

Research Paper

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Morphological and molecular assessment of the diversity of trematode communities in freshwater gastropods and bivalves in Los Tuxtlas tropical rainforest

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Abstract

Trematode life cycles involve molluscs as first intermediate hosts. The identification of trematodes based on the morphology of cercariae released from molluscs is challenging because the identification relies on adult forms obtained from their definitive hosts. Several studies have recently genetically characterized these larval forms establishing a link with adults sampled from their vertebrate hosts, allowing their identification at species level. In Los Tuxtlas tropical rainforest, in south-eastern Mexico, 57 species of trematodes have been reported from wildlife vertebrates; however, studies evaluating the diversity of trematode cercariae in molluscs are lacking. Here, we studied 11 species of molluscs to assess the diversity of cercariae in two lakes of Los Tuxtlas, Veracruz, Mexico; six of the 11 species were infected. Twelve cercarial morphotypes were collected and characterized morphologically. Sequences of the 28S ribosomal RNA gene were generated to allocate each morphotype into a family using the most recent phylogenetic classification of the Digenea as a framework; molecular work revealed 16 genetic lineages; some cercariae were identified up to genus (*Apharyngostrigea*, *Ascocotyle*, *Centrocestus*, *Echinochasmus*, *Lecithodendrium* and *Posthodiplostomum*), and some to species levels (*Gorgoderina rosamondae*, *Langeronia macrocirra*, *Oligogonotylus manteri* and *Phyllodistomum inecoli*) based on their phylogenetic position within the tree, and the genetic distance with respect to other sequenced congeners. Therefore, the cercarial morphotypes in the present study represent at least 16 putative species. Our study contributes to a better understanding of the trematode diversity in an area of high vertebrate species diversity, and to the knowledge of trematode life cycles.

Introduction

Digenetic trematodes are parasitic plathyhelminths with a complex life cycle that involves one or more intermediate host, and vertebrates as definitive hosts for successful completion. Molluscs serve as first intermediate hosts and their distribution accounts for the occurrence of trematode species in a particular area. They are infected by the free-living miracidia through penetration, or by feeding on the trematode eggs. Asexual reproduction occurs within the mollusc (Ginetsinskaya, 1988; Esch *et al.*, 2001; Galaktionov & Dobrovolskij, 2003; Gordy *et al.*, 2016). Cercariae are released from molluscs and, once in the environment, they typically seek and infect the second intermediate host, where they encyst to form the metacercaria (Faltynková *et al.*, 2016).

In the tropical rainforest of Los Tuxtlas, Mexico, a large diversity of trematode species has been reported from different vertebrate groups. At least 57 species of trematodes have been reported from fish, amphibians, reptiles, birds and mammals, and ten of them represented new species (see supplementary table S1). However, with the exception of the recent studies by Velázquez-Urrieta & Pérez-Ponce de León (2020, 2021), no information regarding the life cycle and the morphological characterization of the larval forms of these trematodes is available thus far. The classification of distinct cercarial morphotypes is useful to allocate trematodes into a family, even though in some cases more than one trematode family possess the same cercarial morphotype. For instance, furcocercous cercariae are found in species of Diplostomidae Poirier, 1886, Strigeidae Railliet, 1919 and Clinostomidae Lühe, 1901 (Ginetsinskaja, 1988). Nevertheless, the study of larval forms through genetic data is a valuable source of information to understand the species diversity of trematodes by linking larval forms with adults sampled from their vertebrate definitive hosts (e.g. Locke *et al.*, 2011, 2021); even without a formal recognition of the link, cercarial morphotypes might be diagnosed as independent genetic lineages. This is particularly useful for life-history studies partly resolving an ethical concern and avoiding sampling hosts which might be considered as endangered by

national and international agencies. Alternatively, by studying the diversity of larval forms of trematodes in their intermediate hosts, it is possible to provide a better estimation of the trematode species diversity (e.g. Gordy *et al.*, 2016; Gordy & Hanington, 2018; Selbach *et al.*, 2020); some species might be found in these hosts while the adult forms have not been reported from their natural definitive hosts in the area. The study of terrestrial, aquatic and semi-aquatic molluscs in a region could be a suitable indicator of trematode species diversity based on the premise that higher mollusc diversity leads to higher trematode diversity, although some exceptions are described in the literature (see Blasco-Costa *et al.*, 2014).

Furthermore, the advancement of molecular tools has facilitated the increase of the DNA reference libraries of several parasite groups, allowing the establishment of the link between larval forms and adults, through the sequencing of some molecular markers from different stages of the life cycle – for example, Locke *et al.* (2011) and Blasco-Costa *et al.* (2015). In particular, the nuclear 28S ribosomal RNA (rRNA) gene accounts for the largest representation of sequence data of species of trematodes (Pérez-Ponce de León & Hernández-Mena, 2019). However, there is a paucity of the information on the genetic diversity of cercariae released by molluscs, and the distribution patterns of trematodes in their intermediate hosts in different freshwater systems (Faltynkova *et al.*, 2016). In Mexico, very few studies have characterized the morphology of cercariae in their first intermediate hosts (e.g. Lamothe-Argumedo *et al.*, 1983; Rangel-Ruiz & Lamothe-Argumedo, 1986; Ditrich *et al.*, 1997; Scholz *et al.*, 1996; Barragán-Sáenz *et al.*, 2009). To the best of our knowledge, this is the first study addressing the overall genetic diversity and morphological characterization of trematode cercariae in a particular region of Mexico. The main objectives of this work were twofold: to characterize morphologically and molecularly the cercariae of trematodes released from molluscs in two freshwater systems in the tropical rainforest of Los Tuxtlas; and to determine the phylogenetic position of the cercariae through 28S ribosomal DNA (rDNA) sequences with the aim of achieving their taxonomic identification.

Materials and methods

Specimen collection

Ten species of gastropods and one of bivalve were collected in Laguna Escondida (LE) (18°36'00"N, 95°05'26"W), and Laguna Zacatal (LZ) (18°34'55"N, 95°05' 20"W), Los Tuxtlas, Veracruz, Mexico, during August–September 2018, and January–February 2019 (table 1). Snails and clams were collected by hand on the aquatic vegetation near the shore or using dip nets or a strainer for the sediment at a depth of about 2 m. Specimens were taken alive to the laboratory, placed individually in glass containers with 100 ml of filtered water from the study site, for a period of 16 h of light and 8 h of darkness to stimulate the emergence of cercariae; containers were examined under the stereomicroscope every 6 h for the presence of cercariae; hosts positive to the infection were dissected to search for intra-mollusc trematode stages; snails negative to the infection by cercariae after 36 h were released at the capture site. For morphological study, cercariae were fixed in hot (near boiling) 4% formalin (for the scanning electron microscopy (SEM) study) or in hot (near boiling) tap water; all specimens were preserved in 70% ethanol. For molecular analyses, cercariae were preserved in 100% ethanol. Snails and

clams were identified using specific taxonomic keys (Burch, 1989; Thorp & Covich, 1991). The cercariae were identified using a combination of morphological and molecular data.

Morphological study

A photomicrograph of live specimens representing each cercarial morphotype was taken with a Leica DM500 light microscope (Leica Microsystems, Wetzlar, Germany). After fixation, cercariae were stained with neutral red, cleared with 12% glycerol and mounted in Canada balsam between coverslips. Measurements were obtained for some cercarial traits using a drawing tube attached to the microscope; measurements are presented in micrometres. Vouchers of cercarial morphotypes were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City. Cercariae fixed in 4% formalin were used for SEM. They were dehydrated through an ethanol series, critical-point dried, sputter-coated with gold with Q150R Modular Coating System (Ashford, England), and examined at 15 kV with a Hitachi SU1015 SEM (Hitachi, Tokyo, Japan) and S-2460N SEM (Hitachi, Tokyo, Japan). The criterion for classifying the cercarial morphotypes was based on the recognition of traits such as the tail shape, presence/absence of stylet, eye-spots and virgulate organ, as well as the number of suckers (Ginetsinskaja, 1988).

Molecular study

For molecular analyses, total DNA was extracted from individual cercariae following the DNazol protocol (Molecular Research Center, Cincinnati, OH, USA) (Chomczynski *et al.*, 1997). The combination of primers used for amplification and sequencing for the 28S rDNA gene were: 28SL 5'-AAC AGT GCG TGA AAC CGC CTC-3' (Palumbi, 1996) and URP28S 5'-GCT ATC CTG AGR GAA ACT TCG-3' (Tkach *et al.*, 2000); 28Sy 5'-CTA ACC AGG ATT CCC TCA GTA ACG GCG AGT-3' and 28Sz 5'-AGA CTA CTT TGG TCC GTG TTT CAA GAC-3' (Hillis & Dixon, 1991); and 28Sy 5'-CTA ACC AGG ATT CCC TCA GTA ACG GCG AGT-3' and 28SL 5'-AAC AGT GCG TGA AAC CGC CTC-3' (see supplementary table S2); thermal cycling conditions were 1 min at 94°C, then 35 cycles of 45 s at 92°C, 30 s at 50°C and 1.5 min at 72°C, and a final extension of 10 min at 72°C. Polymerase chain reaction products were sequenced using an ABI 3730xl Genetic Analyzer (Thermo Fisher scientific, Waltham, Massachusetts, USA). Sequences were edited and assembled using the software Geneious 5.1.7 (Biomatters Ltd., Auckland, New Zealand). All newly generated sequences were submitted to GenBank.

Phylogenetic analyses

DNA sequences were aligned using MAFFT (Katoh *et al.*, 2005; Katoh & Standley, 2013), using default parameters; alignments were trimmed to the shortest sequence length. Phylogenetic analyses of the 28S rDNA were performed using representative members of nine digenean families: Cryptogonimidae Ward, 1917; Diplostomidae Poirier, 1886; Echinochasmidae Odhner, 1910; Echinostomatidae Looss, 1899; Gorgoderidae Looss, 1899; Heterophyidae Leiper, 1909; Lecithodendriidae Lühe, 1901; Pleurogenidae Looss, 1899; and Strigeidae Railliet, 1919. Analyses were conducted at family level after an initial overall phylogenetic analysis was performed using the alignment of

Table 1. Molluscs collected and infected with trematode cercariae in Los Tuxtlas tropical rainforest.

