

Integrative taxonomy uncovers *Clinostomum chaacci* and an unidentified congeneric metacercaria infecting *Hoplosternum littorale* (Siluriformes: Callichthyidae) in the Peruvian Amazon Basin

J.D. Chero^a, L.A. Ñacari^b, G.A. Murrieta Morey^c, C.L. Cruces^d, E. Cacique^a, N. Huaman^a, D. Lopez^a, A. Mondragón-Martínez^e, R. Martínez-Rojas^e, J. Yunis-Aguinaga^{f,*}

^a Laboratorio de Zoología de invertebrados, Departamento Académico de Zoología, Facultad de Ciencias Biológicas (FCB), Universidad Nacional Mayor de San Marcos (UNMSM), Av. Universitaria cruce con Av. Venezuela cuadra 34, Lima, Peru

^b Laboratorio de Ecología y Evolución de Parásitos, Facultad de Ciencias del Mar y Recursos Biológicos, Universidad de Antofagasta, 601 Angamos, Antofagasta, Chile

^c Laboratorio de Parasitología y Sanidad Acuicola, Instituto de Investigaciones de la Amazonia Peruana (IIAP), Carretera Iquitos-Nauta, Km 4.5–San Juan Bautista, Iquitos, Loreto 0784, Peru

^d Facultad de Ciencias Biológicas, Universidad Ricardo Palma (URP), Av. Alfredo Benavides 5440 Santiago de Surco, Lima, Peru

^e Laboratorio de Parasitología en Fauna Silvestre y Zoonosis, Facultad de Ciencias Biológicas, Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Peru

^f Universidad Científica del Sur, Carretera Panamericana Sur, Km 19, Lima, Peru

ARTICLE INFO

Keywords:

Molecular phylogenetics
Digenean trematodes
Freshwater fish
Larval morphology
Zoonosis

ABSTRACT

Clinostomum Leidy, 1856 is a cosmopolitan genus of digenean trematodes whose metacercariae commonly infect freshwater fishes and amphibians as second intermediate hosts. In South America, the diversity and taxonomy of *Clinostomum* metacercariae remain poorly understood, due in part to the morphological similarity of larval stages and the scarcity of molecular data. This study provides the first integrative evidence of two phylogenetically distinct *Clinostomum* lineages infecting the callichthyid catfish *Hoplosternum littorale* (Hancock, 1828) (Siluriformes: Callichthyidae), a benthic catfish of commercial and subsistence importance in the Peruvian Amazon. Morphological analyses (light and scanning electron microscopy) combined with molecular data from mitochondrial (cox1) and nuclear (28S rDNA) markers revealed two lineages with clear genetic differentiation. Specimens from Loreto were conspecific with *Clinostomum chaacci* Sereno-Urbe, López-Jiménez, González-García, Ortega-Olivares & García-Varela, 2025, previously reported from several fish hosts across Central and South America, whereas those from Ucayali formed a distinct and highly divergent lineage (11.9–12.7 % cox1 divergence) closely related to *Clinostomum* L1, representing a potentially undescribed species. The detection of *C. chaacci* in *H. littorale* constitutes a new host record and expands the known distribution of this species to the western Amazon Basin. The detection of *Clinostomum* metacercariae in fish muscle tissue raises potential zoonotic concerns, especially in regions where raw or undercooked fish is consumed. These findings underscore the need for integrative taxonomic approaches to unravel hidden

* Corresponding author.

E-mail address: jefyunis@gmail.com (J. Yunis-Aguinaga).

<https://doi.org/10.1016/j.fawpar.2025.e00306>

Received 18 June 2025; Received in revised form 20 November 2025; Accepted 24 November 2025

Available online 27 November 2025

2405-6766/© 2025 Published by Elsevier Inc. on behalf of International Association of Food and Waterborne Parasitology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

diversity in *Clinostomum* and emphasize the need for broader parasitological surveys and public health awareness in Amazonian communities.

1. Introduction

Clinostomum Leidy, 1856 is a cosmopolitan genus of digenean trematodes characterized by a complex three-host life cycle (Kanev et al., 2002). Adult flukes inhabit the oral cavities and upper digestive tracts of piscivorous birds, while larval metacercariae encyst in a variety of aquatic intermediate hosts, including fish and amphibians (Kanev et al., 2002; Shamsi et al., 2013). These metacercariae, often referred to as “yellow grubs” (Choudhary et al., 2022), can cause significant morbidity in aquaculture and may have zoonotic potential, occasionally infecting humans who consume raw or undercooked fish (Park et al., 2009; Mahdy et al., 2024). Currently, the genus *Clinostomum* contains more than 30 nominal species (Kanev et al., 2002); 5 of these species have been recorded in the Americas (Montes et al., 2021). In Peru, Morey et al., 2025a reported the presence of *Clinostomum marginatum* (Rudolphi, 1819) Braun, 1899 metacercariae in the ornamental fish *Poecilia reticulata* (Peters, 1859) based on morphological data. However, accurate identification of larval stages remains challenging, as the underdeveloped internal structures of metacercariae limit the availability of reliable diagnostic characters (Rosser et al., 2013). *Clinostomum marginatum* is a widely reported species of digenean trematode originally described from an unidentified *Ardea* species in Brazil (Rudolphi, 1819). Since its original description, numerous records of *C. marginatum* have been reported from freshwater fish and piscivorous birds in North America (Montes et al., 2021). However, the taxonomic identity of this species remains uncertain, primarily due to the lack of molecular data from South American specimens (Locke et al., 2015; Montes et al., 2021). Recent studies have revealed potential cryptic diversity within what is referred to as *C. marginatum* sensu lato, suggesting that North and South American populations may represent distinct genetic lineages (Montes et al., 2021). Furthermore, in South America multiple *Clinostomum* metacercariae have been recorded, representing distinct genetic lineages (Montes et al., 2023, 2024; Gião et al., 2025). These findings highlight the need for integrative taxonomic approaches, including

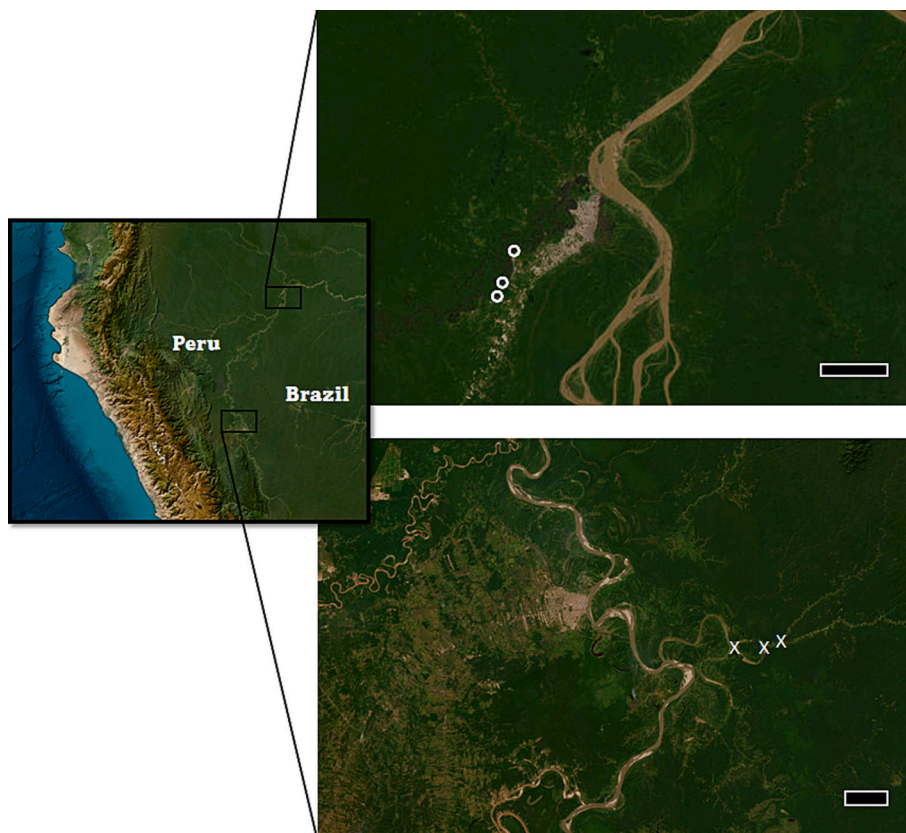


Fig. 1. Sampling locations of fish specimens in the Ucayali and Loreto Regions, Peru. The geographic coordinates of each sampling site are as follows: In the Ucayali Region (X): 08° 26' 41" S - 074° 15' 07" W / 08° 28' 05" S - 074° 10' 36" W / 08° 27' 41" S - 074° 08' 13" W. In the Loreto Region (O): 03° 49' 17" S - 073° 21' 37" W / 03° 48' 42" S - 073° 21' 28" W / 03° 46' 18" S - 073° 19' 59" W. Data from the World Cities Database, provided by Simplemaps (Pareto Software, LLC). Map created using Open Source QGIS. Bar = 2 km.

