

Research Paper

Cite this article: Briosio-Aguilar R, García-Varela M, Hernández-Mena DI, Rubio-Godoy M, Pérez-Ponce de León G (2019). Morphological and molecular characterization of an enigmatic clinostomid trematode (Digenea: Clinostomidae) parasitic as metacercariae in the body cavity of freshwater fishes (Cichlidae) across Middle America. *Journal of Helminthology* **93**, 461–474. <https://doi.org/10.1017/S0022149X18000445>

Received: 8 March 2018

Accepted: 12 April 2018

First published online: 17 May 2018

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Morphological and molecular characterization of an enigmatic clinostomid trematode (Digenea: Clinostomidae) parasitic as metacercariae in the body cavity of freshwater fishes (Cichlidae) across Middle America

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Abstract

The family Clinostomidae Lühe, 1901 contains 29 species allocated to seven genera, of which *Clinostomum* Leidy, 1856 is the most diverse, with c. 14 valid species. The diversity of *Clinostomum* has been assessed, combining morphological and molecular data. The genetic library for species in this genus has increased steadily, although there is little or no information for the other genera included in the family. Molecular phylogenetic relationships among the genera of clinostomids have not been assessed, and their classification is still based on morphological traits. The monotypic *Ithyoclinostomum* was described from a fish-eating bird in Brazil, and its metacercariae have been found in several locations in South America, parasitizing erythrinid freshwater fishes. We collected unusually large metacercariae from the body cavity of cichlids in several locations across Middle America. These metacercariae exhibited some resemblance to *Ithyoclinostomum*, although several differences prevent their inclusion in *Ithyoclinostomum dimorphum*, casting doubt on their taxonomic identification. The main objective of this paper was to characterize the metacercariae collected in cichlids using both morphology and molecular data from three molecular markers, and to assess the molecular phylogenetic relationships among the genera of Clinostomidae to establish the position of the newly generated sequences. We took a conservative position and tentatively placed the metacercariae as belonging to *Ithyoclinostomum*.

Introduction

Members of the cosmopolitan family Clinostomidae Lühe, 1901 are parasites of the buccal cavity, oesophagus or intestine of birds, reptiles and occasionally mammals (Ukoli, 1966; Kanev *et al.*, 2002). The taxonomic status and phylogenetic relationships among the genera allocated to this family have been uncertain. Currently, the family comprises seven genera in four subfamilies according to Kanev *et al.* (2002): Clinostominae Lühe, 1901 with the cosmopolitan genus *Clinostomum* Leidy, 1856 containing c. 14 species parasitizing fish-eating birds as definitive hosts (see Locke *et al.*, 2015; Pérez-Ponce de León *et al.*, 2016; Caffara *et al.*, 2017), *Clinostomatopsis* Dollfus, 1932 (two species), and the monotypic *Clinostomoides* Dollfus, 1959; Euclinostominae Yamaguti, 1958, with *Euclinostomum* Travassos, 1928 containing eight species parasitizing birds (Caffara *et al.*, 2016); Nephrocephalinae Travassos, 1928, with two genera parasitic in crocodilians, i.e. *Odhneriotrema* Travassos, 1928 and *Nephrocephalus* Odhner, 1902, with two and one species, respectively (Woodyard *et al.*, 2017); and Ithyoclinostominae Yamaguti, 1958, with the monotypic *Ithyoclinostomum dimorphum* (Diesing, 1850) Witenberg, 1925, as a parasite of birds. Even though great progress has been made in recent years to increase the generic library for species of *Clinostomum*, the most species-rich genus in the family, in studies aimed at establishing robust species delimitation criteria, few attempts have been made to generate sequence data to perform a phylogenetic analysis at the family level (see Woodyard *et al.*, 2017).

During survey work on the helminth fauna of Middle American freshwater fishes, unusually large clinostomid metacercariae were found unencysted in the body cavity of some cichlid species in several locations of Mexico and Costa Rica. Based on the size of the metacercariae and the position of the genital complex in the posterior fourth of the body, we first identified the species as *Clinostomum heluans* Braun, 1899. An additional piece of information was that *C. heluans* had been previously recorded as a parasite of the great

blue heron, *Ardea herodias* Linnaeus, 1758 in north-eastern Mexico (Bravo-Hollis, 1947). In a recent study, Briosio-Aguilar *et al.* (2018) characterized molecularly the metacercariae of *C. heluans*; in addition, these authors established a molecular link between the metacercariae and the adults and determined that the distributional range of the species extends between northern Mexico and Brazil. However, once we obtained ribosomal and mitochondrial DNA sequences of the unusually large clinostomid metacercariae, we discovered that they are not conspecific with *C. heluans*. The objective of this paper is twofold: to characterize morphologically and molecularly the clinostomid metacercariae found in Middle American cichlids, and to accomplish their identification at genus level, establishing their phylogenetic position within the phylogeny of the family Clinostomidae.

Materials and methods

Specimen collection

Specimens of metacercariae were sampled between 2014 and 2016 in six species of cichlids from six localities, four in Mexico and two in Costa Rica (table 1). In total, 45 individual fish were collected using seine nets and electrofishing, kept alive and transported to the laboratory, pith sacrificed, and examined for parasites under a stereomicroscope. Some specimens were fixed by sudden immersion in hot (near boiling) 4% formalin, subsequently washed in distilled water and stored in 70% ethanol; some specimens were also preserved in vials with 100% ethanol for molecular analysis.

Morphological study

For morphological identification, 29 specimens (14 whole specimens and 15 hologenophores) were stained with Mayer's paracarmine, dehydrated in a graded ethanol series, cleared with methyl salicylate, and mounted on permanent slides with Canada balsam. Voucher specimens were deposited at the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City. All the specimens were examined using a bright-field Axio Zoom V16 microscope (Zeiss, Oberkochen, Germany). Images were obtained through an Axio Cam Mrc5 attached to the microscope, and specimens were measured using the software ZEN-Zeiss Efficient Navigation; measurements are presented in millimeters, with the range followed by the mean in parentheses. Drawings of the metacercariae were made using a drawing tube attached to the microscope. For the scanning electron microscopy (SEM) study, two specimens were dehydrated through a graded series of ethyl alcohol, and then critical-point dried with carbon dioxide, mounted on metal stubs with silver paste, coated with gold, and examined in a Hitachi Stereoscan model SU1510 (Hitachi High-Technologies Mexico S.A. de C.V, Mexico) at 15 kV.

DNA extraction, amplification and sequencing

Seventeen specimens were placed individually in tubes and digested overnight at 56°C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM Na₂ EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. DNA was extracted from the supernatant using DNAzol (Molecular Research Center, Cincinnati, Ohio). Two regions of nuclear ribosomal DNA (rDNA), and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) were

