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Pathology associated with three new *Clinostomum* metacercariae from Argentina with morphological and DNA barcode identification

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Research Paper

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Abstract

In the Laboratory of Parasites of Fishes, Crustaceans and Mollusks (CEPAVE), we undertook a parasitological study on three species of fish from the Espinal and Esteros del Iberá ecoregions of Argentina. Clinostomid metacercariae were found parasitizing *Characidium rachovii*, *Crenicichla vittata* and *Gymnogeophagus balzanii*. In this study, we analysed the damage that these parasites inflict on their hosts through the evaluation of histological sections. In addition, *Clinostomum* metacercariae were identified using morphological characters and DNA barcoding. In the pathological analysis, we observed that muscle tissue was the most affected. The inflammatory response showed vascular congestion areas and infiltration of numerous inflammatory cells, mainly lymphocytes. The molecular and morphological approach supports the presence of three new lineages of clinostomid metacercariae in Argentina. This could lead to the discovery of a high number of lineages or species of *Clinostomum* from South America.

Introduction

Clinostomid metacercariae can be observed on the skin, muscle or internal organs of freshwater fish and amphibians (McAllister, 1990; Lemke *et al.*, 2008; Sutili *et al.*, 2014). The growth of adults in the buccal cavity and oesophagus of piscivorous birds, as well as the presence of members of the Nephrocephalinae subfamily in the oesophagus of reptiles, has been reported (Kanev *et al.*, 2002; Bullard & Overstreet, 2008; Pérez-Ponce de León *et al.*, 2016). Until now, only *Clinostomum complanatum* (Rudolphi, 1809) Braun, 1899 has been recorded as zoonotic in Asian countries. Consumption of raw freshwater fish by humans can cause laryngeal or pharyngeal infections (Park *et al.*, 2009; Hara *et al.*, 2014; Lee *et al.*, 2017; Kim *et al.*, 2019).

Fifteen species of *Clinostomum* Leidy, 1856 have been recognized so far on the basis of morphological and molecular descriptions (Locke *et al.*, 2015, 2019; Rosser *et al.*, 2017; Sereno-Uribe *et al.*, 2018). In Argentina, based only on morphology, *Clinostomum detruncatum* Braun, 1899, *C. complanatum* and *Clinostomum marginatum* (Rudolphi, 1819) Braun, 1899 have been reported (Boero & Led, 1971; Lunaschi *et al.*, 2007; Lunaschi & Drago, 2009).

Matthews & Cribb (1998) and Caffara *et al.* (2014a, b), among others, agree that the validity of species within *Clinostomum* has been problematic. In recent years, molecular analysis has been used to confirm the diagnosis and, together with the morphological features, to help distinguish among species, revealing also a high biodiversity of lineages (Pérez-Ponce de León *et al.*, 2007, 2016; Gustinelli *et al.*, 2010; Caffara *et al.*, 2011, 2014a, b, 2017; Sereno-Uribe *et al.*, 2013; Locke *et al.*, 2015, 2019; Pinto *et al.*, 2015; Acosta *et al.*, 2016; Briosio-Aguilar *et al.*, 2018; Sereno-Uribe *et al.*, 2018).

Metacercariae parasitize a large variety of fish hosts, since they have been found in at least 12 families of freshwater fish: Cichlidae, Percidae, Centrarchidae, Symbranchidae, Eleotridae, Heptapteridae, Profundulidae, Poecilidae, Goodeidae, Characidae, Cyprinidae and Catostomidae (Szidat, 1969; Dias *et al.*, 2003; Pérez-Ponce de León *et al.*, 2007, 2016; Gustinelli *et al.*, 2010; Caffara *et al.*, 2011, 2014a, b, 2017; Morais *et al.*, 2011; Sereno-Uribe *et al.*, 2013, 2018; Locke *et al.*, 2015; Pinto *et al.*, 2015; Acosta *et al.*, 2016; Davies *et al.*, 2016; Briosio-Aguilar *et al.*, 2018).

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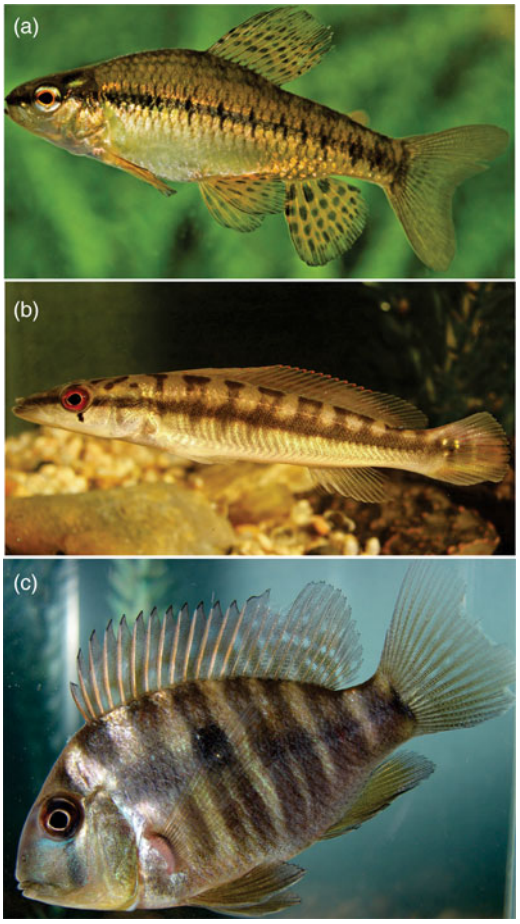

Fig. 1. Fish host harbouring *Clinostomum* sp. metacercariae: (a) *Characidium rachovii*; (b) *Crenicichla vittata*; (c) *Gymnogeophagus balzanii*.

