



Molecular data reveal hidden diversity of the genus *Clinostomum* (Digenea, Clinostomidae) in Argentina, with the description of a new species from *Ardea cocoi* (Ardeidae)

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Abstract

The genus *Clinostomum* has been recently a subject of a large number of molecular phylogenetic studies that have uncovered a larger species diversity than we thought. In Argentina, only two nominal species have been reported, namely *C. detruncatum* and *C. marginatum*. Three putative species represented by metacercariae were recently molecularly diagnosed, and there are at least two additional metacercarial morphotypes diagnosed on morphological grounds. Here, we molecularly characterized specimens of *Clinostomum* sampled from freshwater fishes and fish-eating birds from Argentina through mtDNA cytochrome c oxidase subunit I (COI). Unexpectedly, the phylogenetic analysis uncovered three new additional genetic lineages, two of them corresponding to metacercarial stages and another matching COI sequences of *C. heluans* Braun, 1899, being considered conspecific, whereas the others still require formal description. Additionally, we add a new host species for a lineage molecularly diagnosed in a previous study. The adult specimens recovered from *A. cocoi* in Buenos Aires Province represented a new species clearly distinguished from the two species previously reported in Argentina. *Clinostomum detruncatum* is distinguished from the new species by possessing a characteristic tenoidean uterus, and testes located more posteriorly. *Clinostomum marginatum* is morphologically similar but differs from the new species in having rounded ovary, posterior testis lobated, and lateral cirrus-sac and displacing the anterior testis, and vitelline follicles not extending beyond the caeca end. Our study raises the number of *Clinostomum* species in Argentina up to 10. We describe the new species herein.

Keywords New species · Integrative taxonomy · COI · Argentina · *Ardea cocoi*

Introduction

Digenetic trematodes of the family Clinostomidae Lühe, 1901 are parasites of the oral cavity, pharynx, or oesophagus of piscivorous birds and reptiles (Kanev et al. 2002). Some

isolated reports have been made in mammals, including humans (Kifune et al. 2000; Park et al. 2009; Hara et al. 2014; Lee et al. 2017; Kim et al. 2019). Members of the family possess a complex life cycle where gastropods are the first intermediate hosts, and fish and amphibians act as the second intermediate hosts. The genus *Clinostomum* Leidy, 1856 has been recently subject of a large number of studies, showing an increase in the species diversity. The genetic library of species in the family has increased considerably in the last decade, allowing the recognition of potential new species, and the establishment of a link between metacercariae and adults (e.g., Pérez-Ponce de León et al. 2016). Nevertheless, some records are based on the metacercarial stage sampled in freshwater fishes, and since a complete taxonomic description requires adult forms, where morphological characters are fully developed, metacercariae await the finding of adults in their definitive hosts to accomplish a formal description. However, we acknowledge that some recent studies have described new species based solely on the metacercaria stage by using a

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combination of morphological and molecular data, under the assumption that in this particular genus of trematodes, the recognition of a new species based on the metacercarial stage is not limited by the lack of taxonomically informative morphological characters (see Locke et al. 2019; Caffara et al. 2020).

The genus *Clinostomum* currently contains 22 putative species (Supplementary Table S1). These species were validated either morphologically, molecularly, or using both sources of information through an integrative taxonomy approach (e.g., Sereno-Urbe et al. 2013, 2018; Pérez-Ponce de León et al. 2016; Locke et al. 2019; Caffara et al. 2017, 2019, 2020). Still, nine molecularly recognized species based on metacercarial stages need to be described and named (Locke et al. 2015; Pérez-Ponce de León et al. 2016; Montes et al. 2020). The advancement in the use of molecular tools is allowing a rapid progress in the description of these species. For instance, Locke et al. (2015) revealed the presence of eight lineages of *Clinostomum* from different parts of the world that were considered candidate species. Two of them, i.e., *Clinostomum* sp. 6 and *Clinostomum* sp. 8 (after Locke et al. 2015), were recognized. *Clinostomum* sp. 6 was later identified as *C. heluans* Braun 1899 (Briosio-Aguilar et al. 2018), from South America, and *Clinostomum* sp. 8 was described as *C. sinensis* Locke et al. 2019 from Taiwan (Locke et al. 2019).

The New World clade of *Clinostomum* species (see Locke et al. 2015) contains ten putative species for which DNA sequences have been generated, namely *C. album* Rosser et al. 2017; *C. arqus* Sereno-Urbe et al. 2018; *C. attenuatum* Cort 1913; *C. caffarae* Sereno-Urbe et al. 2018; *C. cichlidorum* Sereno-Urbe et al. 2018; *C. detruncatum* Braun 1899; *C. heluans*; *C. marginatum sl* (Rudolphi 1819) Braun 1901; *C. poteae* Rosser et al. 2018; and *C. tataxumui* Sereno-Urbe et al. 2018 (Caffara et al. 2011, 2013, 2017; Sereno-Urbe et al. 2013, 2018; Fernandes et al. 2015; Locke et al. 2015; Rosser et al. 2017, 2018; Briosio-Aguilar et al. 2018) (Supplementary Table S1). The identity of some species still remains controversial. According to Caballero and Díaz-Ungría (1958), *C. pusillum* Lutz 1928 is a *species inquirendae*. *Clinostomum intermedialis* Lamont 1920 is currently considered a member of the genus *Clinostomatopsis* (Diesing 1850) along with *C. sorbens* (Diesing 1850) (Kanev et al. 2002; Fernandes et al. 2015). Also, the status of *C. marginatum* generates ambiguity. Even though the species was originally described from an unidentified species of *Ardea* in Brazil (no locality was assigned) by Rudolphi, and later re-described by Braun (1901), many records of the species have been published from specimens collected in freshwater fish and fish-eating birds of North America. Considering the apparently wide geographic distribution of the species and the lack of molecular data for South American individuals, Locke et al. (2015) speculated whether or not *C. marginatum* sensu Dzikowski et al. (2004), Caffara

et al. (2011), and Sereno-Urbe et al. (2013) is also present in South America. We agree with the view of these authors and, in this paper, we refer to the species as *C. marginatum* sensu lato (*sl*) (considering the wide distribution of nominal records in the literature across the Americas), and *C. marginatum* sensu stricto (*ss*) for specimens sampled in South America.

