



**UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO**  
**POSGRADO EN CIENCIAS BIOLÓGICAS**

INSTITUTO DE BIOLOGÍA  
SISTEMÁTICA

Posición filogenética de dos especies de clinostómidos (Digenea: Clinostomidae),  
*Ithyoclinostomum* sp. y *Clinostomum heluans*, parásitos de cíclidos y de aves ictiófagas  
en el Continente Americano, utilizando secuencias de ADN.

**TESIS**

QUE PARA OPTAR POR EL GRADO DE:

**MAESTRA EN CIENCIAS BIOLÓGICAS**

PRESENTA:

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INSTITUTO DE ECOLOGÍA, INECOL

CD. MX. MAYO, 2018



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**MÉXICO, CD. MX. MAYO, 2018**



OFICIO CPCB/311/2018

Asunto: Oficio de Jurado para Examen de Grado


Lic. Ivonne Ramírez Wence  
Directora General de Administración Escolar, UNAM  
Presente

Me permito informar a usted que en la reunión del Subcomité por Campo de Conocimiento de Biología Evolutiva y Sistemática del Posgrado en Ciencias Biológicas, celebrada el día 5 de marzo de 2018, se aprobó el siguiente jurado para el examen de grado de **MAESTRA EN CIENCIAS BIOLÓGICAS** de la alumna **BRIOSIO AGUILAR ROSARIO** con número de cuenta **301172327** con la tesis titulada "**Posición filogenética de dos especies de clinostómidos (Digenea: Clinostomidae), *Ithyoclinostomum* sp. y *Clinostomum heluans* parásitos de cíclidos y de aves ictiófagas en el Continente Americano, utilizando secuencias de ADN**", realizada bajo la dirección del **DR. GERARDO PÉREZ PONCE DE LEÓN**:

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Sin otro particular, me es grato enviarle un cordial saludo.

ATENTAMENTE  
"POR MI RAZA HABLARA EL ESPIRITU"  
Cd. Universitaria, Cd. Mx., a 13 de abril de 2018.

  
DR. ADOLFO GERARDO NAVARRO SIGÜENZA  
COORDINADOR DEL PROGRAMA



c.c.p. Expediente del (la) interesado (a).

## **Agradecimientos**

- Al Posgrado en Ciencias Biológicas de la Universidad Nacional Autónoma de México, UNAM por el esfuerzo colectivo que realizan para profesionalizar a sus alumnos.
- Al Consejo Nacional de Ciencia y Tecnología, CONACyT por la beca No. 742934 otorgada durante el Posgrado semestre 2016-2 a 2018-1.
- Al financiamiento otorgado al Programa de apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM) No. IN202617 concedido al Dr. Gerardo Pérez Ponce de León.
- Al Dr. Gerardo Pérez Ponce de León que fungió como Tutor principal en el desarrollo del Posgrado.
- Al Dr. José Martín García Varela y al Dr. Miguel Rubio Godoy, ambos miembros del Comité Tutor.

## Agradecimientos Personales

Hago extensivo mi inmenso agradecimiento a todas las personas que de alguna u otra forma estan involucradas en hacer posible el desarrollo y consumación de este proyecto de investigación

- A los muy estimados doctores que fueron la parte formativa principal de mi proceso educativo Dr. Gerardo Pérez Ponce de León, Dr. José Martín García Varela y Dr. Miguel Rubio Godoy.
- A la Dra. María del Carmen Guzmán Cornejo, el Dr. Carlos Daniel Pinacho Pinacho y el M. en C. Luis García Prieto quienes cortésmente revisaron este escrito y formaron parte del jurado.
- A las técnicas y técnicos académicos que amablemente me facilitaron sus servicios: M. en C. Berenit Mendoza Garfias, M. En C. Laura Margarita Márquez Valderramar Biól. Susana Guzmán Gómez y Biól. María del Carmen Loyola Blanco.
- A la Dra. Ma. Amparo Rodríguez, Karina López, Dr. Hudson Alves Pinto y al M. en C. David Hernández por su trabajo de campo y la disposición para facilitarnos material biológico.
- Asi como a cada una y uno de los docentes que impartieron con compromiso y profesionalidad las clases en el Posgrado: Dra. Alejandra Vasco, Dra. Xitlali Aguirre, Dra. Carolina Granados, M. en C. Laura Márquez, M. en C. Andrea Jiménez, Dra. Lidia Cabrera, Dra. Jeny Sotuyo, Dra. Tania Escalante, Dr. Jose Morrone, Dr. David Gernandt, M. en C. Elkin Noguera, Dr. Erick García y Dr. Alejandro Saldivar.
- A las administrativas y administrativos que cordialmente gestionaron mis trámites.
- Al personal Bibliotecario de la UNAM.
- A los colegas que amablemente me apoyaron en laboratorio, M. en C. Alejandra López, Dra. Brenda Solorzano, M. en C. Leopoldo Andrade y Biól. Eduardo García.
- A todos los que hacen posible reunir el financiamiento de becas CONACyT.

Espero cada uno de ustedes se pueda ver reconocido y valorado en estas sencillas palabras.

A todos ustedes mi más sincero agradecimiento.

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## Resumen

En este trabajo se investigó la posición filogenética de dos especies de tremátodos de la familia Clinostomidae: *Clinostomum heluans* e *Ithyoclinostomum* sp. Entre 2014 y 2016 se realizó trabajo de campo para la obtención de helmintos parásitos de peces dulceacuícolas en diferentes localidades de México y Centroamérica en donde se obtuvieron metacercarias de tremátodos de gran tamaño en la cavidad corporal de algunas especies de cíclidos. Las metacercarias fueron procesadas para estudios morfológicos y moleculares. Para el estudio morfológico, fueron teñidas y montadas en preparaciones permanentes. Para el trabajo molecular, fueron secuenciados tres marcadores, uno mitocondrial (*cox1*) y dos nucleares (ITS1-5.8S-ITS2 y 28S). Para la determinación taxonómica de las metacercarias, se examinó la literatura con respecto al área de distribución y el material depositado en la Colección Nacional de Helmintos (CNHE), llegando a la conclusión que éstas podrían corresponder a la especie *Clinostomum heluans*. Por ello, el presente estudio está dividido en dos partes (capítulos). En el primero, se establece el vínculo molecular entre las metacercarias y los adultos de *C. heluans*, para determinar si las metacercarias recolectadas correspondían a esta especie. Se obtuvieron muestras de tejido de ejemplares tanto adultos como metacercarias de esta especie de clinostómido (de México y Brasil, respectivamente) y se estableció el vínculo a través del gen *cox1*. Esto nos permitió corroborar que nuestros ejemplares no correspondieron con la especie *C. heluans*. En el segundo capítulo se utilizó la reconstrucción filogenética de las metacercarias recolectadas junto con las secuencias disponibles en el GenBank de otros clinostómidos, utilizando para ello los tres genes referidos con anterioridad. Asimismo, se utilizó una aproximación de taxonomía integrativa para tratar de lograr la determinación, incluyendo la asociación hospedatoria, el área de distribución geográfica y la revisión morfológica de los representantes de la familia Clinostomidae. De esta manera, se caracterizó morfológica y molecularmente a estas metacercarias que son parásitos de cíclidos y se ubicaron de manera tentativa dentro del género monotípico *Ithyoclinostomum*. Sin embargo, por algunas características morfológicas y con base en la asociación hospedatoria, no se pudo concluir que nuestros ejemplares correspondieran a la especie *I. dimorphum*. Es necesaria la obtención de datos moleculares de la especie *I. dimorphum* para corroborar que nuestros ejemplares representan una especie diferente, y además se requiere la obtención de adultos en sus hospederos definitivos para poder describir esta potencial especie como nueva.

## Abstract

This study investigates the phylogenetic position of two species of trematodes of the family Clinostomidae: *Clinostomum heluans* and *Ithyoclinostomum* sp. between 2014 and 2016, we conducted fieldwork to obtain helminth parasites from freshwater fishes in different localities of Mexico and Middle America. In six localities, unusually large metacercariae of clinostomids were found in the body cavity of cichlids. Metacercariae were processed for a morphological and molecular study. For the morphological study, specimens were stained and mounted in permanent slides. For molecular work, two nuclear markers (ITS1-5.8S-ITS2 y 28S) and one mitochondrial (*cox1*) were amplified.. For the taxonomic identification of the metacercariae, relevant literature regarding species of clinostomids occurring within that geographical area and comparison to specimens deposited in the National Collection of Helminths were analyzed, leading to the conclusion that they might belong to the species *Clinostomum heluans*. This study is divided in two parts (chapters). In the first one, a molecular link between the metacercariae and adults of *C. heluans* was established to determine if the sampled metacercariae corresponded with this species. Specimens of adults and metacercariae of this species of *Clinostomum* were obtained from Mexico and Brazil, respectively, and the molecular link was established by using *cox1* sequences. We were then able to corroborate that our specimens do not correspond with *C. heluans*. In the second chapter, we tested the phylogenetic position of the unusually large metacercariae considering sequences of clinostomids available in the GenBank for 2 ribosomal and one mitochondrial gene. We followed an integrative taxonomy approach to accomplish the species identification by including data on host association, geographic distribution, and a morphological analysis with respect to all members of the family Clinostomidae. Metacercariae were morphologically and molecularly characterized and tentatively placed within the genus *Ithyoclinostomum*. However, considering some morphological traits and using host association, we conclude that our specimens do not correspond with the species *I. dimorphum*. Further molecular data are necessary to corroborate that our specimens represent a separate species, as well as the collection of adults forms from their definitive hosts to describe this potentially new species.

## I. Introducción

### I.1 Características generales del Phylum Platyhelminthes

El Phylum Platyhelminthes comprende cuatro clases: Trematoda, Monogenea, Cestoda y Turbellaria. Las primeras tres clases contienen principalmente organismos endo y ectoparásitos de invertebrados y vertebrados silvestres. Los platyhelminthes son dorsoventralmente aplanados, carecen de cavidad corporal (acelomados), durante su desarrollo se diferencian tres capas embrionarias, es decir, son tripoblásticos y el orificio oral deriva del blastoporo embrionario por lo que son protostomos. Presentan simetría bilateral y no están segmentados, aunque en la clase Cestoda se observan proglótidos en forma de estróbilo. La boca conduce a una faringe y de ahí a un intestino ciego la mayoría de las veces; y cuando es abierto desemboca en un poro excretor. El intestino puede presentarse desde muy complejo a estar incompleto o incluso ausente. Exhiben un sistema nervioso central compactado en un ganglio cerebral. Como sistema de osmoregulación y excreción presentan protonefridios. Los organismos de este phylum son hermafroditas, aunque también se observan especies dióicas (Brusca & Brusca, 2005).

### I. 2 Características generales de la clase Trematoda Rudolphi, 1808

De acuerdo a Gibson *et al.* 2001, la clase Trematoda comprende dos subclases: Aspidogastrea y Digenea. Esta segunda clase alberga organismos endoparásitos. Presentan un ciclo de vida complejo con dos hospederos intermediarios, el primero un molusco y el segundo un vertebrado, la gran mayoría de las veces. El espécimen sexualmente maduro se encuentra en un vertebrado en la cavidad del cuerpo, en órganos o tejidos (Fig. 1). Presentan dos órganos de fijación: ventosa oral y ventosa ventral. El tegumento es sincicial (células multinucleadas) con o sin espinas o papilas. La boca se presenta como un órgano de fijación en la parte anterior del cuerpo, una prefaringe que se vincula a la boca, la faringe es muscular, puede estar presente o no. El intestino es usualmente bifurcado, a veces con ciegos sencillos, otras veces ramificado y algunas veces con divertículos; los intestinos son frecuentemente ciegos, en ocasiones están abiertos y desembocan en un ano que se encuentra en la pared del cuerpo o en una vesícula excretora. Presentan un único poro excretor terminal. Los digeneos son hermafroditas, aunque existen especies dióicas. El aparato reproductor femenino esta compuesto por un ovario, una glándula de Mehlis y un ootipo. Los huevos se almacenan en el útero. El vitelo se puede observar desde un tejido folicular hasta una masa compacta sencilla. Frecuentemente se observan

dos testículos, aunque pueden presentarse desde uno hasta varios. Cuando la genitalia masculina se encuentra totalmente embebida en un saco muscular, se le nombra saco del cirro; cuando se encuentran tanto el masculino como el femenino dentro del saco muscular, éste se llama saco del *sino* (sinus sac). El aparato reproductor masculino consta de una vesícula seminal, una glándula prostática y un ducto eyaculatorio. Normalmente se exhibe un órgano de almacenamiento espermático. El atrio genital puede o no estar presente. Tanto machos como hembras tienen un conducto reproductor terminal que abre en un poro en común (Gibson *et al.*, 2001). La clase Digenea está constituida por 150 familias, 24 superfamilias y dos grandes subclases, Diplostomida y Plagirochiida (Littlewood *et al.*, 2015). Diplostomida es la subclase más pequeña y contiene tres superfamilias y 19 familias; una de estas es la familia Clinostomidae (Littlewood *et al.*, 2015).

### I.3 Características generales de la familia Clinostomidae Lühe, 1901

Los parásitos de esta familia presentan un cuerpo de tamaño medio a grande, alargado o linguiforme; pueden tener un cuerpo robusto o no, son planos, en forma de lanza o de hoja o marcadamente cónicos en la parte anterior del cuerpo. Usualmente son convexos dorsalmente y ventralmente cóncavos. Su tegumento no está ornamentado, aunque algunos pueden tener finas espinas. La ventosa oral es pequeña o puede estar bien desarrollada. Puede observarse en algunas especies un pliege en forma de collar alrededor de la ventosa oral. La ventosa ventral es muscular, robusta; la cual está situada en la parte anterior media del cuerpo. La prefaringe está pobremente desarrollada o ausente. Tienen una faringe pequeña y escasamente desarrollada. El esófago es corto o está ausente. El intestino es simple, tiene una pared lisa o curvada, puede tener ramas laterales o divertículos. El ovario se encuentra en el espacio intertesticular, puede tener forma esférica, oval o irregular. Los folículos vitelinos están desarrollados en el área lateral de la parte terminal del cuerpo y hasta la parte media de los ciegos intestinales y alcanzan el nivel de la ventosa ventral. El útero es tubular; se observa principalmente en el espacio entre los ciegos intestinales hasta el nivel de la ventosa ventral, después desciende a la derecha hacia el poro genital. Los testículos son lisos o ligeramente lobulados, de forma irregular, en disposición en tandem, a la mitad de la parte media posterior del cuerpo. El saco del cirro y el poro genital se sitúan en el espacio pre-, inter-, post testicular, o lateral al testículo anterior. El poro genital femenino y masculino están juntos y en algunas especies son separados. La vesícula excretora tiene forma de Y (Gibson *et al.*, 2001). Las cercarias de esta familia presentan ocelos, faringe, un furcocerco, un lofocerco con un órgano principal alargado (en forma de cabeza); desarrolla a redia en caracoles pulmonados de agua dulce. Las metacercarias se encuentran en la cavidad abdominal y en

músculo de peces de agua dulce, serpientes, salamandras o caracoles. Los adultos se encuentran principalmente en la cavidad bucal o esófago de aves piscívoras, reptiles y ocasionalmente mamíferos incluyendo humanos. En el ciclo de vida de esta familia de tremátodos las aves depositan los huevos al exterior ya sea a través de las heces o de regurgitaciones; de los huevos eclosiona el miracidio, el cual tiene la potencialidad de producir un gran número de descendientes; el miracidio penetra el primer hospedero intermediario, un caracol planorbido en el cual se desarrolla por reproducción asexual un esperocisto; redia madre y redias hijas la cuales albergan a las cercarias las cuales una vez maduras salen al medio acuático donde entran en contacto con el segundo hospedero intermediario, un pez, una vez dentro del pez desarrollan a metacercarias, para madurar a adultos requieren de la ingesta del segundo hospedero intermediario por parte del hospedero final para que una vez dentro de el, éstas desarrollen a tremátodos adultos y produzcan huevos y el ciclo de vida continúe (Bullard &, Overstreet, 2008) El género tipo es *Clinostomum* Leidy, 1856. (Fig. 1).

