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A morphological and molecular study of adults and metacercariae of *Hysteromorpha triloba* (Rudolpi, 1819), Lutz 1931 (Diplostomidae) from the Neotropical region

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Research Paper

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Abstract

Adults of *Hysteromorpha triloba* (Rudolpi, 1819), Lutz, 1931 inhabit primarily the intestine of cormorants across the globe, whereas metacercariae have been found in the body cavity of freshwater fishes of the families Cyprinidae, Ictaluridae, Ariidae, Pimelodidae and Catostomidae. In this study, adults and metacercariae identified as *H. triloba* were collected from the Neotropical cormorant (*Nannopterum brasilianus*) and from the Mexican tetra fish (*Astyanax mexicanus*) from the Gulf of Mexico and Pacific Ocean slopes in the Neotropical region. Partial DNA sequences of the mitochondrial gene cytochrome *c* oxidase subunit I (*cox* 1) and the internal transcribed spacers (ITS1, 5.8S and ITS2) of nuclear ribosomal DNA were generated for both developmental stages, and were compared with available sequences of *H. triloba* from the Nearctic region. The genetic divergence between metacercariae and adults of *H. triloba* from the Neotropical and Nearctic region (Canada) associated with the double-crested cormorant (*Nannopterum auritus*), ranged from 0 to 5.5% for *cox* 1 and from 0 to 0.2% for ITS. Phylogenetic analyses inferred with both molecular markers using maximum likelihood and Bayesian inference placed the adults and metacercariae in a single clade, confirming that both stages are conspecific. Our data confirmed that *H. triloba* is a widely distributed species across the Americas, parasitizing both the Neotropical and Nearctic cormorants in Argentina, Brazil, Venezuela, Mexico, USA and Canada.

Introduction

Members of the family Diplostomidae Poirier 1886, are endoparasites with a worldwide distribution. Recent morphological and molecular studies have contributed to our understanding of the diversity, evolution and host–parasite interactions of this enigmatic group of diplostomatids (see references in Blasco-Costa & Locke, 2017). However, these studies were focused mainly in the Palaearctic region, leaving gaps in the knowledge of this group of parasites in other biogeographical regions, such as in the Neotropics.

The genus *Hysteromorpha* Lutz, 1931 currently contains two species, the type species *Hysteromorpha triloba* (Rudolphi 1819) Lutz, 1931 and *H. plataleae* Dubinin et Dubinina, 1940 from Russia (see Dubois, 1970). As in other diplostomids, the species *H. triloba* parasitizes the intestines of fish-eating birds of the genera *Phalacrocorax* Brisson, *Ardea* L. and *Nyctanassa* L. across the globe (Hugghins, 1954; Dubois, 1970). In the Americas, metacercariae have been reported primarily in cyprinid fishes, but they have also been reported in four other fish families: Ictaluridae, Catostomidae, Ariidae and Pimelodidae (see Pérez-Ponce de León *et al*., 2007; Drago *et al*., 2011; Locke *et al*., 2011; Monteiro *et al*., 2011), and the planorbid snail *Gyralus hirsutus* Gould has been reported as the first intermediate host of *H. triloba* (Hugghins, 1954).

In Mexico, in the Neotropical region, adults and metacercariae of *H. triloba* were recorded at two localities from Pacific Ocean slopes. However, vouchers of those specimens were not deposited in any collection and the report could not be verified (see Pérez-Ponce de León *et al*., 2007). In the current study, specimen adults and metacercariae identified as *H. triloba* from the Neotropical cormorant (*Nannopterum brasilianus* Gmelin) and Mexican tetra fish (*Astyanax mexicanus* De Filippi) were collected from the Gulf of Mexico and Pacific Ocean slopes. The aims of this study were: (1) to characterize molecularly the adults and metacercariae of *H. triloba*; (2) to link the adult and metacercariae using sequences of both internal transcribed spacers plus 5.8S from nuclear ribosomal DNA, and cytochrome *c* oxidase subunit 1 from mitochondrial DNA; (3) to examine the ultrastructure of the body surface of adults and metacercariae using scanning electron microscopy; and (4) to provide a morphological description of both stages.

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Materials and methods

Specimen collection

A total of 61 birds, including 47 Neotropical cormorants (*N. brasilianus*), and 14 double-crested cormorants (*Nannopterum auritus* Lesson) were collected between June 2005 and March 2015 in 25 localities across Mexico. However, in only two localities, one in the Gulf of Mexico (Tlacotalpan, Veracruz, 18°36′0″N, 95°39′0″W) and the other from Pacific Ocean slopes (La Angostura, Chiapas, 16°11′31″N, 92°59′52″W), two Neotropical cormorants were infected with five and 15 mature adults of *H. triloba* respectively. The intestines were placed in separate Petri dishes with 0.75% saline solution and examined under a dissecting microscope. Avian definitive hosts were identified using the field guides of Howell & Webb (1995) and the American Ornithologists' Union (1998). Freshwater fishes from eight families (Atherinopsidae, Cichlidae, Eleotridae, Gobiidae, Heptapteridae, Mugilidae, Godeidae and Poeciliidae) were examined in search of metacercariae of *H. triloba*. However, only the Mexican tetra fish (*A. mexicanus*) from San Luis Potosi (100° 17′24″N, 99°43′12″W), at a tributary from the Panuco River in the Gulf of Mexico, was positive for the infection. Fish were captured by electrofishing, maintained alive and transported to the laboratory, pith sacrificed and examined immediately. Collected digeneans were fixed, by sudden immersion in hot (steaming) 4% formalin, for morphological comparisons. The fish were identified following Miller *et al.* (2005).