amplified via polymerase chain reaction (PCR). The ITS1, 5.8S and ITS2 regions were amplified using the forward primer BD1, 5'-GTCGTAACAAGGTTTCCGTA-3' and the reverse primer BD2, 5'-ATCTAGACCGGACTAGGCTGTG-3' (Luton *et al.*, 1992). The D1-D3 domains of the 28S rRNA gene were amplified using the primers 502 5'-CAAGTACCGTGAGGGAAAGTTGC-3' (forward) and 536 5'-CAGCTATCCTGAGGGAAA-3' (reverse) (García-Varela & Nadler, 2005). The mitochondrial *cox1* was amplified using the forward primer MplatCOX1dF 5'-TGT AAAACGACGGCCAGTTTWCITTRGATCATAAG-3' and the reverse primer MplatCOX1dR 5'-CAGGAAACAGCTATGACTGAAAYAYAIIGGATCICCACC-3' (Moszczyńska *et al.*, 2009). PCR reactions (25 µl) consisted of 10 µM of each primer, 2.5 µl of 10 X buffer, 1.5 µl of 2 mM MgCl₂, 0.5 µl of dNTPs (10 mM), 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil), 2 µl of the genomic DNA plus 16.7 µl of distilled water. PCR cycling parameters for rDNA amplifications included denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, annealing at 50°C for 1 minute for the three molecular markers, and extension at 72°C for 1 minute, followed by a post-amplification incubation at 72°C for 10 minutes. PCR products for ITS1, 5.8S, ITS2 and 28S rDNA were sequenced with the PCR primers plus the internal primers BD3 5'-GAACATCGACATCTTGAACG-3' and BD4 5'-ATAAGCCGACCCCTCGGC-3' (Hernández-Mena *et al.*, 2013) and 503 5'-CCTTGGTCCGTGTTCAAGACG-3' (forward) (Stock *et al.*, 2001) and 504 5'-CGTCTTGAAACACGGACTAAGG-3' (reverse) (García-Varela & Nadler, 2005), respectively. Sequencing reactions were performed using ABI Big Dye (Applied Biosystems, Boston, Massachusetts, USA) terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA automated sequencer. Contigs were assembled and base-calling differences resolved using Codoncode Aligner version 6.0.2 (Codoncode Corporation, Dedham, Massachusetts, USA). Sequences obtained in the current research for ITS, 28S and *cox1* were aligned with sequences of other genera of clinostomids downloaded from GenBank. Three alignments were built. For ITS and *cox1* alignments, newly generated sequences were aligned along with one to three representative sequences of 23 species/lineages of *Clinostomum*, plus *Odhneriotrema incommodum* (Leidy, 1856) and *Euclinostomum heterostomum* (Rudolphi, 1908). For 28S, an alignment was built considering two newly generated sequences in addition to six validated species of *Clinostomum*, plus newly generated sequences of five genetic lineages of the genus, and one sequence of *Clinostomoides*, two of *Euclinostomum* and one of *Odhneriotrema*. In addition, sequences of the diplostomids *Diplostomum baeri* Dubois, 1937 and *Alaria marciana* (La Rue, 1917) Walton, 1949 were used as outgroups for rooting the trees. A fourth alignment was built to perform a concatenated analysis of 28S-ITS-*cox1*. Due to the number of sequences available for the 28S rRNA gene, the concatenated analysis was performed considering sequences of six validated species of *Clinostomum*, plus those of five genetic lineages, two of *Euclinostomum*, one of *Odhneriotrema*, with *Alaraia marciana* as outgroup.

Phylogenetic analyses

Sequences were aligned with the software Clustal Omega (Sievers *et al.*, 2011), implemented at <https://www.ebi.ac.uk/Tools/msa/clustalo/>. Phylogenetic analysis for each dataset was run under Bayesian inference (BI) and maximum likelihood (ML), employing the nucleotide substitution model GTR + GAMMA, which

Table 1. Localities sampled across Middle America for *Ithyoclinostomum* sp., with the host species and the geographical coordinates of each locality, and the data source.

Locality	Host	Geographical coordinates	Source
<i>Ithyoclinostomum</i> sp. (Metacercaria)			
Mexico			
Tabasco State			
Gregorio Méndez	<i>Mayaheros urophthalmus</i>	17°27'00"N 91°34'10"W	This study
Chiapas State			
El Ocotitalito, Naha	<i>Vieja melanura</i>	17°03'00"N 91°35'49"W	This study
Veracruz State			
Nautla River, Nautla	<i>Herichthys deppi</i>	20°11'18"N 96°44'43"W	This study
Nautla River, Filipinas	<i>Herichthys deppi</i>	20°00'47"N 97°09'50"W	This study
Costa Rica			
Guanacaste Province			
Irigaray River, Liberia	<i>Cribroheros longimanus</i>	10°43'21"N 85°30'38"W	This study
Irigaray River, Liberia	<i>Parachromis managuensis</i>	10°43'21"N 85°30'38"W	This study
Orosí River, Pitahaya	<i>Cribroheros alfari</i>	11°03'05"N 85°24'30"W	This study
Orosí River	<i>Cribroheros alfari</i>	11°02'50"N 85°22'48"W	This study
Nicaragua			
South Caribbean Coast Autonomous Region			
Mahogany River	<i>Cichlasoma managuense</i>	12°03'22"N 83°59'07"W	Aguirre-Macedo <i>et al.</i> , 2001
<i>Ithyoclinostomum dimorphum</i> (Adult)			
Brazil			
Not specified	<i>Ardea cocoi</i>		Braun, 1899
Pará State			
Ilha de Marajó	<i>Ardea cocoi</i>		Lent & Freitas, 1937
Mato Grosso State			
Not specified	<i>Ardea cocoi</i>		Travassos, 1928
Barão de Melgaço	<i>Ardea cocoi</i>	23°30'S 47°30'W	Pinto <i>et al.</i> , 2004
	<i>Ardea alba</i>	23°30'S 47°30'W	Pinto <i>et al.</i> , 2004
	<i>Nycticorax nycticorax</i>	23°30'S 47°30'W	Pinto <i>et al.</i> , 2004
Mato Grosso do Sul State			
Not specified	<i>Ardea cocoi</i>		Travassos, 1941; Travassos & Freitas, 1942, 1943
Paraná River Basin	<i>Ardea cocoi</i>	22°50'–22°70'S 53°15'–53°40'W	Dias <i>et al.</i> , 2003
Barão de Melgaço	<i>Tigrisoma lineatum</i>		Arruda <i>et al.</i> , 2001
São Paulo State			
Pirassununga	<i>Nycticorax nycticorax</i>		Arruda <i>et al.</i> , 2001
Rio Grande do Sul State			
Pelotas	<i>Ardea cocoi</i>	31°46'19'S 52°20'34'W	Fedatto <i>et al.</i> , 2017
<i>Ithyoclinostomum dimorphum</i> (Metacercaria)			
Brazil			
Tocantins State			
Araguaia River, Araguañã	<i>Hoplias malabaricus</i>	06°34'S 48°38'W	Reis, 2014
Rio Grande do Sul State			
Santa Maria	<i>Hoplias malabaricus</i>		Weiblen & Brandão, 1992

(Continued)

Table 1. (Continued.)

Locality	Host	Geographical coordinates	Source
Cachoeira do Sul	<i>Hoplias malabaricus</i>		Gallio et al., 2007
Pirantini River	<i>Hoplias malabaricus</i>	31°30'–34°35' S 53°31'–55°15' W	Rodrigues, 2010
Minas Gerais State			
Rio Doce	<i>Hoplias malabaricus</i>	19°46'53'' S 42°35'57'' W	Belei et al., 2013
Not specified	<i>Hoplias malabaricus</i>		Moreira, 2000
São Francisco Basin	<i>Hoplias malabaricus</i>	18°12'32'' S 45°15'41'' W	Costa et al., 2015
São Francisco Basin	<i>Hoplias intermedius</i>	18°12'32'' S 45°15'41'' W	Costa et al., 2015
Not specified	<i>Hoplerythrinus unitaeniatus</i>		Moreira, 2000
Rio de Janeiro State			
Lajes Reservoir	<i>Hoplias malabaricus</i>	22°42'–22°50' S 44°05'–44°05' W	Paraguassú & Luque, 2007
Espírito Santo State			
Pará State			
Arari Lake, Marajó Island	<i>Hoplias malabaricus</i>	00°39'48'' S 49°10'30'' W	Benigno et al., 2014
Arari Lake, Marajó Island	<i>Hoplerythrinus unitaeniatus</i>	00°39'48'' S 49°10'30'' W	Benigno et al., 2014
Paraná State			
Porto Rico	<i>Hoplias malabaricus</i>		Pavanelli et al., 1990
Porto Rico	<i>Schizodon borelli</i>	22°40'–22°50' S 53°15'–53°40' W	Machado et al., 1996
Argentina			
Tucumán Province	<i>Hoplias malabaricus</i>		Szidat, 1969
Peru			
Alto Amazonas province			
Yurimaguas	<i>Hoplerythrinus unitaeniatus</i>	05°42'03'' S 76°52'03'' W	Delgado et al., 2017

was calculated in the program jModelTest v2.1.10 (Darriba et al., 2012). The concatenated analysis was also run under ML and BI. Bayesian inference was performed in MrBayes v. 3.2.6 (Ronquist et al., 2012), running two independent MCMC of four chains each run (heating parameter = 0.5) for 10 million generations and sampling trees every 1000 generations (printfreq = 1000, samplefreq = 1000, diagnfreq = 10,000), and burn-in periods were set to the first 2500 generations. A 50% majority-rule consensus tree and nodal support (posterior probability values) were calculated from the remaining trees. ML inference (100 replicates), model parameters and bootstrap support (1000 replicates) were estimated with RAxML v. 8.2.X (Stamatakis, 2006). Phylogenetic trees obtained from the analysis were visualized in FigTree v.1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>). Molecular divergence for all markers was estimated using uncorrected *p* distances (p-distances) with the software PAUP* 4.0a (Swofford, 2002).