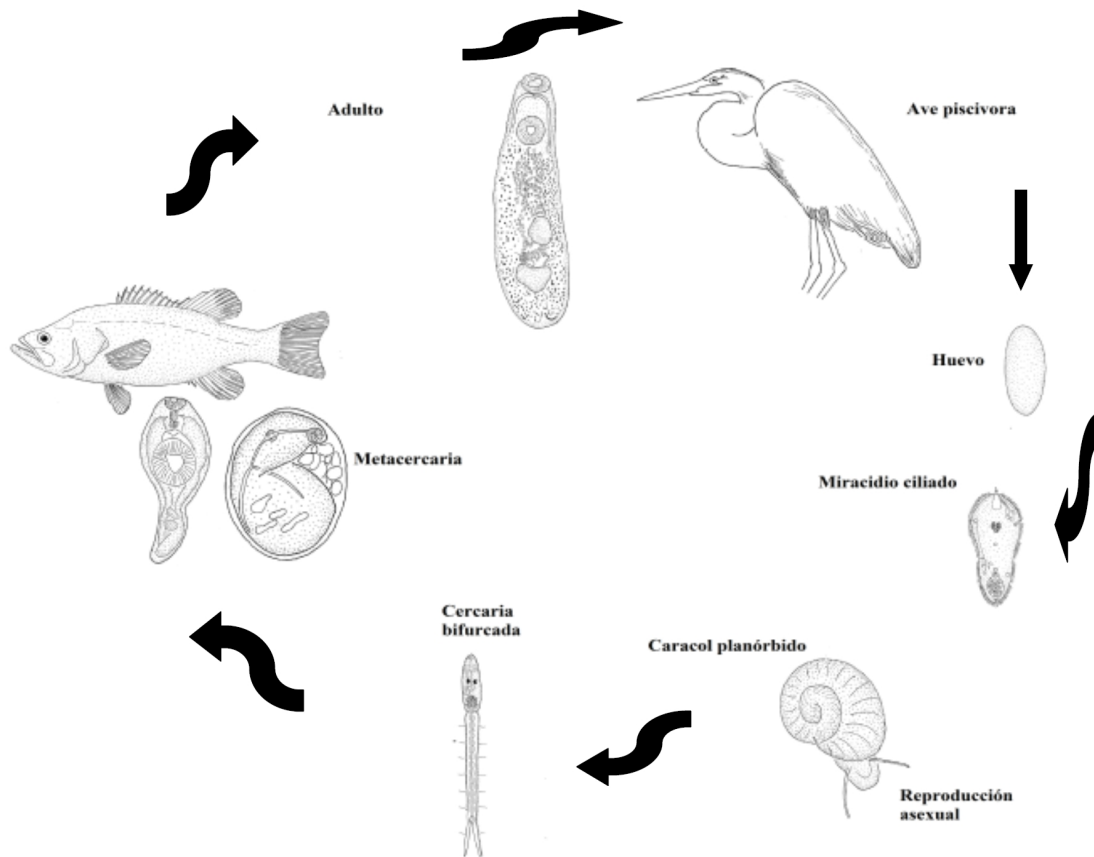


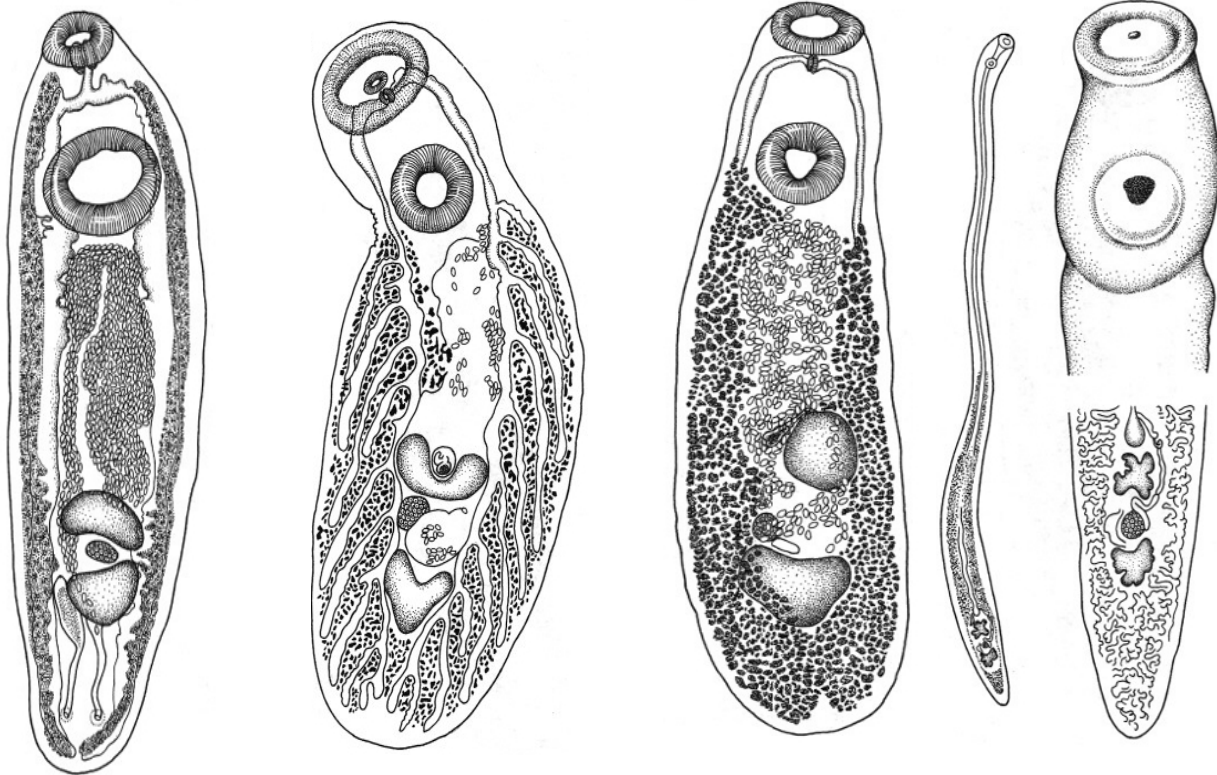
Fig. 1. Ciclo de vida del género *Clinostomum* de la familia Clinostomidae Lühe, 1901. Modificado de Bullard & Overstreet, 2008.



De acuerdo con Kanev *et al.* (2001), la Familia Clinostomidae Lühe, 1901 está compuesta por cuatro subfamilias: Nephrocephalinae Travassos, 1929, Euclinostominae Yamaguti, 1958, Clinostominae Lühe, 1901 e Ithyoclinostominae Yamaguti, 1958. Las características principales que diferencian estas subfamilias se enlistan en la Tabla 1. No se cuenta hasta la fecha con un análisis filogenético de los miembros de esta familia, excepto para algunas especies incluidas en los géneros *Clinostomum*, *Euclinostomum* y *Odnheriotrema*.

Tabla 1. Características representativas de las subfamilias de la familia Clinostomidae Lühe, 1901 según Kanev *et al.* (2001).

<b>Nepthrocephalinae</b> Travassos, 1929.	<b>Euclinostominae</b> Yamaguti, 1958.	<b>Clinostominae</b> Lühe, 1901.	<b>Ithyoclinostominae</b> Yamaguti, 1958.
Los adultos se encuentran en la cavidad esofágica de reptiles	Los adultos se encuentran en cavidad esofágica de aves	Los adultos se encuentran en cavidad esofágica de aves (ocasionalmente de mamíferos)	Los adultos se encuentran en cavidad esofágica de aves
	Ciegos intestinales con numerosas ramificaciones laterales y divertículos	Ciegos intestinales sin ramas laterales ni divertículos	Ciegos intestinales sencillos sin ramas laterales ni divertículos
		Cuerpo robusto, linguiforme entre 5 y 30 mm de largo	Cuerpo delgado, alcanza hasta los 100mm de largo
<i>Nepthrocephalus sessilis</i> Odhner, 1902.	<i>Euclinostomum heterostomum</i> Rudolphi, 1809	<i>Clinostomum complanatum</i> Rudolphi, 1819	<i>Ithyoclinostomum dimorphum</i> Diesing, 1850 (según Braun, 1900).



## II. Objetivos

### II.1 Objetivo general.

- Determinar la posición filogenética de *Ithyoclinostomum* sp. y de *Clinostomum heluans* dentro de la familia Clinostomidae, utilizando secuencias de genes ribosomales y mitocondriales.

### II. 2 Objetivos particulares

- Caracterizar molecularmente a las metacercarias y a los adultos de *C. heluans* obtenidas de sus hospederos intermediarios (Cichlidae) y definitivos (Ardeidae).
- Vincular molecularmente la metacercaria y el adulto de *Clinostomum heluans* en su hospedero intermediario (ciclido) y definitivo (aves).
- Caracterizar morfológicamente a las metacercarias de *Ithyoclinostomum* sp. en cíclidos colectados en México y Costa Rica.
- Caracterizar genéticamente las metacercarias de *Ithyoclinostomum* sp. obtenidas de distintas localidades y de diferentes especies de hospederos.

### III. Materiales y Métodos

#### III.1 Trabajo de campo.

##### III.1.1 Colecta de peces y análisis helmintológico

###### *Ithyoclinostomum* sp.

Entre el año 2014 y 2016 se colectaron peces dulceacuícolas pertenecientes a seis especies de hospederos de la familia Cichlidae en nueve localidades de cuencas hidrológicas de México y Costa Rica (Tabla 2, Fig. 2). Para obtener las muestras, se utilizó equipo de electropesca. Los peces fueron capturados y transportados vivos a la estación de trabajo, donde se identificaron con ayuda de claves especializadas (Miller *et al.*, 2005). Los peces fueron sacrificados para una posterior revisión helmintológica con instrumentos de disección, se obtuvieron los órganos internos y se colocaron en una caja Petri con suero fisiológico al 0.65%. El análisis de cada órgano se realizó con la ayuda de un microscopio estereoscópico. La observación se realizó primero externamente y después de forma interna. Cabe mencionar que las metacercarias se encontraron libres en la cavidad corporal de su hospedero.

###### *Clinostomum heluans*

En el caso de esta especie de tremátodo, las metacercarias fueron recolectadas por Hudson Alves Pinto en *Australoheros* sp. en Pampulha en Belo Horizonte, Minas Gerais, de Brasil, mientras que el adulto fue recolectado por María Amparo Rodríguez-Santiago y Karina López García de la cavidad bucal de *Ardea alba* en la localidad de Palizada del Este, Campeche, México. Asimismo colectaron dos individuos más cuyos hospederos fueron *Ardea herodias* y *Ardea alba* de la misma localidad.

#### III.1.2 Fijación de ejemplares

Los ejemplares encontrados se procesaron de dos maneras de acuerdo al objetivo de análisis. Una vez obtenidos, los ejemplares fueron lavados en solución salina al 0.6 o 0.8%. Para el estudio morfológico, los trematodos fueron fijados en formol caliente al 4%, y posteriormente colocados en un frasco de vidrio con formol al 4% a temperatura ambiente y etiquetado para su identificación taxonómica. Para el análisis molecular, los adultos y metacercarias fueron colocados en un vial con alcohol etílico absoluto, etiquetadas y mantenidas a -20°C hasta su extracción.

Fig. 2 Mapa que muestra la distribución de los ejemplares de *Ithyoclinostomum* sp. y de *C. heluans* utilizados en el presente estudio, incluyendo datos de registros previos en el Continente Americano.

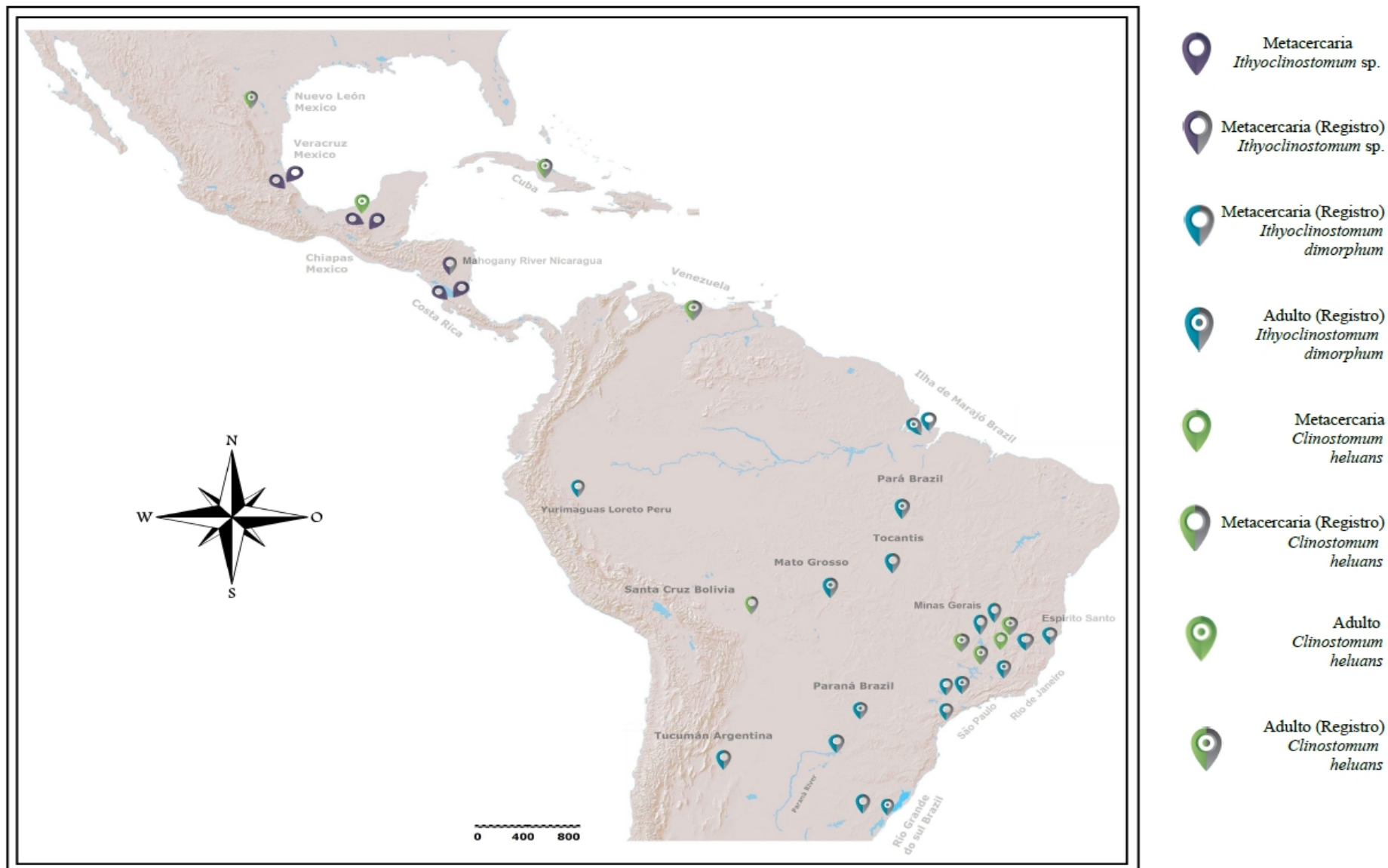


Tabla 2. Registros de metacercarias y adultos de *Ithyoclinostomum* sp., *Ithyoclinostomum dimorphum* y *Clinostomum heluans*.

Localidad	Hospedero	N	W	Autor
<b><i>Ithyoclinostomum</i> sp. (Metacercaria)</b>				
<b>México</b>				
<i>Estado Tabasco</i>				
Gregorio Méndez	<i>Mayaheros urophthalmus</i>	17°27'00"	91°34'10"	Presente estudio
<i>Estado Chiapas</i>				
El Ocotalito, Naha	<i>Vieja melanura</i>	17°03'00"	91°35'49"	Presente estudio
<i>Estado Veracruz</i>				
Nautla River, Nautla	<i>Herichthys deppi</i>	20°11'18"	96°44'43"	Presente estudio
Nautla River, Filipinas	<i>Herichthys deppi</i>	20°00'47"	97°09'50"	Presente estudio
<b>Costa Rica</b>				
<i>Provincia Guanacaste</i>				
Río Irigaray, Liberia	<i>Cribroheros longimanus</i>	10°43'21"	85°30'38"	Presente estudio
Río Irigaray, Liberia	<i>Parachromis managuensis</i>	10°43'21"	85°30'38"	Presente estudio
Río Orosí, Pithaya	<i>Cribroheros alfari</i>	11°03'05"	85°24'30"	Presente estudio
Río Orosí	<i>Cribroheros alfari</i>	11°02'50"	85°22'48"	Presente estudio
<b>Nicaragua</b>				
<i>Región Autónoma de la Costa Caribe Sur</i>				
Río Mahogany	<i>Cichlasoma managuense</i>	12°03'22"	83°59'07"	Aguirre-Macedo <i>et al.</i> , 2001*
<b><i>Ithyoclinostomum dimorphum</i> (Adulto)</b>				
<b>Brasil</b>				
No especificado	<i>Ardea cocoi</i>			Braun, 1899
<i>Estado Pará</i>				
Ilha de Marajó	<i>Ardea cocoi</i>			Lent & Freitas, 1937
<i>Estado Mato Grosso</i>				
No especificado	<i>Ardea cocoi</i>			Travassos, 1928
Barão de Melgaço	<i>Ardea cocoi</i>	23°30'00"	47°30'00"	Pinto <i>et al.</i> , 2004
	<i>Ardea alba</i>	23°30'00"	47°30'00"	Pinto <i>et al.</i> , 2004

	<i>Nycticorax Nycticorax</i>	23°30'00" 47°30'00"	Pinto <i>et al.</i> , 2004
<i>Estado de Mato Grosso do Sul</i>			
No especificado	<i>Ardea cocoi</i>		Travassos, 1941; Travassos & Freitas, 1942, 1943
Cuenca del Río Paraná	<i>Ardea cocoi</i>	22°70'00" 53°40'00"	Dias <i>et al.</i> , 2003
Barão de Melgaço	<i>Tigrisoma lineatum</i>		Arruda <i>et al.</i> , 2001
<i>Estado São Paulo</i>			
Pirassununga	<i>Nycticorax Nycticorax</i>		Arruda <i>et al.</i> , 2001
<i>Estado Rio Grande do Sul</i>			
Pelotas	<i>Ardea cocoi</i>	31°46'19" 52°20'34"	Fedatto <i>et al.</i> , 2017
<b><i>Ithyoclinostomum dimorphum</i> (Metacercaria)</b>		S W	
<b>Brasil</b>			
<i>Estado Tocantins</i>			
Río Araguaia, Araguaianã	<i>Hoplias malabaricus</i>	06°34'00" 48°38'00"	Reis, 2014
<i>Estado Rio Grande do Sul</i>			
Santa Maria	<i>Hoplias malabaricus</i>		Weiblen & Brandão, 1992
Cachoeira do Sul	<i>Hoplias malabaricus</i>		Gallio <i>et al.</i> , 2007
Río Pirantini	<i>Hoplias malabaricus</i>	31°30'00" 53°31'00"	Rodrigues, 2010
<i>Estado Minas Gerais</i>			
Rio Doce	<i>Hoplias malabaricus</i>	19°46'53" 42°35'57"	Belei <i>et al.</i> , 2013
No especificado	<i>Hoplias malabaricus</i>		Moreira, 2000
Cuenca São Francisco	<i>Hoplias malabaricus</i>	18°12'32" 45°15'41"	Costa <i>et al.</i> , 2015
Cuenca São Francisco	<i>Hoplias intermedius</i>	18°12'32" 45°15'41"	Costa <i>et al.</i> , 2015
No especificado	<i>Hoplerythrinus unitaeniatus</i>		Moreira, 2000
<i>Estado Rio de Janeiro</i>			
Reserva Lajes	<i>Hoplias malabaricus</i>	22°42'00" 44°05'00"	Paraguassú & Luque, 2007
<i>Estado Espírito Santo</i>			
	<i>Hoplias malabaricus</i>		Travassos, 1964
<i>Estado Pará</i>			
Lago Arari, Isla Marajó	<i>Hoplias malabaricus</i>	00°39'48" 49°10'30"	Benigno <i>et al.</i> , 2014
Lago Arari, Isla Marajó	<i>Hoplerythrinus unitaeniatus</i>	00°39'48" 49°10'30"	Benigno <i>et al.</i> , 2014
<i>Estado Paraná</i>			
Porto Rico	<i>Hoplias malabaricus</i>		Pavanelli <i>et al.</i> , 1990
Porto Rico	<i>Schizodon borelli</i>	22°40'00" 53°15'00"	Machado <i>et al.</i> , 1996

