco, Veracruz, Mexico	JX977777 (H3)	JX977837					2
	Ardea cinerea	Mwanza, Lake Victoria, Tanzania	MT943768-71, MT943777 (H1, H2, H4, H5, H5)						1
	Ardea herodias	Heron Island, Lake St. Louis, Montreal, Canada	MT943784 (H6)						1
	Biomphalaria straminea	Lagoa Varzea das Flores, Contagem, Minas Gerais, Brazil	MT943766 (H7)						1
	Biomphalaria tenagophila	Belo Horizonte, Minas Gerais, Brazil	MT943767 (H1)	MT974151					1
	Botaurus lentiginosus	Montreal area, Quebec, Canada	MT943782 (H6)		MT679576	MT677870			1
(as *A. cornu*)	*Butoroides virescens*	Tamiahua, Veracruz, Mexico	JX977778 (H2)	JX977838					2
	Nycticorax nycticorax	Montreal area, Quebec, Canada	MT943779-81 (H1, H6, H6)						1
(as *A. cornu*)	*N. nycticorax*	El Huizache, Sinaloa	JX977779 (H1)	JX977839			MF398363	MF398345	2, 3
	Rana pipiens	Tewaukon National Wildlife Refuge, North Dakota, USA	MT943783 (H9)						1
	R. pipiens	Boucherville, Quebec, Canada	HM064883-7 (H1, H1, H2, H6, H6)	HM064966-9					4
	N. nycticorax	North Dakota, USA						JF820597	5
	One or more of *A. cinerea jouyi, Egretta intermedia, N. nycticorax*	South Korea					MT835237		6
Apharyngostrigea simplex									
	Egretta thula	Juancho Pond, Daireaux, Buenos Aires, Argentina	MK570088	MK510081					1
	B. straminea	Belo Horizonte, Minas Gerais, Brazil	MN179319						7
	Poecilia reticulata (exp.)	Belo Horizonte, Minas Gerais, Brazil		MN179273					7
	Cnesterodon decemmaculatus	La Plata, Buenos Aires, Argentina	MH777789-91						7
Apharyngostrigea cornu									
	A. herodias, Catostomus commersoni, Notemigonus crysoleucas, Pimephales notatus	Quebec, Canada	FJ477188, HM064894-901, JF769449-53	HM064969					4
	A. cinerea	Ukraine					AY222092	AF184264	8, 9

Table 2 (*continued*)

Species	Host	Locality	GenBank						Source[b]
			CO1 (haplotypes)	ITS	Mitochondrial genome	rDNA operon	18S	28S	
Apharyngostrigea sp. (as *A. cornu*)	*Nyctanassa violacea*	Cortadura, Veracruz, Mexico	JX977780	JX977840					2
(as *A. cornu*)	*A. alba*	Mexico					MF398562	MF398344	3
(as *A. pipientis*/*A. simplex*)[a]							AY245757		10
Parastrigea diovadena	*Eudicimus albus*	Mexico	JX977808	JX977748			MF398365	MF398348	2,3
Cardiocephaloides medioconiger	*Thalasseus maximus*	Florida, USA			MH536508	MH521247			11
Cotylurus marcogliesei	*Lophodytes cucullatus*	Quebec, Canada			MH536509	MH521248			11
Alaria americana	*Vulpes vulpes*	Nova Scotia, Canada			MH536507	MH521246			11
Hysteromorpha triloba	*Squalius cephalus*	Reggio Emilia, Italy			MH536511	MH521250			11
Diplostomum ardeae	*N. nycticorax*	Yauco, Puerto Rico			MT259035	MT259036			12
Diplostomum baeri	*Salmo trutta*	Northwest Highlands, Scotland			MT302208				13
Diplostomum pseudospathaceum	*Larus ridibundus*	Tovačov, Czech Republic			KR269763	KR269766			14
Diplostomum spathaceum	*L. ridibundus*	Chropyně, Czech Republic			KR269764	KR269765			14
Tylodelphys immer	*Gavia immer*	Quebec, Canada			MH536513	MH521252			11
Posthodiplostomum centrarchi	*A. herodias*	Quebec, Canada			MH536512	MH521251			11
Cyathocotyle prussica	*Gasterosteus aculeatus*	Hamburg, Germany			MH536510	MH521249			11
Clinostomum complanatum	*Squalius cephalus*	Reggio Emilia, Italy			MK814187	MK811210			15

[a] *Apharyngostrigea pipientis* in GenBank record; *A. simplex* in Dzikowski et al. (2003).
[b] 1, present study; 2, Hernández-Mena et al. (2014); 3, Hernández-Mena et al. (2017); 4, Locke et al. (2011); 5, Pulis et al. (2011); 6, Kim et al. (2020); 7, López-Hernández et al. (2019); 8, Olson et al. (2003); 9, Tkach et al. (2001); 10, Dzikowski et al. (2003); 11, Locke et al. (2018); 12, Locke et al. (2020); 13, Landeryou et al. (2020); 14, Brabec et al. (2015); 15, Locke et al. (2019).

coniger (MH521247), *Cotylurus marcogliesei* (MH521248) and *Tylodelphys immer* (MH521252) with default parameters in Geneious. The longest initial assembly (a 4460 bp fragment of 28S) was then used to seed iterative extensions until even and thorough coverage was obtained. The final assembly of the rDNA operon of *A. pipientis* was annotated through alignment with the foregoing sequences, as well as *Diplostomum* spp. (KR269765-6). The barcode sequence of CO1 from another specimen from the same individual *B. lentiginosus* (MT943782) was used to seed iterative extensions of the mitochondrial genome assembly with default parameters in Geneious (attempts to assemble *A. pipientis* reads to the mitochondrial genome of *Cardiocephaloides medioconiger* (MH536508) or *Cotylurus marcogliesei* (MH536509) were unsuccessful). The resulting mt genome assembly was annotated using MITOS2 (refseq 89, translation table = 5, Bernt et al., 2013) and through alignment with mt genomes from *Clinostomum* and diplostomoids (accession numbers are in Table 2).

To build phylogenies based on rDNA operons and mitochondrial genomes of *A. pipientis* and other published data, alignments constructed with MAFFT were stripped of gaps and less reliably aligned columns (identified using GUIDANCE2, Penn et al., 2010), and the GTR + G + I model evolution was selected based on the Bayesian Information Criterion in MEGA-X (Kumar et al., 2018; alignments are provided in Supplementary Data S1, S2). The same approach was used to generate trees based on barcode CO1 fragments and ITS sequences generated from Sanger sequencing, except that alignments were unambiguous and GUIDANCE2 was not used. Trees were constructed with 1000 bootstrap replicates with RAXML (Silvestro and Michalak, 2012; Stamatakis, 2014) and using Bayesian Inference (BI, Ronquist et al., 2012), the latter with four chains of Markov chain Monte Carlo searches sampled every 200 and printed every 1000 generations with 1,100,000 generations and 500 initial trees discarded, and rooted with sequences from *Clinostomum complanatum* (in the case of mt genomes or

rDNA operons) or *Parastrigea diovadena* (CO1 or ITS). Posterior probabilities of BI tree nodes are based on 4158 mitochondrial genome topologies (analysis manually stopped after average standard deviation of split frequencies <0.001) and 10,502 topologies of rDNA operons, CO1 barcodes or ITS sequences (all generations run).

Genetic structure in *A. pipientis* was assessed following the biogeographic divisions of both Wallace (1876) and Holt et al. (2013). However, we mainly follow Wallace (1876) (e.g., Table 1, and see below), because this scheme explained slightly more variation in CO1 sequences. In addition, the regions of Wallace (1876) seem more aligned with global freshwater fish distributions and the transitional, Neotropic-Nearctic, nature of freshwater animals, including fish parasites, in Mexico (Choudhury et al., 2016a, 2016b; Leroy et al., 2019), where some data analyzed here originate. A minimum spanning network of CO1 haplotypes from *A. pipientis* was constructed using PopART (Bandelt et al., 1999), which was also used to test for genetic structure among biogeographic regions using Analysis of Molecular Variance (AMOVA), based on a 413 bp alignment. Geographic structure among CO1 sequences from *A. pipientis* was also assessed by correlation of geographic distances (computed in PASSaGE 2, Rosenberg and Anderson, 2011) with genetic distances computed in MEGA X, and with ANOSIM of CO1 distances within and among biogeographic regions, both in Primer-E (Clarke and Gorley, 2015). These analyses of genetic structure and distance were conducted using CO1 sequences of *A. pipientis* from the present and prior studies (Locke et al., 2011; Hernández-Mena et al., 2014, JX977777-9).

