

Research Paper

Cite this article: Montes MM, García I, Paredes del Puerto JM, Barneche JA, Ibañez Shimabukuro M, Reig Cardarella GF, Martorelli SR and Pérez Ponce de León G (2023). Integrative analysis of new *Clinostomum* metacercariae (Digenea, Clinostomidae) using COI mtDNA and morphology rises the number of lineages found in South American freshwater fishes. *Journal of Helminthology*, 97, e85, 1–9
<https://doi.org/10.1017/S0022149X23000706>

Received: 13 August 2023

Revised: 07 October 2023

Accepted: 08 October 2023

Keywords:









New species; integrative taxonomy; COI; Argentina; *Ardea cocoi*

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Integrative analysis of new *Clinostomum* metacercariae (Digenea, Clinostomidae) using COI mtDNA and morphology rises the number of lineages found in South American freshwater fishes

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Abstract

Clinostomidae is a diverse family of digenean parasitizing fish-eating birds as adults and fishes as metacercariae. The species composition, within the genus *Clinostomum* has been steadily increasing in recent years. In Argentina, four named species of *Clinostomum* have been documented, accompanied by four metacercariae representing distinct genetic lineages whose adults have not been identified. This study focused on examining clinostomids in three fish species – *Australoheros scitulus* (ASI), *Cichlasoma dimerus* (CDIM), and *Pimelodella laticeps* (PLA) – at various localities in Argentina. We conducted both morphological and molecular characterizations of the *Clinostomum* metacercariae collected from these fish species. Molecular phylogenetic analyses using COI mtDNA were performed to determine the placement of these metacercariae within the clinostomid phylogenetic tree. *Clinostomum* ASC represents a distinct lineage, morphologically distinguishable from other sequenced metacercariae due to its body shape (widest anteriorly and becoming slender towards the posterior end); this lineage was found to be closely related to *C. caffrae*. While *Clinostomum* CDIM and *Clinostomum* PLA exhibited morphological differences, they clustered together genetically with metacercariae reported in previous studies as *Clinostomum* L3 and *Clinostomum* CVI. This outcome, coupled with a low genetic distance (0 to 3%), suggests that they are conspecific with metacercariae found in fish across Mexico, Costa Rica, and Argentina. In light of the extensive diversity of fish species in Argentine freshwater ecosystems (over 500 species), and considering the relatively constrained extent of prior investigations, the anticipation of unearthing additional *Clinostomum* species or lineages is plausible.

Introduction

The clinostomids comprise a diverse group of digenean, yet their true diversity remains largely unexplored. In their mature stage, clinostomids act as parasites within the upper respiratory tract and esophagus of piscivorous birds, reptiles (Kanev *et al.* 2002), and mammals including humans (Kifune *et al.*, 2000; Park *et al.*, 2009; Hara *et al.*, 2014; Lee *et al.*, 2017; Kim *et al.*, 2019). DNA investigations have yielded valuable insights into clinostomid taxonomy and distribution. These studies have unveiled a dichotomy between the “New World” and “Old World” clinostomid forms (Locke *et al.*, 2015). Within the Americas, clinostomids exhibit several genetic lineages. Noteworthy contributions have significantly enriched the catalog of identified clinostomid lineages within Argentina (Montes *et al.*, 2020; 2021). In Argentina there have been documented four species, namely *Clinostomum detruncatum* Braun, 1899, *Clinostomum heluans*, Braun, 1899 *Clinostomum marginatum* (Rudolphi 1819) Braun 1901 *sensu stricto*, and *Clinostomum fergaljarii* Montes, Barneche, Pagano, Ferrari, Martorelli, & Pérez Ponce de León, 2021. Additionally, four distinct metacercariae lineages parasitize various fish species: *Clinostomum* sp. CVI and *Clinostomum* sp. GBA in Cichlidae from Esteros del Iberá, Corrientes province, *Clinostomum* sp. CRA in Crenuchidae from Ayui River, Entre Rios province, *Clinostomum* sp. PAU in Lebiasinidae from Timboy River, Corrientes province, and the metacercariae of *C. heluans* in Cichlidae from La Plata, Buenos Aires province (Montes *et al.*, 2020, 2021). In addition to these lineages, *Clinostomum* sp. metacercariae lacking genetic data have been reported, infecting fish

species like *Trigonectes aplocheiloides* Huber, *Hoplosternun littorale* Hancock both from Salta province (Davies et al., 2016), *Neofundulus paraguayensis* Eiganmann and Kennedy from Formosa province (Szidat, 1969); and *Hypostomus plecostomus* (Weyenbergh) from Cordoba province although this host species is not present in Argentina according to Mirande & Koerber (2020). Furthermore, juvenile clinostomids have been reported parasitizing *Tigrisoma lineatum* (Boddaert) from Formosa by Lunaschi & Drago (2009).

In the present study, we have identified *Clinostomum* sp. metacercariae in diverse fish hosts and an adult parasitizing *Ardea cocoi* Linnaeus across several Argentine localities. The principal aim of this study is to present new reports of clinostomids using molecular data, and to discuss the diversity of clinostomids within Argentina.

