Journal of Helminthology

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Research Paper

Cite this article: Gião T, Müller MI, Yamada F, Freitas F, Leite L, da Silva RJ, de Azevedo R and Abdallah V (2025). Morphological and molecular phylogeny of *Clinostomum* sp. (Digenea: Clinostomidae) metacercariae, using DNA barcode from a South American freshwater fish. *Journal of Helminthology*, **99**, e13, 1–10
https://doi.org/10.1017/S0022149X24000993

Received: 29 July 2024
Revised: 16 December 2024
Accepted: 25 December 2024

Keywords:
Freshwater fish; trematodes; *Clinostomum* sp; metacercariae; COI

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Morphological and molecular phylogeny of *Clinostomum* sp. (Digenea: Clinostomidae) metacercariae, using DNA barcode from a South American freshwater fish

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Abstract

Here, we present a comprehensive morphological and molecular phylogenetic analysis of *Clinostomum* sp. (Digenea: Clinostomidae) metacercariae parasitizing two freshwater fish species from Southeast Brazil: *Serrasalmus spilopleura* (piranha) and *Callichthys callichthys* (tambuatá). The morphological examination revealed distinct characteristics of metacercariae in each host. Using the cytochrome c oxidase I (COI) gene barcode region, we obtained DNA sequences that allowed for accurate phylogenetic placement. Phylogenetic analyses revealed that *Clinostomum* sp. HM41 (metacercariae), isolated from *S. spilopleura*, exhibited 86% similarity to *Ithyoclinostomum yamagutii*, while *Clinostomum* sp. HM125 (metacercariae), from *C. callichthys*, showed 98.7% similarity to *Clinostomum* sp. Cr_Ha1. The phylogenetic trees constructed through Bayesian Inference and Maximum Likelihood methods indicated high biodiversity within the *Clinostomum* genus and strong support for distinct lineages. These findings enhance our understanding of the diversity and ecological distribution of *Clinostomum* species in South American freshwater environments.

Introduction

The family Clinostomidae Luhe, 1901 comprises digenetic trematodes, which in their adult stage are predominantly found in the oral cavity and esophagus of birds and reptiles (Kanev *et al.* 2002). These parasites exhibit a complex life cycle with gastropods serving as their first intermediate hosts and fish and amphibians as their second intermediate hosts (Pérez-Ponce De Léon *et al.* 2016). In their metacercariae stage, these trematodes infect a wide variety of fish hosts, having been found in at least 12 families of freshwater fish: Cichlidae, Percidae, Centrarchidae, Symbranchidae, Eleotridae, Heptapteridae, Profundulidae, Poecilidae, Goodeidae, Characidae, Cyprinidae, and Catostomidae (Acosta *et al.* 2016; Briosio-Aguilar *et al.* 2018; Caffara *et al.* 2011, 2014, 2017; Davies *et al.* 2016; Dias *et al.* 2003; Gustinelli *et al.* 2010; Locke *et al.* 2015; Morais *et al.* 2011; Pérez-Ponce de León *et al.* 2007, 2016; Pinto *et al.* 2015; Sereno-Uribe *et al.* 2013, 2018; Szidat 1969).

Based on morphological and molecular descriptions, 23 valid species of *Clinostomum* Leidy, 1856 have been identified thus far, and yet at least eight candidate new species have been registered through DNA sequences but have not yet been described (Briosio-Aguilar *et al.* 2018; Goméz-Ruíz and Lenis 2024; Tavares-Dias et al 2021); however, the taxonomic validity of species within *Clinostomum* has been a contentious issue due to the absence of significant morphological characters that differentiate the valid species from each other, along with the wide distribution of the genus, which is considered cosmopolitan, and its complex life cycle (Caffara *et al.* 2014; Locke *et al.* 2015, 2019; Matthews and Cribb 1998; Rosser *et al.* 2017; Sereno-Uribe *et al.* 2018).

Presently, molecular and morphological analyses have revealed a high biodiversity of lineages and a large database of mitochondrial and nuclear DNA sequences of *Clinostomum* are available, facilitating the correct identification of species, thereby effectively estimating the diversity of this genus (Acosta *et al.* 2016; Briosio-Aguilar *et al.* 2018; Caffara *et al.* 2011, 2014, 2017; Gustinelli *et al.* 2010; Locke *et al.* 2015, 2019; Pérez-Ponce de León *et al.* 2007, 2016; Pinto *et al.* 2015; Sereno-Uribe *et al.* 2013; Sereno-Uribe *et al.* 2018).

During a biodiversity survey of fish parasites in the Paraná river basin, we found Clinostomidae larvae in the musculature of *Serrasalmus spilopleura* Kner 1858 (Characiformes:

Serrasalmidae) and *Callichthys callichthys* (Linnaeus 1758) (Siluriformes: Callichthyidae) from southeast Brazil, and we conducted morphological and molecular analysis with the aim to characterize these metacercariae since many fish parasites larvae can be zoonotic. Additionally, we analyzed the phylogenetic position of these larvae for the first time.

Material and methods

Study area and sampling design

The Ibitinga reservoir is situated within the Tietê River Basin, a part of the larger Paraná River Basin, located in São Paulo state, Brazil. Spanning approximately 12,300 hectares, the reservoir boasts an average depth of 8.6 meters (Vieira *et al.* 2002). It is primarily fed by two main tributaries – namely, the Jacaré-Pepira River (22°30'S; 47°55'W) and the Jacaré-Guaçu River. During sampling near the confluence of the Jacaré-Pepira and Tietê Rivers in Ibitinga city (21°54'46"S; 48°53'14"W), five specimens of *S. spilopleura* were collected.