<b>Argentina</b>					
	Provincia de Tucumán	<i>Hoplias malabaricus</i>			Szidat, 1969
<b>Perú</b>					
	Provincia del Alto amazonas				
	Yurimaguas	<i>Hoplerythrinus unitaeniatus</i>	05°42'03"	76°52'03"	Delgado <i>et al.</i> , 2017
<hr/>					
<b><i>Clinostomum heluans</i> (Adulto)</b>					
<b>México</b>			N	W	
	Estado Campeche,				
	Palizada del Este	<i>Ardea alba</i>	18°15'19"	92°06'32"	En este estudio
	Estado Nuevo León				
	Sabinas Hidalgo	<i>Ardea herodias</i>			Bravo-Hollis, 1947
<b>Brasil</b>			S	W	
	No especificado	<i>Ardea caerulea</i> <i>Nycticorax gardeni</i>			Braun, 1899
	No especificado	<i>Ardea cocoi</i> <i>Nyctanassa violacea</i>			Travassos, 1969
	Estado Minas Gerais	<i>Butorides striata</i>	19°50'50"	43°59'35"	Pinto <i>et al.</i> , 2013
		<i>Tigrisoma lineatum</i>			Fernandes <i>et al.</i> , 2015
	Estado São Paulo	<i>Botaurus pinnatus</i>	24°43'21"	47°32'29"	Werneck <i>et al.</i> , 2017
<b>Cuba</b>		<i>Ardea alba</i>			Pérez-Vigueras, 1955
<b>Venezuela</b>		<i>Ardea alba</i>			Caballero & Diaz-Ungria, 1958
<hr/>					
<b><i>Clinostomum heluans</i> (Metacercaria)</b>					
<b>Brasil</b>					
	Estado Minas Gerais				
	Belo Horizonte	<i>Australoheros sp.</i>	19°50'50"	43°59'35"	En este estudio
	No especificado	<i>Cichla temensis</i>			Vicente <i>et al.</i> , 1978



<i>No especificado</i>	<i>Geophagus proximus</i>		Zago <i>et al.</i> , 2012
<b>Bolivia</b>			
<i>Santa Cruz</i>	<i>Cichlasoma boliviense</i>		Locke <i>et al.</i> , 2015 <sup>o</sup>
<b><i>Clinostomum</i> sp. L5 (Adulto)</b>			
<i>México</i>			
<i>Estado Campeche</i>			
Palizada del Este	<i>Ardea alba</i>	18°15'19" 92°06'32"	En este estudio
	<i>Ardea herodias</i>	18°15'19" 92°06'32"	En este estudio

\* La metacercaria reportada por Aguirre-Macedo *et al.* (2001) fue identificada como *Clinostomum* sp.; en este trabajo se identifica como *Ithyoclinostomum* sp. por las características morfológicas que presenta en común con las metacercarias reportadas en este estudio. <sup>o</sup> El registro reportado por Locke *et al.* (2015) como *Clinostomum* sp. 6 corresponde a *Clinostomum heluans* revelado por análisis filogenéticos con el gen *cox1*.

## III. 2 Estudio morfológico

### III.2.1 Número de ejemplares y técnica de tinción de los organismos

Los ejemplares fijados en formol al 4% fueron lavados con alcohol al 70% y luego en alcohol al 96%, posteriormente se tiñeron utilizando Paracarmín de Meyer y se lavaron en alcohol 96% para retirar el exceso de colorante. Se sumergieron en alcohol acidulado al 2% con ácido clorhídrico y lavados en alcohol al 96% para desactivar el efecto aclarante del alcohol acidulado y deshidratados en alcohol absoluto por 30 minutos, posteriormente fueron aclarados usando salicilato de metilo y montados en preparaciones permanentes con bálsamo de Canadá según Lamothe-Argumedo, 1997 y fueron depositados en la Colección Nacional de Helmintos (CNHE).

### III.2.2 Medición de ejemplares

Las estructuras morfológicas de 14 metacercarias fueron medidas. Para ello, se utilizó un microscopio óptico ZEISS: Axio Zoom V. 16 integrado con una cámara Axio Cam Mrc5. Con el software ZEN-Zeiss Efficient Navigation se obtuvieron fotos en las cuales se llevó a cabo la medición de las estructuras. Los resultados obtenidos fueron utilizados para la caracterización morfológica de *Ithyoclinostomum* sp.

Los organismos se describieron y esquematizaron con ayuda del software GIMP 2.8.16. Para su identificación a nivel específico, se emplearon claves taxonómicas y descripciones originales.

### III.2.3 Obtención de fotografías de Microscopía Electrónica de Barrido (MEB)

Dos ejemplares conservados en formol al 4% fueron lavados en agua destilada y subsecuentemente deshidratados con alcoholes graduales hasta alcohol absoluto posteriormente fueron secados con dióxido de carbono y montados sobre placas de metal con pasta de plata y recubiertos con oro con la finalidad de ser observados en el microscopio electrónico de barrido Hitachi modelo Stereoscan SU1510 (Hitachi High-Technologies México S. A. de C. V, México) a 15 kV.

## III. 3 Estudio Molecular

### III.3.1 Extracción de DNA

Los ejemplares fueron digeridos individualmente en una solución que contuvo 10mM de Tris-

HCl (pH 7.6), 20 mM NaCl, 100 mM Na<sub>2</sub> EDTA (pH 8.0), 1% Sarkosyl y 0.1 mg/ml proteinasa K a 56°C por 24 horas. Posteriormente a la digestión, el ADN se extrajo usando el reactivo DNAzol de acuerdo a las instrucciones de uso de los fabricantes.

### III.3.2 Amplificación de genes nucleares y gen mitocondrial

#### Primers

El ADN ribosomal y mitocondrial se amplificó usando la reacción en cadena de la Polimerasa (PCR) usando los siguientes primers:

Para *ITS1*, *5.8S*, *ITS2* (Luton *et al.*, 1992).

- BD1 5'-GTCGTAACAAGGTTTCCGTA-3' (forward)
- BD2 5'-ATCTAGACCGGACTAGGCTGTG-3' (reverse)

Para *28S* los primers utilizados fueron tomados de García-Varela & Nadler, 2005

- 502 5'-CAAGTACCGTGAGGGAAAGTTGC-3' (forward)
- 536 5'-CAGCTATCCTGAGGGAAA-3' (reverse)

Para el gen Citocromo Oxidasa I (*cox1*) de Moszczyńska *et al.*, 2009

- MplatCOX1dF 5'-TGTAACGACGGCCAGTTTWCITTRGATCATAAG-3' forward
- MplatCOX1dR 5'-CAGGAAACAGCTATGACTGAAAYAAAYAIIGGATCICCACC-3' reverse

#### Condiciones de Amplificación

La reacción en cadena de la polimerasa fue llevada a cabo en volumen total de 25 µl conteniendo 2 µl de DNA genómico, 1 µl de cada primer (10mM) 0.5 µl de dNTP's (10mM), 0.125 µl equivalentes a una media unidad de Taq Polimerasa 2.5 µl de Buffer (10X), 1.5 µl de MgCl<sub>2</sub> y 16.375 µl de agua megapura. Las condiciones de amplificación para cada gen se muestran en la tabla 3.

**Tabla 3. Condiciones de Amplificaciones para primers**

	ITS1-5.8S-ITS2		28S		cox1	
Desnaturalización	94°C	5 min	95°C	5 min	94°C	5min
35 Ciclos	94°C	1min	94°C	1 min	94°C	1min
Alineamiento	50°C	1min	50°C	1 min	50°C	1 min
1a. Extensión	72°C	1min	72°C	1 min	72°C	1 min
Extensión final	72°C	10 min	72°C	10 min	72°C	1 min
Permanencia	4°C		4°C		4°C	

### III.3.3 Reacción de secuenciación

La reacción de secuenciación se realizó con el reactivo Big Dye (Applied Biosystems, Boston Massachusetts) en un volumen total de 10 µl. Los primers usados fueron los mismos para la amplificación, en el caso de los espaciadores internos ITS1, 5.8S, ITS2 y el gen 28S se usaron algunos primers internos:

*Primers internos (ITS1, 5.8S, ITS2):*

- BD3 5'-GAACATCGACATCTTGAACG-3' (forward) (Hernandez-Mena *et al.*, 2013)
- BD4 5'-ATAABCCGACCCTCGGC-3' (reverse) (Hernandez-Mena *et al.*, 2013)

*Primers internos (28S):*

- 503 5'-CCTTGGTCCGTGTTCAAGACG-3' (forward) (Stock *et al.*, 2001)
- 504 5'-CGTCTTGAAACACGGACTAAGG-3' (reverse) (García-Varela & Nadler, 2005)

### III.3.4 Secuenciación y alineamiento

Los purificados fueron secuenciados en un secuenciador ABI Applied Biosystems 3730. Las secuencias fueron ensambladas con el programa Codoncode Aligner version 6.0.2 (Codoncode Corporation). Para cumplir con los objetivos planteados en este trabajo se realizaron seis alineamientos que incluyeron las secuencias generadas en este estudio junto con aquellas obtenidas del GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>). En todos los casos, éstos fueron obtenidos por medio del programa Clustal Omega implementado en el servidor web <https://www.ebi.ac.uk/Tools/msa/clustalo/> y posteriormente corregidos manualmente mediante el programa Bioedit versión 7.2.6.1 (Hall, 1999).

Para el estudio de la posición filogenética de *C. heluans* dentro del género *Clinostomum*, se construyó un alineamiento a partir de secuencias del gen *cox1*. El alineamiento consistió de 51 terminales y 474 caracteres que incluyen cinco especies del género *Clinostomum* reconocidas como especies válidas, además de siete linajes genéticos que representan especies aún por describir, todos estos distribuidos en el Continente Americano, además se incluyeron dos especies del viejo mundo (*C. Complanatum* y *Euclinostomum* sp.) y como grupo externo para enraizar los árboles, a tres especies de diplostómidos (*Diplostomum baeri*, *Austrodiplostomum* sp. y *Alaria marciana*).

Para el caso de *Ithyoclinostomum*, se realizaron cuatro alineamientos con un número variable de

terminales en función de la información disponible para los tres marcadores moleculares ITS1-5.8S-ITS2, 28S y *cox1* (Gustinelli *et al.*, 2010; Caffara *et al.*, 2011, 2013, 2017; Locke *et al.*, 2011, 2015; Sereno-Uribe *et al.*, 2013; Pérez- Ponce de León *et al.*, 2016; Rosser *et al.*, 2017; Woodyard *et al.*, 2017). Se realizaron alineamientos para cada gen individual y concatenados para los tres genes anteriormente mencionados.

*cox1*. El alineamiento consistió de 474 pares de bases (pb) y de 15 secuencias pertenecientes a *Ithyoclinostomum* sp., dos secuencias de *Euclinostomum heterostomum*, tres de *Odhneriotrema incommodum* y una o dos réplicas de cada una de las 11 especies válidas de *Clinostomum*, más una o dos réplicas de cada uno de los 12 linajes descritos por Locke *et al.* (2011); Perez- Ponce de León *et al.* (2016); Caffara *et al.* (2017).

ITS1-5.8S-ITS2. El alineamiento fue de 1142 pb e incluyó 17 secuencias de *Ithyoclinostomum* sp., dos secuencias de *Euclinostomum heterostomum*, dos de *Odhneriotrema incommodum*, y una o dos réplicas de cada una de las 11 especies válidas de *Clinostomum*, más una o dos réplicas de los 12 linajes genéticos descritos por Locke *et al.* (2011); Pérez-Ponce de León *et al.* (2016); Caffara *et al.* (2017).

28S. El alineamiento fue de 1414 pb y solamente incluyó dos secuencias de *Ithyoclinostomum* sp., dos secuencias de *Euclinostomum heterostomum*, una de *Odhneriotrema incommodum*, una o dos réplicas de las seis especies válidas de *Clinostomum* para las cuales hay secuencias disponibles para este marcador, una especie de *Clinostomum* no identificada de Australia y dos réplicas de los cinco linajes de *Clinostomum* de América descritos por Pérez- Ponce de León *et al.* (2016).

Análisis concatenado de los tres marcadores. Consistió de 3094 pb e incluyó dos secuencias de *Ithyoclinostomum* sp., dos secuencias de *Euclinostomum*, una de *Odhneriotrema*, una o dos réplicas de las seis especies válidas de *Clinostomum* y dos réplicas de cinco linajes genéticos de *Clinostomum* descritos por Pérez- Ponce de León *et al.* (2016).

### III. 4 Métodos de reconstrucción filogenética

Se llevaron a cabo análisis de Máxima Verosimilitud (ML) e Inferencia Bayesiana (IB) con los alineamientos anteriormente referidos.

### *Máxima verosimilitud (ML)*

Los análisis de ML fueron realizados en el programa RAxML versión 8.2.X (Stamatakis, 2014). Para ello se hizo una búsqueda del mejor modelo de sustitución nucleotídica en el programa jModeltest versión 2.1.10 (Darriba *et al.*, 2012) de acuerdo al criterio de información de Akaike corregido. Los parámetros de ejecución del programa consistieron en una búsqueda de bootstrap y una subsecuente búsqueda del árbol de ML. Los valores de apoyo de bootstrap consistieron en 1000 repeticiones junto con optimización de longitud de ramas. La Verosimilitud final fue evaluada y optimizada bajo el modelo GAMMA de tasas de heterogeneidad.

### *Inferencia Bayesiana (IB)*

Los análisis de Inferencia Bayesiana fueron elaborados usando el programa MrBayes v. 3.2.6 (Ronquist *et al.*, 2012). El análisis fue diseñado con dos corridas y cuatro cadenas (una fría y tres calientes). Las cadenas de Markov Monte Carlo (MCMC por sus siglas en inglés) fueron corridas por 10 millones de generaciones muestreadas cada 1000 generaciones y las primeras 2500 muestras fueron descartadas como burn-in (25%). La topología consenso y el soporte de los nodos fue evaluado como valores de Probabilidad Posterior (Huelsenbeck *et al.*, 2001). Los resultados fueron examinados con Tracer V 1.4 (Drummond & Rambaut, 2007) para corroborar la convergencia de diferentes parámetros que determinan el número aproximado de generaciones a las cuales los estadísticos se estabilizaron y para identificar el tamaño efectivo de muestra (EES>200). Los árboles resultantes fueron observados y editados en el programa Fig Tree versión 1.4.3 (Rambaut, 2016).

Las divergencias genéticas (distancias “p” no corregidas) fueron calculadas con el programa PAUP versión 4.0a (build 159, Swofford, 2017).

#### **IV. Resultados**

Los resultados de este trabajo se presentan en dos capítulos. El Capítulo 1 incluye la posición filogenética de *C. heluans* dentro del género *Clinostomum* a partir de secuencias del gen mitocondrial *cox1* en donde se utilizó la información molecular para establecer un vínculo entre las metacercarias obtenidas en peces y los adultos en aves ictiófagas. El Capítulo 2 consiste en la caracterización morfológica y molecular de metacercarias del género *Ithyoclinostomum* además del establecimiento de la posición filogenética en la clasificación de la familia Clinostomidae, basados en análisis de secuencias de tres genes, dos nucleares (28S e ITS) y uno mitocondrial (*cox1*).

## IV.1 Capítulo 1

Link Between the Adult and the Metacercaria of *Clinostomum heluans* Braun, 1899 (Trematoda: Clinostomidae) Through DNA Sequences, and its Phylogenetic Position within the Genus *Clinostomum* Leidy, 1856.



## Link Between the Adult and the Metacercaria of *Clinostomum heluans* Braun, 1899 (Trematoda: Clinostomidae) Through DNA Sequences, and its Phylogenetic Position Within the Genus *Clinostomum* Leidy, 1856

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- 71** **ABSTRACT:** The phylogenetic position of *Clinostomum heluans* Braun, 1899 within the genus *Clinostomum* Leidy, 1856 is reported in this study based on sequences of the barcoding region of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*COX1*). Additionally, molecular data are used to link the adult and the metacercariae of the species. The metacercariae of *C. heluans* were found encysted infecting the cichlid fish *Australoheros* sp. in Minas Gerais, Brazil, whereas the adults were obtained from the mouth cavity of the Great White Egret, *Ardea alba*, in Campeche, Mexico. The *COX1* sequences obtained for the Mexican clinostomes and the Brazilian metacercaria were almost identical (0.2% molecular divergence), indicating conspecificity. Similar molecular divergence (0.2–0.4%) was found between sequences of *C. heluans* reported here and *Clinostomum* sp. 6 previously obtained from a metacercaria recovered from the cichlid *Cichlasoma boliviense* in Santa Cruz, Bolivia. Both maximum likelihood and Bayesian inference analyses unequivocally showed the conspecificity between *C. heluans* and *Clinostomum* sp. 6, which form a monophyletic clade with high nodal support and very low genetic divergence. Moreover, tree topology reveals that *C. heluans* occupies a basal position with respect to New World species of *Clinostomum*, although a denser taxon sampling of species within the genus is further required. The metacercaria of *C. heluans* seems to be specific to cichlid fish because both samples from South America were recovered from species of this fish family, although not closely related.
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*Clinostomum* Leidy, 1856 is a cosmopolitan genus of digeneans with complex life cycles; adults of this genus infect the mouth cavity and esophagus of fish-eating birds (Kanev et al., 2002). The genus currently contains 16 described species (see Locke et al., 2015; Pérez-Ponce de León et al., 2016; Rosser et al., 2017), although another 14 putative species recognized through DNA sequences remain to be described (Locke et al., 2015; Pérez-Ponce de León et al., 2016; Caffara et al., 2017). *Clinostomum heluans* is one of the species recognized among the New World species of *Clinostomum* and was originally described by Braun (1899) as a parasite of the Little Blue Heron, *Egretta caerulea*, in Brazil. The

DOI: 10.1645/17-183

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balsam. Voucher specimens were deposited either in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM) (adult, CNHE: no. 10665), or in the collection of the Department of Parasitology, Federal University of Minas Gerais (DPIC) (metacercaria, DPIC: no. 6242). Host taxonomy followed Avibase (<https://avibase.bsc-eoc.org>) and FishBase (<http://www.fishbase.org>). For the molecular study, a fragment of the cytochrome *c* oxidase subunit I gene (*COXI*) was sequenced from 3 adults and 1 metacercaria (paragenophores sensu Pleijel et al., 2008). Individual specimens were digested overnight at 56 °C in a solution containing 10-mM Tris-HCl (pH 7.6), 20-mM NaCl, 100-mM Na<sub>2</sub> EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. Total DNA was extracted using the DNeasy reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. A fragment of *COXI* (~474 bp) was amplified using polymerase chain reaction (PCR) with degenerate forward MplatCOX1dF (5'-TGTAACACGACGGCCAGTTTCITTRGATCATAAG-3') and reverse MplatCOX1dR (5'-CAGGAAACAGCTATGACTGAAAYAAAYAHGGATCCACC-3') primers described by Moszczyńska et al. (2009). PCR reactions, cycling conditions, and sequencing reactions followed procedures as in Pérez-Ponce de León et al. (2016). Contigs were assembled and base-calling differences were resolved using Codoncode Aligner version 3.5.4 (Codoncode Corporation, Dedham, Massachusetts). Phylogenetic analyses were conducted with reference to all species of the genus reported from the Americas, including candidate species that have not yet been described (see Locke et al., 2015; Pérez-Ponce de León et al., 2016). Sequences of *Clinostomum complanatum* and *Euclinostomum* sp., as well as those of *Diplostomum baeri*, *Austrodiplostomum* sp., and *Alaria mustelae*, were also included in the analyses and used as outgroups for rooting the trees. Newly generated sequences were deposited in GenBank, accession numbers: MG860852–MG860855. An alignment was built with sequences of species of *Clinostomum* occurring in the Americas, but the sequences of *C. complanatum* and *Euclinostomum* sp. were also included. Three diplostomid species were used as outgroups for rooting the tree. Phylogenetic analyses were run using maximum likelihood (ML) and Bayesian inference (BI) methods, employing the substitution model GTR + G + I for *COXI*. The models of nucleotide evolution were estimated in jModelTest v2 (Darriba et al., 2012). ML inference (100 replicates), model parameters, and bootstrap support (1,000 replicates) were estimated with RAxML v. 7.0.4 (Stamatakis, 2006). MrBayes v. 3.2.1 (Ronquist et al., 2012) was used to perform BI analysis.