Material and Methods

Specimens used in this study were collected between of 2019 to 2021 from three locations in Argentina (Fig. 1). Freshwater fish species *Australoheros scitulus* Řičan & Kullander (ASC), *Cichlasoma dimerus* (Heckel) (CDIM), and *Pimelodella laticeps* (Eigenmann) (PLA) were obtained through seining procedures conducted at El Palmar National Park, (31°53'39" S and 58°14'05" W, Entre Rios province), Juan Blanco River (35°08'30" S, 57° 26'27" W Buenos Aires province), and Espinillo River (35° 08' 08" S, 57° 42' 37" W Buenos Aires province) (Fig 1). Encysted metacercariae of *Clinostomum* sp. were recovered from both the dorsal and ventral fins of *C. dimerus* and *A. scitulus*, as well as from the axial musculature of *P. laticeps*. These digeneans were subsequently extracted from their cysts, rinsed in 0.65% saline solution, and then preserved in 95% ethanol for later morphological and molecular assessments. Notably, an additional *Clinostomum* sp. specimen was obtained from the esophagus of a deceased heron (*A. cocoi*) found in Santo Tome, Santa Fe Province (31° 41' 58" S, 60° 45' 27" W, Fig. 1) in September 2017. This specific specimen was used for genetic analysis.

Metacercariae were stained with chlorohydric carmine. These samples underwent dehydration through a series of ethanol solutions, followed by cleared and mounting in Canada balsam according to the protocol established by Pritchard & Kruse (1982). Subsequently, each specimen was photographed using an AmScope MU 1000 MP digital camera attached to the Olympus BX51 microscope. Measurements of the specimens were conducted using ImageJ software (Schneider et al., 2012), and drawings were created using a drawing tube coupled with a light microscope.

Molecular analysis involved DNA extraction from entire specimens of metacercariae belonging to *C. dimerus*, *A. scitulus*, *P. laticeps*, and *A. cocoi*. PURO-Genomic DNA (Productos Bio-Lógicos) was employed for DNA extraction, following the manufacturer's protocol. The partial COI-mtDNA gene fragment was amplified through Polymerase Chain Reaction (PCR) using an Eppendorf Mastercycler thermal cycler. The forward primer DICE 1F (5'-ATTAACCCTCACTAAATWC NTTRGATCATAAG-3') and the reverse primer DICE 14R (5'-TAATACGACTCAC TATACCHACMRATAACATATGATG-3') as designed by Van Steenkiste et al. (2015) were employed for amplification. The 50-µl reaction mixture consisted of 25 µl of PB-L master mix (Productos Bio-Lógicos, Argentina), 0.4 µM of each forward and reverse primer, and 4 µL of the template DNA, followed the manufacturer's protocol. Thermocycling conditions were based on those described by Montes et al. (2021). Sequencing of the PCR products was performed by MacroGen Inc. The assembled

