

Research Paper

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Morphological and genetic analysis of a rediscovered *Clinostomum* sp. parasitising *Titanolebias monstrosus* and *Trigonectes aplocheiloides* (Cyprinodontiformes: Rivulidae)

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Abstract

Clinostomids are a group of digeneans in which substantial diversity has been recently discovered, with some metacercariae specific to their fish hosts. Genetic analysis has been instrumental in elucidating species diversity within this genus. Recently, seven COI lineages were reported in Argentina, along with three metacercarial morphotypes lacking molecular data. Two of these were found parasitising Rivulidae fishes. The discovery of *Clinostomum* metacercariae in *Trigonectes aplocheiloides* and *Titanolebias monstrosus* from temporary ponds in the western Chacoan region allowed us to redescribe the metacercariae previously identified by other authors and provide the first sequences of this lineage. The morphology of the metacercariae in both hosts matched previously reported descriptions. Genetic analysis clustered the new lineage with *Clinostomum detruncatum*, *Clinostomum* sp. 7, *Clinostomum* L1, and *Clinostomum* CRA. This discovery leaves only two morphological records of metacercariae to be characterised using DNA sequencing: one in another Rivulidae (*Neofundulus paraguayensis*) and one in a Loricaridae (*Hypostomus* sp.). The present results represent the eighth clinostomid lineage sequenced from Argentina, highlighting the extensive diversity in South America and the many lineages yet to be described or identified, considering that only one of these lineages is formally described based on adult specimens found in the heron *Ardea cocoi*.

Introduction

Species of *Clinostomum* Leidy 1856 have an indirect life cycle involving a snail as the first intermediate host, fish or amphibians as the second intermediate hosts, and fish-eating birds, mammals, or occasionally reptiles as the definitive hosts (Kanev *et al.* 2002). Until recently, species diversity of clinostomids remained largely unknown. This changed with the application of combined morphological and genetic tools, revealing significant species diversity within this family, particularly in the genus *Clinostomum* (e.g., Pérez-Ponce de León *et al.* 2016; Caffara *et al.* 2017; Locke *et al.* 2019; Sereno-Uribe *et al.* 2018; Briosio-Aguilar *et al.* 2018; Montes *et al.* 2020, 2021, 2023). In Argentina, seven COI lineages of *Clinostomum* have been described or reported (Montes *et al.* 2020, 2021, 2023), and phylogenetic analysis supports the division of the genus into two clades: one from the Old World and another from the Americas (Locke *et al.* 2015).

In Argentina, a few metacercariae have been described or cited based solely on morphology, including from *Hypostomus plecostomus* (Linnaeus, 1758) from Córdoba province (Weyenbergh, 1878) and from *Neofundulus paraguayensis* (Eigenmann & Kennedy, 1903) from Formosa province (Szidat, 1969). Additionally, metacercariae were reported in *Trigonectes* sp. (Myers) and *Hoplosternum littorale* (Hancock, 1828) from Salta province, but without molecular data (Davies *et al.* 2016). The records of metacercariae in Rivulidae by Szidat (1969) and Davies *et al.* (2016) are particularly important because of the high value of these fishes to aquarists and their significance as endangered species in the Neotropics (Alonso 2022; Alonso *et al.* 2023, 2024).

Seasonal killifish, members of the Neotropical Rivulidae and African Nothobranchiidae families within the order Cyprinodontiformes, inhabit temporary wetlands that periodically dry up. These fish lay desiccation-resistant eggs that undergo metabolic and developmental arrests

known as diapause, regulated by environmental cues. The eggs hatch when ponds refill with rainwater, whereas adults do not survive the dry period (Podrabsky and Hand 2015; Furness 2016). Seasonal killifish exhibit rapid growth and maturity within a short lifespan, thriving in harsh conditions (Costa 1998; Berois *et al.* 2015). Nearly 48% of killifish species in the Neotropical region are threatened due to their narrow geographic ranges and reliance on seasonal aquatic habitats highly impacted by human activities (Costa 2016; Alonso *et al.* 2018, 2023, 2024).

As part of our studies on killifishes in Argentina, particularly in the western Chacoan region, we discovered *Clinostomum* metacercariae infecting *Trigonectes aplocheiloides* Huber 1995 and *Titanolebias monstrosus* (Huber, 1995). The objectives of this study were to analyse the *Clinostomum* metacercariae found in Rivulidae fish, describe their morphology, and report their COI sequences to evaluate their phylogenetic position.

