Journal of Helminthology

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Assessing the diversity of freshwater fish trematodes from Laguna Escondida, Los Tuxtlas tropical rainforest, Mexico, using morphology and 28S rDNA sequences as barcodes

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Research Paper

Cite this article: Velázquez-Urrieta Y, García-Varela M and Pérez-Ponce de León G (2024). Assessing the diversity of freshwater fish trematodes from Laguna Escondida, Los Tuxtlas tropical rainforest, Mexico, using morphology and 28S rDNA sequences as barcodes. *Journal of Helminthology*, **98**, e67, 1–11
https://doi.org/10.1017/S0022149X2400049X.

Received: 21 April 2024
Revised: 20 June 2024
Accepted: 20 June 2024

Keywords:
Biodiversity; Digenea; DNA; integrative taxonomy; Mexico

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Abstract

Despite a great effort made for almost 90 years, the diversity of freshwater fish trematodes in Mexico is still far from being fully known. The addition of molecular data to the description of trematode diversity in the last two decades added the potential to establish more robust species limits and a more accurate biodiversity estimation, but also led in some instances to the recognition of cryptic species complexes. Here, we used sequences of the large subunit of the nuclear ribosomal gene (28S rRNA) as barcodes, and morphological data, to assess the diversity of freshwater fish trematodes from a lake within a tropical rainforest. Eighty freshwater fish specimens of eight species were studied, and 120 trematode specimens were collected. Morphologically, specimens were allocated into nine genera; molecular phylogenetic analyses along with sequence divergence data provided evidence for recognising 11 trematode taxa, six adults and five metacercariae; six of them were identified to species level. Geographical distribution and host association patterns are briefly discussed for each trematode taxa.

Introduction

Mexico is considered a megadiverse country due to its position between the Nearctic and Neotropical biogeographical regions, resulting in a great variety of environments (Morrone *et al.* 1999). Among vertebrates, fish are the most species rich group with 2,763 species representing near 10% of the total number of known species in the world (Espinosa-Pérez 2014). Freshwater fish are highly diverse, with about 500 species (Lyons *et al.* 2020), with a high percentage of them as endemic components (Miller *et al.* 2005). Their helminth fauna has been intensively studied (Pérez-Ponce de León and Choudhury 2005; Scholz and Choudhury 2014). A recent study on the fish–helminth interactions of continental waters of Mexico reported 483 helminth taxa parasitising 371 fish species in 1,070 localities throughout Mexico (García-Prieto *et al.* 2022). Other studies have shown that some river basins of southeastern Mexico possess higher species richness and endemism levels of freshwater fish helminths (Pérez-Ponce de León and Choudhury 2005; Aguilar-Aguilar *et al.* 2008; Pérez-Ponce de León *et al.* 2011). Still, most records lack genetic information associated to the species of helminth infecting freshwater fishes in Mexico.

Los Tuxtlas tropical rain forest is located in southeastern Mexico, in the coastal plain of the Gulf of Mexico (Von Thaden *et al.* 2020). Laguna Escondida (LE) is a relatively small freshwater reservoir within Los Tuxtlas region; thirteen freshwater fish species have been reported in the locality from the orders Atheriniformes: *Atherinella alvarezi* (Regan); Cichliformes: *Rocio octofasciata* (Regan), *Vieja fenestrata* (Günther); Characiformes: *Astyanax aeneus* (Günther), and *Astyanax finitimus* Bocourt; Cyprinodontiformes: *Belonesox belizanus* Kner, *Gambusia sexradiata* Hubbs, *Heterandria bimaculata* (Heckel), *Poecilia mexicana* Steindachner, and *Xiphophorus helleri* (Heckel); Mugiliformes: *Dajaus monticola* (Bancroft); Siluriformes: *Rhamdia guatemalensis* (Gunther), and *Cathorops aguadulce* (Meek) (Espinosa-Pérez 2017). Irrespective of the large fish diversity, information about their parasite fauna is scarce (see Salgado-Maldonado *et al.* 2005), and no assessment of the genetic diversity has been made. The trematode genetic diversity of LE has been only assessed through molecular studies of cercariae released from mollusks looking for the link between these larval forms and metacercariae with adults for elucidating parasite life cycles (see Velázquez-Urrieta and Pérez-Ponce de León 2020, 2021).

Considering that fish act either as intermediate or definitive hosts of many trematode species, the main objective of this study is to uncover the diversity of trematodes in freshwater fish from

LE using morphology and 28S rDNA sequences to assess the identity, genetic diversity, and phylogenetic position of adults and metacercariae.

Materials and methods

Specimens collection and morphological analyses

Specimens of freshwater fish were collected in Laguna Escondida (18°38'09"N, 95°07'28"W) in January 2019 and 2020. Fish were captured using cast nets, kept alive in containers with water from the collecting site, and transported to the laboratory. Fish euthanisation was carried out in strict accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals: 2020 edition (https//www.avma.org/sites/default/files/2020-02/Guidelines-on-Euthanasia-2020.pdf) and immediately examined for ecto- and endoparasites. Internal organs were separated in Petri dishes with 0.65% saline and analysed under the stereomicroscope. For morphological analysis, specimens were fixed in 4% hot formalin and preserved in ethanol (70%). Some individuals were stained with Gomori's trichrome, dehydrated through ethanol series, cleared in methyl salicylate, and mounted in Canada balsam. Voucher specimens were deposited in the Colección Nacional de Helmintos, Instituto de Biología, UNAM (CNHE). For molecular analyses, worms were preserved in 100% ethanol.

Molecular analyses

For molecular analyses, total DNA was extracted from each individual worm using DNAzol, following the protocol provided by the company (Molecular Research Canter, USA) (Chomczynski *et al.* 1997). Amplification and sequencing of the 28S rRNA gene was carried out using the primes: 28SL 5′ -AAC AGT GCG TGA AAC CGC CTC-3′ (Palumbi 1996) and LO 5′ -GCT ATC CTG AGR GAA ACT TCG-3′ (Tkach *et al.* 2000). Thermal cycling conditions for amplification reactions were 94°C for 1 min, followed by 35 cycles at 92°C for 45 s, 50°C for 40 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Sequencing reactions were accomplished using an ABI 3730xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at the Laboratorio Nacional de Biodiversidad, Instituto de Biología. Universidad Nacional Autónoma de México (LANABIO).

