Parasitology Research (2019) 118:203–217
https://doi.org/10.1007/s00436-018-6168-0

GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Exploring the genetic diversity of *Tylodelphys* (Diesing, 1850) metacercariae in the cranial and body cavities of Mexican freshwater fishes using nuclear and mitochondrial DNA sequences, with the description of a new species

Ana L. Sereno-Uribe[1] · Leopoldo Andrade-Gómez[1,2] · Gerardo Pérez Ponce de León[1] · Martín García-Varela[1]

Received: 30 July 2018 / Accepted: 23 November 2018 / Published online: 14 December 2018

Abstract

Members of the genus *Tylodelphys* Diesing, 1850 are endoparasites of fish-eating birds, particularly ciconiids, anhingids, and podicipedids across the globe. Metacercariae of *Tylodelphys* spp. were collected from the cranial and body cavities of freshwater fishes in central and northern Mexico; adults were recovered from the intestine of two species of freshwater diving birds of the family Podicipedidae, commonly known as grebes, in two locations of central Mexico. Specimens were sequenced for two molecular markers, the internal transcribed spacers (ITS1 and ITS2) plus 5.8S gene of the nuclear ribosomal DNA and of the cytochrome *c* oxidase subunit 1 from mitochondrial DNA. The genetic divergence among the 25 samples (16 metacercariae and 9 adults) and between the newly sequenced specimens and those deposited in the GenBank were estimated. Maximum likelihood and Bayesian inference analyses inferred with each data set revealed the existence of five genetic lineages. Eight metacercariae analyzed in this study were nested in two divergent lineages previously recognized as *Tylodelphys* sp. 5 and *Tylodelphys* sp. 6 (sensu Locke et al., *Int J Parasitol*, 45:841–855, 2015). Five adult specimens recovered from the intestine of the least grebe (*Tachybaptus dominicus* Linnaeus, 1766) in Tecocomulco Lake, Hidalgo State, nested in a single clade with other sequences identified previously as *Tylodelphys aztecae*, expanding its distribution range in other areas of central Mexico. The isolates of the metacercariae found in the cranial cavity of the shortfin silverside, *Chirostoma humboldtianum* Valenciennes, 1835 from Zacapu Lake in central Mexico formed a monophyletic lineage and were recognized as an undescribed species of *Tylodelphys.* The lack of adult specimens of this lineage in our samples prevented a formal description. However, the metacercariae collected in the cranial cavity of the silverside, *Chirostoma jordani* Woolman, 1894 and the adult specimens recovered from the intestine of the western grebe, *Aechmophorus occidentalis* (Lawrence, 1858) from Cuitzeo Lake formed a monophyletic clade, allowing us to link both stages of the life cycle and to describe this as a new species, *Tylodelphys kuerepus* n. sp. The new species represents the eighth species of the genus described in the Americas and the fourth in the Nearctic region. We briefly discuss the ecological associations between the metacercariae and their second intermediate hosts in relation to the genetic diversity patterns uncovered in our study.

Keywords Digenea · *Tylodelphys* · Central Mexico · Species description · Cox 1 · ITS

Handling editor: Julia Walochnik

✉ Martín García-Varela
garciav@unam.mx

[1] Departamento de Zoología, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria, Avenida Universidad 3000, 04510 Mexico, Distrito Federal, Mexico

[2] Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, Avenida Universidad 3000, 04510 Mexico, Distrito Federal, Mexico

Introduction

Adults of the genus *Tylodelphys* Diesing, 1850 are endoparasites of fish-eating birds, particularly ciconiids, anhingids, and podicipedids across the globe (King and Van As 1997; Drago and Lunaschi 2008; Chibwana et al. 2015; Blasco-Costa et al. 2017). As in other diplostomids, members of the genus *Tylodelphys* exhibit a complex life cycle involving a freshwater snail (lymnaeids or planorbids) as the first intermediate host. The metacercaria is found unencysted parasitizing

mainly the eyes, cranial cavity, and body cavity of freshwater fishes that serve as second intermediate hosts (Muzall and Kilroy 2007; Otachi et al. 2015; Chibwana et al. 2015; Blasco-Costa et al. 2017). Currently, the genus *Tylodelphys* contains 21 nominal species; 14 of these species have been recorded in the Americas (see Blasco-Costa et al. 2017). In Mexico, adults of two species of *Tylodelphys*, i.e., *Tylodelphys americana* Dubois, 1936 and *Tylodelphys aztecae* García-Varela, Sereno-Uribe, Pinacho-Pinacho, Hérnandez-Cruz and Pérez Ponce de León, 2016, have been recorded from the intestine of the pied-billed grebe, *Podilymbus podiceps* (Linnaeus, 1758) and the western grebe, *Aechmophorus occidentalis* (Lawrence, 1858) (García-Varela et al. 2016).

Central Mexico is a complex area where the Neotropical and Nearctic faunas overlap and it is considered as a biogeographical transition zone, with high levels of diversification and endemism of different taxa (Morrone 2006), including helminths (Lira-Guerrero et al. 2008; Pérez-Ponce de León and Choudhury 2010; Martínez-Aquino et al. 2014; García-Varela et al. 2017). In this zone, two groups of endemic freshwater fishes (goodeids and atherinopsids) with independent phylogenetic histories, diversified during the Pleistocene (Miller et al. 2005; Domínguez-Domínguez and Pérez Ponce de León 2009). The helminth parasite fauna of both groups of freshwater fishes has been well studied, and the inventory of that group of parasites is well documented (see Lira-Guerrero et al. 2008; Martínez-Aquino et al. 2014). Species in both fish families are infected with diplostomid metacercariae which have been found unencysted either in the cranial cavity or in the body, and their taxonomic status has been controversial since these larval forms have been indistinctly classified either as *Tylodelphys* sp. or *Diplostomum* sp. Recently, García-Varela et al. (2016) described a new species of the genus *Tylodelphys* by characterizing the metacercariae from the body cavity of five species of goodeids, and one species of cyprinid, and the adults recovered from the intestine of the pied-billed grebe (*P. podiceps*). However, metacercariae infecting the cranial cavity of atherinopsids have not been properly studied, and taxonomic identification has not yet been established at the species level. Additionally, in a recent study, Locke et al. (2015) sequenced the cytochrome *c* oxidase subunit 1 from mitochondrial DNA and both internal transcribed spacers plus 5.8S from nuclear ribosomal DNA of 2000 individual diplostomids, including members of the genera *Diplostomum* von Nordmann, 1832, and *Tylodelphys* and *Austrodiplostomum* Szidat and Nani, 1951, from Africa, Europe, Asia, and the Americas. Locke et al. (2015) molecularly validated seven species of *Tylodelphys* and recognized seven additional independent linages, four of which are distributed in freshwater fishes across the Nearctic biogeographical region.

The aims of the present study were (1) to characterize morphologically and molecularly the metacercariae found in the cranial cavity of atherinopsids and in the body cavity of cyprinodontiforms (goodeids and poeciliids), in central Mexico; (2) to establish a link between adults and metacercariae using ITS and *cox 1* DNA sequences; (3) to examine the ultrastructure of the body surface of adults and metacercariae using scanning electron microscopy; and (4) to provide the description of a new species of *Tylodelphys*.

Materials and methods

Specimen collection and taxonomic identification

A total of five species of grebes, including two specimens of *P. podiceps*, two of *Podiceps nigricollis* Brehm, 1831, two of *Tachybaptus dominicus*, five of *A. occidentalis*, and two of *Aechmophorus clarkii* (Lawrence, 1856), were killed with a shotgun in seven localities across the Nearctic biogeographical region of Mexico. Birds were kept on ice and dissected with 2 h of collection. Their viscera were placed in separate Petri dishes with 0.75% saline solution and examined under a dissecting microscope. Fifty-two adult specimens of *Tylodelphys* spp. were collected from two of the sampled species of grebes, with 10 individuals of *Tylodelphys* sp. from *T. dominicus* and 42 from *A. occidentalis*. Avian definitive hosts were identified using the field guides of Howell and Webb (1995) and the American Ornithologists' Union (1998), processed, and deposited in the Colección Nacional de Aves (CNAV), Instituto de Biología, UNAM.

Metacercariae of *Tylodelphys* were collected from the cranial cavity of two species of atherinopsids (*Chirostoma jordani* Woolman, 1894, and *Chirostoma humboldtianum* (Valenciennes, 1835)) and from the body cavity of one species of goodeid (*Allodontichthys tamazulae* Turner, 1946) and of two species of poeciliids (*Poecilia mexicana* Steindachner, 1863, *Gambusia affinis* (Bair and Girard, 1853), and *Gambusia* sp.) (Table 1). Fish were captured with seine nets and electrofishing, maintained alive and transported to the laboratory, pith sacrificed, and immediately examined. Collected digeneans were fixed by sudden immersion in hot (steaming) 4% formalin for morphological comparisons; other specimens were preserved in 100% ethanol for DNA extraction and sequencing. Fish were identified following Miller et al. (2005).