2.3. Data accessibility

DNA sequences reported here are available in the NCBI database under GenBank accession numbers MK510081, MK570088, MT677870, MT679576-86, and MT974151 (nucleotide sequences) and BioProject PRJNA681586 (Illumina reads). Voucher specimens are deposited in the Helminthological Collection of the Museo de La Plata, La Plata, Argentina (MLP-He 7506, MLP-He 7692).

3. Results

3.1. Descriptions and taxonomic remarks

Apharyngostrigea simplex (Johnston, 1904) (Fig. 1, Table 3)

Description of gravid adults. Body slender. Forebody cup-shaped, with large opening and covered with minute spines. Hindbody claviform, dorsally curved. Ratio of forebody length to hindbody length 1: 2.8–3.8. Neck region occupying 39–47% (43%) of hindbody. Suckers well developed, ventral larger than oral. Pharynx absent. Proteolytic gland in intersegmental region. Testes tandem, deeply lobed, in posterior middle of hindbody. Seminal vesicle long and folded. Ovary ovoid. Vitelline follicles similar in size in both parts of body; in forebody with scarce follicles concentrated in base and extending up to acetabular region; in hindbody occupying almost whole width in preovarian region and extending ventrally to testes up to posterior end. Uterus with 8–15 eggs. Copulatory bursa poorly delimited, genital cone well delimited from body parenchyma, oriented obliquely to axis of copulatory bursa and covered with minute papillae; ejaculatory duct and uterus join at base of genital cone forming a hermaphroditic duct; genital atrium with large opening. Excretory vesicle and pore not observed.

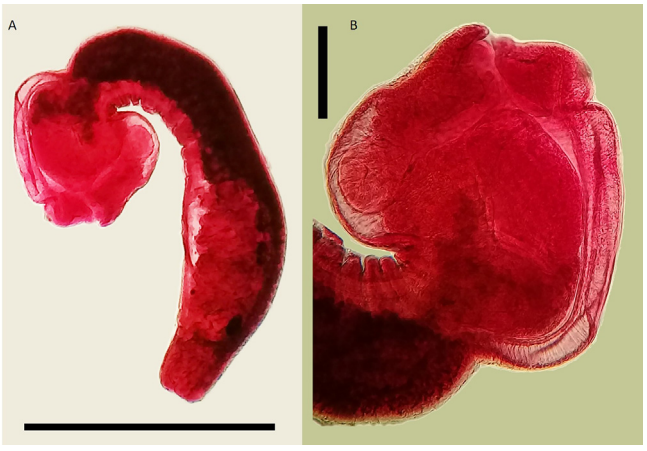

Fig. 1. *Apharyngostrigea simplex* (Johnston, 1904) from *Egretta thula*, Daireux, Argentina. (A) Whole mount, scale bar = 1000 μm; (B) forebody, scale bar = 200 μm.

Host: *Egretta thula* (Molina) (Ardeidae)
Locality: Juancho Pond, Daireaux (36°42′32.4″S; 61°33′00.8″W), Buenos Aires Province, Argentina.
Date of collection: June, 2017.
Site of infection: Intestine.
Voucher specimens: MLP-He 7506.

Remarks: The adults of *A. simplex* found in *E. thula* in the present study are similar to those described by Ostrowski de Núñez (1989) from the same host in Argentina and Dubois and Pearson (1965) in various hosts in Australia, in the shape of testes, position of proteolytic gland and ovary, distribution of vitelline glands in forebody and in most metrical characters (see Table 3).

The CO1 sequences of these adults obtained in the present study (MK570088, MK510081) match data from López-Hernández et al. (2019) (MN179319, MN179273, MH777789-91; see further below) that originate from metacercariae in *Cnesterodon decemmaculatus* (Jenyns) (La Plata, Buenos Aires, Argentina) and experimentally infected *Poecilia reticulata*; and cercariae shed by *Biomphalaria straminea* (Belo Horizonte, Minas Gerais, Brazil). López-Hernández et al. (2019) discussed the similarity of the cercariae and metacercariae to those described by Ostrowski de Núñez (1989) from *C. decemmaculatus* and *B. straminea*.

Apharyngostrigea pipientis (Faust, 1918) Figs. 2, 3; Tables 4–6)

Description of gravid adults. Body virguliform, covered with minute spines. Forebody with median opening. Hindbody strongly recurved. Ratio of forebody length to hindbody length varying with specimen contraction. Suckers well developed. Pharynx absent. Proteolytic gland compact, in intersegmental region, overlapping base of holdfast organ lobe and extending into hindbody. Testes tandem, lobed, located in posterior two thirds of hindbody. Seminal vesicle long and folded. Ovary oval to reniform. Mehlis' gland intertesticular. Laurer's canal opening dorsally. Vitelline follicles similar in size in both parts of body, extending into holdfast organ lobes and body wall to variable extent, up to anterior limit of forebody, occupying nearly entire width of hindbody in preovarian region and extending ventrally to testes up to posterior end, less dense at junction between fore- and hindbody. Uterus with up to 40 eggs. Copulatory bursa poorly delimited, genital cone small, genital atrium with small opening. Excretory pore ventrosubterminal.

Hosts: *Ardea alba egretta* Gmelin, 1789 (Ardeidae), *Ardea herodias* L., *Nycticorax nycticorax* (L.), *Botaurus lentiginosus* (Rackett, 1813); additional sequence-based records in *Ardea cinerea* L. and *Ardea egretta melanorhynchos* Wagler, 1827

Localities: *A. alba egretta* from La Marcela Farm, El Colorado, Formosa, Argentina (26°17′35″S, 59°08′38″W); *A. herodias*, *N. nycticorax*, *B. lentiginosus* from Greater Montreal Area, Quebec, Canada; *A. cinerea* and *A. egretta melanorhynchos* from Mwanza Gulf, Lake Victoria, Tanzania.

Table 3
Comparative measurements of adult *Apharyngostrigea simplex* (Johnston, 1904) from the present and prior studies.

Host	*Egretta thula*		*Egretta novaehollandiae, Egretta garzetta, Ardea intermedia plumifera*
Locality	Daireux, Argentina	Buenos Aires Zoological Garden Argentina	Australia
Source	Present study	Ostrowski de Núñez, 1989	Dubois and Pearson, 1965
Body length (BL)	2269–2369 (2319)	2017–4881 (2823)	2490–3800
Forebody (FB)	469–629 × 600–638 (549 × 619)	462–1215 × 364–1045 (690 × 593)	640–950 × 370–780
Hindbody (HB)	1741–1800 × 425–459 (1770 × 442)	1290–3666 × 316–972 (2153 × 525)	1540–3090 × 320–700
HB/FB	2.8–3.8 (3.3)	2.2–6.9 (5.7)	1.6–4.3 (2.9)
Oral Sucker (OS)	69 × 71	63–185 × 71–168 (129 × 113)	110–165 × 90–110
Ventral Sucker (VS)	119–169 × 145–167 (144 × 156)	126–268 × 134–252 (191 × 186)	150–200 × 130–157
VS width/OS width	2.03	1.5–1.9 [a]	1.43–1.44
Proteolytic gland (PG)	145 × 217	151–378 × 117–252 (261 × 175)	250–400 × 110–220
PG length/FB length	0.31	–	0.28–0.58 (0.40)
BL/PG	15.66	6.3–16.8 (10.6)	–
Ovary	102–130 × 145–179 (116 × 162)	101–268 × 84–412 (147 × 182)	110–260 × 145–340
Anterior testis	251 × 242	269–630 × 210–840 (374 × 384)	190–530 × 200–420
Posterior testis	338 × 251	252–630 × 252–840 (387 × 393)	180–490 × 200–420
Copulatory bursa	275–295 × 266–275 (285 × 270)	–	–
Genital cone	145–266 × 121–126 (205 × 123)	–	160–260 × 130–200
Egg number	8–15 (12)	9–100	
Eggs	86–95 × 52–55 (92 × 54)	90–110 × 50–70	94–110 × 57–68
Ovary position in HB	39–47 (43)%	near midline	41–56 (49)%

[a] Calculated from measurements in Ostrowski de Núñez (1989).