Sequences were edited and assembled using the program Geneious 5.1.7 (Biomatters Ltd. Auckland, New Zealand). All alignments were trimmed to the shortest sequence. The length of the alignment was variable among the analysed taxa. The sequences were first screened thorough the BLAST search (GenBank) for assigning them into the lowest taxonomic level through sequence identity values. Once allocated into family, newly generated sequences were aligned separately with some representative sequences downloaded from GenBank for species allocated in the families Allocreadiidae, Apocreadiidae, Clinostomidae, Cryptogonimidae, Derogenidae, Diplostomidae, Gorgoderidae, Haploporidae, and Heterophyidae; then, phylogenetic analyses were conducted separately. Alignments were built, and the number of base pairs is variable since each alignment was trimmed to the shortest sequence. DNA sequences were aligned using MAFFT (Katoh and Standley 2013), with the default parameters. Uncorrected *p* distances were obtained in MEGA-X (Kumar *et al.* 2018). Phylogenetic analyses were performed through Bayesian inference (BI), using Markov Chain Monte Carlo (MCMC) in Mr. Bayes V 3.1.2 (Ronquist *et al.* 2012), with appropriate model of evolution GTR+1+Γ determined with jModeltest 0.1.1 (Posada 2008). The chains were run for 1,500,000 generations, sampling trees every 1,000 generations; the first 25% of the sampled trees were discarded according to Tracer V 1.5 (htt://beast.bio.ed.ac.uk/ tracer); consensus topology and posterior probability values were calculated from the remaining 75% of the trees.

Results and discussion

Eighty individuals of freshwater fish from eight species were examined for trematodes (Table 1); seventy-three of the 80 analysed hosts were infected with at least one species of trematode. In total, 120 trematode specimens were collected. Morphologically, specimens were allocated into nine genera – namely, *Ascocotyle* Looss, 1899; *Clinostomum* Leidy, 1856; *Crassicutis* Manter, 1936; *Creptotrematina* Yamaguti, 1954; *Genarchella* Travassos, Artigas & Pereira, 1928; *Oligogonotylus* Watson, 1976; *Saccocoelioides* Szidat, 1954; *Posthodiplostomum* Dubois, 1936; and *Phyllodistomum* Braun, 1899. The preliminary nBLAST search of 28S rDNA corroborated the identification to genus level. In most instances, nBLAST search also confirmed conspecificity through a sequence identity higher than 99%, and individuals were identified up to species level. Morphology and 28S rDNA sequence data recognised 11 trematode taxa infecting fish from Laguna Escondida; six of them were identified to species level (Table 1, Figure 1). Of the 11 trematode taxa, seven were adults, and four metacercariae (Table 1). The fish species with the largest trematode diversity was the poecilid *Ga. sexradiata*, with six taxa (Table 1). Along with the nBLAST search, phylogenetic analyses were run through Bayesian inference to test the position of the sequenced individuals in the trematode phylogenetic tree, considering the families where each species is allocated (Figures 2–4).

The following species/taxa were identified (presented by developmental stage and ordered alphabetically):

Metacercariae

***Ascocotyle* sp.**

Metacercariae from the mesenteries of *V. fenestrata* and *G. sexradiata* were allocated to the genus *Ascocotyle*. The newly sequenced isolates were aligned with 28 sequences of *Ascocotyle* spp. Alignment was 967 base pair long and recovered the sequences from Laguna Escondida within a clade containing isolates of *Ascocotyle* from the same locality in Veracruz (sequenced from cercarial stages released from snails; Velázquez-Urrieta and Pérez-Ponce de León 2021), and from cichlids of Lake Nicaragua (Santacruz *et al.* 2022 erroneously identified it as *A. pindoramensis* (Travassos, 1928)), and varied from these sequences only between 0 and 0.55%, indicating they all represent the same species (Figure 2a). At least 14 species of *Ascocotyle* have been reported in Mexico, most of them as larval stages in freshwater and estuarine habitats (Pérez-Ponce de León *et al.* 2007; Espínola-Novelo *et al.* 2023), although no sequence data has been generated for adult forms yet to match with the larval forms from snails and fish. In Veracruz, where the tropical rainforest of Los Tuxtlas lies, six species of *Ascocotyle* have been reported (see Velázquez-Urrieta and Pérez-Ponce de León 2021). The newly sequenced individuals match one of the sequences obtained from snails in Laguna Escondida, although no adults have been found in birds to confirm species identity. Two species of *Ascocotyle* have been reported previously in Laguna Escondida:

https://doi.org/10.1017/S0022149X2400049X Published online by Cambridge University Press

Table 1. Species of trematodes found in freshwater fishes of Laguna Escondida, Los Tuxtlas tropical rainforest, ordered alphabetically by family

Species	Stage	Host	Host family	CNHE	Genbank
Allocreadiidae					
Creptotrematina aguirrepequenoi Jiménez Guzmán, 1973	Adult	*Astyanax aeneus* (Günther)	Characidae	11898	PP862786, 87
Clinostomidae					
Clinostomum sp.	Metcercaria	*Dajaus monticola* (Bancroft) *A. aeneus* (Günther) *Heterandria bimaculata* (Heckel) *Belonesox belizanus* Kner *Poecilia mexicana* Steindachner	Mugilidae Characidae Poeciliidae Poeciliidae Poeciliidae	11899 ------- ------- ------- -------	PP862767, 71 PP862768, 73, 74 PP862769, 65, 72 PP862775, 66 PP862770
Cryptogonimidae					
Oligogonotylus manteri Watson, 1976	Adult	*Gambusia sexradiata*	Poeciliidae	-------	PP862788, 89
Derogenidae					
Genarchella astyanactis (Watson, 1976)	Adult	*G. sexradiata*	Poeciliidae	11900	PP862798, 99
Diplostomidae					
Posthodiplostomum sp. 1	Metacercaria	*H. bimaculata* *Vieja fenestrata*	Poeciliidae Characidae	11901 -------	PP862781 PP862779, 80
Posthodiplostomum sp. 2	Metacercaria	*G. sexradiata* *V. fenestrata* *A. aeneus*	Poeciliidae Characidae Characidae	1902 ------- -------	PP862777 PP862778 PP862776
Gorgoderidae					
Phyllodistomum sp.	Adult	*Xiphophorus helleri* *B. belizanus*	Poeciliidae	------- -------	PP862796 PP862797
Phyllodistomum inecoli Razo-Mendivil, Perez-Ponce de León & Rubio-Godoy, 2013	Adult	*H. bimaculata*	Poeciliidae	11902	PP862790
Haploporidae					
Saccocoelioides orosiensis Curran, Pulis, Andres & Overstreet, 2018	Adult	*D. monticola* *A. aeneus* *H. bimaculata*	Mugilidae Characidae Poeciliidae	11903 ------- -------	PP862792, 93 PP862791 PP862794, 95
Heterophyidae					
Ascocotyle sp.	Metacercaria	*V. fenestrata* *G. sexradiata*	Characidae Poeciliidae	-------	PP862763 PP862764
Megaperidae					
Crassicutis cichlasomae Manter, 1936	Immature adults	*D. monticola* *P. mexicana*	Mugilidae Poeciliidae	------- 11904	PP862782, 84 PP862783, 85