Morphological study

Unflattened digeneans preserved in formalin were stained with Mayer's paracarmine, dehydrated in a graded ethanol series, cleared with methyl salicylate and mounted as permanent slides in Canada balsam. Drawings were made with the aid of a drawing tube. Measurements are given in micrometres (μm) followed by the range.

The following measurements were taken from the specimens studied: body total length, forebody length, forebody width, hindbody length, hindbody width, oral sucker length, oral sucker width, ventral sucker length, ventral sucker width, left pseudosucker length, left pseudosucker width, right pseudosucker length, right pseudosucker width, pharynx length, pharynx width, oesophagus length, oesophagus width, holdfast organ length, holdfast organ width, proteolytic gland length, proteolytic gland width, ovary length, ovary width, anterior testis length, anterior testis width, posterior testis length, posterior testis width, egg length and egg width.

Specimen (paragenophore *sensu* Pleijel *et al.*, 2008) adults and metacercariae were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM), Ciudad de México, México, under numbers: 10631 for the adults and 10632 for the metacercariae. A subsample from these isolates was also fixed in 100% ethanol for molecular work.

Amplification, sequencing of DNA, alignments and phylogenetic analyses

A total of 15 specimens identified as *H. triloba*, 5 metacercariae and 10 adults (5 from the Gulf of Mexico and 5 from the Pacific Ocean slopes), 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_2 EDTA (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. The cytochrome *c* oxidase subunit 1 (*cox* 1) of the mitochondrial DNA and the internal transcribed spacers (ITS1 and ITS2 plus 5.8S) from nuclear ribosomal DNA were amplified using the polymerase chain reaction (PCR). A fragment of *cox* 1 was amplified using the forward primer Plat-diploCOX1F, 5′-CGTTTRAATTATACGGATCC-3′ and the reverse primer Plat-diploCOX1R, 5′-AGCATAG TAATMGCAGCAGC-3′ (Moszczynska *et al.*, 2009). The ITS region was amplified using the forward primer D1, 5′-GTCG TAACAAGGTTTCCGTA-3′ and the reverse primer D2, 5′-ATCTAGACCGGACTAGGCTGTG-3′ (Bowles & McManus, 1993). PCR reactions (25 μl) consisted of 2 μl of genomic DNA, 1 μl of each primer (10 pmol), 2.5 μl of 10× buffer, 1.5 μl 2 mM $MgCl_2$, 0.5 μl of a mix of 10 mM deoxyribonucleoside triphosphates (dNTPs) and 1 U of *Taq* DNA polymerase (Platinum Taq, Invitrogen Corporation, São Paulo, Brazil). PCR cycling parameters consisted of denaturation at 94°C for 1 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 two initial and two internal primers for ITS, BD3 5′-GAACATCGACATCTTGAACG-3′ and BD4 5′-ATAAGCC GACCCTCGGC-3′, and two initial primers for *cox* 1 with 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 5.1.5 (Codoncode Corporation, Dedham, Massachusetts, USA). Sequences were deposited in the GenBank database under numbers MG649464–MG649478 for *cox* 1 and MG649479–MG649493 for ITS. *Cox* 1 and ITS sequences obtained in the current research were aligned with sequences available in GenBank of *H. triloba* and of other genera of diplostomids: *Posthodiplostomum* Dubois 1936, *Mesoophorodiplostomum* Dubois 1936, *Ornithodiplostomum* Dubois 1936, *Diplostomum* von Nordmann 1832, *Austrodiplostomum* Szidat & Nani, 1951 and *Tylodelphys* Diesing, 1850; in addition, sequences of the strigeids *Cardiocephaloides* Sudarikov 1959, *Australapatemom burti* Miller 1923, *Parastrigea diovadena* Dubois & Macko, 1972, *Apharyngostrigea cornu* Ciurea 1927 and *Ichthyocotylurus*, Odening 1969 were used as outgroups, because this family is considered as the sister group of Diplostomidae (see Olson *et al.*, 2003; Hernández-Mena *et al.*, 2017). Sequences were aligned using 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). The best-fit nucleotide substitution models inferred with jModeltest (Posada, 2008) were TIM3 + I + G for the *cox* 1 alignment and TVM + G for the ITS alignment. 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 Markov chain Monte Carlo (MCMC) runs for 10 million generations, sampling every 1000 generations, a heating parameter value of 0.2 and a 'burn-in' of 25%. Trees

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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

