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An integrative taxonomic study reveals a new species of *Tylodelphys* Diesing, 1950 (Digenea: Diplostomidae) in central and northern Mexico

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Abstract

Tylodelphys aztecae n. sp. (Digenea: Diplostomidae) is described from adult specimens obtained from the intestine of the pied-billed grebe (*Podilymbus podiceps*) and the metacercariae found in the body cavity of freshwater fishes of the families Goodeidae and Cyprinidae in eight localities across central and northern Mexico. The new species is mainly distinguished from the other four described species of *Tylodelphys* from the Americas (*T. adulta, T. americana, T. elongata* and *T. brevis*) by having a forebody slightly concave, a larger ventral sucker, two larger pseudosuckers and by having between 2 and 7 eggs in the uterus. Partial DNA sequences of the mitochondrial gene cytochrome *c* oxidase subunit I (*cox1*), and the internal transcribed spacers (ITS1 + 5.8S + ITS2) of the ribosomal DNA, were generated for both developmental stages and compared with available sequences in GenBank of other congeners. The genetic divergence estimated among *Tylodelphys aztecae* n. sp. and other congeneric species varied from 12 to 15% for *cox1*, and from 3 to 11% for ITS. In contrast, the genetic divergence among metacercariae and adults of the new species was very low, ranging between 0 and 1% for *cox1* and between 0 and 0.3% for ITS. Phylogenetic analyses inferred with both molecular markers using maximum likelihood and Bayesian inference placed the adults and their metacercariae in a single clade, confirming that both stages are conspecific. The morphological evidence and the genetic divergence, in combination with the reciprocal monophyly in both phylogenetic trees, support the hypothesis that the diplostomids found in the intestines of the pied-billed grebe bird and the body cavity from goodeid and cyprinid fishes in central and northern Mexico represent a new species.

Introduction

Modern taxonomic practices in helminths combine morphological data and DNA sequences, allowing the establishment of a link between larval and adult stages in different host species in an ecosystem. Recent studies on diplostomid trematodes illustrate the usefulness of such approaches, with the internal transcribed spacers (ITS) of ribosomal DNA and the mitochondrial gene cytochrome *c* oxidase subunit I (*cox1*) as the most popular molecular markers used for the identification and delimitation of species in these trematodes (Chibwana *et al.*, 2013, 2015; Georgieva *et al.*, 2013; Blasco-Costa *et al.*, 2014; García-Varela *et al.*, 2015; Selbach *et al.*, 2015). Further, the development of diplostomid-specific primers flanking the barcode region (*cox1*), allowed the construction of a large barcode library of diplostomids that includes

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populations and species from different regions around the world (Moszczynska *et al.*, 2009; Locke *et al.*, 2010).

The genus *Tylodelphys* Diesing, 1950 contains species whose adults are found parasitizing the intestine of fish-eating birds, such as ciconiids, anhingids and podicipedids, distributed worldwide (King & Van As, 1997; Lunaschi & Drago, 2004). Members of *Tylodelphys*, like other diplostomids, exhibit a three-host life cycle, involving a freshwater snail as the first intermediate host. The metacercaria is found free in the eyes, cranial cavity and body cavity of fish that act as second intermediate hosts (King & Van As, 1997; Muzall & Kilroy, 2007; Chibwana *et al.*, 2015; Otachi *et al.*, 2015). *Tylodelphys* can be distinguished from other genera of Diplostomatidae Poirier, 1866 because they possess a conical hindbody, a tribocytic organ far from the intestinal bifurcation, testes relatively large and anterior testis symmetrical (Niewiadomska, 2002). The genus currently contains 16 species (Lunaschi & Drago, 2004; Drago & Lunaschi, 2008; Otachi *et al.*, 2015). In the Americas only four species have been described from adult specimens: *T. americana* (Dubois, 1936) Dubois, 1937; *T. elongata* (Lutz, 1928) Dubois, 1937; *T. adulta* Lunaschi & Drago, 2004 and *T. brevis* Drago & Lunaschi 2008 (Dubois, 1970; Lunaschi & Drago, 2004; Drago & Lunaschi, 2008).

The species *T. americana* has been recorded from central Mexico, from the intestine of podicipedids, *Podilymbus podiceps* (pied-billed grebe) Linnaeus 1758, in San Pedro Tlaltizapán, Estado de México (León-Règagnón, 1992), and from *P. podiceps* and *Aechmophorus occidentalis* Lawrence, 1858 from Lago Tecocomulco, Hidalgo (Andrade-Rosales, 2012). Additionally, the metacercariae of *Tylodelphys* sp. have been recorded in at least 27 localities from five states across central and northern Mexico, parasitizing 20 species of endemic freshwater fishes, belonging to three families (Goodeidae, Atherinopsidae and Cyprinidae), although they seem to infect goodeids preferentially, since 11 of the 20 species (55%) belong to this family (see Lira-Guerrero *et al.*, 2008; Monks *et al.*, 2013; Martínez-Aquino *et al.*, 2014).

As a part of a research programme designed to compile an inventory of the helminth fauna of birds and fishes in central and northern Mexico, we collected adult specimens of a species of *Tylodelphys* from the intestine of pied-billed grebes, and metacercariae were collected from five fish species belonging to the family Goodeidae and one belonging to the Cyprinidae, in eight localities across central Mexico and one in northern Mexico. Specimens were sequenced for two molecular markers, with the aim of establishing a link between both developmental stages of the life cycle of this diplostomid. Also, sequences were compared with those available in GenBank for other congeneric species; additionally, adults were morphologically compared with species distributed in the Americas. Data derived from morphology and DNA sequences showed the existence of a new species of *Tylodelphys*. The new species is described herein.

Materials and methods

Specimen collection

A total of seven birds were collected between June 2012 and September 2014 in Lago de los Reyes Aztecas in

Table 1. GenBank accession numbers from mainly metacercarial stages, host species, locality and geographical locations. The locality numbers correspond to the numbers in fig. 1.