A. tenuicollis Price, 1935 (= *A. felippei* Travassos, 1928) from *A. aeneus* and from *X. helleri*, and *A. nana* Ransom, 1920 from *X. helleri* (Salgado-Maldonado *et al.* 2005). Both species are widely distributed in Mexico; metacercariae of *A. tenuicollis* have been reported from 24 freshwater fish species, and adults from four species of fish-eating birds, whereas *A. nana* has been found in 23 freshwater fish species and adults in three species (see Scholz *et al.* 2001 and references therein). The lack of sequence data for adults of *Ascocotyle* prevents establishing a link between larval forms and adults to identify the species; unfortunately, very few specimens were recovered from their hosts, and the quality of the material is very poor to achieve the identification following Scholz *et al.* (2001) identification key for larval stages since the number of spines was not established.

***Clinostomum* sp.**

Metacercariae encysted in the mesenteries, gill arches, and fins of *D. monticola, A. aeneus, H. bimaculata, B. belizanus*, and *P. mexicana* were morphologically identified as belonging to *Clinostomum*. These metacercariae are characterised by having a yellow colour, an elongate body, a well-developed and characteristic oral collar, small pharynx, and the genital organs scarcely developed (Figure 1a). The phylogenetic analyses included 11 newly generated sequences, and 10 additional sequences for *Clinostomum* spp. available in GenBan; the alignment was 980 bp long. The phylogenetic tree showed that the new sequences from Los Tuxtlas nested with *Clinostomum* sp. from an ardeid from Catemaco Lake, Veracruz (MH159733) (Figure 2b), a locality close to Laguna Escondida, although with a genetic divergence varying between 1.0% and 1.8%. In the case of *Clinostomum*, molecular markers other than 28S rDNA such as cytochrome c oxidase subunit 1 (*cox1*) and the internal transcribed spacers are better loci for species identification since the genetic library is more complete (see Locke *et al.* 2015a; Pérez-Ponce de León *et al.* 2016). Sereno-Uribe *et al.* (2022) showed that *cox1* is the most appropriate marker for species delimitation for

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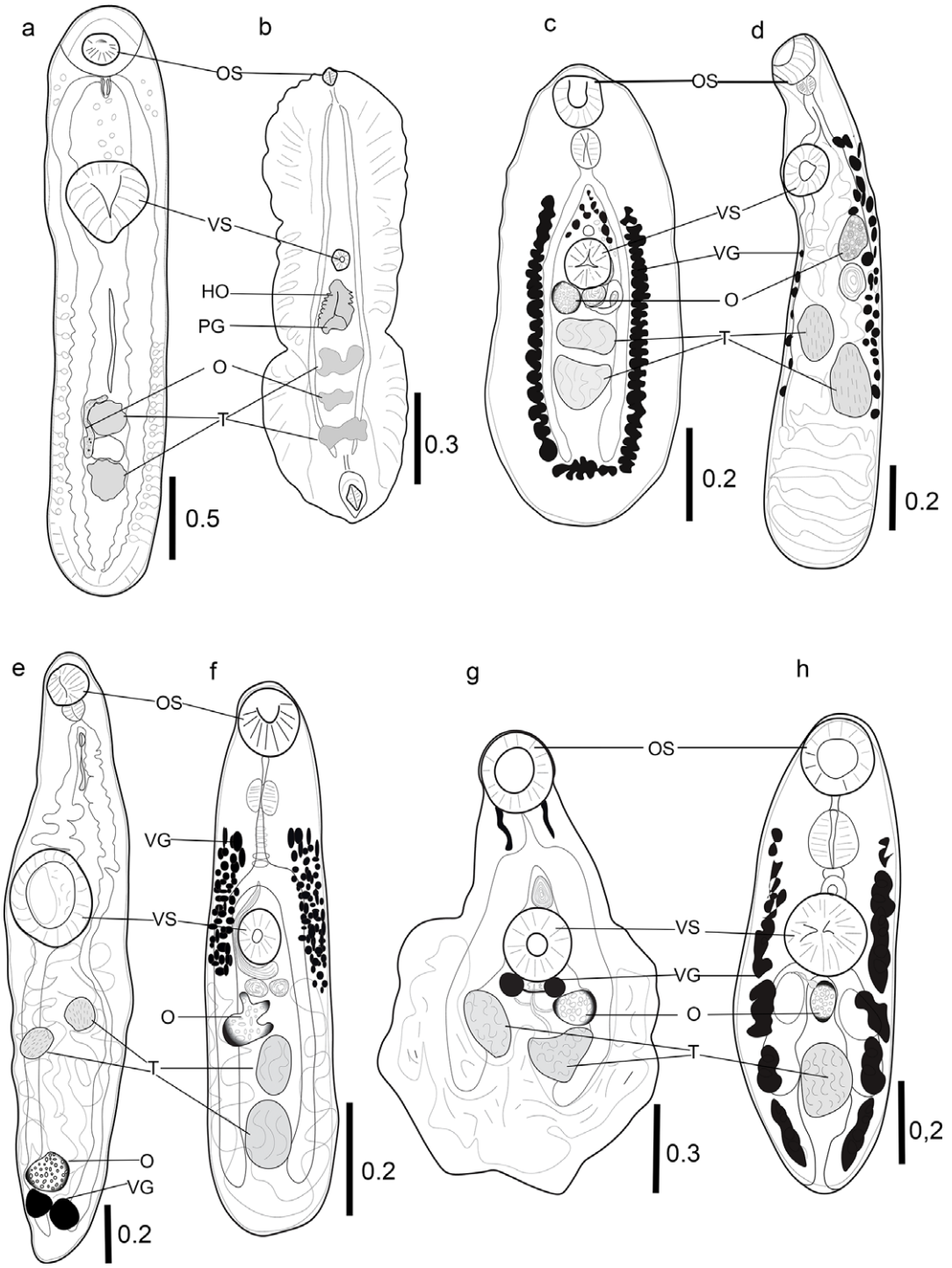