Morphological study of adults and metacercariae of Hysteromorpha triloba

Eight adult worms identified as *H. triloba* obtained from *N. brasilianus* from La Angostura, Chiapas, were measured and morphologically characterized as follows. Body distinctly bipartite with spatulate shape. Total body length 1068–1333 (1220). Forebody trilobated, concave, 441–616 (558) long by 566–754 (665) wide (figs 1a, 2a). Hindbody subtriangular, 534–731 (657) long by 407–540 (465) wide. Oral sucker small, fairly muscular, subterminal, 72–88 (80) long by 77–95 (86) wide; two well-developed lateral pseudosuckers on each side of oral sucker. Left pseudosucker, 82–127 (105) long by 90–142 (120) wide. Right pseudosucker, 88–108 (98) long by 98–168 (128) wide. Ventral sucker oval, 80–100 (90) long by 90–109 (98) wide, situated immediately anterior to holdfast organ and sometimes covered by it (figs 1a, 2a). Prepharynx absent. Pharynx 47–70 (56) long by 40–51 (45) wide, elongate–oval, muscular. Intestinal bifurcation in anterior quarter of forebody. Caeca long, extending to the posterior end of hindbody. Holdfast organ elongate–oval, covered with numerous tiny spines (fig. 2b), 184–376 (286) long by 248–490 (337) wide. Proteolytic gland typically with bipartite appearance, situated at posterior margin of holdfast organ dorsally, 158–235 (207) long by 162–250 (183) wide. Testes in tandem, located in the anterior region of hindbody. Anterior testis ovoid, 130–194 (172) long by 134–227 (194) wide; posterior testis bilobulated, transversally elongated, 136–369 (201) long by 179–455 (366) wide. Ovary pretesticular, subspherical, 104–179 (140) long by 75–156 (105) wide, contiguous with anterior testis. Vitellarium in fore- and hindbody, beginning from anterior margin of holdfast. Vitelline reservoir intertesticular. Uterus distributed from posterior end of holdfast organ to anterior to the genital pore, which is subterminal. Eggs 77–98 (85) long by 46–63 (55) wide. Genital cone absent.

The following characterization is based on five metacercariae obtained from the Mexican tetra fish, *A. mexicanus*, from San Luis Potosi, Mexico. Body distinctly bipartite (figs 1b, 2c–e). Total body length 641–836 (732). Forebody elongate with ventral concavity, covered with numerous tiny papillae, 453–586 (547) long by 498–532 (517) wide (fig. 2d). Hindbody reduced to small conical prominence, 136–251 (188) long by 209–349 (289) wide. Oral sucker small, subterminal, 56–65 (61) long by 43–56 (52) wide. Two well-developed lateral pseudosuckers on each side of oral sucker (figs 1b, 2e). Left pseudosucker 59–90 (71) long by 45–60 (53) wide. Right pseudosucker 57–88 (70) long by 46–66 (55) wide. Ventral sucker oval, fairly muscular, 47–54 (50) long by 69–76 (72) wide; situated immediately anterior to holdfast organ. Prepharynx absent. Pharynx 42–53 (48) long by 24–35 (55) wide, elongate–oval, muscular. Intestinal bifurcation in anterior quarter of forebody. Caeca long, terminating at posterior level of primordial of testes. Holdfast organ 143–195 (179) long by 134–158 (146) wide, elongate–oval, opening longitudinally, lobulated, located near to posterior margin of