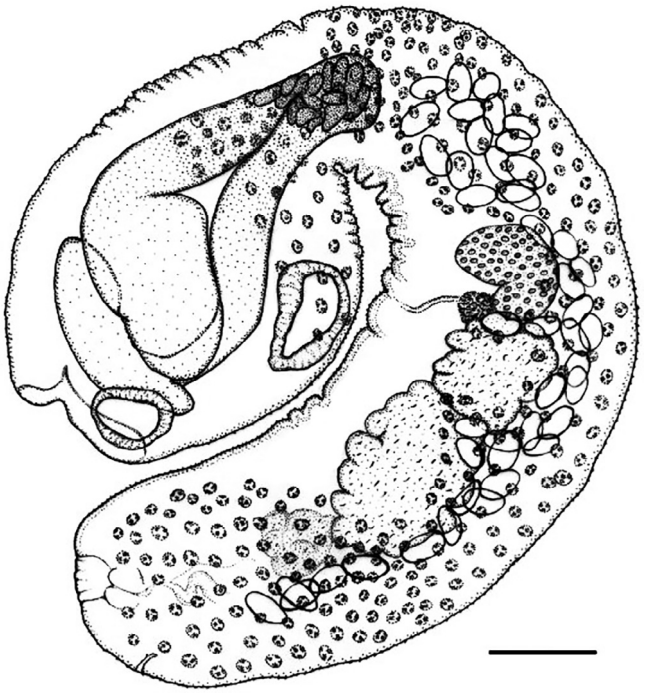

Fig. 2. *Apharyngostrigea pipientis* (Faust, 1918) from *Ardea alba*, Formosa, Argentina. Scale bar = 200 μm.

Site of infection: Intestine.
Voucher specimens: MLP-He 7692 (material from *A. alba egretta*).

See Table 2 for additional records based on molecular links (see below) in Tanzania, Brazil, Canada, United States and Mexico.

Apharyngostrigea pipientis, description of cercariae: Larva of furcocercous type. Body spinous, elongated. Anterior organ trapezoidal. Row of small spines in region of anterior organ. Pharynx small, oval. Caeca elongated, septate, ending after ventral sucker. Ventral sucker rounded, equatorial, bearing a crown of small spines. A pair of unpigmented eyespots in anterior portion of body. Four pairs of penetration glands arranged in two rows anterolateral to ventral sucker. Ducts of penetration glands directed to anterior region and dilated in region of anterior organ. Genital primordium rounded, in posterior region of body. Furcae long and wide, caudal stem swollen and transparent. Caudal stem of variable shape depending on contraction. Larvae produced by elongated sporocyst with evident birth pore.

Hosts and provenance of cercariae: *Biomphalaria tenagophila* (d'Orbigny, 1835) (Planorbidae) (1/12 snails infected at a lake located at Minas Gerais state Administrative Center, Belo Horizonte, in 2019) and *Biomphalaria straminea* (Dunker, 1848) (Planorbidae) (9/16235 snails infected in the Pampulha Reservoir, Belo Horizonte, between 2009 and 2013 and 1/273 snails infected in Várzea das Flores Dam, Contagem, in 2019) from state of Minas Gerais, Brazil.

Larval forms were recovered from *P. reticulata* exposed to cercariae from naturally infected *B. straminea* from Pampulha Reservoir. At 16 days post infection (DPI), larvae were unencysted. Encysted metacercariae were in the abdominal cavity of *P. reticulata* by 25 DPI. General cyst morphology was of the tetracotyle type, including oval shape and thick external cyst wall.

Remarks: Adults of *A. pipientis* were first reported by Olivier (1940), who described worms smaller than those of subsequent reports, including our own, possibly because the specimens were obtained from an experimentally infected and unnatural host. Dubois (1968) noted the ratio of body segment lengths varies greatly depending on specimen contraction, which we suggest likely also affects the total body length and relative positions of the ovary and testes in the hindbody, thus contributing to variability seen across studies (Table 4). Adults we collected possessed a compact, relatively small proteolytic gland originating in the upper portion of the hindbody, which Dubois (1968, 1981) considered to be diagnostic. The specimens of *A. pipientis* we collected from Tanzanian birds were not studied in detail but included both immature, tetracotyle-like forms with slightly developed hindbodies, and adults up to 4 mm in length. Adult worms recently described in detail by Kim et al. (2020) from ardeids in Korea are unusually large but in other respects consistent with *A. pipientis*, except that we observed spines on the tegument, while Kim et al. (2020)

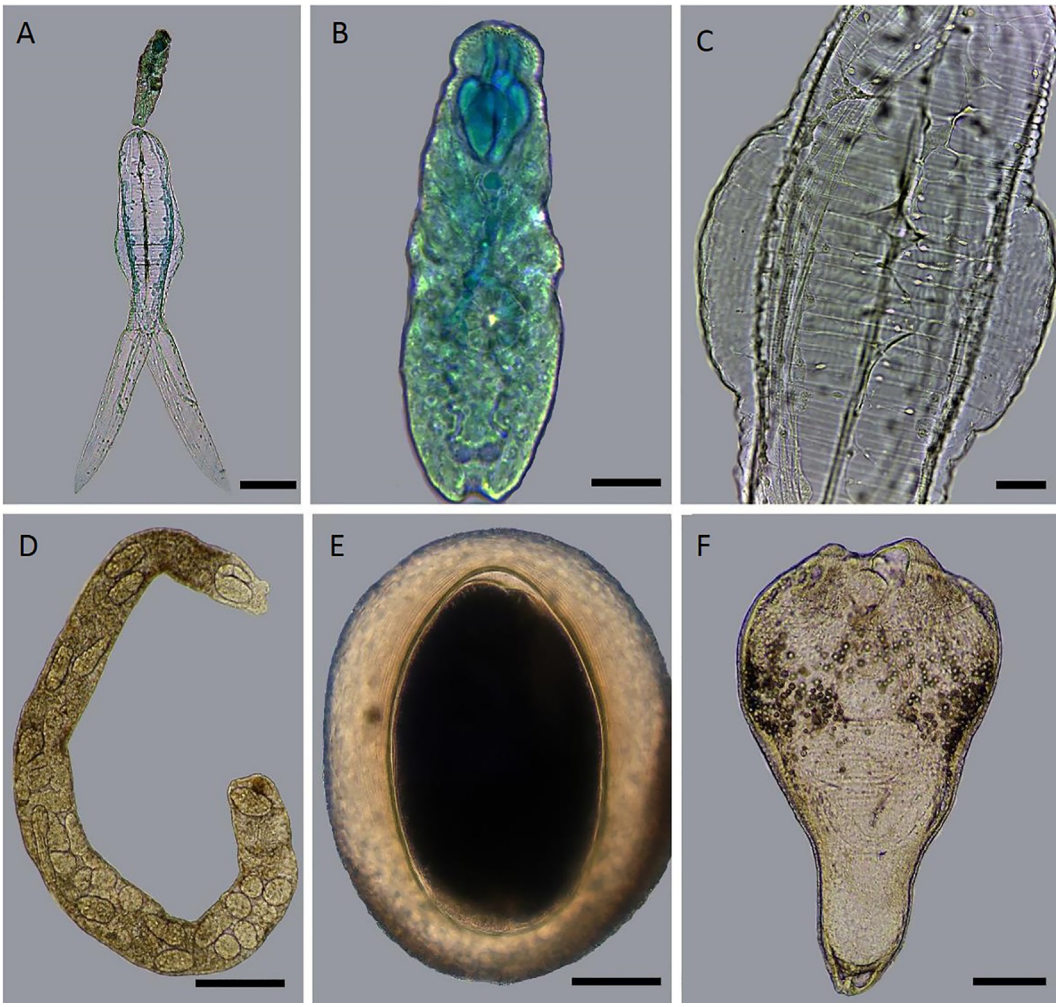

Fig. 3. Larval stages of *Apharyngostrigea pipientis* found in Brazil. Cercaria emerged from naturally infected *Biomphalaria straminea* (A). Detail of body (B) and tail stem (C). Sporocyst (D). Encysted (E) and mechanically excysted metacercariae (F) experimentally obtained in *Poecilia reticulata*. Scale bars: 100 μm (A, D–F), 20 μm (B, C).

reported the tegument to be aspinose; other authors have not commented on the adult tegument.

The morphology of cercariae of *A. pipientis* we collected from *B. straminea* in Brazil agrees with descriptions by Hughes (1928) and Olivier (1940) in the type region. These cercariae also resemble those Lutz (1931) found in *Biomphalaria tenagophila* (as *Biomphlaria immunis*) in Brazil. Interestingly, Lutz (1931) found tetracotyle-type metacercariae in *Poecilia vivipara* and *Leptodactylus latrans* (as *L. ocellatus*) exposed to these cercariae. As others have suggested (Hughes, 1928; Olivier, 1940; Dubois, 1968), Lutz (1931) misidentified this material as *Hysteromorpha triloba* (Rudolphi, 1819), probably due to the subsequent use of naturally infected second intermediate hosts in experimental infections of birds. The general morphology of the cercariae we studied, including the number and arrangement of the penetrating glands and the shape of the tail, also resemble others reported from planorbid snails from the Americas and Africa, including: *Apharyngostrigea* sp. found in *Biomphalaria glabrata* in Guadalupe (Nassi, 1987), *Cercaria hinchicauda* found in *Biomphalaria kuhniana* in Venezuela (Nasir and Diaz, 1973), Furcocercaria sp. III in *Biomphalaria occidentalis* and *B. tenagophila* in Argentina (Ostrowski de Núñez et al., 1991; Fernández et al., 2016), and *Cercaria inflaticauda* found in *Biomphalaria stanleyi* (as *Biomphalaria alexandrina stanleyi*, small morph of *Biomphalaria choanomphala*) in the Democratic Republic of Congo (Fain, 1953). These larvae may be conspecific with *A. pipientis*, but experimental or molecular evidence is required for an unequivocal determination. A report of cercariae of *A. pipientis* from the thiarid snail *Sermyla riqueti* sampled along coastal Thailand (Namchote et al., 2015) appears to represent a member of the superfamily Schistosomatoidea, and differs markedly from *A. pipientis* in the shape and size of the tail and furcae, and in the pigmentation of eyespots.