In contrast, the Jurumirim dam is located on the Paranapanema River, also within the Paraná River Basin (Agostinho *et al.* 1995). Serving as the primary reservoir in a cascade system for downstream river regulation (Henry and Nogueira 1999), it is situated in São Paulo state (23°12'17" S; 49°13'19" W). Twenty specimens of *C. callichthys* were gathered from the Jurumirim reservoir.

Following collection, specimens were transported in plastic bags to an ichthyoparasitology laboratory for analysis. Muscle tissue from the host fish was filleted, and cysts were carefully extracted and examined under a stereomicroscope. Sections were stained using Mayer's carmine alum, mounted on slides, and coverslipped with Canada balsam to facilitate detailed visualization of internal structures (Eiras *et al.* 2006). Indexes of parasite prevalence, intensity, and abundance were calculated according to the method described by Bush *et al.* (1997).

Fish hosts

Serrasalmus spilopleura (Kner 1858), commonly known as piranha or pirambeba, belongs to the Characidae family within the order Characiformes. This species is widely distributed across South America (Braga 1976; Vazzoler and Menezes 1992), predominantly inhabiting lentic environments such as lakes, rivers, and reservoirs (Corredor 2004; Saint-Paul *et al.* 2000). It is known for its predatory behavior and is frequently found in association with aquatic vegetation (Petry *et al.* 2003; Sánchez-Botero *et al.* 2003). Originally native to the Amazon region (Sousa *et al.* 2013), *S. spilopleura* has been introduced into various water bodies across Brazil, including the Tietê, Jacaré-Pepira, and Jacaré-Guaçu Rivers.

Callichthys callichthys (Linnaeus 1758), commonly referred to as tambuatá or tamuatá, belongs to the Callichthyidae family within the order Siluriformes. This species is widely distributed throughout South America, inhabiting freshwater systems from the eastern side of the Andes to as far south as Buenos Aires (Mello *et al.* 2011). *C. callichthys* is known as a benthic feeder, displaying significant seasonal variations in habitat use. During winter, it tends to lead a benthic life (Knoppel 1970; Lowe-McConnell 1964), while in summer, it becomes more active and can be found in neritic zones, engaging in nesting behaviors and providing parental care to its offspring (Carter and Beadle 1931; Lowe-McConnell 1964).

Molecular and phylogenetic analysis

Metacercariae were isolated and fixed in ethanol PA (Merck, Darmstadt, Germany), followed by DNA extraction using the DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA, USA) as per the manufacturer's instructions, resulting in a final volume of 50 µl. Subsequently, DNA samples (5 µl) were subjected to polymerase chain reaction (PCR) amplification using primers specific to the cytochrome c oxidase I (COI) gene barcode region, following the protocol described by Moszczynska *et al.* (2009). The PCR reaction mixture (25 µl) contained puReTaq Ready-to-Go Beads (GE Healthcare, Chicago, IL, USA) supplemented with stabilizers (bovine serum albumin and deoxynucleotide triphosphates) and ≈ 2.5 units of puReTaq DNA polymerase.

PCR thermal cycling conditions consisted of an initial denaturation at 94°C for 30 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 90 s, and a final extension step at 72°C for 7 min. PCR products were visualized on agarose gel stained with GelRed (Phenix Research, Candler, NC, USA), and bands of interest were purified using the QIAquick PCR Purification Kit (QIAGEN).

Purified DNA was then subjected to direct sequencing using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a genetic analyzer (ABI 3500; Applied Biosystems). Sequences were assembled and edited into contigs using Sequencher 5.2.4 (Gene Codes, Ann Arbor, MI, USA), and their identities were verified by comparison using the BLAST program (http://blast.ncbi.nlm.nih.gov).

Sequence alignment was performed using Geneious 7.1.3 (KEARSE *et al.* 2012), and the best-fit model for nucleotide evolution (GTR+G+I) was determined using the Akaike information criterion in jModelTest (Posada 2008). Phylogenetic analyses were conducted using Bayesian Inference (BI) and Maximum Likelihood (ML) methods on the CIPRES Science Gateway (Miller *et al.* 2013). Bayesian analysis utilized the settings: lset nst = 6, rates = invariable, ncat = 4, shape = estimate, inferrates = yes, and basefreq = empirical, with MCMC chains run for 10,000,000 generations, sampling one tree every 1,500 generations. The first 25% of generations were discarded as burn-in, and the consensus tree (majority rule) was computed from the remaining trees, considering nodes with posterior probabilities >90% as well supported.

Maximum Likelihood analysis was performed using RAxML (Stamatakis 2014), with bootstrap support values derived from 1,000 replicates, and nodes supported by bootstrap values >70% considered well supported. The resulting phylogenetic trees were visualized using FigTree v.1.3.1 (Rambaut 2021).

Results

Morphological data

The morphological characteristics of *Clinostomum* sp. metacercariae from *S. spilopleura* will be referred as *Clinostomum* sp. HM41 (Figure 1, Table 1), and the metacercariae found in *C. callichthys* will be referred as *Clinostomum* sp. HM125 (Figure 2, Table 1). All measurements are given in micrometers plus the standard deviation.