molecular tools, to clarify species boundaries and assess the true diversity of *Clinostomum* metacercariae infecting fish hosts in the Neotropical region.

As part of our parasitological surveys on siluriform fishes in Peru, specifically in the northeastern region of the Peruvian Amazon, we collected *Clinostomum* metacercariae infecting *Hoplosternum littorale* (Hancock, 1828) (Callichthyidae), a benthic catfish widely distributed throughout tropical South America (Reis, 1997; Orfinger, 2015). The objectives of this study were to analyze the metacercariae, describe their morphology using light and scanning electron microscopy (SEM), and report their COI and 28S sequences to assess their phylogenetic position.

2. Material and methods

2.1. Collection of samples and morphological study

Between November 2024 and March 2025, a total of 28 *H. littorale* specimens were obtained by local fishermen using gillnets at two distinct localities in the Peruvian Amazon. Of these, 24 individuals were from Zungarococha Lagoon and Santa Clara beach (Lat: 3° 49'S, Lon: 73° 21'W), near to the Nanay River, Loreto, and 4 from "Calleria sector" near to the Ucayali River (Lat: 08° 26' S, Lon: 074° 15' W), Ucayali, Peru (Fig. 1). Live fishes were transported in aerated, water-filled polyethylene bags to a field laboratory, where they were euthanized following Montes et al. (2024). Subsequently, all hosts were subjected to systematic necropsy for the detection and isolation of trematode metacercariae. Host fish were identified according to Dávila et al. (2018).

Encysted metacercariae of *Clinostomum* sp. were isolated from the muscle tissue of *H. littorale*. The parasites were carefully extracted, rinsed in 0.85 % saline solution and fixed in hot 70 % ethanol. Specimens intended for light microscopy were stained with iron acetocarmine, dehydrated through an ascending ethanol series, cleared in methyl salicylate and mounted in Canada balsam. The specimens were examined and photographed using a Nikon™ Eclipse SI compound photomicroscope, and drawings were made with the aid of a drawing tube. Measurements were taken in micrometers, unless otherwise indicated, straight-line distances between extreme points of the structures measured and are expressed as the range followed by the mean and number (n) of structures measured in parentheses. For scanning electron microscopy (SEM), selected specimens were post-fixed in 10 % formalin, dehydrated through a graded ethanol series, subjected to critical point drying using carbon dioxide, sputter-coated with gold, and examined using an Inspect S50-FEI, at an accelerating voltage of 5–10 kV.

Voucher specimens of each morphotype were deposited in the Helminthological Collection of the Museum of Natural History at the San Marcos University (MUSM-HEL), Lima, Peru.

2.2. Molecular analysis

Genomic DNA of the metacercariae were extracted using an TIANamp Genomic DNA Kit (TianGen, Beijing, China) following the manufacturer's protocol. The partial 28S rDNA was amplified by polymerase chain reactions (PCR) using forward primer digl2 (5' – AAG CAT ATC ACT AAG CGG –3') and reverse primer 1500R1 (5'–GCT ACT AGA TGG TTC GAT TAG–3') (Tkach et al., 2002). The mitochondrial DNA cytochrome c oxidase subunit I gene (*cox1*, partial fragment) was amplified using the forward primer Cox 5. Schisto (5'–TCT TTR GAT CAT AAG CG–3') and the reverse primer JB5 (5'–AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG–3') (Lockyer et al., 2003; Derycke et al., 2005; Fernandes et al., 2021). PCR assays were carried out in a total volume of 25 µl containing 12.5 µl of PCR BIO Taq Mix Red (PCR Biosystems), 0.3 µl of each primers with final concentration at 10 µM, 1.2 µl of DNA sample and ultrapure water to complete. An annealing temperature of 55 °C was used for amplification of 28S rDNA and 45 °C for *cox1* genes. PCR products were purified with sparQ PureMag Beads (Quantabio, USA) following the manufacturer's instructions and sequenced in Macrogen Inc. (Seul, Republic of Korea) with the Sanger sequencing method.

Sequences were edited and contigs were assembled using ProSeq 2.9 beta (Filatov, 2002). The National Center for Biotechnology Information (NCBI) sequence database (henceforth 'GenBank') was searched for similar sequences using BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990). The 28S and *cox1* sequences generated in this study were aligned using Clustal W (Larkin et al., 2007) with reference sequences of identified species and putative lineages of *Clinostomum* available in GenBank (Table S1). For the *cox1* alignments, sequences of *Euclinostomum* spp., *Ithyoclinostomum* spp., and *Paralaria mustelae* (Bosma, 1931) Dubois, 1963 were used as outgroup taxa (GenBank accessions in Table S1). Similarly, outgroups for the 28S rDNA dataset included representatives of *Euclinostomum* spp., *Ithyoclinostomum* spp., *Odhneriotrema incommodum* (Leidy, 1856) Lins de Almeida, 1935, and *P. mustelae* (GenBank accessions in Table S2). These taxa were selected as outgroups based on their phylogenetic position within Clinostomidae and related families.

The best-fitting nucleotide substitution models were selected using jModelTest v2.1.6 (Darrriba et al., 2012), under the corrected Akaike Information Criterion (AICc; Posada and Buckley, 2004). The selected models were TIM3 + I + G for the *cox1* dataset and TVM + I + G for the 28S rDNA dataset. Bayesian inference (BI) analyses were performed in MrBayes v3.2.6 (Ronquist et al., 2011), using Metropolis-coupled Markov chain Monte Carlo (MCMC) simulations starting from random trees. The MCMC was run for 5,000,000 generations, with trees sampled every 100 generations, resulting in 50,000 trees per analysis. Convergence and stationarity were assessed by visual inspection of the log-likelihood scores over generations using Tracer v1.7.2. Since likelihood values stabilized before generation 100,000, the first 1000 trees (10 % burn-in) were discarded. The remaining 45,000 trees were used to construct a 50 % majority-rule consensus tree. Maximum likelihood (ML) analyses were conducted using W-IQ-TREE (Nguyen et al., 2015) with 1000 ultrafast bootstrap replicates to assess nodal support. The trees were visualized and edited in FigTree v. 1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Pairwise genetic distances between the sequences of *cox1* mRNA and 28S rDNA were calculated in MEGA X (Kumar et al., 2018) using the Kimura 2-Parameter model (Kimura, 1980).

3. Results

A total of 14 *Clinostomum* metacercariae were recovered from *H. littorale* specimens collected in the Ucayali region (UR), with a prevalence of 50 % (4/4 hosts), a mean abundance of 3.5 ± 3.57 , and a mean intensity of 7 ± 1 . In contrast, five metacercariae were obtained from specimens collected in Loreto region (LR), with a prevalence of 12.5 % (3/24 hosts), a mean abundance of 0.21 ± 0.60 , and a mean intensity of 1.67 ± 0.47 .