Results

Morphology

The clinostomid metacercariae were identified as members of the monotypic genus *Ithyoclinostomum* Witenberg, 1925, based on body size, the presence of an oral collar, simple caeca lacking diverticula, gonads being located in the posterior fourth of the body, and having a pre-testicular genital pore. Even though

body size was variable among collected individuals (11.6–29.5 mm), the position, size and shape of internal organs in proportion to the body length were very similar, particularly for characters related to the reproductive organs (fig. 1).

Ithyoclinostomum sp. (*Metacercariae*)

Taxonomic summary

Hosts. *Mayaheros urophthalmus* (Günther, 1862), *Vieja melanura* (Hubbs, 1935), *Herichthys deppii* (Heckel, 1840), *Cribroheros longimanus* (Günther, 1867), *Cribroheros alfari* (Meek, 1907), *Parachromis managuensis* (Günther, 1867).

Localities. Mexico – Gregorio Méndez, Tabasco (Grijalva river basin); Naha, Chiapas (Usumacinta river basin); Nautla (Nautla river basin); Filipinas Creek (Nautla river basin). Costa Rica – Irigaray River at Liberia, Guanacaste; Orosi River, Guanacaste; Pithaya Creek (Orosi river basin), Guanacaste.

Specimens deposited. Colección Nacional de Helminthos (CNHE), vouchers 10716–10721.

Representative DNA sequences. MH159738–MH159752 (*cox1*), MH159753–MH159770 (ITS), MH159736–MH159737 (28S).

Description. (Based on measurements of 14 specimens; measurements in table 2): Body large, elongate and widest in equatorial region of body. Tegument surface lacking spines. Anterior end forming an oral collar; oral collar with a ventral constriction. Oral

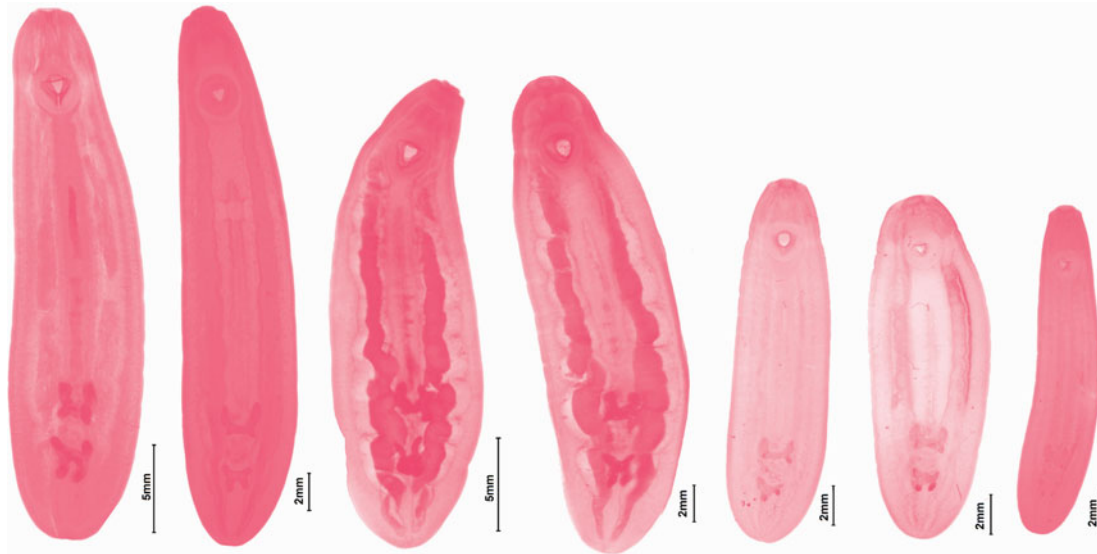


Fig. 1. Metacercariae of *Ithyoclinostomum* sp. obtained from different host species. Processed specimens show the same shape and position of gonads, irrespective of body size.

sucker rounded, terminal, smaller than ventral sucker, embedded in oral collar. Prepharynx absent. Pharynx well developed, leading immediately to intestinal bifurcation. Intestinal caeca run laterally to posterior end of body. Ventral sucker rounded, in anterior fourth of body; triangular aperture. Testes in tandem, in posterior fourth of body, irregularly shaped, with smooth margins, entirely intracaecal. Anterior testis H-shaped, with anterior lobes longer than posterior ones. Posterior testis X-shaped, forming four lobes of same size; inter-testicular space wide. Cirrus sac ovoid, overlapping anterior testis in dextral position. Genital pore pre-testicular, in mid-level of body. Ovary small, rounded, smooth, located in inter-testicular space on right side of body. Uteroduct emerging from ootype, running around left margin of anterior testis to form tubular uterine sac, well developed, extending anteriorly to short distance from posterior border of ventral sucker, descending straight into genital pore; metraterm not observed. Vitellaria undeveloped.

Taxonomic remarks. The metacercariae sampled from Middle American cichlids correspond in general with the diagnosis of the genus *Ithyoclinostomum* following Kanev *et al.* (2002). Our specimens possess a large and elongate body with a small oral sucker surrounded by an oral collar; a considerable space free of internal organs between ventral sucker and anterior testis; simple, long caeca without lateral diverticula; gonads in the posterior fourth of body; and inter-testicular ovary. Overall comparison of morphometric characters showed that our specimens are very similar to those described for *I. dimorphum* (table 2); this species was found in some locations across South America (see Benigno *et al.*, 2014; Costa *et al.*, 2015). In addition, the ultrastructure of the body surface of *I. dimorphum* was previously studied by Dias *et al.* (2003) in adults obtained from *Ardea cocoi* (L.), and by Benigno *et al.* (2014) in metacercariae from *Hoplerlytrinus unitaeniatus*, both in Brazil. Our SEM specimens (fig. 3) are similar, except that that they possess a triangular shaped ventral sucker aperture and a well-defined constriction of the oral collar (see figs 3a, c). Moreover, two main facts prevented us from identifying our specimens as the monotypic *I. dimorphum*. First, we relied solely on the morphology of the metacercarial stage, as we did not

collect the adults from their definitive hosts. Second, our specimens were morphologically different from *I. dimorphum* regarding some traits. For instance, some differences were found in the overall body shape, size and shape of testes, and the position of the genital pore between *I. dimorphum* and our specimens (table 2). In *I. dimorphum*, the body is long and slender, and testes are small and irregularly shaped, whereas in our specimens, the body is elongate and robust, testes are well developed and their form is well defined, the anterior testis is H-shaped and the posterior testis is X-shaped. The cirrus sac in our specimens is located in the mid-level of the anterior testis, and the genital pore is medial; in *I. dimorphum* the cirrus sac is dextral to the right margin of the anterior testis, and the genital pore is dextrally located. A striking difference was found in the body length/body width ratio. On average, in our specimens the ratio is 3.8, whereas in *I. dimorphum* the ratio is around 18 (table 2).

Molecular data. Phylogenetic analyses inferred with three independent datasets (*cox1*, ITS, 28S) through BI and ML unequivocally recovered all the sequenced metacercariae as a monophyletic assemblage, with high bootstrap and posterior probability values (figs 4–6). However, the three datasets yielded different topologies regarding the position of *Ithyoclinostomum* sp. with respect to other clinostomids for which DNA sequences are available.

Cox1. The alignment was 474 bp long and consisted of 15 newly sequenced metacercariae, two sequences of *Euclinostomum heterostomum*, three of *Odhneriotrema incommodum*, and one or two replicates of each of the 11 valid species of *Clinostomum*, plus one or two replicates of each of the 12 genetic lineages of *Clinostomum* not yet described. The phylogenetic analysis suggested that the metacercariae of *Ithyoclinostomum* sp. occupy a basal position with respect to the other genera of clinostomids, as follows: [*Ithyoclinostomum* sp. (*Euclinostomum* (*Odhneriotrema* (*Clinostomum*)))] (fig. 4).