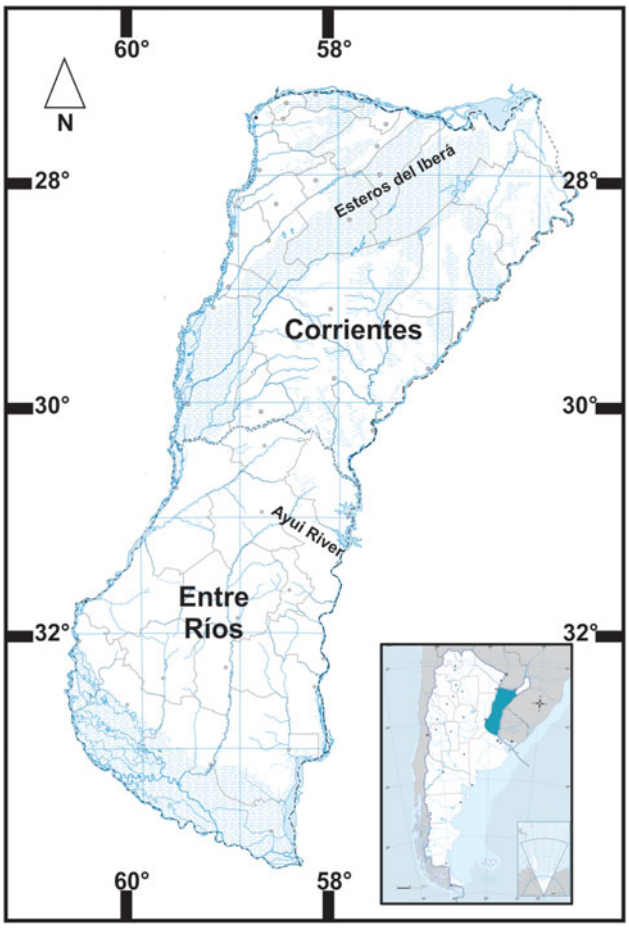

Fig. 2. Sites of collection from Argentina, Ayui River from Concordia (Entre Ríos province) and Iberá Lagoon (Corrientes province).

During investigations on fish parasites from the Espinal and Esteros del Iberá, ecoregions of Argentina, three clinostomid metacercariae were found parasitizing Rachow's Darter Tetra (*Characidium rachovii* Regan, 1913 (Characiformes: Crenuchidae)), the pike cichlid (*Crenicichla vittata* Heckel, 1840 (Perciformes: Cichlidae)) and the Argentine humphead (*Gymnogeophagus balzanii* (Perugia, 1891) (Perciformes: Cichlidae)).

Rachow's Darter Tetra is a freshwater fish that feeds on small invertebrates (Bonetto *et al.*, 1978; Casciotta *et al.*, 2005; Teixeira de Mello *et al.*, 2011; Bastos *et al.*, 2013; Ibarra-Polesel & Poi, 2016). This species is prey to larger fish, such as *Hoplias malabaricus* Bloch, 1794 and *Crenicichla lepidota* Heckel, 1840 (Ibarra-Polesel & Poi, 2016), and birds. The pike cichlid is a benthopelagic freshwater fish that feeds primarily on other fish species (Novakowski *et al.*, 2016), with a distribution in the Paraná River basin, Argentina (Lucena & Kullander, 1992). The Argentine humphead inhabits the Río de la Plata River basin (Casciotta & Gomez, 2000) and feeds on invertebrates from sediments (Wantzen *et al.*, 2002).

The objectives of this manuscript are to analyse the damage that the parasite inflicts on its hosts through the evaluation of histological sections and to identify *Clinostomum* metacercariae using a classical morphological approach together with DNA barcode analysis.

Materials and methods

Specimen sampling

Characidium rachovii (fig. 1a); *C. vittata* (fig. 1b) and *G. balzanii* (fig. 1c) were collected using different nets and a local fisherman. The Rachow's Darter Tetra was collected ($N = 182$, total length range 1.2–5 cm) from the coastal vegetation from Ayui River, Entre Ríos Province (fig. 2). Fish were transported alive to the laboratory, and were kept in aquariums. The pike cichlid ($N = 10$, total length range 25–30 cm) and the Argentine humphead ($N = 10$, total length range 10–15 cm) were collected from Iberá Lagoon, Corrientes Province (fig. 2). Both cichlid fish were transported to the laboratory after being euthanized and examined for digenean parasites in the field. The euthanasia of all specimens was performed quickly by decapitation following the guidelines of the American Fisheries Society (Nickum *et al.*, 2004).

Morphological analysis

Metacercariae were excysted using dissecting needles under a stereomicroscope, and then fixed in 10% formalin. Metacercariae samples were stained using alcoholic hydrochloric acid-carmine (Pritchard & Kruse, 1982). Measurements and digital images of specimens were obtained using an Olympus BX51 microscope (Tokyo, Japan). Illustrations were made with the aid

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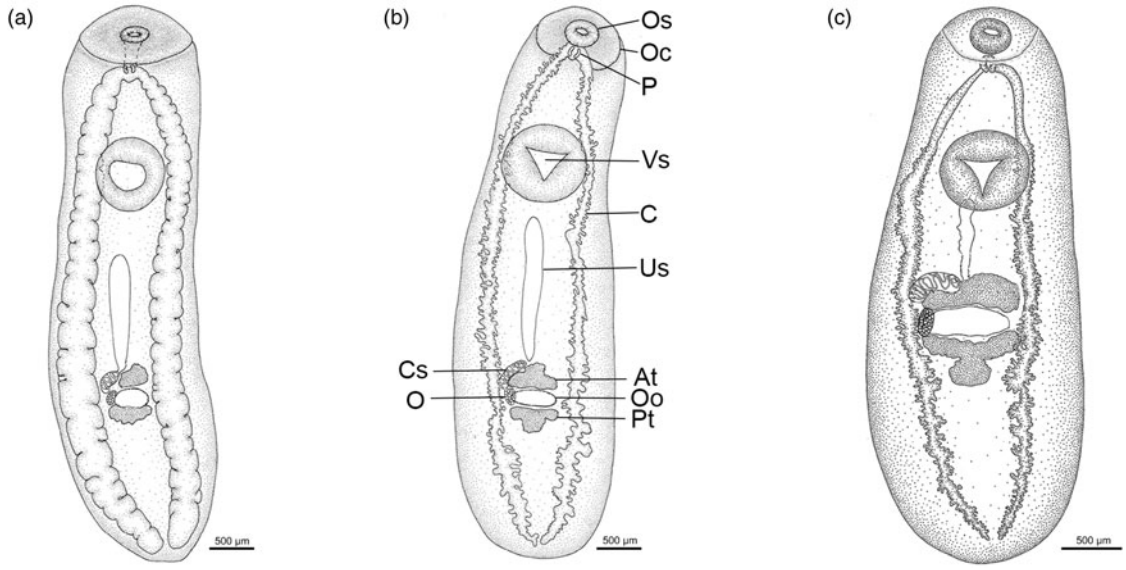

Fig. 3. Clinostomid metacercaria on (a) *Characidium rachovii*; (b) *Crenicichla vittata*; (c) *Gymnogeophagus balzanii*. Abbreviations: At, Anterior testis; C, caecum; Cs, cirrus sac; O, ovary; Oc, oral collar; Oo, ootype; Os, oral sucker; P, pharynx; Pt, posterior testis; Vs, ventral sucker; Us, uterus.

of a drawing tube. The structures were photographed with an AmScope MU 1000 10 MP digital camera (Tokyo, Japan) attached to an Olympus BX51 microscope and measured using ImageJ software (Schneider *et al*., 2012). The measurements are given in micrometres (µm). The type material was deposited in the invertebrate collection of *Museo de La Plata*, Argentina. The mean and the 2.5th and 97.5th percentiles of the distribution of each parameter were calculated with Bayesian statistics using WinBUGS (MRC Biostatistic Unit School of Clinical Medicine, Cambridge Institute of Public Health Forvie Site, Cambridge, UK). This interval was used to represent a 95% Bayesian credible interval. This program was used to generate 100,000 samples from the posterior distributions for each measurement after discarding the initial 10,000 samples as a 'burn in'. The prior probability distribution used was non-informative. A significance level (α) of 5% or less was considered significant ($P \leq 0.05$).