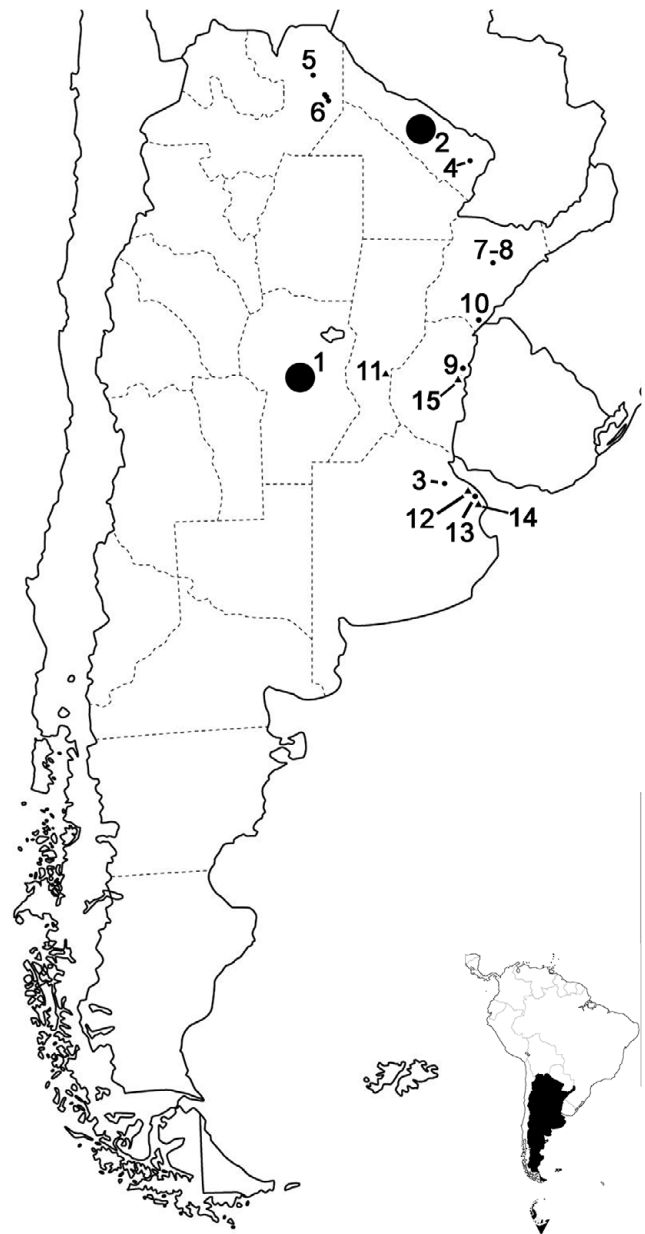


Figure 1. Map of Argentina showing the sampling localities and previous reports of *Clinostomum* spp. (1) Province of Cordoba (Weyenbergh, 1878), (2) Province of Formosa (Szidat, 1969), (3) Uribelarrea city (Boero & Led, 1971), (4) Pirané city, Formosa province (Lunaschi & Drago, 2009), (5) Hickman locality, 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 (Montes et al., 2020). (10) Montecaseros, Corrientes province (Montes et al., 2021). (11) Santo Tome, Santa Fe Province (this study). (12) Juan Blanco stream, Buenos Aires Province (this study). (13) La Balandra, Buenos Aires Province (Sutton & Damborenea, 2000). (14) Espinillo stream, Buenos Aires Province (this study). (15) El Palmar National Park, Corrientes Province (this study). Small circles = previous reports, big circles = diffuse locality, only province, triangle = present study

sequences were then examined for the presence of pseudogenes using the Geneious 5.1.7 program. Alignments of clinostomid species/lineage sequences were carried out using MAFFT 7 (Katoh & Standley, 2013). Outgroups consisted of sequences of *Euclinostomum heterostomum* (Rudolphi 1809), *Ithyoclinostomum yamagutii* Rosser, Woodyard, Mychajlonka, King, Griffin, Gunn &

López-Porras, 2020 and *Odhneriotrema incomodum* (Leidy, 1850). Optimal partitioning schemes and substitution models for each DNA partition were determined using the Bayesian Information Criterion (BIC) with the “greedy” search strategy in Partition Finder v. 1.1.1 (Lanfear *et al.*, 2012). The dataset encompassing barcode fragments was partitioned based on first-, second-, and third-codon positions, each employing the appropriate nucleotide substitution model. The first codon position employed the Tamura-Nei model with estimates of invariant sites and gamma-distributed among-site variation (TrN+I+G), the second codon position utilized the Kimura 1981 model with unequal base frequencies (K81uf), and the third codon position was characterized by the general time-reversible model with gamma-distributed among-site variation (GTR + G). For the Bayesian Inference analyses, the implemented model was GTR for the three positions because the less complex TrN+I+G and K81uf are not implemented in Mr. Bayes. The first with invariant sites and gamma-distributed among-site variation (GTR+I+G) and the second with equal-distributed among-site variation (GTR).

Phylogenetic reconstruction was conducted through Bayesian Inference (BI) using Mr. Bayes 3.2.3 (Ronquist *et al.*, 2012). The construction of phylogenetic trees involved two parallel Metropolis-Coupled Markov Chain Monte Carlo (MCMC) analyses, each spanning 20 million generations. The purpose of these analyses was to estimate the posterior probability (PP) distribution. Topology sampling occurred at intervals of 1,000 generations, with

the average standard deviation of split frequencies remaining below 0.01 by the end of the run, following the recommendations of Ronquist *et al.* (2012). Bayesian posterior probability (PP) was employed to assess clade robustness, with PP values exceeding 0.90 signifying strong support. After discarding the initial 25% of trees as “burn-in,” a majority consensus tree with branch lengths was reconstructed for each run.

Additionally, the uncorrected p-distance was computed using MEGA X (Kumar *et al.*, 2018), and newly generated sequences were submitted to GenBank (Table 1). Specimens that underwent staining were deposited in the Invertebrate Collection of the Museo de La Plata, La Plata, Argentina.

Results

The measurements (Table 2) of *Clinostomum* ASC (FIG 2A), *Clinostomum* PLA (FIG 2B) and *Clinostomum* CDIM (Fig. 2C) were made using 5, 7 and 7 specimens, respectively.

Morphological description

Clinostomidae Lühe, 1901

Clinostomum (ASC) Leidy, 1856

Body elongated spines absent, flattened anterior end with underdeveloped oral collar end. Posterior body end slender. Oral sucker subterminal, rounded, smaller than ventral sucker.

Table 1. Information on clinostomids species/lineages used to construct the cytochrome c oxidase subunit I (COI) phylogenetic tree showed in Fig 1. New sequences in bold