Clinostomidae Luhe, 1901.

Clinostomum Leidy, 1856.

***Clinostomum* sp. UR (isolates M5).**

Host: *Hoplosternum littorale*.

Locality: “Calleria sector” near to the Ucayali River (Lat: 08° 26' S, Lon: 074° 15' W), Ucayali, Peru.

Specimens deposited: XXXXX, voucher.

Representative DNA sequences: PV767868 (*cox1* mRNA) and PV765350 (28S rDNA).

Description (based on four specimens, Figs. 2A– 3A-F).

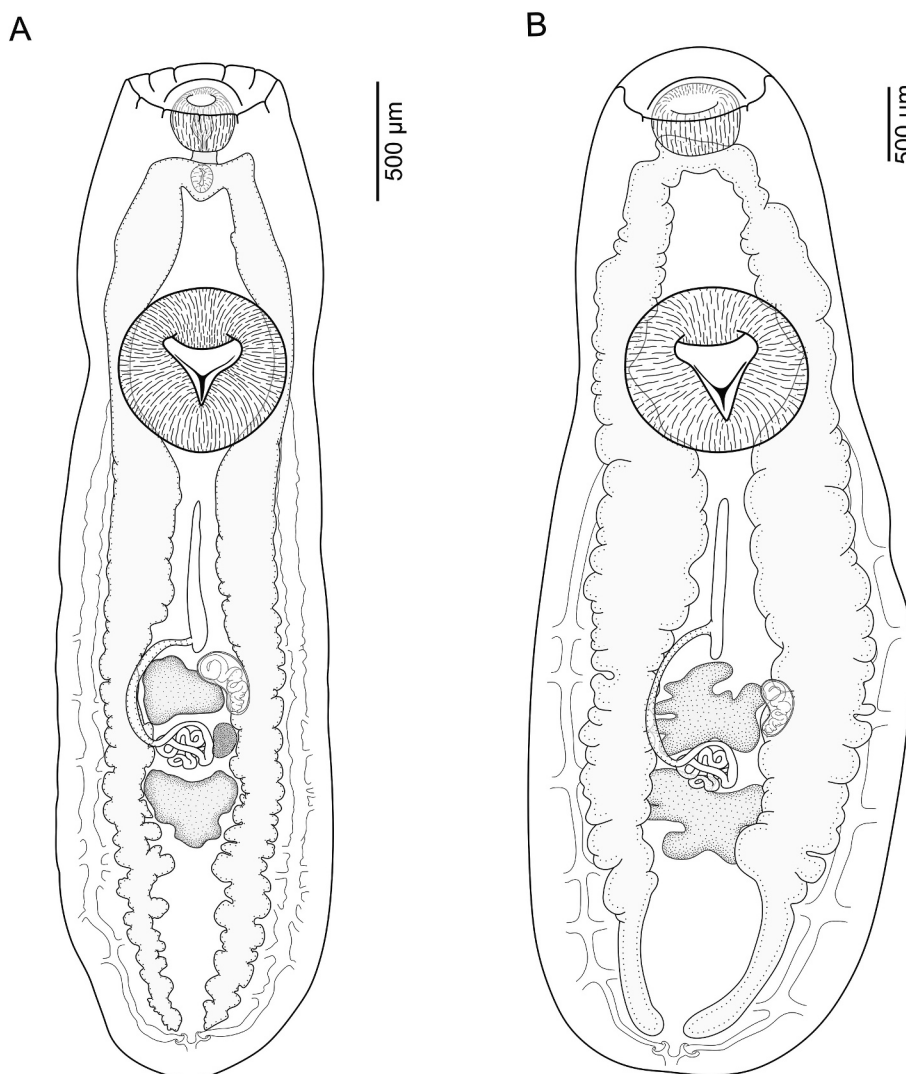


Fig. 2. Line drawing of *Clinostomum* spp. metacercariae from *Hoplosternum littorale* (Hancock, 1828) (Siluriformes: Callichthyidae) from Peruvian Amazon Basin. A. Specimens collected from the Ucayali region (central Amazon). B. Specimens of *Clinostomum chaacci* collected from the Loreto region (northern Amazon).

Body elongate, linguiform, 4.71–5.51 (5.05; $n = 4$) mm long, with two constrictions at ventral sucker level, flattened anterior end with oral collar; greatest width 1.07–1.31 (1.21; $n = 4$) at level of ventral sucker. Body length/body width ratio 3.64–4.39 (4.08; $n = 4$). Tegument with spines. Forebody 1.77–1.86 (1.81; $n = 4$) mm. Hindbody 2.70–2.98 (2.86; $n = 4$) long. Oral collar with conspicuous anterior grooves, $231\text{--}384 \times 603\text{--}764$ (307×688 ; $n = 4$). Oral sucker subterminal, rounded, smaller than ventral sucker, $277\text{--}296 \times 261\text{--}312$ (287×286 ; $n = 4$). Oral sucker width/body width ratio 0.22–0.24 (0.23; $n = 4$). Prepharynx short to absent. Pharynx $95\text{--}114 \times 72\text{--}91$ (105×80 ; $n = 4$). Intestinal caeca deeply diverticulate, lateral to ventral sucker and genital primordium extending to posterior end. Ventral sucker $684\text{--}758 \times 693\text{--}771$ (714×730 ; $n = 4$), with triradiate aperture. Ventral sucker width/body width ratio