Locality	Coordinates	Host samples	Sample number	GenBank accession number (KT)	
				cox1	ITS
1. Lago de los Reyes Aztecas, Tláhuac, Distrito Federal	19°15′58″N, 99°00′24″W	**Podilymbus podiceps*	2328–2335 2337	175315–175322 175323	175388
2. Lago de Xochimilco, Distrito Federal	19°17′13.79″N, 99°6′7.28″W	*Goodea atripinnis*	1900–1903	175324–175327	175370–175371
3. Presa Villa Victoria, Estado de México	19°27′30″N, 99°59′39″W	*Goodea atripinnis* *Girardinichthys multiradiatus*	2284–2288 2238–2239 2241	175328–175332 175333–175334 175335	175383–175384 175372–175373 175385
4. Lago de Almoloya del Río, Estado de México	19°11′20″N, 99°29′30″W	*Girardinichthys multiradiatus*	2279–2283	175336–175340	175381–175382
5. Lago Tiacaque, Estado de México	19°40′20″N, 99°42′12″W	*Goodea atripinnis* *Girardinichthys multiradiatus*	2242–2245 2257–2261 2263–2266	175341–175344 175345–175349 175350–175353	175386–175387 175374
6. Manantial la Luz, Michoacán	19°56′10.4″N, 102°17′57.8″W	*Chapalichthys encaustus*	2232–2233 2235–2236	175354–75355 175356–175357	
7. Lago de Zacapu, Michoacán	19°49′35″N, 101°47′10″W	*Hubbsina turneri* *Skiffia lermae*	2267–2271 2274–2278	175358–175362 175363–175367	175375–175376 175379–175380
8. Rio Guatimape, Durango	24°49′74″N; 104°53′26″W	*Gila conspersa*	2272–2273	175368–175369	175377–175378

*Adult worms recovered from the definitive host.

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Tlahuac in southern Mexico City (table 1, fig. 1). Birds were captured with a shotgun under the collecting permit FAUT 0202 issued by the Mexican government through the Secretaría de Medio Ambiente y Recursos Naturales (SEMARNAT) to M.G.V. Fish were captured with seine nets, minnow traps and electro-fished in eight localities of central and northern Mexico (table 1, fig. 1). Birds were kept on ice, and their intestines examined within 2 h after capture, whereas fish were maintained alive and transported to the laboratory. Individual fish were killed by pithing and examined immediately. Collected digeneans were preserved either in 100% ethanol, for DNA extraction, or in hot (steaming) 4% formalin, for morphology. Avian definitive hosts were identified using the field guides of Howell & Webb (1995) and the American Ornithologists' Union (1998). Fish were identified following Miller *et al.* (2005).

Morphological and molecular analyses

Unflattened specimens preserved in formalin were stained with Mayer's paracarmine, dehydrated in a graded ethanol series, cleared with methyl salicylate, and mounted on microscope slides in Canada balsam. Drawings were made with the aid of a drawing tube. Measurements are given in micrometres (μm), with range followed by mean in parentheses. Specimens were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México, Mexico City. The species identification was conducted following the key of Dubois (1970), and original descriptions (see Lunaschi & Drago, 2004; Drago & Lunaschi, 2008). Additionally, specimens identified as *T. americana* deposited in the CNHE under numbers 1504 and 6856 were compared with our specimens. Some individuals collected in this study were preserved in 4% formalin and dehydrated through a graded series of ethyl alcohol and then critical point dried with carbon dioxide. The specimens were mounted on metal stubs with silver paste, coated with gold and examined in a Hitachi Stereoscan Model SU1510 at 10 kV to obtain micrographs of the body surface.

Forty-six metacercariae and nine adult specimens were placed individually in tubes and digested overnight at 56°C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM Na_2EDTA (pH 8.0), 1% Sarkosyl and 0.1 mg/ml proteinase K. Following digestion, DNA was extracted from the supernatant using the DNAzol reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to the manufacturer's instructions.