Materials and methods

Collection of samples and morphological study

Nine *Tr. aplocheiloides* (TRI) and two *Ti. monstrosus* (TMO) were sampled using hand nets near Hickman on the route to Embarcación city (−23.215479, −63.706228, Salta province). The collection site is the same as the one reported by Davies *et al.* (2016) where they found clinostomids parasitising *H. littorale*. It is also located approximately 100 km in a straight line from Bañados del Quinquicho, where those authors discovered infected *Trigonectes* sp. (Fig. 1).

The fish were transported alive in aerated, water-filled bags to a field laboratory. The hosts were euthanised with an overdose of eugenol anesthetic (30 mg/L) and subjected to necropsy. Internal organs (testes, ovaries, liver, intestine, and mesentery) were excised and fixed in 10% buffered formalin for light microscopic studies. The metacercariae of *Clinostomum* were removed from their cyst using needles, rinsed in 0.85% saline solution, and preserved in 96% ethanol for molecular analysis. Some parasites were fixed in formalin after excysting for morphological analysis.

Molecular analysis

DNA was extracted from whole specimens of metacercariae infecting *Tr. aplocheiloides* and *Ti. monstrosus* using PURO-Genomic DNA (Productos Bio-logicos SA) following the manufacturer's protocol. A fragment of the partial COI-mtDNA gene was amplified using polymerase chain reaction on an Eppendorf Mastercycler thermal cyclor with 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 performed with GoTAQ Master Mix (Promega) following the manufacturer's protocol. The thermocycling conditions followed Montes *et al.* (2023). polymerase chain reaction products were sequenced by Macrogen Inc. (Korea). Sequences were manually edited using the Geneious 11 platform. Nucleotide alignment was checked for pseudogenes by translating sequences into amino acids based on the invertebrate mitochondrial genetic code. Newly sequenced barcode fragments were aligned with COI sequences from GenBank using the MAFFT v.7 program (Kato and Standley 2013).

Sequences of *Euclinostomum heterostomum* (Rudolphi 1809), *Ithyoclinostomum yamagutii* Rosser *et al.* 2020, and *Odhneriotrema*

incomodum (Leidy 1850) were used as outgroups as previously done by Montes *et al.* (2023). Optimal partitioning schemes and substitution models for each DNA partition were determined using the Bayesian Information Criterion 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 used the Tamura-Nei model with estimates of invariant sites and gamma-distributed among-site variation (TrN+I+G). The second codon position utilised the Kimura 1981 model with unequal base frequencies (K81uf), and the third codon position was characterised 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 all three positions because the less complex TrN+I+G and K81uf are not implemented in Mr. Bayes. The first codon with invariant sites and gamma-distributed among-site variation (GTR+I+G) while the second codon position used a model with equal-distributed among-site variation (GTR).

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 using Bayesian Inference through MrBayes v. 3.2.1 (Ronquist *et al.* 2012). Topologies were sampled every 1,000 generations. The first 25% of the sampled trees were discarded as ‘burn in’. The consensus tree was visualised 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, using MEGA 7, with 1,000 bootstrap replicates and a nucleotide substitution (transition + transversions) uniform rate. The obtained sequences were deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov>) (Table 1).

Morphological analysis

Specimens of *Clinostomum* TRI and TMO were stained in hydrochloric carmine, dehydrated through a series of ethanol concentrations, cleared, and mounted in Canada balsam (Pritchard and Kruse 1982). Specimens were photographed with an AmScope MU 1000 MP digital camera attached to an Olympus BX51 microscope and measured using ImageJ software (Schneider *et al.* 2012). Whole specimens were photographed with a Leica DMC 4500 digital camera attached to a Leica M205A stereomicroscope. Voucher specimens were deposited in the Invertebrate Collection of the Museo de La Plata, La Plata, Argentina under the accession numbers MLP-HE 8150 (*Clinostomum* TRI) and MLP-HE 8151 (*Clinostomum* TMO).

Results

A total of 72 digeneans were collected from *Tr. aplocheiloides*, with a prevalence of 78%, an abundance of eight, and an intensity of 14.4. Both *Ti. monstrosus* individuals were infected with more than 100 metacercariae. The parasites in both hosts were attached to different organs and musculature, and at various stages of development, with only those showing more mature genitalia being measured.