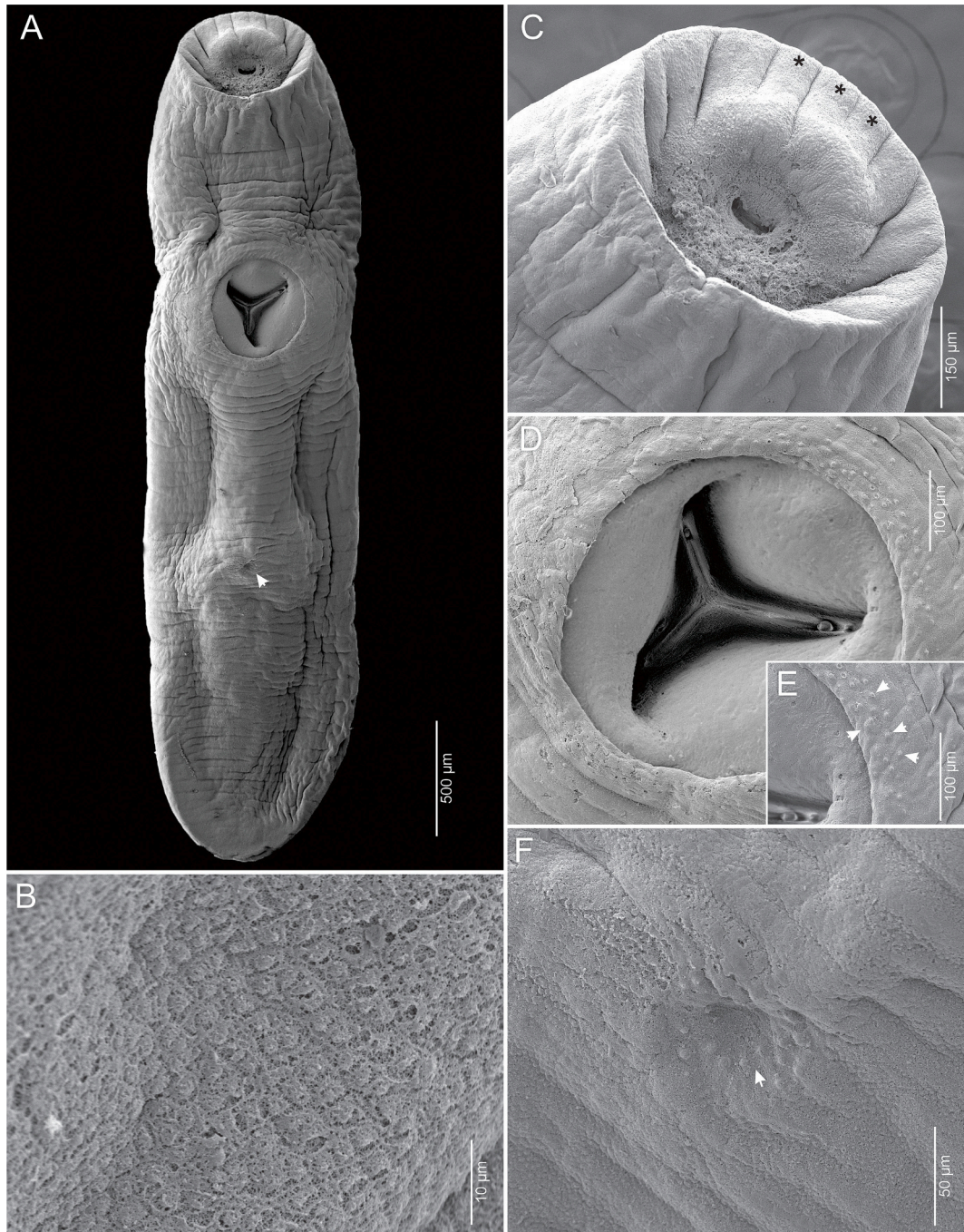


Fig. 3. Scanning electron micrographs of *Clinostomum* sp. (isolate UR) metacercariae from the Ucayali region (central Amazon). (A) Whole body of metacercariae. (B) Body surface. (C) Anterior end showing the oral sucker collar with conspicuous anterior grooves (asterisks). (D) Ventral sucker. (E) Ventral sucker papillae (white arrow). (F) Excretory pore.

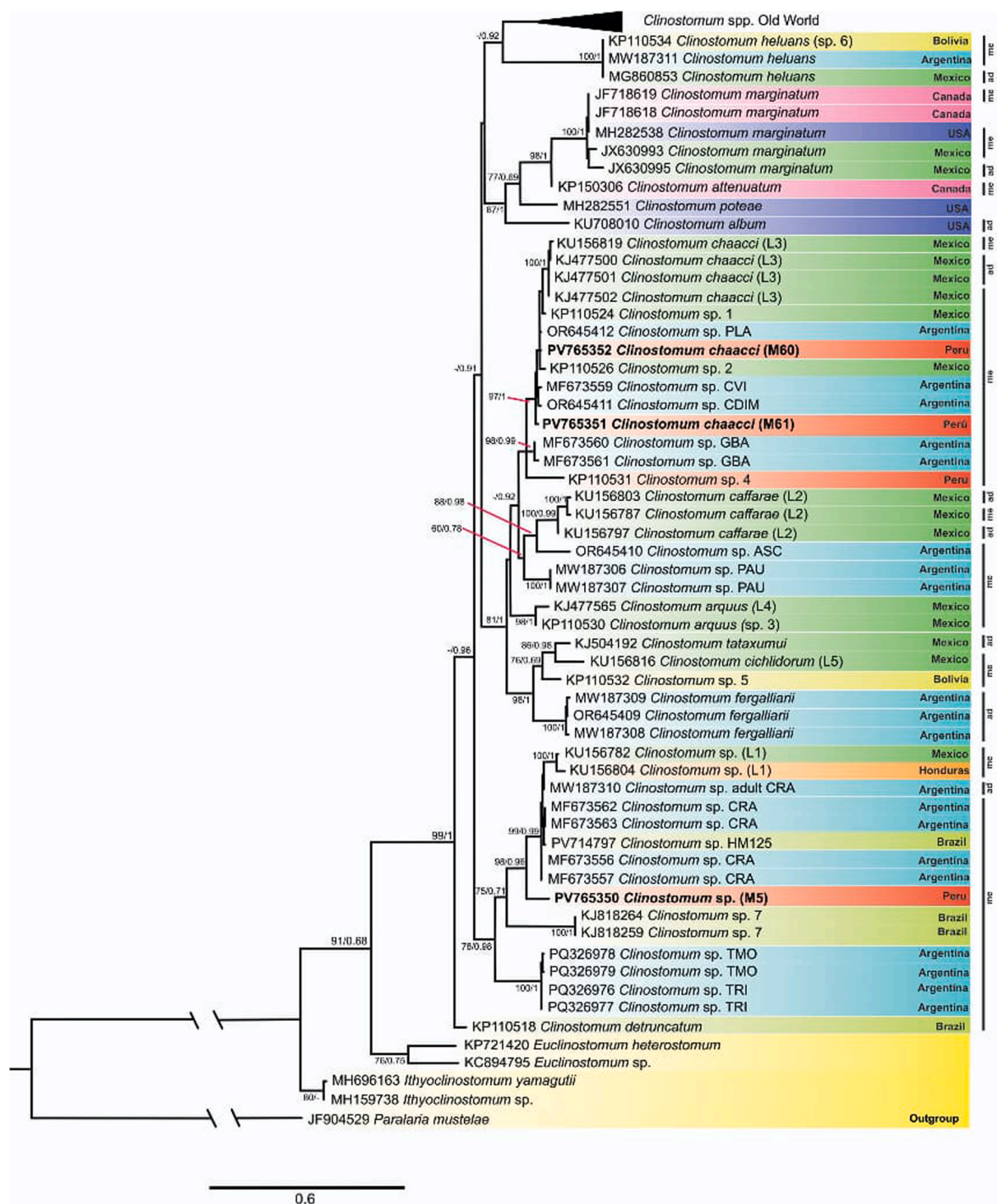


Fig. 4. Phylogenetic tree of species/lineages of clinostomids through the *cox1* gene inferred using Maximum likelihood method and Bayesian Inference (ML/Bi). Sequences from *Clinostomum* species obtained in this study are in blue bold. Scale-bar: average number of nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.57–0.65 (0.60; $n = 4$). Ventral sucker width/oral sucker width ratio 2.47–2.66 (2.56; $n = 4$). Genital primordium in posterior third of body. Testes two, tandem, asymmetrical; anterior testis irregularly lobed, $288\text{--}324 \times 283\text{--}406$ (303×333 ; $n = 4$), anterior testis width/anterior testis length ratio 0.97–1.25 (1.09; $n = 4$); posterior testis almost triangular, $212\text{--}368 \times 344\text{--}454$ (279×392 ; $n = 4$), posterior testis width/posterior testis length ratio 1.23–1.71 (1.47; $n = 4$). Cirrus sac, kidney-shaped, elongated, in right margin of anterior testis, $380\text{--}393 \times 118\text{--}171$ (386×140 ; $n = 4$), cirrus sac length/body length ratio 0.07–0.08 (0.08; $n = 4$). Ovary small, oval, intertesticular and dextral, $122\text{--}165 \times 83\text{--}103$ (144×94 ; $n = 4$), ovary width/ovary length ratio 0.61–0.71 (0.66; $n = 4$). Uterine sac long, tubular, between genital complex and ventral sucker.

3.1. *Clinostomum chaacci* Sereno-Uribe, López-Jiménez, González-García, Sereno-Uribe et al., 2025 (isolate M60 and M61)

Host: *Hoplosternum littorale*.

Locality: Zungarococha Lagoon and Santa Clara beach (Lat: $3^\circ 49'S$, Lon: $73^\circ 21'W$), near to the Nanay River, Loreto, Peru.

Specimens deposited: XXXXX, voucher.

Representative DNA sequences: PV767869–70 (*cox1* mRNA) and PV765351–52 (28S rDNA).

Description (based on four specimens, Fig. 2B).