Three species of *Clinostomum* occur in South America, were originally described from Brazil, and are currently widely distributed, i.e., *C. detruncatum*, *C. heluans*, and *C. marginatum ss.*; *C. detruncatum* has been reported in seven species of fish-eating birds from Brazil, Venezuela, and Argentina; *C. heluans* in eight species of fish-eating birds from Brazil, Bolivia, and Venezuela; and *C. marginatum* in at least 12 species of fish-eating birds from Brazil, Argentina, Colombia, Peru, and Venezuela (Fernandes et al. 2015). Only sequence data of the metacercariae of *C. heluans* and *C. detruncatum* are currently available for South American hosts (Acosta et al. 2016; Briosio-Aguilar et al. 2018). Additionally, Montes et al. (2020) reported the finding of three candidate species of *Clinostomum* parasitizing freshwater fishes in Argentina, which were added to previous reports of the metacercariae of *Clinostomum* sp. from killifishes, *Neofundulus paraguayensis* Eiganmann and Kennedy and *Trigonectes aplocheiloides* Huber, and from the siluriforms *Hoplosternum littorale* Hancock (Szidat 1969; Davis et al. 2016). In this paper, we present morphological and molecular data to describe a new species of *Clinostomum* from the oesophagus of a specimen of the cocoi heron, *Ardea cocoi*, from Argentina, and molecularly characterize metacercariae and an adult specimen of two putative species of *Clinostomum*.

Materials and methods

Collection of samples and morphological study

Specimens used in this study were sampled between 2017 and 2019 in four localities of Argentina. Freshwater fishes representing three species (*Cichlasoma dimerus* (Heckel), *Psalidodon anisitsi* (Eigenmann), and *Pyrrhulina australis* (Eigenmann and Kennedy)) were sampled using a seine net in La Plata (Juan Blanco River 35° 08' 30" S, 57° 26' 27" W, Buenos Aires Province), Concordia (Ayui River 31° 16' 38" S, 58° 0' 5" W, Entre Ríos Province), and Montecaseros (Timboy River 30° 18' 42" S, 57° 43' 32" W, Corrientes Province). Encysted metacercariae of *Clinostomum* sp. were recovered from the caudal fin of *Cichlasoma dimerus*, head muscle tissue of *P. australis*, and axial musculature of *P. anisitsi*. Additionally, one of us (LP) necropsied a moribund female cocoi heron found in Magdalena, Buenos Aires Province (34° 58' 59.7" S, 57° 44' 9.05" W), in September 2017 and another cocoi heron found in Santo Tome, Santa Fe Province (31° 41' 58" S, 60° 45' 27" W) (Fig. 1). The

individuals were dissected, and the buccal cavity and oesophagus examined for parasites. Digeneans were recovered and rinsed in 0.85% saline solution (metacercariae from fishes were released from their cysts), preserved either in nearly boiling 10% formalin for morphological study, or 95% ethanol for molecular work. For the morphological study, adult specimens were stained in chlorohydric carmine, dehydrated through an ethanol series, cleared, and mounted in Canada balsam (Pritchard and Kruse 1982). Each specimen was photographed with an AmScope MU 1000 MP digital camera (Tokyo, Japan) attached to a Olympus BX51 microscope and later measured using ImageJ software (Schneider et al. 2012). In toto specimens were photographed with a Leica DMC 4500 digital camera attached to a Leica M205A glass magnifier. Drawings were made with a drawing tube attached to a light microscope. Measurements of the new species are presented as the mean expressed in micrometers (μm), followed by minimum and maximum values in parentheses. The type specimens were deposited in the Invertebrate Collection of the Museo de La Plata, La Plata, Argentina.

Molecular analysis

DNA was extracted from the distal part of two adult specimens (hologenophores sensu Pleijel et al. 2008), whereas DNA was extracted from whole specimen of metacercariae. For DNA extraction, we used a Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. A fragment of the partial cytochrome c oxidase subunit I (COI)-mtDNA gene was amplified using chain reaction (PCR) on an Eppendorf Mastercycler thermal cycler using the forward primer DICE 1F (5'-ATT AAC CCT CAC TAA ATT WCN TTR GAT CAT AAG-3') and the reverse primer DICE 14R (5'-TAA TAC GAC TCA CTA TAC CHA CMR TAA ACA TAT GAT G-3') (Van Steenkiste et al. 2015). The reaction was carried out with GoTAQ Master Mix (Promega) also according to the manufacturer's protocol. The thermocycling conditions were as follows: 94°C for 2 min; 5 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 in Macrogen Inc. (Korea). Sequences were edited by eye using the platform GENEIOUS 5.1.7 (<http://www.geneious.com>, Kearse et al. 2012). The nucleotide alignment was checked for the presence of pseudogenes in GENEIOUS using the translated amino acid sequences based on the invertebrate mitochondrial genetic code. The newly sequenced barcode fragments were aligned with COI sequences of 17 species of *Clinostomum* and 11 undescribed genetic lineages were downloaded from GenBank with the online version of MAFFT v.7 program (Kato and Standley 2013). Sequences of three species of clinostomids (*Euclinostomum heterostomum* (Rudolphi

1809), *Ithyoclinostomum yamagutii* Rosser et al. 2020, and *Odhneriotrema incomodum* (Leidy 1850)), *Alaria mustelae* Borma 1931, and *Posthodiplostomum* sp. were used as outgroups. The best partitioning scheme and substitution model for each DNA partition was chosen under the Bayesian Information Criterion (BIC; Schwarz 1978) using the “greedy” search strategy in Partition Finder v. 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 positions (Temura and Nei 1993) and K81uf for the second codon position (Kimura 1980).