ITS. The alignment was 1142 bp long and consisted of 17 newly sequenced metacercariae, two sequences of *Euclinostomum heterostomum*, two of *Odhneriotrema incommodum*, and one or two replicates of each of the 11 valid species of *Clinostomum*, plus two

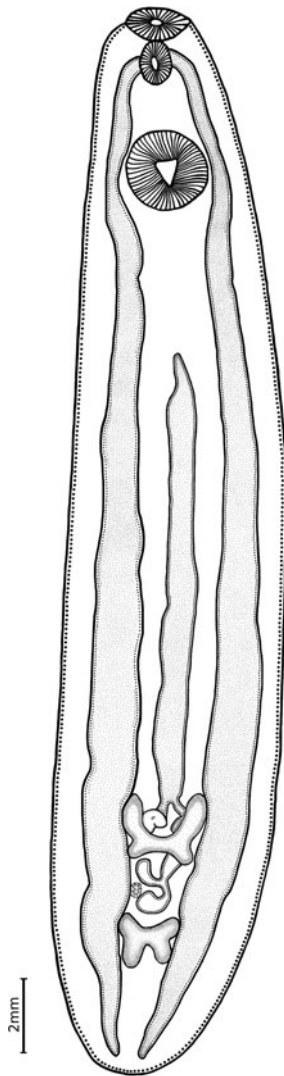


Fig. 2. Line drawing of a metacercaria of *Ithyoclinostomum* sp. from *Herichthys deppi*, Nautla River, Veracruz State, Mexico. Ventral view.

replicates of each of the 12 genetic lineages of *Clinostomum* not yet described. The phylogenetic analysis showed that the metacercariae of *Ithyoclinostomum* sp. are recovered as the sister group of the genus *Odhneriotrema*, as follows: [*Euclinostomum* (*Odhneriotrema* + *Ithyoclinostomum* sp. (*Clinostomum*))] (fig. 5).

28S. The alignment was 1414 bp long, and included only two newly generated sequences of *Ithyoclinostomum* sp., two sequences of *Euclinostomum heterostomum*, one of *Odhneriotrema incommodum*, one or two replicates of six valid species of *Clinostomum* for which this molecular marker has been sequenced and an unidentified species from Australia, two replicates of five newly sequenced genetic lineages of *Clinostomum*, and one sequence of *Clinostomoides brieni* Dollfus, 1950. The phylogenetic analysis revealed that the metacercariae of *Ithyoclinostomum* sp. were recovered as the basal group of the Clinostomidae as follows: [*Ithyoclinostomum* (*Odhneriotrema* (*Euclinostomum* (*Clinostomum*)))] (fig. 6). In this analysis, *C. brieni* nests within the group of *Clinostomum* species that occur in the old world.

Concatenated analysis (cox1 + ITS + 28S). As the mitochondrial gene and the two ribosomal genes yielded different

topologies regarding the position of the newly generated sequences in the phylogeny of Clinostomidae, a fourth alignment was built for a concatenated analysis of the three molecular markers through BI and ML. The final alignment was 3094 bp long and included fewer representative sequences of clinostomids; only species or lineages with sequences of the three markers were included in the analysis. Alignment consisted of two sequences of *Ithyoclinostomum* sp., two of *Euclinostomum*, one of *Odhneriotrema*, one or two replicates of six valid species of *Clinostomum*, and two replicates of five genetic lineages of *Clinostomum*. The concatenated tree recovered *Odhneriotrema incommodum* as the basal member of the group, as the sister taxon of the metacercariae of *Ithyoclinostomum* sp. plus *Euclinostomum* and *Clinostomum*, as follows: [*Odhneriotrema* (*Ithyoclinostomum* sp. (*Euclinostomum* (*Clinostomum*)))] (fig. 7).

Genetic divergence. The genetic distance estimated through uncorrected *p* distances showed a high level of divergence between the sequences of *Ithyoclinostomum* sp. and the other clinostomids for the three molecular markers. On average, for the 28S rRNA gene, our metacercariae from *Euclinostomum*, *Odhneriotrema* and *Clinostomum* varied 3.69–9.97%, 7.78% and 6.02–7.92%, respectively; for ITS they varied 19.72%, 15.82% and 17.15–20.14%, and for *cox1* divergence values varied 18.14%, 19.62% and 16.66–23.62% with respect to the species included in the aforementioned genera. Intraspecific divergence among isolates of our metacercariae was very low or null (0.00–0.85% for *cox1*, 0.00–0.09% for ITS, and 0.0% for 28S), indicating that irrespective of body size (see fig. 1) and geographical location (Mexico or Costa Rica), all metacercariae are conspecific.

Discussion

The morphology of the specimens reported in our study resembled that of members of the genus *Ithyoclinostomum*. Molecular results confirm that our specimens form a monophyletic assemblage and that they represent an independent genetic lineage, not closely related with species of *Clinostomum*, the most species-rich genus within the family Clinostomidae; moreover, the phylogenetic relationships of the metacercariae with other clinostomids remain uncertain, as the three molecular markers used in this study resolved contradictory sister-group relationships (figs 4–6). Even though we hypothesized that the metacercariae recovered from Middle American cichlids belong in the genus *Ithyoclinostomum*, morphologically, they cannot be considered conspecific with *I. dimorphum*. Several differences show that our specimens may actually represent an undescribed species. In addition to morphology, three pieces of information were useful to complement the species differentiation: habitat, host specificity, and geographical distribution. In terms of habitat, records of *I. dimorphum* as a metacercaria in most locations of South America indicate the body cavity (mesentery) of their hosts is the preferential habitat; more rarely, metacercariae were found in the musculature, opercula, stomach and gills. It is unclear, however, if the metacercariae were always encysted in the different habitats where they occurred. For instance, Szidat (1969) found metacercariae encysted in the gill arches of their hosts in Tucumán, Argentina; Gallio *et al.* (2007) recovered them encysted in the musculature of their hosts in Rio Grande do Sul, Brazil; and Belei *et al.* (2013) found them encysted in the visceral cavity of their hosts in Minas Gerais, Brazil. In

Table 2. Morphometric data for the metacercariae of *Ithyoclinostomum* sp. from Middle American cichlids sampled in this study, and comparison with published descriptions of metacercariae and adults of *I. dimorphum*. Measurements are expressed as a range, in mm; mean values are in parentheses. * represents ratios not provided in the original description/redescription; these were calculated as reference values for comparative purpose only.

	<i>Ithyoclinostomum dimorphum</i>		<i>Ithyoclinostomum dimorphum</i>					<i>Ithyoclinostomum</i> sp.	
	(Adult)		(Metacercaria)					(Metacercaria)	
	<i>Ardea cocoi</i>	<i>Ardea cocoi</i>	<i>Hoplias malabaricus</i>	<i>Hoplerythrinus unitaeniatus</i>	<i>Hoplias malabaricus</i>	<i>Hoplias intermedius</i>	<i>Hoplerythrinus unitaeniatus</i>	<i>Cichlasoma managuense</i>	Cichlidae
	Braun, 1901	Lent & Freitas, 1937	Szidat, 1969	Benigno <i>et al.</i> , 2014	Reis, 2014	Costa <i>et al.</i> , 2015	Delgado <i>et al.</i> , 2017	Aguirre-Macedo <i>et al.</i> , 2001	(This paper)
BL	60–100	90	13	23.55	47.20	15.0–33.0 (22)	50	39	11.60–29.55 (18.71)
BW	2–6	5	2.3	1.9	3.75	1.50–4.00 (2.39)	4	11	2.34–8.21 (5.25)
BL/BW (ratio)	10–16.6*	18*	5.65*	12.39*	12.59*	8.25–10 (9.20)*	12.5*	3.54*	2.12–4.95 (3.85)
OCL									0.32–1.54 (0.74)
OCW									0.97–2.85 (2.05)
OSL	0.5 (diameter)	0.763	0.2 (diameter)	0.36	0.24	0.30–0.32 (0.31)		0.65	0.32–0.99 (0.53)
OSW		0.579		0.40	0.31	0.52–0.57 (0.53)		0.90	0.23–2.35 (1.25)
OSW/BW (ratio)	0.08*	0.11*	0.08*	0.21*	0.08*	0.14–0.34 (0.22)*		0.08*	0.08–0.43 (0.26)
PhL				0.32	0.61				0.49–0.91 (0.70)
PhW				0.23	0.37				0.45–0.83 (0.63)
VSL	1.6 (diameter)	1.8 (diameter)	0.75 (diameter)	1.15	0.69	0.90–1.20 (0.98)		3.5 (diameter)	1.20–2.85 (1.97)
VSW				1.25	1.21	0.92–1.42 (1.05)			1.215–2.82 (2.02)
VSW/OSW (ratio)	3.2*	3.10*	3.75*	3.12*	3.90*	1.76–2.49 (1.98)*		1: 3.88	1: 1.05–2.36 (1.56)
VSL/OSL (ratio)	3.2*	2.35*	3.75*	3.19*	2.875*	3–3.75 (3.16)*		5.38*	2.14–5.42 (3.91)
VSW/BW (ratio)	0.26*	0.36*	1.63*	0.65*	0.32*	0.35–0.61 (0.43)*		0.32*	0.31–0.52 (0.41)
OSL/VSL (ratio)	0.31*	0.42*	0.26*	0.31*	0.34*	0.26–0.9 (0.31)*		0.18*	0.18–0.47 (0.27)
OSW/VSW (ratio)	0.31*	0.32*	0.26*	0.32*	0.25*	0.40–0.56 (0.50)*		0.26*	0.16–0.95 (0.62)
DBS									1.32–3.39 (2.16)
DOC&VS									1.14–3.03 (1.71)
ATL		3.021		0.47	1.35		1.625		0.68–2.52 (1.38)
ATW		0.815		0.34	1.12		0.775		0.59–2.22 (1.41)
ATW/ATL (ratio)		0.27*		0.47*	0.82*		0.48*		0.75–1.47 (1.03)
PTL		2.893		0.45	1.65		0.775		0.62–2.50 (1.23)
PTW		1.709		0.23	1.35		0.650		0.65–2.31 (1.24)
PTW/PTL (ratio)		0.59*		0.51*	0.81*		0.84*		0.73–1.76 (1.06)
DBVS&AT									5.73–15.30 (8.88)