Body robust, linguiform, 4.10–6.50 (5.32; $n = 4$) mm long, with two discrete constrictions at ventral sucker level, flattened anterior end with oral collar; greatest width 1.20–2.00 (1.66; $n = 4$) mm at level testicular. Body length/body width ratio 3.14–3.25 (3.20). Tegument lacking spines. Forebody 1.32–1.43 (1.38; $n = 4$) mm long. Hindbody 1.90–2.43 (2.11; $n = 4$) mm long. Oral collar, $160\text{--}200 \times 170\text{--}250$ (180×220 ; $n = 4$). Oral sucker subterminal, rounded, smaller than ventral sucker, $246\text{--}288 \times 278\text{--}296$ (282×314 ; $n = 4$). Oral sucker width/body width ratio 0.88–0.97 (0.89; $n = 4$). Prepharynx short to absent. Pharynx $98\text{--}128 \times 76\text{--}96$ (84×80 ; $n = 4$). Intestinal caeca robust, deeply diverticulate, lacking diverticula at distal end. Ventral sucker $619\text{--}727 \times 687\text{--}743$ ($755 \times$

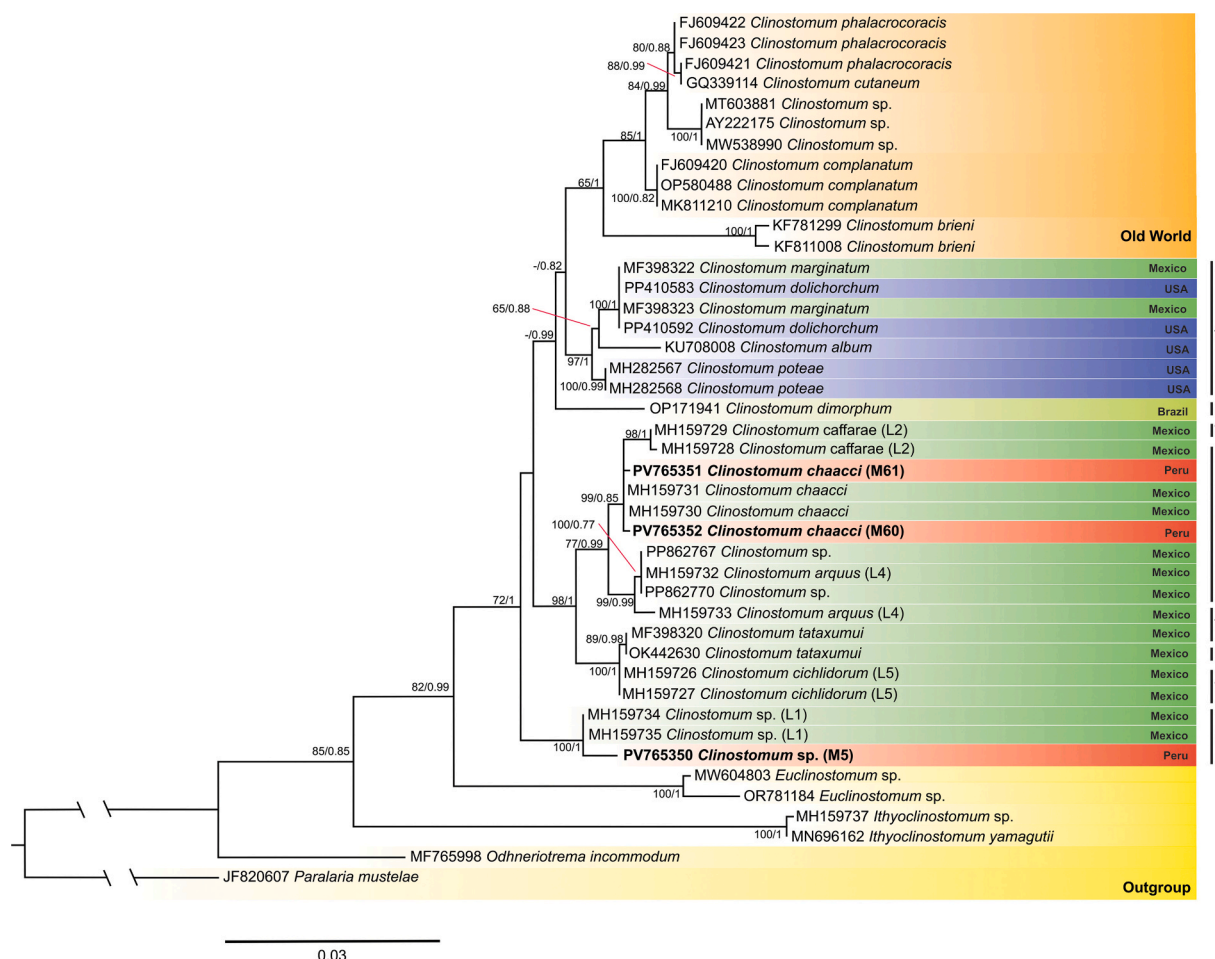


Fig. 5. Phylogenetic tree of species/lineages of clinostomids through 28S rRNA gene inferred using Maximum likelihood method and Bayesian Inference (ML/BI). Sequences from *Clinostomum* species obtained in this study are in blue bold. Scale-bar: average number of nucleotide substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

779; $n = 4$), with triradiate aperture. Ventral sucker width/body width ratio 0.58–0.37 (0.46; $n = 4$). Ventral sucker width/oral sucker width ratio 2.47–2.51 (2.48; $n = 4$). Genital primordium in posterior third of body. Testes two, tandem, asymmetrical, strongly lobulated; anterior testis, $170\text{--}274 \times 379\text{--}452$ (241×451 ; $n = 4$), anterior testis width/anterior testis length ratio 2.22–1.64 (0.53; $n = 4$); posterior testis $195\text{--}260 \times 261\text{--}515$ (246×422 ; $n = 4$), posterior testis width/posterior testis length ratio 1.33–1.99 (1.71; $n = 4$). Cirrus sac, kidney-shaped, in dextralateral to anterior testis, $390\text{--}400 \times 120\text{--}185$ (395×155 ; $n = 4$), cirrus sac length/body length ratio 0.06–0.08 (0.08; $n = 4$). Ovary small, intertesticular and dextral, $130\text{--}170 \times 86\text{--}110$ (150×96 ; $n = 4$), ovary width/ovary length ratio 0.60–0.70 (0.65; $n = 4$). Uterine sac long, tubular, between genital complex and ventral sucker.

3.2. Molecular analyses

In this study, we generated *cox1* mtDNA and 28S rDNA sequences from three *Clinostomum* metacercariae infecting *H. littorale*, collected from Ucayali ($n = 1$) and Loreto ($n = 2$), Peru.

Cox1: The final alignment comprised 443 bp and 88 terminals, including the new sequences and five outgroup taxa representing other clinostomid and diplostomid species (Fig. 3). The Ucayali specimen (*Clinostomum* M5) formed a sister lineage to the clade containing *Clinostomum* L1, HM125, and CRA, with strong nodal support (ML = 98; BI = 0.96). Genetic divergence between M5 and its closest relatives ranged from 8.7 % to 12.7 % (36–51 bp), being lowest with *Clinostomum* CRA (8.7 %–9.7 %) and highest with *Clinostomum* L1 (11.9 %–12.7 %). In contrast, our sequences from Loreto (M60 and M61) clustered with high support (ML = 97; BI = 1) in a clade that also included *Clinostomum* CDIM, CVI, sp1, sp2, PLA, and *Clinostomum chaacchi* Sereno-Urbe, López-Jiménez, González-García, Ortega-Olivares & García-Varela, 2025 (ex L3), indicating that our specimens correspond to *C. chaacchi*. The two isolates, M60 and M61, differed by 1.37 % (6 bp). Isolate M60 was most closely related to *Clinostomum* PLA (0.45 %, 2 bp), whereas M61 showed slightly higher divergence (1.37 %) from isolates PLA, CDIM, and CVI.

28S: The final alignment was 1081 bp in length and included 43 terminals. As with the mitochondrial data, M5 clustered closely with *Clinostomum* L1, receiving full support (ML = 100; BI = 1), with a genetic divergence of just 0.47 % (5 bp) (Fig. 4). In contrast, the Loreto specimens M60 and M61 grouped together with strong support (ML = 99; BI = 0.85), in a clade that included *C. chaacchi* (ex L3), confirming also that they correspond to this species. The two isolate differed slightly from each other (0.19 %, 2 bp) and both were nearly identical to *C. chaacchi*, with only 0.09 % divergence (1 bp). (See Fig. 5.)