The phylogenetic trees were reconstructed using two parallel analyses of Metropolis-coupled Markov chain Monte Carlo (MCMC) for 20×10^6 generations each, to estimate the posterior probability (PP) distribution using Bayesian Inference through MrBayes v. 3.2.1 (Ronquist et al. 2012). Topologies were sampled every 1000 generations. The first 25% of the sampled trees were discarded as “burn in.” The consensus tree was visualized in FigTree 1.4.2 (Rambaut 2014). The proportion (p) of absolute nucleotide sites (p-distance) (Nei and Kumar 2000) was obtained to compare the genetic distance among and between lineages. The p-value matrix was obtained using MEGA v.6.0 (Tamura et al. 2013), with 1000 bootstrap replicates and with a nucleotide substitution (transition + transversions) uniform rate. The obtained sequences were deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov>) (Table 1). The remaining parts of the specimens (hologenophores) used for DNA extraction were also processed as mentioned above and deposited in Invertebrate Collection of the Museo de La Plata, La Plata, Argentina.

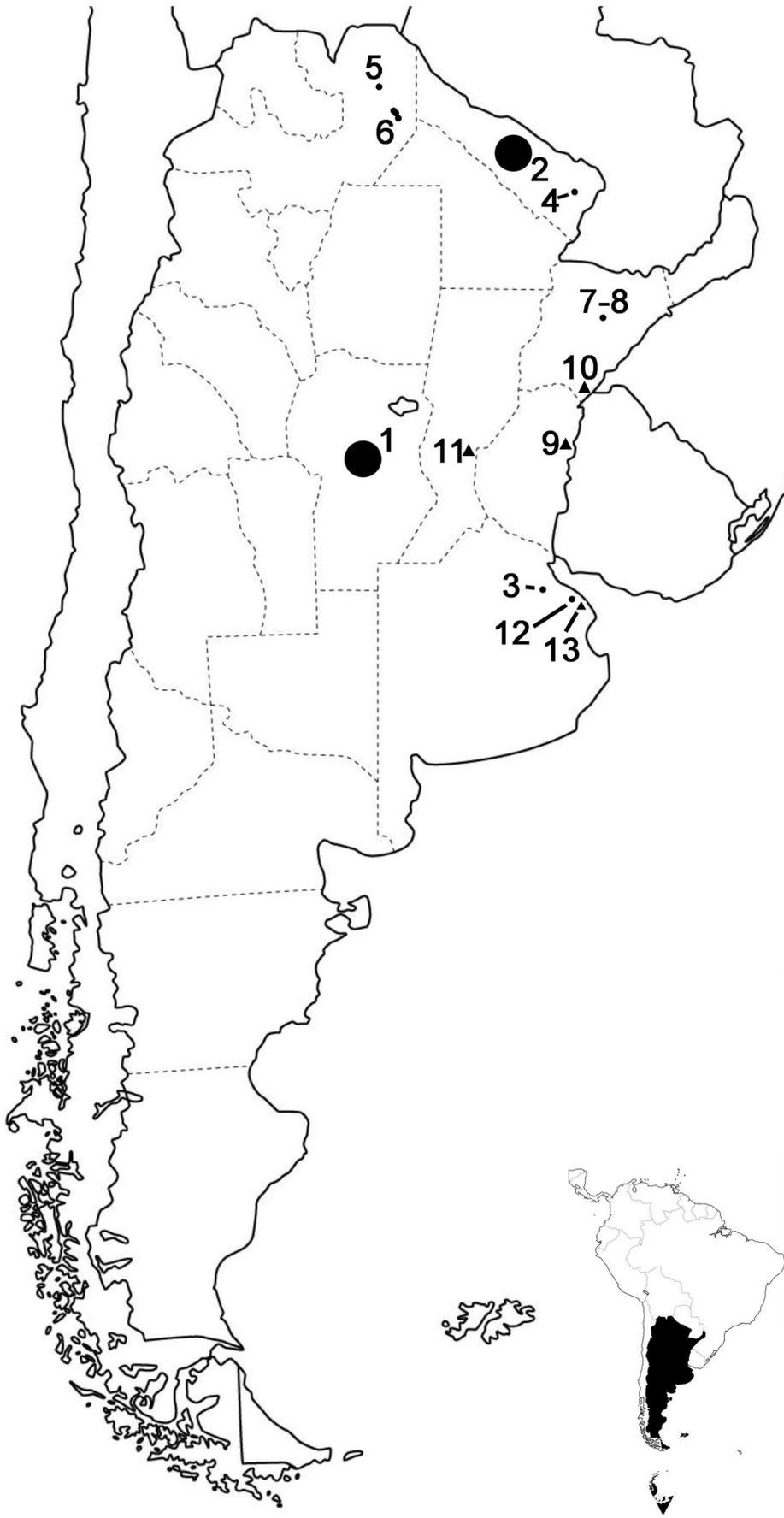
Results

Clinostomidae Lühe, 1901

Clinostomum Leidy, 1856

Clinostomum fergalliarii n. sp. (Fig. 2, Supplementary Fig. 1))