Clinostomidae Lühe, 1901

Clinostomum Leidy, 1856

Clinostomum TRI (Fig. 2a), TMO (Fig. 2b)

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 Ocotes, México	Pérez Ponce de León <i>et al.</i> (2016)
<i>Clinostomum</i> ASC	OR645410	Cichliformes, Cichlidae	<i>Australoheros scitulus</i>	Entre Ríos, Argentina	Montes <i>et al.</i> (2023)
<i>Clinostomum attenuatum</i>	KP150305	Anura, Ranidae	<i>Lithobates</i> sp.	Quebec, Canada	Locke <i>et al.</i> (2015)
<i>Clinostomum brieni</i>	MH253044	Siluriformes, Clariidae	<i>Clarias gariepinus</i>	Limpopo, South Africa	Caffara <i>et al.</i> (2019)
<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>	Rio Las Vueltas, Costa Rica	
<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”	MF673556–57	Characiformes, Crenuchiidae	<i>Characidium rachovii</i>	Entre Ríos Argentina	Montes <i>et al.</i> (2020)
<i>Clinostomum</i> “Cra”	MF673562–63	Characiformes, Characidae	<i>Hyphessobrycon anisitsi</i>	Entre Ríos Argentina	Montes <i>et al.</i> (2020)
<i>Clinostomum</i> “CDIM”	OR645411	Cichliformes, Cichlidae	<i>Cichlasoma dimerus</i>	Bueno Aires, Argentina	Montes <i>et al.</i> (2023)
<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</i> “Cvi”	MF673558–59	Cichliformes, Cichlidae	<i>Crenicichla vitatta</i>	Corrientes, Argentina	Montes <i>et al.</i> (2020)
<i>Clinostomum detruncatum</i>	KP110518	Synbranchiformes, Synbranchidae	<i>Synbranchus marmoratus</i>	Guaira, Brazil	Locke <i>et al.</i> (2015)
<i>Clinostomum fergalliarrii</i>	MW187308–09	Pelecaniformes, Ardeidae	<i>Ardea Cocoi</i>	Buenos Aires, Argentina	Montes <i>et al.</i> (2021)
	OR645409			Santa Fe, Argentina	Montes <i>et al.</i> (2023)
<i>Clinostomum</i> “Gba”	MF673560–61	Cichliformes, Cichlidae	<i>Gymnogeophagus balzanii</i>	Corrientes, Argentina	Montes <i>et al.</i> (2020)
<i>Clinostomum heluans</i>	KP110534		<i>Cichlasoma boliviense</i>	Santa Cruz, Bolivia	Locke <i>et al.</i> (2015)
	MG860853	Pelecaniformes, Ardeidae	<i>Ardea alba</i>	Mexico	Briosio-Aguilar <i>et al.</i> (2018)
	MG860852	Perciformes, Cichlidae	<i>Australoheros</i> sp.	Brazil	
	MW187311		<i>Cichlasoma dimerus</i>	Buenos Aires, Argentina	Montes <i>et al.</i> (2021)
<i>Clinostomum</i> L1	KU156782	Siluriformes, Pimelodidae	<i>Rhamdia guatemalensis</i>	Rio San Juan, México	Pérez Ponce de León <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (2011)
	JF718619	Perciformes, Centrarchidae	<i>Lepomis gibbosus</i>		
	MH282538		<i>Lepomis macrochirus</i>	Mississippi, USA	Rosser <i>et al.</i> (2018)
	JX630993	Cypriniformes, Catostomidae	<i>Catostomus nebuliferus</i>	Durango, Mexico	Sereno-Uribe <i>et al.</i> (2013)
	JX630995	Pelecaniformes, Ardeidae	<i>Ardea alba</i>	Veracruz, Mexico	

(Continued)

Table 1. (Continued)

Species/lineages	COI sequence	Host order and family	Host species	Locality	Sequence from
<i>Clinostomum</i> TMO	PQ326978–79	Cyprinodontiformes, Rivulidae	<i>Titanolebias monstrosus</i>	Salta, Argentina	This study
<i>Clinostomum</i> “Pau”	MW187306–07	Characiformes, Lebiasinidae	<i>Pyrrhulina australis</i>	Corrientes, Argentina	Montes et al. (2021)
<i>Clinostomum phalacrocoracis</i>	KP110522	Pelecaniformes, Ardeidae	<i>Ardea cinerea</i>	Central, Kenya	Locke et al. (2015)
<i>Clinostomum philippiense</i>	KP110523	Perciformes, Osphronemidae	<i>Trichogaster microlepis</i>	Thailand	Locke et al. (2015)
<i>Clinostomum</i> PLA	OR645412	Cichliformes, Cichlidae	<i>Pimelodella laticeps</i>	Buenos Aires, Argentina	Montes et al. (2023)
<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 guatemensis</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</i> sp. 7	KJ818259	Cyprinodontiformes, Poeciliidae	<i>Poecilia reticulata</i>	Minas Gerais, Brazil	Pinto et al. (2015)
<i>Clinostomum sinensis</i>	MK801713	Cypriniformes, Cyprinidae	<i>Candidia barbata</i>	Taiwan	Caffara et al. (2019)
<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</i> TRI	PQ326976–77	Cyprinodontiformes, Rivulidae	<i>Trigonectes aplocheiloides</i>	Salta, Argentina	This study
<i>Clinostomum ukolii</i>	MN044350	Siluriformes, Mochokidae	<i>Synodontis batensoda</i>	Anambra River, Nigeria	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)