Table 1

Measurements of *Clinostomum* spp. infecting *Hoplosternum littorale* from Peru compared with those reported for Davies et al. (2016).

Species	<i>Clinostomum</i> sp.	<i>Clinostomum chaacchi</i>	<i>Clinostomum</i> sp.
Host	<i>Hoplosternum littorale</i>	<i>Hoplosternum littorale</i>	<i>Hoplosternum littorale</i>
Locality	Ucayali region, Peru	Loreto region, Peru	Salta Province, Argentina
<i>n</i>	4	4	7
Reference	Present study	Present study	Davies et al. (2016)
Body length (BL)	4713–5514 (5052)	4100–6500 (5320)	3398–4032 (3662)
Body width (BW)	1074–1311 (1212)	1200–2000 (1660)	835–1152 (1012)
BL/BW	3.64–4.39 (4.08)	3.14–3.25 (3.20)	3.62
Forebody length	1773–1863 (1816)	1326–1430 (1384)	–
Hindbody length	2706–2980 (2868)	1902–2435 (2111)	–
Collar length	231–384 (307)	160–200 (180)	–
Collar width	603–764 (688)	170–250 (220)	–
Oral sucker length (OSL)	277–296 (287)	246–288 (282)	187–285 (229)
Oral sucker width (OSW)	261–312 (286)	278–296 (314)	246–442 (355)
OSW/BW	0.22–0.24 (0.23)	0.88–0.97 (0.89)	0.35
Pharynx length	95–114 (105)	98–120 (110)	–
Pharynx width	72–91 (80)	76–96 (84)	–
Ventral sucker length (VSL)	684–758 (714)	619–727 (755)	540–717 (613)
Ventral sucker width (VSW)	693–771 (730)	687–743 (779)	520–687 (603)
VSW/OSW	2.47–2.66 (2.56)	2.47–2.51 (2.48)	1.7
VSW/BW	0.57–0.65 (0.60)	0.58–0.37 (0.46)	0.6
Distance between suckers	640–778 (722)	638–679 (721)	98–314 (188)
Anterior testis length (ATL)	288–324 (303)	170–274 (241)	265–324 (295)
Anterior testis width (ATW)	283–406 (333)	379–452 (451)	285–353 (303)
ATW/ATL	0.97–1.25 (1.09)	2.22–1.64 (0.53)	1.03
Posterior testis length (PTL)	212–368 (279)	195–260 (246)	226–363 (274)
Posterior testis width (PTW)	344–454 (392)	261–515 (422)	295–373 (339)
PTW/PTL	1.23–1.71 (1.47)	1.33–1.99 (1.71)	1.24
Distance between testes	199–271 (233)	281–288 (308)	98–196 (142)
Ovario length (OL)	122–165 (144)	130–170 (150)	137–187 (160)
Ovario width (OW)	83–103 (94)	86–110 (96)	98–147 (121)
OW/OL	0.61–0.71 (0.66)	0.60–0.70 (0.65)	0.76
Cirrus sac length (CSL)	380–393 (386)	390–400 (395)	167–275 (226)
Cirrus sac width (CSW)	118–171 (140)	120–185 (155)	88–137 (102)
CSL/BL	0.07–0.08 (0.08)	0.06–0.08 (0.07)	0.06

Values based on data from Montes et al. (2024).

4. Discussion

Our integrative approach, combining morphological and molecular data, revealed two phylogenetically distinct lineages of *Clinostomum* metacercariae infecting *H. littorale* in different regions of the Peruvian Amazon Basin: Ucayali (central Amazon) and Loreto (northern Amazon). This finding expands our current understanding of clinostomid diversity in the Neotropics and underscores the importance of molecular tools for detecting cryptic lineages, as highlighted by Montes et al. (2023) and Serrano-Uribe et al. (2025).

Previous report of *Clinostomum complanatum* (Rudolphi, 1814) Braun, 1899 infecting *H. littorale* in Brazil (Dias et al., 2003) lack morphometric data and voucher specimens, raising doubts about their validity, especially considering that *C. complanatum* is geographically restricted to the western Palearctic (Locke et al., 2019). More recent findings in Argentina (Davies et al., 2016) and Peru (Morey et al., 2025b) described unidentified *Clinostomum* metacercariae in *H. littorale*, but without genetic data, precluding confident species assignment. Morphologically, our Loreto specimens (M60 and M61) resemble those reported by Morey et al. (2025b), but differ from the Ucayali specimens (*Clinostomum* sp. UR) by having a more robust body, thicker intestinal caeca without distal diverticula, and deeply lobed testes. Although this variation could reflect developmental or host-related plasticity, the consistent genetic divergence supports the recognition of distinct evolutionary lineages. While Davies et al. (2016) did not generate molecular data, our comparative analysis revealed that the metacercariae from Argentina differ morphometrically from both lineages. The Peruvian specimens were generally larger and showed higher ratios for body length/body width, sucker spacing, and cirrus sac length, while having smaller ratios for oral sucker/body width and ovary width/length (Table 1). These differences likely represent genuine interpopulation divergence, potentially reflecting distinct lineages, although this cannot be confirmed in the absence of molecular evidence. Based on the *cox1* marker, the Loreto specimens (isolate M60 and M61) clustered with *C. chaacsi*, and with other metacercariae previously reported from Central (Mexico) and South America (Argentina), including *Clinostomum* PLA, sp1, sp2, CVI, and CDIM, exhibiting minimal intraspecific divergence (0.09–1.37 %). These results indicate that all of these metacercariae together with our two isolates, are conspecific and correspond unequivocally to *C. chaacsi*. Metacercariae of *C. chaacsi* have been reported infecting siluriform fishes such as *Rhamdia guatemalensis* (Günther, 1864) and *R. rogersi* Regan, 1907 (identified as *Clinostomum* sp1. and *Clinostomum* L3), and the perciform *Sicydium salvini* Ogilvie-Grant, 1884 (as *Clinostomum* sp2.) in Mexico (Locke et al., 2015; Pérez-Ponce De León et al., 2016). In Argentina, metacercariae of this species have been recorded in cichliform hosts, including *Crenicichla vittata* Heckel, 1840 (as *Clinostomum* CVI) and *Cichlasoma dimerus* (Heckel, 1840) (as *Clinostomum* CDIM), as well as in the siluriform *Pimelodella laticeps* Eigenmann, 1917 (as *Clinostomum* PLA) (Montes et al., 2020, 2023). In the present study, we report a new host record for the metacercaria of *C. chaacsi*, the callichthyid catfish *H. littorale* from Peru. Our findings clarify the taxonomic status of these records, reduce uncertainty in larval identifications within the genus, and suggest that *C. chaacsi* represents a widespread lineage across central and South America, characterized by low host specificity in the second intermediate hosts.

A persistent challenge in clinostomid taxonomy is the lack of adult specimens for many lineages. Several, including *C. chaacsi* and *C. tataxumui*, were initially known only from larvae and later linked to adults through molecular matches with parasites from piscivorous birds (Serrano-Uribe et al., 2025). *Clinostomum chaacsi* was originally described from the oral cavity of two ardeid host in the Neotropical region of Mexico, the great blue heron *Ardea herodias* Linnaeus, 1758 (type host), and the barethroated tiger-heron *Tigrisoma mexicanum* Swainson, 1834 (Serrano-Uribe et al., 2025). Of these, *A. herodias* has a distribution largely restricted to North and Central America, while *T. mexicanum* extends further south into South America, reaching northern Peru, specifically the coastal region of Tumbes. However, *T. mexicanum* does not occur in the Amazonian lowlands around Iquitos, where our specimens were collected, a region biogeographically distinct from the Pacific coastal zone. This suggests that other heron species of the Ardeidae, more widely distributed in the Amazon Basin, may serve as definitive hosts of *C. chaacsi* in this part of South America. Consequently, targeted sampling of ardeid birds in the Peruvian Amazon is essential to elucidate the complete life cycle and confirm the host associations of these taxa.