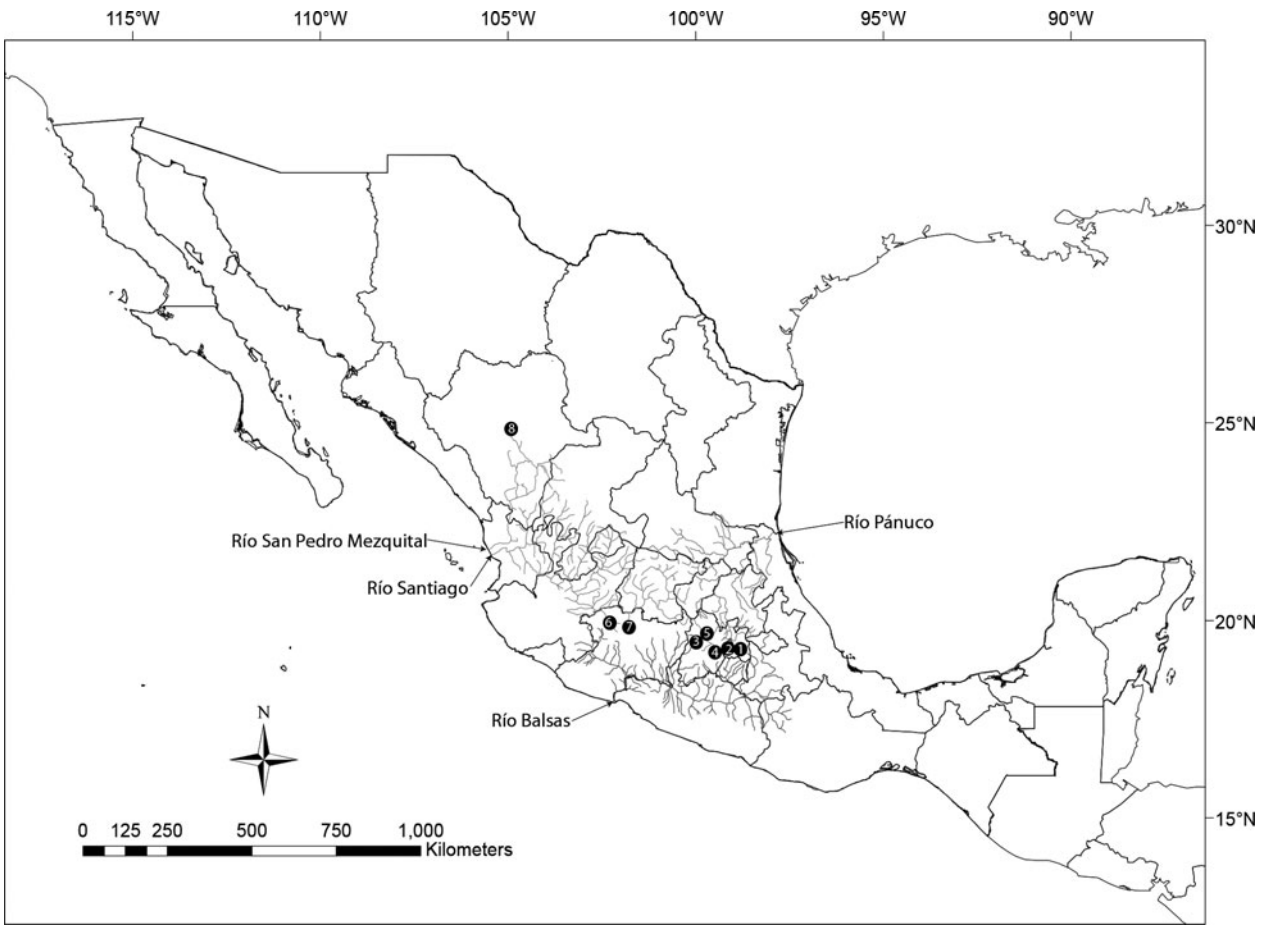

Fig. 1. Sampling sites (see table 1) of avian and fish hosts infected with *Tylodelphys aztecae* n. sp., from central and northern Mexico.

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The cytochrome oxidase subunit 1 (*cox1*) of the mitochondrial DNA was amplified using the polymerase chain reaction (PCR) with the forward primer Plant-diploCOXF, 5′-CGTTTRAATTATACGGATCC-3′, and the reverse primer Plant-diploCOXR, 5′-AGCATAGTAATMGCAG-CAGC-3′ (Moszczynska *et al.*, 2009). The ITS region was amplified using the forward primer BD1 5′-GTC-GTAACAAGGTTTCCGTA-3′ and the reverse primer BD2 5′-ATCTAGACCGGACTAGGCTGTG-3′ (Bowles & McManus, 1993). PCR reactions (25 μl) consisted of 10 μM of each primer, 2.5 μl of 10 × buffer, 2 mM $MgCl_2$, 0.5 μl of deoxynucleoside triphosphates (dNTPs) (10 mM) and 1 U of *Taq* DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). PCR cycling conditions for both molecular markers included a step of denaturation at 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, annealing at 50°C for 1 min, 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, USA) 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 5.0.2 (Codoncode Corporation, Dedham, Massachusetts, USA). Sequences were deposited in GenBank (table 1). ITS and *cox1* sequences obtained in the current research were aligned with sequences of the genera of diplostomids downloaded from GenBank – *Posthodiplostomum* Dubois 1936, *Ornithodiplostomum* Dubois 1936, *Diplostomum* von Nordmann 1832 and *Tylodelphys*. In addition, sequences of the strigeids *Cardiocephaloides* Sudarikov 1959, *Australapatemom burti* Miller 1923, *Parastrigea diovadena* Dubois and Macko, 1972, *Apharyngostrigea cornu* Ciurea 1927 and *Ichthyocotylurus*, Odening 1969 were used as an outgroup, since this family is considered to be the sister group of Diplostomidae (see Olson *et al.*, 2003). Sequences were aligned using the software Clustal W (Thompson *et al.*, 1997). Maximum likelihood (ML) and Bayesian inference analyses (BI) were performed for each dataset. The ML tree was inferred using RAxML 7.0.4. (Stamatakis, 2006). Modeltest program version 3.0 (Posada & Crandall, 1988) was used for inferring the best model of nucleotide substitution. For both alignments the best model selected was GTR + G + I. Tree searches were performed using 1000 (ML) random taxon addition heuristic searches. Clade support was assessed by bootstrap resampling with 10,000 replicates. Bayesian analyses were performed with MrBayes version 3.1.2 (Huelsenbeck & Ronquist, 2001). Settings were two simultaneous runs of the Markov chain (MCMC) for 10 million generations, sampling every 1000 generations, a heating parameter value of 0.2 and a 'burn-in' of 25%. Trees were drawn using FigTree version 1.3.1 (Rambaut, 2006). The genetic divergence among taxa was estimated using uncorrected 'p' distances with the program MEGA version 6 (Tamura *et al.*, 2013).

Results

Description of Tylodelphys aztecae n. sp.

Morphological description: adults

Description (table 2) was based on ten adult specimens. Body linguiform. Forebody slightly spatulate, bearing

Table 2. Comparative morphometrics (in μm) of adult worms of *Tylodelphys aztecae* n. sp., with congeneric species from the Americas.

Characteristics	*T. adulta* Lunaschi & Drago, 2004	*T. brevis* Drago & Lunaschi, 2008	*T. elongata* Dubois, 1970	*T. americana* Dubois, 1970	*T. americana* This study	*T. aztecae* n. sp. This study
Body (L)	1123–1464	570–851	1500–2350	900–2400	1272–1755	874–1135
Forebody (L)	790–950	371–507	800–1120	550–1500	800–1063	626–763
Hindbody (L)	1269–528	371–507	450–650	310–900	451–750	370–467
Oral sucker (L)	71–97	40–67	80–100	48–87	76–90	80–101
Oral sucker (W)	83–103	44–69	90–104	48–95	62–87	63–100
Pharynx (L)	71–110	45–57	63–73	49–79	50–73	57–95
Pharynx (W)	53–74	22–31	60–68	33–72	43–60	33–53
Ventral sucker (L)	60–80	24–36	70–90	33–76	82–108	32–103
Ventral sucker (W)	78–97	27–54	99–100	36–108	88–115	80–142
Tribocytic organ (L)	195–250	69–131	160–210	115–390	151–279	127–206
Tribocytic organ (W)	178–274	50–102	–	110–510	168–294	123–234
Oesophagus (L)	25	10.0–15	–	40	38	38–48
Pseudosucker (L)	145–216	48–74	110–210	50–112	96–150	130–243
Pseudosucker (W)	74–126	29–59	80–130	68–80	32–51	32–97
Anterior testis (L)	120–121	41–71	100–140	110–300	90–123	65–115
Anterior testis (W)	216–494	133–226	445–460	270–575	270–321	150–440
Posterior testis (L)	115–168	34–83	110–180	110–290	91–172	50–255
Posterior testis (W)	211–427	121–202	400–445	240–520	230–289	212–350
Ovary (L)	73–83	34–53	75–125	63–135	89–105	57–110
Ovary (W)	73–97	29–78	95–200	80–90	80–90	60–100
Eggs, number	1–20	1–2	15	30	10	2–7
Egg (L)	87–99	83–102	90–97	84–103	83–96	89–113
Egg (W)	51–59	45–64	60–66	53–63	64–68	45–77
Hindbody/ forebody (L) ratio	0.28–0.64	0.4–0.8	0.55–0.72	0.38–0.80	0.58–0.73	0.57–0.70

L, Length; W, width.