Two species of *Clinostomum* were found in our samplings. Morphologically, one corresponds to an undescribed species (*Clinostomum* sp. Lineage 5 after Pérez-Ponce de León et al., 2016), and a second species corresponds to *C. heluans*. The metacercariae were also identified as belonging to *C. heluans*. Both the metacercaria and the adult correspond with *C. heluans* because they possess a relatively large body (between 10 and 20 mm in the adult), 2 testes occupying the posterior third of the body, an intertesticular ovary, and a genital pore located pretesticularly (Braun, 1901; Pérez-Vigueras, 1955; Travassos et al., 1969). The *COXI* sequences obtained for both developmental stages of *C. heluans* evaluated in the present study were almost identical (molecular divergence of 0.2%), confirming the con-

specificity between the metacercariae found in freshwater fishes from Brazil and the adults recovered from fish-eating birds from southeastern Mexico, irrespective of geographic distance (about 7,000 km). The newly generated sequences of *C. heluans* form a monophyletic clade along with the sequence of *Clinostomum* sp. 6 (GenBank KP110534, after Locke et al., 2015), and these relationships are highly supported by bootstrap and posterior probability values found in ML and BI analyses, respectively (Fig. 1). Overall, the genetic divergence between *C. heluans* and *Clinostomum* sp. 6 varies from 0.2 to 0.4%. These results show that *Clinostomum* sp. 6 is conspecific with *C. heluans*. This species is the basal taxon of the New World clade of *Clinostomum* species (Fig. 1), and it seems to be the most widely distributed, since its distribution range extends between Brazil and Bolivia in South American and northern Mexico in Central America. The position of this species as verified in the present study contrasts with that of previous molecular phylogenetic analyses (see Locke et al., 2015; Pérez-Ponce de León et al., 2016); however, corroboration of this hypothesis requires a denser taxon sampling to include other species of *Clinostomum* and also requires other molecular markers. Unfortunately, we were unable to successfully amplify a nuclear marker such as the internal transcribed spacers (ITSs), which are commonly used in phylogenetic analyses of clinostomids. Additionally, Figure 1 shows molecular confirmation that the specimens of *Clinostomum* recovered from *A. alba* and *A. herodias* in this study correspond to the undescribed species referred as Lineage 5 in Pérez-Ponce de León et al. (2016). This species was recorded as a parasite from the mouth cavity of the Bare-Throated Tiger Heron, *Tigrisoma mexicanum*, and the Boat-Billed Heron, *Cochlearius cochlearis*, in several localities across southeastern Mexico (Pérez-Ponce de León et al., 2016). This result is interesting because the metacercariae of *Clinostomum* Lineage 5 seem to be host-specific to cichlid fish across a wide geographic range in Middle America, parasitizing at least 10 cichlid species (see table 1 in Pérez-Ponce de León et al., 2016). The record of *Clinostomum* Lineage 5 in *A. alba* and *A. herodias* from Laguna de Términos, Campeche, Mexico, represents a new locality record, and even though large numbers of cichlids have been studied in that geographic region, metacercariae of *C. heluans* have been not found, even though the adults were obtained from fish-eating birds.

The genetic library for species of clinostomids has steadily increased in recent years, and substantial progress has been made in our understanding of the evolutionary relationships and biogeographical history of this group of fish-eating bird digenaeans (e.g., Caffara et al., 2014, 2016, 2017; Acosta et al., 2016). More importantly, molecular information used in combination with morphology has been instrumental in establishing more robust species limits and increasing our understanding of species diversity within the cosmopolitan genus *Clinostomum* (see Caffara et al., 2011, 2017; Sereno-Urbe et al., 2013; Locke et al., 2015; Pérez-Ponce de León et al., 2016). The molecular information generated in this study was useful in assessing the phylogenetic position of *C. heluans* and establishing a link between the metacercariae and the adults. In a large-scale molecular survey of *Clinostomum*, Locke et al. (2015) used molecular data from 2 molecular markers, *COXI* and ITS, to delineate 8 putative species within the genus. The sequence data of one of them, *Clinostomum* sp. 6, was generated from a specimen of metacercaria collected from the cichlid *C. boliviense* from Santa Cruz, Bolivia.



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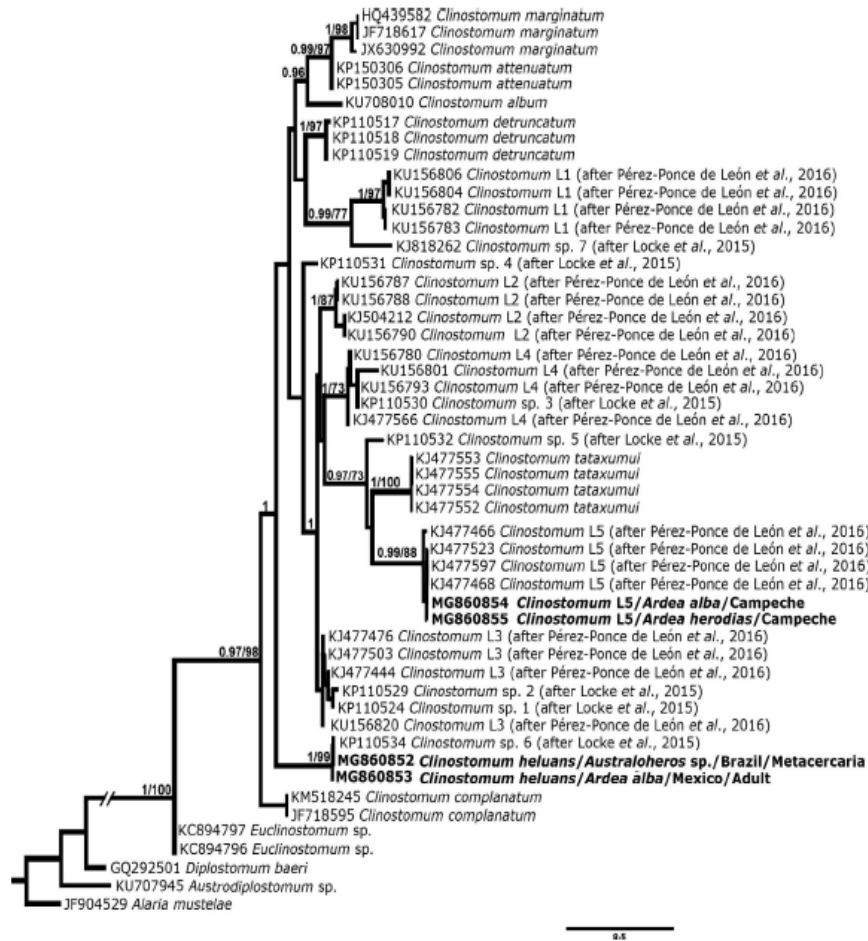


FIGURE 1. Phylogenetic relationships between *Clinostomum heluans* (in bold) and New World *Clinostomum* spp. inferred through sequences of the cytochrome *c* oxidase subunit 1 gene by Bayesian inference (BI) and maximum likelihood (ML) analyses. The numbers above branches represent posterior probability and bootstrap values obtained by BI and ML, respectively. Sequences of *Clinostomum* Lineage 5 (after Pérez-Ponce de León et al., 2016) are also in bold.

Interestingly, our *COXI* sequence of the metacercariae of *C. heluans* was obtained from another species of cichlid, *Australoheros* sp. in Minas Gerais, Brazil, and our data demonstrate that the species uncovered in the Locke et al. (2015) study should be referred as *C. heluans*. *Clinostomum* sp. 6 (after Locke et al., 2015) was recognized molecularly, but, since no morphological traits were reported, it was not possible to correlate the putative species with a previously recognized species such as *C. heluans*. Morphologically, this species is clearly separated from other *Clinostomum* species, even in the metacercarial stage, and our study provided that information. We acknowledge, however, that distinguishing among other metacercarial forms in this group, as well as in other groups of digenans such as diplostomids and strigeids, is practically impossible.

The molecular sequences here obtained for *C. heluans* may also help to elucidate the snail involved in the transmission of the parasite as the first intermediate host. The cercariae found in species of *Biomphalaria* from Brazil, and described by Lutz (1934) as the larvae of *C. heluans*, do not correspond to this species, given the fact that the metacercariae experimentally obtained by Pinto et al. (2015) show sexual structures located between the middle and posterior third of body, as verified in an undescribed species (*Clinostomum* sp. 7 after Locke et al., 2015). Thus, the snail intermediate host and the morphology of the cercariae of *C. heluans* remain unknown. We need to keep collecting molecular data from a wider array of species of *Clinostomum* around the world to fully understand the diversity patterns of this species-rich group of digenans. *Clinostomum heluans* exhibits a wide



geographic range that extends from southern Brazil to northern Mexico, and it possesses a host-specificity pattern that involves several species of ardeids as definitive hosts and cichlids as second intermediate hosts. Additional molecular information associated with morphological assessments of all stages of the life cycle will be instrumental in this endeavor. A large effort has been made in increasing the genetic library of this digenean genus, but molecular data from regions such as Australia and South America are still needed.

The authors thank David Hernández for his help processing samples to obtain DNA. Laura Márquez provided technical assistance with the sequencer. This study was partially funded by the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM IN20617 and IN206716) to G.P.P.L. and M.G.V., respectively. This paper partially fulfills the requirements of the M.Sc. degree for R.B.A. within the Posgrado en Ciencias Biológicas of UNAM. R.B.A. thanks CONACyT for providing a scholarship to complete her M.Sc. program.

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## **Capítulo 2**

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

### 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

--Manuscript Draft--

Manuscript Number:	H4571R1
Full Title:	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
Article Type:	Research Article
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Abstract:	The family Clinostomidae Lühe, 1901 contains 29 species allocated into seven genera; Clinostomum Leidy, 1856 being 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 in the last years, although the information for the other genera included in the family is very scarce or null. 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 of 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 with Ithyoclinostomum, although several differences prevent their inclusion in I. 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.



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3 1 Morphological and molecular characterization of an enigmatic clinostomid trematode (Digenea:  
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5 2 Clinostomidae) parasitic as metacercariae in the body cavity of freshwater fishes (Cichlidae)  
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7  
8 3 across Middle America  
9  
10 4 Rosario Briosio-Aguilar<sup>1,2</sup>, Martín García-Varela<sup>1</sup>, David. I. Hernández-Mena<sup>1</sup>, Miguel Rubio-Godoy<sup>3</sup>,  
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#### 37 15 Abstract

38  
39 16 The family Clinostomidae Lühe, 1901 contains 29 species allocated into seven genera; *Clinostomum*  
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42 17 Leidy, 1856 being the most diverse, with *c.*14 valid species. The diversity of *Clinostomum* has been  
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44 18 assessed combining morphological and molecular data. The genetic library for species in this genus has  
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47 19 increased steadily in the last years, although the information for the other genera included in the family  
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49 20 is very scarce or null. Molecular phylogenetic relationships among the genera of clinostomids have not  
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52 21 been assessed, and their classification is still based on morphological traits. The monotypic  
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54 22 *Ithyoclinostomum* was described from a fish-eating bird in Brazil, and its metacercariae have been  
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57 23 found in several locations of South America, parasitizing erythrinid freshwater fishes. We collected  
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59 24 unusually large metacercariae from the body cavity of cichlids in several locations across Middle

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3 25 America. These metacercariae exhibited some resemblance with *Ithyoclinostomum*, although several  
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5 26 differences prevent their inclusion in *I. dimorphum*, casting doubt on their taxonomic identification.  
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7  
8 27 The main objective of this paper was to characterize the metacercariae collected in cichlids using both,  
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10 28 morphology and molecular data from three molecular markers; and to assess the molecular  
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12 29 phylogenetic relationships among the genera of Clinostomidae to establish the position of the newly  
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14 30 generated sequences. We took a conservative position and tentatively placed the metacercariae as  
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16 31 belonging to *Ithyoclinostomum*.  
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## 22 33 Introduction

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25 34 Members of the cosmopolitan family Clinostomidae Lühe, 1901 are parasites of the buccal cavity,  
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27 35 oesophagus or intestine of birds, reptiles, and occasionally mammals (Ukoli, 1966; Kanev *et al.*, 2002).  
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29  
30 36 The taxonomic status and phylogenetic relationships among the genera allocated into this family have  
31  
32 37 been uncertain. Currently, the family comprises seven genera included in four subfamilies according to  
33  
34 38 Kanev *et al.* (2002): Clinostominae Lühe, 1901 with the cosmopolitan genus *Clinostomum* Leidy, 1856  
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36  
37 39 containing *c.* 14 species parasitizing fish-eating birds as definitive hosts (see Locke *et al.*, 2015; Pérez-  
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39 40 Ponce de León *et al.*, 2016; Caffara *et al.*, 2017), *Clinostomatopsis* Dollfus, 1932 (two species), and the  
41  
42 41 monotypic *Clinostomoides* Dollfus, 1959; Euclinostominae Yamaguti, 1958, with *Euclinostomum*  
43  
44 42 Travassos, 1928 containing eight species parasitizing birds (Caffara *et al.*, 2016); Nephrocephalinae  
45  
46  
47 43 Travassos, 1928, with two genera parasitic in crocodylians, i.e., *Odhneriotrema* Travassos, 1928 and  
48  
49 44 *Nephrocephalus* Odhner, 1902, with two and one species, respectively (Woodyard *et al.*, 2017); and  
50  
51  
52 45 Ithyoclinostominae Yamaguti, 1958, with the monotypic *Ithyoclinostomum dimorphum* (Diesing, 1850)  
53  
54 46 Witenberg, 1925, as a parasite of birds. Even though great progress has been made in recent years to  
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56  
57 47 increase the generic library for species of *Clinostomum*, the most species-rich genus in the family, in  
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59 48 studies aimed at establishing robust species delimitation criteria, few attempts have been made to  
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3 49 generate sequence data to perform a phylogenetic analysis at the family level (see Woodyard *et al.*,  
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5 50 2017).  
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8 51 During survey work on the helminth fauna of Middle American freshwater fishes, unusually  
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10 52 large clinostomid metacercariae were found unencysted in the body cavity of some cichlid species in  
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12  
13 53 several locations of Mexico and Costa Rica. Based on the size of the metacercariae and the position of  
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15 54 the genital complex in the posterior fourth of the body, we first identified the species as *Clinostomum*  
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18 55 *heluans* Braun, 1899. An additional piece of information was that *C. heluans* had been previously  
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20 56 recorded as a parasite of the great blue heron, *Ardea herodias* Linnaeus, 1758 in northeastern Mexico  
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22 57 (Bravo-Hollis, 1947). In a recent study, Briosio-Aguilar *et al.* (2018) characterized molecularly the  
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25 58 metacercariae of *C. heluans*; in addition, these authors established a molecular link between the  
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27 59 metacercariae and the adults and determined that the distributional range of the species extends  
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30 60 between northern Mexico and Brazil. However, once we obtained ribosomal and mitochondrial DNA  
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32 61 sequences of the unusually large clinostomid metacercariae, we discovered that they are not conspecific  
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35 62 with *C. heluans*. The objective of this paper is two-fold, to characterize morphologically and  
36  
37 63 molecularly the clinostomid metacercariae found in Middle American cichlids, and to accomplish their  
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40 64 identification at genus level establishing their phylogenetic position within the phylogeny of the family  
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42 65 Clinostomidae.

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## 46 67 **Materials and methods**

### 49 68 *Specimen collection*

50  
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52 69 Specimens of metacercariae were sampled between 2014 and 2016 in six species of cichlids from six  
53  
54 70 localities, four in Mexico and two in Costa Rica (table 1). In total, 45 individual fish were collected  
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56  
57 71 using seine nets and electrofishing, kept alive and transported to the laboratory, pith sacrificed, and  
58  
59 72 examined for parasites under a stereomicroscope. Some specimens were fixed by sudden immersion in  
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3 73 hot (near boiling) 4% formalin, subsequently washed in distilled water and stored in 70% ethanol; some  
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5 74 specimens were also preserved in vials with 100% ethanol for molecular analysis.  
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8 75 *Morphological study*  
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10 76 For morphological identification, 29 specimens (14 whole specimens and 15 hologenophores) were  
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12 77 stained with Mayer's paracarmine, dehydrated in a graded ethanol series, cleared with methyl  
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15 78 salicylate, and mounted on permanent slides with Canada balsam. Voucher specimens were deposited  
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18 79 at the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional  
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20 80 Autónoma de México, Mexico City. All the specimens were examined using a bright-field Zeiss Axio  
21  
22 81 Zoom V16 microscope. Images were obtained through an Axio Cam Mrc5 attached to the microscope,  
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24  
25 82 and specimens were measured using the software ZEN-Zeiss Efficient Navigation; measurements are  
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27 83 presented in millimeters with the range followed by the mean in parenthesis. Drawings of the  
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30 84 metacercariae were made using a drawing tube attached to the microscope. For the scanning electron  
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32 85 microscopy (SEM) study, two specimens were dehydrated through a graded series of ethyl alcohol, and  
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34  
35 86 then critical-point dried with carbon dioxide, mounted on metal stubs with silver paste, coated with  
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37 87 gold, and examined in a Hitachi Stereoscan model SU1510 (Hitachi High-Technologies Mexico S.A.de  
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40 88 C.V, Mexico) at 15 kV.