Molluscs species	Total		Laguna Zacatal		Laguna Escondida	
	Collected	Infected	Collected	Infected	Collected	Infected
<i>Pomacea flagellata</i> (Say)	305	0	150	0	155	0
<i>Melanooides tuberculata</i> (Müller)	810	20	410	20	0	0
<i>Aroapyrgus alleei</i> Marrison	1310	51	0	0	1310	51
<i>Planorbella foveale</i> (Menke)	233	0	233	0	0	0
<i>Biomphalaria helophila</i> (d'Orbigny)	410	11	810	11	0	0
<i>Physella mexicana</i> (Phillippi)	512	0	0	0	512	0
<i>Pyrgophorus coronatus</i> (Pfeiffer)	420	0	0	0	420	0
<i>Pachychilus turati</i> (Villa & Villa)	560	0	0	0	560	0
<i>Ferrissia</i> sp.	774	15	0	0	774	15
<i>Hebetancylus excentricus</i> (Morelet)	400	10	0	0	400	10
<i>Pisidium</i> sp.	700	13	700	13	0	0
Total	6434	120	2303	44	4131	76

Pérez-Ponce de León & Hernández-Mena (2019). Family-level relationships were assessed to better depict the relationships of the newly generated sequence data. Species chosen as outgroups for rooting the trees were selected using the analysis of Pérez-Ponce de León & Hernández-Mena (2019). Two phylogenetic approaches were performed, Bayesian Inference and Maximum likelihood; Bayesian Inference analyses were conducted using Markov Chain Monte Carlo in Mr Bayes V 3.1.2 (Ronquist *et al.*, 2012), with the model of evolution GTR+I+ Γ , obtained in jModeltest 0.1.1 (Posada, 2008). The chains were run for 1,500,000 generations, sampling trees every 1000 generations. The first 25% of trees were discarded as burn-in and the resulting trees were used to obtain a 50% majority-rule consensus tree. Tracer v1.7.1 (Rambaut *et al.*, 2018) was used to assess convergence of independent runs. Nodal support was estimated as posterior probabilities. Maximum likelihood analyses were performed in RaxML 6.0 (Stamatakis, 2006) with the appropriate evolution model (GTR+I+ Γ) obtained in jModeltest 0.1.1 (Posada, 2008). Genetic distances were estimated through uncorrected *p*-distances using MEGA-X (Kumar *et al.*, 2018).

The procedure to achieve the identification of cercariae followed that used previously by several authors (Kudlai *et al.*, 2015; O'Dwyer *et al.*, 2015; Donald & Spencer, 2016; Chontanarith *et al.*, 2017; Gordy & Hanington, 2018; Huston *et al.*, 2018; Cribb *et al.*, 2019; Onaca *et al.*, 2020). DNA sequences were generated for individuals representing cercarial morphotypes (and sometimes their intra-molluscan stages), and first screened through the BLAST tool available in GenBank to check that only parasite DNA was amplified, and to preliminary assign each morphotype to a superfamily or family-level group; in some cases, a link was established and morphotypes were assigned to genus and even to species levels. Then, phylogenetic analyses were run for each superfamily or family-level analyses, and the position and sister-group relationships of each morphotype within the tree was established.

Results

A total of 6434 molluscs belonging to two classes, eight families and 11 species were sampled and analysed for infections with

digenean trematodes. We examined 4131 individuals from LE and 2303 from LZ (table 1). Seven species of molluscs were sampled in LE and five in LZ. The most abundant were the planorbid gastropod *Biomphalaria helophila* in LZ, and the cochliopid gastropod *Aroapyrgus alleei* in LE. The ampullariid gastropod *Pomacea catemacensis* was the only one found in both localities, although it was not infected with trematodes. Of the 6434 analysed hosts, only 120 harboured trematode infections, resulting in an overall prevalence of 1.86%. Furthermore, only six of the 11 species harboured trematode infections – namely, *Melanooides tuberculata*, *A. alleei*, *B. heliophila*, *Hebetancylus excentricus*, *Ferrissia* sp. and *Pisidium* sp. (see table 1). The species with the highest trematode species richness was *A. alleei*, with five species.

Twelve cercarial morphotypes we recovered (Furcocercous cercaria I, II, III; Distome cercaria I, II; Xiphidiocercaria, Virgulate cercaria, Ophthalmocercaria, Pleurolophocercous cercaria, Monostome cercaria and Cysticercous cercaria I, II). However, multiple infections were not found; each individual mollusc was parasitized only by one species of trematode at the same time in our samples. Regarding the locality, eight cercarial morphotypes were found in LE and five in LZ.

Morphological descriptions of cercarial morphotypes

Furcocercous cercaria I

Furcocercous cercaria (figs 1a, 2a and 4a) Description based on ten specimens: body small, elongate, 120.6–123.4 (122.1) long, 40.6–41.4 (41.2) wide at equatorial region; tegument spinose, blunt spines distributed as follows: the first third of body without spines, the second possess a circular band and in the third spines are homogeneous; eye-spots spherical, pre-equatorial. Oral sucker subterminal, oval, with two pair of glandular cells. Pharynx large, muscularized. Oesophagus long, narrow; intestinal caeca short. Five pairs of robust penetration glands in two lateral fields, in posterior half of body. Ventral sucker small, spherical, post-equatorial. Excretory vesicle V-shaped. Tail elongated, longer than body, spined, blunt spines, with distribution homogeneous; bifurcated, with longitudinal muscular fibres along central axis,

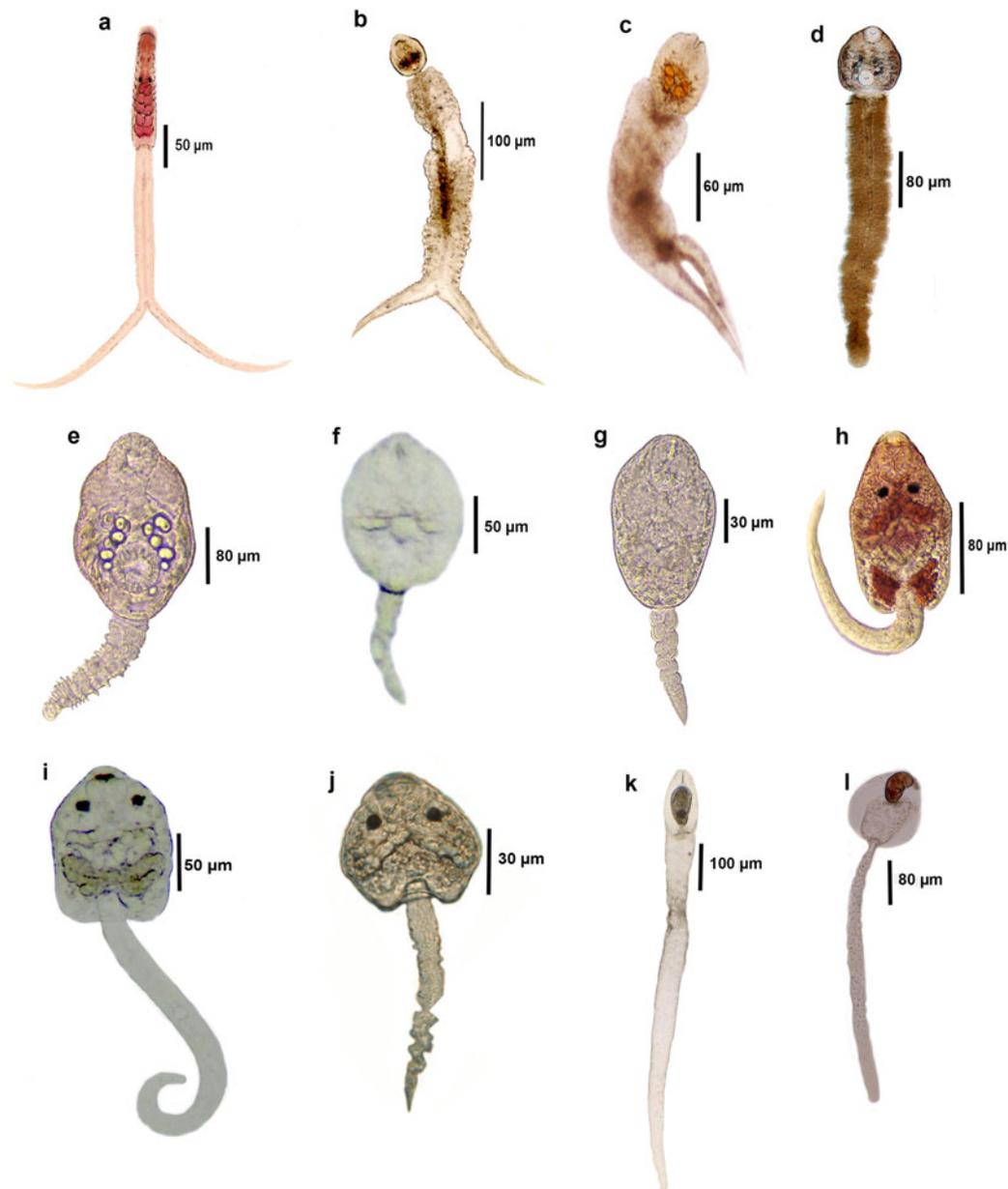


Fig. 1. Photomicrographs of the cercarial morphotypes released from molluscs in two lakes of Los Tuxtlas, Veracruz. (a) Furcocercous cercaria I; (b) Furcocercous cercaria II; (c) Furcocercous cercaria III; (d) Distome cercaria I; (e) Distome cercaria II; (f) Xiphidiocercaria; (g) Virgulate cercaria; (h) Ophthalmocercaria; (i) Pleurolophocercous cercaria; (j) Monostome cercaria; (k) Cysticercous cercaria I; (l) Cysticercous cercaria II.

275.9–285 (284) long, 34–37.1 (35.1) wide; furca length shorter than tail length. In *Ferrisia* sp., LE.