We collected 62 specimens of *Clinostomum* sp. HM41 metacercariae (Figure 1) from *Serrasalmus spilopleura*. These metacercariae were predominantly found infecting various anatomical sites including the musculature, ocular cavity, palate, operculum, and lower jaw. Additionally, we collected 10 specimens of *Clinostomum*

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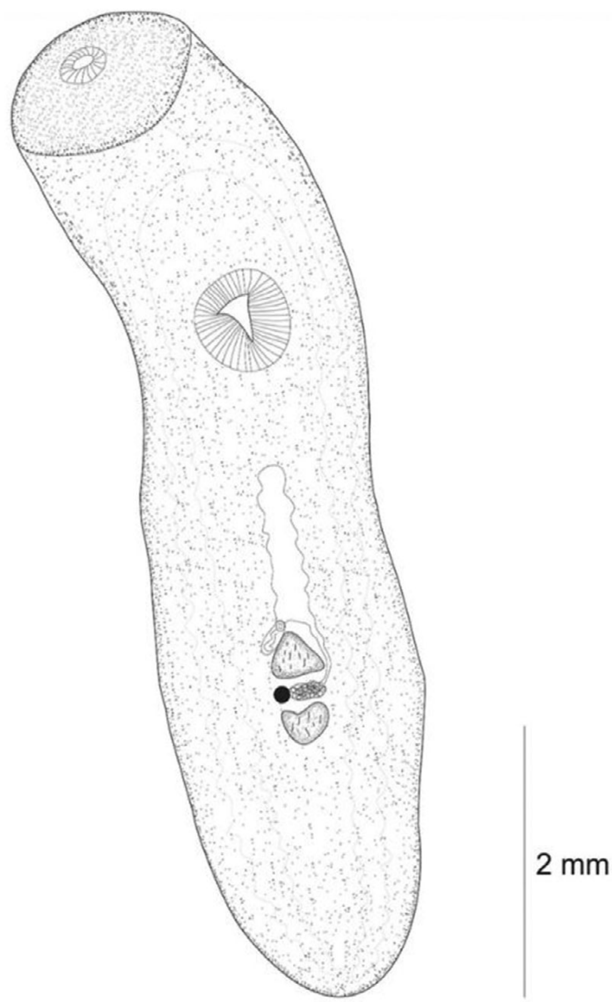

Figure 1. *Clinostomun* sp. HM41 metacercarie from *Serrasalmus spilopleura* (Kner, 1858) collected in the Ibitinga reservoir in the state of São Paulo, Brazil (scale: 2mm).

sp. HM125 (Figure 2) from *Callichthys callichthys*, with the primary site of infection localized in the musculature.

Clinostomum sp. HM41

Taxonomy Summary

Host: Serrasalmus spilopleura specimens collected had mean weight of 209.08 ± 26.42 kg and mean length of 15.2 ± 3.09 cm.

Locality: The Ibitinga reservoir in the state of São Paulo, Brazil (22°30'S; 47°55'W)

Epidemiological data: Prevalence of 80% and mean intensity of 15.5 ± 3.88 parasites per fish, and mean abundance of 12.4 ± 3.02 parasites (range, 4–36 parasites)

Specimens deposit:

Representative DNA: Sequences will be deposited in GenBank after the manuscript acceptance.

Description:

A morphometric description of specimens found in *S. spilopleura* hosts (n=10) is provided: the oral suction cup exhibited a mean length of 393.64 ± 98.89 and a mean width of 303.07 ± 137.02. Meanwhile, the ventral suction cup had a mean length of 1007.60 ± 235.17 and a mean width of 959.20 ± 185.26. The intestinal cecum measured on average 7287.02 ± 1336.19 in length. The uterus displayed a mean length of 1749.30 ± 377.60 and a mean width of 637.56 ± 260.43. The cirrus sac had a mean length of 314.80 ± 28.56 and a mean width of 299.70 ± 28.07. The ovary measured on average 154.25 ± 48.99 in length and 86.77 ± 39.77 in width. The anterior testis exhibited a mean length of 483.84 ± 161.44 and a mean width of 263.53 ± 71.40, while the posterior testis had a mean length of 473.91 ± 127.79 and a mean width of 363.64 ± 141.31 (Table 1).

Clinostomum sp. HM125

Taxonomy Summary

Host: Callichthys callichthys specimens collected had mean weight of 57.29 ± 12.06 kg and mean length of 12.69 ± 1.01.

Locality: Jurumirim reservoir, São Paulo state (23°12'17" S; 49°13'19" W)

Epidemiological data: The parasites presented prevalence of 15%, mean intensity of 3.33 ± 1.20 (range, 1–5 parasites).

Specimens deposit:

Representative DNA: Sequences will be deposited in GenBank after the manuscript acceptance.

Description:

A morphometric description of specimens found in *C. callichthys* hosts (n=3) is provided: the oral suction cup averaged 371.93 ± 68.94 in length and 411.18 ± 112.89 in width, while the ventral suction cup measured 828.09 ± 97.21 in length and 834.31 ± 128.87 in width. The intestinal cecum exhibited an average length of 7020.33 ± 1190.95. The uterus showed dimensions with a mean length of 1233.29 ± 452.53 and a mean width of 220.74 ± 162.63. The cirrus sac had a mean length of 356.12 ± 54.83 and a mean width of 164.85 ± 11.45. The ovary's average length was 162.65 ± 75.35, and its width was 108.01 ± 70.72. Lastly, the anterior testis measured 345.68 ± 68.02 in length and 325.34 ± 49.80 in width, while the posterior testis had a mean length of 351.47 ± 124.28 and a mean width of 361.76 ± 104.90.

Molecular data

Two mitochondrial COI gene sequences were obtained, measuring 593 base pairs for *Clinostomum* sp. HM41 (metacercariae) and 595 base pairs for *Clinostomum* sp. HM125 (metacercariae). Upon performing BLASTn local alignments, *Clinostomum* sp. HM41 (metacercariae) exhibited 86% similarity to *Ithyoclinostomum yamagutii* (MN696164) which was the closest related taxa while *Clinostomum* sp. HM125 (metacercariae) showed 98.7% similarity to *Clinostomum* sp. Cr_Ha1 (MF673562).