Histological methods

In order to evaluate the histopathological lesions of the infected tissues, sections with encysted metacercariae from the mesentery of *C. vittata* and muscle of *C. rachovii* and *G. balzanii* were fixed in 10% buffered formalin. After dehydration and paraffin embedding, the samples were sectioned at 5 µm and then stained with haematoxylin and eosin (H&E), Masson's trichrome and Giemsa techniques to study the histology and cellular infiltration at the site of encysted metacercariae. The stained slides were observed and photographed under an Olympus CX31 microscope equipped with an Olympus U-CMAD3 camera (Tokyo, Japan).

Molecular data

For the genetic analysis, the total genomic DNA of the whole metacercariae fixed in 96% alcohol was extracted using a Wizard® Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol.

A fragment of the partial mitochondrial gene cytochrome oxidase subunit I (COI-mtDNA gene) was amplified from individual worms, and no hologenophore specimens were preserved. Amplification was conducted by polymerase chain reaction (PCR) on an Eppendorf Mastercycler thermal cycler (Hamburg, Germany) using forward primers DICE 1F (5′ –ATT AAC CCT CAC TAA ATT WCN TTR GAT CAT AAG- 3′) and DICE 14R (5′ –TAA TAC GAC TCA CTA TAC CHA CMR TAA ACA TAT GAT G- 3′) as proposed by Van Steenkiste *et al*. (2015). The reactions were made with GoTAQ Master Mix (Promega) according to the manufacturer's protocol. Thermocycling conditions were as follows: 94°C for 2 min; five cycles of 95°C for 30 s, 48°C for 40 s, 72°C for 1 min; followed by a re-amplification of 40 cycles of 94°C for 30 s, 56°C for 40 s, 72°C for 1 min; and a final extension at 72°C for 10 min.

The PCR products were sequenced using an ABI 3730XLs sequencer (Macrogen Inc., Seoul, Korea) and the primers DICE 1F/DICE 14R.

Phylogenetic analysis

Sequences were edited by eye, checking the nucleotide alignment for the presence of pseudogenes using the translated amino acid sequences based on the invertebrate mitochondrial genetic on the platform GENEIOUS 5.1.7 (Kearse *et al*., 2012). According to the last works on clinostomids (Locke *et al*., 2015, 2019; Pinto *et al*., 2015; Pérez-Ponce de León *et al*., 2016; Caffara *et al*., 2017; Rosser *et al*., 2017, 2018; Sereno-Uribe *et al*., 2018), one or two sequences representing each species or lineages deposited in the GenBank database were selected. Barcode fragment alignments were assembled using MAFFT version 7 (Katoh & Standley, 2013). The best partitioning scheme and substitution model for each DNA partition was chosen under the Bayesian information criterion (Schwarz, 1978) using the 'greedy' search strategy in Partition Finder version 1.1.1 (Lanfear *et al*., 2012). The barcode fragment dataset was partitioned into first-, second- and third-codon positions with the appropriate nucleotide substitution model implemented for each codon position (TrN + I + G for the first and third codon position (Tamura & Nei, 1993); and K81uf for the second codon position (Kimura, 1981).

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Table 1. Measurements of *Clinostomum* sp. metacercariae parasites on *Characidium rachovii*, *Crenicichla vittata* and *Gymnogeophagus balzanii*.

	Ayui River, Concordia (Entre Ríos)	Laguna Iberá (Corrientes)	
	Clinostomum sp. on *C. rachovii* (*N* = 12)	*Clinostomum* sp. on *C. vittata* (*N* = 12)	*Clinostomum* sp. on *G. balzanii* (*N* = 5)
Body length	5603–8163 (6714)	3614–5215 (4398)	3667–4821 (4384)
Body width	1483–1824 (1647)	1037–1617 (1377)	1329–1927 (1556)
Body length/width	3.78–4.47 (4.08)	2,69–3.74 (3.13)	2.5–3.25 (2.84)
Oral collar width	1028–1355 (1200)	774–994 (913)	901–1117 (1036)
Forebody	4020–6023 (4740)	2525–3645 (3120	2437–3339 (2892)
Oral sucker length	203–326 (286)	232–310 (266)	162–265 (216)
Oral sucker width	246–413 (327)	236–327 (295)	208–299 (262)
OS width/body width	0.17–0.23 (0.20)	0.20–0.23 (0.21)	0.19–0.22 (0.21)
Prepharynx	86–221 (151)	49–96 (72)	54–71 (64)
Pharynx length	117–183 (142)	98–132 (120)	100–122 (112)
Pharynx wide	100–132 (113)	77–123 (103)	71–87 (80)
Ventral sucker length	751–908 (832)	692–890 (784)	608–707 (649)
Ventral sucker width	748–894 (816)	654–921 (821)	748–1038 (886)
VS width/OS width	2.11–3.04 (2.54)	2.55–3.9 (2.94)	2.5–4.19 (3.35)
VS width/body width	0.44–0.55 (0.50)	0.51–0.59 (0.56)	0.54–0.59 (0.56)
Uterine sac length	1120–1347 (1265)	489–650 (576)	552–1171 (862)
Uterine sac wide	127–237 (189)	79–104 (92)	96–120 (108)
Caeca length	5028–7477 (5988)	3162–4433 (3885)	3264–4199 (3817)
Caeca wide	232–387 (308)	126–389 (243)	162–303 (225)
Anterior testis length	154–308 (244)	178–275 (227)	234–255 (245)
Anterior testis width	242–349 (317)	468–619 (546)	591–786 (658)
AT width/length	0.98–1.55 (1.25)	2.2–2.95 (2.49)	2.32–2.55 (2.43)
Posterior testis length	150–335 (231)	237–341 (289)	302–562 (432)
Posterior testis width	238–443 (369)	493–646 (581)	627–1103 (785)
PT width/length	0.89–2.63 (1.78)	1.63–2.46 (2.03)	1.26–2.08 (1.67)
Distance between testes	238–295 (270)	199–297 (243)	208–237 (223)
Ovary length	107–162 (138)	140–185 (166)	166
Ovary width	81–109 (90)	129–171 (150)	85
Ovary width/length	0.54–0.76 (0.64)	0.74–1.08 (0.91)	0.51
Cirrus sac length	254–373 (302)	355–400 (377)	343–461 (402)
Cirrus sac width	157–205 (184)	118–131 (126)	111–120 (116)
Cirrus sac length/body length	0.04–0.06 (0.04)	0.09–0.13 (0.11)	0.09–0.11 (0.10)