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scattered papillae on the ventral surface of tegument (fig. 2a, c). Total length 874–1135 (1080). Oral sucker relatively small, fairly muscular, terminal, longer than wide, 80–101 (89) long by 63–100 (78) wide; two well-developed pseudosuckers on each side of oral sucker, 130–243 (172) long, 32–97 (57) wide (figs 2b, 3a). Ventral sucker oval, fairly muscular, wider than long, 80–142 (115) wide by 32–103 (64) long. Tribocytic organ, oval, 127–206 (175) long by 123–234 (181) wide. Prepharynx absent; relatively large pharynx, oval, 57–95 (74) long, 33–53 (40) wide; oesophagus long 32–48 (42); two blind intestinal caeca, extending to the tribocytic organ. Testes in tandem, extending transversally, occupying almost all the width of hindbody; anterior testis 65–115 (90) long by 150–440 (295) wide; posterior testis 50–255 (155) long by 212–350 (280) wide. Ovary pretesticular, spherical 57–110 (71) by 60–100 (75), contiguous with anterior testis. Mehlis' gland lateral anterior testis. Vitelline follicles surround tribocytic organ extending to hindbody. Uterus extending from the ovario-testicular area posteriorly to the genital pore opening at the end of hindbody (fig. 3b, c). Eggs 89–113 (105) long by 45–77 (54) wide.

Taxonomic summary: adults

Type host. *Podilymbus podiceps* (Linnaeus, 1758) (pied-billed grebe), Podicipedidae.

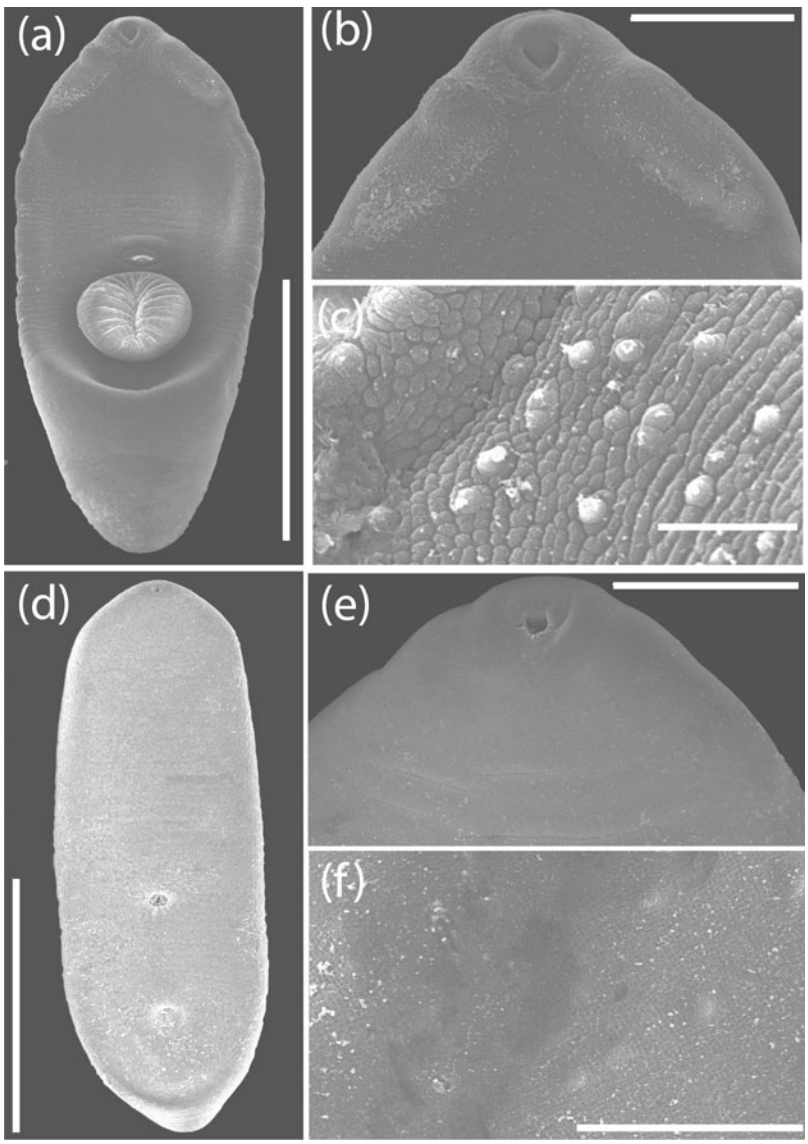

Fig. 2. Scanning electron micrographs of *Tylodelphys aztecae* n. sp. (a–c) Adult: (a) entire specimen from *Podilymbus podiceps* from Lago de los Reyes Aztecas Tlahuac, Mexico City; (b) anterior region showing pseudosuckers; (c) tegument of the ventral surface of forebody showing papillae. (d–f) Metacercariae from *Goodea atripinnis:* (d) entire specimen; (e) anterior region; (f) tegument. Scale bars: (a) 400 μm; (b, e) 100 μm; (c) 10 μm; (d) 500 μm; (f) 20 μm.