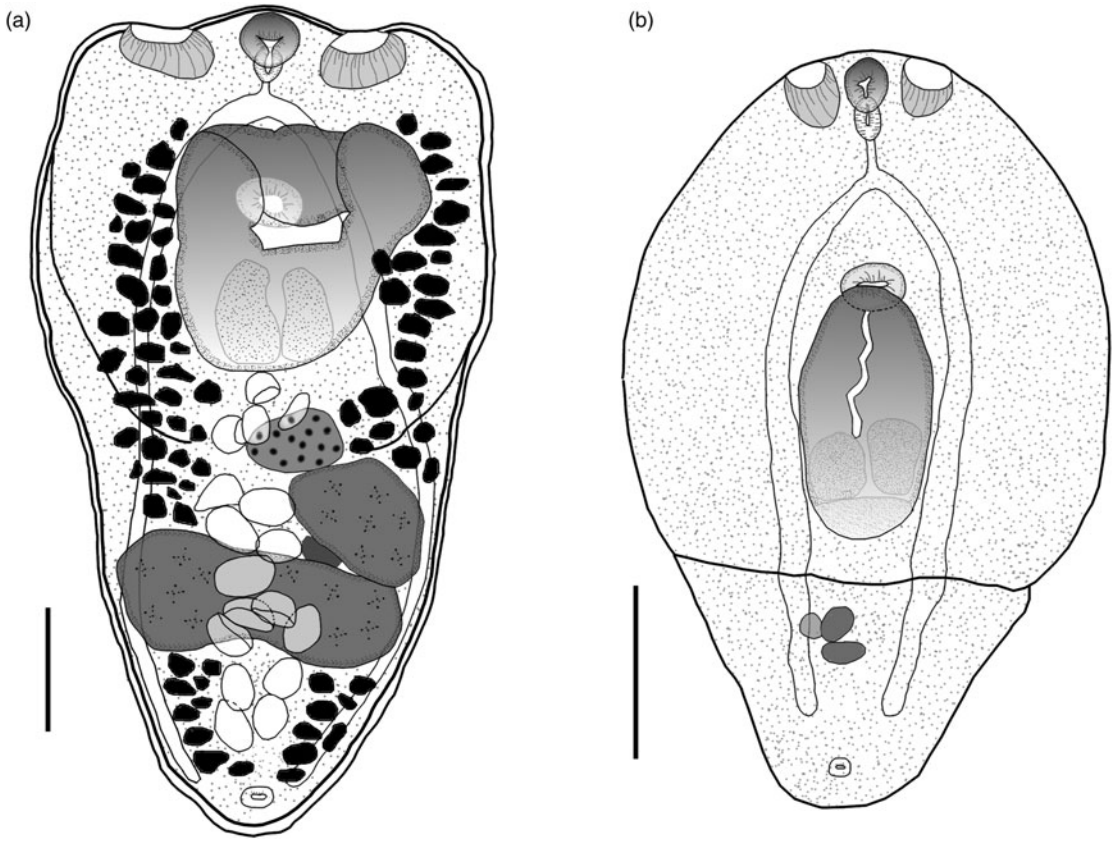

Fig. 1. *Hysteromorpha triloba* (Rudolpi, 1819), Lutz, 1931. (a) Adult obtained from the intestine of *Nannopterum brasilianus*. (b) Metacercaria obtained from the body cavity of *Astyanax mexicanus*. Scale bars: 200 µm.

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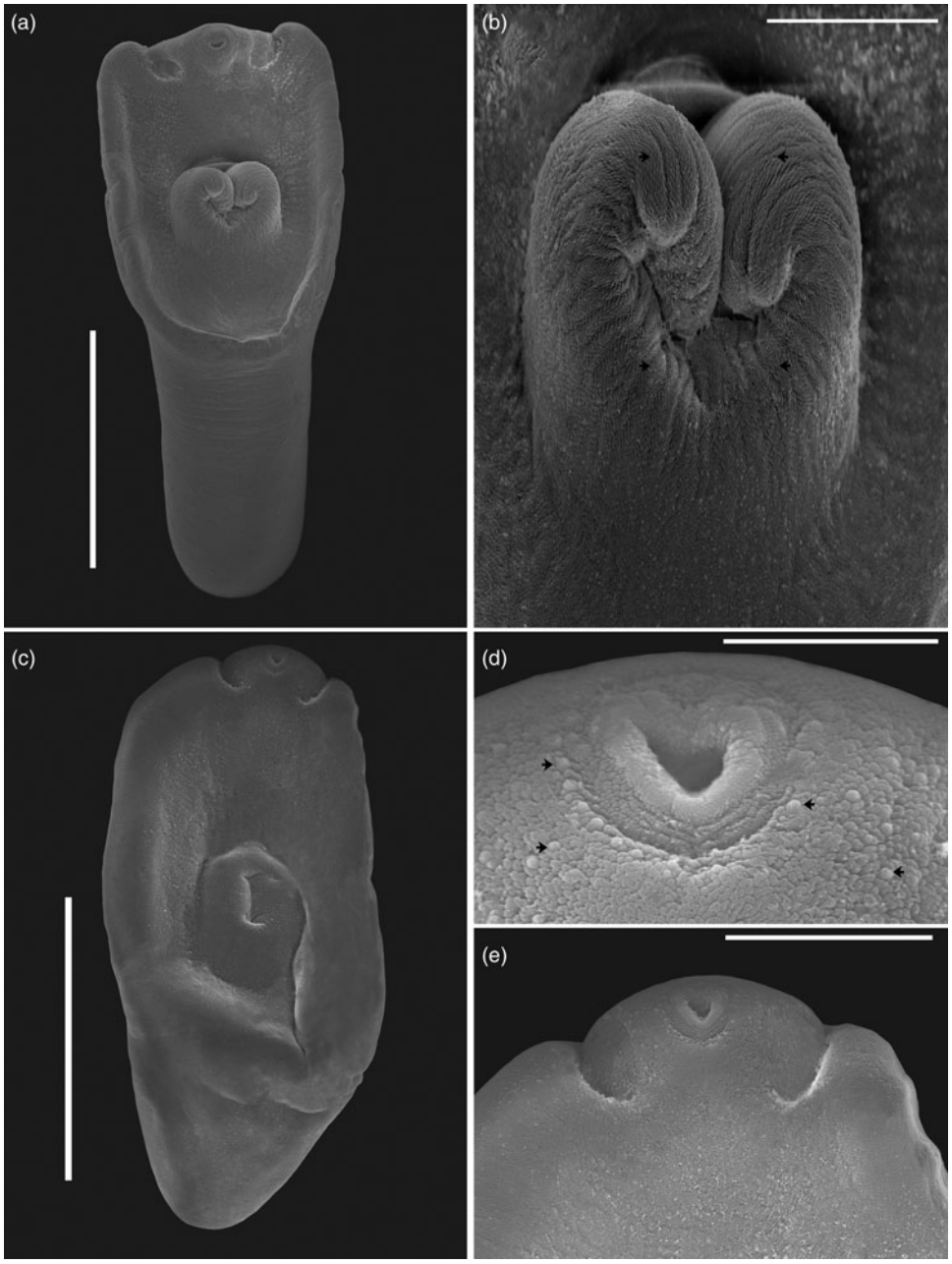