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42 89 *DNA extraction, amplification and sequencing*  
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44 90 Seventeen specimens were placed individually in tubes and digested overnight at 56 °C in a solution  
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46  
47 91 containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM Na<sub>2</sub> EDTA (pH 8.0), 1% Sarkosyl, and  
48  
49 92 0.1 mg/ml proteinase K. DNA was extracted from the supernatant using the DNAzol (Molecular  
50  
51  
52 93 Research Center, Cincinnati, Ohio). Two regions of nuclear ribosomal DNA (rDNA), and the  
53  
54 94 mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) were amplified *via* the polymerase chain  
55  
56  
57 95 reaction (PCR). The ITS1, 5.8S and ITS2 region was amplified using the forward primer BD1, 5'-  
58  
59 96 GTCGTAACAAGGTTTCCGTA-3' and the reverse primer BD2, 5'-  
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61 97 ATCTAGACCGGACTAGGCTGTG-3' (Luton *et al.*, 1992). The D1-D3 domains of the 28S rRNA

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3 98 gene were amplified using the primers 502 5'-CAAGTACCGTGAGGGAAAGTTGC-3' (forward) and  
4  
5 99 536 5'-CAGCTATCCTGAGGGAAA-3' (reverse) (García-Varela & Nadler, 2005). The mitochondrial  
6  
7  
8 100 *cox1* was amplified using the forward primer MplatCOX1dF 5'-  
9  
10 101 TGTA AACGACGGCCAGTTTWCITTRGATCATAAG-3' and the reverse primer MplatCOX1dR  
11  
12 102 5'-CAGGAAACAGCTATGACTGAAAYAAAYAIIGGATCICCACC-3' (Moszczyńska *et al.*, 2009).  
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15 103 PCR reactions (25 µl) consisted of 10 µM of each primer, 2.5 µl of 10 X buffer, 1.5 µl of 2 mM MgCl<sub>2</sub>,  
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17 104 0.5 µl of dNTP's (10 mM), 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São  
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20 105 Paulo, Brazil) plus 2 µl of the genomic DNA plus 16.7 µl of distilled water. PCR cycling parameters  
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22 106 for rDNA amplifications included denaturation at 94 °C for 5 min; followed by 35 cycles of 94 °C for 1  
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24 107 min, annealing at 50°C for 1 min for the three molecular markers, and extension at 72 °C for 1 min,  
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27 108 followed by a post-amplification incubation at 72 °C for 10 min. PCR products for ITS1, 5.8S, ITS2  
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30 109 and 28S rDNA were sequenced with the PCR primers plus the internal primers BD3 5'-  
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32 110 GAACATCGACATCTTGAACG-3' and BD4 5'-ATAAGCCGACCCTCGGC-3' (Hernandez-Mena *et*  
33  
34 111 *al.*, 2013) and 503 5'-CCTTGGTCCGTGTTCAAGACG-3' (forward) (Stock *et al.*, 2001) and 504 5'-  
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37 112 CGTCTTGAAACACGGACTAAGG-3' (reverse) (García-Varela & Nadler, 2005), respectively.  
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40 113 Sequencing reactions were performed using ABI Big Dye (Applied Biosystems, Boston,  
41  
42 114 Massachusetts) terminator sequencing chemistry and reaction products were separated and detected  
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44 115 using an ABI 3730 capillary DNA automated sequencer. Contigs were assembled and base-calling  
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46  
47 116 differences resolved using Codoncode Aligner version 6.0.2 (Codoncode Corporation, Dedham,  
48  
49 117 Massachusetts). Sequences obtained in the current research for ITS, 28S and *cox1* were aligned with  
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51 118 sequences of other genera of clinostomids downloaded from GenBank. Three alignments were built.  
52  
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54 119 For ITS and *cox1* alignments, newly generated sequences were aligned along with one to three  
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57 120 representative sequences of 23 species/lineages of *Clinostomum*, plus *Odhneriotrema incommodum*  
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59 121 (Leidy, 1856) and *Euclinostomum heterostomum* (Rudolphi, 1908). For 28S, an alignment was built  
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3 122 considering two newly generated sequences in addition to six validated species of *Clinostomum*, plus  
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5 123 newly generated sequences of five genetic lineages of the genus, and one sequence of *Clinostomoides*,  
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7  
8 124 two of *Euclinostomum* and one of *Odhneriotrema*. In addition, sequences of the diplostomids  
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10 125 *Diplostomum baeri* Dubois, 1937, and *Alaria marciana*e (La Rue, 1917) Walton, 1949 were used as  
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12 126 outgroups for rooting the trees. A fourth alignment was built to perform a concatenated analysis of  
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14 127 28S-ITS-cox1. Due to the number of sequences available for the 28S rRNA gene, the concatenated  
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16 128 analysis was performed considering sequences of six validated species of *Clinostomum*, plus those of  
17  
18 129 five genetic lineages, two of *Euclinostomum*, one of *Odhneriotrema*, with *Alaria marciana*e as  
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20 130 outgroup.

### 21 131 *Phylogenetic analyses*

22 132 Sequences were aligned with the software Clustal Omega (Sievers *et al.*, 2011), implemented in the  
23  
24 133 website <https://www.ebi.ac.uk/Tools/msa/clustalo/>. Phylogenetic analysis for each data set was run  
25  
26 134 under Bayesian Inference (BI) and Maximum Likelihood (ML), employing the nucleotide substitution  
27  
28 135 model GTR+GAMMA, which was calculated in the program jModelTest v2.1.10 (Darriba *et al.*, 2012).  
29  
30 136 The concatenated analysis was also run under ML and BI. Bayesian inference was performed in  
31  
32 137 MrBayes v. 3.2.6 (Ronquist *et al.*, 2012), running two independent MCMC of four chains each run  
33  
34 138 (heating parameter= 0.5) for 10 million generations and sampling trees every 1000 generations  
35  
36 139 (printfreq=1000 samplefreq=1000 diagnfreq=10000), and Burn-in periods were set to the first 2500  
37  
38 140 generations. A 50% majority-rule consensus tree and nodal support (posterior probability values) were  
39  
40 141 calculated from the remaining trees. ML inference (100 replicates), model parameters and bootstrap  
41  
42 142 support (1,000 replicates) were estimated with RAxML v. 8.2.X (Stamatakis, 2014). Phylogenetic trees  
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44 143 obtained from the analysis were visualized in FigTree v.1.4.3. (Rambaut, 2016). Molecular divergence  
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46 144 for all markers was estimated using uncorrected *p* distances (*p*-distances) with the software PAUP\*  
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48 145 4.0a (Swofford, 2002).

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3 147 Results  
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5 148 Morphology  
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8 149 The clinostomid metacercariae were identified as members of the monotypic genus *Ithyoclinostomum*  
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10 150 Witenberg, 1925, based on body size, the presence of oral collar, simple caeca lacking diverticula,  
11  
12 151 gonads being located in the posterior fourth of body, and having a pre-testicular genital pore. Even  
13  
14 152 though body size was variable among collected individuals (11.6–29.5 mm), the position, size and  
15  
16 153 shape of internal organs in proportion to the body length was very similar, particularly for characters  
17  
18 154 related with the reproductive organs (fig. 1).  
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22 155 *Ithyoclinostomum* sp.  
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24 156 (Metacercariae)  
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26 157 (figs. 2, 3a-d)  
27  
28 158 *Hosts*: *Mayaheros urophthalmus* (Günther, 1862), *Vieja melanura* (Hubbs, 1935), *Herichthys deppii*  
29  
30 159 (Heckel, 1840), *Cribroheros longimanus* (Günther, 1867), *Cribroheros alfari* (Meek, 1907),  
31  
32 160 *Parachromis managuensis* (Günther, 1867).  
33  
34  
35 161 *Localities*: Mexico–Gregorio Méndez, Tabasco (Grijalva River basin); Naha, Chiapas (Usumacinta  
36  
37 162 River basin); Nautla (Nautla River basin); Filipinas Creek (Nautla River basin). Costa Rica–Irigaray  
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39 163 River at Liberia, Guanacaste; Orosi River, Guanacaste; Pithaya Creek (Orosi River basin), Guanacaste.  
40  
41  
42 164 *Specimens deposited*: Colección Nacional de Helmintos (CNHE), vouchers 10716-10721.  
43  
44 165 *Representative DNA sequences*: MH159738-MH159752 (*cox1*), MH159753-MH159770 (*ITS*),  
45  
46 166 MH159736-MH159737 (28S)  
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49 167 *Description*. (Based on measurements of 14 specimens; measurements in table 2): Body large, elongate  
50  
51 168 and widest in equatorial region of body. Tegument surface lacking spines. Anterior end forming an oral  
52  
53 169 collar; oral collar with a ventral constriction. Oral sucker rounded, terminal, smaller than ventral  
54  
55 170 sucker, embedded in oral collar. Prepharynx absent. Pharynx well-developed leading immediately to  
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57  
58 171 intestinal bifurcation. Intestinal caeca run laterally to posterior end of body. Ventral sucker rounded, in  
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3 172 anterior fourth of body; triangular aperture. Testes in tandem, in posterior fourth of body, irregularly  
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5 173 shaped, with smooth margins, entirely intracaecal. Anterior testis H-shaped, with anterior lobes longer  
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7  
8 174 than posterior ones. Posterior testis X-shaped, forming four lobes of same size; inter-testicular space  
9  
10 175 wide. Cirrus-sac ovoid, overlapping anterior testis in dextral position. Genital pore pre-testicular, in  
11  
12 176 mid-level of body. Ovary small, rounded, smooth, located in inter-testicular space on right side of  
13  
14  
15 177 body. Uteroduct emerging from ootype, running around left margin of anterior testis to form tubular  
16  
17 178 uterine sac, well-developed, extending anteriorly to short distance from posterior border of ventral  
18  
19  
20 179 sucker, descending straight into genital pore; metraterm not observed. Vitellaria undeveloped.  
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25 181 Taxonomic remarks

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27 182 The metacercariae sampled from Middle American cichlids correspond in general with the diagnosis of  
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29  
30 183 the genus *Ithyoclinostomum* following Kanev *et al.* (2002). Our specimens possess a large and elongate  
31  
32 184 body with a small oral sucker surrounded by an oral collar, a considerable space free of internal organs  
33  
34 185 between ventral sucker and anterior testis, simple, long caeca without lateral diverticula, gonads in the  
35  
36  
37 186 posterior fourth of body, and inter-testicular ovary. Overall comparison of morphometric characters  
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40 187 showed that our specimens are very similar to those described for *I. dimorphum* (table 2); this species  
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42 188 was found in some locations across South America (see Benigno *et al.*, 2014; Costa *et al.*, 2015). In  
43  
44 189 addition, the ultrastructure of the body surface of *I. dimorphum* was previously studied by Dias *et al.*  
45  
46  
47 190 (2003) in adults obtained from *Ardea cocoi* (L.), and by Benigno *et al.* (2014) in metacercariae from  
48  
49 191 *Hopleryttrinus unitaeniatus*, both in Brazil. Our SEM specimens (fig. 3) are similar, except by the fact  
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51  
52 192 that they possess a triangular shaped ventral sucker aperture and a well-defined constriction of the oral  
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54 193 collar (see figs. 3a, 3c). Moreover, two main facts prevented us from identifying our specimens as the  
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57 194 monotypic *I. dimorphum*. First, we relied solely on the morphology of the metacercarial stage, since we  
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59 195 did not collect the adults from their definitive hosts. Second, our specimens were morphologically  
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61 196 different from *I. dimorphum* regarding some traits. For instance, some differences were found on the

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3 197 overall body shape, testes size and shape, and the position of the genital pore between *I. dimorphum*  
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5 198 and our specimens (table 2). In *I. dimorphum*, the body is long and slender, and testes are small and  
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7  
8 199 irregularly shaped, while in our specimens, the body is elongate and robust, testes are well-developed,  
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10 200 and their form is well-defined, the anterior testis is H-shaped and the posterior testis is X-shaped. The  
11  
12 201 cirrus-sac in our specimens is located in the mid-level of the anterior testis, and the genital pore is  
13  
14 202 medial; in *I. dimorphum* the cirrus-sac is dextral to the right margin of the anterior testis, and the  
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16 203 genital pore is dextrally located. A striking difference was found in the body length/body width ratio.  
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19 204 On average, in our specimens the ratio is 3.8, while in *I. dimorphum* the ratio is around 18 (see table 2).  
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#### 23 206 *Molecular data*

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25 207 Phylogenetic analyses inferred with three independent datasets (*cox1*, ITS, 28S) through BI and ML  
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27 208 unequivocally recovered all the sequenced metacercariae as a monophyletic assemblage, with high  
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30 209 bootstrap and posterior probability values (figs. 4-6). However, the three datasets yielded different  
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32 210 topologies regarding the position of *Ithyoclinostomum* sp. with respect to other clinostomids for which  
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35 211 DNA sequences are available.  
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39 212 *Cox1*. The alignment was 474 bp long and consisted of 15 newly sequenced metacercariae, two  
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42 213 sequences of *Euclinostomum heterostomum*, three of *Odhneriotrema incommodum*, and one or two  
43  
44 214 replicates of each of the 11 valid species of *Clinostomum*, plus one or two replicates of each of the 12  
45  
46  
47 215 genetic lineages of *Clinostomum* not yet described. The phylogenetic analysis suggested that the  
48  
49 216 metacercariae of *Ithyoclinostomum* sp. occupy a basal position with respect to the other genera of  
50  
51 217 clinostomids, as follows: [*Ithyoclinostomum* sp. (*Euclinostomum* (*Odhneriotrema* (*Clinostomum*)))]  
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54 218 (fig. 4).  
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56  
57 219 ITS. The alignment was 1142 bp long and consisted of 17 newly sequenced metacercariae, two  
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59 220 sequences of *Euclinostomum heterostomum*, two of *Odhneriotrema incommodum*, and one or two  
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3 221 replicates of each of the 11 valid species of *Clinostomum*, plus two replicates of each of the 12 genetic  
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5 222 lineages of *Clinostomum* not yet described. The phylogenetic analysis showed that the metacercariae of  
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8 223 *Ithyoclinostomum* sp. are recovered as the sister group of the genus *Odhneriotrema*, as follows:  
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10 224 [*Euclinostomum* (*Odhneriotrema* + *Ithyoclinostomum* sp. (*Clinostomum*))] (fig. 5).

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13 225 28S. The alignment was 1414 bp long, and only included two newly generated sequences of  
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15 226 *Ithyoclinostomum* sp., two sequences of *Euclinostomum heterostomum*, one of *Odhneriotrema*  
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17 227 *incommodum*, one or two replicates of six valid species of *Clinostomum* for which this molecular  
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20 228 marker has been sequenced and an unidentified species from Australia, two replicates of five newly  
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22 229 sequenced genetic lineages of *Clinostomum*, and one sequence of *Clinostomoides brieni* Dollfus, 1950.  
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24  
25 230 The phylogenetic analysis revealed that the metacercariae of *Ithyoclinostomum* sp. were recovered as  
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27 231 the basal group of the Clinostomidae as follows: [*Ithyoclinostomum* (*Odhneriotrema* (*Euclinostomum*  
28  
29 232 (*Clinostomum*))] (fig. 6). In this analysis, *C. brieni* nests within the group of *Clinostomum* species that  
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32 233 occur in the old world.

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34 234 Concatenated analysis (*cox1*+*ITS*+*28S*). Since the mitochondrial gene, and the two ribosomal  
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37 235 genes yielded different topologies regarding the position of the newly generated sequences in the  
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39 236 phylogeny of Clinostomidae, a fourth alignment was built for a concatenated analysis of the three  
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42 237 molecular markers through BI and ML. The final alignment was 3094 bp long and included fewer  
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44 238 representative sequences of clinostomids; only species or lineages with sequences of the three markers  
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47 239 were included in the analysis. Alignment consisted of two sequences of *Ithyoclinostomum* sp., two of  
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49 240 *Euclinostomum*, one of *Odhneriotrema*, one or two replicates of six valid species of *Clinostomum*, and  
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51  
52 241 two replicates of five genetic lineages of *Clinostomum*. The concatenated tree recovered  
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54 242 *Odhneriotrema incommodum* as the basal member of the group, as the sister taxon of the metacercariae  
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56 243 of *Ithyoclinostomum* sp. plus *Euclinostomum* and *Clinostomum*, as follows: [*Odhneriotrema*  
57  
58 244 (*Ithyoclinostomum* sp. (*Euclinostomum* (*Clinostomum*))] (fig. 7).