Figure 1. Line drawings of the trematodes of freshwater fishes from Laguna Escondida, Los Tuxtlas, Veracruz. *Clinostomum* sp. (a); *Posthodiplostomum* sp. 1 (b); *C. cichlasomae* (c); *C. aguirrepequenoi* (d); *G. astyanactis* (e); *O. manteri* (f); *P. inecoli* (g); *S. orosiensis* (h).

species in the genus. Still, novel 28S sequences of *Clinostomum* obtained in our study from five species of freshwater fishes show they are almost identical. Pérez-Ponce de León *et al.* (2016) identified at least three putative species of *Clinostomum* from samples obtained from Catemaco Lake. Two of them were described as *Clinostomum caffarae* Sereno-Uribe, García-Varela, Pinacho-Pinacho & Pérez-Ponce de León, 2018 and *Clinostomum arquus* Sereno-Uribe, García-Varela, Pinacho-Pinacho & Pérez-Ponce de León, 2018 based on adults sampled from the snowy egret, *Egretta thula* Molina. However, no 28S rDNA sequences were generated for these species, and we cannot compare them at this time. *Clinostomum complanatum* (Rudolphi, 1814) was previously reported by Salgado-Maldonado *et al.* (2005) as a parasite of *P. mexicana*; however, this represents a misidentification by the authors since it has been proven that *C. complanatum* is not distributed in the Americas (Pérez-Ponce de León *et al.* 2016). The identification of

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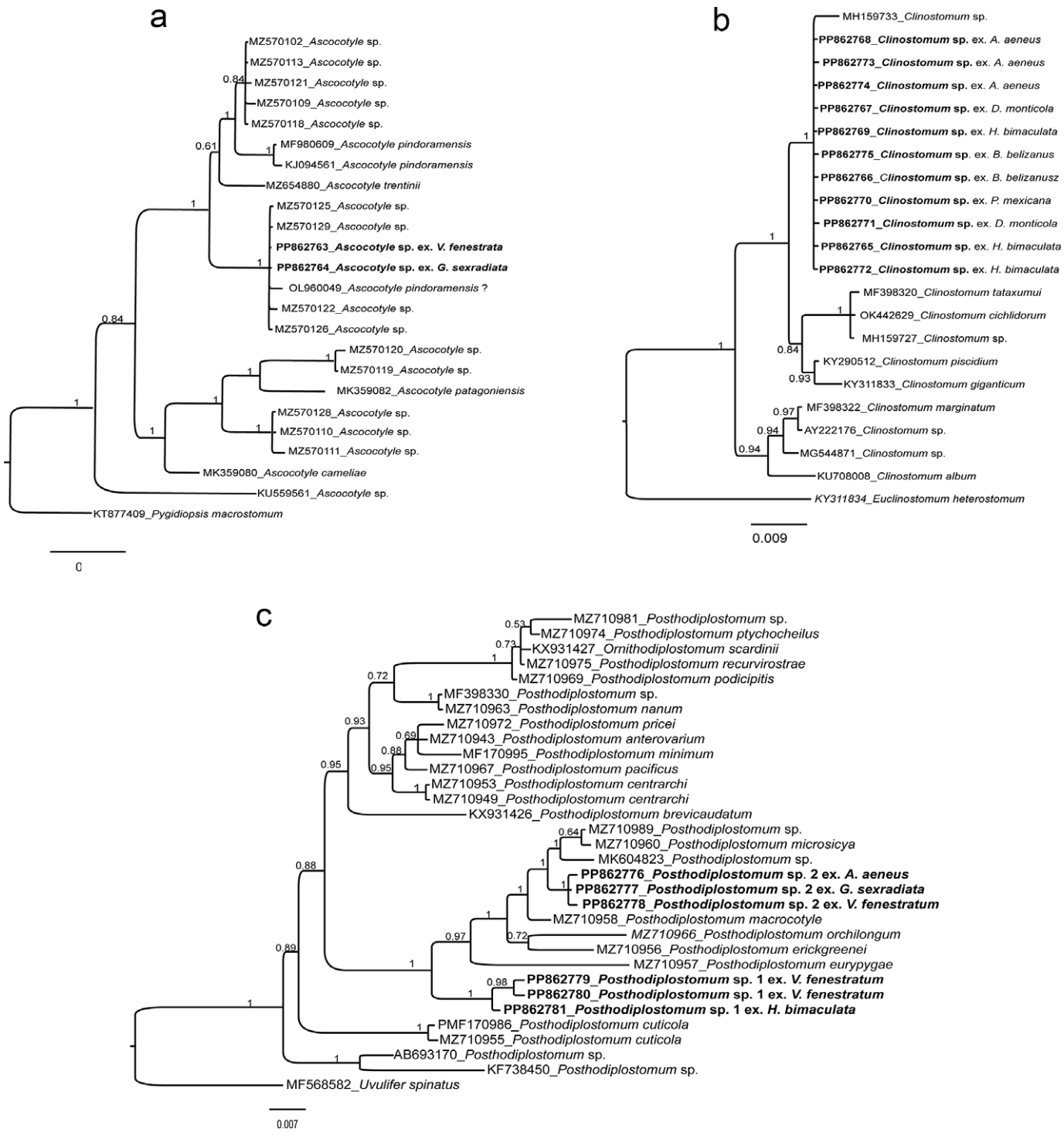

Figure 2. Bayesian phylogenetic trees of the 28S rDNA showing the phylogenetic positions of trematodes sequences from Laguna Escondida, Los Tuxtlas. *Ascocotyle* spp. (a); *Clinostomum* spp. (b); and *Posthodiplostomum* spp. (c).

this larval form at species level is pending until DNA sequences are obtained for other molecular markers.