Fig. 2. Scanning electron micrographs of *Hysteromorpha triloba* (Rudolpi, 1819), Lutz, 1931. (a) Adult obtained from the intestine of *Nannopterum brasilianus*; (b) forebody region showing holdfast with spines, shown with black arrows. (c) Metacercaria from the body cavity of *Astyanax mexicanus*; (d) forebody showing the oral sucker recovered with papillae, shown with black arrows; (e) oral sucker and pseudosucker. Scale bars: (a) 400 μm; (b, e) 100 μm; (c) 300 μm; (d) 30 μm.

forebody. Proteolytic gland typically with bipartite appearance, situated at posterior margin of holdfast organ dorsally, 48–71 (54) long by 106–136 (118) wide. Reproductive system poorly developed, with primordia of two oval testes. Anterior testis 27–74 (43) long by 36–75 (59) wide; posterior testis 22–38 (29) long by 32–67 (54) wide. Primordial ovary 29–48 (40) long by 69–86 (77) wide, placed at same level of anterior testis, elongate–oval. Genital pore subterminal, dorsal.

Remarks

Following the original description of Lutz (1931) and subsequent descriptions (Ciurea, 1930; Goss, 1940; Gupta, 1963; Ostrowski de Nuñez, 1970; Dubois, 1970), our specimens collected in two cormorants (*N. brasilianus*) from Mexico possess features that are consistent with the diagnosis of *H. triloba*: a distinctly bipartite body; forebody concave, trilobated; hindbody subtriangular; oral sucker with lateral lobes separated by two pseudosuckers; prepharynx absent; pharynx small; ventral sucker small; holdfast organ lobulated and covered with tiny spines; proteolytic gland bipartite; testes in tandem, anterior testis ovoid, smaller than posterior testis, which is bilobulated and transversally elongated; ovary pretesticular. However, our specimens show some level of morphological intraspecific variation. For instance, some meristic data of newly collected material had lower limits with respect to previous descriptions for the following characters: proteolytic

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gland width (162–250 vs. 180–270), anterior testis length (130–194 vs. 130–385), posterior testis width (179–455 vs. 280–630), ovary width (75–156 vs. 91–250) and egg width (46–63 vs. 48–78). Likewise, the newly collected material had higher limits for posterior testis length (136–369 vs. 80–299). In addition, we provided new measurements for each pseudosucker (see table 1).

Molecular characterization

In this study, *cox* 1 sequences of 15 individuals of *H. triloba* (5 metacercariae and 10 adults) from the Neotropical region were generated and aligned with a *cox* 1 dataset that contained 24 isolates of *H. triloba* from the Nearctic region plus sequences of the following genera of diplostomids: *Posthodiplostomum, Ornithodiplostomum, Diplostomum, Austrodiplostomum* and *Tylodelphys*. The strigeids *Cardiocephaloides*, *Australapatemon*, *Parastrigea, Apharyngostrigea* and *Ichthyocotylurus* were used as outgroups. The alignment consisted of 97 sequences with 466 nucleotides. The genetic divergence among the genera of Diplostomatidae *Posthodiplostomum*, *Ornithodiplostomum, Diplostomum, Tylodelphys* and *Austrodiplostomum* ranged from 11 to 23%, and among species of the same genus ranged from 10 to 15%. The genetic divergence among 15 individuals of *H. triloba* (5 metacercariae and 10 adults) from the Neotropical region

Table 1. Comparative morphometrics (in microns) of adult worms of *Hysteromorpha triloba* (Rudolphi 1819) Lutz, 1931.