Species/Lineages	COI sequence	Host Order and Family	Host species	Locality	Sequence from
<i>Clinostomum album</i>	MH282542	Planorbidae	<i>Planorbella trivolvis</i>	Mississippi, USA	Rosser <i>et al.</i> (2018)
<i>Clinostomum arquus</i>	KJ477565	Cyprinodontiformes, Poeciliidae	<i>Pseudoxiphophorus jonesii</i>	Presa Los Ocotés, México	Pérez Ponce de León <i>et al.</i> (2016)
<i>Clinostomum brienii</i>	MH253044	Siluriformes, Clariidae	<i>Clarias gariepinus</i>	Limpopo, South Africa	Caffara <i>et al.</i> (2019)
<i>Clinostomum</i> ASC	OR645410	Cichliformes, Cichlidae	<i>Australoheros scitulus</i>	Entre Ríos, Argentina	This Study
<i>Clinostomum attenuatum</i>	KP150305	Anura, Ranidae	<i>Lithobates</i> sp.	Quebec, Canada	Locke <i>et al.</i> (2015)
<i>Clinostomum caffarae</i>	KU156797	Pelecaniformes, Ardeidae	<i>Egretta thula</i>	Lago de Catemaco, México	Pérez Ponce de León <i>et al.</i> (2016)
<i>Clinostomum cichlidorum</i>	KU156816	Perciformes, Cichlidae	<i>Archocentrus siquia</i>	Río Las Vueltas, Costa Rica	
<i>Clinostomum</i> “Cra”	MF673556-57	Characiformes, Crenuchiidae	<i>Characidium rachovii</i>	Entre Ríos Argentina	Montes <i>et al.</i> (2020)
<i>Clinostomum</i> “Adult-Cra”	MW187310	Pelecaniformes, Ardeidae	<i>Ardea cocoi</i>	Santa Fe, Argentina	Montes <i>et al.</i> (2021)
<i>Clinostomum</i> “Cra”	MF673562-63	Characiformes, Characidae	<i>Hyphessobrycon anisitsi</i>	Entre Ríos Argentina	
<i>Clinostomum</i> “CDIM”	OR645411	Cichliformes, Cichlidae	<i>Cichlasoma dimerus</i>	Bueno Aires, Argentina	This study
<i>Clinostomum</i> “Cvi”	MF673558-59		<i>Crenicichla vitatta</i>	Corrientes, Argentina	Montes <i>et al.</i> (2020)
<i>Clinostomum complanatum</i>	KM518246	Caudata: Salamandridae	<i>Triturus carnifex</i> or <i>Lissotriton vulgaris</i>	Italy	Caffara <i>et al.</i> (2014)
<i>Clinostomum cutaneum</i>	KP110515	Pelecaniformes, Ardeidae	<i>Ardea cinerea</i>	Central, Kenya	Locke <i>et al.</i> (2015)
<i>Clinostomum detruncatum</i>	KP110518	Synbranchiformes, Synbranchidae	<i>Synbranchus marmoratus</i>	Guaira, Brazil	
<i>Clinostomum fergallarii</i>	MW187308-09	Pelecaniformes, Ardeidae	<i>Ardea Cocoi</i>	Buenos Aires, Argentina	Montes <i>et al.</i> (2021)
	OR645409			Santa Fe, Argentina	This study

(Continued)

Table 1. (Continued)

Species/Lineages	COI sequence	Host Order and Family	Host species	Locality	Sequence from
<i>Clinostomum</i> "Gba"	MF673560-61	Cichliformes, Cichlidae	<i>Gymnogeophagus balzanii</i>	Corrientes, Argentina	Montes et al. (2020)
<i>Clinostomum</i> L1	KU156782	Siluriformes, Pimelodidae	<i>Rhamdia guatemalensis</i>	Rio San Juan, México	Pérez Ponce de León et al. (2016)
<i>Clinostomum</i> L3	KJ477500	Pelecaniformes, Ardeidae	<i>Tigrisoma mexicanum</i>	Emiliano Zapata, México	
<i>Clinostomum</i> M1	KY865681	Siluriformes, Schilbeidae	<i>Schilbe intermedius</i>	South Africa	Caffara et al. (2017)
<i>Clinostomum</i> M2	KY865662	Osteoglossiformes, Mormyridae	<i>Marcusenius macrolepidotus</i>		
<i>Clinostomum</i> M3	KY865667	Siluriformes, Amphiliidae	<i>Amphilius uranoscopus</i>		
<i>Clinostomum</i> M4	KY865661	Cypriniformes, Cyprinidae	<i>Barbus trimaculatus</i>		
<i>Clinostomum marginatum sensu lato</i>	JF718618	Anura, Ranidae	<i>Rana clamitans</i>	Quebec, Canada	Caffara et al. (2011)
	JF718619	Perciformes, Centrarchidae	<i>Lepomis gibbosus</i>		
	MH282538		<i>Lepomis macrochirus</i>	Mississippi, USA	Rosser et al. (2018)
	JX630993	Cypriniformes, Catostomidae	<i>Catostomus nebuliferus</i>	Durango, Mexico	Sereno-Urbe et al. (2013)
	JX630995	Pelecaniformes, Ardeidae	<i>Ardea alba</i>	Veracruz, Mexico	
<i>Clinostomum phalacrocoracis</i>	KP110522		<i>Ardea cinerea</i>	Central, Kenya	Locke et al. (2015)
<i>Clinostomum philippiense</i>	KP110523	Perciformes, Osphronemidae	<i>Trichogaster microlepis</i>	Thailand	
<i>Clinostomum</i> "Pau"	MW187306-07	Characiformes, Lebiasinidae	<i>Pyrrhulina australis</i>	Corrientes, Argentina	Montes et al. (2021)
<i>Clinostomum</i> PLA	OR645412	Cichliformes, Cichlidae	<i>Pimelodella laticeps</i>	Buenos Aires, Argentina	This study
<i>Clinostomum poteae</i>	MH282551	Suliformes, Phalacrocoracidae	<i>Nannopterum auritus</i>	Mississippi, USA	Rosser et al. (2018)
<i>Clinostomum</i> sp. 1	KP110524	Siluriformes, Pimelodidae	<i>Rhamdia guatemalensis</i>	Yucatan, Mexico	Locke et al. (2015)
<i>Clinostomum</i> sp. 2	KP110526	Perciformes, Gobiidae	<i>Sicydium salvini</i>	Oaxaca, Mexico	
<i>Clinostomum</i> sp. 3	KP110530	Cyprinodontiformes, Poeciliidae	<i>Poecilia mexicana</i>	Veracruz, Mexico	Pérez Ponce de León et al. (2016)
<i>Clinostomum</i> sp. 4	KP110531	Cichliformes, Cichlidae	<i>Apistogramma</i> sp.	Iquitos, Peru	Locke et al. (2015)
<i>Clinostomum</i> sp. 5	KP110532		<i>Cichlasoma boliviense</i>	Santa Cruz, Bolivia	
<i>Clinostomum heluans</i>	KP110534		<i>Cichlasoma boliviense</i>	Santa Cruz, Bolivia	
	MG860853	Pelecaniformes, Ardeidae	<i>Ardea alba</i>	Mexico	Briosio-Aguilar et al. (2018)
	MG860852	Perciformes, Cichlidae	<i>Australoheros</i> sp.	Brazil	
	MW187311		<i>Cichlasoma dimerus</i>	Buenos Aires, Argentina	Present Study
<i>Clinostomum sinensis</i>	MK801713	Cypriniformes, Cyprinidae	<i>Candidia barbata</i>	Taiwan	Caffara et al. (2019)
<i>Clinostomum</i> sp. 7	KJ818259	Cyprinodontiformes, Poeciliidae	<i>Poecilia reticulata</i>	Minas Gerais, Brazil	Pinto et al. (2015)
<i>Clinostomum tataxumui</i>	KJ504192	Pelecaniformes, Ardeidae	<i>Tigrisoma mexicanum</i>	Laguna Manialtepec, Mexico	Pérez Ponce de León et al. (2016)
<i>Clinostomum tilapiae</i>	KY649357	Siluriformes, Mochokidae	<i>Synodontis batensoda</i>	Anambra River, Nigeria	Caffara et al. (2017)
<i>Clinostomum ukolii</i>	MN044350				Caffara et al. (2020)
<i>Euclinostomum heterostomum</i>	KP721420	Cichliformes, Cichlidae	Cichlids	Lake Kinneret, Israel	Caffara et al. (2016)
<i>Ithyoclinostomum yamagutii</i>	MN696163	Pelecaniformes, Ardeidae	<i>Ardea herodias</i>	Mississippi, USA	Rosser et al. (2020)
<i>Odhneriotrema incommodum</i>	MF766002	Crocodylia, Alligatoridae	<i>Alligator mississippiensis</i>	Mississippi, USA	Woodyard et al. (2017)