Based on the holotype and five paratypes. Measurements from adult and mature worms are shown in Table 2: body, linguiform with, smooth tegument, widest at gonads level; body constriction at ventral sucker level. Oral sucker, small subterminal, surrounded by wide cephalic collar. Pharynx present. Intestinal caeca with small projections (diverticules), bifurcate after a short prepharynx and extending to almost reach posterior body end. Caeca with inner and outer surface. Ventral sucker round, three times larger than oral sucker and, located in anterior third of the body. Testes, tandem, smooth, postequatorial. Anterior testis lobed medial compressed. Posterior testis medial kidney-shaped. Cirrus-sac slightly bent,



◀ **Fig. 1** Map of Argentina showing the sampling localities and previous reports of *Clinostomum* spp. (1) Province of Cordoba (Weyenbergh 1878), (2) Province of Formosa (Szidat 1969), (3) Uribelarrea city (Boero and Led 1971), (4) Pirané city, Formosa province (Lunaschi and Drago 2009), (5) Hickman locality, Salta province (Davies et al. 2016), (6) Quinquicho Wetland, Salta province (Davies et al. 2016), (7, 8) Ibera Lagoon, Corrientes province (Montes et al. 2020), (9) Concordia city, Entre Rios Province (this study), (10) Montecaseros, Corrientes province (this study), (11) Santo Tome, Santa Fe Province (this study), (12) Juan Blanco River, Buenos Aires Province (this study), (13) La Balandra, Buenos Aires Province (Sutton and Damborenea 2000). Small circles = previous reports, big circles = diffuse locality, only province, triangle = present study

overlapping (not displacing) right margin of anterior testis. Genital pore opening with little digitations (see supplementary Fig. 2). Ovary ovoid, intertesticular, dextral, longer than wide. Uterus from intertesticular space, ascending through left margin of anterior testis before opening into a uterine sac. Uterine sac extending between anterior testis and ventral sucker. Vitelline follicles widely extended in hindbody, from the mid-level of ventral sucker to the posterior body end. Eggs, small yellowish, with a fine rectangular projection on one end.

Taxonomic summary

Type-host: cocoi heron, *Ardea cocoi* Linnaeus (Pelecaniformes: Ardeidae).

Site of infection: Upper oesophagus

Type-locality and collection date: Magdalena, Buenos Aires, Argentina. 34° 58' 59.7" S, 57° 44' 9.05" June 2018.

Prevalence and mean intensity: 8 worms were found in the only bird examined from Magdalena, 1 worm from one bird examined from Santa Fe.

Specimens deposited: Holotype MLP-He 7715, Paratypes MLP-He 7716, and hologenophore MLP-He 7717.

Zoobank No.: 31A5D433-13AC-4363-BFDC-2B8AC67627C2

Etymology: The species epithet “fergalliaris” is in honor to Dr. Fernando Galliaris, a recognized zoologist from Argentina and dear friend of Dr. Montes. This is a recognition for his company and support during our first years as PhD students.

Taxonomic remarks

In this study, we describe *C. fergalliaris* n. sp. as a new species from Argentina. Considering the current distribution patterns of species of *Clinostomum* and the fact that no species from the Palearctic, Afrotropical, Indomalayan, and Australian biogeographical regions is shared either with the Nearctic and Neotropical regions, the new species is only compared with the 10 species from the Americas. Sereno-Urbe et al. (2018) argued that describing new species of *Clinostomum* is challenging because the

morphological characters commonly used to separate species can be considered of low utility, and not reliable. Besides, no single character can be taken to discriminate among species; it is necessary to use a combination of characters to establish more robust species limits. Characters related with the structure of the genital complex such as the position of the gonads along the body, testes shape, position of the cirrus-sac regarding the anterior testis, and the position of the genital pore relative to the anterior testis were found to be very important. Still, other characters were found to be useful to distinguish the new species from the other congeners from the Americas.

The new species can be readily distinguished from the other three species occurring in South America, namely *C. detruncatum*, *C. heluans*, and *C. marginatum*. The new species is distinguished from *C. detruncatum* by lacking a tenoidean uterine sac, i.e., with lateral digitations, and the position of testes, which is between the middle and posterior third of the body (see Acosta et al. 2016). From *C. heluans*, the new species differs by having a smaller size (7820–8580 vs. 14000–15800) (see Table 2), diverticulate caeca rather than smooth, an uterine sac almost reaching the posterior border of the ventral sucker (vs. an uterine sac reaching half the distance between the ventral sucker and the anterior testis), and a more equatorial position of testes rather than in the posterior end of the body. The comparison of the new species with *C. marginatum sensu stricto* deserves more detailed consideration. The new species is morphologically very similar. We compared the new species with *C. marginatum sensu stricto*, i.e., specimens described from South American birds (Table 2). For instance, in *C. fergalliaris* n. sp., the cirrus-sac ventrally overlaps the right margin of the anterior testis (not displacing it) (vs. a cirrus-sac either slightly displacing the anterior testis, or immediately contiguous with the anterior border, see Figs 4 and 8 in Braun 1901), the ovary is elongated in the anteroposterior axis of the body (vs. rounded to transversely ovoid), and the vitelline follicles extend posteriorly beyond the end of caeca, to reach the end of the body (vs. vitelline follicles not extending beyond the end of caeca). Additionally, the new species possesses smooth testes, whereas in *C. marginatum*, testes are slightly to deeply lobed (this is particularly evident in Fig. 20 of Braun 1901).