Phylogenetic analyses using Maximum Likelihood and Bayesian methods were conducted on a 412 base pair alignment containing 52 taxa, resulting in similar tree topologies. The phylogenetic tree revealed two main clades labeled as A and B (Figure 3). Clade A included *Ithyoclinostomum yamagutii* (MN696163-64), along with *Ithyoclinostomum* species from North and Central America, and *Clinostomum* sp. HM41 (metacercariae), albeit with low node support (bootstrap 54, posterior probability 0.8) (Figure 3). The genus *Euclinostomum* showed robust node support, and clade B encompassed a diverse assemblage of *Clinostomum* species (Figure 3), where *Ithyoclinostomum dimorphum* (OP174427) clustered within, aligning with previous findings (Simões *et al.* 2022) regarding its classification as *Clinostomum* sp.

Clinostomum sp. HM125 (metacercariae) clustered closely with species from South America, specifically from Argentina and Brazil, with strong node support (Figure 3). It appears to belong to the same species group as *Clinostomum* sp. Cr Ha1 (MF673562), *Clinostomum* sp. Cr Ha2 (MF673563), *Clinostomum* sp. Adult-Cra (MW187310), *Clinostomum* sp. Cra1 (MF673556), and *Clinostomum* sp. Cra1 (MF673557), exhibiting less than 2% intraspecific variation according to BLASTn results. Furthermore, *Clinostomum* sp. 43 (KJ818259), originally found as metacercariae in guppy (*Poecilia reticulata*) in Brazil, clustered prominently

https://doi.org/10.1017/S0022149X24000993 Published online by Cambridge University Press

Table 1. Comparative measurements of *Clinostomum* sp. HM41 and *Clinostomum* sp. HM125 and other species reported from fish and also fish-eating birds. Measurements are show in μm with the mean and standard deviation when available

Species	*Clinostomum* sp. HM41	*Clinostomum* sp. HM125	*Clinostomum* sp.			*I. yamagutii* n. sp.	C. fergalliari
Host	*S. spilopleura* (Kner, 1858)	*C. callicthys* (Linnaeus, 1758)	*C. rachovii* (Regan, 1913)	*C. vittata* (Heckel, 1840)	*G. balzanii* (Perugia, 1891)	*Ardea herodias* (Linnaeus, 1758)	*Ardea cocoi* (Linnaeus, 1766)
Locality	Ibitinga reservoir, São Paulo, Brazil	Jurumirim reservoir, São Paulo, Brazil	Ayui River, Concordia, Argentina	Laguna Iberá, Corrientes, Argentina		Mississippi, USA	Magdalena, Buenos Aires, Argentina
Reference	This study.	This study.	Montes *et al.* 2020	Montes *et al.* 2020		Rosser *et al.* 2020	Montes *et al.* 2021
VOL	571.4–272.3 (393.64 ± 8.89)	294.34–477.13 (371.93 ± 68.94)	203–326 (286)	232–310 (266)	162–265 (216)	429–641 (535)	289–390 (339)
VOW	461.8–123.5 (303.07 ± 137.02)	318.09–586,94 (411.18 ± 112.89)	246–413 (327)	236–327 (295)	208–299 (262)	807–839 (823)	295–436 (372)
VVL	1,144.9–620 (1,007.60 ± 235,17)	735.76–970.69 (828.09 ± 97.21)	751–908 (832)	692–890 (784)	608–707 (649)	2.219–2.436 (2.327)	969–1091 (1023)
VVW	1,176.8–713.6 (959.2 ± 185.26)	64361–1,015.45 (834.31 ± 128.87)	748–894 (816)	654–921 (821)	748–1038 (886)	2.108–2.462 (2,285)	951–1059 (1006)
CIL	9,063.5–5,264.4 (7,287.02 ± 1,336.19)	5,034.00–8,461.00 (7,020.33 ± 1190.95)					
CIW							
UL	2,219.1–1,171.5 (1,749.30 ± 377.6)	815.93–2,100.00 (1,233.29 ± 452.53)	1120–1347 (1265)	489–650 (576)	553–1171 (862)	11.166–11.560 (11,363)	
UW	1,035.7–439.7 (637.56 ± 260.43)	63.00–438.00 (220.74 ± 162.63)	127–327 (189)	79–104 (92)	96–120 (108)		
SCL	355.2–314.8 (314.8 ± 28.56)	288.14–438.41 (356.12 ± 54.83)	254–273 (302)	355–400 (377)	343–461 (402)	639–649 (644)	541–838 (657)
SCW	339.4–299.7 (299.7 ± 28.07)	151.75–183.63 (164.85 ± 11.45)	157–205 (184)	118–131 (126)	111–120 (116)	742–776 (759)	180–314 (246)
OL	172.9–73.5 (154.25 ± 48.99)	103.05–270.84 (162.65 ± 75.35)	107–162 (138)	140–185 (166)	166	630–698 (664)	264–616 (437)
OW	130–40.2 (86.77 ± 39.77)	47.99–186.66 (108.01 ± 70.72)	81–109 (90)	129–171 (150)	85	493–591 (542)	173–260 (229)
TAL	701.6–258.3 (483.84 ± 161.44)	296.12–463.91 (345.68 ± 68.02)	154–308 (244)	178–275 (227)	234–255 (245)	1.995–2.024 (2.010)	359–850 (612)
TAW	339.1–175.8 (263.53 ± 71.4)	266.18–387.07 (325.34 ± 49.80)	242–349 (317)	468–619 (546)	591–786 (658)	2.661–2.708 (2.685)	798–1317 (1026)
TPL	603.2–268.7 (473.91 ± 127.79)	190.48–491.24 (351.47 ± 124.28)	150–335 (231)	237–341 (289)	302–562 (432)	1.502–2.000 (1.751)	412–752 (566)
TPW	655.2–219.6 (363.64 ± 141.31)	221.31–449.22 (361.76 ± 104.90)	238–443 (369)	493–646 (581)	627–1103 (785)	1.944–2.084 (2.014)	1001–1392 (1117)