Morphological analyses

Unflattened specimens preserved in formalin were stained with Mayer's paracarmine, dehydrated in graded ethanol series, cleared in methyl salicylate, and mounted as permanent slides using Canada balsam. All the specimens were examined using a bright-field Leica DM 1000 LED microscope (Leica, Wetzlar, Germany). Measurements

Table 1 Specimens of *Tylodephys* sampled in this study in different locations of Mexico and sequenced for two molecular markers

Locality	Coordinates	Host	Life cycle stage	GenBank accession number *cox 1*	GenBank accession number ITS	Taxon name
1. Lago de Cuitzeo, Michoacan	19° 56′ 54.12″ N 101° 10′ 28.7″ W	*Aechmophorus occidentalis* Lawrence, 1858	A	MK172806–809	MK177831–34	*Tylodelphys kuerepus* n. sp.
		Chirostoma jordani Woolman, 1894	M	MK172810–814	MK177835–36	
2.Lago de Tecocomulco, Hidalgo	19° 51′ 26.05″ N 98° 23′ 13.507″ W	*Tachybaptus dominicus* Linnaeus, 1766	A	MK172790–794	MK177837–840	*Tylodelphys aztecae*
3. Lago de Zacapu, Michoacan	19° 49′ 23.7″ N 101° 47′ 16.2″ W	*Chirostoma humboldtianum* Valenciennes, 1835	M	MK172803–805	MK177841–42	*Tylodelphys* sp. A.
4. Rio Conchos, Tamaulipas	24° 45′ 56.1″ N 97° 59′ 55.5″ W	*Gambusia affinis* Bair and Girard, 1853	M	MK172802	MK177843	*Tylodelphys* sp. 5 sensu Locke et al. (2015)
5. La Azufrosa, Tamaulipas	22° 58′ 50 ″ N 98° 08′ 7.55″ W	*Poecilia mexicana* Steindachner, 1863	M	MK172801	MK177848	*Tylodelphys* sp. 6 sensu Locke et al. (2015)
6. Rio Tamazula, Jalisco	19° 43′ 22.7″ N 103° 12′ 08.5″ W	*Allodontichthys tamazulae* Turner, 1946	M	MK172800	MK177847	*Tylodelphys* sp. 6 sensu Locke et al. (2015)
7. Rio SanPedro Meoqui, Chihuahua	28° 15′ 42.183″ N 105° 29′ 3.89″ W	*Gambusia* sp.	M	MK172795–799	MK177844–46	*Tylodelphys* sp. 6 sensu Locke et al. (2015)

A adult, *M* metacercaria

were taken using the Leica Application Suite microscope software; the descriptions are presented in micrometers with the range followed by the mean in parenthesis. Drawings were made with the aid of a drawing tube attached to the microscope. Some of the adult individuals preserved in 4% formalin were dehydrated through a graded series of ethyl alcohol and then critical-point dried with carbon dioxide. These specimens were mounted on metal stubs with silver paste, coated with gold, and examined in a Hitachi Stereoscan model SU1510 (Hitachi High-Technologies Mexico S.A.de C. V, Mexico) at 15 kV. Type and voucher specimens were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City.

DNA extraction, PCR amplification, sequencing, and phylogenetic analyses

Twenty-five individuals of *Tylodelphys* spp. (16 metacercariae and 9 adults) were placed individually in tubes and digested overnight at 56 °C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM Na2-EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. Following digestion, DNA was extracted using DNAzol reagent (Molecular Research Center, Cincinnati, Ohio) according to the manufacturer's instructions. The ITS1, ITS2 region and 5.8S gene from nuclear ribosomal DNA (rDNA) was amplified using the forward primer D1, 5′-GTCGTAACAAGGTTTCCGTA-3′ and the reverse primer D2, 5′-ATCTAGACCGGACT AGGCTGTG-3′ (Bowles and McManus 1993). The cytochrome *c* oxidase subunit 1 (*cox 1*) of the mitochondrial DNA was amplified using the forward primer Plat-diploCOXF, 5′-CGTTTRAATTATACGGATCC-3′ and the reverse primer Plat-diploCOXR, 5′-AGCA TAGTAATMGCAGCAGC-3′ (Moszczynska et al. 2009). PCR reactions (25 μl) consisted of 10 μM of each primer, 2.5 μl of 10× buffer, 1.5 μl of 2 mM $MgCl_2$, 0.5 μl of dNTP's (10 mM), 1 U of Taq DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil) plus 2 μl of the genomic DNA plus 16.7 μl of distillated water. PCR cycling parameters for both amplifications included denaturation at 94 °C for 5 min and followed by 35 cycles of 94 °C for 1 min, annealing at 50 °C for 1 min for both molecular markers, and extension at 72 °C for 1 min, followed by a post-amplification incubation at 72 °C for 10 min. Sequencing reactions were performed using ABI Big Dye (Applied Biosystems, Boston, Massachusetts) terminator sequencing chemistry and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contigs were assembled and base-calling differences resolved using Codoncode Aligner version 5.1.2 (Codoncode Corporation, Dedham, Massachusetts). Sequences obtained in the current research for ITS, and *cox 1* was aligned with sequences of other species of the genus *Tylodelphys* plus sequences of species of the genus *Diplostomum*, downloaded from GenBank. In addition,

sequences of the strigeids *Australapatemon* Sudarikov, 1959, *Parastrigea* Szidat, 1928, and *Apharyngostrigea* Ciurea, 1927 were used as outgroups. Sequences of each molecular marker were aligned separately using the software Clustal W (Thompson et al. 1997). Nucleotide substitution models were selected for each molecular marker using jModelTest v0.1.1 (Posada and Crandall 2001) and by applying the Akaike criterion; for the ITS data set, selected model was TVM +I+G for Bayesian analysis, and GTRGAMMAI model was used for all maximum likelihood (ML) analyses. For the *cox 1*, data set selected model was TPM3uf +I+G. Phylogenetic trees were constructed through ML with the program RAxML v7.0.4 (Stamatakis 2006). A GTRGAMMAI substitution model was used, and 10,000 bootstrap replicates were run to assess nodal support. We also estimated gene trees using MrBayes 3.2.2 (Huelsenbeck and Ronquest 2001), with two runs of the Markov chain (MCMC) for 10 million generations, sampled every 1000 generations, a heating parameter value of 0.2 and burn-in (25%). Trees were drawn using FigTree version 1.4.0 (Rambaut 2006). The genetic divergence among taxa was estimated using uncorrected "*p*" distances with the program MEGA version 6 (Tamura et al. 2013).

Results

Molecular characterization and phylogenetic analyses

The alignment of new sequenced individuals of *Tylodelphys* spp. (16 metacercariae and 9 adults) for the mtDNA *cox 1* along with sequences of other congeneric species of *Tylodelphys*, seven species of *Diplostomum*, and three species of strigeids, *Australapatemom burti* (Miller, 1923), *Parastrigea diovadena* (Dubois and Macko, 1972), and *Apharyngostrigea cornu* (Zeder, 1800) used as outgroups, included 466 characters with 104 sequences. The phylogenetic trees inferred with ML and Bayesian inference (BI) recovered similar topologies and placed the 25 new isolates from Mexico in five independent clades within *Tylodelphys*, with moderate to high bootstrap support and high values of Bayesian posterior probabilities (Fig. 1).

Clade I

The first clade contained a single metacercaria recovered from the cranial cavity of the poeciliid *G. affinis* from northern Mexico, along with other two isolates from eleotrids previously recognized as *Tylodelphys* sp. 5 sensu Locke et al. (2015), from the fat sleeper, *Dormitator latrifons* (Richardson, 1844) (GenBank KR271520), and the Pacific sleeper, *Gobiomorus maculatus* (Günther, 1859) (GenBank KR271521) from estuaries of the Pacific slope of Mexico (Fig. 1).

Fig. 1 Maximum likelihood tree inferred with *cox 1* data set. Numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI)

Clade II

The second clade included five adult specimens collected from the intestine of the least grebe, *T. dominicus* from Tecocomulco Lake, Hidalgo State, in central Mexico; those specimens correspond with the species *T. aztecae* because they nested in a single clade and the sequences showed a low level of genetic divergence ranging from 0 to 0.7% (Fig. 1).

Clade III

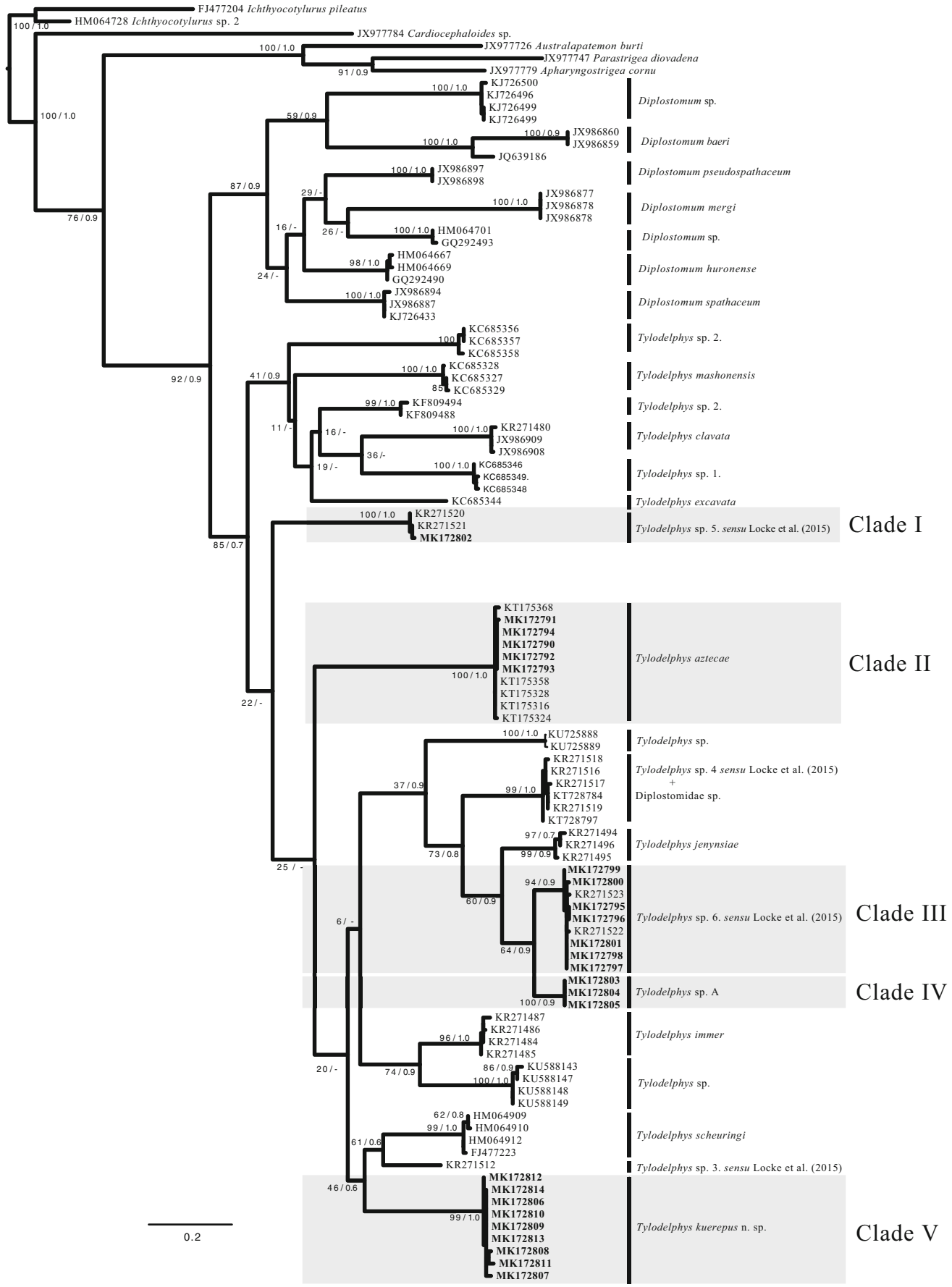
The third clade was formed by six metacercariae from the body cavity of two species of Poeciliid (*P. mexicana* and *Gambusia* sp.) fishes and one from the goodeid *A. tamazulae* Turner, 1946. These isolates nested with two isolates previously recognized as *Tylodelphys* sp. 6 sensu Locke et al. (2015) (GenBank KR271522–523) recovered from *Poecilia latipinna* Lesueur, 1821 from Mississippi, USA (Fig. 1).