Furthermore, the new species can be also easily distinguished from the seven species that occur in Middle and North America. *Clinostomum attenuatum* was described from a metacercariae in frogs, and later, it has been found infecting several species of amphibians. Since adults have not been described, we cannot compare *C. attenuatum* with the new species. From *C. tataxumui* the new species differs by having diverticulated caeca along the body (vs. only diverticulated in the post testicular region), smooth testes (vs. irregularly shaped), the posterior extent of vitelline follicles beyond the end of caeca (vs. not reaching the end of caeca), and different

Table 1 Cytochrome c oxidase subunit I (COI) sequences of clinostomids obtained and downloaded from GenBank used in the present study

	Host order and family	Host species	Locality	Sequence from	GenBank
<i>Aalaria mustelae</i>	Anura, Ranidae	<i>Lithobates clamitans</i>	New Hampshire, USA	Locke et al. (2011)	JF904528
<i>Clinostomum album</i>	Planorbidae	<i>Planorbella trivolvis</i>	Mississippi, USA	Rosser et al. (2018)	MH282542
<i>Clinostomum arcus</i>	Cyprinodontiformes, Poeciliidae	<i>Pseudoxiphophorus jonesii</i>	Presa Los Ocotés, Mexico	Pérez-Ponce de León et al. (2016)	KJ477565
<i>Clinostomum brieni</i>	Siluriformes, Clariidae	<i>Clarias gariepinus</i>	Limpopo, South Africa	Caffara et al. (2019)	MH253044
<i>Clinostomum attenuatum</i>	Anura, Ranidae	<i>Lithobates</i> sp.	Quebec, Canada	Locke et al. (2015)	KP150305
<i>Clinostomum caffarae</i>	Pelecaniformes, Ardeidae	<i>Egretta thula</i>	Lago de Catemaco, Mexico	Pérez-Ponce de León et al. (2016)	KU156797
<i>Clinostomum cichlidorum</i>	Perciformes, Cichlidae	<i>Archocentrus siquia</i>	Rio Las Vueltas, Costa Rica	Montes et al. (2020)	KU156816
<i>Clinostomum</i> "Cra"	Characiformes, Crenuchiidae	<i>Characidium rachovii</i>	Entre Ríos Argentina	This study	MF673556-57
<i>Clinostomum</i> "Adult-Cra"	Pelecaniformes, Ardeidae	<i>Ardea cocoi</i>	Santa Fe, Argentina		MW187310
<i>Clinostomum</i> "Cra"	Characiformes, Characidae	<i>Psaltidodon anisitsi</i>	Entre Ríos Argentina		MF673562-63
<i>Clinostomum</i> "Cvi"	Cichliformes,	<i>Crenicichla vitatta</i>	Corrientes, Argentina	Montes et al. (2020)	MF673558-59
	Cichlidae				
<i>Clinostomum complanatum</i>	Caudata: Salamandridae	<i>Triturus carnifex</i> or <i>Lissotriton vulgaris</i>	Italy	Caffara et al. (2014)	KM518246
<i>Clinostomum cutaneum</i>	Pelecaniformes, Ardeidae	<i>Ardea cinerea</i>	Central, Kenya	Locke et al. (2015)	KP110515
<i>Clinostomum detrunccatum</i>	Synbranchiformes, Synbranchidae	<i>Synbranchius marmoratus</i>	Guaira, Brazil		KP110518
<i>Clinostomum fergalliarui</i> n. sp.	Pelecaniformes, Ardeidae	<i>Ardea cocoi</i>	Buenos Aires, Argentina	This study	MW187308-09
<i>Clinostomum</i> "Gba"	Cichliformes, Cichlidae	<i>Gymnogeophagus balzanii</i>	Corrientes, Argentina	Montes et al. (2020)	MF673560-61
<i>Clinostomum</i> L1	Siluriformes, Pimelodidae	<i>Rhamdia guatemalensis</i>	Rio San Juan, Mexico	Pérez-Ponce de León et al. (2016)	KU156782
<i>Clinostomum</i> L3	Pelecaniformes, Ardeidae	<i>Tigrisoma mexicanum</i>	Emiliano Zapata, Mexico		KJ477500
<i>Clinostomum</i> M1	Siluriformes, Schilbeidae	<i>Schilbe intermedius</i>	South Africa	Caffara et al. (2017)	KY865681
<i>Clinostomum</i> M2	Osteoplossiformes, Mormyridae	<i>Marcusenius macrolepidotus</i>			KY865662
<i>Clinostomum</i> M3	Siluriformes, Amphiliidae	<i>Amphilius uranoscopus</i>			KY865667
<i>Clinostomum</i> M4	Cypriniformes, Cyprinidae	<i>Barbus trimaculatus</i>			KY865661
<i>Clinostomum marginatum</i> sensu lato	Anura, Ranidae	<i>Rana clamitans</i>	Quebec, Canada	Caffara et al. (2011)	JF718618
	Perciformes, Centrarchidae	<i>Lepomis gibbosus</i>			JF718619
	Perciformes, Centrarchidae	<i>Lepomis macrochirus</i>	Mississippi, USA	Rosser et al. (2018)	MH282538
	Cypriniformes, Catostomidae	<i>Catostomus nebuliferus</i>	Durango, Mexico	Sereno-Urbe et al. (2013)	JX630993
	Pelecaniformes, Ardeidae	<i>Ardea alba</i>	Veracruz, Mexico		JX630995
	Pelecaniformes, Ardeidae	<i>Ardea cinerea</i>	Central, Kenya	Locke et al. (2015)	KP110522
<i>Clinostomum phalacrocoracis</i>	Perciformes, Osphronemidae	<i>Trichogaster microlepis</i>	Thailand	Locke et al. (2015)	KP110523
<i>Clinostomum philippiense</i>	Characiformes, Lebiasinidae	<i>Pyrrhulina australis</i>	Corrientes, Argentina	This study	MW187306-07
<i>Clinostomum "Pau"</i>	Suliformes, Phalacrocoracidae	<i>Nannopterum auritus</i>	Mississippi, USA	Rosser et al. (2018)	MH282551
<i>Clinostomum poteae</i>	Siluriformes, Pimelodidae	<i>Rhamdia guatemalensis</i>	Yucatan, Mexico	Locke et al. (2015)	KP110524
<i>Clinostomum</i> sp. 1	Perciformes, Gobiidae	<i>Sicydium salyini</i>	Oaxaca, Mexico		KP110526
<i>Clinostomum</i> sp. 2	Cyprinodontiformes, Poeciliidae	<i>Poecilia mexicana</i>	Veracruz, Mexico	Pérez-Ponce de León et al. (2016)	KP110530
<i>Clinostomum</i> sp. 3	Cichliformes, Cichlidae	<i>Apistogramma</i> sp.	Iquitos, Peru	Locke et al. (2015)	KP110531
<i>Clinostomum</i> sp. 4					