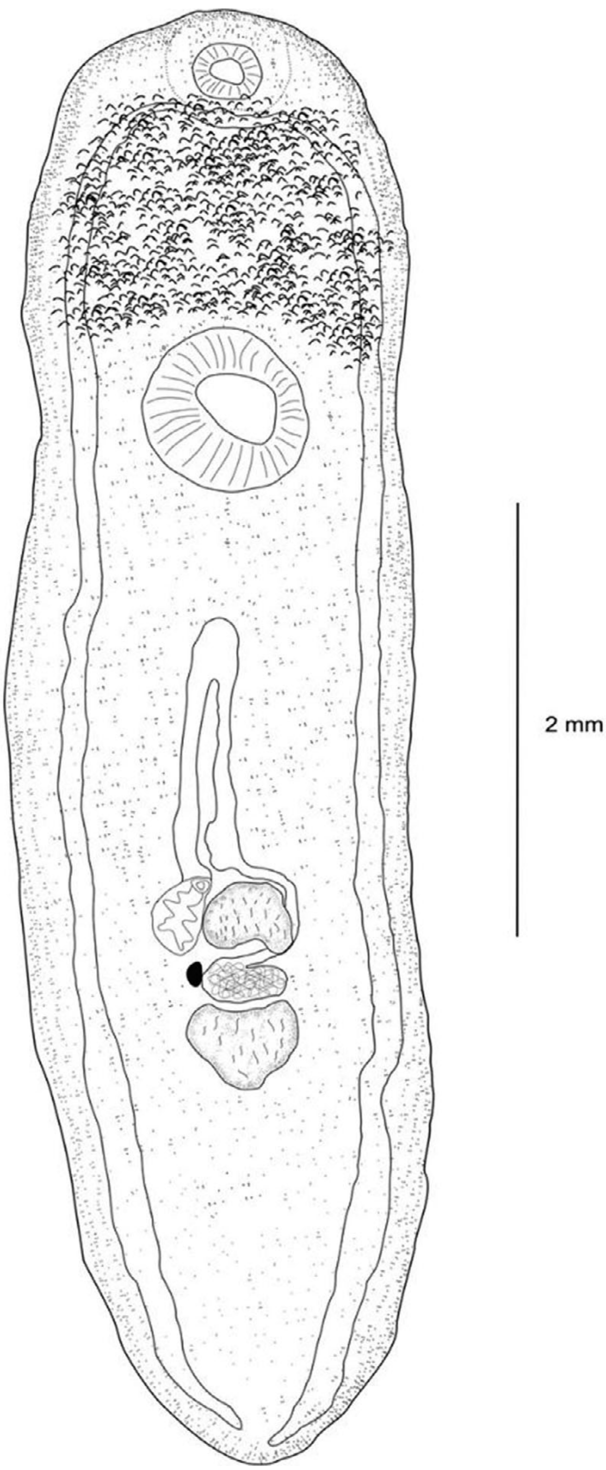

Figure 2. *Clinostomun* sp. HM125 metacercarie from *Callichthys callichthys* (Linnaeus, 1758), collected in the Jurumirim reservoir, São Paulo state, Brazil (scale: 2mm).

within the same clade as *Clinostomum* sp. HM125 and related species from Argentina (Figure 3).

Discussion

Identification of *Clinostomum* metacercariae solely based on morphological attributes can lead to misidentifications. To enhance the accuracy of *Clinostomum* systematics and deepen our understanding of the life cycle of these digenetic trematodes, molecular techniques have become indispensable (Bonette *et al.* 2011; Caffara *et al.* 2011; Gustinelli *et al.* 2010; Locke *et al.* 2015; Matthews and Cribb 1998; Sereno-Uribe et al, 2013; Simões *et al.* 2022). Despite the scarcity of morphological studies that differentiate species, molecular DNA sequencing has proven crucial for accurate species differentiation within the genus (Caffara *et al.* 2011; Locke *et al.* 2015; Rosser *et al.* 2016).

In 2005, the concept of integrative taxonomy was officially introduced as a comprehensive method for the description of taxa, integrating different data sources and methodologies to reach the final result (Dayrat 2005; Will *et al.* 2005). Data on conspecific specimens generated by different researchers can be analyzed comparatively (Patterson *et al.* 2010; Satler *et al.* 2013; Schlick-Steiner *et al.* 2007). Within this concept, molecular analysis drives the continuous evolution of taxonomic tools, complementing the fields of morphology, ecology, natural history, and statistics (Knapp 2008). Pante *et al.* (2014) argue that integrative taxonomy should be encouraged and developed within the formal description of species, highlighting it as a tool for enhancing and improving the quality of hypotheses concerning species and their descriptions. In Brazil, there are records of at least five species of *Clinostomum* in larval stage parasitizing fishes (Table 2).

However, the vast majority of these records are based on morphological identifications rather than molecular analyses, making it challenging to characterize the distribution of species within the genus in Brazil and South America (Tavares-Dias *et al.* 2021). In our work, we are not describing as new species since we do not have the adult phase.