The phylogenetic reconstruction was carried out using Bayesian inference through MrBayes version 3.2.1 (Ronquist *et al.*, 2012). The phylogenetic trees were reconstructed using two parallel analyses of Metropolis-coupled Markov chain Monte Carlo for 20×10^6 generations each to estimate the posterior probability distribution. Topologies were sampled every 1000 generations. The first 25% of the sampled trees were discarded as 'burn in'. Additionally, the proportion (p) of absolute nucleotide sites (p-distance) (Nei & Kumar, 2000) was obtained to compare the genetic distance among and between lineages, with and without outgroups. The P-value matrix was obtained using MEGA version 6.0 (Tamura *et al.*, 2013) with variance estimation, using the bootstrap method (500 replicates) and with a nucleotide substitution (transition + transversions) uniform rate. The final trees were visualized in FigTree software version 1.4.3 (Rambaut, 2014).

The six sequences obtained were deposited in GenBank under the accession numbers MF673556–MF675361 (supplementary table S1).

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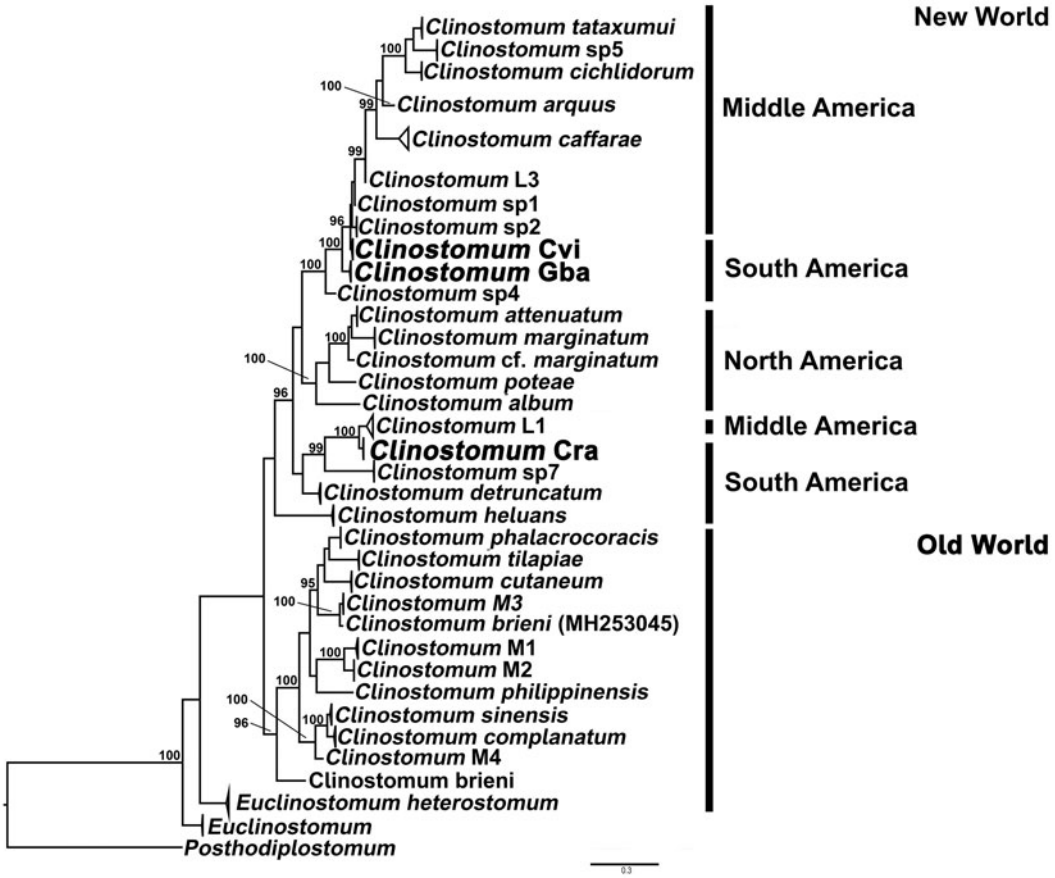

Fig. 4. Phylogenetic position of the new lineages of *Clinostomum* sp. Values above branches represent Bayesian posterior probavility values higher than 95%. Abbreviations: *Clinostomum* sp1–sp8 (*sensu* Locke *et al*., 2015), *Clinostomum* L1–L5 (*sensu* Pérez-Ponce de León *et al*., 2016), *Clinostomum* M1–M4 (*sensu* Caffara *et al*., 2017). *Clinostomum* sp. Cra on *Characidium rachovii*; *Clinostomum* sp. Cvi on *Crenicichla vittata*; and *Clinostomum* sp. Gba on *G. balzanii*. Scale bar shows number of subtitutions per site.

Results

Morphological data

The morphological characteristics of the *Clinostomum* metacercariae on *C. rachovii*, *C. vittata* and *G. balzanii* (*Clinostomum* Cra, Cvi and Gba, respectively) (fig. 3 and supplementary fig. S1) were apparently similar. The measurements are presented in table 1.

Clinostomum sp. Cra

Taxonomic summary

Host. *Characidium rachovii* Regan, 1913.
Locality. Ayui River (Concordia), 31°16′38″S, 58°0′5″W, Entre Ríos Province, Argentina.
Specimens deposited. MLP-He 7623.
Representative DNA sequences. MF673556–MF673557.

Description (based on 12 specimens) (fig. 3a). Body elongated, devoid of spines, flattened anterior end with oral collar. Oral sucker subterminal, rounded, smaller than the ventral sucker. Pharynx short. Intestinal caeca lateral to ventral sucker and genital primordium to posterior end of body. Intestinal wall diverticulated. Ventral sucker 2–3 times larger than oral sucker, with rounded, almost triangular opening. Testes slightly triangular, with the base facing each other and smooth, concave. Anterior testis mostly rounded, almost oval, and posterior testis more elongated. Apex posterior testis directed to the posterior end, poorly defined and irregular margin. Cirrus sac, kidney-shaped, in right margin of anterior testis, opening into genital atrium. Ovary small, oval, intertesticular and dextrally. Uterine sac tubular between genital complex and ventral sucker.

Molecular data

The fragments of the partial COI mtDNA gene from *Clinostomum* sp. Cra measured 570 and 588 bp. The BLASTN analysis of those sequences shows a similarity with *Clinostomum* sp7 and *Clinostomum* L1 (94–96 and 84%, respectively).