***Posthodiplostomum* spp.**

Encysted metacercariae from the mesenteries of *H. bimaculata*, *A. aeneus*, *G. sexradiata*, and *V. fenestratum* were identified as belonging to the genus *Posthodiplostomum*; they are mainly characterised by having a body divided in two segments, a well-developed tribocytic organ in the anterior segment, and gonads poorly developed in the posterior segment (Figure 1b, *Posthodiplostomum* sp.1). Six isolates of the newly sequenced specimens were aligned with 25 sequences of *Posthodiplostomum* spp. available in GenBank for the 28S rRNA gene. Alignment was 1,015 bp. The phylogenetic tree yielded sequences from Laguna Escondida in two reciprocally monophyletic clades, showing they represent two separate species (Figure 2c). At this time, 28S rDNA sequences are only useful to recognise two separate

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lineages of *Posthodiplostomum* in Laguna Escondida, although none of them correspond to a previously described species. The nBLAST search did not yield a match with a previously described species. As in the case of *Clinostomum*, the genetic library for species of *Posthodiplostomum* is increasing steadily, and other molecular markers as *cox1* seem to be more accurate for species delimitation (see Locke *et al.* 2015b; Pérez-Ponce de León *et al.* 2022). Morphologically, metacercarial stages cannot be separated, and this represents a practical problem that can only be overcome by using DNA sequences. In a previous study, Salgado-Malonado *et al.* (2005) reported *P. minimum* (McCallum, 1921) parasitising *V. fenestrata* in Laguna Escondida; however, molecular analyses have shown that the genus *Posthodiplostomum* is constituted by at least six genetic lineages infecting freshwater fishes of Mexico, and none of them represents the species *P. minimum*. Our study revealed that *V. fenestrata* is, in fact, infected with two genetic lineages of *Posthodiplostomum* in Laguna Escondida, and neither of them correspond to *P. minimum*.

Adults

Crassicutis cichlasomae **Manter, 1936**

Trematodes from the intestines of *D. monticola* and *P. mexicana* were morphologically identified as *C. cichlasomae* because they have an oval body, pretesticular uterus, and a body surface without spines (Figure 1c); they were, however, juveniles. Due to the unusual host association (the species is a cichlid specialist, see Choudhury *et al.* 2017), identification was corroborated through 28S rDNA sequences. Four newly sequenced isolates were aligned with 13 sequences of four species of *Crassicutis* available in GenBank. Alignment was 999 bp long. The phylogenetic tree showed that the new sequences nested in a moderately supported clade with C. *cichlasomae* from cichlid fish of Mexico; sequence divergence was null, showing conspecificity, and appeared as the sister taxa of *C. choudhuryi* Perez-Ponce de León, Razo-Mendivil, Rosas, Mendoza & Mejia, 2008 from cichlids of Nayarit Mexico, with a divergence value of 1.3% (Figure 3a). Species of *Crassicutis* are members of the core parasite fauna of cichlids (*sensu* Pérez-Ponce de León

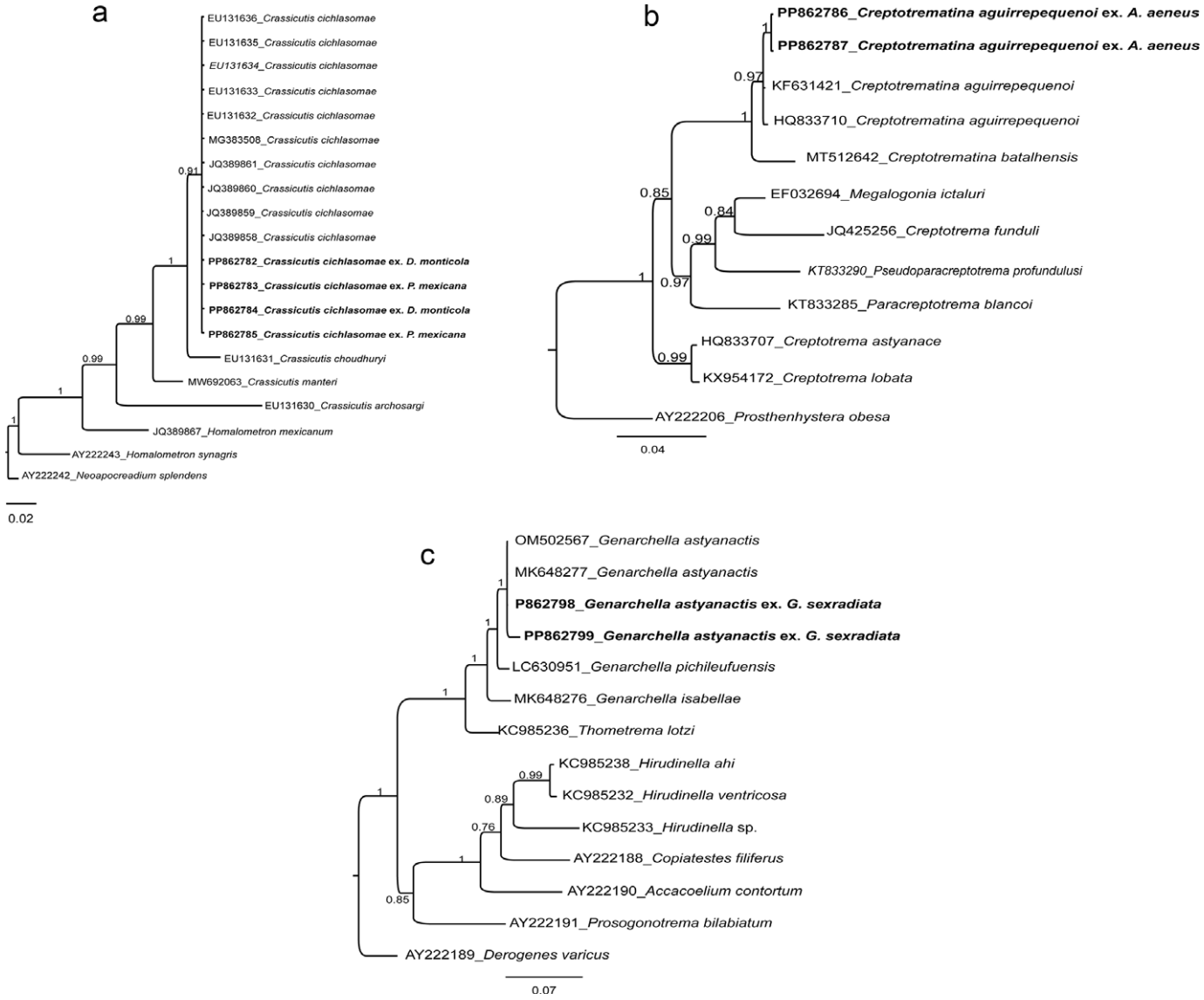

Figure 3. Bayesian phylogenetic trees of the 28S rDNA showing the phylogenetic positions of trematodes sequences from Laguna Escondida, Los Tuxtlas. *Crassicutis* spp. (a); *Creptotrematina* (b); and *Genarchella* spp. (c).