Encystment of metacercariae of *A. pipientis* in experimentally infected guppies (none encysted 16 DPI, all encysted 25 DPI) was consistent with the 18–23 DPI onset of encystment in experimentally infected *R. pipiens* (Olivier, 1940). In *A. simplex*, Ostrowski de Núñez (1989) recorded encystment in *C. decemmaculatus* 16 DPI. In contrast, in *P. vivipara* exposed to cercariae resembling *A. pipientis*, Lutz (1931) reported encystment just 1 DPI, suggesting the fish used in experiments may have harbored pre-existing, natural infections. Cysts and metacercariae of *A. pipientis* in the present study were morphometrically consistent with accounts by Faust (1918) and Hughes (1928), except for a wider tribocytic organ and somewhat greater total length in excysted worms. In the same reservoir where we collected *B. straminea* used in experimental infections, Pinto and Melo (2012) reported 8/60 guppies were naturally infected with metacercariae of *Apharyngostrigea* sp. that are morphometrically consistent with those of *A. pipientis*. Metacercariae of *A. pipientis* that we obtained experimentally, as well as those of Pinto and Melo (2012), were larger than metacercariaee

Table 4
Morphometric data from adult *Apharyngostrigea pipientis* (Faust, 1918) in the present and prior studies. Dimensions in μm given as range (mean), *n* measured.

Host	*Ardea alba egretta, Ardea herodias, Botaurus lentiginosus, Nycticorax nycticorax*	Domestic pigeon (exp.)	*Botaurus lentiginosus*	*Egetta tricolor* (as *Hydranassa tricolor*)	Ardeidae	*Ardea alba egretta* (as *Egretta alba egretta*)	*Ardea cinerea jouyi*, *N. nycticorax, Egretta intermedia*
Locality	Formosa, Argentina (*A. alba*), Quebec, Canada (all other hosts above)	Michigan, USA	Wisconsin and Michigan, USA	Louisiana, USA		Valparaiso, Chile	Republic of Korea
N specimens	10 (6 hologenophores, 4 paragenophores)	4	≈20			5 adults, 4 juveniles	
Note			As *Aphryngostrigea tenuis*		Includes data from *Apharyngstrigea duboisi* (Dubois, 1980)		
Source	Present study	Olivier, 1940	Dubois and Rausch, 1950	Lumsden and Zischke, 1963	Dubois, 1968	Dubois, 1981	Kim et al., 2020
Body Length (L)	1575–4000 (2906), 7	1650-1950[a]	2100–3650	2894[b] –4744	Up to 3650	1500–2200	4100–4800
Forebody (FB) L	650–1000 (823), 9	420–500	370–750	370–750	370–750		1000–12000
FB Width (W)	476–677 (577), 8	340–470	390–560	390–560	340–720		800–900
Hindbody (HB) L	850–3000 (2074), 7	1230–1450	1740–3150	1950[b]	1100–3150		3200–3600
HB W	300–551 (412), 8	230–280	300–480	313[b]	230–610		430–460
HB L/FB L	1.2–3.2 (2.5), 7	3.9[a]	3.6–6.3	2.5[b]	1.7–6.3		2.7–3.7[c]
Oral sucker L	103–153 (134), 4	length > width	96–110	182[b]	96–140		diameter 104–132
Oral sucker W	50–174 (99), 5	70–90	80–90	120[b]	70–125		
Ventral sucker L	120–251 (166), 6		120–190	148[b]	120–215		diameter 185–214
Ventral sucker W	104–172 (143), 6		110–145	170[b]	110–195		
Proteolytic gland L	121–320 (212), 4	110–130	215–315	388[b]	110–340	210	246[c] –353
Proteolytic gland W	183–270 (222), 4		155–200	183[b]	110–200	175	138[c] –177
Ovary L	160, 1	120–160	165–250	130[b]	80–190		260
Ovary W	87, 1	90–100	110–190	114[b]	120–250		96
Ovary position in HB	50 /100, 1	33–38 /100[a]	30–47 (35) /100	58 /100[b]	30–47/100		
Anterior testes L	238–338 (294), 4	150–190	315–530	195[b]	150–530		130
Anterior testes W	222–436 (291), 5	130–140	200–320	195[b]	130–320		260
Posterior testes L	238–413 (332), 5	150–200	340–630	268[b]	150–630		174
Posterior testes W	160–436 (283), 5	130–140	200–320	182[b]	130–320		243
Copulatory bursa W	260–464 (359), 4			216[b]			
N Eggs	0–40 (12), 4	6-11[a]			few to many		fairly numerous
Eggs	64–103 (85) × 45–60 (54), 13 eggs, 7 worms, 4 birds		85–95 × 52–70		78–95 × 47–70	87–95 × 48–55	96 × 62

[a] Estimated from Figs. 25, 28, 29 and 30 in Olivier, 1930.
[b] Estimated from Fig. 6 in Lumsden and Zischke, 1963.
[c] Estimated from Fig. 1A, 3E and 4A in Kim et al., 2020.

Table 5
Morphometrics of cercariae and sporocysts of *Apharyngostrigea pipientis* (Faust, 1918) from naturally infected snails in Brazil, with data from Olivier (1940) for comparison. Data given as range in μm with mean in parenthesis. L, length; W, width.

		Present study	Olivier, 1940
Locality		Brazil	Douglas Lake Michigan, USA
Host		*Biomphalaria straminea*	*Planorbula armigera*
Body	L	157–204 (173)	93–174 (134)
	W	33–63 (52)	50–81 (57)
Oral sucker	L	37–52 (44)	36–59 (44)
	W	18–27 (23)	27–45 (31)
Ventral sucker	L	18–25 (21)	18–27 (23)
	W	17–25 (20)	–
Tail stem	L	239–389 (333)	174–391 (298)
	W	102–177 (135)	80–180 (130)
Furcae	L	116–253 (212)	118–229 (184)
	W	23–58 (42)	–
Sporocysts	L	1375–2923 (2149)	–
	W	102–184 (135)	–

of *A. simplex* studied by Ostrowski de Núñez (1989) (length 302–874, mean 481 × width 311–462, mean 347) and López-Hernández et al. (2019) (originally as *Apharyngostrigea* sp., but now linked to *A. simplex*, see Section 3.2).

3.2. Analysis of partial CO1 and nuclear rDNA sequences

Sequences of CO1 and ITS from *A. simplex* in the present study from *Egretta thula* in Argentina matched data obtained by López-Hernández et al. (2019) from cercariae from *B. straminea*, metacercariae from experimentally infected *P. reticulata* in Belo Horizonte, MG, Brazil, and metacercariae from naturally infected *C. decemmaculatus* in La Plata, BA, Argentina (see Table 7 for p-distances, and Figs. 4 and 5 for phylogenies).

Sequences of CO1 and ITS from *A. pipientis* adults, metacercariae and cercariae from Tanzania, Brazil, USA and Canada (see Table 2) matched previously published data from *A. pipientis* from *R. pipiens* in Montreal, QC, Canada (Locke et al., 2011) as well as three sequences identified as *Apharyngostrigea cornu* (JX977777-9) from Hernández-Mena et al. (2014) from ardeids in Mexico; as discussed below, we consider the latter to be *A. pipientis* misidentified as *A. cornu*. Sequences (JX977780 and JX977840) from another isolate (DNA1006) from the same study also identified as *A. cornu* are divergent from available data from *Apharyngostrigea* (including *A. cornu*), also suggesting misidentification of another, undetermined species of *Apharyngostrigea*.

The diversity of CO1 haplotypes of *A. pipientis* was not markedly different in any of the three biogeographic regions sampled, when sampling effort was considered, indicating no support for a recent expansion in any of these regions. Fourteen variable positions occurred in a 428 bp alignment of CO1 sequences from 29 *A. pipientis* sampled in Nearctic, Neotropic, and Afrotropic regions. More variable positions occurred in sequences from regions where more specimens were sequenced (Nearctic n = 11, eight variable sites; Afrotropic n = 11, five variable sites; Neotropic n = 7, two variable sites).