In the phylogram (fig. 4), the sequences of *Clinostomum* Cra were grouped with *Clinostomum* L1 (reported by Pérez-Ponce de León *et al*., 2016) and *Clinostomum* sp7 reported by Pinto *et al*. (2015). *Clinostomum* L1 was found in Honduras and Mexico (Middle America), and *Clinostomum* sp7 was recorded in Brazil (South America).

The genetic distance between *Clinostomum* sp. Cra and the *Clinostomum* sp. from the Old World is (range, mean ± standard deviation) 16–19 (17 ± 1)%, and from the New World is 5–18 (15 ± 3)% (supplementary table S2). The closest species to *Clinostomum* Cra is *Clinostomum* L1, with a *p*-distance of 5% (table 2).

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Taxonomic remarks

The *Clinostomum* sp. Cra can be compared with the genetically closest species, *Clinostomum* L1 (parasite on *Rhamdia* sp. Bleeker described by Sereno-Uribe *et al.* (2018)) and *Clinostomum* sp7 (parasite on *Poecilia reticulata* Peters, 1859).

The *Clinostomum* sp. Cra specimens found have similar measurements to *Clinostomum* L1, but they differ in several features. The integument of *Clinostomum* L1 is covered with spines that are absent in *Clinostomum* sp. Cra. The oesophageal bulb is inconspicuous in *Clinostomum* L1, but conspicuous in *Clinostomum* sp. Cra. In *Clinostomum* L1, the intestinal caeca have irregular margins from the posterior edge of the ventral sucker to the posterior body end, whereas in *Clinostomum* sp. Cra the intestinal caeca have irregular margins anterior and posterior to the ventral sucker towards the anterior and posterior body ends. Compared to *Clinostomum* sp7, *Clinostomum* sp. Cra has greater body length, oral sucker width, ventral sucker length and width, anterior and posterior testes and cirrus sac is longer.

Davies *et al.* (2016) and Lunaschi & Drago (2009) described *Clinostomum* sp. metacercariae and immature specimens, respectively, from Argentina only using morphological analysis. The body length, cirrus sac width and distance between the anterior and posterior testes are greater in *Clinostomum* sp. Cra than in the two *Clinostomum* sp. metacercariae found parasitizing *Hoplosternum littorale* Hanckock, 1828 (Siluriformes, Callichthyidae) and *Trigonectes* spp. Myers, 1925 (Cyprinodontiformes, Rivulidae). *Clinostomum* sp. Cra has a larger body, ventral sucker, greater ovary width and smaller anterior testis compared with the immature specimens identified by Lunaschi & Drago (2009) as *C. marginatum*. Additionally, *C. marginatum* have long caeca with small lateral diverticula in the last quarter of the body, a feature not observed in our specimens.

Pathological analysis

Specimens of *C. rachovii* appeared in a lethargic state near the riverside; they showed macroscopic whitish-looking cysts localized mainly in the axial musculature and with raised scales around the cyst area. The histological analysis revealed that metacercariae were invading the muscle tissue (fig. 5a) and were encapsulated by a thin fibrotic tissue layer of fibroblast-like cells with abundant blood capillaries (fig. 5b). Although the underlying tissue appeared normal, an interruption of the muscle tissue was observed and its fibres had undergone a rearrangement around the capsule of the cyst. Also, in the muscle tissue, vascular congestion areas and the infiltration of numerous inflammatory cells, mainly lymphocytes (figs 5c, d), were observed.

Clinostomum sp. Cvi

Taxonomic summary

Host. *Crenicichla vittata* Heckel, 1840.
Locality. Iberá Lagoon, 28°32′12″S, 57°10′17″W, Corrientes Province, Argentina.
Specimens deposited. MLP-He 7624.
Representative DNA sequences. MF673558–MF673559.

Description (based on 12 specimens) (fig. 3b). Body elongated, flattened anterior end with oral collar. Integument smooth. Oral sucker subterminal, rounded, smaller than the ventral sucker. Pharynx short, close to oral sucker. Intestinal caeca, lateral to ventral sucker and genital complex, reaching the end of body, with small diverticula along the wall. Ventral sucker with triangular

Table 2. Interspecific genetic distance (expressed in percentage) among Clinostomid metacercariae found in the present study against species and lineages of the world.

		Cra	Cvi	Gba
Clinostomum from the New World	atte	15	15	15
	cf_marg	16	14	15
	marg	18	15	16
	potae	15	15	14
	cichli	15	13	12
	tata	16	13	12
	sp5	15	13	11
	L3	15	4	7
	sp1	15	2	6
	sp2	15	3	7
	Cvi	**16**	–	**5**
	Gba	**14**	**5**	–
	arq	15	10	10
	caffa	14	10	10
	sp4	15	10	9
	dent	13	13	12
	album	17	15	15
	L1	5	18	16
	Cra	–	**16**	**14**
	sp7	15	18	18
	heluans	18	16	17
	sin	17	16	15
Clinostomum from the Old World	comp	18	16	16
	M4	17	15	15
	cuta	16	18	16
	phala	17	17	15
	tila	17	17	17
	M3	17	16	15
	M1	18	17	16
	M2	19	16	16
	phil	17	18	17
	brieni	17	14	12
	E_het	19	20	19
	E	18	19	17

album, *Clinostomum album*; arq, *Clinostomum arquus*; AT, anterior testis; atte, *Clinostomum attenuatum*; brieni, *Clinostomum brieni*; cafa, *Clinostomum caffarae*; cf_marg, *Clinostomum* cf. *marginatum*; cichli, *Clinostomum cichlidorum*; comp, *Clinostomum complanatum*; Cra, *Clinostomum* sp. on *Characidium rachovii*; cuta, *Clinostomum cutaneum*; Cvi, *Clinostomum* sp. on *Crenicichla vitatta*; dent, *Clinostomum detruncatum*; E, *Euclinostomum* sp.; E_het, *Euclinostomum heterostomum*; Gba, *Clinostomum* sp. on *Gymnogeophagus balzanii*; heluans, *Clinostomum heluans*; L1 and L3, *Clinostomum* sp. *sensu* Pérez-Ponce de León *et al.* (2016); M1–M4, *Clinostomum* sp. *sensu* Caffara *et al.* (2017); marg, *Clinostomum marginatum*; OS, oral sucker; phala, *Clinostomum phalacrocoracis*; phil, *Clinostomum philippinensis*; potae, *Clinostomum potae*; PT, posterior testis; sp1, sp2, sp4, sp5, *Clinostomum sensu* Locke *et al.* (2015); sp7, *sensu* Pinto *et al.* (2015); sin, *Clinostomum sinensis*; tata, *Clinostomum tataxumui*; tila, *Clinostomum tilapiae*; VS, ventral sucker.