In our morphological analysis, *Clinostomum* sp. HM41 (metacercariae) exhibited morphological characteristics typical of *Clinostomum* species rather than *Ithyoclinostomum* spp., yet molecular analysis grouped it within the *Ithyoclinostomum* clade (low support), and with more taxa added to the database, this result can possibly change. This discrepancy underscores the need for further sequencing of additional taxa to validate the taxonomy within the genus *Ithyoclinostomum*. *Ithyoclinostomum* species, known for their larger body size compared to *Clinostomum*, were historically distinguished based on morphological features such as cirrus-sac position, testes shape, gonad position, and the free area between the ventral sucker and anterior testis (Rosser *et al.* 2020; Simões *et al.* 2022). However, recent molecular data challenge these distinctions. For instance, Simões *et al.* (2022), evaluating the phylogenetic position of *Ithyoclinostomum dimorphum* compared to *Clinostomum* spp. in Brazil, demonstrated that size alone is not a reliable morphological criterion to differentiate *Clinostomum* spp. from *Ithyoclinostomum* spp. These observations show that, despite recent advances and applications of molecular techniques in taxonomic studies in South America, trematode species whose descriptions are based solely on morphological characters need to be revisited and analyzed regarding their phylogenetic positions compared to species from other genera, even if morphologically distinct. There are still several issues related to the availability of a molecular database, particularly concerning genera of trematodes, most of whose species have taxonomic descriptions based solely on morphological characters and have not yet been sequenced (Poulin *et al.* 2020; Simões *et al.* 2022).

Clinostomum sp. HM125 (metacercariae), found in *Callichthys callichthys* from Jurumirim reservoir, clustered together with Argentinean species, suggesting conspecificity with minor intraspecific variation. Phylogenetic analyses highlight a distinct

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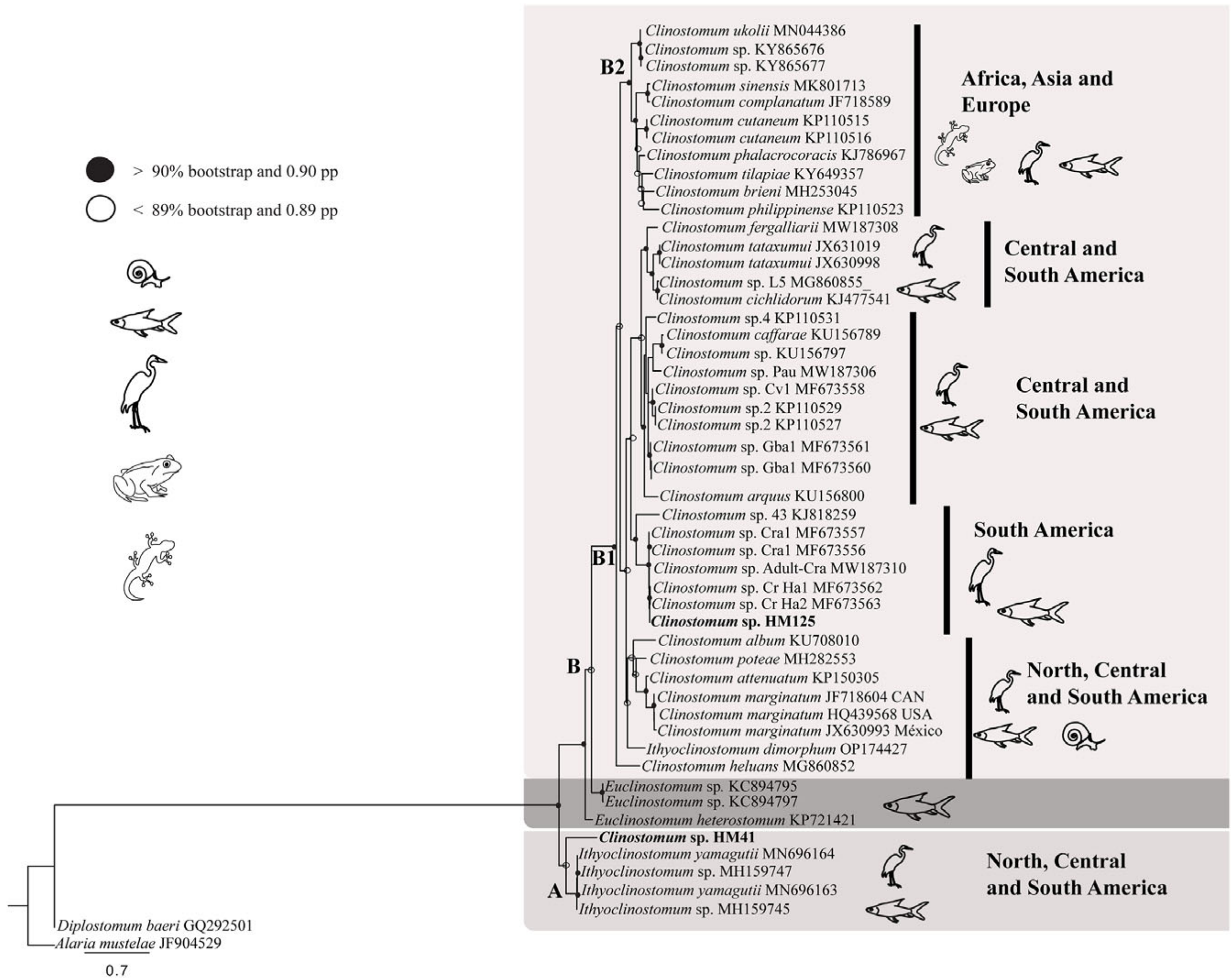

Figure 3. Maximum Likelihood tree based on barcoding COI sequences of trematodes clinostomids worldwide. GenBank accession numbers are given after species names. Nodal supports values are shown bootstrap (B) and posterior probability (PP), respectively. Values for weakly supported nodes (<0.9 PP and <90 B) are not shown. Gray scale rectangles differentiate main clades and their schematic host figures.