Description (based on five specimens found on *Tr. aplocheiloides* [TRI] and eight specimens on *Ti. monstrosus* [TMO], measurements Table 2). Body elongated, devoid of spines, flattened anterior end with oral collar. Oral sucker subterminal, rounded, smaller than ventral sucker. Developed prepharynx. Short pharynx. Intestinal caeca lateral to ventral sucker and genital primordium extending to posterior end. Diverticulated (TRI) or slightly diverticulated (TMO) intestinal wall. Ventral sucker 2–3 times larger than oral sucker, rounded, almost triangular opening. Genital complex posterior to body middle plane (TRI) or in posterior end (TMO). Testes slightly triangular, points rounded, apex of triangle wide but smaller than elongated base with an almost irregular margin. Concave base of testes facing the ootype. Posterior testis transversely elongated (in TMO). Kidney-shaped cirrus sac in right margin of anterior testis. Small, oval ovary, intertesticular and dextrally located. Tubular uterine sac not observed (TRI), tubular uterine sac, long, between genital complex and ventral sucker (TMO).

Remarks

Despite minor morphological differences (such as pharynx size and cirrus sac length), both metacercariae found in *Tr. aplocheiloides* and *T. monstrosus* constitute a single biological entity and may be identical to those described by Davies et al. (2016). The metacercariae described by these authors were obtained from the same host (*Tr. aplocheiloides*) and *H. littorale*, near the same sampling site as in the present study. The range of measurements and features is quite similar. The main difference observed in the measurements is the body length/body weight ratio, which is smaller in *Trigonectes* sp. compared to those reported here or in *H. littorale* by Davies et al. (2016). Although the oral sucker width falls within the reported range, it is larger in both hosts found by Davies et al. (2016). This measurement affects the ratios of oral sucker width/body width and ventral sucker width/oral sucker width, which are larger and smaller, respectively, in Davies et al. (2016). The distance between the suckers is smaller in both hosts reported by Davies et al. (2016). The anterior