https://doi.org/10.1017/S0022149X2400049X Published online by Cambridge University Press

and Choudhury 2005). For instance, Pérez-Ponce de León *et al.* (2007) reported *C. cichlasomae* in 21 species of cichlids belonging to eight genera across the country. Since we sampled juvenile individuals from the intestine of a mugilid (*D. monticola*) and a poecilid (*P. mexicana*), we consider those as accidental infections. Both fish species are omnivorous and may have fed upon snails. Snails of the genus *Pyrgophorus coronatus* act as first and second intermediate host of *Crassicutis* (Scholz *et al.* 1995); most likely, even though *D. monticola* and *P. mexicana* were infected, the parasite did not reach sexual maturity. We did not sample cichlids in our study, but *V. fenestrata* occurs in the locality (Espinosa-Pérez 2017), and, in fact, *C. cichlasomae* was previously reported from that cichlid species in Laguna Escondida by Salgado-Maldonado *et al.* (2005).

***Creptotrematina aguirrepequenoi* Jiménez Guzmán, 1973**

Trematodes from intestines of *A. aeneus* were morphologically very similar to *C. aguirrepequenoi*, which are characterised by the presence of an elongated body, ventral sucker larger than the oral sucker, large number of small eggs, the uterus extending to the posterior end of the body, and the oral sucker with a pair of ventrolateral muscular lobes (Figure 1d). The 28S rDNA phylogenetic analysis consisted of 11 sequences of allocreadiids; alignment was 1,044 bp long. The newly generated sequences nested together with those of *C. aguirrepequenoi* (specimens from Mexico and Costa Rica) with high nodal support; our sequences differed from those in GenBank between 0.1 and 0.2%, indicating conspecificity (Figure 3b). All these sequences nest as the sister taxa of *C. batalhensis* Dias & Abdhalla, 2020 described from *Astyanax* spp. in Brazil (Dias *et al.* 2020). *Creptotrematina aguirrepequenoi* is apparently widely distributed across Middle America. The species was originally described by Jiménez-Guzman (1973) from *Astyanax mexicanus* (De Filippi) in northern Mexico, but it has been reported from *Astyanax* spp. from across Mexico, including Veracruz, but also in Costa Rica (Curran *et al.* 2011; Razo-Mendivil *et al.* 2014). A previous record of the species *Magnivitellinum simplex* Kloss, 1966 as a parasite of *A. aeneus* in Laguna Escondida was made by Salgado-Maldonado *et al* (2005). The species we report here is morphologically similar to *M. simplex*, and we tried to corroborate the identification by studying museum specimens since Salgado-Maldonado *et al.* (2005) mentioned that specimens were deposited in the Colección Nacional de Helmintos; however, they were actually not deposited, and we could not confirm if that was a misidentification.

***Genarchella astyanactis* (Watson, 1976)**

Specimens from the stomachs of *Ga. sexradiata* were morphologically identified as *G. astyanactis.* They are characterised by having an elongated body, curved seminal vesicle and vitellarium formed by two compact masses lying in the posterior end of the body (Figure 1e). The new sequences were aligned with other those of other species of derogenids, hirudinellids and didymozoids; the alignment consisted of 14 sequences and was 1,085 bp long. In the phylogenetic tree the new sequences were nested in a well-supported clade with specimens of *G. astyanactis* from Yucatán, Mexico and Lake Nicaragua, Nicaragua, and sequences were almost identical, varying 0–0.9%; this clade was recovered as the sister group of *G. pichileufuensis* Tsuchida, Urabe, Viozzi, Rauque & Flores, 2021 from a siluriform in Argentina, with divergence of 2.6% (Figure 3c) (Tsuchida *et al.* 2021). Another species of *Genarchella* was recently described by Moravec & Prouza (2024), *G. venezuelensis*, as a parasite of a loricariid siluriform in Venezuela, although is clearly differentiated from *G. astyanactis* by having a distinct median papilla-like lobe the anterior margin of the ventral sucker. The species was originally described from a characid in Lake Nicaragua (Watson, 1976), but has been found in *Astyanax* spp. in Mexico (Pérez-Ponce de León *et al.* 2007). Interestingly, the species seems to be specific to characids of the genus *Astyanax*, but the species was reported here in a poecilid (*G. sexradiata*). We studied specimens of the characid *A. aeneus* (Günther) but we were unable to obtain samples of this species. In this case, since adults were sexually mature, we cannot consider it represents an accidental infection.

***Oligogonotylus manteri* Watson, 1976**

Some adults from the intestines of *G. sexradiata* were identified as belonging to the cryptogonimid genus *Oligogonotylus* Watson, 1976 by having a longitudinal row of five to nine sucker-like gonotyls, and vitelline follicles extending between the pharynx level and anterior margin of the ovary (Figure 1f). Particularly, they match with the description of *O. manteri*. The phylogenetic tree included sequences of other cryptogonimids for an alignment of 14 terminals and 1,069 bp (Figure 4a). The newly sequenced individuals nested in a well-supported clade with those of *O. manteri* from Yucatán, Mexico, and from Lake Nicaragua, with null or very low sequence divergence values (0–0.1%). This species was recovered as the sister taxa of *O. mayae* Razo-Mendivil, Rosas-Valdez & Pérez-Ponce de León, 2008 from Mexico, with divergence values varying from 1.7% to 1.9%. No sequence data for the 28S rRNA gene were available for comparison for the species *O. andinus* Vélez-Sampedro, Uruburu & Lenis, 2022 described from poeciliids and cichlids in Colombia (Vélez-Sampedro *et al.* 2022). Adults of *O. manteri* have been reported in Mexico from 10 species of cichlids allocated in six genera. The metacercaria has been reported from the eyes, fins, gills, heart, intestine, mesentery, opercula, rectum, scales, and spleen of 10 species of cichlids, but also it has been found in another eight fish species, exhibiting low host specificity at this level (see Pérez-Ponce de León *et al.* 2007). Still, this represents the first report of adults of the species parasitising the intestine of a poecilid fish. Since all specimens are juvenile, we consider this an accidental infection.