Table 1 (continued)

	Host order and family	Host species	Locality	Sequence from	GenBank
<i>Clinostomum</i> sp. 5	Cichliformes, Cichlidae	<i>Cichlasoma boliviense</i>	Santa Cruz, Bolivia		KP110532
<i>Clinostomum heluans</i>	Cichliformes, Cichlidae	<i>Cichlasoma boliviense</i>	Santa Cruz, Bolivia		KP110534
	Pelecaniformes, Ardeidae	<i>Ardea alba</i>	Mexico	Brioso-Aguilar et al. (2018)	MG860853
	Perciformes, Cichlidae	<i>Australoheros</i> sp.	Brazil		MG860852
<i>Clinostomum sinensis</i>	Cypriniformes, Cyprinidae	<i>Cichlasoma dimerus</i>	Buenos Aires, Argentina	Present study	MW187311
<i>Clinostomum</i> sp. 7	Cyprinodontiformes, Poeciliidae	<i>Candidia barbata</i>	Taiwan	Caffara et al. (2019)	MK801713
<i>Clinostomum tataxumui</i>	Pelecaniformes, Ardeidae	<i>Poecilia reticulata</i>	Minas Gerais, Brazil	Pinto et al. (2015)	KJ818259
<i>Clinostomum tilapiae</i>	Siluriformes, Mochokidae	<i>Tigrisoma mexicanum</i>	Laguna Manialtepec, Mexico	Pérez-Ponce de León et al. (2016)	KJ504192
<i>Clinostomum ukolii</i>		<i>Synodontis batensoda</i>	Anambra River, Nigeria	Caffara et al. (2017)	KY649357
<i>Euclinostomum heterostomum</i>	Cichliformes, Cichlidae	Cichlids	Lake Kinneret, Israel	Caffara et al. (2020)	MN044350
<i>Ithyoclinostomum yamagutii</i>	Pelecaniformes, Ardeidae	<i>Ardea herodias</i>	Mississippi, USA	Caffara et al. (2017)	KP721420
<i>Odhnertiorema incommodum</i>	Crocodylia, Alligatoridae	<i>Alligator mississippiensis</i>	Mississippi, USA	Rosser et al. (2020)	MN696163
<i>Posthodiplostomum</i> sp.	Perciformes, Percidae	<i>Perca flavescens</i>	Quebec, Canada	Woodyard et al. (2017)	MF766002
				Locke et al. (2010)	HM064865

measurements of structures such as the oral collar, ventral sucker, posterior testis, and cirrus-sac. Additionally, the cirrus-sac in the new species overlaps the anterior testis, whereas in *C. tataxumui* the anterior testis is displaced by the cirrus-sac.

The new species differs from three recently described species from Middle America, namely *C. caffarae*, *C. arquus*, and *C. cichlidorum*, mainly by the posterior extent of the vitelline follicles and the position of the cirrus-sac with respect to the anterior testis. In the three species, vitelline follicles do not extend beyond the end of caeca, and cirrus-sac displaces the anterior testis to the left. Additionally, *C. arquus* and *C. caffarae* lack diverticulate caeca, and the uterine sac extends to mid-way between the ventral sucker and anterior testis, whereas in the new species the uterine sac extends to almost reach the posterior margin of ventral sucker. The new species shares with *C. cichlidorum* the presence of diverticulate caeca along the body, but they are readily distinguished because in the new species testes are smooth (vs. deeply lobed), and cirrus-sac is at the level of anterior testis (vs. intertesticular).

Finally, from *C. album* and *C. poteae*, two species recently described in the USA, the new species differs in overall body size, since these two species are smaller. Additionally, *C. fergalliaris* n. sp. differs from *C. album* by having smooth testes (vs. irregularly shaped), by the anterior extent of vitelline follicles reaching the mid-level of the ventral sucker (vs. some distance from posterior margin of the ventral sucker), and by having diverticulate caeca along the entire extension (vs. diverticulate caeca only in the forebody). From *C. poteae*, the new species differs further by the position of the cirrus-sac overlapping the anterior testis (vs. having a cirrus-sac contiguous and displacing the anterior testis), and by the posterior extent of vitelline follicles towards the posterior end of the body (vs. follicles not reaching the end of caeca).