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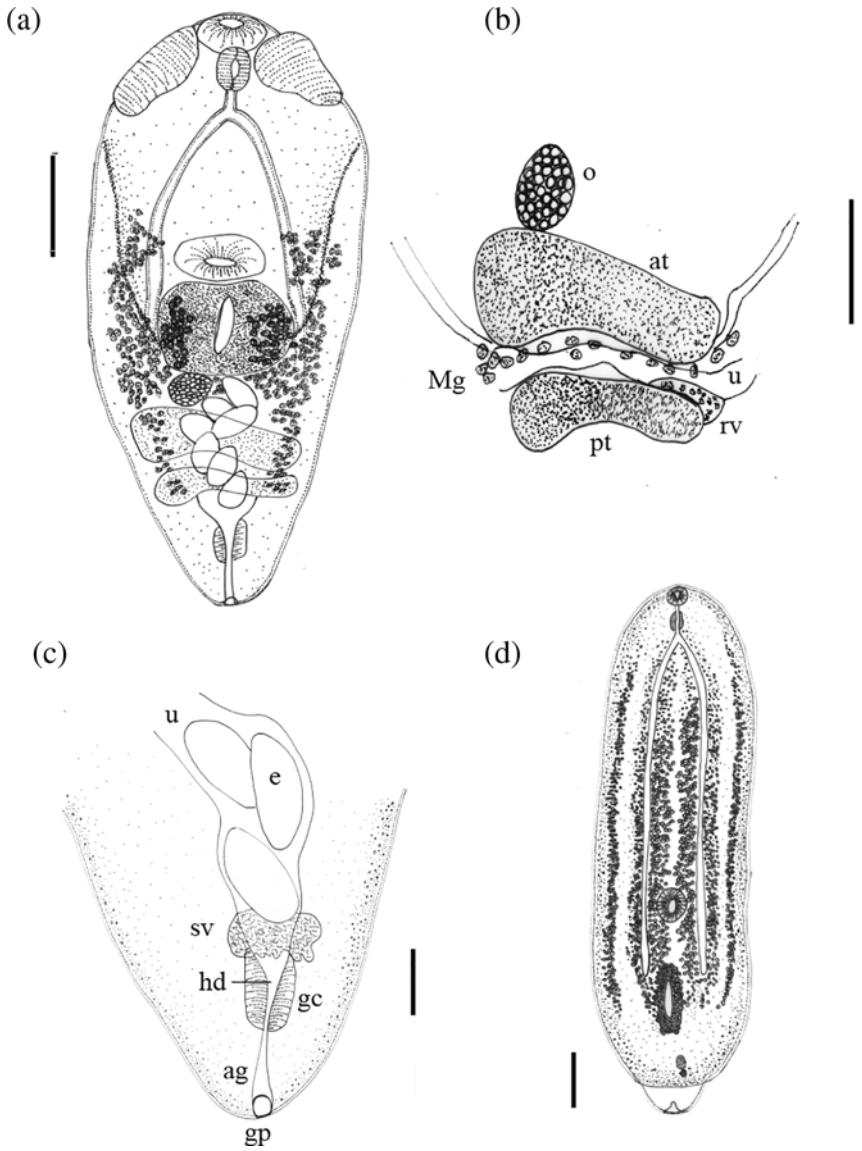

Fig. 3. *Tylodelphys aztecae* n. sp. (holotype) from *Podilymbus podiceps* from Lago de los Reyes Aztecas Tlahuac, Mexico City. (a–c) Adult: (a) entire specimen; (b) enlarged dorsal view of proximal female genitalia; (c) enlarged lateral view of terminal genitalia. (d) Metacercaria from body cavity of *Goodea atripinnis* from from Lago de los Reyes Aztecas Tlahuac, Mexico City. Abbreviations: ag, genital atrium; at, anterior testis; e, egg; gc, genital cone; gp, genital pore; hd, hermaphroditic duct; Mg, Mehlis'gland; o, ovary; pt, posterior testis; rv, vitelline reservoir; sv, seminal vesicle; u, uterus. Scale bars: (a, d) 200 μm; (b) 100 μm; (c) 50 μm.

Site of infection. Intestine.

Type locality. Lago de los Reyes Aztecas Tlahuac, Mexico City (19°15′58″N, 99°00′24″W).

Type material. Holotype CNHE: 9777; paratypes CNHE: 9778.

Etymology. The specific epithet refers to both the Azteca, a Mesoamerican civilization that dominated central Mexico during the early 13th century and founded Tenochtitlan (where currently Mexico City is settled), and the locality where the adults were found, Lago de los Reyes Aztecas, an area used for recreation by the Aztec emperors.

Morphological description: metacercariae

The metacercariae of the new species are commonly found free and active in the body cavity of their second intermediate host. The following characterization is based on 30 specimens obtained from *Goodea atripinnis* from the Lago de los Reyes Aztecas, Tlahuac, Mexico City (figs 2d and 3d). Body linguiform, 940–1530 (1231) long by 290–590 (444) wide. Entire body containing calcareous corpuscles

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forming at least six rows along body. Hindbody reduced to a small conical prominence. Papillae absent on the surface of tegument (fig. 2f). Lateral pseudosuckers lacking (fig. 2d, e). Oral sucker small, terminal, almost rounded, 40–80 (55) long by 30–72 (45) wide (fig. 2e). Ventral sucker small, fairly muscular 45–157 (83) long by 37–90 (64) wide. Prepharynx very small; oval pharynx 22–60 (32) long by 12–22 (17) wide; intestinal caeca long, extending posteriorly to level of anterior border of tribocytic organ; tribocytic organ 170–245 (207) long by 67–140 (88) wide. Reproductive system poorly developed (fig. 3d).

Taxonomic summary: metacercariae

Type host. Goodea atripinnis Jordan, 1880, Goodeidae.

Site of infection. Body cavity.

Type locality. Lago de los Reyes Aztecas Tlahuac, Mexico City (19°15′58″N, 99°00′24″W).

Voucher material. CNHE: 9779.