Clades IV–V

The fourth clade included three metacercariae from the cranial cavity of the shortfin silverside, *C. humboldtianum* from Zacapu Lake, also in central Mexico. Finally, the fifth clade was formed by five metacercariae recovered from the cranial cavity of the charal, *C. jordani*, and four adult specimens recovered from the intestine of the western grebe, *A. occidentalis*, from Cuitzeo Lake in central Mexico (Fig. 1).

Sequences of 10 metacercariae and eight adults of *Tylodelphys* spp. were generated for the ITS 1, ITS 2, and 5.8 gene and were also aligned with sequences of *Tylodelphys*, *Diplostomum*, *Ornithodiplostomum* sp., *Posthodiplostomum* sp. and the strigeids *A. burti*, *P. diovadena*, and *A. cornu* and *Cardiocephaloides* sp. that were used as outgroups. The ITS data set included 1158 characters with 86 sequences. The ITS phylogenetic trees inferred with ML and BI recovered the same topology as the *cox 1* trees, and the five lineages for the new sequenced individuals were obtained, although with partially different interrelationships with other members of *Tylodelphys* (Fig. 2).

In summary, of the five lineages where the new sequenced individuals are placed within the phylogenetic tree of *Tylodelphys*, adult specimens were also sampled from the definitive host for one of them (clade V), allowing the formal description of a new species, and establishing the molecular link between adults and


FJ477204 Ichthyocotylurus pileatus
HM064728 Ichthyocotylurus sp. 2
JX977784 Cardiocephaloides sp.
JX977726 Australapatemon burti
JX977747 Parastrigea diovadena
JX977779 Apharyngostrigea cornu
KJ726500
KJ726496
KJ726499
KJ726499
Diplostomum sp.
JX986860
JX986859
JQ639186
Diplostomum baeri
JX986897
JX986898
Diplostomum pseudospathaceum
JX986877
JX986878
JX986878
Diplostomum mergi
HM064701
GQ292493
Diplostomum sp.
HM064667
HM064669
GQ292490
Diplostomum huronense
JX986894
JX986887
KJ726433
Diplostomum spathaceum
KC685356
KC685357
KC685358
Tylodelphys sp. 2.
KC685328
KC685327
KC685329
Tylodelphys mashonensis
KF809494
KF809488
Tylodelphys sp. 2.
KR271480
JX986909
JX986908
Tylodelphys clavata
KC685346
KC685349.
KC685348
Tylodelphys sp. 1.
KC685344
Tylodelphys excavata
KR271520
KR271521
MK172802
Tylodelphys sp. 5. sensu Locke et al. (2015)
Clade I
KT175368
MK172791
MK172794
MK172790
MK172792
MK172793
KT175358
KT175328
KT175316
KT175324
Tylodelphys aztecae
Clade II
KU725888
KU725889
Tylodelphys sp.
KR271518
KR271516
KR271517
KT728784
KR271519
KT728797
Tylodelphys sp. 4 sensu Locke et al. (2015)
+
Diplostomidae sp.
KR271494
KR271496
KR271495
Tylodelphys jenynsiae
MK172799
MK172800
KR271523
MK172795
MK172796
KR271522
MK172801
MK172798
MK172797
Tylodelphys sp. 6. sensu Locke et al. (2015)
Clade III
MK172803
MK172804
MK172805
Tylodelphys sp. A
Clade IV
KR271487
KR271486
KR271484
KR271485
Tylodelphys immer
KU588143
KU588147
KU588148
KU588149
Tylodelphys sp.
HM064909
HM064910
HM064912
FJ477223
Tylodelphys scheuringi
KR271512
Tylodelphys sp. 3. sensu Locke et al. (2015)
MK172812
MK172814
MK172806
MK172810
MK172809
MK172813
MK172808
MK172811
MK172807
Tylodelphys kuerepus n. sp.
Clade V
0.2

metacercariae. We present the description of the new species herein, and we characterize morphologically the metacercariae of *Tylodelphys* sp. A representing clade IV, for which adults were not obtained.

Family Diplostomidae Poirier, 1886

Genus *Tylodelphys* Diesing, 1850

Tylodelphys kuerepus n. sp.

Description based on the holotype and 16 paratypes; two specimens for SEM; measurements taken from stained and mounted adult specimens (Table 2, Figs. 3 and 4):

Body linguiform, forebody slightly spatulate, concave ventrally; hindbody conical (Figs. 3a and 4a, b). Total length 1854–2318 (2082). Tegument covered with pectinate spines (Fig. 4e). Oral sucker small, fairly muscular, terminal, 89–153 (118) long, 115–161 (137) wide; two well-developed pseudosuckers on each side of oral sucker, 109–196 (146) long, 70–123 (88) wide. Ventral sucker oval, fairly muscular wider than long, 90–140 (120) long, 112–152 (134) wide. Holdfast organ oval, 271–452 (351) long, 146–280 (199) wide, covered with triangular spines (Fig. 4f). Prepharynx absent; relatively large pharynx, oval, 112–157 (135) long, 67–117 (95) wide; esophagus long 40–44 (42); intestinal ceca extending laterally to reach holdfast organ. Testes in tandem, extending transversally occupying almost all the width of hindbody; anterior testis 219–436 (308) long, 434–598 (517) wide; posterior testis 184–440 (320) long, 369–461 (434) wide. Ovary pretesticular, spherical 88–200 (130) long, 86–209 (137) wide, contiguous with anterior testis. Mehlis' gland lateral to anterior testis. Vitelline follicles distributed in two fields alongside the forebody extending from anterior margin of ventral sucker to posterior end of hindbody. Vitelline reservoir in intertesticular space. Seminal vesicle subspherical contiguous with posterior testis, the copulatory bursa enclosing a small genital cone with a muscular hermaphroditic duct opening terminally to genital pore (Fig. 3b). Uterus extending from the ovario-testicular area posteriorly to the genital pore, opening terminally in hindbody. Eggs 62–94 (78) long, 52–88 (62) wide.

Host: *Aechmophorus occidentalis* (Lawrence, 1858) (Western grebe), Podicipedidae

Site of infection: Intestine

Locality: Cuitzeo Lake, Michoacan, Mexico (19° 56′ 0″ N 101° 5′ 0″ W)

Type-material: CNHE: 10941 (holotype); 10942 (paratypes)

Prevalence of infection: 100%

Representative DNA sequences: MK172806-809 (*cox 1*), MK177831-34 (ITS)

Etymology: The specific epithet refers to common name of the fish that serve as second intermediate host in the Purhépecha language. The Purhépechas is an indigenous group that inhabits central Mexico mainly in the Michoacan State.

Fig. 2 Maximum likelihood tree inferred with ITS 1, ITS 2, and 5.8S data set. Numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI)

Metacercaria

Description (based on measurements of eight stained and mounted specimens, and two specimens for SEM) (Figs. 3 and 4):

Body linguiform, slightly concave ventrally, 1027–1247 (1168) long, 313–386 (350) wide. Tegument devoid of spines or papillae (Fig. 4c). Hindbody reduced to a small conical prominence. Lateral pseudosuckers lacking. Oral sucker small, terminal, almost rounded, 42–56 (49) long, 25–45 (38) wide. Ventral sucker small, fairly muscular 52–64 (57) long, 46–65 (55) wide. Prepharynx inconspicuous; pharynx oval, 36–49 (40) long, 15–25 (19) wide; intestinal ceca long, extending posteriorly to level of anterior border of holdfast organ; holdfast organ 129–169 (142) long, 67–87 (74) wide. Calcareous corpuscles forming at least six rows extending from anterior region from body to anterior margin of holdfast organ. Reproductive system poorly developed.