Molecular analyses

In this study, we provided new COI sequences of eight specimens of *Clinostomum* from Argentina, either representing adults or metacercariae. The final COI alignment was 800 bp long and consisted of 57 terminals including isolates of the newly sequenced specimens of *Clinostomum*, and four sequences of other clinostomids and diplostomids used as outgroups (Fig. 3). The phylogenetic tree resolved *Clinostomum* as a monophyletic genus sister to *Euclinostomum heterostomum* (Rudolphi, 1809). Two major clades were formed (as shown in previous studies), one representing the Old World and the other the New World samples. All the newly provided sequences were nested in the New World clade occupying different positions within the tree and representing potential candidate species either as

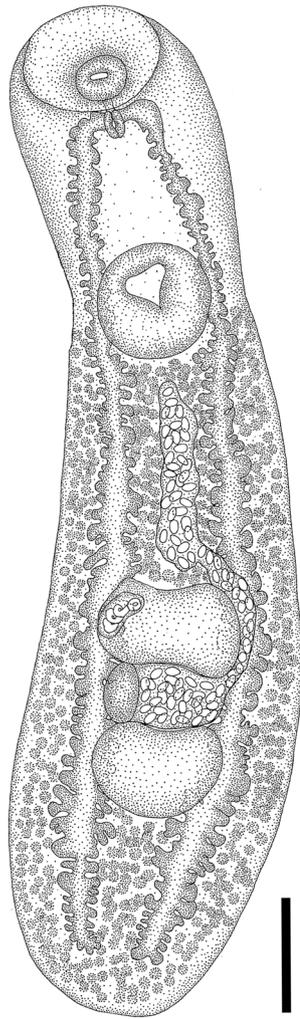


Fig. 2 *Clinostomum fergalliaris* n. sp. adult from *A. cocoi*, Magdalena, Buenos Aires province, Argentina. Ventral view of the holotype. Scale bar = 1000 μ m

metacercarial stages or adults, with the exception of the specimens from *Cichlasoma dimerus* from Juan Blanco River, which was nested within *C. heluans*, showing conspecificity.

Four lineages, mainly represented by metacercariae (with one exception), were resolved as independent evolutionary units. One of them contained sequences from *C. rachovii*, *P. anistisi*, and a single individual adult sequenced from the oesophagus of *Ardea cocoi* from Santa Fe Province, with very low or null COI divergence (0–1%). This lineage was recovered as sister to *Clinostomum* Lineage 1 (after Pérez-Ponce de León et al. 2016) from Middle American heptapterid catfishes, and COI divergence between this lineage and sampled from Argentina varied from 3 to 5%. Another lineage (sampled from *P. australis*) was recovered as the sister species of *C. caffarae*, a species described from southeastern Mexico, and these two as a sister taxa of another lineage sampled from *Gymnogeophagus balzanii* (Perugia). Genetic divergence among these three lineages/species varied from 9 to 12%. The

fourth lineage was resolved as basal lineage of a clade consisting of eight putative species of *Clinostomum*, mostly from Middle American freshwater fishes, particularly heptapterids and cichlids. Interestingly, adults recovered from *A. cocoi* from the Buenos Aires Province were resolved within this clade as the sister species of *Clinostomum* sp. 5 (after Locke et al. 2015) from a cichlid of Bolivia, plus *C. tataxumui* and *C. cichlidorum* from cichlids and eleotrids from Mexico and Costa Rica. The COI divergence between the new species and their sister taxa varies between 10 and 12% (Supplementary Table S2). Reciprocal monophyly of these newly sampled adults and genetic divergence values further corroborated the distinction of the new species.

Discussion

This study and a previously published uncovered six genetic lineages of *Clinostomum* from Argentina based on information on the COI barcode. This fragment of the mtDNA has been useful for discriminating among congeneric species (e.g., Gustinelli et al. 2010; Caffara et al. 2011, 2017, 2020; Sereno-Uribe et al. 2013; Locke et al. 2015, 2019; Acosta et al. 2016; Pérez-Ponce de León et al. 2016; Rosser et al. 2017, 2018). One of the uncovered genetic lineages was described as a new species, *Clinostomum fergalliaris* n. sp., following an integrative taxonomy approach and establishing a morphological differentiation with two nominal species occurring in birds in Argentina, but also comparing the new species with all the congeners currently described in the New World. The other five molecularly recognized lineages were mostly based on metacercarial stages, and we took a conservative position to await until adults are found in fish-eating birds to properly describe and name these species. We are aware that some authors have described species solely based on metacercarial morphology complemented with strong genetic evidence (Locke et al. 2019; Caffara et al. 2020).

Molecular data have increased our capacity to document more accurately parasite species diversity in taxonomic groups with subtle morphological differences. The species in the genus *Clinostomum* represent such problematic groups, where species differentiation is sometimes challenging and where taxonomic history is rather complex (see Ukoli 1966, and description in Caffara et al. 2011). However, the molecular library has increased notably in the last decade, allowing more accurate species delimitation, and linking larval stages with adults (e.g., Pinto et al. 2015; Fernández et al. 2016) to obtain a better understanding, not only on the distribution patterns of the species, but also on their life cycle characteristics. Life history traits can be also used to draw conclusions on the separation of species once they are correlated with the potential co-distribution of intermediate hosts and potential host specificity (see discussion in Pérez-Ponce de León et al.