The Ucayali specimen (isolate M5) formed a highly supported and divergent clade alongside *Clinostomum* L1. The mitochondrial divergence between M5 and related taxa ranged from 11.9 % to 12.7 %, exceeding the interspecific thresholds commonly reported for *Clinostomum* (typically >7–10 %) (Serrano-Uribe et al., 2025). This level of divergence strongly suggests that the Ucayali specimen represents a distinct, previously unrecognized taxon. The fact that both lineages infect the same fish host, *H. littorale*, in different regions without evidence of co-infection may reflect ecological or geographic barriers restricting gene flow, or potentially host-parasite coevolution shaped by basin-specific dynamics. Montes et al. (2023) reported similar patterns of divergence among metacercariae from different hosts and localities, including undescribed lineages in Argentina that share phylogenetic affinities with sequences from Mexico and Central America. These authors also highlighted how morphological convergence and host-related plasticity can obscure species boundaries, reinforcing the importance of molecular markers, especially *cox1*, for accurate identification. Their study, like ours, demonstrates that many metacercariae cannot be reliably assigned to species without genetic data and comprehensive comparative frameworks.

The detection of *Clinostomum* metacercariae encysted in the musculature of *H. littorale* raises potential public health concerns, especially in Amazonian regions where raw or undercooked fish dishes are traditionally consumed. Several digenaeans trematodes, including members of *Clinostomum*, are recognized zoonotic agents capable of infecting humans through ingestion of metacercariae in inadequately prepared fish. After ingestion, the parasites may excyst in the stomach and migrate to the oropharyngeal region, causing symptoms characteristic of human clinostomiasis, such as acute pharyngitis or laryngitis (Park et al., 2009; Lee et al., 2017). While most documented cases involve *C. complanatum* in Asia and Europe, the potential for zoonotic transmission in South America should not be overlooked. This is particularly relevant in light of the growing number of reports of *Clinostomum* metacercariae in edible fish species (Montes et al., 2023), including the present findings, where *H. littorale*, a widely consumed species, harbored encysted larvae within muscle tissue.

In conclusion, this study presents the first integrative evidence of two phylogenetically distinct *Clinostomum* lineages, *C. chaacci* and *Clinostomum* sp. (isolated UR), infecting *H. littorale* in the Peruvian Amazon, revealing unexpected diversity within Neotropical clinostomids. The combined morphological and molecular approach exposed limitations of larval morphology for species identification and highlighted the taxonomic complexity of the group. Additionally, the detection of metacercariae in edible muscle tissues raises concerns about zoonotic transmission, reinforcing the need for public health awareness. Future studies should prioritize the sampling of definitive hosts, link larval and adult stages through molecular data, and expand regional surveys to better understand the diversity, distribution, and epidemiological relevance of these parasites in the Amazon and beyond.

CRediT authorship contribution statement

J.D. Chero: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **L.A. Nacari:** Methodology, Formal analysis, Data curation. **G.A. Murrieta Morey:** Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **C.L. Cruces:** Methodology, Investigation, Formal analysis, Data curation. **E. Cacique:** Formal analysis, Data curation. **N. Huaman:** Formal analysis, Data curation. **D. Lopez:** Formal analysis, Data curation. **A. Mondragón-Martínez:** Project administration, Methodology, Investigation, Data curation, Conceptualization. **R. Martínez-Rojas:** Methodology, Funding acquisition, Formal analysis, Data curation. **J. Yunis-Aguinaga:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Ethical standards

The authors declare that all applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study is part of the project titled “Caracterización morfológica, ultraestructural y mitogenómica de helmintos con potencial zoonótico en peces de interés comercial en la Amazonia peruana,” which was approved by the Ethics Committee of the Faculty of Biological Sciences of the Universidad Nacional Mayor de San Marcos (Approval Letter No. 000063–2024-CB-D-FCB/UNMS).

Financial support

This work was funded by the Consejo Nacional de Ciencia, Tecnología e Innovación Tecnológica (CONCYTEC) and the Programa Nacional de Investigación Científica y Estudios Avanzados (PROCIENCIA) under the call “E041-2024-03 Proyectos de Investigación Básica” [PE501087644–2024]. The “Universidad Científica del Sur” supported paying the article processing charge for this work.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper. No financial, personal, or professional relationships with other people or organizations could inappropriately influence or bias the content of this work.

Acknowledgments

The authors are thankful to Danayra Cristhel Cruz Espinoza, staff of the Laboratory of Specialized Equipment from UNMSM, for the support in SEM observations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fawpar.2025.e00306>.

References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).
- Choudhary, K., Ray, S., Shamsi, S., Agrawal, N., 2022. Characterization of *Clinostomum* (Digenea: Clinostomidae) spp. in India. *Parasitol. Res.* 121, 3083–3089. <https://doi.org/10.1007/s00436-022-07644-y>.
- Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods* 9, 772. <https://doi.org/10.1038/nmeth.2109>.
- Davies, D., Ostrowski de Nuñez, M., Ramallo, G., Nieva, L., 2016. Nuevos hospedadores y localidades de colecta de *Clinostomum* sp. (Strigeida: Clinostomidae). *Acta Zool. Liloana*. 60, 89–94.
- Dávila, C.R.G., Riveiro, H.S., Silva, M.A.F., de Loayza, J.E.M., Chávez, C.A.C.A., Ruiz, D.C., Renno, J.F., 2018. Peces de consumo de la Amazonía peruana. In: Instituto de Investigaciones de la Amazonía Peruana (IIAP), Iquitos, Perú.
- Derycke, S., Remerie, T., Vierstraete, A., Backeljau, T., Vanfleteren, J., Vincx, M., Moens, T., 2005. Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. *Mar. Ecol. Prog. Ser.* 300, 91–103. <https://doi.org/10.3354/meps300091>.
- Dias, M.L.G.G., Eiras, J.C., Machado, M.H., Souza, G.T.T., Pavanelli, G.C., 2003. The life cycle of *Clinostomum complanatum* Rudolphi, 1814 (Digenea, Clinostomidae) on the floodplain of the high Paraná river, Brazil. *Parasitol. Res.* 89, 506–508. <https://doi.org/10.1007/s00436-002-0796-z>.