Table 2. Comparative measurements of *Clinostomum* metacercariae reported in the present manuscript. Measurements are shown in μm with the mean followed by the range (when available). Abbreviation: ASC = *Australoheros scitulus*, CDIM = *Cichlasoma dimerus*, PLA = *Pimelodella laticeps*

	ASC	CDIM	PLA
N° specimens measured	5	7	7
Body length	3557 (3295–3902)	4378 (3837–4947)	4566 (2669–6496)
Body width	870 (805–946)	1215 (1107–1352)	1148 (812–1445)
Hindbody length	973 (912–1067)	1337 (1144–1566)	1464 (909–1943)
Forebody length	2584 (2375–2835)	3041 (2693–3526)	3102 (1760–4553)
Cephalic collar length	397 (355–433)	827 (688–892)	752 (538–954)
Cephalic collar width	210 (174–228)	409 (365–443)	422 (207–596)
Oral sucker length	157 (134–170)	250 (213–292)	232 (144–304)
Oral sucker width	169 (116–185)	238 (196–294)	210 (157–309)
Prepharynx	53 (45–67)	72 (61–82)	84 (60–100)
Pharynx length	75 (67–80)	148 (106–177)	144 (151–173)
Pharynx width	68 (52–78)	121 (93–153)	149 (141–206)
Caeca length	3194 (2920–3498)	3787 (3242–4400)	3993 (2231–5783)
Caeca width	178 (109–222)	206 (144–299)	345 (70–617)
Ventral sucker length	444 (420–468)	695 (622–805)	708 (504–932)
Ventral sucker width	451 (415–488)	731 (656–844)	686 (536–871)
Anterior testis length	60 (49–74)	173 (135–235)	288 (238–367)
Anterior testis width	145 (99–183)	474 (354–577)	362 (303–477)
Posterior testis length	46 (39–54)	173 (120–211)	287 (191–390)
Posterior testis width	107 (87–147)	479 (371–554)	372 (326–514)
Cirrus–sac length	108 (108–108)	371 (297–430)	351 (245–426)
Cirrus–sac width	57 (57–57)	150 (123–167)	143 (101–189)
Ovary length	70 (59–78)	131 (86–184)	163 (106–238)
Ovary width	29 (26–31)	123 (71–166)	105 (60–143)
Uterine sac length	732 (474–1298)	513 (386–655)	581 (343–787)
Uterine sac width	30 (17–44)	194 (105–317)	152 (99–236)

Prepharynx short. Intestinal caeca slightly diverticulated, lateral to ventral sucker and genital primordium extending to posterior end of body. Ventral sucker 2–3 times larger than oral sucker, with almost triangular opening. Primordium of genital complex in posterior end of body. Anterior testis slightly triangular. Posterior testis transversely elongated. Cirrus sac, kidney-shaped in right margin of anterior testis. Ovary small, oval, intertesticular and dextral. Uterine sac tubular, long, between genital complex and almost reaching ventral sucker.