Furcocercous cercaria II

Furcocercous cercaria II (figs 1b and 2b) Description based on ten specimens: body small, oval, 77.7–79 (78.3) long, 50.3–60.3 (55) wide at equatorial region; tegument spinose, blunt spines homogeneously distributed; eye-spots absent; oral sucker terminal, spherical. Pre-pharynx short and narrow. Pharynx wide. Oesophagus long; intestinal caeca short. Six pairs of spherical penetration glands, distributed in two lateral fields, one on each side of ventral sucker. Ventral sucker small, spherical, post-equatorial, smaller at oral sucker. Excretory vesicle V-shaped. Tail wider and longer than the body, spined, blunt spines, with homogeneous

distribution; bifurcated, with longitudinal muscles fibres along the central axis of the tail, 298.2–304 (314) long, 56–65.3 (81) wide; furca length shorter than tail length. In *H. excentricus*, LE.

Furcocercous cercaria III

Furcocercous cercaria III (figs 1c and 2c) Description based on ten specimens: body small, oval, 76.4–86.3 (81) long, 46.32–50.2 (48.2) wide at equatorial region; tegument spinose, blunt spines with homogeneous distribution; eye-spots absent; oral sucker terminal, spherical, with one pair of glandular cells. Pre-pharynx long and narrow. Pharynx small. Oesophagus short; intestinal caeca long and wide. Four pairs of large penetration glands, distributed in two lateral fields, one on each side of body. Ventral sucker small, spherical, post-equatorial, smaller to oral sucker.

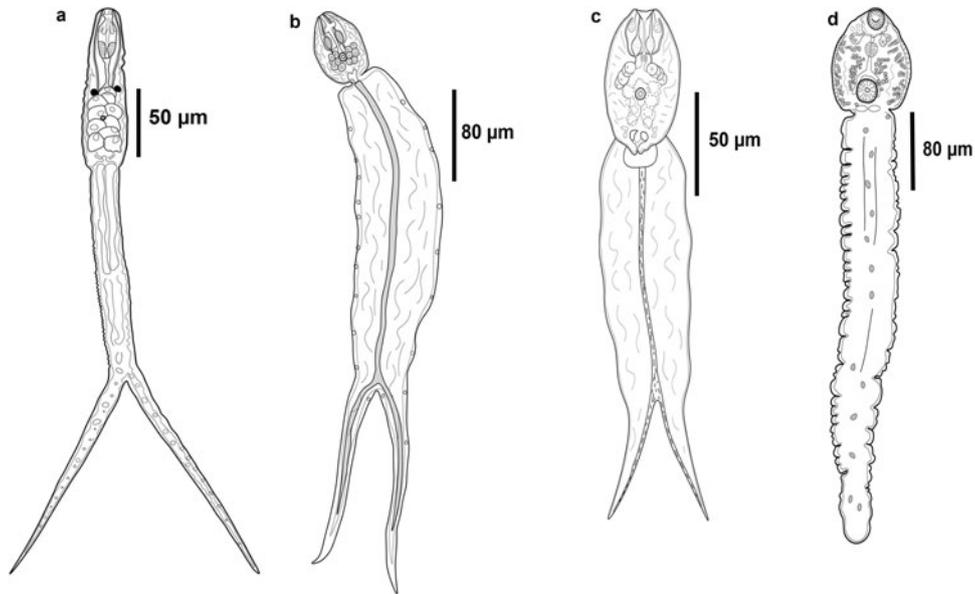


Fig. 2. Line drawings of the cercarial morphotypes released from molluscs in two lakes of Los Tuxtlas, Veracruz. (a) Furcocercous cercaria I; (b) Furcocercous cercaria II; (c) Furcocercous cercaria III; (d) Distome cercaria I.

Cystogenous glands post-equatorial, overlapping with caeca. Excretory vesicle V-shaped. Tail wider and longer than the body, spined, blunt spines, with homogeneous distribution; bifurcated, with longitudinal muscles fibres along the central axis of the tail, 113.7–228.1 (180.7) long, 73.2–88.3 (80.5) wide; furca length shorter than tail length. In *H. excentricus*, LE.

Distome cercaria I

Distome cercaria I (figs 1d, 2d and 4b, c) Description based on ten specimens: body spherical, 80.23–96.54 (89) long, 60.56–69.3 (65) wide at equatorial region; tegument smooth. Eye-spots absent. Oral sucker subterminal, spherical, opening directed ventrally. Oral opening with eight nail-shaped spines on anterior border and festoons. Pre-pharynx long; pharynx spherical; oesophagus long. Intestinal caeca short. Two pairs of small penetration glands at pharynx level, distributed in two lateral fields one on each side of body. Ventral sucker spherical, near posterior end, larger than oral sucker; ventral opening surrounded by 37 nail-shaped spines and festoons; three pairs of papillae on internal surface. Cystogenous gland cells distributed in two lateral rows, from pharynx to posterior margin of ventral sucker. Excretory bladder saccular or transversely oval. Tail robust, approximately five times longer than body, 440–501.3 (467.3) long, 80.2–98.3 (89.5) wide. In *A. alleei*, LE.

Distome cercaria II

Distome cercaria II (figs 1e, 3a and 4d) Description based on ten specimens: body oval, smooth, 97.3–110 (103) long, 50.56–65.3 (59.25) wide at equatorial region; spines and eye-spots absent. Oral sucker subterminal, spherical, opening directed ventrally. Oral opening with ten nail-shaped spines on anterior border and festoons. Pre-pharynx short; pharynx spherical; oesophagus long. Intestinal caeca short. Three pairs of small penetration glands at level of pharynx in two lateral fields on each side of body. Ventral sucker spherical near posterior end, equal size to oral sucker; ventral opening surrounded by 25 nail-shaped spines and festoons. Cystogenous gland cells well-developed, in two lateral rows, between pharynx and ventral sucker. Excretory vesicle

saccular. Tail simple, equal size to body length, 80–101 (90.4) long, 20.56–28.3 (24.2) wide. In *Biomphalaria helophila*, LZ.

Xiphidiocercaria

Xiphidiocercaria (figs 1f and 3b) Description based on ten specimens: body small, elongate, 90.6–91.7 (91.3) long, 44.3–61.8 (55.5) wide at equatorial region; tegument without spines. Eye-spots absent. Oral sucker subterminal, spherical, opening ventrally. Oral stylet well-developed, lanceolate. Pharynx globular. Oesophagus inconspicuous, intestinal caeca short. Three pairs of large penetration glands, equatorial, distributed in two lateral fields on each side of body. Ventral sucker spherical, smaller than oral sucker, post-equatorial. Cystogenous gland cells in the posterior end; excretory vesicle Y-shape. Tail small, simple, 31.3–48.8 (42.8) long, 20.2–27.2 (23.1) wide. In *A. alleei*, LE.

Virgulate cercaria

Virgulate cercaria (figs 1g and 3c) Description based on 20 specimens: body small, elongate, 128.3–204.2 (159.9) long, 62.3–90.5 (76.5) wide at equatorial region; tegument spinose, blunt spines, with homogeneous distribution; small ciliated papillae among spines; eyes-spots absent. Oral sucker subterminal, spherical, oral aperture opening ventrally, one pair of virgulae present. Oral stylet well-developed, lanceolate, needle shaped. Pharynx globular. Oesophagus inconspicuous; intestinal caeca short. Three pairs of penetration glands at level of ventral sucker, in two lateral fields on each side of body. Ventral sucker spherical, smaller than oral sucker, post-equatorial. Excretory vesicle Y-shaped in posterior extremity of body. Tail simple, shorter than body, without spines, 76.4–153.2 (115.9) long, 14–33.1 (21.8) wide. In *A. alleei*, LE.

Ophthalmocercaria

Ophthalmocercaria (figs 1h, 3d and 4e, i) Description based on ten specimens: body small, elongate, 120.1–138 (127) long, 70.3–75.9 (73.3) wide at equatorial region; tegument spinose, spines blunt, with homogeneous distribution; four pairs of