Source	Ciurea, 1930	Goss, 1940	Gupta, 1963	Ostwowsky de Nuñez, 1970	Dubois, 1970	This study
Locality	Romania	Australia	India	Argentina		La Angostura, Chiapas, Mexico
Host	*Phalacrocorax carbo*	*P. sulcirostris*	*P. carbo*	*Nannopterum brasilianus*	*N. auritus*	*N. brasilianus*
Body total (L)	780–1910	1230	–	851–1739	2170	1068–1333
Forebody (L)	390–1050	600	876–1170	–	360–1250	441–616
Forebody (W)	–	610	774–1096	–	350–1090	566–754
Hindbody (L)	340–620	630	651–755	–	250–1400	534–731
Hindbody (W)	–	380	581–866	–	220–860	407–540
Oral sucker (L)	77–130	86	66–83	52–104	34–130	72–88
Oral sucker (W)		–	94–117	65–117	42–130	77–95
Ventral sucker (L)	111–117	80	81–92	52–117	60–167	80–100
Ventral sucker (W)		–	125–199	84–156	67–199	90–109
Pseudosucker (L)	55–170	–	–	–	–	–
Left pseudosucker (L)	–	–	–	–	–	82–127
Left pseudosucker (W)	–	–	–	–	–	90–142
Right pseudosucker (L)	–	–	–	–	–	88–108
Right pseudosucker (W)	–	–	–	–	–	98–168
Pharynx (L)	55–88	43–55	58–61	39–65	–	47–70
Pharynx (W)	37–57	–	58–64	26–65	–	40–51
Holdfast organ (L)	220–340	–	290–418	260–585	180–418	184–376
Holdfast organ (W)	170–380	–	351–651	208–325	170–651	248–490
Proteolytic gland (L)	150–300	–		–	150–300	158–235
Proteolytic gland (W)	210–270	–	–	–	180–270	162–250
Anterior testis (L)	–	210–160	246–385	130–260	120–300	130–194
Anterior testis (W)	–	–	213–250	156–338	120–450	134–227
Posterior testis (L)	80–240	340–170	190–241	117–299	80–330	136–369
Posterior testis (W)	280–490	–	432–617	299–520	280–630	179–455
Ovary (L)	40–120	160	81–102	65–130	40–176	104–179
Ovary (W)	100–240	100	149–215	91–208	100–250	75–156
Eggs (L)	97–99	86	79–96	91–104	75–99	77–98
Eggs (W)	55–62	85	60–71	52–78	48–75	46–63

L, Length; W, width.

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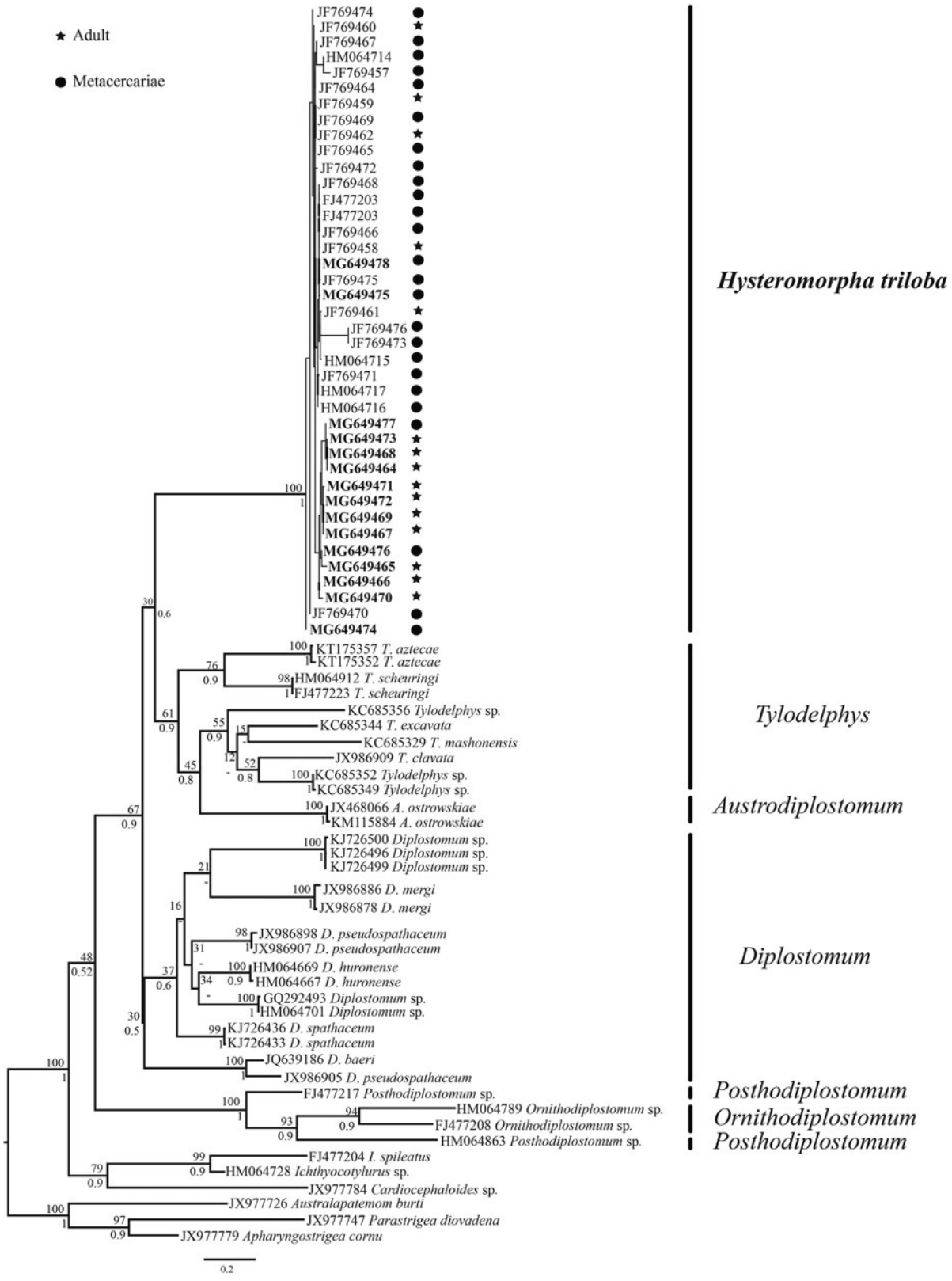