Remarks

The new species belongs to *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 & Lunaschi, 2008). Only 11 species of the genus *Tylodelphys* have been reported from the Americas, four as adults and seven as metacercariae (*Tylodelphys destructor* Szidat et Nani, 1951; *T. barilochensis* Quaggiotto et Valverde 1992; *T. crubensis* Quaggiotto et Valverde 1992; *T. argentinus* Quaggiotto et Valverde 1992; *T. jenynsiae* Szidat 1969; *T. cardiophilus* Szidat 1969 and *T. scheuringi* Hughes 1929) (Hughes, 1929; Lunaschi & Drago, 2004; Muzzal & Kilroy, 2007; Drago & Lunaschi, 2008). Considering that no adults have been described for those species, we decided to consider these seven species as *incertae sedis*. The other four species are *T. adulta*, a parasite of the great grebe (*Podiceps major* Boddaert 1783) from Argentina; *T. brevis*, a parasite of the wood stork (*Mycteria americana* Linnaeus 1758) from Argentina; *T. elongata*, a parasite of the least grebe (*Podiceps dominicus* Linnaeus 1766) from Cuba, Venezuela and Brazil; and *T. americana* a parasite of the wood stork (*M. americana*) and pied-billed grebe (*Podilymbus podiceps*) from Brazil, Venezuela and Mexico. *Tylodelphys aztecae* n. sp. can be differentiated from three of the other species from the Americas (*T. brevis*, *T. elongata* and *T. americana*) by having a spatulate and slightly concave forebody, with a larger ventral sucker and two larger pseudosuckers, and by having a small number of eggs (2–7) in the uterus. However, *T. aztecae* n. sp. resembles *T. adulta* morphologically, in the body shape and the tribocytic organ size, but the new species differs from *T. adulta* in the distribution of the vitelline follicles, in the size of the pseudosuckers and in the number and size of the eggs (table 2).

Molecular characterization and phylogenetic analyses

In this study, sequences of the *cox1* of 55 individuals of *T. aztecae* n. sp. (9 adults and 46 metacercariae) from central and northern Mexico (table 1, fig. 1) were aligned with a *cox1* dataset containing sequences of the genera of diplostomids *Posthodiplostomum, Ornithodiplostomum, Diplostomum* and *Tylodelphys*; and strigeids *Cardiocephaloides*, *Australapatemon*, *Parastrigea* and *Ichthyocotylurus* used as the outgroup. The alignment consisted of 110 sequences with 466 nucleotides. The genetic divergence among the genera of Diplostomatidae *Posthodiplostomum*, *Ornithodiplostomum, Diplostomum* and *Tylodelphys* ranged from 11 to 23%, and among the species of *Diplostomum* (*D. pseudospathaceum* Niewiadomska 1984, *D. baeri* Dubois 1937, *D. mergi* Dubois 1932, *D. huronense* La Rue 1927, *Diplostomum* sp. and *D. spathaceum* Rudolphi 1819) from 10 to 15%. Among species of *Tylodelphys* (*T. mashonense* Beverley-Burton 1963, *T. scheuringi* Hughes 1929, *T. clavata* von Nordmann 1832, *T. excavata* Rudolphi 1803 and *T. aztecae* n. sp.) genetic divergence varied from 12 to 15%, whereas the genetic divergence among the 54 isolates of *T. aztecae* n. sp. was very low and ranged from 0 to 1%.

The ITS sequences of *T. aztecae* n. sp. (1 adult and 18 metacercariae) were aligned with the same genera of Diplostomatidae for which sequences are available, i.e. *Posthodiplostomum, Ornithodiplostomum, Tylodelphys* and *Diplostomum,* and sequences from other genera of strigeids available in GenBank, i.e. *Parastrigea, Apharyngostrigea, Australapatemon* and *Cardiocephaloides*, which were also used as outgroups. The alignment consisted of 63 sequences with 1151 nucleotides. The genetic divergence among the genera *Posthodiplostomum*, *Ornithodiplostomum, Diplostomum* and *Tylodelphys* ranged from 14 to 18%, and among species of *Diplostomum* (*D. baeri, D. mergi, D. huronense, D. pseudospathaceum, D. spathaceum, Diplostomum* sp., *D. indistinctum* and *D. paracaudatum*) from 2 to 3.5%. Meanwhile, among species of *Tylodelphys* (*T. clavata, T. excavata T. mashonense, Tylodelphys* sp. and *T. aztecae* n. sp.) divergence values varied from 3 to 11%, whereas the genetic divergence among the 19 isolates of the new species was very low, from 0 to 0.3 %.

Maximum likelihood (ML) analysis inferred with *cox1* and ITS datasets each yielded a single tree that was very similar in topology to the Bayesian inference (BI) consensus tree (figs 4 and 5). The ML and Bayesian consensus trees inferred with *cox1* (fig. 4) and ITS (fig. 5), showed that all the sequences of *T. aztecae* n. sp. are nested together within a monophyletic clade, with strong bootstrap support and Bayesian posterior probability values (99/1.0 with *cox1* and 100/1.0 with ITS). The *cox1* tree shows the new species as the sister taxon to *T. scheuringi*, for which sequences of the metacercariae are available, and although no information on the morphology of the adults has been published thus far, both species are distributed in the Nearctic biogeographical region. On the other hand, in the ITS tree the new species appears as the sister taxon of an unidentified metacercaria of *Tylodephys* sp. collected from western Siberia (GenBank number KF477191). Unfortunately, no sequences of ITS are available for *T. scheuringi*, and no *cox1* sequences are available for the species from Siberia.

Discussion

The phylogenetic trees obtained in this study show that both *Diplostomum* and *Tylodelphys* are monophyletic, with