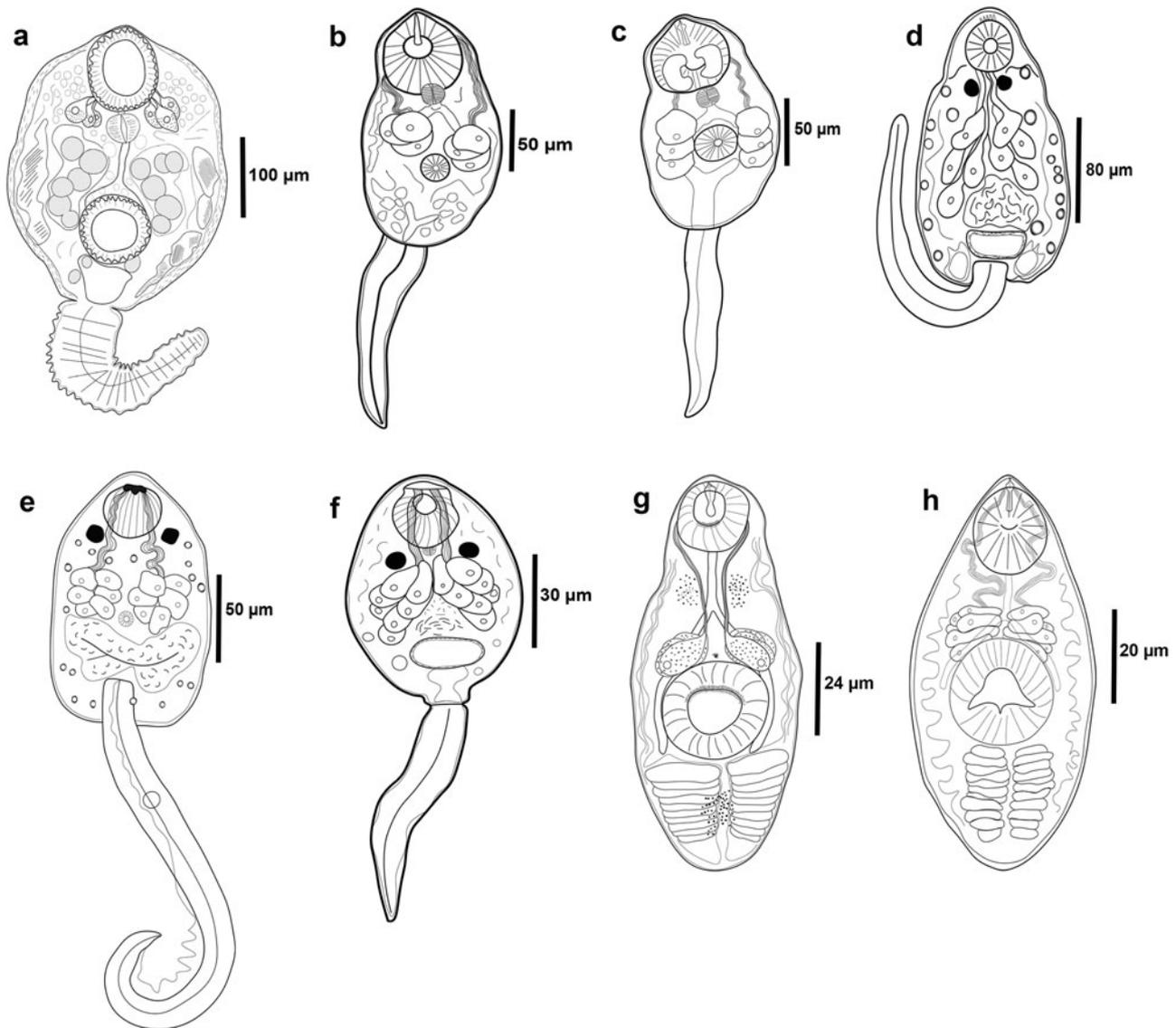


Fig. 3. Line drawings of the cercarial morphotypes released from molluscs in two lakes of Los Tuxtlas, Veracruz. (a) Distome cercaria II; (b) Xiphidiocercaria; (c) Virgulate cercaria; (d) Ophthalmocercaria; (e) Pleurolophocercous cercaria; (f) Monostome cercaria; (g) Cysticercous cercaria I; (h) Cysticercous cercaria II.

small ciliated papillae on ventral region; eyes-spots large, spherical to oval. Oral sucker subterminal, spherical, oral aperture opening ventrally; nine hooks on dorsal border of oral opening; six papillae on ventral border of oral opening. Pharynx inconspicuous, globular. Oesophagus short, intestinal caeca short. Four pairs of large penetration glands, oval, in two lateral fields on each side of body. Ventral sucker absent. Cystogenous gland cells distributed along body. Excretory vesicle oval-shaped, in posterior extremity of body. Tail simple without spines, equal large than body 161.6–182.2 (170.1) long, 21.8–34.7 (26.8) wide. In *M. tuberculata*, LZ.

Pleurolophocercous cercaria

Pleurolophocercous cercaria (figs 1i, 3e and 4f, j) Description based on ten specimens: body small, bell-shaped, 140.3–169.6 (155.4) long, 50–100.2 (80.2) wide at equatorial region; tegument spinose in anterior half from anterior end to level of eye-spots, spines blunt; eyes-spots large, oval to spherical. Oral sucker subterminal, spherical. Ten large circumoral spines on dorsal border

of oral opening. Pharynx, oesophagus and intestinal caeca not discernible in our specimens. Ventral sucker weakly developed, spherical, equatorial. Six pairs of penetration glands in two lateral fields, one on each side of body. Cystogenous glands distributed along body. Excretory vesicle large, Y-shaped. Tail longer than body, curved dorsoventrally, with a lateral fin-fold, 310–390 (337.3) long, 20.3–38.9 (27.3) wide. In *A. alleei*, LE.

Monostome cercaria

Monostome cercaria (figs 1j, 3f and 4g, k) Description based on ten specimens: body small, elongate, 98.9–110.5 (103.5) long, 48.23–50.7 (49.5) wide at mid-hindbody; tegument spinose, spines lanceolate, with homogeneous distribution; two large eyes-spots, spherical. Oral sucker subterminal, spherical, oral aperture directed ventrally, with four conspicuous hooks on antero-dorsal border of oral opening in one horizontal row. Pharynx globular, small. Oesophagus and intestinal caeca inconspicuous. Seven pairs of large penetration glands, in two lateral fields on each side of body, forming two clusters. Ventral sucker absent.

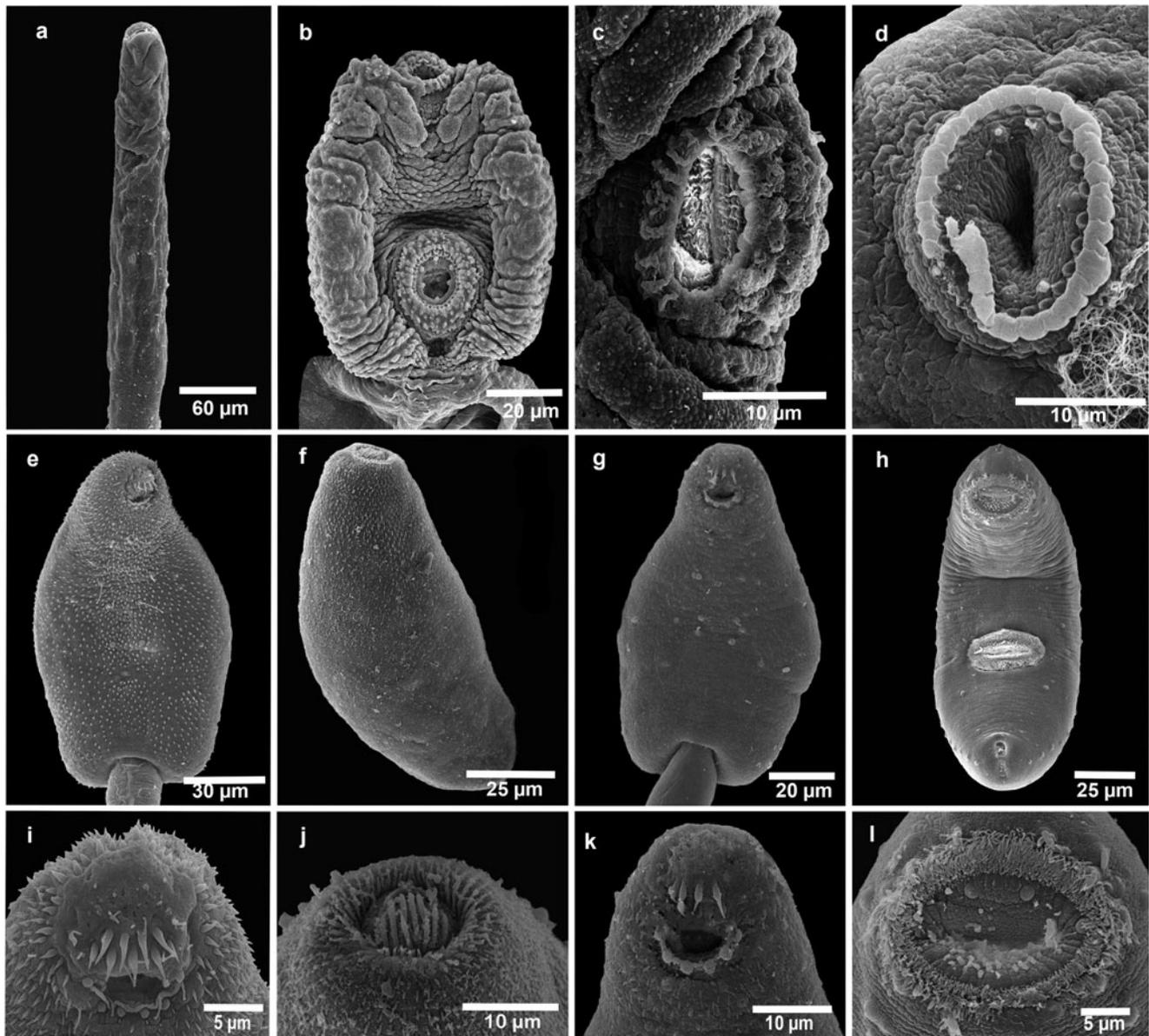


Fig. 4. SEM photomicrographs of the cercarial morphotypes released from molluscs in two lakes of Los Tuxtlas, Veracruz. (a) Body of *Furcocercous* cercaria I; (b) body of *Distome* cercaria I; (c) oral sucker of *Distome* cercaria I; (d) ventral sucker of *Distome* cercaria II; (e) body of *Ophthalmocercaria*; (f) body of *Pleurolophocercous* cercaria; (g) body of *Monostome* cercaria; (h) body of *Cysticercous* cercaria I; (i) oral sucker of *Ophthalmocercaria*; (j) oral sucker of *Pleurolophocercous* cercaria; (k) oral sucker of *Monostome* cercaria; (l) oral sucker of *Cysticercous* cercaria I.

Excretory vesicle oval. Tail simple, shorter than body, without spines, 80.45–87.2 (83.7) long, 14.5–17.45 (15.7) wide. In *A. alleei*, *B. helophila* and *M. tuberculata*, LE and LZ.

Cystocercous cercaria

Cystocercous cercaria (figs 1k, 3g and 4h, l) Description based on ten specimens: anterior chamber hollow, oval, with an opening in anterior region. Total length 1090.5–1500.2 (1370.3). Apparently without prominent structures. A pyramidal mass of granular cells arising from the base at junction with the tail. Tail longer than the chamber, cylindrical, tapering from anterior end, almost equal to that of the chamber to form a bluntly rounded posterior end; transparent, with nuclei in greater concentration near the anterior end. Cercaria body small, elongate, 136.3–148.4 (145.4) long, 55–57.2 (55.1) wide at equatorial region; tegument lacking spines; eye-spots lacking. Sensorial papillae distributed ventrally

along body. Oral sucker subterminal, spherical, oral aperture opening ventrally, with numerous sensorial bristles along exterior border. Dumb-bell-shaped papillae surrounding oral sucker opening. Oral stylet well-developed, lanceolate, rounded posteriorly. Pharynx absent. oesophagus long. Intestinal caeca short reaching posterior border of ventral sucker. Six pairs of penetration glands, pre-acetabular, in two lateral clusters on each side of body. Ventral sucker spherical, larger than oral sucker, post-equatorial. Excretory bladder Y-shaped, cytogenous gland cells arranged in two clusters. Excretory pore at posterior end. In *Pisidium* sp., LZ.