Nine CO1 haplotypes occurred among the 29 sequences of *A. pipientis* (Table 2, Fig. 4). Two common CO1 haplotypes of *A. pipientis* (5/29 and 6/29 sequences) occurred in all three biogeographic regions sensu Wallace (1876); two equally common haplotypes were unique to either Nearctic (5/29 sequences) or Afrotropic regions (6/29 sequences). Most Nearctic and Afrotropic CO1 haplotypes were unique to those regions, while the majority of Neotropic haplotype diversity was shared with other regions. Uncorrected CO1 p-distances were greater between specimens that were collected further apart (Spearman ρ = 0.271, P = 0.0005), a tendency driven by comparisons among biogeographic regions, in that the relationship was not significant if limited to comparisons within biogeographic regions (Spearman ρ = 0.167, P = 0.084). A similar magnitude of genetic structure between biogeographic regions was indicated by AMOVA of aligned haplotypes (Φ_{ST} = 0.364, P < 0.001) and by Analysis of Similarities (ANOSIM) of uncorrected p-distances (R = 0.385, P = 0.0002). Pairwise ANOSIMs of CO1 distances indicated Afrotropic *A. pipientis* were more distinct from those in the Americas (Afrotropic-Neotropic R = 0.500, P = 0.0007, Afrotropic-Nearctic R = 0.459, P = 0.0001), with less differentiation between Nearctic and Neotropic sequences (pairwise R = 0.198, P = 0.036). This is also illustrated by large proportion of haplotypes found only in the Afrotropic region (Fig. 4B, C).

Table 6
Morphometrics of metacercariae of *Apharyngostrigea pipientis* (Faust, 1918) from laboratory-raised *Poecilia reticulata* exposed to cercariae from naturally infected *Biomphalaria straminea* in Brazil. Data also reported for the species by Faust (1918) and Hughes (1928), from naturally infected frogs, and from metacercariae of *Apharyngostrigea* sp. from Pinto and Melo (2012). Data given as range in μm with mean in parentheses. DPI, days post-infection; L, length; W, width.

		Present study	Pinto and Melo (2012)	Faust (1918)	Hughes (1928)
Locality		Pampulha Reservoir, Belo Horizonte, Brazil	Pampulha Reservoir, Belo Horizonte, Brazil	Chicago, Illinois, USA	Chicago, Illinois, USA
		Poecilia reticulata (experimental)	*P. reticulata*	*Rana pipiens*	*R. pipiens*
Cyst	L	25 DPI 828–1093 (939)	516–894 (779)	760–1000	Large morph 720–885 (818) Small morph 525–585 (557)
		40 DPI 791–877 (842)			
		60 DPI 791–1066 (911)			
	W	25 DPI 645–865 (725)	722–997 (870)	500–700	Large morph 600–705 (666) Small morph (360–435 (387)
		40 DPI 550–722 (645)			
		60 DPI 653–946 (749)			
Metacercariae (excysted)					
Body	L	567–1117 (760)	618–790 (680)	500	525–720 (617)
	W	361–602 (462)	412–499 (461)	370	360–420 (282[a])
Oral sucker	L	51–77 (60)	51–77 (65)	Diameter 75	Diameter 57–78 (70)
	W	60–92 (69)	61–92 (74)		
Ventral sucker	L	86–111 (97)	86–103 (93)	Diameter 80	69–96 (80)
	W	86–120 (100)	86–111 (99)		87–123 (98)
Tribocytic organ	L	273–423 (350)	287–409 (341)		240–313 (270)
	W	252–307 (279)	237–307 (290)		93–135 (111)

[a] Mean outside range, as reported by Hughes (1928).

Table 7
Genetic distances within and between species of *Apharyngostrigea* (uncorrected p (%), using all alignment sites).

		Intraspecific		Interspecific	
		Mean	Range	Mean	Range
CO1					
	Apharyngostrigea pipientis [a] (*n* = 36)	0.76	0–1.94	9.67	6.13–14.29
	Apharyngostrigea simplex (*n* = 5)	0.88	0.21–1.30	12.81	11.14–14.29
	Apharyngostrigea sp. (JX977780, DNA1006, *n* = 1)	n/a	n/a	7.88	6.13–12.30
	Overall (*n* = 53)	0.74	0–1.94	9.87	6.13–14.29
ITS [b]					
	A. pipientis (*n* = 7) [c]	0.10	0–0.34	1.72	0.12–3.42
	A. simplex (*n* = 2)	0	0–0	3.08	2.78–3.42
	Apharyngostrigea sp. (JX977840, DNA1006, *n* = 1)	n/a	n/a	0.80	0.12–3.12
	Overall (*n* = 12)	0.09	0–0.34	1.87	0.12–3.42

[a] Including records JX977777-9, identified as *Apharyngostrigea cornu*.
[b] Excluding HM064968, a 297-bp sequence of *A. pipientis*.
[c] Including records JX977837-9, identified as *A. cornu*.

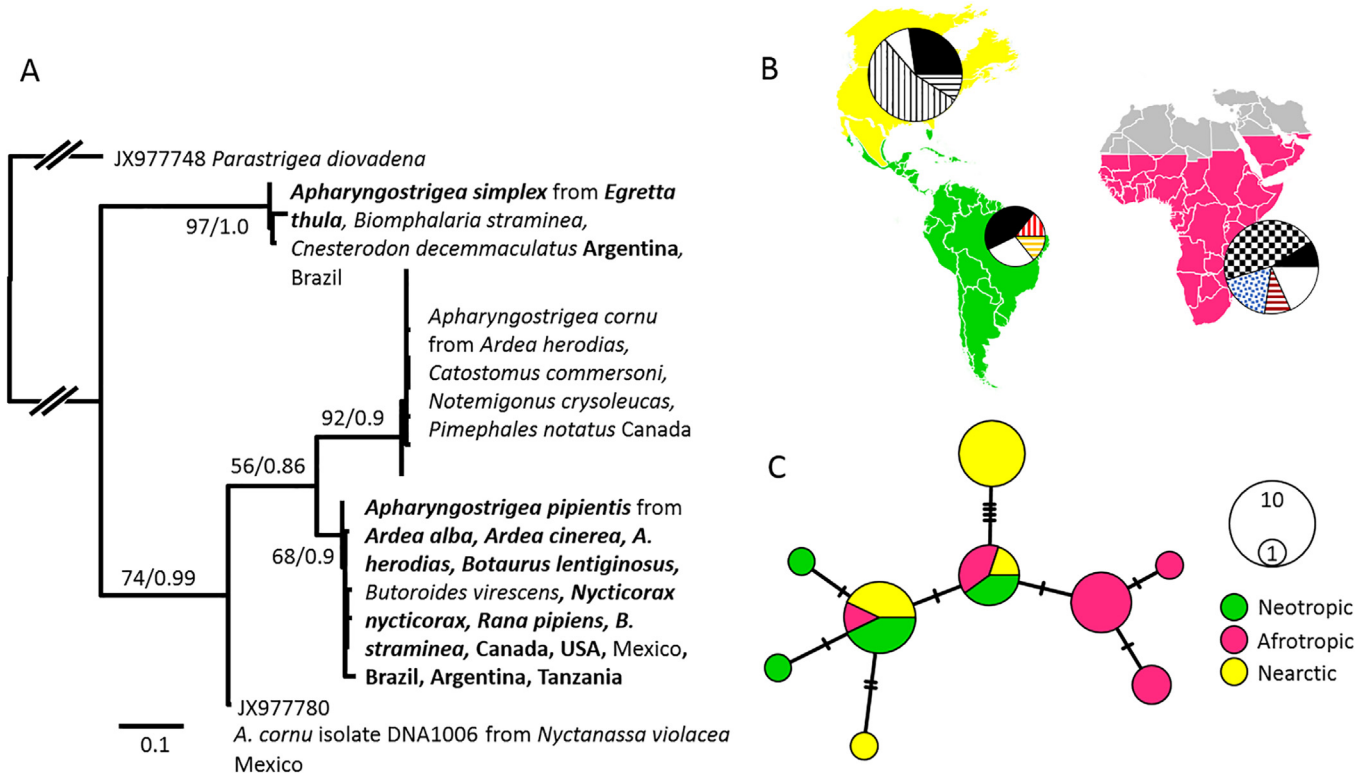

Fig. 4. Phylogenetic analyses of non-redundant partial sequences of cytochrome *c* oxidase 1 (CO1) from *Apharyngostrigea* in the present and other studies. A Maximum Likelihood topology is annotated with bootstrap support in 1000 pseudoreplicates/posterior probability in separate Bayesian Inference analysis; sequences in the tree are (*n* replicates of identical sequences in parentheses): JX977748, MK570088, MH777791(2), MH777789, HM064895-6, HM064899(2), HM064900(2), HM064901, JF769450-2, MT943783, MT943766, JX977777(19), MT943772, MT943769(2), MT943778(5), HM064885(8), JX977780 (A). Bold labels for species, hosts and countries in (A) indicate some data were generated in the present study. Pie charts show CO1 haplotype distribution among Nearctic (yellow), Neotropic (green) and Afrotropic (pink) regions; solid black or white haplotypes are shared among biogeographic regions, others are unique to biogeographic regions (B). A minimum spanning network of CO1 haplotype variation is color coded as in B (C). Circle sizes in B and C are proportionate to number of haplotype replicates.