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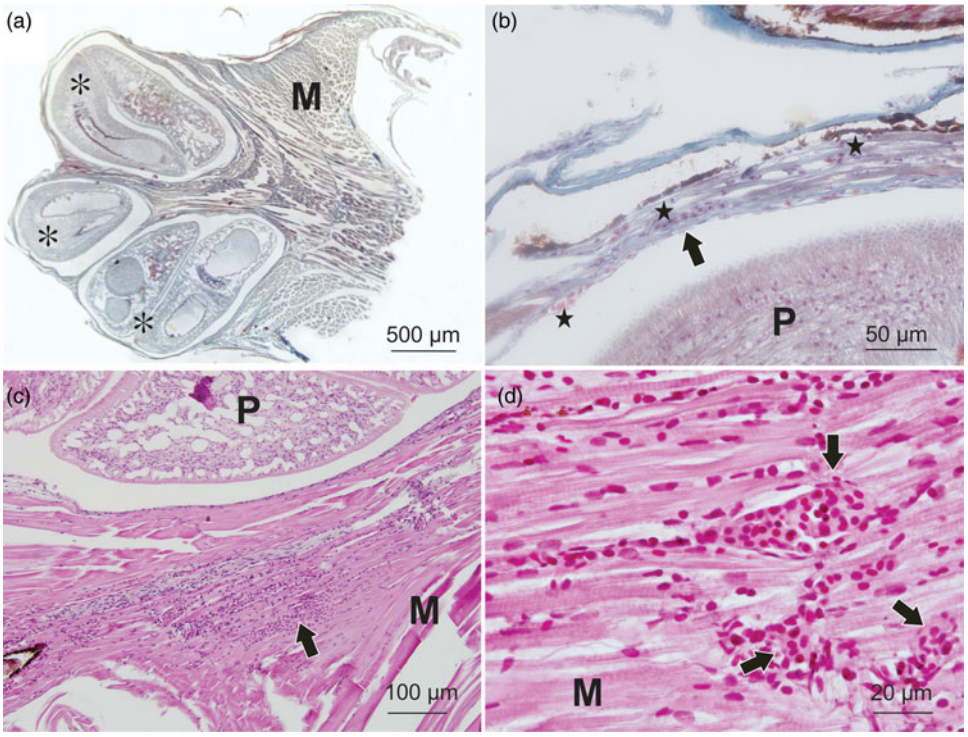

Fig. 5. Musculature axial of *Characidium rachovii* infected with *Clinostomum* metacercariae. (a) General view of the cysts with metacercaria (asterisks) within the musculature axial, Masson's trichrome. (b) Capsule of a thin fibrotic tissue (arrow) with abundant blood capillaries (stars), Masson's trichrome. (c) Infiltration of inflammatory cells (arrow) in the musculature that surrounded to the cyst, H&E. (d) Detail of the vascular congestion areas (arrows) in the muscle tissue, H&E. Abbreviations: M, musculature; P, parasite.

opening. Testes, slightly triangular, irregular, with the base facing each other and slightly concave. Cirrus sac, kidney-shaped, overlapping right margin of the anterior testis opening into genital atrium. Ovary oval, dextral, between testes. Uterine sac tubular and not reaching ventral sucker.

Molecular data

The fragments of the partial COI mtDNA gene from the *Clinostomum* sp. Cvi measured 561 and 570 bp. The BLASTN analysis of those sequences show similarity (98%) with *Clinostomum* sp2 and sp1 on *Sicydium salvini* Ogilvie-Grantand, 1884 and *Rhamdia guatamensis* from Mexico reported by Locke *et al.* (2015).

In the phylogram (fig. 4), the sequences of *Clinostomum* sp. Cvi grouped with species from South and Middle America.

The genetic distance between *Clinostomum* sp. Cvi and the *Clinostomum* sp. from the Old World is 14–18 (16 ± 1)%, and from the New World is 2–18 (12 ± 5)% (supplementary table S2). The closest species to *Clinostomum* sp. Cvi are *Clinostomum* sp1, sp2 and L3, with a *p*-distance of 2, 3 and 5%, respectively. Between the new species reported here, the most similar to *Clinostomun* sp. Cvi is *Clinostomum* sp. Gba, with a genetic distance of 5% (table 2).

Taxonomic remarks

The *Clinostomum* sp. Cvi can be compared with the genetically closest species, *Clinostomum arquus* Sereno-Uribe *et al.*, 2018, *Clinostomum caffarae* Sereno-Uribe *et al.*, 2018, *Clinostomum cichlidorum* Sereno-Uribe *et al.*, 2018, *Clinostomum tataxumui* Sereno-Uribe *et al.*, 2013, *Clinostomum* L3 *sensu* Sereno-Uribe *et al.* (2018), *Clinostomum* sp1, sp2, sp4, sp5 *sensu* Locke *et al.* (2015) and *Clinostomum* sp. Gba (reported below). Unfortunately, the metacercariae reported by Locke *et al.* (2015) were sequenced, but a morphological approach was not applied.

Clinostomum sp. Cvi specimens have triangular testes, three-lobed with irregular margins, whereas in *C. arquus* the anterior and posterior testes are smooth and rounded, in *C. caffarae* the anterior testis has smooth margins with irregularly shape and in *C. tataxumui* the testes have smooth margins and the posterior testis appears triangular (according to the drawing provided by Sereno-Uribe *et al.* (2013)). Also, the anterior testis of *C. cichlidorum* metacercariae is displaced to the right side of the body, but is not displaced in *Clinostomum* sp. Cvi.

In the metacercariae of *C. arquus* and *C. caffarae*, the intestinal caeca are smooth, while in *C. tataxumui* they have slightly indented margins. *Clinostomum* L3 have diverticulated margins from the posterior margin of the ventral sucker to the posterior body end; whereas, in *Clinostomum* sp. Cvi, the intestinal caeca are highly irregular. In *C. cichlidorum* the intestinal caeca have diverticula more prominent from the ventral sucker to the posterior body end, but those are absent in *Clinostomum* sp. Cvi.

The two metacercariae described by Davies *et al.* (2016), despite the similarities they share, both have smaller cirrus sac length compared with *Clinostomum* sp. Cvi. The immature specimens of *C. marginatum* have a larger anterior testis, cirrus sac width and narrow ovary compared with *Clinostomum* sp. Cvi. In addition, the immature specimens of *C. marginatum* have long caeca with small lateral diverticula in the last quarter of the body, but the lateral diverticula in our specimens are in all the length of the body.