(Continued)

Table 2. (Continued.)

	<i>Ithyoclinostomum dimorphum</i> (Adult)	<i>Ithyoclinostomum dimorphum</i> (Metacercaria)	<i>Ithyoclinostomum</i> sp. (Metacercaria)
DBT			0.54–2.03 (1.24)
OL	0.842	0.18	0.19–0.50 (0.31)
OW	1.183	0.12	0.19–0.50 (0.27)
OW/OL (ratio)	1.40*	0.67*	0.55–1.28 (0.84)
CSL	0.579	0.45	
CSW	0.920	0.27	

Abbreviations: BL, body length; BW, body width; OCL, oral collar length; OCW, oral collar width; OSW, oral sucker length; OSV, oral sucker width; PhL, pharynx length; PhW, pharynx width; VSL, ventral sucker length; VSW, ventral sucker width; DBS, distance between suckers; DOC&VS, distance between oral collar and ventral sucker; ATL, anterior testis length; ATW, anterior testis width; PTL, posterior testis length; PTW, posterior testis width; DBVS&AT, distance between ventral sucker and anterior testis; DBT, distance between testis; OL, ovary length; OW, ovary width; CSL, cirrus sac length; CSW, cirrus sac width.

contrast, some authors have reported metacercariae of *I. dimorphum* from the mesentery/musculature, coelomic cavity/stomach, and body cavity of their fish hosts, although none of them mentioned specifically whether or not the metacercariae were encysted (Benigno *et al.*, 2014; Costa *et al.*, 2015; Delgado *et al.*, 2017). In our study, the metacercariae of *Ithyoclinostomum* sp. were found exclusively in the body cavity of their cichlid hosts, and in all cases they were unencysted, and actively moving when collected.

Furthermore, geographical distribution and host specificity patterns were also used to differentiate *I. dimorphum* from the specimens sampled in this study. The metacercariae of *I. dimorphum* show a strong host specificity, having been reported only from three species of Characiform fishes (Family Erythrinidae Valenciennes, 1847): *Hoplias malabaricus* Bloch, 1794, *Hoplias intermedius* (Günther, 1864) and *Hopleryttrinus unitaeniatus* (Spix & Agassiz, 1829) in Brazil, Peru and Argentina (Szidat, 1969; Travassos *et al.*, 1969; Pavanelli *et al.*, 1990; Weiblen & Brandão, 1992; Paraguassú & Luque, 2007; Gallio *et al.*, 2007; Belei *et al.*, 2013; Benigno *et al.*, 2014; Costa *et al.*, 2015; Delgado *et al.*, 2017). The species has also been found as a parasite of *Schizodon borrelli* (Boulenger, 1900) in the Paraná River, Brazil (Machado *et al.*, 1996), a member of the Anostomidae Günther, 1864. Erythrinids and anostomids are both Characiforms, although they are not closely related (see Oliveira *et al.*, 2011). In this sense, *I. dimorphum* seems to be restricted to these particular groups of characiforms in South America. In contrast, our specimens are host-specific to cichlids and are apparently restricted to Middle America; they have only been found unencysted in the body cavity of their hosts.

Taking together all the pieces of information discussed above, it is possible that the specimens from cichlids represent an undescribed species; however, sampling adults from their definitive hosts is a requirement to present a complete species description for this potentially new species. The single specimen reported in Aguirre-Macedo *et al.* (2001) as *Clinostomum* sp. 2, from the body cavity of *Parachromis managuensis* (Günther, 1867), in the South Atlantic area of Nicaragua, in Central America, corresponds fully with the morphology of the metacercariae we describe in this study (see table 2), even though the single specimen collected by those authors from the cichlid is larger (39 mm long); therefore, these specimens have to be further considered as *Ithyoclinostomum* sp. Interestingly, Belei *et al.* (2013) reported the presence of the metacercariae of *I. dimorphum* in *H. malabaricus* from the Parque Estadual do Rio Doce, Brazil and, as their specimens were hardened due to fixation in formalin, the body was macerated and those authors reported the presence of eggs in the metacercariae, arguing the possibility that the digenean larval stage acquired sexual maturity due to permanent absence of the definitive hosts. In our specimens, even though gonads were well developed, there was no evidence of the development of the uterine sac.

Adults of *Ithyoclinostomum* have been found in the buccal cavity of fish-eating birds in some locations of Brazil. The original record (as *Clinostomum dimorphum* Diesing, 1850) was made as a parasite of *Ardea cocoi*. According to Lent & Freitas (1937) the species was transferred to *Ithyoclinostomum* by Witenberg (1925), but as a generic diagnosis was not provided, Lent & Freitas formally described the genus for the first time. Since it was first described, *I. dimorphum* has also been reported as a parasite of *Ardea alba* (Linnaeus, 1758), *Nycticorax nycticorax* (Linnaeus, 1758) and *Tigrisoma lineatum* (Boddaert, 1783) (see Benigno *et al.*, 2014; Fernandes *et al.*, 2015). The genus *Ithyoclinostomum* is unique