Clinostomum (CDIM) Leidy, 1856

Body elongated spines absent, flattened anterior end with oral collar. Oral sucker subterminal, rounded, smaller than ventral sucker. Prepharynx short. Intestinal caeca slightly diverticulated lateral to ventral sucker and genital primordium extending to posterior end of body. Ventral sucker 2–3 times larger than oral sucker, with almost triangular opening. Primordium of genital complex postequatorial. Anterior testis transversally elongated with irregular margins. Posterior testis triangular, base transversely elongated, apex rounded, irregular margin. Cirrus sac, kidney-shaped, elongated, in right margin of anterior testis. Ovary small,

oval, intertesticular and dextral. Uterine sac tubular, short between genital complex and almost reaching ventral sucker.

Clinostomum (PLA) Leidy, 1856

Body elongated spines absent, flattened anterior end with oral collar. Oral sucker subterminal, rounded, smaller than ventral sucker. Prepharynx short. Intestinal caeca deeply diverticulate lateral to ventral sucker and genital primordium extending to posterior end of body. Ventral sucker 2–3 times larger than oral sucker, with almost triangular opening. Primordium of genital complex in posterior third of body. Anterior testis slightly triangular. Posterior testis slightly triangular-shaped and almost irregular margin. Cirrus sac, kidney-shaped, elongated, in right margin of anterior testis. Ovary small, oval, intertesticular and dextral. Uterine sac tubular, not observed.

Molecular analysis

Partial COI mtDNA sequences were obtained for one specimen of *Clinostomum* sp. ASC, *Clinostomum* PLA, *Clinostomum* CDIM, and an adult of *C. fergallarii* collected from a heron. The final COI

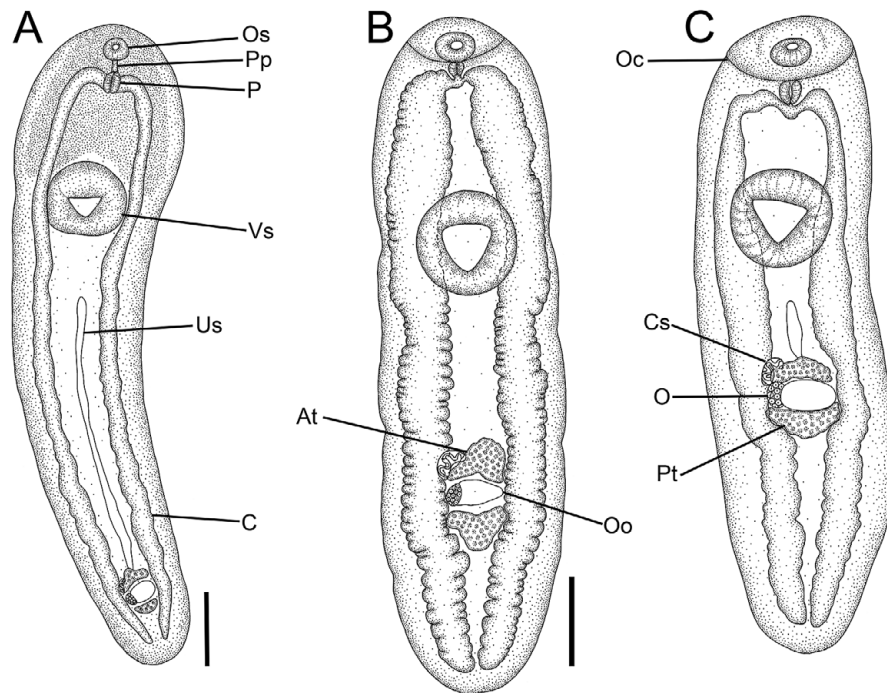


Figure 2. Clinostomid metacercariae infecting (A) *Australoheros scitulus*; (B) *Pimelodella laticeps*; (C) *Cichlasoma dimerus*. Abbreviations: At = Anterior testis; C = caecum; Cs = cirrus sac; O = ovary; Oc = oral collar; Oo = ootype; Os = oral sucker; P = pharynx; Pp = Prepharynx; Pt = posterior testis; Vs = ventral sucker; Us = uterus. Scale bars = 500 μ m.

alignment was 603 bp long and consisted of 56 terminals including isolates of the newly sequenced specimens of *Clinostomum* and three sequences of other clinostomids used as outgroups (Fig. 3) downloaded from GenBank. The new sequences nested within the New World clade in the phylogenetic tree.

The metacercaria of *Clinostomum* ASC represents a potential candidate of a new species. This lineage was recovered as the sister species of *Clinostomum caffarae* Sereno-Uribe, García-Varela, Pinacho-Pinacho & Pérez Ponce de León, 2018 (Fig. 3) with high posterior probability support value (PP=0.98). The genetic distance between *Clinostomum* ASC and the other closely related *Clinostomum* specie/lineages such as *C. caffarae*, *Clinostomum* L3, *Clinostomum* sp. 1, *Clinostomum* PLA and *Clinostomum* GBA was 10% (Supplementary table S1).