Host: *Chirostoma jordani* Woolman, 1894, Atherinopsidae

Site of infection: Cranial cavity

Locality: Cuitzeo Lake, Michoacan, Mexico (19° 56′ 0″ N 101° 5′ 0″ W)

Specimen deposited: CNHE: 10943

Prevalence of infection: 100%

Representative DNA sequences: MK172810-814 (*cox 1*), MK177835-36 (ITS)

Tylodelphys sp. A (clade IV)

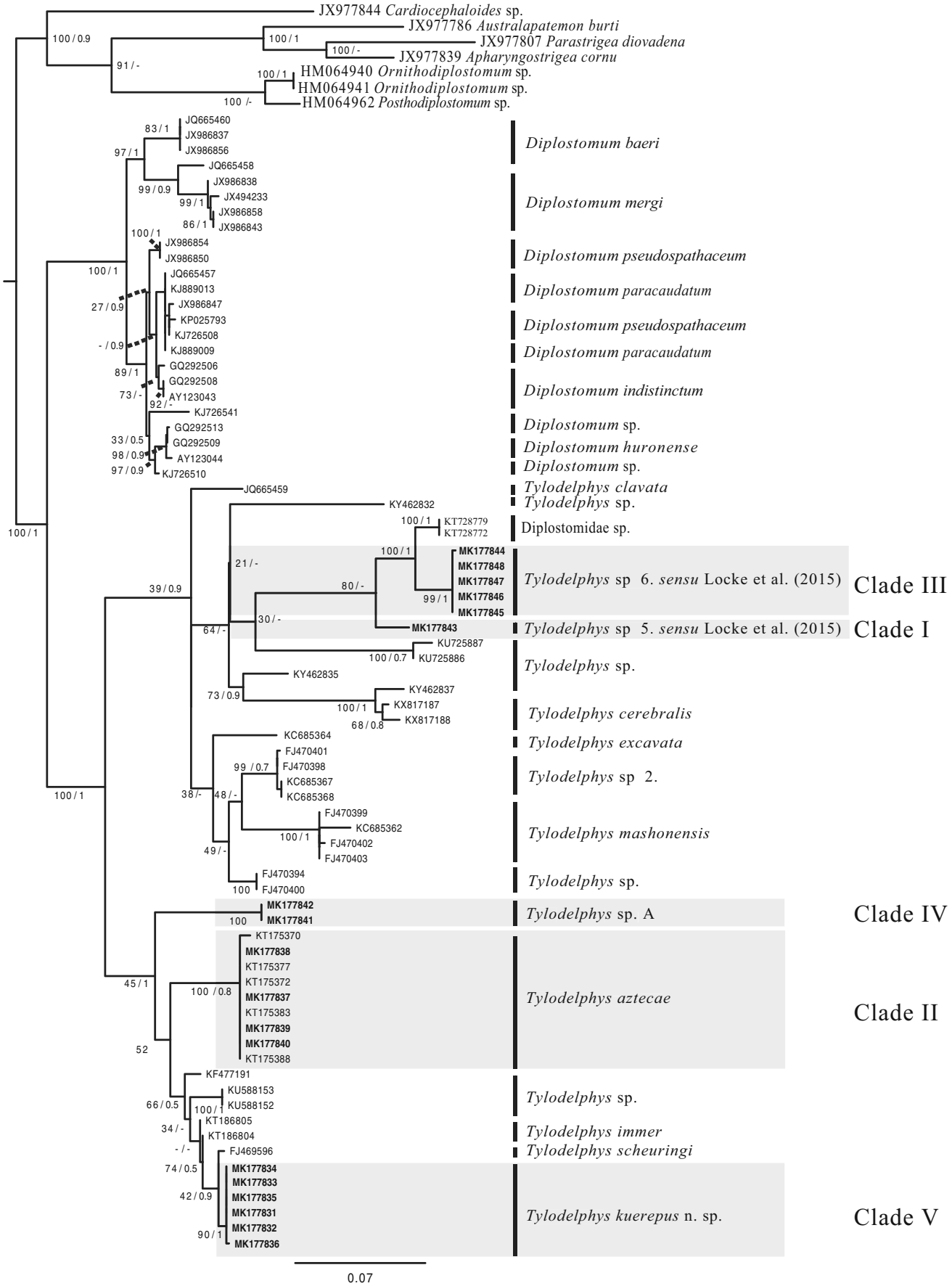
(Metacercaria)

Description (Based on measurements of seven stained and mounted specimens and two specimens for SEM) (Figs. 3 and 4):

Body linguiform, slightly concave ventrally, 1533–1930 (1816) long, 548–631 (596) wide. Tegument devoid of spines or papillae (Fig. 4d). Hindbody reduced to a small conical prominence. Lateral pseudosuckers lacking. Oral sucker small, terminal, almost rounded, 81–90 (85) long, 61–78 (67) wide. Ventral sucker small, fairly muscular 75–102 (88) long, 70–106 (83) wide. Prepharynx small; pharynx oval, 42–67 (49) long, 20–27 (24) wide; intestinal ceca long, extending posteriorly to level of anterior border of holdfast organ; holdfast organ 220–256 (239) long, 101–120 (107) wide. Calcareous corpuscles forming at least six rows extending from anterior region from body to anterior margin of holdfast organ. Reproductive system poorly developed

Host: *Chirostoma humboldtianum* (Valenciennes, 1835)

Site of infection: Cranial cavity


JX977844 Cardiocephaloides sp.
JX977786 Australapatemon burti
JX977807 Parastrigea diovadena
JX977839 Apharyngostrigea cornu
HM064940 Ornithodiplostomum sp.
HM064941 Ornithodiplostomum sp.
HM064962 Posthodiplostomum sp.
JQ665460
JX986837
JX986856
Diplostomum baeri
JQ665458
JX986838
JX494233
JX986858
JX986843
Diplostomum mergi
JX986854
JX986850
Diplostomum pseudospathaceum
JQ665457
KJ889013
Diplostomum paracaudatum
JX986847
KP025793
Diplostomum pseudospathaceum
KJ726508
KJ889009
Diplostomum paracaudatum
GQ292506
GQ292508
AY123043
Diplostomum indistinctum
KJ726541
Diplostomum sp.
GQ292513
GQ292509
AY123044
KJ726510
Diplostomum huronense
Diplostomum sp.
Tylodelphys clavata
JQ665459
KY462832
Tylodelphys sp.
KT728779
KT728772
Diplostomidae sp.
MK177844
MK177848
MK177847
MK177846
MK177845
Tylodelphys sp 6. sensu Locke et al. (2015)
Clade III
MK177843
Tylodelphys sp 5. sensu Locke et al. (2015)
Clade I
KU725887
KU725886
Tylodelphys sp.
KY462835
KY462837
KX817187
KX817188
Tylodelphys cerebralis
KC685364
Tylodelphys excavata
FJ470401
FJ470398
KC685367
KC685368
Tylodelphys sp 2.
FJ470399
KC685362
FJ470402
FJ470403
Tylodelphys mashonensis
FJ470394
FJ470400
Tylodelphys sp.
MK177842
MK177841
Tylodelphys sp. A
Clade IV
KT175370
MK177838
KT175377
KT175372
MK177837
KT175383
MK177839
MK177840
KT175388
Tylodelphys aztecae
Clade II
KF477191
KU588153
KU588152
Tylodelphys sp.
KT186805
KT186804
Tylodelphys immer
FJ469596
Tylodelphys scheuringi
MK177834
MK177833
MK177835
MK177831
MK177832
MK177836
Tylodelphys kuerepus n. sp.
Clade V
0.07

Locality: Zacapu Lake, Michoacan, Mexico (24° 45′ 56.1″ N; 97° 59′ 55.5″ W).

Specimens deposited CNHE: 10944

Prevalence of infection: 100%

Representative DNA sequences: MK172803-805 (*cox 1*), MK177841-842 (ITS)

Taxonomic remarks The new species belongs to the genus *Tylodephys* because it possesses an indistinctly bipartite body, well-developed pseudosuckers, non-trilobate anterior extremity, and a copulatory bursa enclosing a small genital cone with a hermaphroditic duct opening terminally (see Drago and Lunaschi 2008). To date, only seven species of *Tylodelphys* have been described based on adult specimens in the Americas, i.e., *Tylodelphys elongata* (Lutz, 1928) from *T. dominicus* in Cuba, Venezuela, and Brazil; *T. americana* (Dubois, 1936) from *Mycteria americana* Linnaeus, 1758 and *P. podiceps* in Brazil, Venezuela, and Mexico; *Tylodelphys immer* Dubois, 1961 from *Gavia immer* (Brunnich, 1764) and *Strix varia* Barton, 1799 in the USA; *Tylodelphys podicipina robrauschi* Dubois, 1969 from *Podiceps grisegena* (Boddaert, 1783) in Canada; *Tylodelphys adulta* Lunachi and Drago, 2004, from *Podiceps major* (Boddaert, 1783) in Argentina; *Tylodelphys brevis* Drago and Lunachi, 2008 from *M. americana* in Argentina; and *T. aztecae* from *P. podiceps* in Mexico. *T. kuerepus* n. sp. can be differentiated from these seven species by their large overall body size and by having a spatulate and slightly concave forebody. Morphometric comparisons of the new species with congeneric species from the Americas (Table 2) show that the new species exhibits the higher limits for the following morphometric characters: oral sucker length and width; pharynx length and width; ventral sucker length and width; holdfast organ large; anterior and posterior testes length and width; ovary length and width and hindbody/forebody length. In addition, the new species can also be distinguished from the other seven species by having a

Table 2 Morphological comparison among adult species of *Tylodelphys* from the Americas. Measurements in micrometers