Cystocercous cercaria II

Cystocercous cercaria II (figs 1l and 3h) Description based on ten specimens: anterior chamber hollow, spherical, without anterior opening. Total length 905.3–960.2 (921.1). Without prominent structures. A rectangular mass of granular cells arises from the

base at junction with the tail. Tail cylindrical, tapering posteriorly; bluntly rounded posterior end. Transparent body. The chamber contains cercarial body 115.2–139.2 (127.2) long, 147–136.4 (141.9) wide. Body small, elongate to oval, 65.2–80.4 (73.6) long, 42.3–49.2 (44.8) wide at equatorial region; tegument without spines, with small papillae; eyes-spots lacking. Oral sucker subterminal, spherical, oral aperture directed ventrally, sensorial bristles not observed. Oral stylet well-developed, lanceolate. Pharynx absent. Oesophagus long. Intestinal caeca short. Six pairs of transversally elongated penetration glands, pre-acetabular, in two lateral clusters on each side of body. Ventral sucker spherical, post-equatorial, sensorial bristles not observed, larger than oral sucker. Excretory vesicle Y-shaped. Excretory pore at posterior end. In *Pisidium* sp., LZ.

Molecular identification of cercarial morphotypes

A preliminary BLAST search of the GenBank nucleotide database showed that the newly generated sequences belong to eight families of digeneans, namely Diplostomidae, Strigeidae, Echinochasmidae, Gorgoderidae, Heterophyidae, Lecithodendriidae, Pleurogenidae and Cryptogonimidae. To further corroborate the allocation of each morphotype into a genus, sequences were included in a comprehensive phylogenetic analysis of the Digenea. Morphotypes recognized morphologically were allocated into ten genera (table 2): *Apharyngostrigea* Ciurea, 1927, *Ascocotyle* Looss, 1899, *Centrocestus* Looss, 1899, *Echinochasmus* Dietz, 1909, *Gorgoderina* Looss, 1899, *Langeronia* Caballero & Bravo, 1949, *Lecithodendrium* Looss, 1896, *Oligogonotylus* Watson, 1976, *Phyllodistomum* Braun, 1899 and *Posthodiplostomum* Dubois, 1936. To further identify the cercarial morphotypes to the lowest taxonomic level, particular phylogenetic analyses were built for each family group using available 28S rDNA sequences from GenBank. The 12 cercarial morphotypes diagnosed morphologically resulted in the recognition of 16 putative trematode species based on molecular phylogenetic analyses of the 28S rRNA gene, ten as parasites of birds, three of freshwater fish, two of amphibians and one of mammals (table 2).

The alignment of the phylogenetic analyses of the families Diplostomidae and Strigeidae consisted of 1303 bp, including the newly generated sequences and those available in GenBank for some members of the superfamily Diplostomoidea (fig. 5a). *Mesostephanus microbursa* Caballero, Grocott & Zerecero, 1953 and *Braunina cordiformis* Wolf, 1903 were selected as outgroups. The sequence of the furcocercous cercariae type I (ex *Ferrissia* sp., one isolate) was placed as sister of *Posthodiplostomum* sp. Genetic divergence among congeners was 3.2%. The sequences of the furcocercous cercariae type II (*H. excentricus*, one isolate) and III (two isolates) appeared as sister taxa of several species of *Apharyngostrigea* and *Parastrigea* Szidat, 1928, with a very low genetic divergence among the novel sequences and *Apharyngostrigea* (0.23%) (fig. 5a).

In the phylogenetic analysis of the family Echinochasmidae, the alignment consisted of 1225 bp, including the newly generated sequences and those available in GenBank for some members of the superfamily Echinostomatoidea (fig. 5b); sequences of the Distome cercaria I (ex *A. alleei*, one isolate) and Distome cercaria II (ex *B. helophila*, four isolates) appeared as sister taxa of species of the genera *Echinochasmus* Dietz, 1909 and *Stephanoprora* Odhner, 1902, showing 0.5–0.6% of genetic divergence.

For the phylogenetic analysis of the families Lecithodendriidae and Pleurogenidae, the alignment consisted of 1290 bp, including

the newly generated sequences and those available in GenBank for some members of superfamily Microphalloidea (fig. 6a); sequences of Xiphidiocercaria (ex *A. alleei*, three isolates) appeared as the sister species of *Lecithodendrium* spp., with a genetic divergence of 2.2%. Instead, sequences of the virgulate cercaria (ex *A. alleei*, four isolates) were nested in a clade with *Langeronia macrocirra* (= *Loxogenes macrocirra*) Caballero & Bravo, 1949, with null genetic divergence and showing conspecificity (fig. 6a).

The phylogenetic analysis of the family Gorgoderidae included an alignment of 1293 bp, considering the newly generated sequences and those available in GenBank for some members of the superfamily Gorgoderoidea (fig. 6b). Sequences of the Cystocercous cercaria I were identical to *Gorgoderina rosamondae* Velázquez-Urrieta & Pérez-Ponce de León, 2020, whereas the single sequence of the Cystocercous cercaria type II (ex *Pisidium* sp., three isolates) corresponded genetically with the genus *Phyllodistomum*; however, one isolate showed a very low genetic divergence with *Phyllodistomum inecoli* Razo-Mendivil, Pérez-Ponce de León & Rubio-Godoy, 2013 (0.2%) and was considered conspecific (fig. 6b). The other two isolates of that cercarial morphotype represented a separate species.

Finally, the phylogenetic analysis cercarial morphotypes included in the families Cryptogonimidae and Heterophyidae consisted of 1298 bp, including the newly generated sequences and those available in GenBank for some members of superfamily Opisthorchioidea (fig. 7); sequences of the Pleurolophocercous cercaria (ex *A. alleei*, one isolate) corresponded genetically with *Oligogonotylus manteri* Watson, 1976; sequences of the Ophthalmocercaria (ex *M. tuberculata* two isolates) were identical to *Centrocestus formosanus* Nishigori, 1924. Twenty-eight sequences of the Monostome cercaria were obtained, including four isolates from *B. heliophila*, five isolates from *M. tuberculata* and 19 isolates from *A. alleei*. All these sequences were retrieved as four independent monophyletic clades and appeared as sister taxa of different species of *Ascocotyle* Looss, 1899, although conspecificity with some species was not evidenced for either of them because genetic divergence values varied were very high and varied between 5 and 7%. Overall, molecular phylogenetic analyses of the cercarial morphotypes yielded the identification of eight to genus level and four to species level (*G. rosamondae*, *L. macrocirra*, *O. manteri*, *P. inecoli*).

Discussion

The present study represents a fine-scale morphological and molecular analysis of the trematode diversity in two freshwater habitats of Los Tuxtlas tropical rainforest. Overall, of the 11 species of molluscs studied, six harboured trematode infections; however, our results showed that each locality possesses their own mollusc and digenean fauna. Traditionally, the recognition of cercarial diversity rely on detailed morphological descriptions, sometimes accompanied with the description of intra-molluscan stages (e.g. Scholz *et al.*, 2001; Gilardoni *et al.*, 2011; Veeravechskij *et al.*, 2018). The modern approach to describe larval trematode diversity has been successfully used by several authors (see Kudlai *et al.*, 2015; O'Dwyer *et al.*, 2015; Donald & Spencer, 2016; Chontanarith *et al.*, 2017; Gordy & Hanington, 2018; Huston *et al.*, 2018; Cribb *et al.*, 2019; Onaca *et al.*, 2020). We followed such approach and were able to allocate the 12 cercarial morphotypes into 16 putative species, using a combination of morphological and molecular data along with other sources of information such as the previous records of trematodes in the surrounding area, host association and geographical distribution.

Table 2. Summary data of cercarial morphotypes in molluscs of the tropical rainforest of Los Tuxtlas, Veracruz, Mexico.