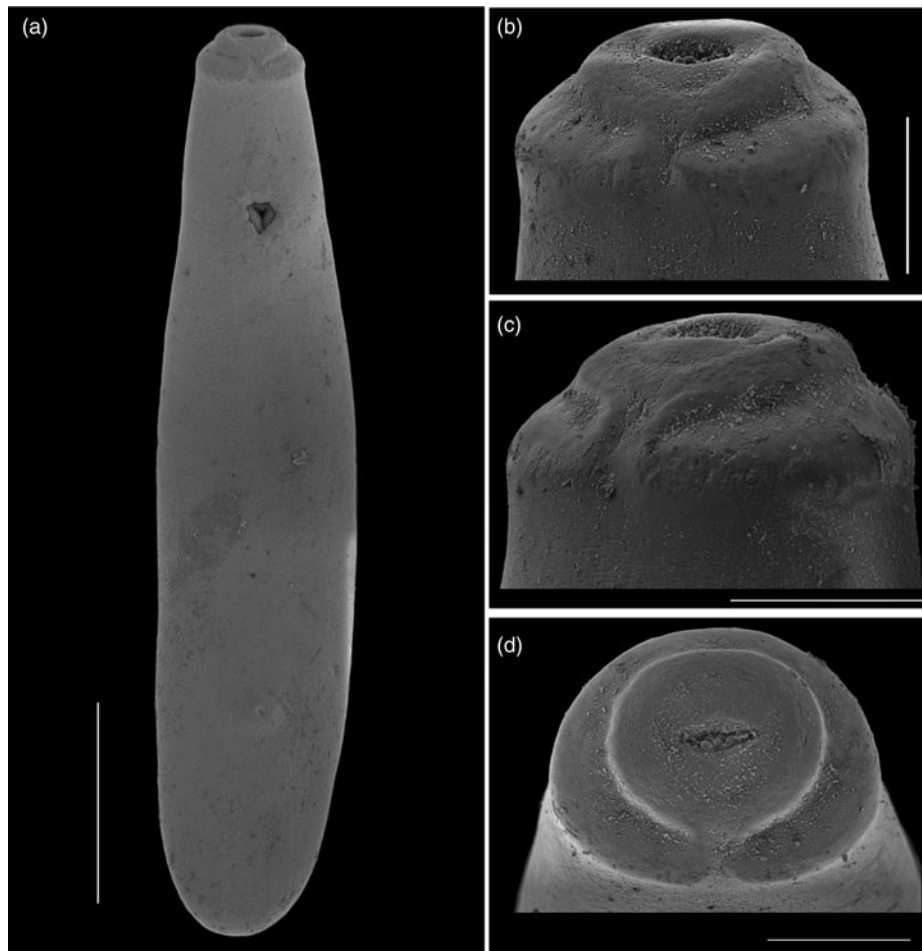


Fig. 3. Scanning electron microscopy images of *Ithyoclinostomum* sp. from *Criboheros alfari*, Oroqui River, Costa Rica. (a) Entire worm; scale bar = 2 mm. (b) Oral collar, showing ventral constriction; ventral view; scale bar = 0.5 mm. (c) Oral collar, showing ventral constriction; ventro-lateral view; scale bar = 0.5 mm. (d) Oral collar, showing ventral constriction and oral aperture; en face view; scale bar = 0.5 mm.

among clinostomids because of the species' body size: they are 'the largest species among the so far known Clinostomidae, reaching sizes which are rarely encountered among the trematodes: 60–100 mm³' (Braun, 1901). *Clinostomum heluans* Braun, 1899, a species widely distributed in the Americas as a parasite of fish-eating birds (Briosio-Aguilar *et al.*, 2018), is also characterized by its larger size, although it is not as large as *I. dimorphum*; adults of *C. heluans* are usually 15–20 mm long (see Werneck *et al.*, 2017 and references therein); likewise, the specimens of *C. heluans* reported by Bravo-Hollis (1947) from the buccal cavity of *Ardea herodias* (Linnaeus, 1758) in northern Mexico are probably the largest reported, reaching 20.7–26.1 mm. The report by Bravo-Hollis (1947) led us to consider, initially, that our specimens corresponded with *C. heluans*, as they were 11.6–29.5 mm long; also, our samples coincided with the geographical range of the species (Briosio-Aguilar *et al.*, 2018). However, morphologically, our specimens resembled more closely *Ithyoclinostomum* than *C. heluans*; the molecular evidence gathered in this study confirmed unequivocally that our specimens did not nest within the clade formed by *Clinostomum* species. Metacercariae of *I. dimorphum* are variable in length, reaching 15–50 mm (table 2). On average, our specimens lie within the length range of that species, although in the lower limit (18.7 mm), and as mentioned above, the body length/width ratio is much greater in *I. dimorphum*, which means that specimens of that species are more elongated.

The definitive hosts of the metacercariae characterized herein still remain unknown. Considering the most parsimonious

explanation of sister-group relationships among clinostomids shown by the concatenated phylogenetic analysis (fig. 7), the possibility that crocodylians are the definitive hosts of this trematode cannot be ruled out, especially as we have analysed for helminths *c.* 377 fish-eating bird individuals in the last few years, including species of ardeids, pelicans, cormorants, anhingids and threskiornithids, and we have been unable to recover adult parasites corresponding with the species we characterize in this study. We have to keep looking at a wider diversity of birds and whenever possible at crocodylians to try to obtain adult forms of this trematode species. Previous classification schemes of the family Clinostomidae considered *Ithyoclinostomum* to be part of the subfamily Clinostominae (see Skrjabin, 1947; Feizullaev & Mirzoeva, 1983) or the monotypic subfamily Ithyoclinostominae (see Yamaguti, 1971); these classifications included only species whose adults were found in birds. The current classification scheme (Kanev *et al.*, 2002) also includes the subfamily Nephrocephalinae in the family Clinostomidae; their members are parasites of crocodylians. Our molecular phylogenetic analysis provides additional support to the classification scheme proposed by Kanev *et al.* (2002), which is based solely on morphology.

As our specimens did not conform entirely to the diagnosis of *I. dimorphum* and *C. heluans*, we compared our material with the other genera included in separate subfamilies of the family Clinostomidae (see Kanev *et al.*, 2002). Molecularly, our specimens were closely related to the genera *Euclinostomum* and *Odhneriotrema*, the only members other than *Clinostomum* for which sequences are

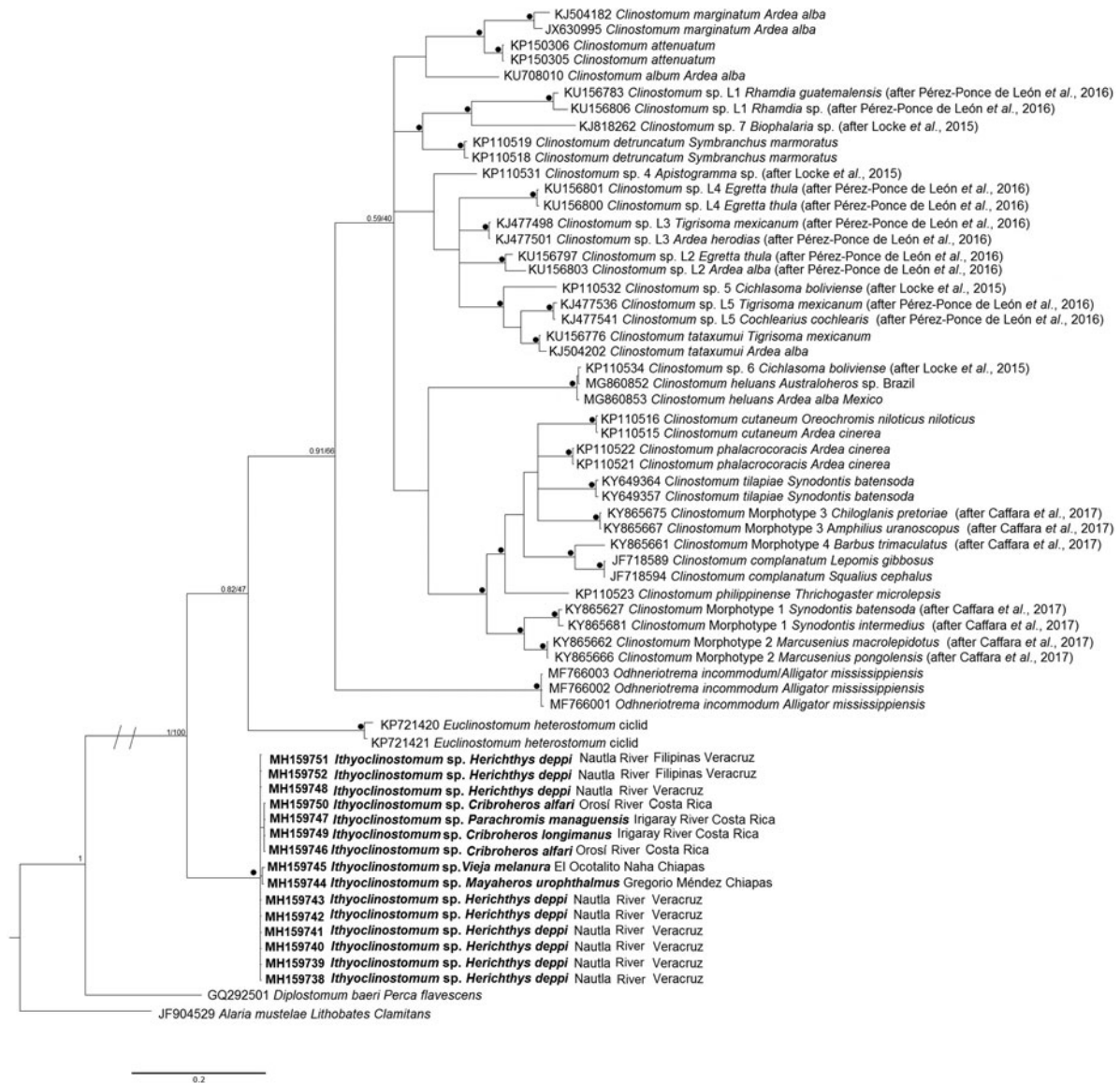


Fig. 4. Bayesian inference tree for *Ithyoclinostomum* sp. based on the cytochrome c oxidase subunit 1 gene (*cox1*) dataset. Dots above branches represent posterior probability values and bootstrap values higher than 0.8/80%. Newly generated sequences of *Ithyoclinostomum* sp. in this study are in bold.