Fig. 3. Maximum likelihood tree inferred with the *cox* 1 dataset; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI). The GenBank accession numbers in bold were generated in this study.

ranged from 0 to 2.5%, whereas the genetic divergence among the 24 isolates of *H. triloba* from the Nearctic region ranged from 0 to 4.7%. Finally, the genetic divergence among 39 individual of *H. triloba* ranged from 0 to 5.5%. Maximum likelihood (ML) analysis yielded a single tree that was identical in topology to the Bayesian inference (BI) consensus tree (fig. 3). The ML and Bayesian consensus trees showed that all the sequences of *H. triloba* generated in this study (10 adults and 5 metacercariae) are nested within a

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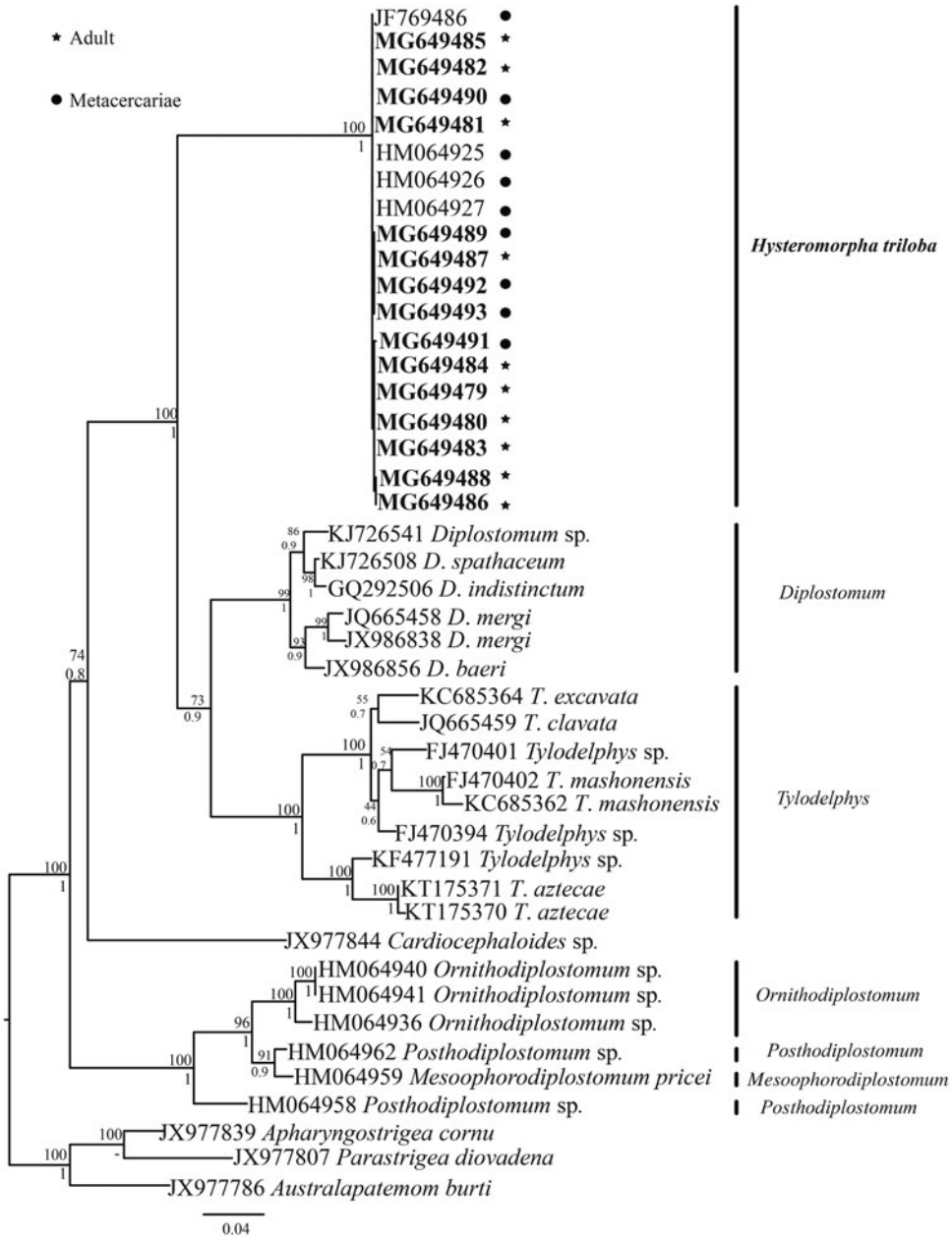

Fig. 4. Maximum likelihood tree inferred with the ITS1, 5.8S and ITS2 dataset; numbers near internal nodes show ML bootstrap clade frequencies and posterior probabilities (BI). The GenBank accession numbers in bold were generated in this study.

monophyletic clade, with strong bootstrap support and Bayesian posterior probability values (100/1.0). This clade also included the sequence of *H. triloba* from the double-crested cormorant from the Nearctic region (fig. 3).