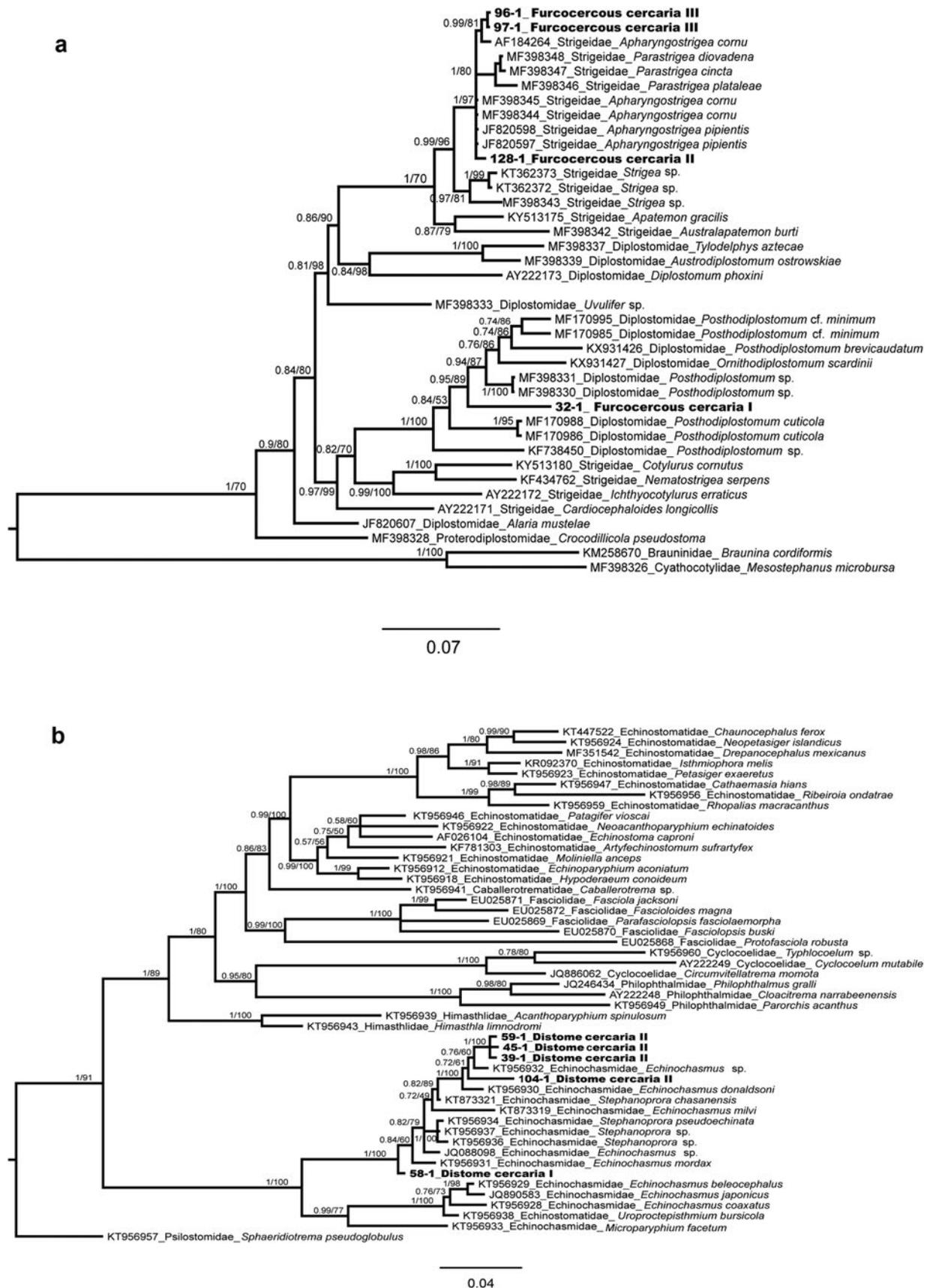
Trematode	Cercarial morphotype	Host species	Locality	GenBank accession no.
Family Diplostomidae				
<i>Posthodiplostomum</i> sp.	Furcocercous cercaria I	<i>Ferrissia</i> sp.	LE, Los Tuxtlas, Veracruz	MZ570083
Family Strigeidae				
<i>Apharyngostrigea pipientis</i>	Furcocercous cercaria II	<i>Hebetancylus excentricus</i>	LE, Los Tuxtlas, Veracruz	MZ570086
<i>Apharyngostrigea</i> sp.	Furcocercous cercaria III	<i>Hebetancylus excentricus</i>	LE, Los Tuxtlas, Veracruz	MZ570084, 85
Family Echinochasmidae				
<i>Echinochasmus</i> sp.1	Distome cercaria I	<i>Aroapyrgus alleei</i>	LE, Los Tuxtlas, Veracruz	MZ570089
<i>Echinochasmus</i> sp. 2	Distome cercaria II	<i>Biomphalaria helophila</i>	LZ, Los Tuxtlas, Veracruz	MZ570087, 88, 90, 91
Family Lecithodendriidae				
<i>Lecithodendrium</i> sp.	Xiphidiocercaria	<i>Aroapyrgus alleei</i>	LE, Los Tuxtlas, Veracruz	MZ570096–98
Family Pleurogenidae				
<i>Langeronia macrocirra</i>	Virgulate cercaria	<i>Aroapyrgus alleei</i>	LE, Los Tuxtlas, Veracruz	MZ570092–95
Family Heterophyidae				
<i>Centrocestus formosanus</i>	Ophthalmocercaria	<i>Melanoides tuberculata</i>	LZ, Los Tuxtlas, Veracruz	MZ570131
Family Cryptogonimidae				
<i>Oligogonotylus manteri</i>	Pleurolophocercous cercaria	<i>Aroapyrgus alleei</i>	LE, Los Tuxtlas, Veracruz	MZ570132
<i>Ascocotyle</i> sp. 1	Monostome cercaria	<i>Biomphalaria helophila</i>	LZ, Los Tuxtlas, Veracruz	MZ570112, 119, 120, 124
<i>Ascocotyle</i> sp. 2	Monostome cercaria	<i>Melanoides tuberculata</i>	LZ, Los Tuxtlas, Veracruz	MZ570110, 111, 123, 127, 128
<i>Ascocotyle</i> sp. 3	Monostome cercaria	<i>Melanoides tuberculata</i>	LZ, Los Tuxtlas, Veracruz	MZ570122, 125, 126, 129
<i>Ascocotyle</i> sp. 4	Monostome cercaria	<i>Aroapyrgus alleei</i>	LE, Los Tuxtlas, Veracruz	MZ570102–109, 113–118, 121
Family Gorgoderidae				
<i>Gorgoderina rosamondae</i>	Cystocercous cercaria I	<i>Pisidium</i> sp.	LZ, Los Tuxtlas, Veracruz	MW616452, 53
<i>Phyllostomum</i> sp. 1	Cystocercous cercaria II	<i>Pisidium</i> sp.	LZ, Los Tuxtlas, Veracruz	MZ570099, 100
<i>Phyllostomum</i> sp. 2	Cystocercous cercaria II	<i>Pisidium</i> sp.	LZ, Los Tuxtlas, Veracruz	MZ570101

LE, Laguna Escondida; LZ, Laguna Zacatal.

Families Diplostomidae and Strigeidae

In the phylogenetic tree (fig. 5a), three morphotypes were resolved as members of the families Diplostomidae and Strigeidae – that is, the Furcocercous cercariae types I, II and III. The first one was nested within a group of species of the genera *Posthodiplostomum* Dubois, 1936 and *Ornithodiplostomum* Dubois, 1936, with high nodal support value. In Veracruz, at least eight species of freshwater fish have been reported as hosts of the metacercariae of *Posthodiplostomum minimum* (see Pérez-Ponce de León *et al.*, 2007); adults of *P. minimum* were reported from ardeids of Lake

Catemaco, a locality 31 km south of LE (Lamothe-Argumedo & Pérez Ponce de León, 1986). Sequences of the 28S rRNA gene of those specimens are not available yet and are required to confirm they are conspecific with our samples. However, due to the phylogenetic position on the tree, we concluded that our specimens belong in the genus *Posthodiplostomum*. We cannot ascertain if the specimens from Los Tuxtlas belong to the species *P. minimum*. An ongoing study on the genetic diversity of larval *Posthodiplostomum* in Middle America shows higher richness of species in the genus as previously considered.



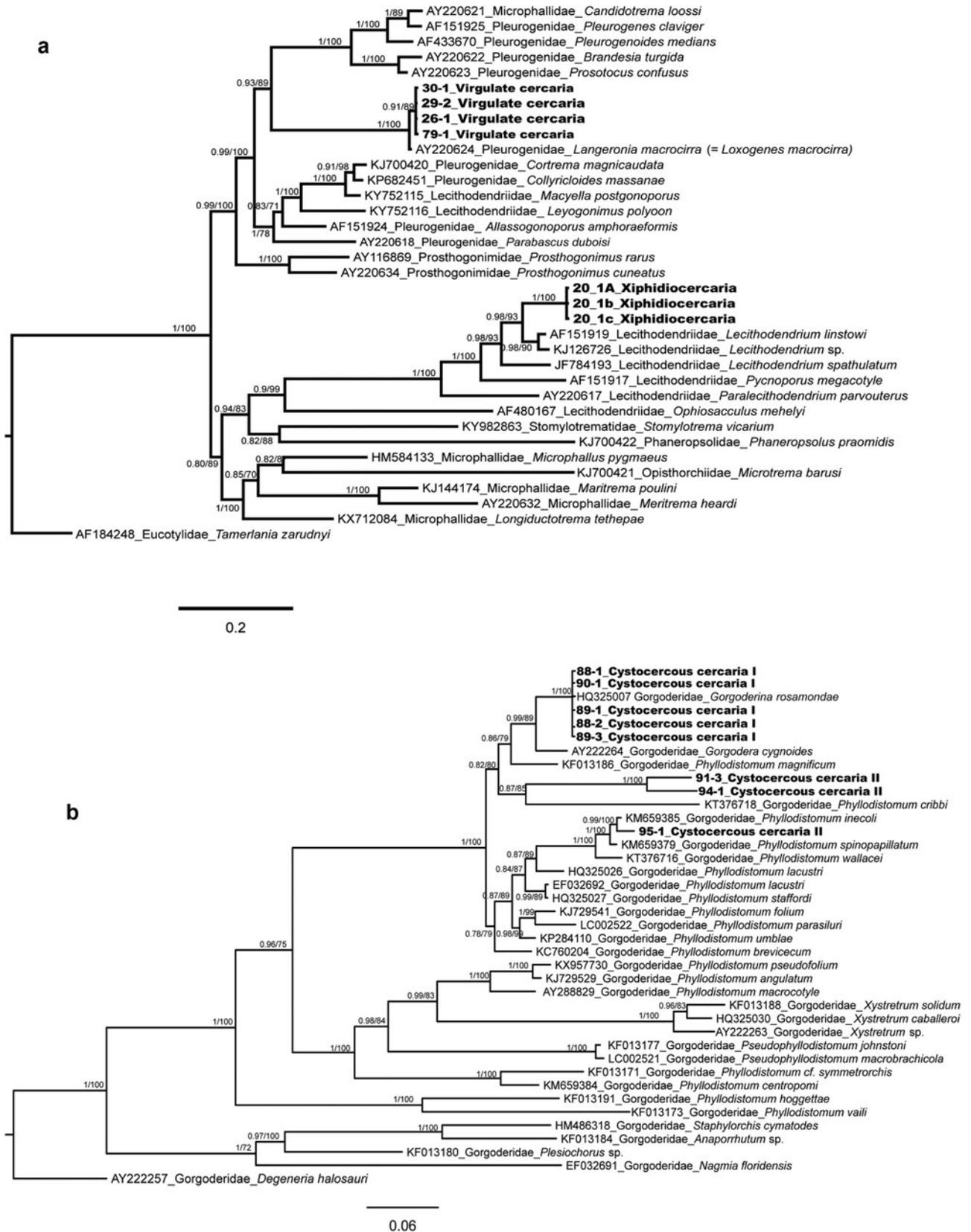


Fig. 6. Bayesian phylogenetic trees of the 28S rRNA showing the phylogenetic position of Virgulate cercaria, Xiphidiocercaria and Cystocercous cercaria morphotypes released by snails or clams from Los Tuxtlas. (a) Partial phylogenetic tree of superfamily Microphalloidea; (b) partial phylogenetic tree of the superfamily Gorgoderioidea.