Using the biogeographic regions of Holt et al. (2013), three sequences (JX977777-9) from Hernández-Mena et al. (2014) originating in Mexico are classified in the Nearctic rather than the Neotropical region, as in Wallace (1876). Genetic structure based on the realms of Holt et al. (2013) yielded similar results overall (AMOVA Φ_{ST} = 0.25, P < 0.001, ANOSIM global R = 0.263, P = 0.0003), but this was driven solely by the distinctness of Afrotropic sequences in pairwise ANOSIMs (Afrotropic-Neotropic R = 0.487, P = 0.0009, Afrotropic-Nearctic R = 0.333, P = 0.0002; Nearctic-Neotropic R = 0.047, P = 0.285).

In phylogenetic analysis of partial CO1 sequences, *A. pipientis*, *A. cornu* and *A. simplex* were each monophyletic, and *A. simplex* was the earliest divergent species in the genus (Fig. 4). The topology of phylogenies based on ITS sequences was similar in that *A. simplex* was basal, but *A. pipientis, A. cornu* and *Apharyngostrigea* sp. (JX977840, DNA1006 of Hernández-Mena et al. 2014) were not resolved (Fig. 4). *Apharyngostrigea pipientis* differs from *A. cornu* by mean 8.23 (range 7.26–9.47%) in CO1, compared with up to 1.94% variation within these species. The ITS sequences of *A. pipientis* (excluding HM064968, a short sequence) differ from those

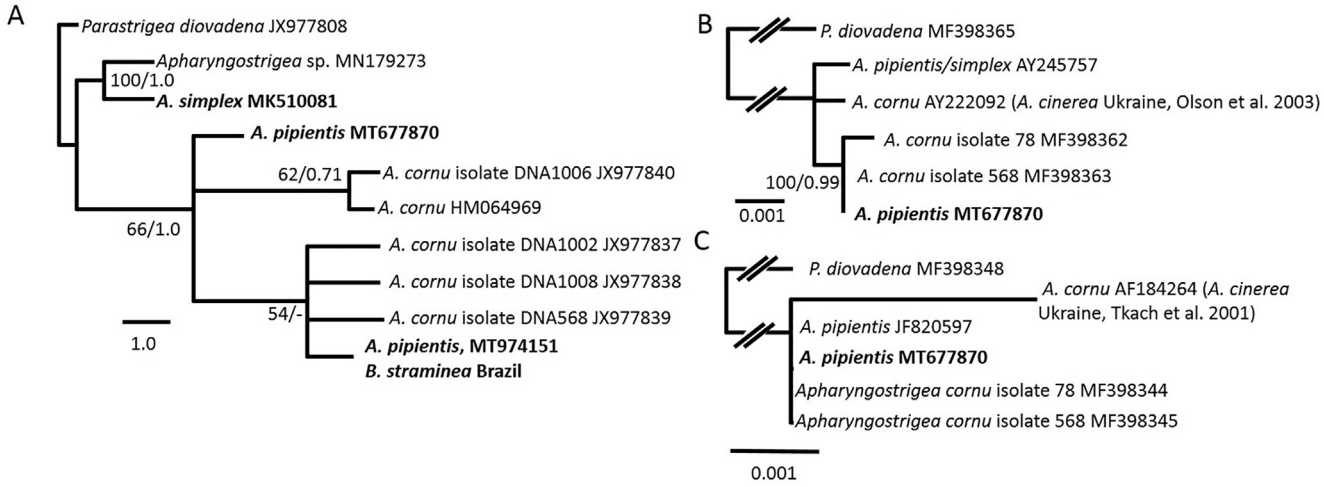

Fig. 5. Phylogenetic analysis of partial nuclear rDNA sequences of *Apharyngostrigea* Ciurea, 1924 from the present and prior studies: internal transcribed spacer (A); 18S (B); 28S (C). Nodes of Maximum Likelihood topologies are annotated with bootstrap support in 1000 pseudoreplicates/posterior probability in separate Bayesian Inference analyses.

Table 8
Mitochondrial genome of *Apharyngostrigea pipientis* (GenBank accession number MT679576).

	Type	Start	End	Length	Initial/Terminal codon
COX3	CDS	1	697	697	ATG/T(AA)
tRNA-His	rTNA	693	748	56	
CYTB	CDS	762	1871	1110	ATG/TAG
ND4L	CDS	1872	2135	264	ATG/TAG
ND4	CDS	2096	3391	1296	ATG/TAG
tRNA-Gln	tRNA	3401	3462	62	
tRNA-Phe	tRNA	3679	3738	60	
tRNA-Met	tRNA	3765	3832	68	
ATP6	CDS	3922	4440	519	ATG/TAG
ND2	CDS	4436	5352	917	ATG/T(AA)
tRNA-Val	tRNA	5352	5414	63	
tRNA-Ala	tRNA	5430	5493	64	
tRNA-Asp	tRNA	5498	5562	65	
ND1	CDS	5563	6468	906	GTG/TAG
tRNA-Pro	tRNA	6473	6534	62	
tRNA-Asn	tRNA	6543	6606	64	
tRNA-Ile	tRNA	6607	6673	67	
tRNA-Lys	tRNA	6684	6754	71	
ND3	CDS	6761	7117	357	ATG/TAG
tRNA-Trp	tRNA	7204	7268	65	
COX1	CDS	7274	8857	1584	ATG/TAG
tRNA-Ser	tRNA	7139	7200	62	
tRNA-Thr	tRNA	8872	8933	62	
Large subunit	rRNA	9335	9958	936	
tRNA-Cys	tRNA	9921	9985	65	
Small subunit	rRNA	9983	10,711	729	
COX2	CDS	10,748	11,350	603	ATG/TAA
ND6	CDS	11,363	11,818	456	ATG/TAG
tRNA-Tyr	tRNA	11,828	11,891	64	
tRNA-Leu1	tRNA	11,896	11,960	65	
tRNA-Ser2	tRNA	11,961	12,027	67	
tRNA-Leu2	tRNA	12,046	12,110	65	
tRNA-Arg	tRNA	12,131	12,197	67	
ND5	CDS	12,198	13,787	1590	GTG/TAA
tRNA-Glu	tRNA	14,000	14,071	72	
tRNA-Gly	tRNA	14,277	14,343	67	

of *A. cornu* by mean 0.62 (range 0.46–0.72%), compared with up to 0.34% variation within these species.

3.3. Phylogenomic analysis of mitochondrial genomes and rDNA operons

The mt genome assembly of *A. pipientis* comprised 0.17% (146687/85788258 reads) of the read pool and was 14,348 bp in length with mean coverage of 1545 (range 1047–9006) reads per site (MT679576, Table 8). Modifications to MITOS2 annotations included shortening the 3′ends of *cox3* and *nad2*, addition of tRNA-His, and lengthening and joining two short, separate blocks of 16S rRNA annotations. A 9397 bp rDNA operon assembly that comprised 0.6% (596,309 reads) of the read pool (with mean of 9226, range 5731–19,574, reads per site) was trimmed to a 7500 bp sequence (MT677870) including mainly the sequences