Table 2 Comparative measurements of *Clinostomum fergalharrii* n. sp. and other species of *Clinostomum* reported from fish-eating birds in South America (excepting *C. marginatum* which is also found in North America). Measurements are shown in μm with the mean followed by the range (when available)

	<i>C. fergalharrii</i> n. sp.	<i>C. marginatum</i> sensu stricto (from Lunaschi and Drago 2009, immature specimens)	<i>C. marginatum</i> sensu stricto (from Braun 1901)	<i>C. detrunctatum</i> (from Travassos et al. 1969)	<i>C. detrunctatum</i> (from Boero and Led 1971)	<i>C. heluans</i> (from Fernandes et al. 2015)
BL	7840 (6180–8580)	4700 (2800–5800)	4000–8000	6000–14000	6000–6030	14000–15800
BW	2250 (1990–2640)	1100 (800–1400)	2000–2300	2000–3000	1800	1400–1800
BL/BW	3.43 (3.11–3.79)					
HBL	2220 (1810–2660)	2800 (1500–3500)				
FBL	5660 (4170–6260)	1100 (800–1500)				
CoL	1230 (1060–1380)					840–1000
CoW	810 (970–1100)					1000–1440
OSL	339 (289–390)	236 (197–278)	200–370		576	640–800
OSW	372 (295–436)	299 (240–336)	210–300			580–700
OSW/BW	0.16 (0.12–0.20)					
VSL	1023 (969–1091)	677 (605–720)				880–1100
VSW	1006 (951–1059)	667 (614–720)			864	880–1100
VSW/OSW	2.76 (2.22–3.59)	1:2.3 (1:2.1–2.6)	570–1070	1000–1500		
VSW/BW	0.44 (0.38–0.48)	1:6.7 (1:4.7–8.7)	570–970			
DBS	1060 (940–1290)					
ATL	612 (359–850)	384 (336–475)			388	860–900
ATW	1026 (798–1317)	442 (350–523)				1700–1840
ATW/ATL	1.77 (1.34–2.22)					
PTL	566 (412–752)	360 (288–480)				640–840
PTW	1117 (1001–1392)	464 (432–480)			340	1440–1740
PTW/PTL	2 (1.75–2.43)					
DBT	650 (412–752)					
CSL	657 (541–838)					
CSW	246 (180–314)					
CSL/BL	0.1 (0.09–0.11)					
OL	437 (264–616)	153 (134–173)				349–523
OW	229 (173–260)	122 (106–144)			274	276–485
OW/OL	0.55 (0.42–0.66)					
EL	121 (113–126)		104–140	110–62	108–114	123 (114–133)
EW	76 (71–78)		55–75			76 (69–88)



Fig. 3 Phylogenetic tree inferred using Bayesian Inference derived from cytochrome c oxidase subunit I (COI) gene dataset. Numbers in the nodes represent posterior probability (<94% are not shown). *Clinostomum*

species observed in this study are in bold and with a strong black bar; those reported in other reports from Argentina are in a white bar with black lines

2016). This is particularly important in bird trematodes due to an increase in dispersal capabilities.

Of the 23 valid species of *Clinostomum*, 11 are distributed in the New World, four in South America (*C. detruncatum*, *C. heluans*, *C. marginatum* sensu stricto, and *C. fergalliaris* n. sp.), five in Middle America (*C. tataxumui*, *C. arquus*, *C. caffarae*, *C. cichlidorum*, and *C. heluans*), and four in North America (*C. attenuatum*, *C. album*, and

C. poteae—plus *C. marginatum* sensu lato) (Supplementary Table S1). Following a convention, Dzikowski et al. (2004), Caffara et al. (2011), and Sereno-Uribe et al. (2013) provided morphological evidence showing that *C. marginatum* occurs in the Nearctic biogeographical region and referred the species as the “American type.” The species was differentiated from the type-species *C. complanatum* (the “European type”), occurring in the Palearctic. These and several other reports

created a large confusion regarding the distribution of *C. marginatum* across the Americas; however, as previously stated, sequences of individuals of *C. marginatum* are not yet available from South American specimens, where the species was originally described by Rudolphi from the intestine of *Ardea* sp. in Brazil (see Braun 1901, p. 25). Locke et al. (2015) raised the question whether or not the North American forms could be also found in South America. Our results provide further evidence to point out that the records of specimens identified as *C. marginatum* across North America (Caffara et al. 2011; Sereno-Urbe et al. 2013) are questionable because of the wide geographical distribution range that the species would have, from Canada southwards to Argentina, and the distributional gap in tropical Middle America, where the species has not been reported (Pérez-Ponce de León et al. 2016). The records of *C. marginatum* as either metacercarial or adult stages in South America (e.g., Fernandes et al. 2015; Murrieta-Morey and de Oliveira-Malta 2016, 2017, 2018; Fedatto Bernardon et al. 2017; Pereira Negreiros and Tavares-Dias 2019; Chagas de Souza et al. 2020a, b) require further validation through DNA sequence data.

With the uncovered genetic lineages of our study and the description of a new species, the number of nominal species of *Clinostomum* in the New World raises to 11 (Supplementary Table S1), whereas the number of genetic lineages awaiting a full taxonomic description raises to 12. Based on the results by Dzikowski et al. (2004), Caffara et al. (2011), and Sereno-Urbe et al. (2013) showing that *C. complanatum* does not occur in the Americas, the verification of the reports of the species in the Americas also requires further verification. A search on the ISI Web of Knowledge for the term “*Clinostomum complanatum*” for the last 20 years yielded 77 records. Twenty-five out of the 77 reports of *C. complanatum* in fish or birds are from Argentina, Brazil, Nicaragua, Costa Rica, Mexico, the USA, and Canada. Most likely all these records correspond to *C. marginatum* sensu lato.

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Code availability Not applicable

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Data availability All the material will be deposited in Museums and the sequences deposited on GenBank.

Declarations

Ethics approval 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.

Consent to participate All the authors give their consent to participate in this work

Consent for publication All the authors give their consent to the publication of this work.

Conflict of interest The authors declare no competing interests.

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