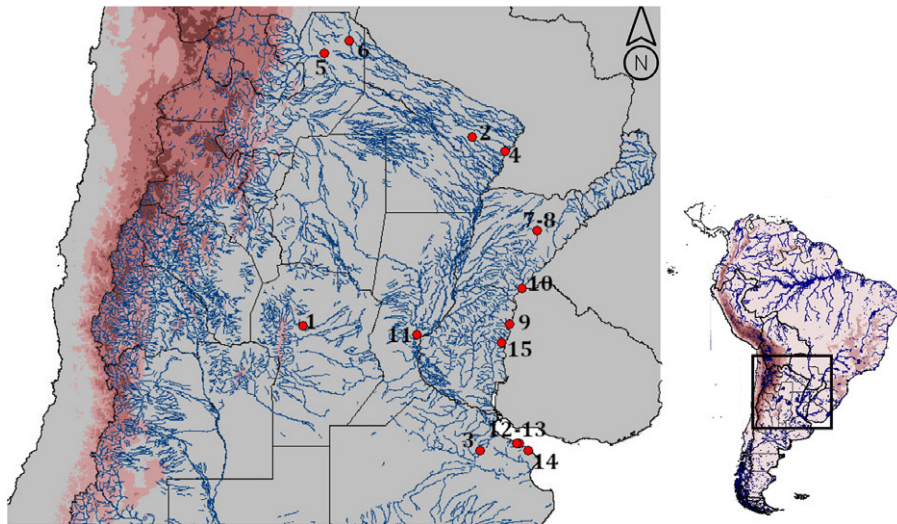


Figure 1. Map of Argentina showing the sampling localities and previous reports of *Clinostomum* spp. Dots: Samples sites without genetic information. **1** Province of Cordoba (Weyenbergh 1878). **2** Province of Formosa (Szidat 1969). **3** Uribelarrea city (Boero and Led 1971). **4** Pirané city, Formosa province (Lunaschi and Drago 2009). **5** Quinquicho Wetland, Salta province (Davies et al. 2016). **6** Hickman locality, Salta province (this study). **7, 8** Ilibera Lagoon, Corrientes province (Montes et al. 2020). **9** Concordia city, Entre Ríos Province (Montes et al. 2021). **10** Montecaseros, Corrientes province (Montes et al. 2021). **11** Santo Tome, Santa Fe Province (Montes et al. 2021). **12** Juan Blanco stream, Buenos Aires Province (Montes et al. 2021). **13** La Balandra, Buenos Aires Province (Sutton and Damborenea 2000). **14** Espinillo stream, Buenos Aires Province (Montes et al. 2023). **15** El Palmar National Park, Corrientes Province (Montes et al. 2023).

and posterior testicle width/length ratios are larger in *Trigonectes* sp. from Davies et al. (2016), where the testicles are wider than they are long. The distance between the testes is also greater in *Trigonectes* sp. from Davies et al. (2016) compared to that found in *Tr.*

aplocheiloides in this study. These observed differences may reflect variation in the maturation stage of the metacercariae.

Molecular analysis

Four partial COI mtDNA fragments were recovered (Table 1), two from *Tr. aplocheiloides* and two from *Ti. monstrosus*. The sequences measured 612 bp for *Clinostomum* TRI and 542–606 bp for *Clinostomum* MON. The final COI alignment was 624-bp long and consisted of 63 terminals, including the newly sequenced *Clinostomum* specimens and outgroups. Metacercariae from both rivulid hosts fell within the New World clade in the phylogenetic tree (Fig. 3), clustering with a p-distance of 0.52% (Supplementary material 1). Phylogenetic analysis showed this lineage as a sister species to the node formed by *Clinostomum* sp. 7, *Clinostomum* L1, and *Clinostomum* CRA. The smallest p-distances were with *Clinostomum arqus* García-Varela, Pinacho-Pinacho & Pérez-Ponce de León, 2018 and *Clinostomum* sp. 3 (10.91%–11.17%), *Clinostomum* L3 (12.50–12.76%), *Clinostomum* GBA (13.12%–13.38%), and *Clinostomum caffarae* Sereno-Uribe, García-Varela, Pinacho-Pinacho & Pérez-Ponce de León, 2018 (13.25%–13.77%).

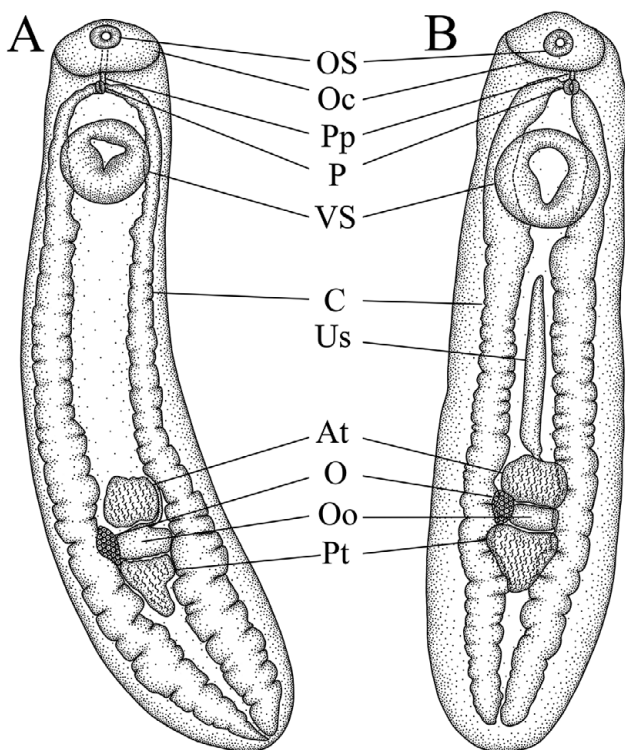


Figure 2. Clinostomid metacercaria from A) *Trigonectes aplocheiloides*; B) *Titanolebias monstrosus*. Abbreviations: At, anterior testis; C, caecum; Cs, cirrus sac; O, ovary; Oc, oral collar; Oo, ootype; Os, oral sucker; P, pharynx; Pp, Prepharynx; Pt, posterior testis; Vs, ventral sucker; Us, uterus. Scale bars = 500 μ m.