***Phyllodistomum* spp.**

The specimens collected from the urinary bladder of the poeciliids *X. helleri, B. belizanus*, and *H. bimaculata* were morphologically identified as members of the genus *Phyllodistomum*; specimens were characterised by having a foliated hindbody, two compact and post-acetabular vitelline masses, and the uterus strongly coiled and extending in the hindbody (Figure 1g). Three individuals were sequenced and aligned with 42 sequences representing 28 species of *Phyllodistomum.* Alignment was 1,041 bp long; the phylogenetic tree yielded one of the newly generated sequences nested within *P. inecoli* Razo-Mendivil, Pérez-Ponce de León & Rubio-Godoy, 2013, a parasite of poeciliids from Mexico, with very low genetic divergence (0.1%) indicating conspecificity; two additional sequences were recovered as an independent clade, as the sister group to a clade containing sequences of *P. wallacei* Pérez-Ponce de León, Martínez-Aquino & Mendoza-Garfias, 2015 and *P. spinopapillatum* Pérez-Ponce de León, Pinacho-Pinacho, Mendoza-Garfias & García-Varela, 2015, *P. simonae* Pinacho-Pinacho, Sereno-Uribe, Hernández-Orts, García-Varela & Pérez-Ponce de León, 2021 + *P. inecoli* Razo-Mendivil, Pérez-Ponce de León & Rubio-Godoy, 2013, albeit with moderate nodal support value (0.89) (Figure 4b). The genetic divergence between the new sequenced individuals and the three species mentioned above varied from 0.6 to 1.5%. In this case, 28S rDNA sequences are not very useful in establishing species limits in a group of species of *Phyllodistomum* infecting cyprinodontiform fishes (see Pinacho-Pinacho *et al.* 2021). These authors found a low genetic divergence

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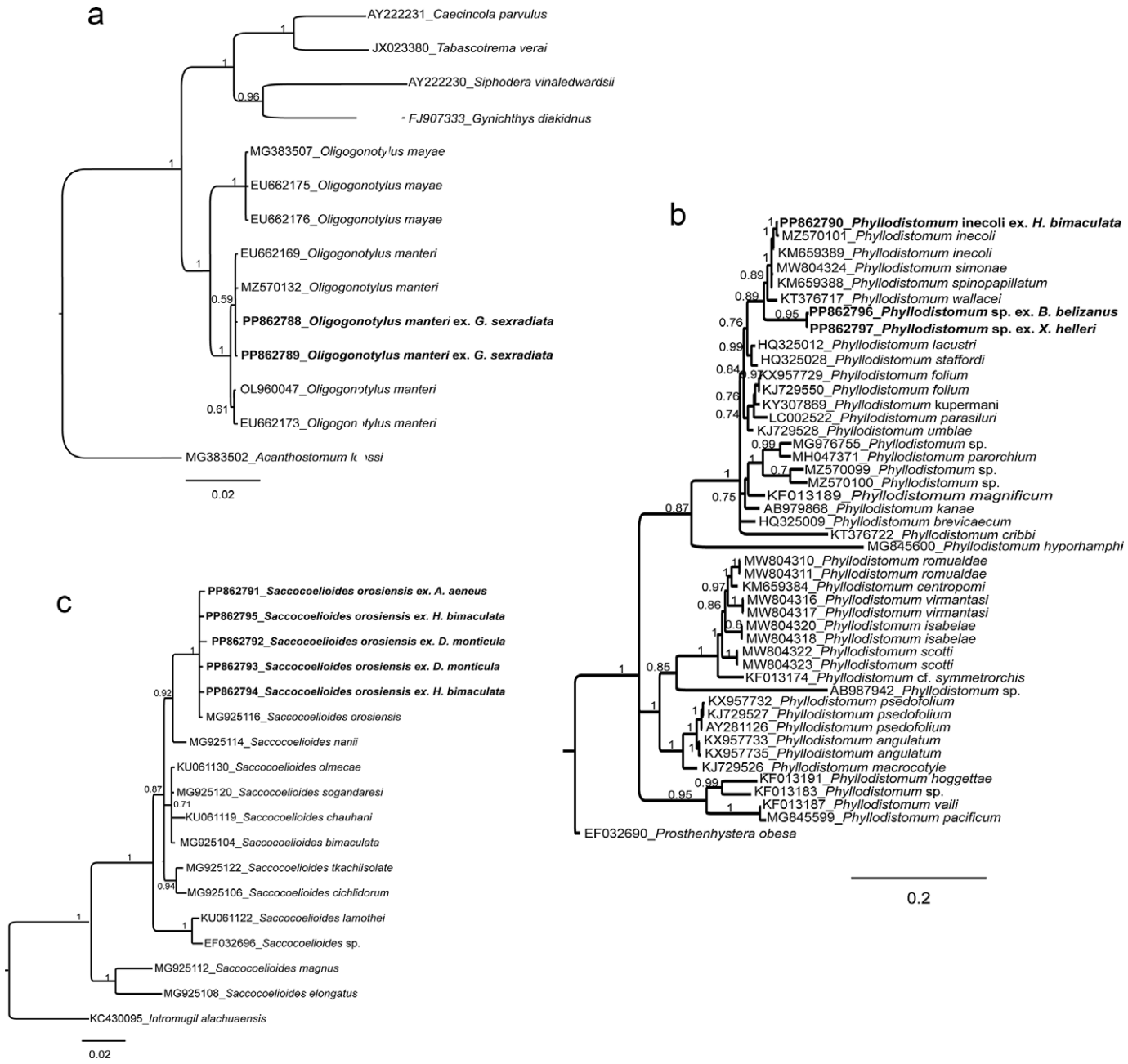

Figure 4. Bayesian phylogenetic trees of the 28S rDNA showing the phylogenetic positions of trematodes sequences from Laguna Escondida, Los Tuxtlas. *Oligogonotylus* spp. (a); *Phyllodistomum* spp. (b); *Genarchella* spp. (c); and *Saccocoelioides* (d).

among the species contained in that clade, varying from 0.1 to 1.4% for the 28S rRNA gene, although estimated divergence of *cox1* between the same species varied between 5.1 and 10.7%. Criteria to consider these species as independent units also considered host association and geographical distribution – that is, *P. inecoli* is a parasite of poeciliids in river basins draining to the Gulf of Mexico coast; *P. spinopapillatum* is found in profundulids in river basins draining to the Pacific coast, in Oaxaca; *P. simonae* infect an endemic species of profundulid in a close basin in the highlands of Chiapas; and *P. wallacei* is found in an endemic goodeid in the Cuzalapa and Ayuqila river basins in Michoacán, which drain to the Pacific coast (Razo-Mendivil *et al.* 2013; Pérez-Ponce de León *et al.* 2015a; Pérez-Ponce de León *et al.* 2015b; Pinacho-Pinacho *et al.* 2021). The position in the phylogenetic tree and the sequence divergence of the two newly sequenced individuals sampled from *X. helleri* and *B. belizanus* from Laguna Escondida suggest they represent an undescribed species. Unfortunately, we sampled very few individuals, preventing the proper description of the new species; furthermore, it would be necessary to obtain *cox1* sequences to establish a more robust species delimitation by comparing with sequences of species available in GenBank.