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3 245 Genetic divergence. The genetic distance estimated through uncorrected *p* distances showed a  
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5 246 high divergence levels between the sequences of *Ithyoclinostomum* sp. and the other clinostomids for  
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8 247 the three molecular markers. On average, for the 28S rRNA gene, our metacercariae varied from  
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10 248 *Euclinostomum*, *Odhnerioretrema* and *Clinostomum* from 3.69-9.97%, 7.78%, and 6.02-7.92%,  
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12  
13 249 respectively; for ITS, they varied 19.72%, 15.82%, and 17.15-20.14%, and for *cox1* divergence values  
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15 250 varied 18.14%, 19.62%, and 16.66-23.62% with respect to the species included in the aforementioned  
16  
17 251 genera. Intraspecific divergence among isolates of our metacercariae was very low or null (0.00-0.85%  
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19 252 for *cox1*, 0.00-0.09% for ITS, and 0.0% for 28S), indicating that irrespective of body size (see fig. 1)  
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21 253 and geographical location (Mexico or Costa Rica), all metacercariae are conspecific.  
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## 27 255 Discussion

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29 3256 The morphology of the specimens reported in our study resembled that of members of the genus  
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31 3257 *Ithyoclinostomum*. Molecular results confirm that our specimens formed a monophyletic assemblage  
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33 3258 and that they represented an independent genetic lineage, not closely related with species of  
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35 3259 *Clinostomum*, the most species-rich genus within the family Clinostomidae; moreover, the  
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37 3260 phylogenetic relationships of the metacercariae with other clinostomids remain uncertain, since the  
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39 3261 three molecular markers used in this study resolved contradictory sister-group relationships (figs 4-6).  
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41 3262 Even though we hypothesized that the metacercariae recovered from Middle American cichlids belong  
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43 3263 in the genus *Ithyoclinostomum*, morphologically, they cannot be considered conspecific with *I.*  
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45 3264 *dimorphum*. Several differences showed that our specimens may actually represent an undescribed  
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47 3265 species. In addition to morphology, three pieces of information were useful to complement the species  
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49 3266 differentiation: habitat, host-specificity, and geographic distribution. In terms of habitat, records of *I.*  
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51 3267 *dimorphum* as a metacercaria in most of locations of South America indicate the body cavity  
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53 3268 (mesentery) of their hosts as the preferential habitat, and more rarely metacercariae were found in the  
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369 musculature, opercula, stomach, and gills. It is unclear, however, if the metacercariae were always  
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570 encysted in the different habitats where they occurred. For instance, Szidat (1969) found metacercariae  
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871 encysted in the gill arches of their hosts in Tucumán, Argentina; Gallio *et al.* (2007) recovered them  
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1072 encysted in the musculature of their hosts in Rio Grande Do Sul, Brazil; and Belei *et al.* (2013) found  
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1273 them encysted in the visceral cavity of their hosts in Minas Gerais, Brazil. In contrast, some authors  
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1574 reported the metacercariae of *I. dimorphum* from the mesentery/musculature, coelomic cavity/stomach,  
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1775 and body cavity of their fish hosts, although none of them refer specifically if the metacercariae were  
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2076 encysted or not (Benigno *et al.*, 2014; Costa *et al.*, 2015; Delgado *et al.*, 2017). In our study, the  
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2277 metacercariae of *Ithyoclinostomum* sp. were exclusively found in the body cavity of their cichlid hosts  
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2578 and in all cases, they were unencysted, and actively moving when collected.  
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2779       Furthermore, geographic distribution and host specificity patterns were also used to differentiate  
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3080 *I. dimorphum* from the specimens sampled in this study. The metacercariae of *I. dimorphum* shows a  
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3281 strong host specificity since they have only been reported from three species of Characiform fishes  
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3582 (Family Erythrinidae Valenciennes, 1847), including *Hoplias malabaricus* Bloch, 1794, *H. intermedius*  
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3783 (Günther, 1864), and *Hoplerythrinus unitaeniatus* (Spix & Agassiz, 1829) in Brazil, Peru, and Argentina  
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4084 (Travassos *et al.*, 1969; Szidat, 1969; Pavanelli *et al.*, 1990; Weiblen & Brandao, 1992; Paraguassú &  
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4285 Luque, 2007; Gallio *et al.*, 2007; Belei *et al.*, 2013; Benigno *et al.*, 2014; Costa *et al.*, 2015; Delgado *et*  
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4486 *al.*, 2017). Additionally, the species has been found also as a parasite of *Schizodon borreli* (Boulenger,  
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46  
4787 1900) in the Paraná River, Brazil (Machado *et al.*, 1996), a member of the Anostomidae Günther, 1864.  
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4988 Erythrinids and Anostomids are both Characiforms, although they are not closely related (see Oliveira  
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5289 *et al.*, 2011). In this sense, *I. dimorphum* seems to be restricted to these particular groups of  
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5590 characiforms in South America. In contrast, our specimens are host-specific to cichlids and are  
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5791 apparently restricted to Middle America; they have been only found unencysted in the body cavity of  
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5992 their hosts.  
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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, correspond fully with the morphology of the metacercariae we describe in this study (see table 2), even though the single specimen collected by these 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, since their specimens were hardened due to fixation in formalin, the body was macerated and these 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 are well-developed, no evidence of the development of the uterine sac is observed.

The 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 since a generic diagnosis was not provided, these authors formally described the genus for the first time. After first described, *I. dimorphum* has been reported also as a parasite of *Ardea alba* (Linnaeus, 1758), *Nycticorax nycticorax* (Linnaeus, 1758), and *Tigrisoma lineatum* (Boddaert, 1783) (see Benigno *et al.*, 2014; Fernandez *et al.*, 2015). The genus *Ithyoclinostomum* is unique among clinostomids because of their body size: they are “the largest species among the so far known Clinostomidae, reaching sizes which are rarely encountered among the trematodes: 60-100 mm” (Braun, 1901). *Clinostomum heluans* Braun, 1899, a species widely

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317 distributed in the Americas as a parasite of fish-eating birds (Briosio-Aguilar *et al.*, 2018), is also  
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518 characterized by its larger size, although it is not as large as *I. dimorphum*; adults of *C. heluans* are  
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819 usually 15–20 mm long (see Werneck *et al.*, 2017 and references therein); likewise, the specimens of  
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1020 *C. heluans* reported by Bravo-Hollis (1947) from the buccal cavity of *Ardea herodias* (Linnaeus, 1758)  
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1321 in northern Mexico are probably the largest reported, reaching between 20.7 and 26.1 mm. The report  
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1522 by Bravo-Hollis (1947) led us to consider, initially, that our specimens corresponded with *C. heluans*  
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1823 since they were 11.6–29.5 mm long; also, our samples coincided with the geographic range of the  
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2024 species (Briosio-Aguilar *et al.*, 2018). However, morphologically, our specimens resembled more  
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2225 closely *Ithyoclinostomum* than *C. heluans*; the molecular evidence gathered in this study, unequivocally  
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2526 confirmed that our specimens did not nest within the clade formed by *Clinostomum* species.  
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2727 Metacercariae of *I. dimorphum* are variable in length, since they reach 15–50 mm long (see table 2). On  
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3028 average, our specimens lie within the length range of that species, although in the lower limit (18.7  
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3229 mm), and as mentioned above, the body length/width ratio is much greater in *I. dimorphum*, which  
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3530 means that specimens of that species are more elongated.

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3731 The definitive hosts of the metacercariae herein characterized still remain unknown.  
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4032 Considering the most parsimonious explanation of sister-group relationships among clinostomids  
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4233 shown by the concatenated phylogenetic analysis (fig. 7), the possibility that crocodilians are the  
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4434 definitive hosts of this trematode cannot be ruled out, especially since we have analyzed for helminths  
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4735 around 377 fish-eating bird individuals in the last few years, including species of ardeids, pelicans,  
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4936 cormorants, anhingids, and theskiornithids, and we have been unable to recover adult parasites  
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5237 corresponding with the species we characterize in this study. We have to keep looking at a wider  
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5438 diversity of birds and whenever possible at crocodilians to try to obtain adult forms of this trematode  
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5739 species. Previous classification schemes of the family Clinostomidae considered *Ithyoclinostomum* to  
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5940 be part of the subfamily Clinostominae (see Skrjabin, 1947; Feizullaev and Mirzoeva, 1983) or to the  
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6141 monotypic subfamily Ithyoclinostominae (see Yamaguti, 1971); these classifications included only

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342 species whose adults were found in birds. The current classification scheme (Kanev *et al.*, 2002) also  
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543 include the subfamily Nephrocephalinae in the family Clinostomidae; their members are parasites of  
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344 crocodilians. Our molecular phylogenetic analysis provided additional support to the classification  
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1045 scheme proposed by Kanev *et al.* (2002) which is based solely on morphology.  
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1346 Since our specimens did not conform entirely to the diagnosis of *I. dimorphum* and *C. heluans*,  
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1547 we compared our material with the other genera included in separate subfamilies of the family  
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1748 Clinostomidae (see Kanev *et al.*, 2002). Molecularly, our specimens were closely related to the genera  
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2049 *Euclinostomum* and *Odhneriotrema*, the only members other than *Clinostomum* for which sequences  
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22350 are available in GenBank; genetic divergence values and reciprocal monophyly indicated that our  
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2551 specimens were independent evolutionary significant units. Morphologically, species included in these  
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27352 genera are different. *Euclinostomum* is the only genus in the subfamily Euclinostominae (Kanev *et al.*,  
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29  
3053 2002); adults are parasites of ardeids and metacercariae are commonly found in cichlids in the old  
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32354 world (see Caffara *et al.*, 2016); adults and metacercariae of species of *Euclinostomum* are  
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34  
3555 characterized by having 6-15 blind diverticula extending latero-posteriorly to the main ceca (Kanev *et*  
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37356 *al.*, 2002; Caffara *et al.*, 2016); this character, and the body size of the organisms are the main traits  
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4057 that distinguish our specimens of *Ithyoclinostomum* sp. from the species in that genus. Species of the  
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42358 genus *Odhneriotrema*, a member of the Nephrocephalinae along with *Nephrocephalus*, are parasites of  
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4459 the buccal cavity and oesophagus of crocodilians in the Americas. They differ from *Ithyoclinostomum*  
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4760 sp. in the small size of the oral sucker, the position of the ovary and cirrus-sac with respect to testes, the  
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49361 size of the cirrus-sac, and the large inter-testicular space.  
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5262 The genus *Clinostomatopsis* Dollfus, 1932 belongs to the Clinostomatinae (Kanev *et al.*, 2002),  
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54363 and shows some resemblance with the metacercariae from the present study because of the lobated  
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5764 nature of the testes. Unfortunately, no sequence data was available for this species, and the comparison  
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59365 relied solely on morphology. Currently, the genus contains two species known to parasitize the  
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366 oesophagus of Neotropical birds (Lunaschi & Drago, 2009): *Clinostomatopsis intermedialis* Lamont,  
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567 1920, recorded as a parasite of cormorants, *Phalacrocorax brasilianus* (Gmelin, 1789) from Venezuela  
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368 (Lamont, 1920), and *P. penicillatus* (Brandt, 1837) from northeastern Mexico (Bravo-Hollis, 1947).  
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1369 The second species, *Clinostomatopsis sorbens* (Braun, 1899) Dollfus, 1932, is a parasite of *Mycteria*  
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1370 *americana* (Linnaeus, 1758), *Ardea coccoi* and *Jabiru mycteria* (Lichtenstein, 1819) in Brazil (see  
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1371 Benigno *et al.*, 2014; Fernandes *et al.*, 2015, and references therein) and has been found in *Tigrisoma*  
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1372 *lineatum* in Argentina (Lunaschi & Drago, 2009). Metacercariae was recorded from the mesentery of  
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2373 the erythrinids *H. malabaricus* and *H. unitaeniatus* in Brazil by Benigno *et al.* (2014), who included  
21  
2374 SEM micrographs of the specimens. The metacercariae of *C. sorbens* are different from the ones we  
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24  
2375 characterize in this study because they are relatively smaller (<10 mm), they have an inter-testicular  
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2376 cirrus-sac and genital pore (which is a diagnostic trait for the genus), and even though they possess  
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3377 deeply lobed testes, testes shape is very different; anterior testis formed by five irregular lobes, and  
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3378 posterior testis by six lobes, two directed anteriorly, and four directed posteriorly (see Figure 1 in  
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3379 Benigno *et al.*, 2014). Finally, the metacercariae of *Ithyoclinostomum* sp. are also different from the  
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3380 monotypic *Clinostomoides*. *Clinostomoides brieni* Dollfus, 1950 infects the oesophagus of herons and  
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3381 their metacercariae are more commonly found in siluriform fishes in Asia and Africa (Kanev *et al.*,  
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4382 2002); this species is characterized by possessing an elongated body and gonads are also located in the  
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4383 posterior fourth of the body; however, it can be easily distinguished from our metacercariae because the  
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4384 cirrus-sac is inter-testicular and the genital pore is post-testicular according with the diagnosis by  
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3385 Kanev *et al.* (2002), although a recent description of the metacercariae from *Clarias gariepinus*  
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5386 (Burchell, 1822) from Botswana, Africa (van Rensburg *et al.*, 2013) refers to a “genital pore just  
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5387 submedian to right, immediately anterior to posterior testis”. The fact that the sequenced specimen of  
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5388 this species (from a metacercariae obtained from a siluriform fish in India –KF781299– as a direct  
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5389 submission) is nested within a group of species of *Clinostomum* that includes the cosmopolitan *C.*  
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390 *complanatum*, indicates a possible misidentification, and requires further verification.



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391 In conclusion, the metacercariae characterized in this study were included in the genus  
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592 *Ithyoclinostomum* because they share some morphological traits; however, the inclusion of our  
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893 specimens in the genus is tentative until sequences of the adults or metacercariae of the species *I.*  
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1094 *dimorphum* are provided from specimens sampled from their natural distributional range in South  
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1395 America. Molecular data will provide confirmation or rejection of this hypothesis. Even if our  
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1596 specimens belong to a different genus, they represent an undescribed species; to accomplish the proper  
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1797 species description, and naming of the species, we need to collect adult forms from their definitive  
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1998 hosts, either fish-eating birds or crocodilians, and characterize them morphologically and molecularly,  
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2299 to establish a link between the larval forms and the adults in their definitive hosts.  
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26  
27401 **Acknowledgements.** Thanks are due to Luis García for the loan of specimens from the Colección  
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29  
30402 Nacional de Helminthos (CNHE, Mexico City). We also thank Susana Guzmán and Berenit Mendoza-  
31  
32403 Garfias for their assistance with the image acquisition through light microscopy, scanning electron  
33  
34404 microscopy, respectively. Laura Márquez provided technical assistance with the sequencer. We also  
35  
36  
37405 thank Arturo Angulo from the Universidad de Costa Rica for support to sample freshwater fish in Costa  
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39406 Rica.  
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44408 **Financial support.** This work has been supported by the Programa de Apoyo a Proyectos de  
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46  
47409 Investigación e Innovación Tecnológica (PAPIIT-UNAM) through the grants No. IN202617 and  
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49410 IN206716 to G.P.P.L. and M.G.V, respectively.  
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5412 **Conflict of interest.** None.  
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59414 **Ethical standards.** Specimens in Mexico were collected under the Cartilla Nacional de Colector  
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3 415 Científico (FAUT 0202 and 0057) issued by the Secretaría del Medio Ambiente y Recursos Naturales  
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5 416 (SEMARNAT), to M.G.V. and G.P.P.L., respectively. Specimens in Costa Rica were obtained under  
6  
7 417 the collector permit issued to Arturo Angulo from the Universidad de Costa Rica.  
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 51 579 **Figure legends**  
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 54 580  
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 56 581 Figure 1. Metacercariae of *Ithyoclinostomum* sp. obtained from different host species. Processed  
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 58 582 specimens show the same shape and position of gonads, irrespective of different body sizes.  
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584 Figure 2. Line drawing of the metacercariae of *Ithyoclinostomum* sp. from *Herichthys deppi* Nautla  
585 River, Veracruz state. Ventral view.

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587 Figure 3. Scanning Electron Microscopy of *Ithyoclinostomum* sp. from *Cribroheros alfari*, Orosi River,  
588 Costa Rica. a) entire worm, *scale bar* = 2 mm; b) oral collar showing ventral constriction, ventral view,  
589 *scale bar* = 0.5 mm; c) oral collar showing ventral constriction, ventro-lateral view, *scale bar* = 0.5  
590 mm; d) oral collar showing ventral constriction and oral aperture, em face view, *scale bar* = 0.5 mm.

591 Figure 4. Bayesian inference tree for *Ithyoclinostomum* sp. based on the cytochrome c oxidase subunit  
592 1 gene (*cox1*) dataset. Dots above branches represent posterior probability values and bootstrap values  
593 higher than 0.8/80%. Newly generated sequences of *Ithyoclinostomum* sp. in this study are in bold.