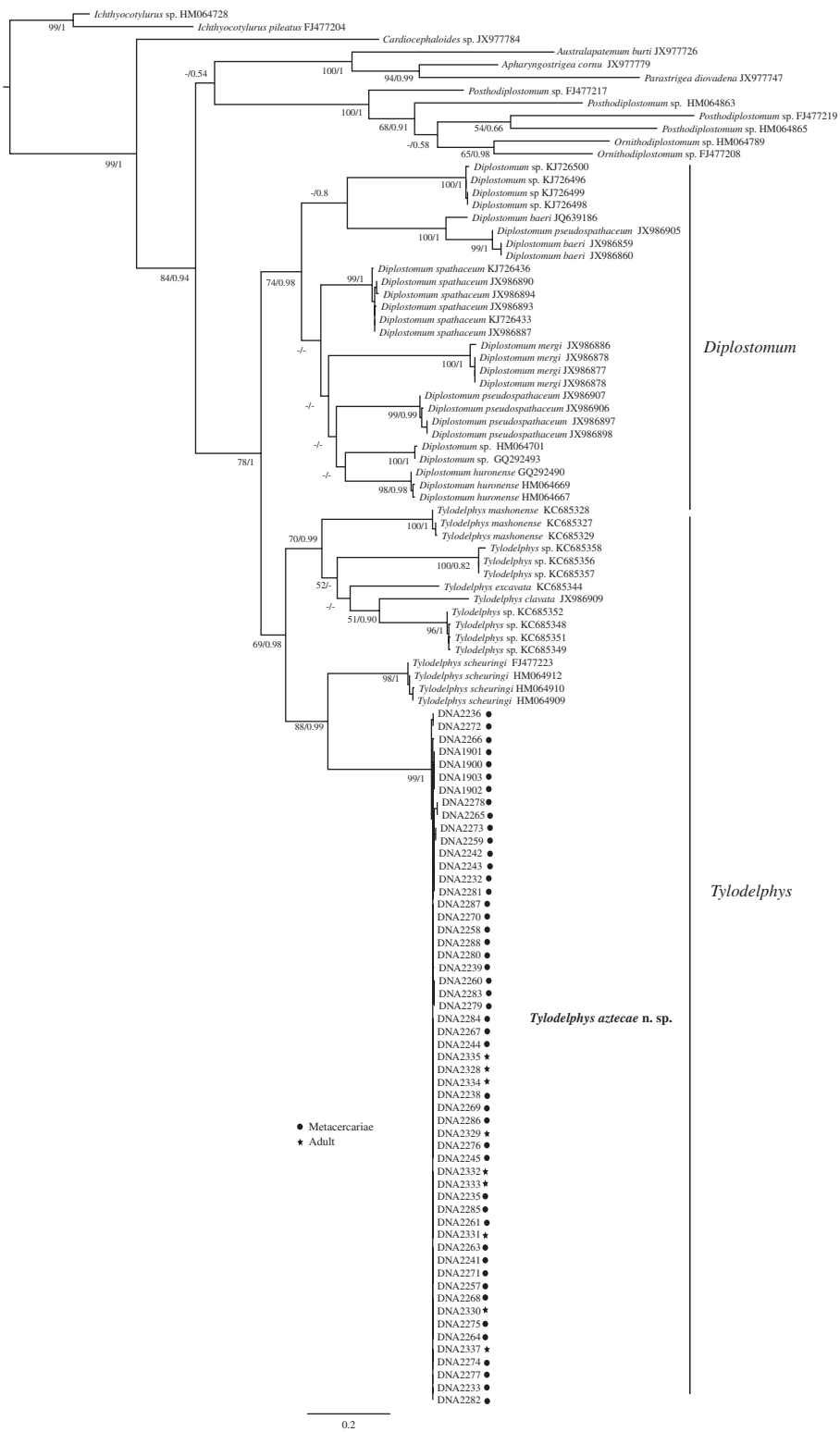

Fig. 4. Maximum likelihood tree and consensus Bayesian inference trees inferred with *cox1* datasets; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

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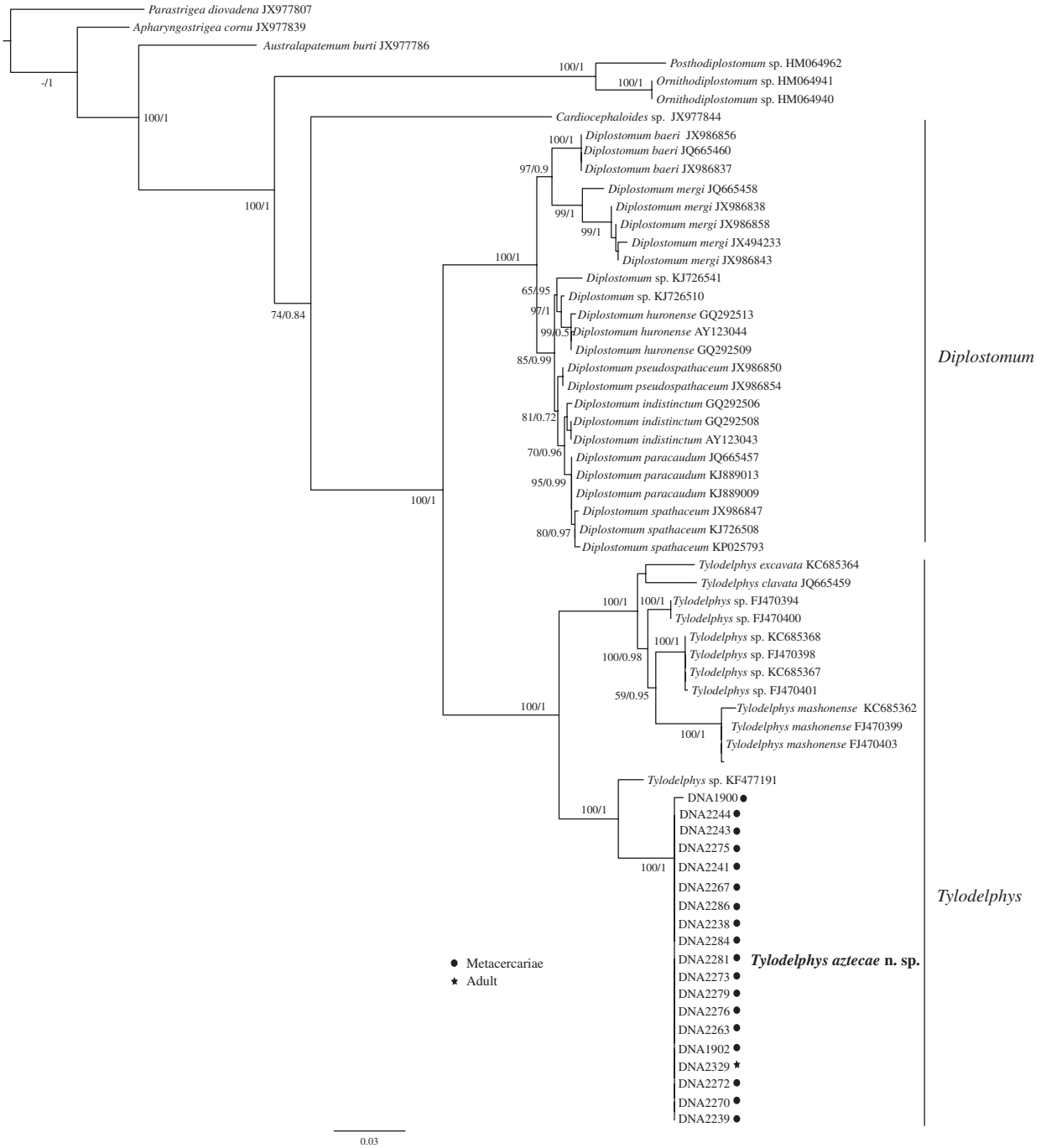