***Saccocoelioides orosiensis* Curran, Pulis, Andres & Overstreet, 2018**

Trematodes from the intestines of *D. monticola*, *A. aeneus*, and *H. bimaculata* were initially allocated in the genus *Saccocoelioides* by having a small body with the tegument entirely covered by minute spines, dispersed eye-spot remnants in anterior half of the body, and sac-like caeca (Figure 1h); however, identification to

https://doi.org/10.1017/S0022149X2400049X Published online by Cambridge University Press

species level relied on molecular data since members of the genus are difficult to identify due to their small body size. The 28S rDNA phylogenetic analyses of *Saccocoelioides* included five newly sequenced isolates and 12 specimens of 11 congeneric species for which 28S rDNA sequences are available; the alignment was 1061 bp long. In the phylogenetic tree, the new sequences nested in a highly supported monophyletic clade with *S. orosiensis* from poeciliids of Mexico, varying only between 0 and 0.1%, showing conspecificity. The species was yielded nested as the sister species of *S. nani* Szidat 1954 from Argentina with a divergence of 1.18% (Figure 4c). *Saccocoelioides orosiensis* was originally described by Curran *et al.* (2018) as a parasite of the poecilid *Poecilia gilli* (Kner) in northwestern Costa Rica. Interestingly, Curran *et al.* (2018) also described a new species of *Saccocoelioides* from *A. aeneus* from the same locality in Costa Rica. We sampled specimens from three host species, including the poecilid *H. bimaculata*, the mugilid *D. monticola*, and the characid *A. aeneus*; our sequenced individuals from *A. aeneus* correspond with *S. orosiensis.* Also, *S. chauhani*, which was first described by Lamothe (1974) from *Astyanax aeneus* from Catemaco Lake (a nearby locality to Laguna Escondida), is also clearly separated in the phylogenetic analysis. *Saccocoelioides* cf. *sogandaresi* was reported by Salgado-Maldonado *et al.* (2005) as a parasite of *P. mexicana.* Clearly, the authors were not totally convinced about the identification since they recognised that the species was morphologically like *S. sogandaresi.* In our study, we report *S. orosiensis* also as a parasite of a poecilid from the same locality based on morphological and molecular data; the record by Salgado-Maldonado *et al.* (2005) most likely corresponds to the species *S. orosiensis.*

Final considerations

This study reports 11 taxa of trematodes infecting freshwater fish in a relatively small reservoir (958 by 267 m) enclaved in Los Tuxtlas tropical rainforest, and identifications are validated by using both morphological and molecular data; this reservoir drains to the Río Máquinas, which opens into the Gulf of Mexico. Most of the taxa identified correspond to adult forms (64%); six of the seven adult species have been reported across southeastern Mexico. Some of them, even in other areas of Central America, correspond to a neotropical component which is the result of the distribution of their hosts, primarily cichlids, characids, and poeciliids (Choudhury *et al.* 2016, 2017). Only four taxa were found as metacercariae; all of them complete their life cycle when fish are consumed by fish-eating birds, primarily herons, and are generalist species in freshwater fishes since they are commonly found in a wide array of fish species (see Scholz *et al.* 2001; Pérez-Ponce de León *et al.* 2007).

Our study reinforces the importance of using DNA sequence data to accomplish a more accurate species delimitation and then a better estimation and understanding of trematode diversity. 28S rDNA sequences are useful as barcodes to identify trematodes, although we acknowledge that in some cases, as shown in the genera *Clinostomum*, *Posthodiplostomum*, and *Phyllodistomum* in the present study, it is necessary to consider other molecular markers more variable than 28S rDNA. In the genera mentioned above, the genetic library has been increased in the last years for other markers such as the internal transcribed spacers (ITS1-5.8-ITS2) and the mitochondrial *cox1*. Still, in our opinion for a general investigation about trematode diversity, sequences of the 28S rRNA gene can be used as an initial step to accomplish the goal. This approach is more important when studies are centered in the first and second intermediate hosts, especially if a molecular link can be established with sequences of adults. The 28S rDNA genetic library of trematodes increases steadily; this is the molecular marker upon which the current classification scheme of trematodes is based on (Olson *et al.* 2003; Pérez-Ponce de León & Hernández-Mena, 2019). In some cases, a thorough study with other molecular markers, in the context of integrative taxonomy, will be necessary to detect cryptic species complexes through a molecular prospecting approach.

Acknowledgements. This paper was part of the fulfilments of YVU to complete her PhD program in the Posgrado en Ciencias Biológicas UNAM. YVU thanks CONAHCYT (Consejo Nacional de Humanidades, Ciencia y Tecnología) for granting a scholarship; we thank Laura Márquez and Nelly López (LANABIO) for their help with the use of automatic sequencer. Special thanks are due to Rosamond Coates, Chief of the Estación de Biología Tropical Los Tuxtlas, for the facilities and permission to collect in Los Tuxtlas Biological Station; we also thank Dario Velasco for his help during fieldwork. We sincerely thank two anonymous reviewers whose comments greatly improved the quality of our manuscript.

Financial support. This project was partially funded by the Consejo Nacional de Humanidades, Ciencia y Tecnología (CONAHCyT) A1-S-21694, and by the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (PAPIIT-UNAM) IN212621 and 200824 to GPPL, and IN201122 to MGV.

Competing interest. None.

Ethical standard. Specimens were collected under the Cartilla Nacional de Colector Científico (FAUT 0057) issued by the Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT) to GPPL, and under permission of the Estación de Biología Tropical de Los Tuxtlas, UNAM.

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