The ITS sequences of *H. triloba* (10 adults and 5 metacercariae) were aligned with four isolates of *H. triloba* from the Nearctic region and with sequences of other genera of Diplostomatidae. The alignment consisted of 44 sequences with 1138 nucleotides. The genetic divergence among the genera *Posthodiplostomum*, *Ornithodiplostomum, Diplostomum, Hysteromorpha* and *Tylodelphys* ranged from 14 to 18%, and among congeneric species of *Diplostomum* and *Tylodelphys* from 3 to 11%. In comparison, the genetic divergence among the 19 isolates of *H. triloba* was very low, from 0 to 0.2%. Maximum likelihood (ML) analysis yielded a single tree that was identical in topology to the Bayesian inference (BI) consensus tree (fig. 4). The ML and Bayesian consensus trees showed that all the sequences of *H. triloba* generated in this study (10 adults and 5 metacercariae) are nested within a clade, with strong bootstrap support and Bayesian posterior probability values (100/1.0). This clade also included four sequences (JF769486, HM064925–927) of *H. triloba* from the double-crested cormorant from the Nearctic region (fig. 4).

Discussion

The phylogenetic trees obtained with both molecular markers placed the metacercariae found in the body cavity from the

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Mexican tetra fish and the adults from the Neotropical cormorant in a single clade, confirming that both stages of the life cycle are conspecific. The genetic divergence estimated among 15 individuals of *H. triloba* from the Neotropical region (10 adults and 5 metacercariae) with the *cox* 1 dataset was very low, ranging from 0 to 2.5%, and among specimens of *H. triloba* from the Nearctic region ranging from 0 to 5.5%. These values of genetic divergence are higher than those found at the intraspecific level in other diplostomatid species. For instance, the genetic divergence among isolates of *Tylodelphys* sp., *T. mashonense, T. excavata, T. aztecae* and *T. scheuringi* ranged from 0 to 1.4% (Chibwana *et al*., 2013, 2015; Otachi *et al*., 2015; García-Varela *et al*., 2016; Blasco-Costa *et al*., 2017), and among isolates of *Diplostomum mergi, D. pseudospathaceum* and *D. baeri* from 0 to 1.01% (Georgieva *et al*., 2013; Selback *et al*., 2015). Finally, genetic divergence among isolates of *Uvulifer spinatus* ranged from 0 to 1.8% (López-Jimenez *et al*., 2017). With respect to ITS1, 5.8S and ITS2, the genetic divergence estimated among the 19 isolates (5 metacercariae, 10 adults from the Neotropical region plus 4 isolates from the Nearctic region) of *H. triloba* was very low, ranging from 0 to 0.2%. This range of genetic divergence is similar to those described previously for congeneric diplostomatids. For example, genetic divergence ranged from 0 to 1.4% between *Tylodelphy*s sp. and *T. mashonense* (see Chibwana *et al*., 2013, 2015), from 0 to 0.03% among specimens of *T. aztecae* (García-Varela *et al*., 2016), from 0.2 to 1.2% among isolates of *Tylodelphys* spp. (Blasco-Costa *et al*., 2017) and from 0 to 0.4% among isolates of *D. baeri* (see Blasco-Costa *et al*., 2014). Finally, among isolates of *Uvulifer spinatus* genetic divergence ranged from 0 to 1.4% (López-Jimenez *et al*., 2017).

Currently, *Hysteromorpha* is a genus distributed across the globe as parasite of fish-eating birds (see Hugghins, 1954; Dubois, 1970; Ostrowski de Nuñez, 1970). The taxonomic history of the type species (*H. triloba*) has been unstable since its erection. For instance, it was described as *Distomum trilobum*, and was later transferred to the genera *Hemistomum* and *Proalaria* (see Dubois, 1970). Finally, Lutz (1931) evaluated morphological characters based on its life cycle and transferred it to the new genus *Hysteromorpha*. Since then, *H. triloba* has been recorded in several countries, such as Argentina, Brazil, Venezuela, Mexico, USA and Canada in the Americas, associated with the Neotropical cormorant *N. brasilianus* and the double-crested cormorant *N. auritus*, suggesting that *H. triloba* can be regarded as a member of the 'core' helminth fauna of these two fish-eating bird species (Fedynich *et al*., 1997; Drago *et al*., 2011; Locke *et al*., 2011; Monteiro *et al*., 2011; O´Hear *et al*., 2014; Sheehan *et al*., 2016). In contrast, the metacercaria of *H. triloba* exhibits low host specificity, since it has been recorded in at least eight species of freshwater fishes from unrelated families, such as Cyprinidae, Characidae, Catostomidae, Ictaluridae, Ariidae and Pimelodidae (see Pérez-Ponce de León *et al*., 2007; Locke *et al*., 2011).

Blasco-Costa & Locke (2017) pointed out the great progress that has been made in recent years in the understanding of the diversity and evolution of diplostomatids, mainly in the Palaearctic region. This progress has left a big gap in the knowledge of this enigmatic group of parasites in the Neotropical region. Therefore, the current study contributes to our understanding of the genetic diversity, host–parasite interactions and life cycle of *H. triloba* in this biogeographical region.

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