Discussion

The metacercariae found in *Tr. aplocheiloides* and *Ti. monstrosus* belong to the same entity and belong to the *Clinostomum* genus.

Previous findings (Montes et al. 2020, 2021, 2023) revealed a wide range of second intermediate hosts of clinostomids in Argentina, including Characidae, Cichlidae, Crenuchiidae, Heptapteridae, and Lebiasinidae. This study is the first to sequence a *Clinostomum* lineage from the host belonging to the Rivulidae. The *Clinostomum* found here represents the eighth lineage identified in Argentina, with only one formally described as a new species so far (*Clinostomum fergalliari* Montes, Barneche, Pagano, Ferrari, Martorelli & Pérez-Ponce de León, 2021). Some metacercariae found are specific to their fish hosts such as *Clinostomum* ASC

Table 2. Measurements of *Clinostomum* sp. infecting Rivulidae host from Embarcacion (Salta, Argentina) compared with those reported for Davies et al. (2016)

	This study		Davies et al. (2016)	
	<i>Trigonectes aplocheiloides</i>	<i>Titanolebias monstrosus</i>	<i>Hoplosternum littorale</i>	<i>Trigonectes</i> sp.
BL	4039 (3499–5942)	4545 (4080–5247)	3,662 (3,398–4,032)	3,542 (2,966–4,752)
BW	1014 (784–1336)	1225 (1060–1384)	1,012 (835–1,152)	1,231 (778–1,930)
BL/BW	3.97 (3.45–4.46)	3.72 (3.31–3.95)	3.62*	2.88*
FbL	1004 (876–1127)	934 (808–1,018)	n/c	n/c
HbL	3,035 (2,453–4,890)	36,11 (3,105–4,273)	n/c	n/c
CoL	332 (286–416)	373 (294–423)	n/c	n/c
CoW	658 (532–810)	617 (512–652)	n/c	n/c
OsL	159 (89–197)	177 (135–229)	229 (187–285)	230 (157–363)
OsW	159 (113–202)	196 (158–230)	355 (246–442)	334 (147–579)
OsW/BW	0.16 (0.08–0.20)	0.16 (0.12–0.21)	0.35*	0.27*
PL	97 (89–108)	83 (70–87)	n/c	n/c
PW	99 (92–104)	70 (62–79)	n/c	n/c
VsL	678 (602–782)	642 (546–713)	613 (540–717)	681 (560–805)
VsW	641 (583–734)	650 (534–744)	603 (520–687)	664 (540–835)
VsW/OsW	4.28 (3.17–6.50)	3.44 (3.03–4.29)	1.70*	2.03*
VsW/BW	0.64 (0.55–0.75)	0.52 (0.45–0.58)	0.6*	0.54*
DbS	429 (239–589)	401 (361–448)	188 (98–314)	56 (128–206)
ATL	265 (250–285)	343 (220–453)	295 (265–324)	275 (167–393)
ATW	270 (209–310)	305 (190–431)	303 (285–353)	355 (265–520)
ATW/ATL	1.02 (0.84–1.22)	0.93 (0.54–1.22)	1.03*	1.3*
PTL	237 (190–293)	343 (244–463)	274 (226–363)	226 (147–304)
PTW	261 (197–317)	346 (230–487)	339 (295–373)	376 (250–599)
PTW/PTL	1.11 (0.92–1.44)	1.04 (0.77–1.38)	1.24*	1.66*
Dbt	129 (103–159)	202 (158–295)	142 (98–196)	191 (167–216)
CsL	175 (140–205)	283 (237–319)	226 (167–275)	211 (167–255)
CsW	77 (66–107)	119 (101–136)	102 (88–137)	88 (79–98)
CsL/BL	0.04 (0.03–0.06)	0.06 (0.06–0.07)	0.06*	0.06*
OL	120 (107–136)	152 (103–217)	160 (137–187)	132 (59–196)
OW	86 (64–108)	113 (84–172)	121 (98–147)	126 (59–285)
OW/OL	0.72 (0.60–0.96)	0.75 (0.56–0.95)	0.76*	0.95*

In bold are measurements of the new specimens. Measurements are shown in μm with the mean followed by the range.