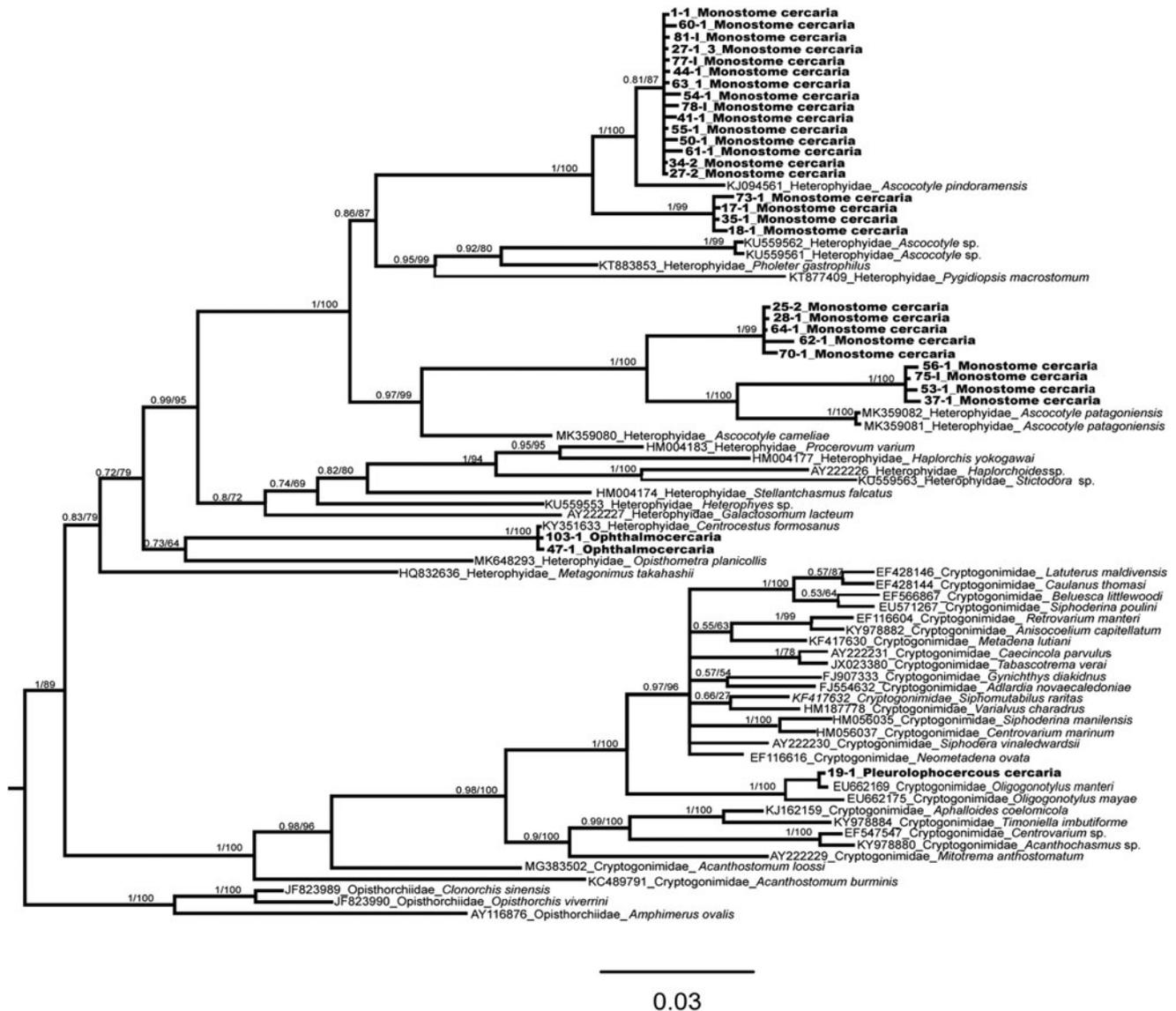


Fig. 7. Bayesian phylogenetic tree of the 28S rRNA of the superfamily Opisthorchioidea (in part), showing the phylogenetic position of Monostome cercaria, Ophthalmocercaria and Pleurolophocercus cercaria released by snails from Los Tuxtlas.

The second and third morphotypes (Furcocercous cercariae type II and III) were resolved within the family Strigeidae *sensu lato*, in a group of species of the genera *Apharyngostrigea* Ciurea, 1927, and *Parastrigea*; these relationships were also supported with a high nodal support value. In Veracruz, two species have been reported and sequenced from ardeid fish-eating birds (Hernández-Mena *et al.*, 2014, 2017), *Apharyngostrigea cornu* (Zeder, 1800) and *Parastrigea diovadena* Dubois & Macko, 1972. Particularly in the region of Los Tuxtlas, adult forms of *Apharyngostrigea* sp. from *Egretta thula* were recorded by Lamothe-Argumedo *et al.* (1993). Figure 5a depicts the interrelationships among *A. cornu*, *A. pipientis* (Faust, 1918) and *P. diovadena* in an unresolved polytomy, although the genetic distance between our samples and *A. cornu* is the lowest (0.23%). We identified our specimens as members of *Apharyngostrigea*. A recent study (Locke *et al.*, 2021) revealed that previous sequences identified as *A. cornu* from Mexico match those of *A. pipientis*, a species widely distributed across the world. Most likely, the furcocercous cercariae released from *H. excentricus* characterized in our study correspond with *A. pipientis*; still, DNA

sequences of adult forms from fish-eating birds are required to confirm the hypothesis.

Family Echinochasmidae

In the phylogenetic tree (fig. 5b), two cercarial morphotypes were resolved as members of the family Echinochasmidae: Distome cercaria I and II. Both morphotypes were nested within a group of species of the genera *Echinochasmus* and *Stephanoprora*, which were not yielded as monophyletic, with high nodal support value. The Distome cercaria I was nested within species of *Echinochasmus* spp. The metacercariae of *Echinochasmus leopoldinae* Scholz, Ditrich & Vargas, 1996 has been reported infecting several freshwater fish species from Los Tuxtlas tropical rainforest and neighbouring areas (see Scholz *et al.*, 1996; Pérez-Ponce de León *et al.*, 2007). The species was sampled from Balzapote, approximately 6.3 km from LE; however, sequences of the 28S rRNA gene of that species are not currently available for comparison. Whether or not they

correspond to *E. leopoldinae* considering the previous reports based on morphology needs to be determined when sequences of the 28S rRNA gene and other molecular markers become available. Sequences of the Distome cercaria II occupy a different position within the phylogenetic tree, but still belong in *Echinochasmus*; these sequences show a small genetic distance (0.5–0.6%) with respect to *Echinochasmus* sp. and *E. donaldsoni*; the latter species was described from *Podilymbus podiceps* L. in the USA (Tkach *et al.*, 2015). We cannot confirm that our sequences belong in one of these taxa. Interestingly, the two putative species of *Echinochasmus* were sampled from different snail species and different localities.

Families Lecithodendriidae and Pleurogenidae

In the phylogenetic tree (fig. 6a), two cercarial morphotypes were resolved as belonging to the families Lecithodendriidae and Pleurogenidae: the Xiphidiocercaria and the Virgulate cercaria. The first morphotype appeared as the sister species of *Lecithodendrium* spp., and these relationships were supported with a high nodal support values. Species of Lecithodendriidae are parasites of insectivorous vertebrates and they use prosobranch molluscs as first intermediate hosts. Cercariae released from the snails encyst as metacercariae in aquatic insect larvae; these insects and then ingested by an insectivorous host and the life cycle is completed (Enabulele *et al.*, 2018). Species of *Lecithodendrium* are parasites of insectivorous bats, although no species has been reported in Mexico thus far (Pérez-Ponce de León *et al.*, 2007). Ditrich *et al.* (1997) reported Xiphidiocercaria sp. 3 as probably belonging to Lecithodendriidae, with no further discussion. Reports of at least three species of the genus *Paralecithodendrium* Travassos, 1921 parasitic in bats are available in localities of central Mexico. Since our sequences nest in a monophyletic clade highly supported along with other species of *Lecithodendrium*, we inferred that our specimens belong in that genus, although we cannot confirm the identification at species level based on the genetic distance with respect to these species. The Xiphidiocercaria sp. 3 reported by Ditrich *et al.* (1997) as Lecithodendriidae? is morphologically different from the one reported in our study. Our specimens possess three pairs of large penetration glands, and the ventral sucker is located posteriorly to these glands; instead, Xiphidiocercaria sp. 3 possesses four pairs of penetration glands, and the ventral sucker is located anteriorly to penetration glands. Furthermore, the topology of the phylogenetic analysis demonstrate also that they do not belong in *Paralecithodendrium*, which is resolved as the sister group of *Lecithodendrium*, confirming the results obtained by Enabulele *et al.* (2018).

The second morphotype, the Virgulate cercaria, is resolved within the Pleurogenidae as the sister taxa of *L. macrocirra* (= *L. macrocirra*), with high nodal support. The report of this cercarial morphotype was recently published by Velázquez-Urrieta & Pérez Ponce de León (2020), who inferred the life cycle of *L. macrocirra* in Los Tuxtlas tropical rainforest. The molecular and morphological information was used to link the larval forms with the adult stage of the trematode, and the transmission pathway of the species was elucidated. Adults of *L. macrocirra* have been reported in frogs in LE (Paredes-Calderón *et al.*, 2004; Paredes-León *et al.*, 2008; Martínez-Salazar & León-Règagnon, 2010).

Family Gorgoderidae

Two morphotypes were resolved as members of the family Gorgoderidae (fig. 6b): the Cystocercous cercaria type I and II.