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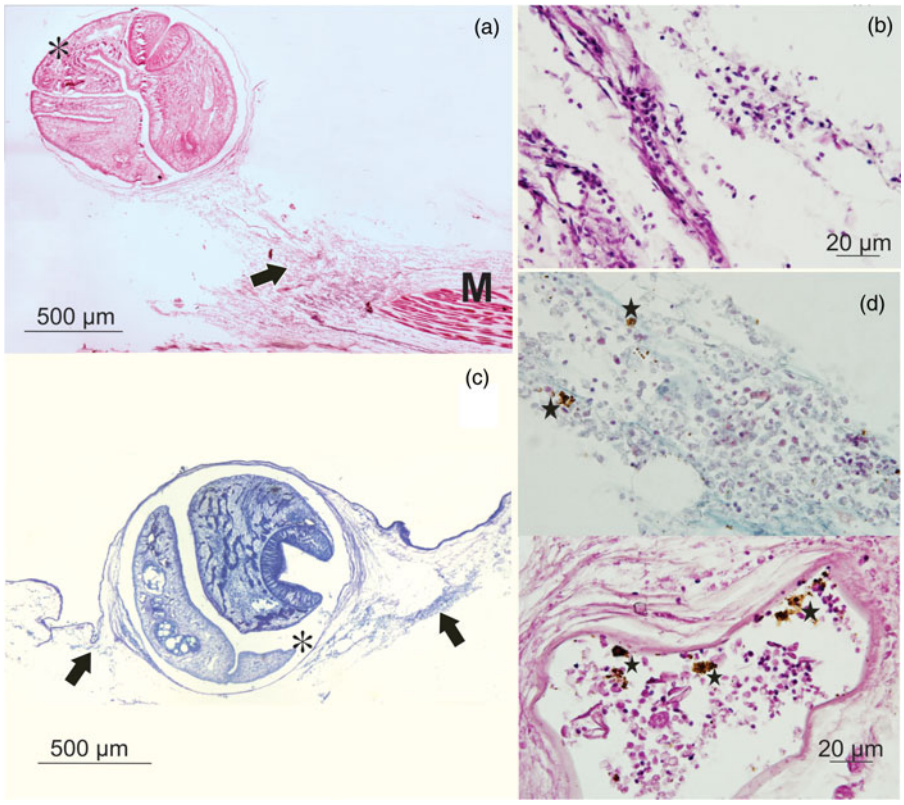

Fig. 6. Cysts with *Clinostomum* metacercariae in *Crenicichla vittata* infected. (a) General view of the cyst with metacercaria (asterisk) encapsulated into the fascia of the muscle tissue, with abundant leukocyte infiltration and desquamation cells (arrow), H&E. (b) Leukocyte infiltration in the fascia, H&E. (c) Metacercaria cyst (asterisk) in the mesentery with infiltration of lymphocytes and desquamation cells (arrows), Giemsa. (d) Connective tissue with lymphocytic infiltration, abundant cellular detritus and pigment granules (stars), Masson's trichrome and H&E. M, musculature.

Pathological analysis

Specimens of *C. vittata* showed no obvious macroscopic signs. When performing the inspection in search of parasites, cysts with encapsulated metacercariae were found in two body sites. At the first site, in the palate, the digenean was found in the fascia of the muscle tissue (fig. 6a), and leukocyte infiltration was also observed (fig. 6b). The other cyst was found in the mesentery (fig. 6c); it was also surrounded by its capsule but, unlike in the other cases, leukocyte infiltration was not abundant, and in the connective tissue some desquamation of cells and the occurrence of pigment granules could be observed (fig. 6d).

Clinostomum sp. Gba

Taxonomic summary

Host. Gymnogeophagus balzanii (Perugia, 1891).
Locality. Iberá Lagoon, 28°32′12″S, 57°10′17″W, Corrientes Province, Argentina.
Specimens deposited. MLP-He 7625 (one specimen).
Representative DNA sequences. MF673560– MF673561.

Description (based on four specimens) (fig. 3c). Body elongated, flattened anterior end with oral collar. Integument without spines. Oral sucker subterminal, rounded, smaller than the ventral sucker. Pharynx short, close to oral sucker. Intestinal caeca with diverticulated margins, evident in post acetabular regions. Caeca lateral to the ventral sucker and genital complex, reaching end of body. Ventral sucker with triangular opening. Testes slightly triangular, irregular margin, with the base facing each other and slightly concave. Cirrus sac, kidney-shaped, in overlapping right margin on the anterior testis, opening into genital atrium. Ovary small, oval, dextral and intertesticular. Uterine sac tubular, and overlapped with the posterior end of the ventral sucker.

Molecular data

The fragments of the partial COI mtDNA gene from the *Clinostomum* sp. Gba measured 564 and 579 bp. The BLASTN analysis of those sequences show a similarity with *Clinostomum* sp2 and sp1 (98%) of *S. salvini* and *R. guatamensis* from Mexico reported by Locke *et al.* (2015).

In the phylogram (fig. 4), the sequences of *Clinostomum* sp. Gba were grouped with species from South and Middle America. The closest species are *Clinosotmum* sp1, sp2, sp4, L3 and Cvi.

The genetic distance between *Clinostomum* sp. Gba and the *Clinostomum* sp. from the Old World is 12–17 (16 ± 2)%, and from the New World is 5–18 (12 ± 4)% (supplementary table S2). Between the new species reported here, the most similar to *Clinostomun* sp. Gba is *Clinostomum* sp. Cvi, with a genetic distance of 5%. The other closest species to *Clinostomum* sp. Gba are *Clinostomum* sp1, sp2 and L3, with a *p*-distance of 5, 7 and 7%, respectively (table 2).

Taxonomic remarks

In our study, the closest metacercaria to *Clinostomum* sp. Gba is *Clinostomum* sp. Cvi, not only in the phylogram, but also in their morphology. The main differences between them are the size of the oral sucker, which is larger in *Clinostomum* sp. Cvi, and the posterior testis, which is larger in *Clinostomum* sp. Gba.