Abbreviations: AT, anterior testicle; B, body; Co, collar; Cs, Cirrus sac; DbS, distance between suckers; Dbt, distance between testicles; Fb, forebody; Hb, hindbody; L, length; n/c, not calculated; O, ovary; Os, oral sucker; P, pharynx; PT, posterior testicle; Vs, ventral sucker; W, width.

*Calculated from the measurements provided by Davies et al. (2016).

on *Australoheros scitulus* Řičan & Kullander 2003, *Clinostomum* GBA on *Gymnogeophagus balzanii* (Perugia, 1891), and *Clinostomum* PAU on *Pyrhulina australis* (Eigenmann and Kennedy, 1903), all of them from Argentina. Others have a broader range of fish hosts, such as *Clinostomum* CRA in *Characidium rachovii* Regan 1913 and *Psalidodon anisitsi* 1907 (Eigenmann), *Clinostomum heluans* Braun, 1899, on *Australoheros* sp., and *Cichlasoma dimerus* (Heckel, 1840) (in Argentina), and *Clinostomum* L3 on *Gobiomorus maculatus* (Günther, 1859), *Rhamdia guatemensis* (Günther, 1864), *Rhamdia laticauda* (Kner, 1858) and *Sicydium salvini* Ogilvie-Grant, 1884. This last lineage, until the analysis of

Montes et al. (2023), was considered separate from others found in Argentina, like *Clinostomum* CVI on *Crenicichla vittata* Heckel, 1840, *C. dimerus* (CDIM); and *Clinostomum* PLA on *Pimelodella laticeps* (Eigenmann, 1917). However, genetic distance among these and other metacercariae (*Clinostomum* sp1 and *Clinostomum* sp2) from the Americas indicates they belong to the same lineage but exhibit different morphologies. The conspecificity among *Clinostomum* L3, *Clinostomum* sp1, and *Clinostomum* sp2 was previously established by Perez Ponce de Leon et al. (2016).

The new *Clinostomum* lineage appears to be less host-specific, parasitising different hosts from the same locality and