The Cystocercous cercaria I is the larval form of *G. rosamondae* Velázquez-Urrieta & Pérez-Ponce de León, 2021. This life cycle of the species was recently inferred by Velázquez-Urrieta & Pérez-Ponce de León (2021). In the region of Los Tuxtlas, three additional species of *Gorgoderina* have been reported – namely, *Gorgoderina festoni* Mata-López & León Règagnon, 2005, *Gorgoderina parvicava* (Travassos, 1922) and *Gorgoderina* sp. (reported as *Gorgoderina attenuata* (Stafford, 1902) (Guillén-Hernández *et al.*, 2000; Paredes-Calderón *et al.*, 2004; Mata-López & León-Règagnon, 2005). The second morphotype, Cystocercous cercaria type II, was nested within a group of species of *Phyllodistomum*, with high nodal support value; it appears as sister taxon of *P. inecoli*. This species has been reported as adult in freshwater fishes, particularly poeciliids in Veracruz, but also in other states across south-eastern Mexico (Razo-Mendivil *et al.*, 2013); the 28S rDNA sequences are very similar to *P. inecoli*, exhibiting low genetic distance (0.2%). This lead us to conclude that our specimens represent the cercariae of *P. inecoli*.

Families Cryptogonimidae and Heterophyidae

In the phylogenetic tree (fig. 7), the Pleurolophocercous cercaria morphotype was resolved as a member of the family Cryptogonimidae and corresponded genetically with *O. manteri* because sequences were identical to those downloaded from GenBank. This species of trematode is commonly found in Middle American freshwater fishes of the family Cichlidae (Watson, 1976; Pérez-Ponce de León *et al.*, 2007; Razo-Mendivil *et al.*, 2008), which serve both as the second intermediate and definitive hosts (Scholz *et al.*, 1994). Morphologically, the cercariae of *O. manteri* is very similar to that described by Scholz *et al.* (1994), except by the fact that these authors reported the presence of seven pairs of penetration glands, and we only observed six in our specimens. Another difference is the first intermediate host. Scholz and co-workers elucidated the life cycle of the species in the Yucatan peninsula, and *Pyrgophorus coronatus* Pfeiffer was recognized as the intermediate host; in our study, we report the species in the gastropod *A. alleei*.

Two additional morphotypes were uncovered in our phylogenetic analysis (fig. 7), the Ophthalmocercaria and a Monostome cercaria; both yielded as members of the family Heterophyidae. The Ophthalmocercaria was genetically identical to *C. formosanus*. The metacercariae of *C. formosanus* has been reported in more than 60 freshwater fish species across Mexico, and represents an invasive species introduced to Mexico along with its first intermediate host, the thiarid snail *M. tuberculata* (Pérez-Ponce de León *et al.*, 2007; Aguilar-Aguilar *et al.*, 2009). In the area of Los Tuxtlas, the metacercariae of *C. formosanus* has been reported in five species of freshwater fishes of the families Mugilidae, Poeciliidae and Cichlidae (Salgado-Maldonado *et al.*, 2005).

The third morphotype, the Monostome cercaria, was nested within a group of species of the genus *Ascocotyle*, with high nodal support values. As adults, species of *Ascocotyle* are parasites of fish-eating birds. Even though we observed no discernible morphological differences among specimens of this cercarial morphotype, they formed four separate clades, probably corresponding to four species; one appears as the sister species of *Ascocotyle patagoniensis* Hernández, Montero, Crespo, García, Raga & Aznar, 2012, albeit with a large genetic distance (5.2–6.2%); our specimens are clearly a separate species. These two are the sister group of a clade formed by five individuals that seem to represent a separate species, diverging from the other two by 6.9–7%.

Another clade is formed by two subclades, one formed by four sequences, representing a separate species, sister to *Ascocotyle pindoramensis* Scholz, Santos & Portes, 2006, and 15 additional sequences of the Monostome cercaria, which also represent a separate species. The genus *Ascocotyle* is particularly species-rich in México. Twelve species allocated into three subgenera (i.e. *Ascocotyle*, *Leighia* and *Phagicola*) have been reported México, all as metacercariae, and seven also as adults (see Pérez-Ponce de León *et al.*, 2007). Unfortunately, no sequences of any of these species have been generated in previous studies. Three species – namely, *Ascocotyle (Ascocotyle) tenuicollis* Price, 1935, *Ascocotyle (Phagicola) diminuta* Stunkard & Haviland, 1924 and *Ascocotyle (Phagicola) nana* Ransom, 1920 – are widely distributed in freshwater fishes and fish-eating birds across south-eastern Mexico. We cannot ascertain whether the sequences generated in our study correspond to any of the species previously reported until DNA sequences of adults are generated and the molecular link between the cercariae and the adults is established. In Veracruz alone, where the tropical rainforest of Los Tuxtlas lies, six species of *Ascocotyle* have been reported (see supplementary table S1).

Trematode species diversity in Los Tuxtlas

Several parasite surveys have been conducted in Los Tuxtlas tropical rainforest; at least 57 trematode species have been reported in 35 species of vertebrates (supplementary table S1); this might be considered a large species richness because the number of analysed host species only represents 3.3% of the vertebrate fauna of the region. In total, 877 species of vertebrates have been reported, including 80 freshwater fishes, 44 amphibians, 118 reptiles, 507 birds and 128 mammals (González *et al.*, 1997; López, 2017). Of the 57 species of trematodes, only two cercariae have been morphologically and genetically characterized (Velázquez-Urrieta & Pérez-Ponce de León, 2020, 2021). Our study reported 16 species of trematodes, of which only four corresponded with adult forms previously reported in vertebrates of the area – namely, *L. macrocirra*, *G. rosamondae*, both in amphibians, *C. formosanus* and *O. manteri* in freshwater fishes. The remaining 12 putative species were allocated up to genus level, and their identity requires further molecular work; still, adults of the 12 species must be in the study region since their cercariae are infecting molluscs in the two freshwater habitats. Our study increased the trematode species diversity of Los Tuxtlas to 73 species; of these, 23 are parasites of freshwater fish, 19 of amphibians, 16 of birds, five of reptiles and ten of mammals. However, our expectation was to find a larger trematode species diversity in the studied molluscs, including not just a higher prevalence of infection and species richness, but also showing correspondence with the adults that have been found in vertebrates of the tropical rainforest. We speculate that there must be a seasonality in the distribution of cercariae among individual molluscs because we noticed a different composition of cercariae in the two sampling seasons. However, more freshwater habitats need to be sampled within the tropical rainforest to fully assess the diversity of molluscs that would serve as first intermediate hosts of trematodes. Clearly, the inventory of the trematode fauna in the region is far from complete. Still, many species of vertebrates remain unstudied.

Most of the species uncovered in this study exhibit a classic three-host life cycle, with the exception of *O. manteri*, which has been shown to have the capacity to abbreviate its life cycle since fish may serve as second intermediate host and definitive

host (Scholz *et al.*, 1994). Most of the species we report in this study use a wide variety of invertebrates and vertebrates as second intermediate host. For instance, species in the genera *Apharyngostrigea*, *Ascocotyle*, *Centrocestus*, *Echinochasmus* and *Oligogonotylus* encyst in fish; *Langeronia*, *Phyllodistomum* and *Lecithodendrium* encyst in aquatic insect larvae; and *Gorgoderina* encyst in tadpoles and odonates (Scholz *et al.*, 1994, 1996; Scholz & Salgado-Maldonado, 2000; Bolek *et al.*, 2009; Locke *et al.*, 2011; Besprozvanynkh *et al.*, 2017; Pinto *et al.*, 2018; Velázquez-Urrieta & Pérez-Ponce de León, 2020, 2021). Two novel investigations are worth mentioning. Selbach *et al.* (2020) and Duan *et al.* (2021) conducted comprehensive studies of cercarial diversity in freshwater ecosystems in Europe. The first one analysed 5347 snails of six species (from six lakes) and reported 36 species of trematodes, whereas the second analysed 5657 snails belonging to ten species (from 21 lakes) and reported 22 trematode species. Compared with our study, where we analysed 6434 molluscs belonging to two classes and 11 species, and reported 16 trematode species, it seems that species richness is lower in the tropical rainforest, where overall species diversity is much higher than that of temperate areas. Overall prevalence of infection is also contrasting. In our study, overall prevalence was very low – 1.8% – whereas in the studies by Selbach *et al.* (2020) and Duan *et al.* (2021) prevalence was 19.6 and 12.6%, respectively. Irrespective of the causes that determine such difference between habitats, the two studies mentioned above were designed not just to investigate the trematode diversity, but to provide relevant data on transmission pathways as well as trophic interactions in the ecosystems. For instance, considering the uneven distribution and abundance of snails among waterbodies and after controlling for snail body size, Selbach *et al.* (2020) found no statistically significant differences in prevalence of infection between localities, seasons and years of sampling; these data allowed authors to show that trematode communities were temporarily and spatially stable. Ultimately, these authors discovered that the composition of snail populations was the main factor shaping the trematode community structure. This pattern was also discussed by Gordy *et al.* (2016), who suggested that parasite diversity is not determined by snail host diversity but is determined by their species composition and abundance. Our results agree with such a pattern because only six of the 11 species of molluscs analysed (bivalves and gastropods) were infected, and one of the species, *A. alleei*, reached the highest trematode species richness, with five species. This cochliopid gastropod, along with *B. heliophila*, are the most abundant molluscs in each of the studied localities.

To the best of our knowledge, this is the first study to address the trematode larval diversity in molluscs of a tropical rainforest. Onaca *et al.* (2020) characterized and identified the digenean larval stages in a neotropical floodplain in Brazil; however, they only analysed 26 specimens of one snail species, *Aylacostoma chloroticum* (Scott), and although they found an overall prevalence of infection of 73.08%, only three digenean species were reported. The approach we followed once again demonstrated to be a very precise way to advance our knowledge of trematode life cycles (Kudlai *et al.*, 2015). In addition, the approach is very useful for providing a better estimate of the trematode species diversity in the area, and for providing baseline data for the further monitoring of ecosystem health.

Supplementary material. To view supplementary material for this article, please visit <https://doi.org/10.1017/S0022149X21000407>

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