available in GenBank; genetic divergence values and reciprocal monophyly indicated that our specimens were independent evolutionarily significant units. Morphologically, species included in these genera are different. *Euclinostomum* is the only genus in the subfamily Euclinostominae (Kanev *et al.*, 2002); adults are parasites of ardeids and metacercariae are commonly found in cichlids in the old world (see Caffara *et al.*, 2016); adults and metacercariae of species of *Euclinostomum* are characterized by having 6–15 blind diverticula extending latero-posteriorly to the main caeca (Kanev *et al.*, 2002; Caffara *et al.*, 2016); this character, and the body size of the organisms are the main traits that distinguish our specimens of *Ithyoclinostomum* sp. from the species in that genus. Species of the genus *Odhneriotrema*, a member of the Nephrocephalinae along with *Nephrocephalus*, are parasites of the buccal cavity and oesophagus of crocodilians in the Americas. They differ from *Ithyoclinostomum* sp. in the small size of the oral sucker, the position of the ovary and cirrus sac with respect to testes, the size of the cirrus sac, and the large inter-testicular space.

The genus *Clinostomatopsis* Dollfus, 1932 belongs to the Clinostomatinae (Kanev *et al.*, 2002), and shows some resemblance to the metacercariae from the present study because of the lobated nature of the testes. Unfortunately, no sequence data were available for this species, and the comparison relied solely on morphology. Currently, the genus contains two species known to parasitize the oesophagus of Neotropical birds (Lunaschi & Drago, 2009): *Clinostomatopsis intermedialis* Lamont, 1920, recorded as a parasite of the cormorants *Phalacrocorax brasilianus* (Gmelin, 1789) from Venezuela (Lamont, 1920), and *P. penicillatus* (Brandt, 1837) from north-eastern Mexico (Bravo-Hollis, 1947). The second species, *Clinostomatopsis sorbens* (Braun, 1899) Dollfus, 1932, is a parasite of *Mycteria americana* (Linnaeus, 1758), *Ardea cocoi* and *Jabiru mycteria* (Lichtenstein, 1819) in Brazil (see Benigno *et al.*, 2014; Fernandes *et al.*, 2015, and references therein) and has been found in *Tigrisoma lineatum* in Argentina (Lunaschi & Drago, 2009). Metacercariae were recorded from the mesentery of the

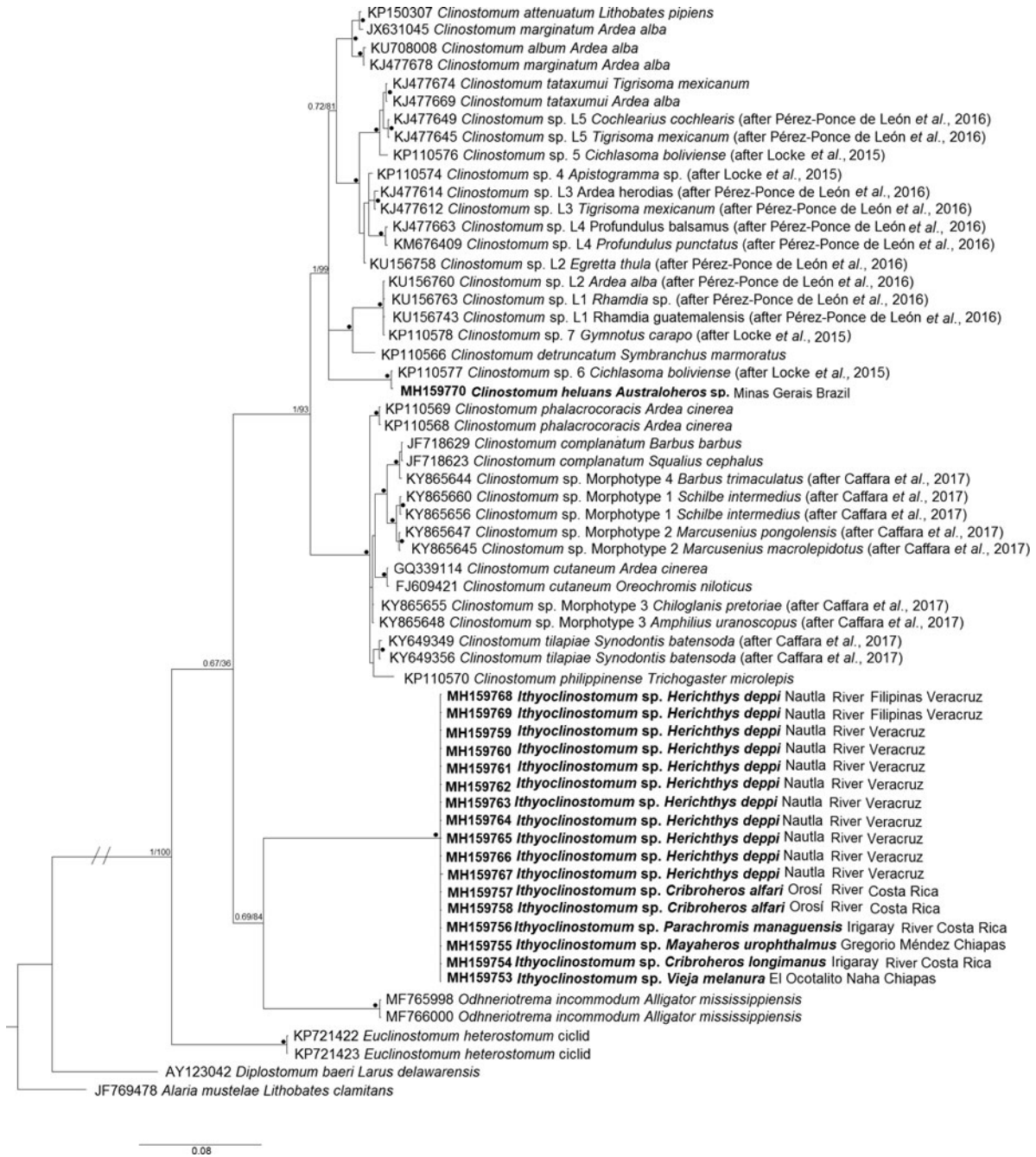


Fig. 5. Bayesian inference tree for *Ithyoclinostomum* sp. based on the internal transcribed spacers (ITS1–5.8S–ITS2) dataset. Dots above branches represent posterior probability values and bootstrap values higher than 0.8/80%. Newly generated sequences of *Ithyoclinostomum* sp. in this study are in bold.

erythrinids *H. malabaricus* and *H. unitaeniatus* in Brazil by Benigno *et al.* (2014), who included SEM micrographs of the specimens. The metacercariae of *C. sorbens* are different from the ones we characterize in this study because they are relatively smaller (< 10 mm), they have an inter-testicular cirrus sac and genital pore (which is a diagnostic trait for the genus), and although they possess deeply lobed testes, the shape of the testes is very different: the anterior testis is formed by five irregular lobes, and the posterior testis by six lobes, two directed anteriorly and four directed posteriorly (see Figure 1 in Benigno *et al.*, 2014). Finally, the metacercariae of *Ithyoclinostomum* sp. are also different from the monotypic *Clinostomoides*. *Clinostomoides brieni* Dollfus,

1950 infects the oesophagus of herons and their metacercariae are more commonly found in siluriform fishes in Asia and Africa (Kanev *et al.*, 2002); this species is characterized by an elongated body, with gonads also located in the posterior fourth of the body; however, it can be easily distinguished from our metacercariae because the cirrus sac is inter-testicular and the genital pore is post-testicular according to the diagnosis by Kanev *et al.* (2002), although a recent description of the metacercariae from *Clarias gariepinus* (Burchell, 1822) from Botswana, Africa (van Rensburg *et al.*, 2013) refers to a ‘genital pore just submedian to right, immediately anterior to posterior testis’. The fact that the sequenced specimen of this species (from a metacercaria