Characteristics	*T. immer* Dubois 1961	*T. podicipina robrauschi* Dubois, 1969	*T. elongata* Dubois, 1970	*T. americana* Dubois, 1970	*T. adulta* Lunaschi and Drago, 2004	*T. brevis* Drago and Lunaschi, 2008	*T. americana* García-Varela et al. 2016	*T. aztecae* García-Varela et al. 2016	*T. kuerepus* n. sp. This study
Body (L)	1840	1600	1500–2350	900–2400	1123–1464	570–851	1272–1755	874–1135	1854–2318
Forebody (L)	690–1140	550–990	800–1120	550–1500	790–950	371–507	800–1063	626–763	934–1367
Hindbody (L)	330–790	260–630	450–650	310–900	1269–528	371–507	451–750	370–467	868–1090
Oral sucker (L)	72–120	70–135	80–100	48–87	71–97	40–67	76–90	80–101	89–153
Oral sucker (W)	80–115	57–120	90–104	48–95	83–103	44–69	62–87	63–100	115–161
Pharynx (L)	60–89	52–122	63–73	49–79	71–110	45–57	50–73	57–95	112–157
Pharynx (W)	48–70	52–102	60–68	33–72	53–74	22–31	43–60	33–53	67–117
Ventral sucker (L)	70–100	80–120	70–90	33–76	60–80	24–36	82–108	32–103	90–140
Ventral sucker (W)	80–122	100–155	99–100	36–108	78–97	27–54	88–115	80–142	112–152
Holdfast organ (L)	190–270	120–320	160–210	115–390	195–250	69–131	151–279	127–206	271–452
Holdfast organ (W)	100–210	110–265	–	110–510	178–274	50–102	168–294	123–234	146–280
Esophagus (L)	5–52	0–50	–	40	25	10.0–15	38	38–48	40–44
Pseudosucker (L)	–	150–245	110–210	50–112	145–216	48–74	96–150	130–243	109–196
Pseudosucker (W)	–	75–140	80–130	68–80	74–126	29–59	32–51	32–97	70–123
Anterior testis (L)	90–200	80–210	100–140	110–300	120–121	41–71	90–123	65–115	219–436
Anterior testis (W)	250–380	270–610	445–460	270–575	216–494	133–226	270–321	150–440	434–598
Posterior testis (L)	90–195	100–240	110–180	110–290	115–168	34–83	91–172	50–255	184–440
Posterior testis (W)	215–350	250–530	400–445	240–520	211–427	121–202	230–289	212–350	369–461
Ovary (L)	95–115	85–117	75–125	63–135	73–83	34–53	89–105	57–110	88–200
Ovary (W)	80–145	110–170	95–200	80–90	73–97	29–78	80–90	60–100	86–209
Eggs number	3–17	25	15	30	1–20	1–2	10	2–7	14–25
Egg (L)	85–104	85–98	90–97	84–103	87–99	83–102	83–96	89–113	62–94
Egg (W)	54–68	54–63	60–66	53–63	51–59	45–64	64–68	45–77	52–88
Ratios: hindbody/forebody (L)	0.45–0.76	0.41–0.66	0.55–0.72	0.38–0.80	0.28–0.64	0.4–0.8	0.58–0.73	0.57–0.70	0.69–0.94

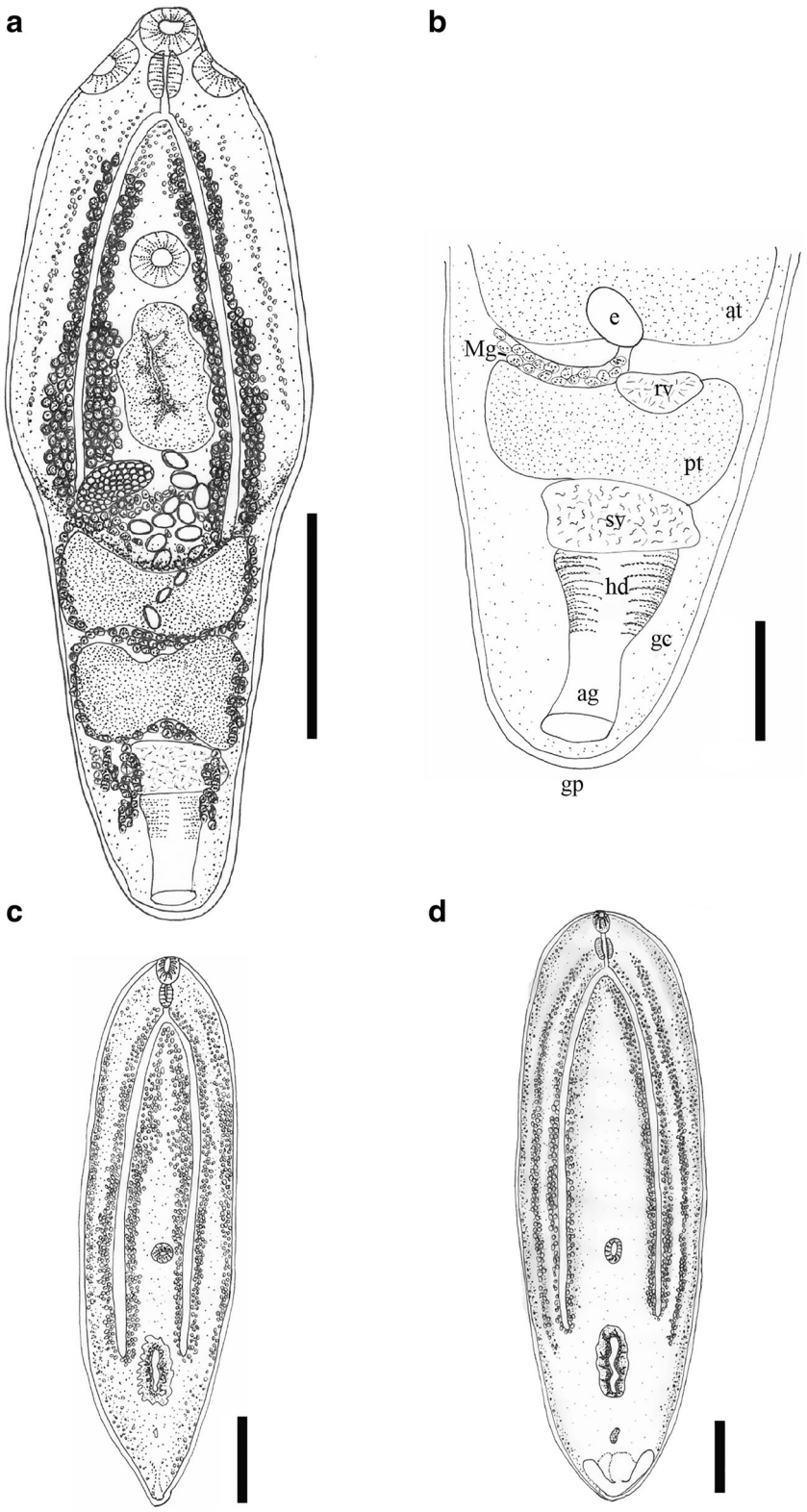


Fig. 3 *Tylodelphys kuerepus* n. sp. **a** Adult (holotype) from *Aechmophorus occidentalis*, from Cuitzeo, Lake, Michoacan, ventral view. **b** Enlarged lateral view of terminal genitalia. **c** Metacercaria (paratype) from cranial cavity of *Chirostoma jordani* from Cuitzeo, Lake, Michoacan, ventral view. **d** Metacercaria of *Tylodelphys* sp. A (voucher) from cranial cavity of *Chirostoma humboldtianum* from Zacapu, Lake, Michoacan, ventral view. ag, genital atrium; at, anterior testis; e, egg; gc, genital cone; gp, genital pore; hd, hermaphroditic duct; Mg, Mehlis'gland; pt, posterior testis; rv, vitelline reservoir; sv, seminal vesicle. Scale bars, 500 μm (**a**); 200μm (**b**–**d**).

tegument covered with pectinate spines and by having triangular spines on the holdfast organ (Fig. 4e, f).

The metacercariae of species of *Tylodelphys* parasitize usually the eyes and brain (cranial cavity) of freshwater fishes across of the world (see Chibwana et al. 2015; Otachi et al. 2015; Blasco-Costa et al. 2017). The habitat where we recorded the metacercariae of the new species is not uncommon. Morphologically, the metacercaria of *T. kuerepus* n. sp. is very