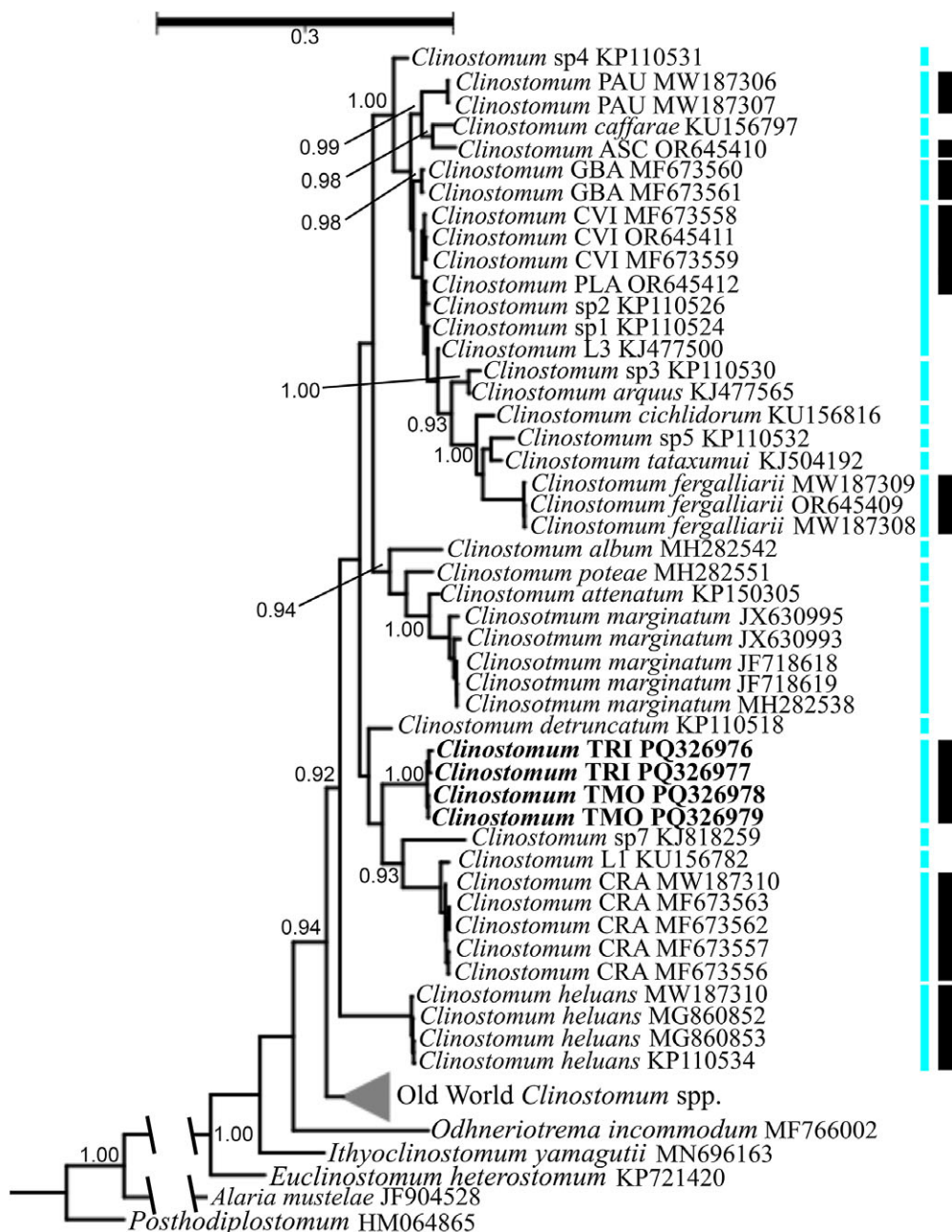


Figure 3. Phylogenetic tree inferred using Bayesian Inference derived from cytochrome c oxidase subunit I (COI) gene dataset. Numbers in the nodes represent posterior probability (<0.9 are not shown). The *Clinostomum* species observed in this study are in bold. Reports from Argentina are indicated with a strong black bar, whereas the turquoise bars represent lineages or species recognised in the New World.

environment. This lineage seems endemic to the Western Chacoan region, particularly in temporary ponds. The parasite appears adapted to the hydrology of these ponds, filling with water in summer from rains and drying in autumn and winter (Alonso *et al.* 2016). In addition, this shallow and confined environment might allow cercariae to parasitise secondary hosts more efficiently.

We found *Clinostomum* metacercariae displaying different degrees of development, from short individuals with underdeveloped genitalia to larger ones with developed testes, particularly in *Tr. aplocheiloides* (Supplementary material 1). Variations in developmental stages could lead to misidentification of distinct lineages without genetic analysis. Accurate description of metacercariae requires ‘mature’ specimens.

The genetic study has reduced the number of morphological-only descriptions (or citations without genetic information) of *Clinostomum* in Argentine fishes, to those found by Weyenbergh (1878) in *H. plecostomus* from Córdoba, which is not a species present in Argentina (Mirande and Koerber 2020, Bogan *et al.* 2024), and that may actually be *Hypostomus commersoni* Valenciennes 1836 or *Hypostomus cordovae* (Günther, 1880); and by Szidat (1969) in *N. paraguayensis*; and juveniles found in bird throats by Lunaschi and Drago (2009) and Lunaschi *et al.* (2007). Without genetic data, it is impossible to determine if these represent previously reported lineages.

The importance of this new metacercariae relies on several aspects. Parasites play vital roles in ecosystems as part of

biodiversity, controlling host populations, and participating in energy flow within food chains (Lafferty *et al.* 2006; Timi and Poulin, 2020). This study of parasites, particularly this lineage, is crucial. Many aquarists value rivulids captured in nature, and the ‘yellow grub’ disease (caused by several *Clinostomum* species) could pose a significant problem if they also collect snails for their biotopes. We have observed that some fishermen use rivulids as bait, which could lead to the translocation of their parasites. Conservation issues related to Rivulidae could result in biodiversity loss in temporary ponds and affect hidden diversity within fish. Changes in rainfall, temperature (Allen and Ingram 2002; Karl and Trenberth 2003) and habitat surroundings, such as large soy plantations and agriculture, could endanger these pools. These habitats are susceptible to fire, agrochemical contamination, and other ecosystem changes (Alonso *et al.* 2018). Limited knowledge of parasite biodiversity and life cycles is crucial for protecting host fishes and ecosystems. Parasites can detect environmental changes, showing more sensitive responses than their hosts and indicating biogeographical shifts (Parmar *et al.* 2016). This underscores the importance of describing this and other parasite species or lineages. Finally, a very interesting line of investigation on the coevolution of those parasites and their seasonal killifish host inhabiting temporary wetlands seems to be a promising avenue for future research.

Supplementary material. The supplementary material for this article can be found at <http://doi.org/10.1017/S0022149X24000658>.

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

Availability of data and material. All the material will be deposited in Museums and the sequences deposited on GenBank

Code availability. Not applicable

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