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

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

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

table 1. Localities sampled across Middle America for *Ithyoclinostomum* sp., state for each locality, host and geographical coordinates; and records of adults and metacercariae of *I. dimorphum* reported in fish-eating birds and freshwater fishes South America

Locality	Host	N	W	Author
<b><i>Ithyoclinostomum</i> sp. (Metacercaria)</b>				
<b>Mexico</b>				
<i>Tabasco State</i>				
Gregorio Méndez	<i>Mayaheros urophthalmus</i>	17°27'00"	91°34'10"	This study
<i>Chiapas State</i>				
El Ocotalito, Naha	<i>Vieja melanura</i>	17°03'00"	91°35'49"	This study
<i>Veracruz State</i>				
Nautla River, Nautla	<i>Herichthys deppi</i>	20°11'18"	96°44'43"	This study
Nautla River, Filipinas	<i>Herichthys deppi</i>	20°00'47"	97°09'50"	This study
<b>Costa Rica</b>				
<i>Guanacaste Province</i>				
Irigaray River, Liberia	<i>Cribroheros longimanus</i>	10°43'21"	85°30'38"	This study
Irigaray River, Liberia	<i>Parachromis managuensis</i>	10°43'21"	85°30'38"	This study
Orosí River, Pitahaya	<i>Cribroheros alfari</i>	11°03'05"	85°24'30"	This study
Orosí River	<i>Cribroheros alfari</i>	11°02'50"	85°22'48"	This study
<b>Nicaragua</b>				
<i>South Caribbean Cost Autonomous Region</i>				
Mahogany River	<i>Cichlasoma managuense</i>	12°03'22"	83°59'07"	Aguirre-Macedo <i>et al.</i> , 2001
<b><i>Ithyoclinostomum dimorphum</i> (Adult)</b>				
<b>Brazil</b>				
Not specified	<i>Ardea cocoi</i>			Braun, 1899
<i>Pará State</i>				
Ilha de Marajó	<i>Ardea cocoi</i>			Lent & Freitas, 1937
<i>Mato Grosso State</i>				
Not specified	<i>Ardea cocoi</i>			Travassos, 1928
Barão de Melgaço	<i>Ardea cocoi</i>	23°30'	47°30'	Pinto <i>et al.</i> , 2004
	<i>Ardea alba</i>	23°30'	47°30'	Pinto <i>et al.</i> , 2004
	<i>Nycticorax Nycticorax</i>	23°30'	47°30'	Pinto <i>et al.</i> , 2004

<i>Mato Grosso do Sul State</i>					
	Not specified	<i>Ardea cocoi</i>			Travassos, 1941; Travassos & Freitas, 1942, 1943
	Paraná River Basin	<i>Ardea cocoi</i>	22°50' -22°70'	53°15'-53°40'	Dias <i>et al.</i> , 2003
	Barão de Melgaço	<i>Tigrisoma lineatum</i>			Arruda <i>et al.</i> , 2001
<i>São Paulo State</i>					
	Pirassununga	<i>Nycticorax Nycticorax</i>			Arruda <i>et al.</i> , 2001
<i>Rio Grande do Sul State</i>					
	Pelotas	<i>Ardea cocoi</i>	31°46'19"	52°20'34"	Fedatto <i>et al.</i> , 2017
<b><i>Ithyoclinostomum dimorphum (Metacercaria)</i></b>					
<b>Brazil</b>					
			S	W	
<i>Tocantins State</i>					
	Araguaia River, Araguaianã	<i>Hoplias malabaricus</i>	06°34'	48°38'	Reis, 2014
<i>Rio Grande do Sul State</i>					
	Santa Maria	<i>Hoplias malabaricus</i>			Weiblen & Brandão, 1992
	Cachoeira do Sul	<i>Hoplias malabaricus</i>			Gallio <i>et al.</i> , 2007
	Pirantini River	<i>Hoplias malabaricus</i>	31°30'-34°35'	53°31'-55°15'	Rodrigues, 2010
<i>Minas Gerais State</i>					
	Rio Doce	<i>Hoplias malabaricus</i>	19°46'53"	42°35'57"	Belei <i>et al.</i> , 2013
	Not specified	<i>Hoplias malabaricus</i>			Moreira, 2000
	São Francisco Basin	<i>Hoplias malabaricus</i>	18°12'32"	45°15'41"	Costa <i>et al.</i> , 2015
	São Francisco Basin	<i>Hoplias intermedius</i>	18°12'32"	45°15'41"	Costa <i>et al.</i> , 2015
	Not specified	<i>Hoplerythrinus unitaeniatus</i>			Moreira, 2000
<i>Rio de Janeiro State</i>					
	Lajes Reservoir	<i>Hoplias malabaricus</i>	22°42'-22°50'	44°05'-44°05'	Paraguassú & Luque, 2007
<i>Espírito Santo State</i>					
		<i>Hoplias malabaricus</i>			Travassos, <i>et al.</i> , 1964
<i>Pará State</i>					
	Arari Lake, Marajó Island	<i>Hoplias malabaricus</i>	00°39'48"	49°10'30"	Benigno <i>et al.</i> , 2014
	Arari Lake, Marajó Island	<i>Hoplerythrinus unitaeniatus</i>	00°39'48"	49°10'30"	Benigno <i>et al.</i> , 2014
<i>Paraná State</i>					
	Porto Rico	<i>Hoplias malabaricus</i>			Pavanelli <i>et al.</i> , 1990
	Porto Rico	<i>Schizodon borelli</i>	22°40'-22°50'	53°15'-53°40'	Machado <i>et al.</i> , 1996
<b>Argentina</b>					

<i>Tucumán Province</i>	<i>Hoplias malabaricus</i>			Szidat, 1969
<b>Peru</b>				
Alto amazonas province				
Yurimaguas	<i>Hoplerythrinus unitaeniatus</i>	05°42'03"	76°52'03"	Delgado <i>et al.</i> , 2017

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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 in mm as a range; measurements in parenthesis for some columns represent the mean value. Measurements with an asterisk represent ratios not provided in the original description/redescription; these ratios were calculated as a reference value with a 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	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		0.763		0.36	0.24	0.30 – 0.32 (0.31)		0.65	0.32 – 0.99 (0.53)
OSW	0.5 (diameter)	0.579	0.2 (diameter)	0.40	0.31	0.52 – 0.57 (0.53)		0.90	0.23 – 2.35 (1.25)
OSW/BW	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		1.8	0.75	1.15	0.69	0.90 – 1.20 (0.98)		3.5	1.20 – 2.85 (1.97)
VSW	1.6 (diameter)	(diameter)	(diameter)	1.25	1.21	0.92 – 1.42 (1.05)		(diameter)	1.215 – 2.82 (2.02)
VSW/OSW	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	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	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	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	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		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		0.59*		0.51*	0.81*		0.84*	0.73 – 1.76 (1.06)
DBVS&AT								5.73 – 15.30 (8.88)
DBT								0.54 – 2.03 (1.24)
OL		0.842		0.18			0.400	0.19 – 0.50 (0.31)
OW		1.183		0.12			0.375	0.19 – 0.50 (0.27)
OW/OL		1.40*		0.67*			0.875*	0.55 – 1.28 (0.84)
CSL		0.579		0.45	1.27			
CSW		0.920		0.27	1.5			

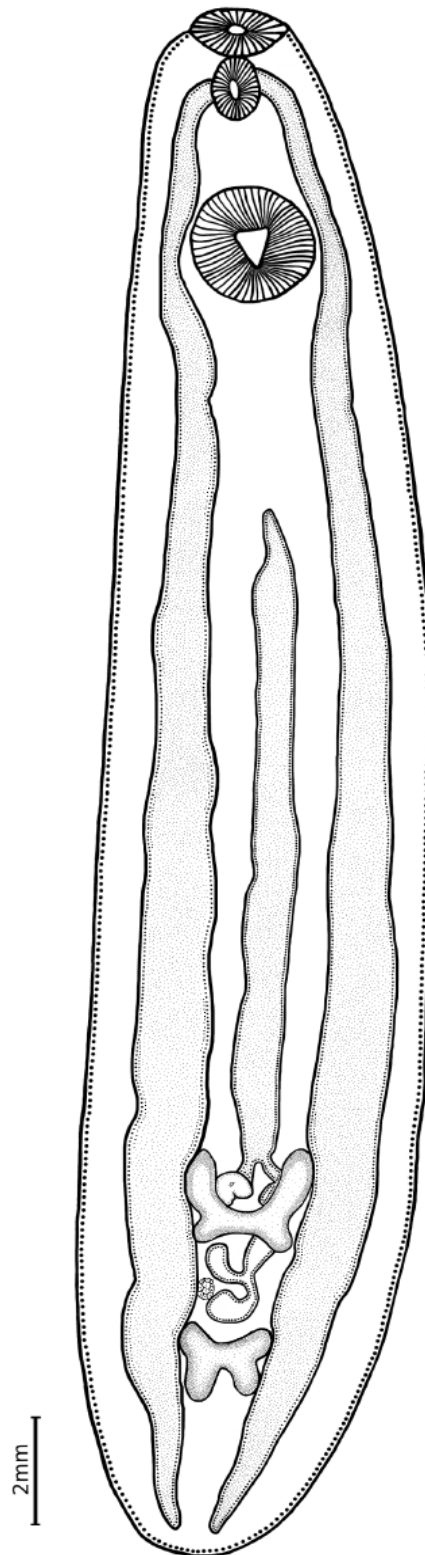
Abbreviations: Body length (BL), Body width (BW), ratio BL/BW, oral collar length (OCL), oral collar width (OCW), oral sucker length (OSL), oral sucker width (OSW), ratio OSW/BW, Pharynx length (PhL), Pharynx width (PhW), ventral sucker length (VSL), ventral sucker width (VSW), ratio VSW/OSW, ratio VSL/OSL, ratio VSW/BW, ratio OSL/VSL, ratio OSW/VSW, distance between suckers (DOS/VS), distance between oral collar and ventral sucker (DOC&VS), anterior testis length (ATL), anterior testis width (ATW), ratio ATW/ATL, posterior testis length (PTL), posterior testis width (PTW), ratio PTW/PTL, distance between ventral sucker and anterior testis (DBVS&AT), distance between testis (DBT), Ovary length (OL), Ovary width (OW), ratio OW/OL, cirrus sac length (CSL), cirrus sac width (CSW).

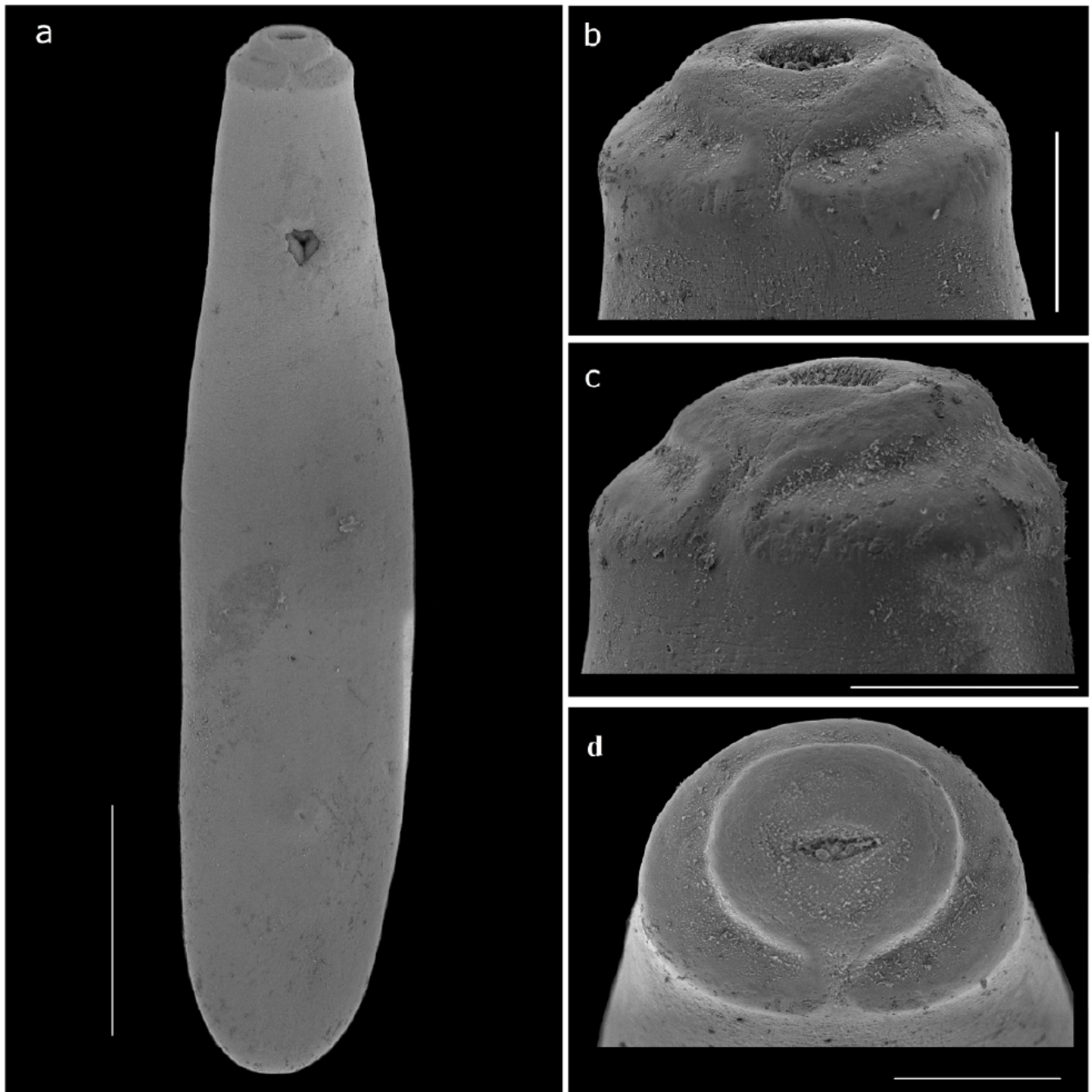


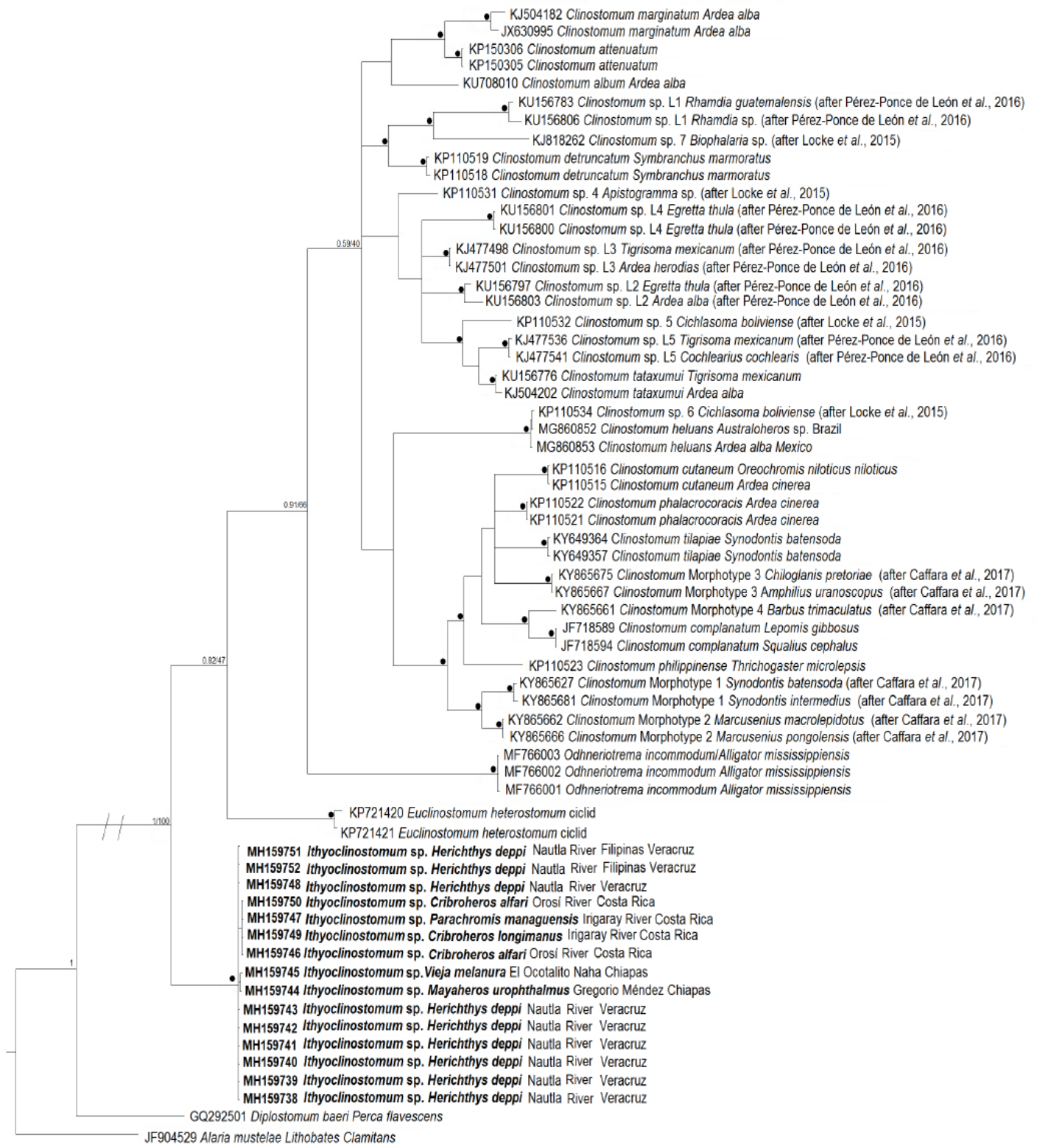
Figure 1

[Click here to download Figure Fig1 CMYK1200.tif](#)











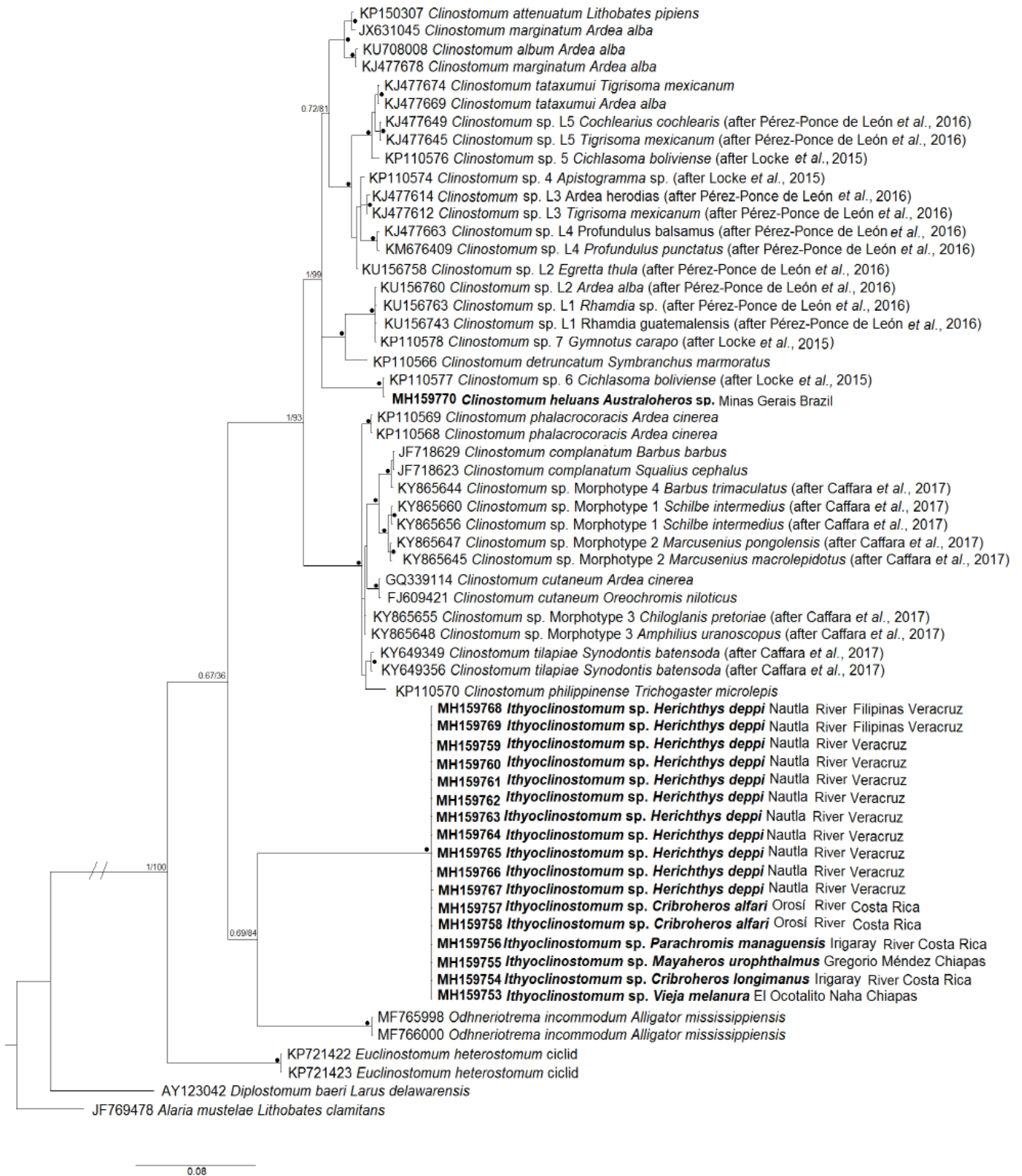


Figure 6

[Click here to download Figure Fig 6 28S \(1\).tif](#)