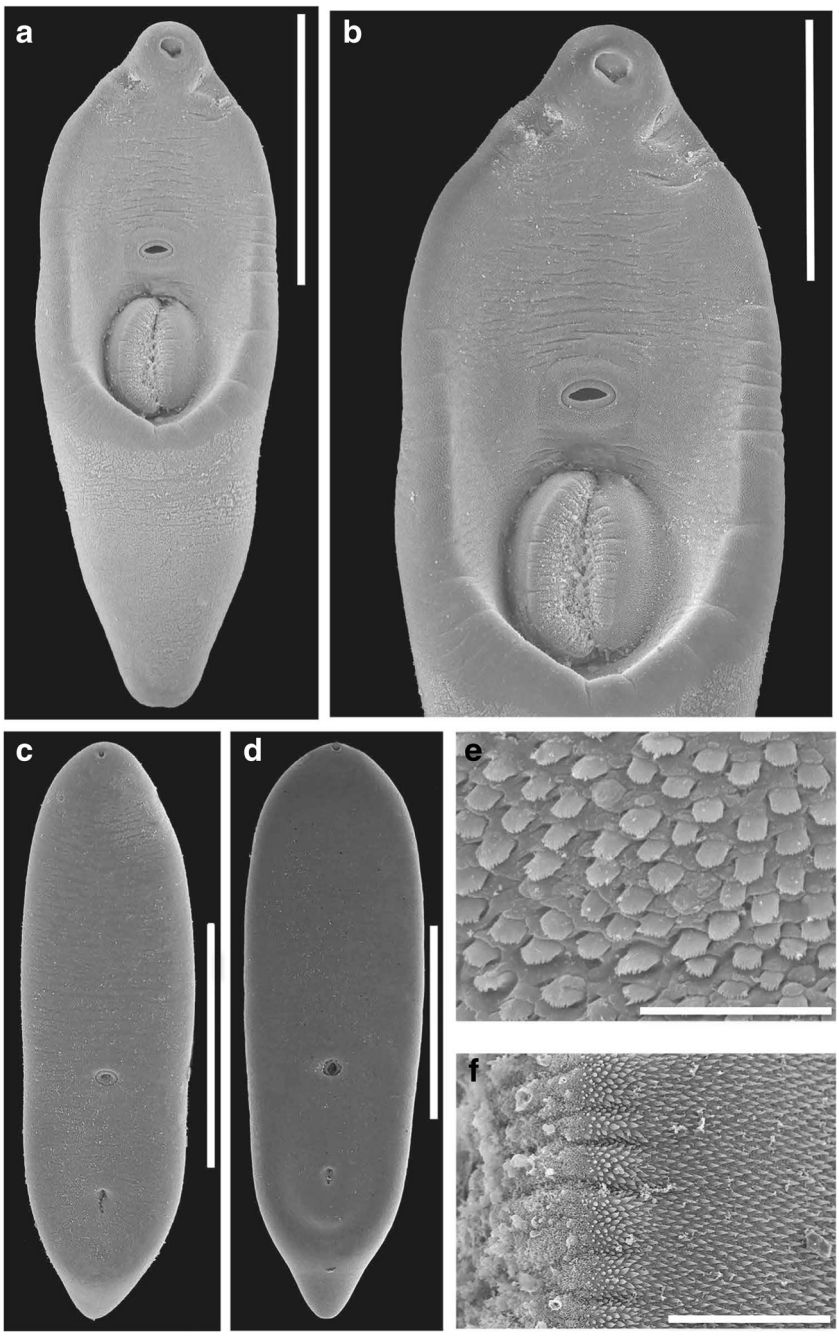


Fig. 4 Scanning electron micrographs of *Tylodelphys kuerepus* n. sp. **a** Adult specimen from *Aechmophorus occidentalis*, from Cuitzeo, Lake, Michoacan. **b** Anterior region. **c** Metacercaria of *T. kuerepus* n. sp. from cranial cavity of *Chirostoma jordani* from Cuitzeo, Lake, Michoacan. **d** Metacercaria of *Tylodelphys* sp. A from cranial cavity of *Chirostoma humboldtianum* from Zacapu, Lake, Michoacan. **e** Tegument from adult of *T. kuerepus* n. sp. **d** Tegument around holdfast organ from adult of *T. kuerepus* n. sp. Scale bars, 400 μm (**a**, **d**); 300 μm (B); 400 μm (**c**); 10 μm (E); 30 μm (**f**)

similar to that of *Tylodelphys* sp. A. However, the metacercaria of *T. kuerepus* n. sp. differs from *Tylodelphys* sp. A, by having an overall smaller body size. For instance, the body size of the new species is 1027–1247 long by 313–386 wide while in *Tylodelphys* sp. A the body is 1533–1930 long by 548–631 wide; the internal organs of the new species are also smaller, e.g., oral sucker 42–56 vs 81–90 long by 25–45 vs 61–78 wide; ventral sucker 52–64 vs 75–102 long by 46–65 vs 70–106 wide; pharynx 36–49 vs 42–67 long by 15–25 vs 20–27 wide; holdfast organ 129–169 vs 220–256 long by 67–87 vs 101–120 wide. The metacercaria of the new species is morphologically similar to *T. aztecae*, the other species occurring in central Mexico, but can be differentiated because is also smaller for most morphological traits: oral sucker 42–56 vs 40–80 long by 25–45 vs 30–72 wide; ventral sucker 52–64 vs 45–157 long by 46–65 vs 37–90 wide; pharynx 36–49 vs 22–60 long; holdfast organ 129–169 vs 170–245. The three metacercariae morphologically characterized from freshwater fishes occurring in central Mexico (*T. kuerepus* n. sp., *Tylodelphys* sp. A, and *T. aztecae*) lack lateral pseudosuckers and show a strict habitat and host specificity pattern, i.e., *T. kuerepus* n. sp., and *Tylodelphys* sp. A were found unencysted

in the cranial cavity of atherinopsid fishes, *C. jordani* in Cuitzeo Lake, and *C. humboldtianum* in Zacapu Lake, respectively. The metacercariae of *T. aztecae* has been found also unencysted in the body cavity of goodeids in central Mexico.

Previous records of *Tylodelphys* sp. were made by Guzmán-Cornejo and García-Prieto (1999) from the same host and locality (Cuitzeo Lake), although the record was made as *Diplostomum* (*Tylodelphys*) sp. and later corrected in Pérez-Ponce de León et al. (2007) and Lira-Guerrero et al. (2008). Guzmán-Cornejo and García-Prieto (1999) reported the presence of an unidentified species of *Tylodelphys* from the brain of *C. jordani* and from the body cavity of the blackfin goodea, *Goodea atripinnis* Jordan, 1880. We observed the specimens deposited at the CNHE and were able to identify that the specimens from the goodeid corresponded with *T. aztecae* although no specimens from the *C. jordani* were deposited. Additionally, in the same study two specimens of the white great egret, *Ardea alba* Linnaeus, 1758 collected in Cuitzeo Lake were necropsied. In one of them, a single specimen of *Tylodelphys* was recovered from the intestine of the bird; however, due to the poor quality of the specimen and the fact that it was immature, the taxonomic identification was not completed. Even though species of *Tylodelphys* have been found as adults in members of the families Accipitridae, Ardeidae, and Podicipedidae (see Niewiadomska 2002), herons are not the most common definitive hosts for congeneric species (see Dubois 1970); considering this and the low intensity of infection of the parasite in *A. alba*, this might be considered as an accidental infection.

Discussion

cox 1 phylogenetic analysis

The phylogenetic trees inferred with the mitochondrial *cox 1* gene generated in this study showed that the 25 new sequenced specimens of *Tylodelphys* spp. from Mexico formed five independent lineages supported by high bootstrap (ML) and posterior probability (BI) values. The interspecific genetic divergence based on *cox 1* among the lineages is relatively high, varying 5% between *Tylodelphys* sp. 6 sensu Locke et al. (2015) and *Tylodelphys* sp. A sampled in fish from Zacapu Lake and 12–14% between *Tylodelphys* sp. 5 sensu Locke et al. (2015) and *T. aztecae*. The genetic divergence among the new species and the two species of *Tylodelphys* occurring in central Mexico varied from 13 to 15%. Finally, the genetic divergence among *T. kuerepus* n. sp. and their sister taxa, *Tylodelphys* sp. 3 sensu Locke et al. (2015), and *Tylodelphys scheuringi* (Hughes, 1929) varied from 9 to 10%. These values of genetic divergence are similar to those found among other species of *Tylodelphys* with values between 8 and 16.5% (see Chibwana et al. 2015; Otachi et al. 2015; García-Varela et al. 2016; Blasco-Costa et al. 2017). Our study confirmed the existence of a new species of *Tylodelphys* because all isolates from one locality, Cuitzeo Lake, formed a reciprocally monophyletic clade; the adult and metacercarial stages of *T. kuerepus* n. sp. were linked molecularly and were characterized using an integrative taxonomy approach, combining morphology and DNA sequence data along with host association and geographical distribution. The metacercariae were found unencysted in the cranial cavity of the charal, *C. jordani*, and the adults were recovered from intestine of the western grebe, *A. occidentalis*, in the same locality, Cuitzeo Lake, one of the largest lakes in central Mexico. The genetic divergence between isolates of both developmental stages was very low and ranged between 0 and 1.4% for *cox 1*. The low level of intraspecific genetic divergence is similar to that found among isolates of other species of *Tylodelphys*; Chibwana et al. (2013) found intraspecific divergence levels of 0.50 and 0.61% among isolates of two species of *Tylodelphys* from African freshwater fish. Otachi et al. (2015) found intraspecific variation between 0 and 2.4% among isolates from African freshwater fish, while García-Varela et al. (2016) uncovered intraspecific levels between 0 and 1% among isolates of *T. aztecae* and Blasco-Costa et al. (2017) reported an intraspecific level of 0.8% among isolates of *Tylodelphys* from New Zealand freshwater fish.

ITS phylogenetic analysis

The phylogenetic trees inferred with the ITS data set (Fig. 2) contained fewer taxa than *cox 1* trees (Fig. 1) and was used to corroborate the findings obtained through the *cox 1* barcodes. Even though the internal transcribed spacers exhibit lower variation levels, a subsample of isolates of the five lineages recovered through the analysis of *cox 1* was analyzed along with available sequences of species of *Tylodelphys* for that molecular marker. The phylogenetic trees based on ITS not only showed less resolution but also recovered the five lineages yielded by the *cox 1* trees (Fig. 2), although no sequences of ITS were obtained in the study by Locke et al. (2015). The interspecific genetic divergence among the lineages inferred with the internal transcribed spacers data set ranged between 3 and 8%. These ranges of genetic divergence are similar to those found among species of *Tylodelphys* varying 2.5–10.2% (Chibwana et al. 2013), 3–11% (García-Varela et al. 2016), and 1.1–7.7% (Blasco-Costa et al. 2017). The intraspecific genetic divergence for ITS between metacercariae and adults of *T. kuerepus* n. sp. was very low and ranged from 0 to 0.08%. This range of intraspecific genetic divergence is also similar to those found in *Tylodelphys cerebralis* from India (0.007%, Choudhary et al. 2017).

Similarly, no intraspecific variability was found for the nuclear markers between the two samples of *Tylodelphys* sp. from New Zealand (Blasco-Costa et al. 2017).

Taxonomic implications

T. kuerepus n. sp. (clade V in Figs. 1 and 2) represents the eighth species of the genus described in the Americas and the fourth in the Nearctic region, although *T. americana* and *T. elongata* are more widely distributed and have been recorded in both the Nearctic and the Neotropical biogeographical regions (see León-Règagnón 1992; García-Varela et al. 2016), probably as a result of the migrating patterns of their definitive hosts. Still, the fact that they are apparently the same species across a wide geographical range needs to be determined by sequencing specimens from South America. Thus far, eight species of *Tylodelphys* in the Americas have been described morphologically with adult specimens (Table 2), and only two of them, have morphological data to corroborate DNA sequences. However, other congeneric species such as *T. scheuringi*, *T. argentinus*, *T. barilochensis*, *T. cardiophilus*, *T. crubensis*, *T. destructor*, and *T. jenynsiae* were described only with characters of the metacercariae, but not with adults obtained from their definitive hosts; some of these species (excepting those for which genetic data are available) have been considered as incertae sedis by some authors (see García-Varela et al. 2016; Blasco-Costa et al. 2017). Notwithstanding, a comprehensive DNA barcode survey was recently conducted by Locke and co-workers to explore the diversity and host specificity patterns in larval Diplostomidae collected from the eyes of freshwater fish across the globe (Locke et al. 2015). The study included representative samples of *Tylodelphys* from the Americas and other regions of Europe, Asia, and Africa, among species of other diplostomid genera; authors of that study validated seven putative species of *Tylodelphys* through *cox 1* barcodes and recognized six additional genetic lineages within the genus (referred as *Tylodephys* sp. 1–6), based on *cox 1* sequences of metacercariae stages; of these, two candidate species were found in African freshwater fish in (previously recognized by Chibwana et al. 2015 and Otachi et al. 2015) and four in North American freshwater fish. Even though most of the unidentified putative species distinguished genetically in the study by Locke et al. (2015) were supported by at least one additional line of evidence, metacercariae were not morphologically characterized. We compared the new sequenced metacercariae with data from all previous studies on *Tylodelphys* in order to establish the interrelationships among the sequenced members of the genus for two molecular markers.