Pathological analysis

Specimens of *G. balzanii* also showed macroscopic cysts in the axial musculature of the trunk and tail. The inflammatory

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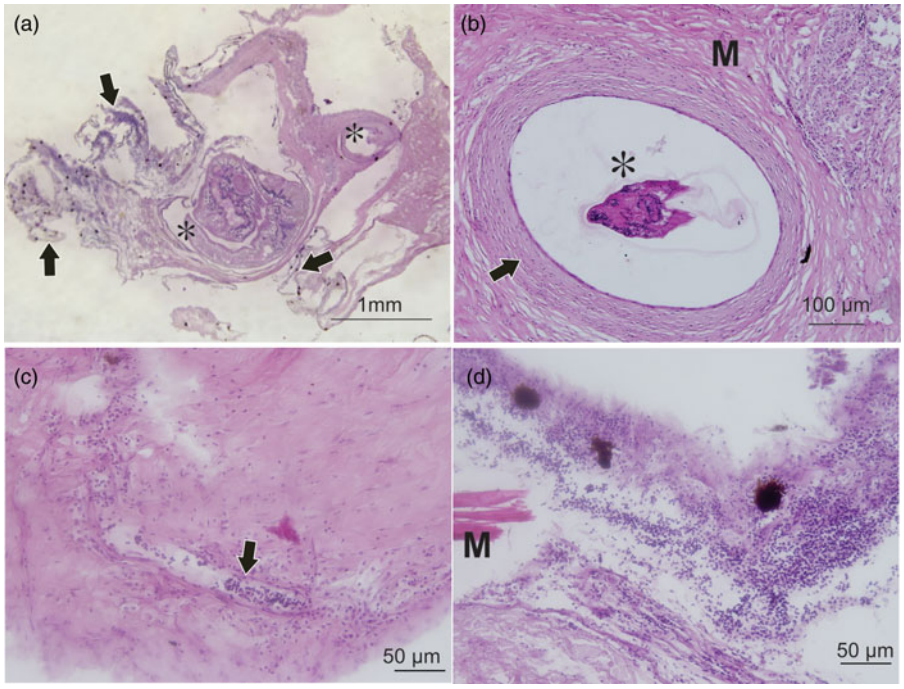

Fig. 7. Musculature axial of *Gymnogeophagus balzanii* infected with *Clinostomum* metacercariae. (a) General view of muscle tissue showing a metacercaria with oral sucker (asterisks) and abundant leucocyte infiltration with numerous melanophores (arrows), H&E. (b) Detail of the oral sucker (asterisk) – note the thick layer of connective tissue arranged concentrically within the muscle tissue (arrow), H&E. (c) Dilated lymphatic vessels with infiltration of lymphocytes (arrow), H&E. (d) Leukocyte infiltration in the muscle tissue, mainly lymphocytes and numerous melanophores, H&E. M, musculature.

response showed characteristics similar to those described for *C. rachovii*, but leucocyte infiltration was more abundant (fig. 7a). The muscle tissue where the metacercariae were attached by the oral sucker showed signs of damage and was replaced by a thick layer of connective tissue arranged concentrically (fig. 7b). In areas near the cyst, dilated lymphatic vessels with infiltration of lymphocytes and disruption of the muscle tissue occurred (fig. 7c). In addition, in *G. balzanii*, an onset of melanosis was observed; numerous melanophores invaded the connective tissue (fig. 7d), possibly in response to the presence of the metacercariae.

Discussion

The three metacercariae under study represent new lineages, different from the ones known until now in South America and the world (Pérez-Ponce de León *et al.*, 2007, 2016; Gustinelli *et al.*, 2010; Caffara *et al.*, 2011, 2014a, b, 2017; Morais *et al.*, 2011; Sereno-Uribe *et al.*, 2013, 2018; Locke *et al.*, 2015; Pinto *et al.*, 2015; Acosta *et al.*, 2016; Davies *et al.*, 2016; Briosio-Aguilar *et al.*, 2018). The *p*-distance supports the independence from all the other sequences, confirming their status as new lineages or species.

In keeping with the previous works based on molecular analysis (Pérez-Ponce de León *et al.*, 2007, 2016; Gustinelli *et al.*, 2010; Caffara *et al.*, 2011, 2014a, b, 2017; Sereno-Uribe *et al.*, 2013, 2018; Locke *et al.*, 2015, 2019; Pinto *et al.*, 2015; Acosta *et al.*, 2016; Briosio-Aguilar *et al.*, 2018), our results confirm the differentiation into two groups of clinostomids, one from the New World and another from the Old World. Our analysis complements the information about the clinostomids around the world, by reporting the presence of three new lineages from South America and the first molecular analysis of species from Argentina. But, further studies in South America must be encouraged. If we observe the phylogram, sub-nodes inside the New World start to differentiate: one from Middle America, another from North America and others in South America, with a few species occurring all throughout the Americas. We hope that in the future, new species and sequences could shed light on this incipient arrangement of the species and prove it or disprove it.

The identification of the *Clinostomum* sp. reported from Argentina until now was only based on morphology. The specimens reported by Lunaschi & Drago (2009) as *C. marginatum* should be studied taking into consideration the new information and analysed based on an integrative analysis.

Clinostomum complanatum is a species found only in the Old World (Locke *et al.*, 2015), and the record of this parasite by Lunaschi *et al.* (2007) in specimens collected in La Balandra, Buenos Aires, and deposited in the helminthological collection of the Museo de La Plata could be a misidentification. *Clinostomum detruncatum* was found in Argentina (Buenos Aires Province) by Boero & Led (1971), but only adult specimens were reported. These species were not included in the comparison because the metacercariae described by Acosta *et al.* (2016) are genetically distant from the metacercariae under study.

In light of our discovery of three new metacercariae with two different lineages from the same collection site (Iberá Lagoon), we believe that perhaps the clinostomid metacercariae reported until now in Argentina that were identified only by morphology should be studied by molecular methods to be able to determine the real biodiversity of the Clinostomidae family in Argentina.

The results of the histopathological analysis of the metacercariae found in *C. rachovii*, *C. vittata* and *G. balzanii* were similar: in all three cases, the most affected tissue was the muscle. Several researchers have studied clinostomid metacercariae (Kalantan *et al.*, 1987; Adeyemo & Agbede, 2008; Purivirojkul & Sumontha, 2013) and they have reported that this parasite causes damage to the viscera and musculature. As in other infections by digeneans described by Bullard & Overstreet (2008), clinostomid cysts were surrounded by a connective tissue capsule and an evidently severe mononuclear inflammatory response. This infiltration was mainly composed of lymphocytes and macrophages

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that expanded to the adjacent musculature, as has also been observed by Shamsi *et al.* (2013) in infected piscivorous birds. According to Roca *et al.* (2007), the recruitment of lymphocytes suggests a role in the regulation of early immune responses to infection. Ferguson (1989) has observed that the presence of metacercariae may or may not induce a melanin response. In the cases presented in this study, only in *G. balzanii* an onset of melanosis was observed, with melanophores invading the connective tissue close to the musculature.

In conclusion, in the present work we propose the presence of three new lineages, different from those reported from Middle America or even the rest of the world. The only remaining doubts arise in the comparisons made with the clinostomid metacercariae reported from Argentina, as these lack genetic studies. This could lead to the discovery of a large number of lineages or species of *Clinostomum* from South America. Besides, despite the variety of organs infected by the clinostomid metacercariae, it is evident that the most affected one is the muscle tissue. It could be of interest to study and correlate the effect of the parasite load on the host's mobility, its response to predators or search for food.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X20000292

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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