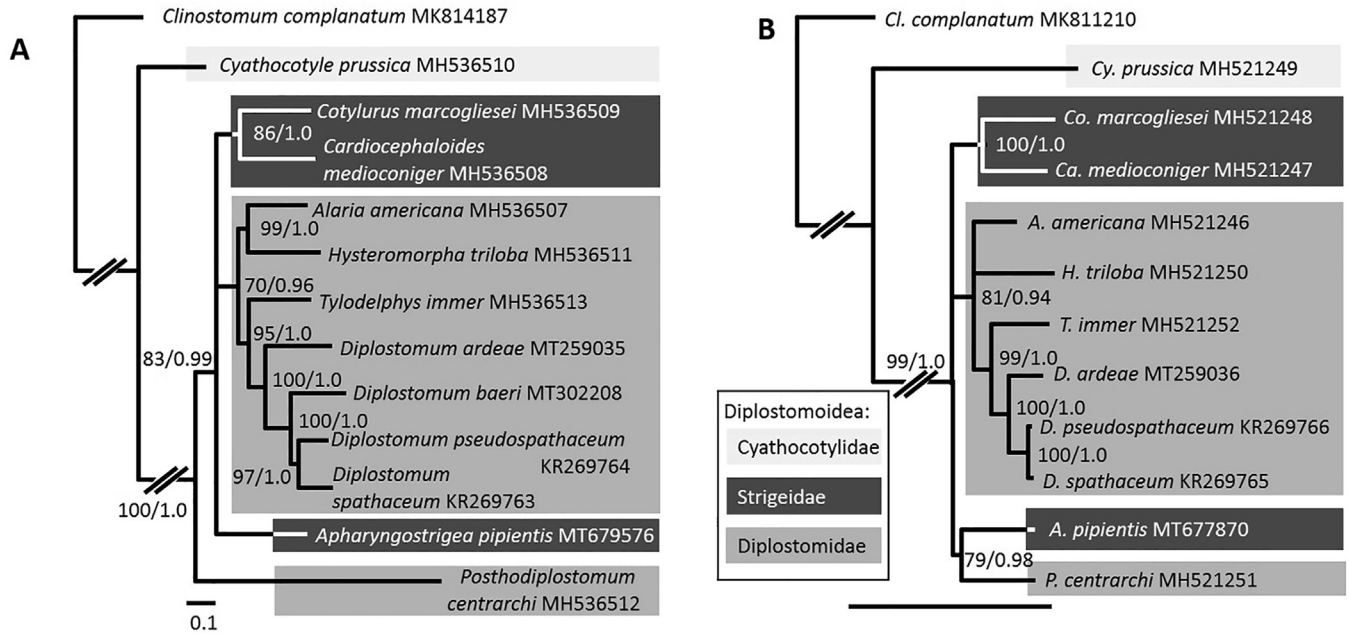

Fig. 6. . Phylogenetic analysis of *Apharyngostrigea pipientis* (Faust, 1918). (A) Mitochondrial genomes (10,035 bp alignment with 5677 variable sites) and (B) rDNA operons (7051 bp alignment with 1650 variable sites). Nodes of the Maximum Likelihood topology shown are annotated with bootstrap support in 1000 pseudoreplicates/posterior probability in separate Bayesian Inference analysis. Nodes in which bootstrap support <50% or posterior probability <0.5 are collapsed. See Supplementary Fig. S1 for a completely resolved topology.

of the rDNA subunits and internal transcribed spacers (7249 bp in total length, 18S 1980 bp, ITS1 680 bp, 5.8S 157 bp, ITS2 292 bp, 28S 4212 bp), flanked by portions of the external transcribed spacers, with mean coverage of 9041 (range 6882–11416) reads per site.

Phylogenetic analyses of both mt genomes and rDNA operons did not support the monophyly of the Strigeidae, but also did not converge on a clear alternative. The rDNA operon of *A. pipientis* fell within a moderately supported lineage with *Posthodiplostomum centrarchi*, separate from strigeids *Cardiocephaloides medioconiger* and *Cotylurus marcogliesei* (Fig. 6). In analysis of mt genomes, however, *A. pipientis* was not allied with *P. centrarchi* and its position was unresolved with respect to other strigeids and diplostomids. Resolved consensus topologies place *A. pipientis* in clades separate from other strigeids, albeit with weak support in the mt genome phylogeny (Supplementary Fig. S1).

Phylogenetic analyses and distance-comparisons using the rDNA operon of *A. pipientis* and ITS, partial 18S and partial 28S sequences from the present and other studies did not clarify relationships among or identifications of *Apharyngostrigea* spp. (Fig. 5), other than the ITS-based support for the basal position of *A. simplex* also seen in the CO1 phylogeny. Lack of interspecific resolution among rDNA sequences from *Apharyngostrigea* is attributable to the small magnitude of variation in ITS (0–7 differences over 1168 bp, excluding *A. simplex*), 18S (0–3 differences over 1681 bp) and 28S (0–3 differences over 1168 bp) sequences from specimens of uncertain identification. For example, an 18S GenBank record (AY245757) labelled *A. pipientis* is called *A. simplex* in a paper by authors of the sequence (Dzikowski et al., 2003). An 18S sequence of *A. pipientis* (MT835237) from Korea (Kim et al., 2020) matched (1428/1428 identities) the data that were obtained in the present study from a specimen from *B. lentiginosus* in Quebec (MT677870), but the Korean sequence was excluded from phylogenetic analysis to maximize alignment length. In addition, ITS obtained from Sanger sequencing of cercariae of *A. pipientis* in Brazil in the present study was an early divergent member of a clade composed of both *A. cornu* (based on adults and metacercariae in Locke et al. (2011) with divergent CO1 sequences) and *A. pipientis* ITS generated from Illumina sequencing, as well as ITS *A. cornu* records of Hernández-Mena et al. (2014) believed to be *A. pipientis* based on their CO1 sequences (see above, and Fig. 5).

4. Discussion

Molecular data gathered here confirm prior records of *A. pipientis* throughout the Americas (Dubois, 1968, 1981) and reveal its presence in the Afrotropic region for the first time. Comparable distributions are known in few other sequenced digeneans associated with fresh water (Table 1, Supplementary Table S1). As argued below, the evidence suggests the wide geographic range of *A. pipientis* is not a result of human-mediated introduction, although more study is needed to confirm this.

The distribution of CO1 haplotypes of *A. pipientis* shows significant isolation in biogeographic regions, but also that this isolation is incomplete or recent. Sequences of CO1 are not more uniform in any particular region, such as might be expected if the species had recently expanded its range. The greater isolation of Afrotropic populations of *A. pipientis* can be attributed to its separation from Neotropic and Nearctic regions by an uninterrupted oceanic barrier, and by a physically greater distance, both of which likely impede dispersal by birds. The African isolates of *A. pipientis* are unlikely to represent infections acquired in the Americas, even if from wide ranging hosts (*A. cinerea*, *A. alba*) with broad distributions. *Ardea cinerea* is an Old World heron, only occasionally observed in American coastal areas (Kushlan and Hancock, 2005; Renner and Linager, 2007), suggesting transatlantic journeys to Africa by *A. cinerea* harboring American *A. pipientis* are infrequent. Similarly, while *Ardea alba* consists of four geographically disjunct subspecies (Gill and Donsker, 2020), the most reliable division is that between the American *A. alba egretta* and the remaining three subspecies found in different parts of the Old World (Pratt, 2011), a distinction based partly on molecular evidence (Sheldon, 1987, and see also Sasikala et al., 2012). The infection levels and presence of immature worms in Tanzanian birds also suggest local transmis-

sion. Vagrant birds probably occasionally spread parasites across the Atlantic, maintaining a low level of interbreeding among established metapopulations of *A. pipientis* in the Americas and in Africa. The reduced level of genetic structure among New World *A. pipientis* suggests higher connectivity among populations of *A. pipientis* in the Americas. Several New World ardeids migrate or have overlapping ranges in the Americas (Kushlan and Hancock, 2005) that could provide *A. pipientis* with continua or stepping stones of genetic mixing. Several other freshwater digeneans (*Clinostomum heluans*, *Drepanocephalus spathans*, *Austrodiplostomum compactum*, *T. querquedulae*, Table 1) are likely dispersed throughout the Americas in this manner. Unlike some scenarios based on intermediate hosts discussed below, this view of the wide distribution of *A. pipientis* emphasizes natural dispersal by birds and invokes no anthropogenic introductions of exotic host species. However, this view also implies that *A. pipientis* was undetected or misidentified in Africa until now.

The broad distribution of *A. pipientis* should be considered together with the constraints imposed by the geographic ranges of intermediate hosts, which often limit parasite distributions (Table 1). Our data comprise sequences from adults of *A. pipientis* in three biogeographic regions, as well as Nearctic metacercariae and Neotropic cercariae. Sequencing the missing links in Afrotropic and Neotropic life cycles could shed new light on how *A. pipientis* attained and maintains its wide distribution, particularly in Africa. If *A. pipientis* is recovered from native intermediate hosts, this parasite can be parsimoniously assumed to be native to the same biogeographic region. For example, the present new records of *A. pipientis* in *B. straminea* and *B. tenagophila* suggest the parasite has long been present in the Neotropical region. Snails in the genus *Biomphalaria* are closely related to *Planorbula armigera*, first intermediate host of *A. pipientis* in the type region (Olivier, 1940; Albrecht et al., 2007). If future work uncovers *A. pipientis* from African species of *Biomphalaria*, as seems plausible, this would provide further evidence of it being a long-established, overlooked species in the region. Notably, a cercaria strongly resembling that of *A. pipientis* was recorded by Fain (1953) from *B. stanleyi* in the Democratic Republic of Congo.