Our study also showed that some of the sequenced isolates of *Tylodelphys* spp. corresponded with two of the four putative species referred by Locke et al. (2015) as *Tylodelphys* sp. 5 and *Tylodelphys* sp. 6 (as clades I and III, respectively; see Figs. 1 and 2), expanding the geographic distribution and host range of these two species. Unfortunately, adult forms have not been found in their definitive hosts, and these species cannot be described yet. The phylogenetic trees inferred with the *cox 1* gene yielded that the single metacercaria recovered from the cranial cavity of *G. affinis* from a locality in northern Mexico is nested in a reciprocally monophyletic clade along with two metacercariae (GenBank KR271520–21) from localities along the Pacific slope of Mexico, corresponding with *Tylodelphys* sp. 5 sensu Locke et al. (2015). The genetic divergence among the three isolates for that molecular marker was very low, varying from 0.04 to 0.07%. Since only one specimen was collected in our study and was used for DNA extraction, the metacercariae representing this lineage was not morphologically characterized. Furthermore, the seven metacercariae recovered from the body cavity of two species of poeciliids, i.e., *P. mexicana* and *Gambusia* sp., and one species of goodeid, *A. tamazulae*, and the two isolates referred as *Tylodelphys* sp. 6 sensu Locke et al. 2015, from the poeciliid *P. latipinna* from Mississippi, USA (GenBank KR271522–523), also formed a reciprocally monophyletic clade. The genetic divergence among these nine isolates was also very low, ranging between 0 and 0.7%. In this case too, the entire individual metacercariae collected from poeciliids and goodeids were sequenced, and no specimens were processed for morphological analyses, preventing the morphological characterization of these larval forms.

Five adult specimens recovered from the intestine of the least grebe (*T. dominicus*) from Tecocomulco Lake, formed a monophyletic clade with other five specimens of the species *T. aztecae* (herein represented as clade II, see Figs. 1 and 2), including four metacercariae (GenBank KT175368, KT175358, KT175324, KT175328,) and one adult specimen (GenBank KT175316). The genetic divergence among the 10 isolates for the *cox 1* gene was also very low and ranged from 0 to 0.7%, indicating conspecificity and expanding the geographic distribution range of *T. aztecae* in other areas of central Mexico, since the species was originally described from the Lago Los Reyes Aztecas, in Mexico City (García-Varela et al. 2016).

The three metacercariae collected from the cranial cavity of the shortfin silverside, *C. humboldtianum* in Zacapu Lake in Central Mexico were identical genetically and formed an independent lineage highly supported by bootstrap and posterior probability values; this lineage represents a yet unidentified species of *Tylodelphys* (represented as clade IV in Figs. 1 and 2). Since a larger number of individuals were collected from their fish hosts, the metacercaria was morphologically and molecularly characterized in this study. However, none of the sampled adults from grebes corresponded with this

species, and a molecular link was not established, preventing the description of this lineage as a potentially new species of *Tylodelphys*; actually, we necropsied two individuals of the pied-billed grebe, *P. podiceps* from the same locality (Zacapu Lake) but no *Tylodelphys* adults were observed.

Host specificity and geographical distribution patterns

T. kuerepus n. sp. seems to be highly host specific. The new species was found, as metacercariae, in the cranial cavity of *C. jordani*, and as an adult in the intestine of the western grebe (*A. occidentalis*), in the same locality in central Mexico. *C. jordani* is one of the species of atherinopsid silversides more widely distributed in water bodies across central Mexico; among the 36 species comprising the endemic genus *Chirostoma*, the species *C. jordani* holds the highest helminth species richness, with 21 species (see Lira-Guerrero et al. 2008). The results of our study indicate that two additional species of trematodes are part of the core helminth parasite fauna of atherinopsids (sensu Pérez-Ponce de León and Choudhury 2005). Besides the new species, we also found metacercariae of an undescribed species of *Tylodelphys* unencysted in the cranial cavity of the shortfin silverside, *C. humboldtianum* in Zacapu Lake, where the species had been previously found (Lira-Guerrero et al. 2008). The other putative species uncovered in our study showed also a strict host specificity towards certain groups of second intermediate hosts. *Tylodelphys* sp. 5 and *Tylodelphys* sp. 6 (sensu Locke et al. 2015) were mainly found parasitizing poeciliid in several localities across Mexico.

Biogeographically, there seems to be a gap in the distribution of *Tylodelphys* metacercariae in Central American freshwater fishes, since no records of their presence have been published thus far, even though parasite surveys have been conducted with relative intensity in the area, analyzing several host families (e.g., Aguirre-Macedo et al. 2001; Sandlund et al. 2010; Pinacho-Pinacho et al. 2015, 2018; Pérez Ponce de León et al. 2016; López-Jimenez et al. 2017; Briosio-Aguilar et al. 2018). Other species of diplostomids, i.e., *Austrodiplostomum compactum* Lutz, 1928 and *Posthodiplostomum minimum* MacCallum, 1921 have been found in some species of freshwater fishes (Aguirre-Macedo et al. 2001; Sandlund et al. 2010), but no specimens of *Tylodelphys* yet. The current distribution of species of *Tylodelphys* in the Americas suggests they inhabit more temperate areas and represent two separate assemblages, one occurring in South America, with the adults of *T. adulta* and *T. brevis* having been described in Argentina, and a second assemblage in North America, with the species *T. immer*, *T. podicipina robrauschi*, and *T. aztecae* and the new species we described in this study. Additionally, two species, *T. americana* and *T. elongata*, have been recorded in both South America (Brazil, Argentina, and Venezuela, see Dubois 1970; Fernandes et al. 2015) and North America (USA and Mexico, see Dubois 1970; León-Règagnón 1992); however, a molecular phylogenetic analysis is pending to corroborate whether they represent the same species or not; the lack of *Tylodelphys* metacercariae in freshwater fishes in Central America may support the contention that they are separate species. This disjunct and amphi-temperate distribution pattern has been described in other parasite species. For instance, the acanthocephalan genus *Pomphorhynchus* Monticelli, 1905 exhibits the same distribution pattern, with some species occurring in South America, and other species occurring in North America, with no species of the genus present in Middle-America (García-Varela et al. 2017). The fact that apparently the same species of *Tylodelphys* occurs in both North and South America might be due to dispersal capacity through the birds acting as definitive hosts that can disperse the parasites during migration. In contrast, the autogenic nature of *Pomphorhynchus* species determines that the entire life cycle has to be completed in the aquatic environment, limiting the dispersal capabilities to geological processes; additionally, species of *Pomphorhynchus* mainly parasitizes temperate freshwater fish and this might account for the limited distribution in both extremes of the Americas.

Finally, Blasco-Costa et al. (2017) discussed that the species diversity of *Tylodelphys* should be very different from what we know today, mainly because a large proportion of the species are currently distinguished based on the morphology of the metacercariae. The entire genus *Tylodelphys* require a taxonomic revision and, most importantly, molecular sequence data from other congeneric species to link larval forms with adults from their definitive hosts to support current species designations. The database of DNA sequences of diplostomids, including *Tylodelphys*, is steadily accumulating, and in some genera as *Diplostomum*, at a very fast rate, with sequence data from specimens from across the globe; therefore, comprehensive phylogenetic analyses will provide a better understanding of the interrelationships among species and genera and surely will provide a better estimate of the species diversity of this interesting group of trematodes.

Acknowledgements We thank Luis García Prieto for providing specimens deposited at the CNHE, Berenit Mendoza for her help in obtaining the scanning electron microphotographs, and Laura Márquez for her help with the automatic sequencer. Specimens were collected under the Cartilla Nacional de Colector Científico (FAUT 0202 and 0057) issued by the Secretaría del Medio Ambiente y Recursos Naturales (SEMARNAT) to MGV and GPPL, respectively. The permission to use a shotgun to collect birds was issued by the Secretaría de la Defensa Nacional. This research was partially supported by grants from the Programa de Apoyo a Proyectos de Investigación e Inovación Tecnológica (PAPIIT-UNAM) IN206716 and IN202617 to MGV and GPPL, respectively; LAG thanks the support of the Programa de Posgrado en Ciencias Biológicas, UNAM, and CONACYT for granting a scholarship to complete his PhD program.