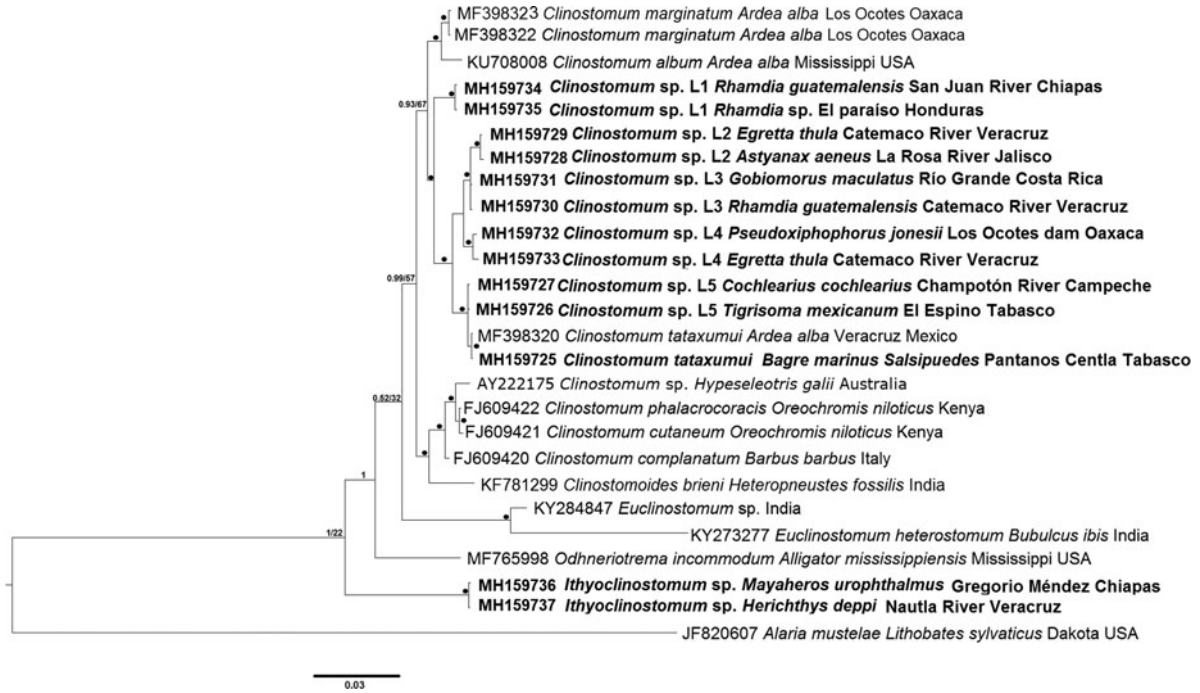


Fig. 6. Bayesian inference tree for *Ithyoclinostomum* sp. based on the 28S rRNA gene dataset. Dots above branches represent posterior probability values and bootstrap values higher than 0.8/80%. Newly generated sequences in this study for *Ithyoclinostomum* sp., as those for Lineages 1–5 (sensu Pérez-Ponce de León et al., 2016) are in bold.

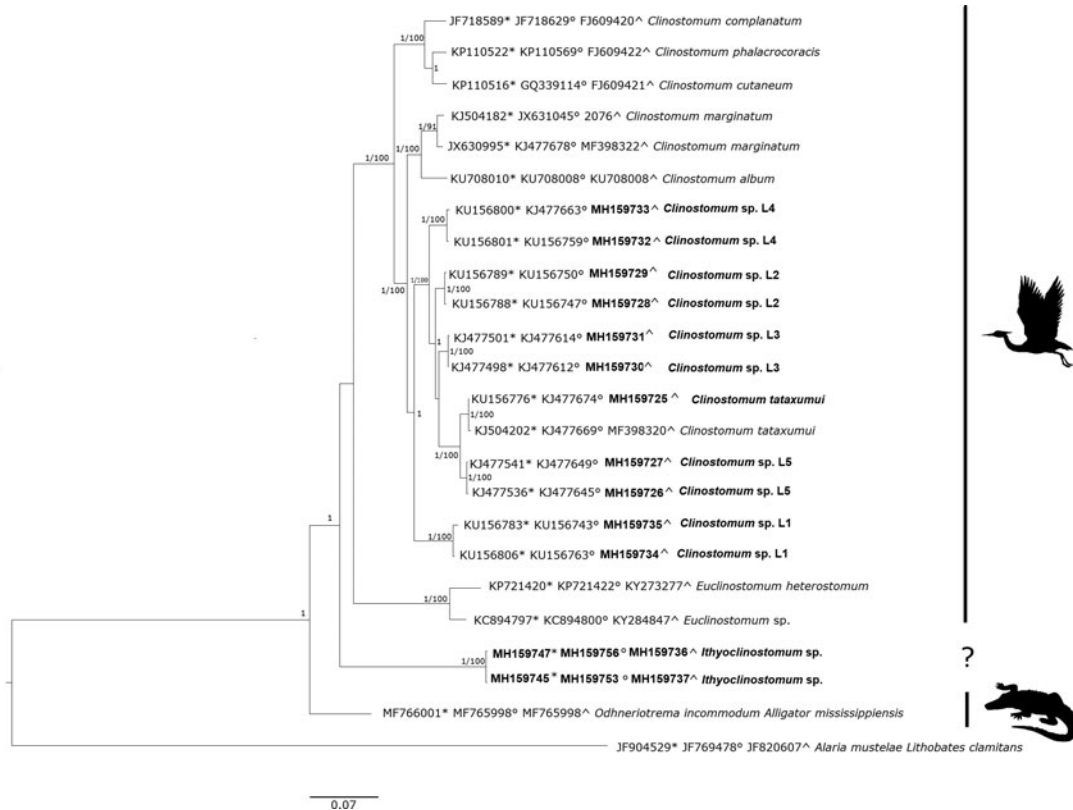


Fig. 7. Bayesian inference tree for *Ithyoclinostomum* sp. based on the concatenated dataset (28S + ITS1–5.8S–ITS2 + cox1). Numbers above branches represent posterior probability values and bootstrap values. The definitive host, either a fish-eating bird or a crocodylian, for each species/genetic lineage of clinostomid is included in the figure. * cox1 sequences; ° ITS sequences; ^ 28S sequences.

obtained from a siluriform fish in India – KF781299 – as a direct submission) is nested within a group of species of *Clinostomum* that includes the cosmopolitan *C. complanatum* indicates a possible misidentification, and requires further verification.

In conclusion, the metacercariae characterized in this study were included in the genus *Ithyoclinostomum* because they share some morphological traits; however, the inclusion of our specimens in the genus is tentative until sequences of the adults or metacercariae of the species *I. dimorphum* are provided from specimens sampled from their natural distributional range in South America. Molecular data will provide confirmation or rejection of this hypothesis. Even if our specimens belong to a different genus, they represent an undescribed species; to accomplish the proper species description, and naming of the species, we need to collect adult forms from their definitive hosts, either fish-eating birds or crocodilians, and characterize them morphologically and molecularly, to establish a link between the larval forms and the adults in their definitive hosts.

Acknowledgements. Thanks are due to Luis García for the loan of specimens from the Colección Nacional de Helminthos (CNHE, Mexico City). We also thank Susana Guzmán and Berenit Mendoza-Garfias for their assistance with image acquisition through light microscopy and scanning electron microscopy, respectively. Laura Márquez provided technical assistance with the sequencer. We also thank Arturo Angulo from the Universidad de Costa Rica for support in sampling freshwater fish in Costa Rica.

Financial support. This work has been supported by the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM) through the grants IN202617 and IN206716 to GPPL and MGCV, respectively.

Conflict of interest. None.

Ethical standards. In Mexico, specimens were collected under the Cartilla Nacional de Colector Científico (FAUT 0202 and 0057) issued by the Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT), to MGCV and GPPL, respectively. In Costa Rica, specimens were obtained under the collector permit issued to Arturo Angulo from the Universidad de Costa Rica.

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