Fig. 5. Maximum likelihood tree and consensus Bayesian inference trees inferred with ITS1, 5.8S and ITS2 datasets; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI).

a relationship strongly supported by bootstrap and posterior probability values. Trees inferred with both molecular markers placed the metacercariae found in the body cavity of goodeids and cyprinids from central and northern Mexico, and the adults from the intestine of the pied-billed grebe in a single clade, confirming that both stages of the life cycle are conspecific. The genetic divergence estimated among 55 individuals of *T. aztecae* n. sp. (9 adults and 46 metacercariae) with the *cox1* dataset was very low, ranging between 0 and 1.0%. These low values of genetic divergence are similar to those found at the intraspecific level in other diplostomatid species. For instance, the genetic divergence among isolates of *Tylodelphys* sp., *T. mashonense, T. excavata* and *T scheuringi*

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ranged from 0 to 1.4%, and among isolates of *Diplostomum mergi, D. pseudospathaceum* and *D. baeri* divergence ranged from 0 to 1.01% (Chibwana *et al.*, 2013, 2015; Georgieva *et al.*, 2013; Otachi *et al.*, 2015; Selback *et al.*, 2015). With respect to ITS1, 5.8S and ITS2, the genetic divergence estimated among the 19 isolates of *T. aztecae* n. sp. (1 adult and 18 metacercariae), was also very low (0–0.3%). These ranges of genetic divergence are also similar to those previously described for congeneric diplostomatids, e.g. *Tylodelphy*s sp. and *T. mashonense* showed a divergence between 0 and 1.4% (Chibwana *et al.*, 2013, 2015), and among isolates of *D. baeri* the divergence ranged from 0 to 0.4% (Blasco-Costa *et al.*, 2014).

Currently, *Tylodelphys* contains 16 described species, which are distributed worldwide. Seven of these species were described solely from metacercariae from the Americas (*T. destructor*, *T. barilochensis*, *T. crubensis*, *T. argentinus T. jenynsiae*, *T. cardiophilus* and *T. scheuringi*) and no adults were included in their descriptions (Hughes, 1929; Drago & Lunaschi, 2008). The four species of *Tylodelphys* adults distributed in the Americas apparently show some level of host specificity. Two species, *T. brevis* and *T. americana,* are parasites of ciconiid and podicipedid birds from Argentina, Brazil, Venezuela and Mexico (León-Règagnón, 1992; Drago & Lunaschi, 2008; Andrade-Rosales, 2012), whereas *T. elongata*, *T. adulta* and *T. aztecae* n. sp. are only found in podicipedids. The distribution pattern of these species shows that three species occur in the Neotropical region (*T. adulta* and *T. brevis* in Argentina, and *T. elongata* in Cuba, Venezuela and Brazil), one species in the Nearctic region (*T. aztecae* n. sp.), and apparently one species possesses a larger distribution range (*T. americana*) since it has been recorded in both biogeographical regions (Brazil, Venezuela and Mexico). The other species distributed in the Americas, *T. scheuringi*, was described from metacercariae from freshwater fishes in North America (Hughes, 1929), and no adults have been obtained to establish a proper species description. Apparently, the metacercariae exhibit very low host specificity, since they have been recorded in more than 20 species of freshwater fishes allocated to 14 families (Margolis & Arthur, 1979; McDonald & Margolis, 1995; Gibson, 1996; Hoffman, 1999). Interestingly, the *cox1* tree shows that *T. scheuringi* is the sister species of *T. aztecae* n. sp. and, considering that the current distribution of the new species extends from central to some areas of northern Mexico, it is plausible to postulate that their relationship is due to the fact that both inhabit birds in the Nearctic biogeographical region. More thorough sampling is required in areas of northern Mexico (where the Nearctic freshwater fish component is very abundant; Miller *et al.*, 2005) to corroborate the possibility that *T. scheuringi* is also found in Mexico.

The taxonomy of metacercariae of diplostomid species found unencysted in the brain and body cavity of freshwater fishes in Mexico has been controversial, since these larval forms have been indistinctly classified either as *Tylodelphys* sp. or *Diplostomum* sp. (Monks *et al.*, 2005; Pérez-Ponce de León *et al.*, 2007; Lira-Guerrero *et al.*, 2008; Martínez-Aquino *et al.*, 2014) and have been found in species allocated to unrelated families such as Cichlidae, Characidae, Heptapteridae, Goodeidae, Atherinopsidae, Poeciliidae and Cyprinidae. Both species conform with the description of a diplostomulum-like larva, and both have a linguiform body, with a hindbody reduced to a small conical prominence, and possess lateral pseudosuckers on the anterior end of forebody (Hoffman, 1999). Interestingly, we discovered that the metacercariae of the new species possess poorly developed, or actually indistinguishable, pseudosuckers; even at the ultrastructural level, no evidence of the structures was found (fig. 2e), which contrasts with their evident presence in the adults (fig. 2b). We looked at all the metacercariae of *Diplostomum* sp. and *Tylodelphys* sp. deposited at the Colección Nacional de Helmintos (CNHE) and confirmed that the development of this structure is variable in the larval forms. Our findings clearly indicate that the taxonomic status of the diplostomulum-like larvae found in freshwater fishes of Mexico needs to be re-evaluated by using a combination of morphological and molecular data, shedding light on the life cycle of these species by linking the metacercariae with the adults found in aquatic birds, which require more intensive sampling to obtain adults, with the potential for discovery of a hidden diversity of this group of trematodes.

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Conflict of interest

None.

Ethical standards

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