clustering pattern of these haplotypes across South America. Figure 3 illustrates how South American *Clinostomum* species within clade B1 are grouped, also incorporating species from Central and North America. Presently, 23 species are validated within the genus *Clinostomum*, with 11 found in the New World. These species exhibit distribution patterns across South America: *C. marginatum* (Rudoplhi 1819), *C. detrucatum* (Braun 1899), *C. heluans* (Braun 1899), and *C. fergalliarii* (Montes, Barneche, Pagano, Ferrari, Martorelli, and Pérez-Ponce de León 2021); Central America: *C. heluans* (Braun 1899), *C. tataxumui* (Sereno-Uribe, Pinacho-Pinacho, García-Varela & Pérez-Ponce de León 2013), *C.arquus* (Sereno-Uribe, García-Varela, Pinacho-Pinacho & Pérez-Ponce de León 2018), *C. caffarae* (Sereno-Uribe, García-Varela, Pinacho-Pinacho and Pérez-Ponce de León 2018), and *C. cichlidorum* (Sereno-Uribe, García-Varela, Pinacho-Pinacho & Pérez-Ponce de León 2018); and North America: *C. attenuatum* (Cort 1913), *C. album* (Rosser Alberson, Woodyard, Cunningham, Pote and Griffin 2017), *C. poteae* (Rosser, Baumgartner, Alberson, Noto, Woodyard, King, Wise and Griffin 2018), and *C. marginatum* (Montes, Barneche, Pagano, Ferrari, Martorelli, and Pérez-Ponce de León 2021).

The expanding occurrence of *Clinostomum* parasites in new localities suggests an increase in their distribution range, likely facilitated by migratory piscivorous birds (Antonucci *et al.* 2015). This study underscores ongoing taxonomic challenges in trematode diagnostics and emphasizes the pivotal role of molecular tools in encompassing these issues. Furthermore, it broadens the understanding of *Clinostomum* spp. metacercariae distribution in South America while raising pertinent questions regarding the validity of the genus *Ithyoclinostomum.*

In conclusion, our study underscores the complexity of *Clinostomum* taxonomy and the necessity for integrating molecular tools alongside traditional morphological approaches to accurately identify and differentiate species within this genus. The unexpected molecular clustering of *Clinostomum* sp. HM41 (metacercariae) within the *Ithyoclinostomum* clade can change in the future studies, while more taxa will be added to the database, also highlighting unresolved taxonomic issues regarding *Ithyoclinostomum* characterization and calling for further investigations involving a broader range of taxa. The phylogenetic insights gained from our study, particularly regarding *Clinostomum* sp. HM125 (metacercariae) and its clustering with Argentinean species, contribute to understanding regional species diversity, distribution, and evolutionary dynamics within *Clinostomum.* Moving forward, continued collaborative efforts across disciplines will be essential to elucidate the intricate relationships among *Clinostomum* species, refine species

Table 2. Records of species and GenBank entries of the genus *Clinostomum* and their respective hosts worldwide

Parasite Species/ Genbank	Host	Locality	Reference
Clinostomum ukolii MN044386	*Synodontis batensoda* (fish)	Africa	Caffara *et al.* 2020
Clinostomum sp. KY865676 / KY865677	*Schilbe intermedius* (fish)	Africa	Caffara *et al.* 2017
Clinostomum sinensis MK801713	*Candidia barbata* (fish)	Asia	Locke *et al.* 2019
Clinostomum complanatum JF718589	*Lepomis gibbosus* (fish)	Europe	Caffara *et al.* 2011
Clinostomum cutaneum KP110515	*Ardea cinerea* (bird)	Africa	Locke *et al.* 2015
Clinostomum cutaneum KP110516	*Ardea cinerea* (bird) *Oreochromis niloticus* (fish)	Africa	Locke *et al.* 2015
Clinostomum phalacrocoracis KJ786967	*Sarotherodon galilaeus* (fish) *Oreochromis aureus* (fish) *Tilapia zillii* (fish)	Israel	Caffara *et al.* 2014
Clinostomum tilapiae KY649357	*Synodontis batensoda* (fish)	Africa	Caffara *et al.* 2017
Clinostomum brieni MH253045	*Clarias gariepinus* (fish) *C. ngamensis* (fish)	Africa	Caffara *et al.* 2018
Clinostomum philippinense KP110523	*Trichopodus microlepis* (fish)	Thailand	Locke *et al.* 2015
Clinostomum fergalliarii MW187308	*Ardea cocoi* (bird)	Argentina	Montes *et al.* 2021
Clinostomum tataxamui JX630998/ JX631019	*Dormitator maculatus* (fish) *Ardea alba* (bird)	Mexico	Sereno-Uber *et al.* 2013
Clinostomum sp. L5 MG860855	*Ardea Herodias* (bird)	Mexico	Briosio-Aguilar *et al.* 2018
Clinostomum cichlidorum KJ477541	*Cochlearius cochlearisa* (bird)	Mexico	Pérez-Ponce de Léon *et al.* 2016
Clinostomum sp. 4 KP110531	*Apistogramma* sp. (fish)	Peru	Locke *et al.* 2015
Clinostomum caffarae KU156789	*Astyanax aeneus* (fish)	Mexico	Pérez-Ponce de Léon *et al.* 2016
Clinostomum sp. KU156797	*Egretta thulaa* (bird)	Mexico	Pérez-Ponce de Léon *et al.* 2016
Clinostomum sp. Pau MW187306	*Pyrrhulina australis* (fish)	Argentina	Montes *et al.* 2021
Clinostomum sp. Cv1 MF673558	*Crenicichla vittata* (fish)	Argentina	Montes *et al.* 2020

(*Continued*)

Table 2. (*Continued*)