Clinostomum PLA was clustered in a clade with *Clinostomum* L3, and *Clinostomum* sp1 and sp2, albeit with low posterior probability support value (Fig. 3). The genetic distance between *Clinostomum* PLA and the other lineages included in the same clade (*Clinostomum* L3, sp1, sp2, CVI and CDIM) varied between 1–2%. The metacercariae of *Clinostomum* CDIM was yielded in a node with *Clinostomum* L3, sp1, sp2, PLA, and CVI with low support value (Fig. 3, Supplementary table S1). The distance between those metacercariae varied from 0–2%. The newly generated sequence of an adult of *Clinostomum* was nested within the clade of *C. fergallarii* and sequences were identical, showing conspecificity.

Discussion

As a part of a comprehensive study aimed at describing the freshwater fish parasite fauna from the Northeast and Middle regions of Argentina, we have found and reported new clinostomid metacercariae. DNA analysis revealed unexpected findings, including the identification of a new lineage recognized as *Clinostomum* ASC. A

feature that distinguishes these metacercariae from others found in freshwater fishes in Argentina is their body shape, which is widest anteriorly and becoming slender posteriorly. Similar metacercariae have been found by Murrieta-Morey *et al.* (2022) in *Apistogramma* sp., *Cichlasoma amazonarum* (Kullander) and *Pterophyllum scalare* (Schultze) from Peru. These host species (such as *A. scitulus* reported here) belong to the Cichlidae family. Furthermore, the site of infection, i.e., the fins, is consistent across all the infected fishes. In addition to *Clinostomum* ASC, other species reported in Argentina from cichlids include *Clinostomum* CVI (see below), *Clinostomum* CDIM (see below), *Clinostomum* GBA, and *C. heluans* (Montes *et al.*, 2020; 2021). The presence of *C. fergallarii* in *A. coqui* from Santa Fe (Fig. 1) represents a new locality record, thereby expanding the distribution of this species 500 km to the north.

The *Clinostomum* CDIM found in cichlid fish shows a low genetic difference compared to the metacercariae *Clinostomum* PLA (reported here in a siluriform fish), *Clinostomum* CVI, *Clinostomum* L3, *Clinostomum* sp. 1 and *Clinostomum* sp. 2. According to Pérez Ponce de León *et al.* (2016), *Clinostomum* L3 exhibits a low divergence value compared to *Clinostomum* sp. 1 and *Clinostomum* sp. 2 indicating that they may be congeners.

In a previous study, Montes *et al.* (2020) compared the morphology of *Clinostomum* CVI with *Clinostomum* L3 of Sereno-Uribe *et al.* (2018) and concluded that the only difference observed was the diverticulate margins of caeca; the genetic difference was 4%. This value does not represent a threshold for considering them as either a different entity or the same lineage found by Pérez Ponce de León *et al.* (2016). Based on our findings of *Clinostomum* PLA and *Clinostomum* CDIM, in the context of the new phylogenetic analysis, we consider them conspecific with *Clinostomum* sp1, *Clinostomum* Sp2, *Clinostomum* L3 and *Clinostomum* CVI reported by Locke *et al.* (2015), Pérez Ponce de León *et al.* (2016), Sereno-Uribe *et al.* (2018) and Montes *et al.* (2020), respectively. Interestingly, the