- Fernandes, T.F., Dos Santos, J.N., de Vasconcelos Melo, F.T., Achatz, T.J., Greiman, S.E., Bonilla, C.C., Tkach, V.V., 2021. Interrelationships of *Anenterotrema* (Digenea: Dicrocoeliidae) from Neotropical bats (Mammalia: Chiroptera) with description of a new species from *Molossus molossus* in Brazil. *Parasitol. Res.* 120, 2003–2016. <https://doi.org/10.1007/s00436-021-07133-8>.
- Filatov, D.A., 2002. Proseq: a software for preparation and evolutionary analysis of DNA sequence data sets. *Mol. Ecol. Notes* 2, 621–624. <https://doi.org/10.1046/j.1471-8286.2002.00313.x>.
- Gião, T., Müller, M.I., Yamada, F., Freitas, F., Leite, L., da Silva, R.J., de Azevedo, R., Abdallah, V., 2025. Morphological and molecular phylogeny of *Clinostomum* sp. (Digenea: Clinostomidae) metacercariae, using DNA barcode from a south American freshwater fish. *J. Helminthol.* 99. <https://doi.org/10.1017/S0022149X24000993> e13, 1–10.
- Kanev, I., Radev, V., Fried, B., 2002. Family Clinostomidae Lühe, 1901. In: Gibson, D.I., Jones, A., Bray, R.A. (Eds.), *Keys to the Trematoda*. CABI Publishing and the Natural History Museum, London, pp. 113–120. <https://doi.org/10.1079/9780851995472.0113>.
- Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Molecul. Evol.* 16 (2), 111–120.
- Kumar, S., Stecher, G., Li, M., Niyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547. <https://doi.org/10.1093/molbev/msy096>.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948. <https://doi.org/10.1093/bioinformatics/btm404>.
- Lee, G.S., Park, S.W., Kim, J., Seo, K.S., You, K.W., Chung, J.H., Moon, H.C., Hong, G.Y., 2017. A case of endoscopically treated Laryngopharyngitis resulting from *Clinostomum complanatum* infection. *Korean J. Gastroenterol.* 69, 177–180. <https://doi.org/10.4166/kjg.2017.69.3.177>.
- Locke, S.A., Caffara, M., Marcogliese, D.J., Fioravanti, M.L., 2015. A large-scale molecular survey of *Clinostomum* (Digenea: Clinostomidae). *Zool. Scr.* 44, 203–217. <https://doi.org/10.1111/zsc.12096>.
- Locke, S.A., Caffara, M., Barčák, D., Sonko, P., Tedesco, P., Fioravanti, M.L., Li, W., 2019. A new species of *Clinostomum* Leidy, 1856 in East Asia based on genomic and morphological data. *Parasitol. Res.* 118, 3253–3265. <https://doi.org/10.1007/s00436-019-06536-y>.
- Lockyer, A.E., Olson, P.D., Ostergaard, P., Rollinson, D., Johnston, D.A., Attwood, S.W., Southgate, V.R., Horak, P., Snyder, S.D., Le, T.H., Agatsuma, T., McManus, D. P., Carmichael, A.C., Naem, S., Littlewood, D.T., 2003. The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858. *Parasitology* 126, 203–224. <https://doi.org/10.1017/S0031182002002792>.
- Mahdy, O.A., Abdel-Magood, S.Z., Abdelsalam, M., Salem, M.A., 2024. A multidisciplinary study on *Clinostomum* infections in Nile tilapia: micro-morphology, oxidative stress, immunology, and histopathology. *BMC Vet. Res.* 20, 60. <https://doi.org/10.1186/s12917-024-03901-7>.
- Montes, M.M., Plaul, S.E., Croci, Y., Waldbillig, M., Ferrari, W., Topa, E., Martorelli, S.R., 2020. Pathology associated with three new *Clinostomum* metacercariae from Argentina with morphological and DNA barcode identification. *J. Helminthol.* 94, 1–11. <https://doi.org/10.1017/S0022149X23000706>.
- Montes, M.M., Barneche, J., Pagano, L., Ferrari, W., Martorelli, S.R., Ponce, Pérez, de León, G., 2021. Molecular data reveal hidden diversity of the genus *Clinostomum* (Digenea, Clinostomidae) in Argentina, with the description of a new species from *Ardea coccy* (Ardeidae). *Parasitol. Res.* 120, 2779–2791. <https://doi.org/10.1007/s00436-021-07234-4>.
- Montes, M.M., García, I., Paredes del Puerto, J.M., Barneche, J.A., Ibañez Shimabukuro, M., Reig, Cardarella G.F., Martorelli, S.R., 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. *J. Helminthol.* 97 (e85), 1–9. <https://doi.org/10.1017/S0022149X23000706>.
- Montes, M.M., Alonso, F., Terán, G.E., Serra Alanis, S.W., Waldbillig, M., Cavallo, M.G., Balcazar, D., Reig Cardarella, G.F., 2024. Morphological and genetic analysis of a rediscovered *Clinostomum* sp. parasitising *Titanolebias monstrosus* and *Trigonectes aplocheiloides* (Cyprinodontiformes: Rivulidae). *J. Helminthol.* 20, 98, e74. <https://doi.org/10.1017/S0022149X24000658>.
- Morey, G.A.M., Llanos, S.G.R., Chu, L.A.R., Dreyfus, A.C.T., Solsol, L.A.G., Sánchez, R.F.C., Calizaya, E.Y.L., 2025b. Zoonotic Parasites in *Hoplosternum littorale* Acquired in the Market of Santa Clara, in the City of Iquitos, Loreto-Peru. *Acta Parasitol.* 70 (2), 1–6.
- Morey, G.A.M., Llanos, S.G.R., Chu, L.A.R., Taricuarima, A., García, L., Chuquipiondo, R., Llasaca, E., 2025a. Zoonotic parasites in *Hoplosternum littorale* acquired in the market of Santa Clara, in the City of Iquitos. *Loreto-Peru. Acta Parasitol.* 70, 78. <https://doi.org/10.1007/s11686-025-01019-w>.
- Nguyen, L.T., Schmidt, H.A., Von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32, 268–274. <https://doi.org/10.1093/molbev/msu300>.
- Orfinger, A.B., 2015. Selected population characteristics of the non-native catfish *Hoplosternum littorale* (Hancock, 1828) in Central Florida, USA. *Int. J. Aquat. Biol.* 3, 258–262. <https://doi.org/10.22034/ijab.v3i4.104>.
- Park, C.-W., Kim, J.-S., Joo, H.-S., Kim, J., 2009. A human case of *Clinostomum complanatum* infection in Korea. *Korean J. Parasitol.* 47, 401–404. <https://doi.org/10.3347/kjp.2009.47.4.401>.
- Pérez-Ponce De León, G., García-Varela, M., Pinacho-Pinacho, C.D., Sereno-Urbe, A.L., Poulin, R., 2016. Species delimitation in trematodes using DNA sequences: middle-American *Clinostomum* as a case study. *Parasitology* 143, 1773–1789. <https://doi.org/10.1017/S0031182016001517>.
- Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. *Syst. Biol.* 53, 793–808. <https://doi.org/10.1080/10635150490522304>.
- Reis, R.E., 1997. Revision of the neotropical catfish genus *Hoplosternum* (Ostariophysi: Siluriformes: Callichthyidae), with the description of two new genera and three new species. *Ichthyol. Explorat. Freshwaters.* 7, 299–326.
- Ronquist, F., Huelsenbeck, J., Teslenko, M., 2011. Draft MrBayes version 3.2 manual: tutorials and model summaries [software]. <http://brahms.biology.rochester.edu/software>. Html.
- Rosser, T.G., Woodyard, E.T., Mychajlonka, M.N., King, D.T., Griffin, M.J., Shamsi, S., Halajian, A., Tavakol, S., Mortazavi, P., Boulton, J., 2013. Pathogenicity of *Clinostomum complanatum* (Digenea: Clinostomidae) in piscivorous birds. *Res. Vet. Sci.* 95, 537–539. <https://doi.org/10.1016/j.rvsc.2013.06.018>.
- Rudolphi, C.A., 1819. *Entozoorum Synopsis Cui Accedunt Mantissa Duplex et Indices Locupletissimi*. Augusti Rücker, Berolini, p. 811.
- Serrano-Urbe, A.L., López-Jiménez, A., González-García, M.T., Ortega-Olivares, M.P., García-Varela, M., 2025. A phylogeographic approach of three species of *Clinostomum* Leidy, 1856, (Trematoda: Clinostomidae) from the Neotropical region of Mexico, with the description of a new species from *Ardea herodias* (Ardeidae). *Parasitol. Int.* 108, 103067. <https://doi.org/10.1016/j.parint.2025.103067>.
- Shamsi, S., Gasser, R., Beveridge, I., 2013. Description and genetic characterisation of *Hysterothylacium* (Nematoda: Raphidascarididae) larvae parasitic in Australian marine fishes. *Parasitol. Int.* 62 (3), 320–328.
- Tkach, V.V., Lotz, J., Swiderski, Z., Esteban, J.G., 2002. On the systematic position of *Ophiosacculus* Macy, 1935 (Digenea: Lecithodendriidae), with the erection of the Ophiosacculinae n. subfam. *Syst. Parasitol.* 53, 159–167. <https://doi.org/10.1023/A:1021199108887>.