Parasite Species/ Genbank	Host	Locality	Reference
Clinostomum sp. 2 KP110529/ KP110527	*Sicydium salvini* (fish)	Mexico	Locke *et al.* 2015
Clinostomum sp. Gba1 MF673560 MF673561	*Gymnogeophagus balzanii* (fish)	Argentina	Montes *et al.* 2020
Clinostomum arquus KU156800	*Egretta thulaa* (bird)	Mexico	Pérez-Ponce de Léon *et al.* 2016
Clinostomum sp. 43 KJ818259	*Poecilia reticulata* (fish)	Brazil	Pinto *et al.* 2015
Clinostomum sp. Cra1 MF673557	*Characidium rachovii* (fish)	Argentina	Montes *et al.* 2020
Clinostomum sp. Cra1 MF673556	*Characidium rachovii* (fish)	Argentina	Montes *et al.* 2020
Clinostomum sp. Adult-Cra MW187310	Ardea cocoi (bird)	Argentina	Montes *et al.* 2021
Clinostomum sp. Cr Ha1 MF673562	*Psalidodon anisitsi* (fish)	Argentina	Montes *et al.* 2021
Clinostomum sp. Cr Ha2 MF673563	*Psalidodon anisitsi* (fish)	Argentina	Montes *et al.* 2021
Clinostomum sp. HM125	*Callichthys callichthys* (fish)	Brazil	This study
Clinostomum album KU708010	*Ardea alba*	USA	Rosser *et al.* 2017
Clinostomum poteae MH282553	*Phalacrocorax auritus* (bird)	USA	Rosser *et al.* 2018
Clinostomum attenuatum KP150305	*Lithobates clamitans* (frog) *Lithobates pipiens* (frog)	Canada USA	Locke *et al.* 2015
Clinostomum marginatum JF718604	*Ardea Herodias* (bird)	Canada	Caffara *et al.* 2011
Clinostomum marginatum HQ439568	*Eurycea tynerensis* (salamander) *Micropterus salmoides* (fish) *Lepomis macrochirus* (fish)	USA	Bonnet *et al.* 2011
Clinostomum marginatum JX630993	*Catostomus nebuliferus* (fish)	Mexico	Sereno-Uribe *et al.* 2013
Clinostomum heluans MG860852	*Australoheros* sp. (fish) *A.alba* (bird) *A. herodias* (bird)	Brazil Mexico	Briosio-Aguilar *et al.* 2018
Clinostomum sp. HM 41	*Serrasalmus spilopleura* (fish)	Brazil	This study
Euclinostomum sp. KC894795	*Trichopsis vittata* (fish)	Thailand	Senapin *et al.* 2014
Euclinostomum sp. KC894797	*Trichopsis vittata* (fish)	Thailand	Senapin *et al.* 2014

(*Continued*)

https://doi.org/10.1017/S0022149X24000993 Published online by Cambridge University Press

Table 2. (*Continued*)

Parasite Species/ Genbank	Host	Locality	Reference
Euclinostomum heterostomum KP721421	*Astatotilapia flavijosephii* (fish) *Oreochromis aureus* (fish) *Sarotherodon galilaeus* (fish) *Tilapia zillii* (fish) *Tristramella simonis* (fish)	Israel	Caffara *et al.* 2016
Ithyoclinostomum yamagutii MN696164	*Ardea herodias* (bird)	USA	Rosser *et al.* 2020
Ithyoclinostomum sp. MH159747	*Mayaheros urophthalmus* (fish) *Vieja melanura* (fish) *Herichthys deppii* (fish) *Cribroheros longimanus* (fish) *Parachromis managuensis* (fish)	Mexico Costa Rica	Briosia-Aguilar *et al.* 2018
Ithyoclinostomum yamagutii MN696163	*Ardea Herodias* (bird)	USA	Rosser *et al.* 2020
Ithyoclinostomum sp. MH159745	*Mayaheros urophthalmus* (fish) *Vieja melanura* (fish) *Herichthys deppii* (fish) *Cribroheros longimanus* (fish) *Parachromis managuensis* (fish)	Mexico Costa Rica	Briosio-Aguilar *et al.* 2018
Diplostomum baeri GQ292501	*Percina caprodes* (fish)	Canada	Locke *et al.* 2009
Alaria mustelae JF904529	*Neovison vison* (mammal) *Lithobates pipiens* (frog) *Lithobates clamitans* (frog)	Canada	Locke *et al.* 2011

boundaries, and deepen our understanding of their ecological roles and impacts on host communities worldwide.

Supplementary material. The supplementary material for this article can be found at http://doi.org/10.1017/S0022149X24000993.

Availability of data and material. All data supporting the findings of this study are available within the paper.

Authors contribution. Conceptualization: FFJ, RKA, VDA. Methodology: FFJ, MIM, FHY, VDA. Formal analysis: MIM, FHY. Investigation: FFJ, LARL, VDA. Writing – Original Draft: TG, FFJ, LARL, VDA. Writing – Review & Editing: LARL, MIM, FHY, RJS, RKA, VDA. Supervision: VDA.

Funding. The authors thank the São Paulo Research Foundation (*Fundação de Amparo à Pesquisa do Estado de São Paulo* – FAPESP) for financial support (process nº 2016/21040-9); the Coordination of Superior Level Staff Improvement (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* – CAPES) M.I.M. grant number AUX-PE-PNPD 3005/2010; The Young Researcher Program (*Programa Jovem Pesquisador* – PROPE-UNESP 02/2016; and the National Council for Scientific and Technological Development (*Conselho Nacional de Desenvolvimento Científico e Tecnológico* – CNPq) F.H.Y. processes nº 304502/2022-7 and 174814/2023-2).

Consent to participate. Not applicable.

Consent for publication. Not applicable.

Competing interest. The authors declare no competing interests.

Ethical standards. Not applicable.

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