The role of second intermediate hosts in the wide geographic range of *A. pipientis* also awaits further data from naturally infected hosts in Neotropic and Afrotropic regions, but the present results are suggestive. Reports of metacercariae of *A. pipientis* in hylid and ranid anurans of North America (Olivier, 1940; Ulmer, 1970; McAlpine, 1997; Goldberg et al., 2002; Schotthoefer et al., 2009; Locke et al., 2011; Pulis et al., 2011) suggest frogs in the same families could support the parasite in other regions. Notably, in northeastern North America, McAlpine (1997) recorded *A. pipientis* in both *R. pipiens* and *Rana catesbeiana*. The latter frog is globally invasive (Lowe et al., 2000) and is established throughout the Americas, including Argentina and Brazil (Kraus, 2008). While unknown in Africa (Measey et al., 2017 doubt a report of *R. catesbeiana* in Namibia by Rueda-Almonacid, 1999), introductions of this anuran species could have contributed to the wide distribution of *A. pipientis* uncovered in our study. Our experimental infections also imply that another globally introduced host, the guppy *P. reticulata*, might have a role in the maintenance or spread of *A. pipientis*. Guppies are widely introduced for mosquito control and via aquarium release (Deacon, A.E., 2011. The behavioural ecology of the Trinidadian guppy, *Poecilia reticulata*, as an invasive species. PhD thesis, University of St Andrews, Scotland) and may have facilitated the introduction of other helminth parasites in Africa (Tavakol et al., 2017). In the same reservoir in Belo Horizonte where the material used to infect guppies was collected, Pinto and Melo (2012) reported metacercaraie resembling *A. pipientis* in naturally infected guppies. This suggests our experimental results may reflect naturally occurring infections. Even if *P. reticulata* plays little role in transmission of *A. pipientis*, a capacity to infect fish is potentially significant. A second intermediate host spectrum including both fish and amphibians would facilitate the spread and establishment of *A. pipientis* in new habitats, whether through recent co-introductions in these hosts, or in colonisations resulting from natural dispersal by avian definitive hosts.

A recent report of *A. pipientis* in ardeids in Korea (Kim et al., 2020), with 18S identical to that of a North American isolate in the present study, suggests this parasite might be cosmopolitan. In our view, this record requires confirmation with molecular markers more suitable to species level analysis, but the possibility of *A. pipientis* in Asia is interesting in light of the introduction of *B. straminea* in the region (Habib et al., 2018). Much additional work will be necessary to learn how and when *A. pipientis* achieved its broad distribution. For example, biogeographic histories of two well-studied freshwater digeneans include both well supported and less clear events. The arrival of African *Schistosoma mansoni* in the New World is linked to the slave trade (Morgan et al., 2005; Crellen et al., 2016), and European colonial expansion was also thought to have brought *Fasciola hepatica* to the New World (e.g., Mas-Coma et al., 2001). However, molecular analysis of eggs in coprolites in Patagonia suggests *F. hepatica* was in South America long before Europeans (Beltrame et al. 2020). The expansion of both these freshwater digeneans was facilitated by the presence of native compatible snail hosts in the Americas, and by the lack of a required second intermediate host, in contrast to the three hosts in the life cycle of *A. pipientis*.

On balance, our interpretation is that *A. pipientis* is likely native to the Americas (molecular records in naturally infected, native snails, frogs and birds, and historical records in Faust, 1918; Olivier, 1940; Lumsden and Zischke, 1963; Dubois, 1968, 1980, 1981; Sepúlveda et al., 1999; plausible dispersal through avian migration). The history of *A. pipientis* in Africa is less clear due to the lack of historical records or sequences from naturally infected intermediate hosts. However, the population genetics of *A. pipientis*, although based on few samples, do not suggest recent expansion, and are consistent with a long established, wide-ranging, and geographically structured species.

Ukoli (1967) and Ostrowski de Núñez (1989) commented on the difficulty of distinguishing species of *Apharyngostrigea*, which adds plausibility to the possibility of *A. pipientis* being overlooked or misidentified in Africa. The history of *A. pipientis* also attests to this difficulty. Faust (1918) described it as *Tetracotyle pipientis* from metacercariae from *R. pipiens* near Chicago, Illinois, USA, i.e., the same host and region from which some genetic data originate in the present and prior studies (Locke et al., 2011; Pulis et al., 2011). In Michigan, USA, Olivier (1940) described all developmental stages of *A. pipientis*: cercariae from naturally infected *P. armigera*, metacercariae from naturally and experimentally infected *R. pipiens*, and adults from experimentally infected domestic pigeons. Dubois (1968) considered *Apharyngostrigea gundlachi* Pérez Vigueras, 1944 and *Apharyngostrigea duboisi* Pérez Vigueras, 1944 to be synonyms of *A. pipientis* but later, Dubois (1980) revalidated both the latter species, and reassigned figure 10 of *A. pipientis* to *A. duboisi* in Dubois' (1968) widely consulted monograph. Together with the fact that the first description of adult *A. pipientis* was from a non-natural host (Olivier, 1940), which may affect morphology (Blankespoor, 1974), this situation makes unequivocal determination of *A. pipientis* based on the literature challenging. Our hope is that the present morphological information from adults of *A. pipientis* from naturally infected ardeids, genetically linked to the original intermediate host taxon and region of description, will prove useful for future identifications and revisionary work in this genus.

Apharyngostrigea pipientis and *A. cornu* differ little if at all in nuclear ribosomal DNA but are distinguished by moderate variation in mitochondrial CO1, suggesting recent divergence and a sis-

ter relationship. However, *Apharyngostrigea cornu* was described in Europe (Zeder, 1800), and although the wide distribution of *A. pipientis* provides a form of analogous support for the possible presence of *A. cornu* in North America (Locke et al., 2011), comparative CO1 or other mitochondrial sequences from Europe are needed to clarify the status of Nearctic *A. cornu*. The differences between the 18S and 28S sequence from Ukrainian *A. cornu* (Tkach et al., 2001; Olson et al., 2003) and those of any other N. American isolates of *Apharyngostrigea* (Fig. 5) suggest the North American isolates might be a different species.

Samples of *A. simplex* in the present study and those with matching CO1 sequences in López-Hernández et al. (2019) are from the same hosts and region as those studied by Ostrowski de Núñez (1989), who described all developmental stages. Using miracidia from adult *A. simplex* in *E. thula* at the Buenos Aires Zoological Garden, Ostrowski de Núñez (1989) experimentally infected *B. straminea* and then *C. decemmaculatus*. However, *A. simplex* was originally described from samples collected in Australia, where Johnston (1904) described it as *Holostomum simplex* from specimens in *Egretta* (*Ardea*) *novaehollandiae* (Latham). Here again, therefore, it would be desirable to obtain comparative sequences from Australia to corroborate present and past records of *A. simplex* in South America (present study; Ostrowski de Núñez, 1989), and additional sequencing could clarify records in North America (Sepúlveda et al., 1996; Dronen and Chen, 2002; Flowers et al., 2004) and Africa (Dubois, 1968, considered Ukoli's 1967 account of *A. simplex* in Ghana to be *Apharyngostrigea ibis* Azim, 1935).

Consistent with prior work, in phylogenetic analysis *A. pipientis* was not placed in the same lineage as *Cotylurus* or *Cardiocephaloides*, although all belong to the Strigeidae (Hernández-Mena et al., 2014; Blasco-Costa and Locke, 2017). The separation between these two strigeid clades was poorly supported by mitogenomes, however. Nonetheless, given the topology of the rDNA operon phylogeny, and the more taxonomically representative analyses by Blasco-Costa and Locke (2017) and Hernández-Mena et al. (2014) showing the same two-clade division of the Strigeidae, this scenario seems supported overall. This division within the Strigeidae is not easily reconciled with known morphological distinctions. For example, analyses of morphological and life-history characters led Shoop (1989) and Zarzornova and Sysoev (1993) to place *Cardiocephaloides* with *Apharyngostrigea* in one clade, and *Cotylurus* in another, within the monophyletic Strigeidae. The topologies in Fig. 6 and Supplementary Fig. S1 also indicate the most obvious feature distinguishing current concepts of the Diplostomidae and Strigeidae—forebody flattened or cup-shaped—do not differ reliably among clades in the Diplostomoidea. The mitogenomic and rDNA operon data provided here will be useful for future comparisons with additional taxa to further clarify the status of the Strigeidae, as well as relationships among and identifications of species of *Apharyngostrigea*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2020.12.006.

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