References

Aguirre-Macedo ML, Scholz T, González-Solís D, Vidal-Martínez VM, Posel P, Arjona-Torres G, Dumailo S, Siu-Estrada E (2001) Some adult endohelminths parasitizing freshwater fishes from the Atlantic drainages of Nicaragua. Com Parasitol 68:190–195

American Ornithologists' Union (AOU) (1998) Check-list of North American birds, 7th edition 829 pp. Washington, D.C.,

Blasco-Costa I, Poulin R, Presswell B (2017) Morphological description and molecular analyses of *Tylodelphys* sp. (Trematoda: Diplostomidae) newly recorded from the freshwater fish *Gobiomorphus cotidianus* (common bully) in New Zealand. J Helminthol 91:332–345. https://doi.org/10.1017/S0022149X16000298

Bowles J, McManus DP (1993) Rapid discrimination of *Echinococcus* species and strains using a PCR-based method. Mol Biochem Parasitol 57:231–239

Briosio-Aguilar R, García-Varela M, Hernández-Mena DI, Rubio-Godoy M, Pérez Ponce de León G (2018) Morphological and molecular characterization of an enigmatic clinostomid trematode (Digenea: Clinostomidae) parasitic as metacercariae in the body cavity of freshwater fishes (Cichlidae) across Middle America. J Helminthol:17:1–17:1714. https://doi.org/10.1017/S0022149X18000445

Chibwana FD, Blasco-Costa I, Goergieva S, Hosea KM, Nkwengulila G, Scholz T, Kostadinova A (2013) A first insight into the barcodes for African diplostomids (Digenea: Diplostomidae): brain parasites in *Clarias gariepinus* (Siluriformes: Clariidae). Infect Genet Evol 17: 62–70. https://doi.org/10.1016/j.meegid.2013.03.037

Chibwana FD, Nkwengulila G, Locke SA, McLughlin JD, Marcogliese DJ (2015) Completion of the life cycle of *Tylodelphys mashonense* (Sudarikov, 1971) (Digenea: Diplostomidae) with DNA barcodes and rDNA sequences. Parasitol Res 114:3675–3682. https://doi.org/10.1007/s00436-015-4595-8

Choudhary A, Tripathi R, Gupta S, Singh HS (2017) First report on molecular evidence of *Tylodelphys cerebralis* (Diplostomum cerebralis) Chakrabarti, 1968 (Digenea: Diplostomidae) from snakehead fish *Channa punctata*. Acta Parasitol 62:386–392. https://doi.org/10.1515/ap-2017-0046

Domínguez-Domínguez O, Pérez-Ponce de León G (2009) La mesa central de México es una provincia biogeográfica. Análisis descriptivo basado en componentes bióticos dulceacuícolas. Rev Mex Biodivers 80:835–852

Drago FB, Lunaschi LI (2008) Description of a new species of *Tylodelphys* (Digenea, Diplostomidae) in the wood stork, *Mycteria americana* (Aves, Ciconiidae) from Argentina. Acta Parasitol 53: 263–267. https://doi.org/10.2478/s11686-008-0038-3

Dubois G (1961) Le genre Diplostomum von Nordmann 1832 (Trematoda: Strigeida). Bull Soc Neuchâtel Sci Nat 84:113–124

Dubois G (1969) Les Strigeata (Trematoda) de la collection Elizabeth M. Boyd. Bull Soc Neuchâtel Sci Nat 92:5–12

Dubois G (1970) Synopsis des Strigeidae et des Diplostomatidae (Trematoda). Mem Soc Sci Nat Neuchatel 10:257–727

Fernandes BMM, Justo MCN, Cárdenas MQ, Cohen SC (2015) South American trematodes parasites of birds and mammals. Oficina de Livros, Rio de Janeiro

García-Varela M, Sereno-Uribe AL, Pinacho-Pinacho CD, Hernández-Cruz E, Pérez-Ponce de León G (2016) An integrative taxonomic study reveals a new species of *Tylodelphys* Diesing, 1850 (Digenea: Diplostomidae) in central and northern Mexico. J Helminthol 4:1–12. https://doi.org/10.1017/S0022149X15000917

García-Varela M, Mendoza-Garfias B, Choudhury A, Pérez-Ponce de León G (2017) Morphological and molecular data for a new species of *Pomphorhynchus* Monticelli, 1905 (Acanthocephala: Pomphorhynchidae) in the Mexican redhorse *Moxostoma austrinum* Bean (Cypriniformes: Catostomidae) in central Mexico. Syst Parasitol 94:989–1006. https://doi.org/10.1007/s11230-017-9756-y

Guzmán-Cornejo M del C, García-Prieto L (1999) Trematodiasis en algunos peces del lago de lago de Cuitzeo, Michoacán, México. Rev Biol Trop 47:593–596 https://revistas.ucr.ac.cr/index.php/rbt/article/view/19211/26036

Howell SNG, Webb S (1995) A guide to the birds of Mexico and northern Central America. Oxford, New York

Huelsenbeck JP, Ronquest F (2001) MrBayes: Bayesian inference of phylogeny. Biometrics 17:754–755

King PH, Van AGJ (1997) Description of the adult and larval stages of *Tylodelphys xenopi* (Trematoda: Diplostomidae) from southern Africa. J Parasitol 83:287–295

León-Règagnón V (1992) Fauna helmintológica de algunos vertebrados acuáticos de la cienága de Lerma, Estado de México. An Inst Biol UNAM 63:151–153

Lira-Guerrero G, García-Prieto L, Pérez Ponce de León G (2008) Helminth parasites of atherinopsid freshwater fishes (Osteichthyes: Atheriniformes) from central Mexico. Rev Mex Biodivers 79:325–331

Locke SA, Al-Nasiri FS, Caffara M, Drago F, Kalven M, Lapierre AR, McLaughlin JD, Nie P, Overstreet RM, Souza GTR, Takemoto RM, Marcogliese DJ (2015) Diversity, specificity and speciation in larval Diplostomidae (Platyhelminthes: Digenea) in the eyes of freshwater fish, as revealed by DNA barcodes. Int J Parasitol 45:841–855. https://doi.org/10.1016/j.ijpara.2015.07.001

López-Jimenez A, Pérez-Ponce de León G, García-Varela M (2017) Molecular data reveal high diversity of *Uvulifer* (Trematoda: Diplostomidae) in Middle America, with the description of a new species. J Helminthol 11:1–15. https://doi.org/10.1017/S0022149X17000888

Lunaschi LI, Drago FB (2004) Descripción de una especie nueva de *Tylodelphys* (Digenea:Diplostomidae) parásita de *Podiceps major* (Aves: Podicepedidae) de Argentina. An Inst Biol UNAM 75:245–252

Martínez-Aquino A, Mendoza-Palmero CA, Aguilar-Aguilar R, Pérez Ponce de León G (2014) Checklist of helminth parasites Goodeinae (Osteichthyes: Cyprinodontiformes: Goodeidae), an endemic subfamily of freshwater fishes from Mexico. Zootaxa 3856: 151–191. https://doi.org/10.11646/zootaxa.3856.2.1

Miller RR, Minckley WL, Norris SM (2005) Freshwater fishes of Mexico. The University of Chicago Press

Morrone J (2006) Biogeographical areas and transition zones of Latin America and the Caribbean island based on pan-biogeographic and cladistics analyses of the entomofauna. Ann Rev 51:467–494

Moszczynska A, Locke SA, McLaughlin JD, Marcogliese DJ, Crease TJ (2009) Development of primers for the mitochondrial cytochrome c oxidase I gene in digenetic trematodes (Platyhelminthes) illustrates the challenge of barcoding parasitic helminthes. Mol Ecol Resour 9: 75–82. https://doi.org/10.1111/j.1755-0998.2009.02634.x

Muzall MP, Kilroy AL (2007) *Tylodelphys scheuringi* (Diplostomidae) infecting the brain of the central mudminnow, *Umbrina limi*, in Silver Creek, Michigan, U.S.A. Com Parasitol 74:164–166. https://doi.org/10.1654/4278.1

Niewiadomska K (2002) Family Diplostomidae Poirier, 1886. In: Gibson DI, Jones A, Bray RA (eds) Keys to the Trematoda, Vol. 1. CABI Publishing and London, The Natural History Museum, Wallingford, pp 167–196

Otachi EO, Locke SA, Jirsa F, Fellner-Frank C, Marcogliese DJ (2015) Morphometric and molecular analyses of *Tylodelphys* sp. metacercariae (Digenea:Diplostomidae) from the vitreous humour

of four fish species from Lake Naivasha, Kenya. J Helminthol 4:1–11. https://doi.org/10.1017/S0022149X14000170

Pérez Ponce de León G, García Prieto L, Mendoza Garfías B (2007) Trematode parasites (Platyhelminthes) of wildlife vertebrates in Mexico. Zootaxa 1534:1–247

Pérez Ponce de León G, García-Varela M, Pinacho-Pinacho CD, Sereno-Uribe AL, Poulin R (2016) Species delimitation in trematodes using DNA sequences: Middle-American *Clinostomum* as a case study. Parasitol 143:1773–1789. https://doi.org/10.1017/S0031182016001517

Pérez-Ponce de León G, Choudhury A (2005) Biogeography of helminth parasites of freshwater fishes in Mexico: the search for patterns and processes. J Biogeogr 32:645–659. https://doi.org/10.1111/j.1365-2699.2005.01218.x

Pérez-Ponce de León G, Choudhury A (2010) Parasite inventories and DNA-based taxonomy: lessons from helminths of freshwater fishes in a megadiverse country. J Parasitol 96:236–244

Pinacho-Pinacho CD, García-Varela M, Hernández-Orts JS, Mendoza-Palmero CA, Sereno-Uribe AL, Martínez-Ramírez E, Andrade-Gómez L, López-Jiménez A, Hernández-Cruz E, Pérez-Ponce de León G (2015) Checklist of the helminth parasites of the genus *Profundulus* Hubbs, 1924 (Cyprinodontiformes, Profundulidae), an endemic family of freshwater fishes in Middle-America. Zookeys 523:1–30. https://doi.org/10.3897/zookeys.523.6088

Pinacho-Pinacho CD, García-Varela M, Sereno-Uribe AL, Pérez-Ponce de León G (2018) A hyper-diverse genus of acanthocephalans revealed by tree-based and non-tree-based species delimitation methods: ten cryptic species of *Neoechinorhynchus* in Middle American freshwater fishes. Mol Phylogenet Evol 127:30–45. https://doi.org/10.1016/j.ympev.2018.05.023

Posada D, Crandall KA (2001) Selecting the best-fit model of nucleotide substitution. Syst Biol 50:580–601

Rambaut A (2006) FigTree v1.3.1. Institute of Evolutionary Biology. University of Edinburgh

Sandlund OT, Daverdin RH, Choudhury A, Brooks DR, Diserud HD (2010) A survey of freshwater fishes and their macroparasites in the Guanacaste Conservation Area (ACG), Costa Rica. Norwegian Institute for Nature Research (NINA) Report 635, Trondheim

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725–2729

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F (1997) The Clustal windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882