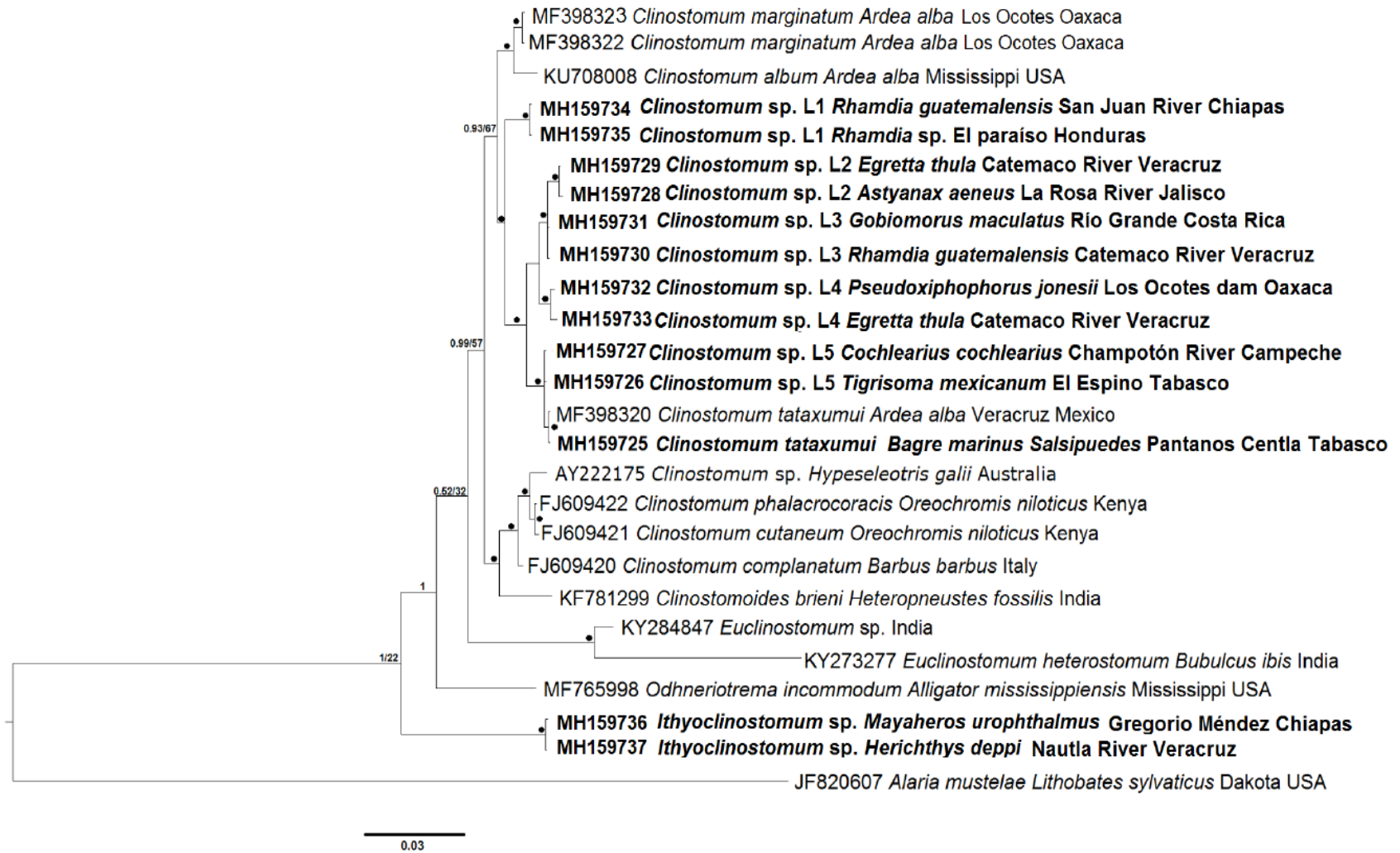
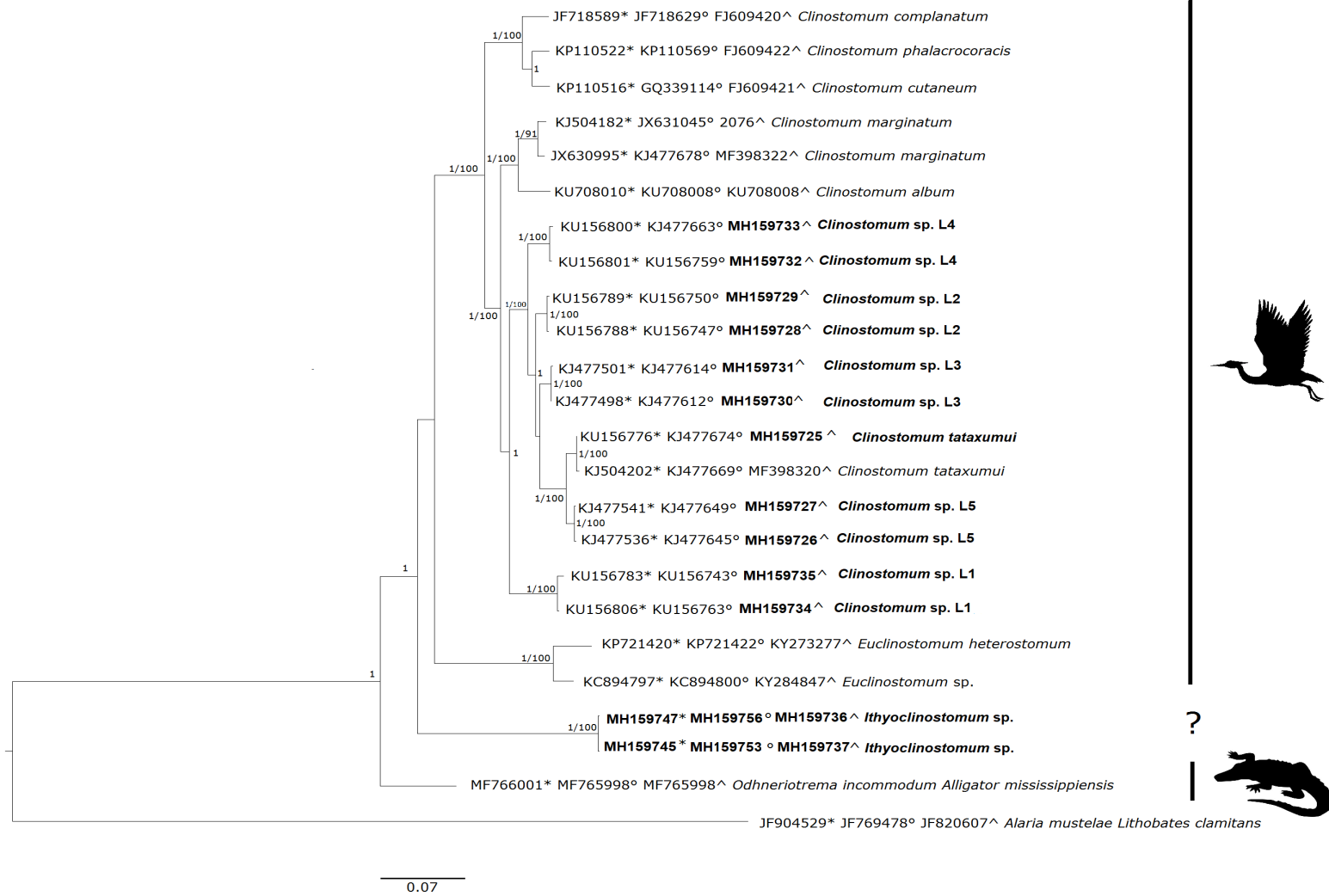




Figure 7

[Click here to download Figure Fig 7 concatenado \(1\).tif](#)



## V. Discusión general

En el presente trabajo se estudió la metacercaria de un tremátodo perteneciente a la familia Clinostomidae cuya determinación taxonómica requirió el uso de una aproximación integrativa donde se consideraron caracteres moleculares, morfología, patrones de asociaciones ecológicas y de distribución geográfica. Desde que los ejemplares fueron recolectados en cíclidos de localidades de México y Costa Rica surgió una interrogante sobre la identificación de estos tremátodos. En primera instancia se consideró que podrían pertenecer a *Clinostomum heluans*, una especie originalmente descrita de la cavidad bucal de la garza azul *Ardea caerulea* en Brasil (Braun, 1899), pero que en la actualidad se ha encontrado parasitando al menos a siete especies de ardeidos de Brasil, Venezuela, Cuba y México (Bravo-Hollis, 1947; Pérez-Vigueras, 1955; Caballero y Diaz-Ungría, 1958; Travassos *et al.*, 1969; Pinto *et al.*, 2013; Fernandes *et al.*, 2015; Werneck *et al.*, 2017). Adicionalmente, existe un registro de esta especie para la República Checa y el este de Rusia (Sitko, 2012); sin embargo, estos registros requieren verificación ya que al parecer *C. heluans* solo se distribuye en el Continente Americano.

*Clinostomum heluans* exhibe gran tamaño alcanzando hasta los 26 mm de largo y 3.2 mm de ancho (Bravo-Hollis, 1947), presenta las gónadas terminales y su distribución geográfica abarca desde el norte de México (Sabinas Hidalgo, Nuevo León) hasta Minas Gerais, Brasil (ver Fig. 2). Por ello se consideró preliminarmente que las metacercarias podrían corresponder con *C. heluans*. Sin embargo, revisiones morfológicas de especímenes adultos de esta especie mostraron diferencias considerables y los análisis moleculares confirmaron que las metacercarias no pertenecen al género *Clinostomum*. Cabe destacar que Locke *et al.* (2015) colectó una metacercaria de *Cichlasoma boliviense* en Santa Cruz, Bolivia y la reportó como *Clinostomum* sp. 6; en los análisis filogenéticos aquí presentados con el marcador molecular *cox1*, esta especie presentó baja variación genética (0.2-0.4%) con respecto a *C. heluans*, lo cual nos indicó que *Clinostomum* sp.6 corresponde a *Clinostomum heluans* (ver resultados del Capítulo 1 del presente trabajo).

Las metacercarias reportadas en este estudio también comparten características morfológicas con el género *Ithyoclinostomum* aunque hay claras diferencias a la especie monotípica *Ithyoclinostomum dimorphum*.

El género *Ithyoclinostomum* es característico en la familia Clinostomidae debido a que la especie tipo alcanza un tamaño poco frecuente entre las especies de tremátodos, 60-100 mm de largo

en individuos adultos y 13-50 mm en metacercarias. En este trabajo las metacercarias midieron entre 11 y 29.5 mm de largo y 2.3-8.4 mm de ancho; en el caso de *I. dimorphum* el ancho del cuerpo varía entre 1.5 y 3.75, lo cual indica que éstos presentan un cuerpo más delgado y alargado con respecto a las metacercarias de Costa Rica y México. Aguirre-Macedo *et al.* (2001) reportaron un ejemplar de metacercaria recolectada de la cavidad corporal de un cíclido en Nicaragua de mayores dimensiones (39 mm de largo x 11 mm de ancho). Morfológicamente, nuestros ejemplares corresponden con el género *Ithyoclinostomum*, principalmente en la posición pretesticular y anterolateral del poro genital y de la bolsa del cirro, además de ser el género que incluye a la especie más grande de tremátodo conocido. No obstante, las metacercarias de nuestro estudio difieren morfológicamente de *I. dimorphum* con respecto al hábitat, la relación hospedatoria y la distribución geográfica. Las metacercarias de *I. dimorphum* han sido registradas enquistadas o libres en la cavidad del cuerpo y en diferentes órganos de peces caraciformes, principalmente de las familias Erythrinidae y Anostomidae, con una distribución amplia en Brasil, aunque también hay reportes en Perú y Argentina (Arruda *et al.*, 2001; Belei *et al.*, 2013; Benigno *et al.*, 2014; Braun, 1899; Dias *et al.*, 2003; Delgado *et al.*, 2017; Fedatto *et al.*, 2017; Gallio *et al.*, 2007; Lent & Freitas, 1937; Reis, 2014; Machado *et al.*, 1996; Moreira, 2000; Rodrigues, 2010; Travasos, 1928, 1941; Travasos & Freitas, 1942, 1943, 1964; Paraguassú & Luque, 2007; Pavanelli *et al.*, 1990; Pinto *et al.*, 2004; Weiblen & Brandão, 1992; Szidat, 1969). Mientras que *Ithyoclinostomum* sp. se encontró libre en la cavidad corporal y únicamente se ha reportado en cíclidos de Centroamérica y México (Tabla 2).

La revisión morfológica comparativa de las especies de los géneros restantes de la familia Clinostomidae mostró de igual manera diferencias sustanciales. Con respecto a los géneros *Clinostomatopsis* y *Clinostomoides* (subfamilia Clinostominae) varios atributos difieren de los especímenes aquí estudiados; *Clinostomatopsis sorbens* presenta el saco del cirro y el poro genital inter-testicular, testículos muy grandes y profundamente lobulados; el testículo anterior presenta cinco lóbulos irregulares mientras que el testículo posterior presenta seis (Benigno *et al.*, 2014). Las metacercarias de *C. intermedialis* y *C. sorbens* han sido encontradas en erytrinidos de Brasil y los adultos en suliformes del Norte de México, Centro y Sudamérica y pelicaniformes y ciconiiformes de Brasil y Argentina, respectivamente. El género monotípico *Clinostomoides* (*Clinostomoides brieni*) presenta la bolsa del cirro en posición intertesticular y el poro genital está situado en posición posterior al testículo posterior; sus metacercarias se encuentran enquistadas en branquias y presentan papilas y espinas en la superficie corporal, parasitando a peces siluriformes y ardeidos de África y Asia (Kanev

*et al.*, 2002).

Con relación a la subfamilia Nephrocephalinae, ésta incluye los géneros *Nephrocephalus* y *Odhneriotrema*; se han encontrado metacercarias de *Odhneriotrema incommodum* parasitando el tejido ovárico y testicular de peces lepisosteiformes comúnmente llamados "pejelagartos" y los especímenes adultos han sido encontrados en la cavidad bucal y esofágica de caimanes de Florida (Leigh, 1978; Woodyard *et al.*, 2017). En contraste, las metacercarias de *Ithyoclinostomum* sp. fueron encontradas en cíclidos de México y Centroamérica, mientras que el hospedero definitivo es aún desconocido, aunque si bien éste debe ser un ave ictiófaga, no descartamos la posibilidad de que sea alguna especie de cocodrilo. Si este fuera el caso, es probable incluso que nuestros ejemplares pudieran representar un género diferente a *Ithyoclinostomum*. En cuanto a las características morfológicas de los integrantes del género *Nephrocephalus*, éstos presentan poros genitales femenino y masculino separados y la bolsa del cirro posttesticular. Finalmente, en el caso del género *Euclinostomum*, aunque las metacercarias también se han registrado como parásitos de cíclidos y los adultos son encontrados en la cavidad bucal y esofágica de ardeidos del viejo mundo (Caffara *et al.*, 2016); morfológicamente son muy diferentes ya que los ciegos intestinales son ramificados y presentan divertículos característicos de este género, que están ausentes en *Ithyoclinostomum*.

Los resultados moleculares de nuestro estudio confirman que las metacercarias representan un linaje genético claramente diferenciado de otros géneros de la familia Clinostomidae para los cuales hay secuencias disponibles en el GenBank. Los análisis de inferencia filogenética de tres marcadores genéticos, dos nucleares y uno mitocondrial, aunque fueron discrepantes, mostraron claramente a través de un análisis concatenado de los tres marcadores que las metacercarias que identificamos tentativamente como pertenecientes al género *Ithyoclinostomum* sp. conforman un grupo monofilético, aunque su relación con otros clinostómidos requiere necesariamente la adición de secuencias de más representantes. La topología del análisis concatenado sugiere además que *Odhneriotrema*, parásito de cocodrilos, es el grupo basal de clinostómidos, mientras que *Ithyoclinostomum* es el grupo hermano de *Euclinostomum* y *Clinostomum*. La confirmación de nuestra hipótesis podrá realizarse cuando se cuente con secuencias, tanto de *I. dimorphum* como de otros clinostómidos, principalmente de aquellos que se distribuyen en Sudamérica, incluyendo representantes del género *Clinostomatopsis*.

## VI. Conclusiones

Se estableció molecularmente el vínculo entre el adulto de *Clinostomum heluans* obtenido de la cavidad bucal de ardeidos del sureste de México y la metacercaria recolectada de un cíclido de Brasil.

Los análisis filogenéticos con el gen mitocondrial *cox1* nos permitieron corroborar que el linaje descrito por Locke *et al.* (2015) como *Clinostomum* sp. 6 corresponde con la especie *C. heluans*.

Las metacercarias que se recolectaron en la cavidad corporal de diferentes especies de cíclidos de México y Centroamérica fueron identificadas como pertenecientes al género *Ithyoclinostomum* principalmente por la posición del complejo genital y la del poro genital.

Las metacercarias analizadas no corresponden con la especie *Ithyoclinostomum dimorphum* por la longitud del cuerpo (relación largo/ancho), el hábitat y los patrones de distribución geográfica y hospedatoria.

El hospedero definitivo de *Ithyoclinostomum* sp. aún se desconoce.

La identificación de los ejemplares dentro del género *Ithyoclinostomum* es tentativa. Se requiere obtener secuencias de ADN de las formas adultas en sus hospederos definitivos para corroborar la identificación taxonómica.

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