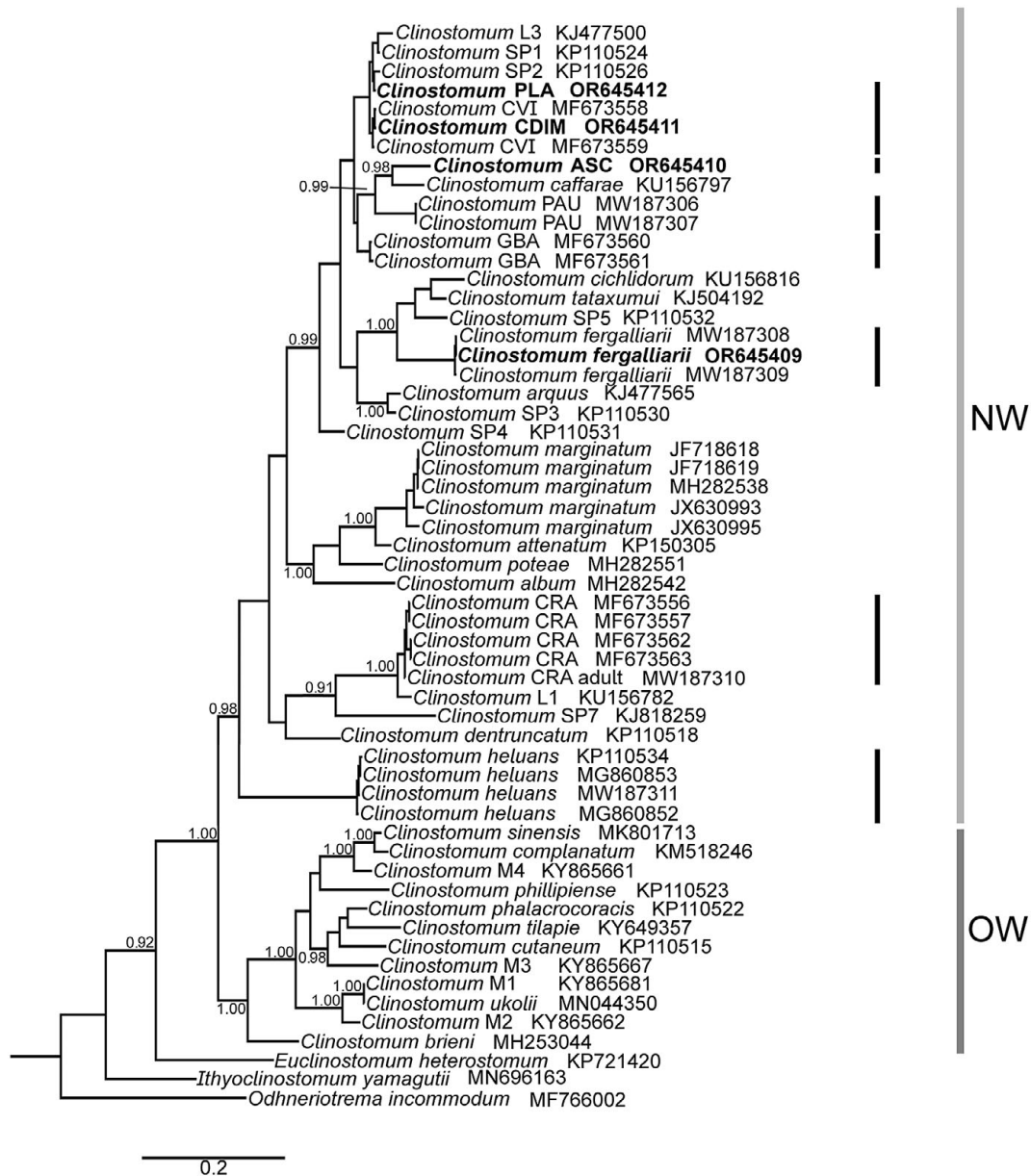


Figure 3. Phylogenetic tree of species/lineages of clinostomids through the cytochrome c oxidase subunit I (COI) gene inferred using Bayesian Inference. Numbers in the nodes represent posterior probability (<90% are not shown). *Clinostomum* species sequenced in this study are in bold and the black bars indicate species reported from Argentina.

morphology of these metacercariae shows some variation depending on the infected host or the development stage of the larvae. This lineage, herein named *Clinostomum* L3 (the name used when morphologically described), exhibits a wide distribution range in the Neotropical biogeographical region since it has been reported from Mexico, Costa Rica, and Argentina. Although this species infects a siluriform fish (Heptapteridae) in Mexico and Argentina, it has been reported in Gobiiformes (Eleotridae) in Costa Rica, and now in Cichliformes (Cichlidae) in Argentina. Other fish may host this digenean in other regions of South America independently of the order or family of their host. The wide distribution of these parasites is the result of their low specificity as metacercariae towards the second intermediate hosts and even their definitive host. The use of several hosts allowed *Clinostomum* L3 to present a wide geographical range that extends between Mexico and Argentina.

Additionally, this digenean does not show specificity for the site of infection. For example, they have been found in the mesentery and fascia of the muscle tissue in *Crenicichla vittata* Heckel (Montes *et al.*, 2019), in the muscle of *P. laticeps* (*Clinostomum* PLA), and in the fins of *C. dimerus* (*Clinostomum* CDIM). The final host species as reported by Sereno-Uribe *et al.* (2018) are the great blue heron, *Ardea herodias* L., and the bare-throated tiger heron, *Tigrisoma mexicanum* Swainson, both members of the family Ardeidae. These authors were only able to sequence the digenean and did not have enough samples to formally describe the species. Other Ardeidae species within Argentina could be infected with that clinostomid. The large distribution of this digenean across the Americas is not unexpected, as this distribution range has been reported for other digenean such *Austrodiplostomum compactum* (Lutz, 1928) Dubois, 1970 (Ostrowski de Núñez, 2017

The accumulation of information on clinostomids, including the new sequences reported in this study corroborates the presence of two nodes evolving in the New and the Old World. However, we must continue to monitor the wildlife vertebrates, as there is a possibility of *Clinostomum* sp. from Old World being present in the Americas. This is supported by the potential for natural migration of birds from Africa/Europe as documented in the past for *Bulbucus ibis* (L.) (Cosby, 1972).

Similar long-distance natural migrations have been reported in other continents, for example, another heron species, *Egretta garzetta* (L.) was captured in Trinidad by Downs (1959) after being banded six months earlier in Spain 4000 miles across the Atlantic.

Considering the large number of freshwater fish species reported in Argentina [over 500 according to Mirande & Koerber (2020)], we suspect that there may be more species of clinostomids awaiting discovery either in freshwater fish or fish-eating birds. Furthermore, conducting new studies on bird parasites could lead to the discovery of adult forms that are necessary for the description of new species. Based on our current knowledge, at least five lineages are awaiting formal description in Argentina. Overall, this paper contributes to the knowledge of clinostomid diversity, highlighting the importance of molecular information for accurate identification, and showing the implications of these findings for the understanding of parasite-host interactions in the region.

Supplementary material. The supplementary material for this article can be found at <http://doi.org/10.1017/S0022149X23000706>.

Acknowledgements. We are grateful to Marcia Montes for the line drawings, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and to CEPAVE for the provision of facilities and equipment.

Financial support. This work was partially supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET PIP 1713), Agencia Nacional de Promoción Científica y Técnica (PICT 2020 SERIE A-01531) and Universidad Nacional de La Plata (PPID N902 to S.R.M.).

Competing interest. On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical standard. 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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