

## Research Article

**Metacercariae of *Haplorchis pumilio* (Looss, 1896) in *Carassius auratus* (Linnaeus, 1758) from Mérida City, Yucatán, Mexico: a co-introduced parasite**

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**OPEN ACCESS****Abstract**

The invasive alien species (IAS) and adult trematode parasite of reptiles, birds and mammals (including humans), *Haplorchis pumilio* (Looss, 1896), is recorded for the first time from Yucatán state in Mexico. The ornamental freshwater fish species, *Carassius auratus* (“goldfish”) from pet shops from Mérida City, were necropsied to search for parasites. The trematode was identified based on molecular and morphological data of metacercariae collected from seven out of ten pet shops sampled. Partial sequences of the 28S ribosomal and COI mitochondrial genes corroborate the identifications based on morphological characters. DNA sequences from GenBank showed low genetic divergence in both genes from *H. pumilio* associated with three different host groups during larval and adult stages around the world, e.g., cercariae from *Melanoides tuberculata* (“red-rimmed melania”), from *Trichogaster trichopterus* (“three spot gourami”) and trematode adults from *Homo sapiens*. We infer that this trematode was likely introduced by accident through pet shop commerce together with the complex of the IASs, *M. tuberculata* and *C. auratus*. As Yucatán is the second major ornamental fish producing state in Mexico, accidental release of these IASs into the aquatic environment could result in the introduction of *H. pumilio* into regional freshwater bodies. The introduction of *H. pumilio* in Yucatán is probably relatively recent and due to the paucity of studies directed at this parasite, its distribution is not well known, but since its potential pathogenicity can cause an emergence of diseases, not only in wildlife, but also in local human populations, particular attention should be given to the findings of this study.

**Key words:** COI, emergence of infectious diseases, parasites of ornamental fishes, 28S

**Introduction**

The trematode parasite *Haplorchis pumilio* (Looss, 1896) is an invasive alien species (IAS) in Mexico, and part of a complex of IASs that form an ecological web in freshwater environments, because their first intermediate

hosts (e.g., the snails *Melanooides tuberculata* (Müller, 1774) and *Tarebia granifera* (Lamarck, 1822)) are globally distributed IAS (Huston et al. 2014; Albrecht et al. 2018). The larval stages of *H. pumilio* (i.e., cercariae as parasites of snails, and metacercariae as parasites of freshwater fishes) are distributed in many hydrological systems of the world, e.g., Africa, Australia, China, India, Mexico, Palestine, USA and Venezuela (Huston et al. 2014; Krailas et al. 2014; Dechruksa et al. 2017). Furthermore, *H. pumilio* is recorded as an adult parasite in reptiles, birds and mammals, including humans (Hung et al. 2013, 2015; Chai and Jung 2017). The wide geographic distribution of *H. pumilio*, associated with the global distribution of their first intermediate hosts, is ecological evidence to consider the parasite as a threat to human health (Arya et al. 2016). Their highly invasive nature as an opportunistic parasite, that is likely to keep moving northward as a result of climate change, means that they constitute a threat to the biodiversity of aquatic wildlife such as endemic freshwater fish species.

To date, the metacercariae of *H. pumilio*, only considering the south of Mexico, were recorded as parasites of seven freshwater fish species of seven genera included in three families, i.e., Cichlidae: *Cichlasoma salvini* (Günther), *Parachromis managuensis* (Günther) (as *Cichlasoma managuense*), *Petenia splendida* (Günther) and *Thorichthys helleri* (Steindachner) (as *Cichlasoma helleri*); Eleotridae: *Dormitator latifrons* (Richardson) and *Gobiomorus maculatus* (Günther, 1859), and Poeciliidae: *Poecilia mexicana* (Steindachner) (Scholz et al. 2001; for hosts synonyms see Miller et al. 2005). Based on these records, *H. pumilio* was included in the list of invasive aquatic species of Mexico (Salgado-Maldonado and Rubio-Godoy 2014). It is possible that the records of *H. pumilio* in Mexican endemic freshwater fish species are related to the presence of *M. tuberculata* (Scholz and Salgado-Maldonado 2000; Scholz et al. 2001; Salgado-Maldonado and Rubio-Godoy 2014). *Haplorchis pumilio* could possibly be found in *T. granifera* as well (e.g., Huston et al. 2014), since this snail has been recorded in Mexican hydrological systems (Naranjo-García and Olivera-Carrasco 2014), albeit to date, parasitological records from *T. granifera* from Mexico are lacking.

*Carassius auratus* (Linnaeus, 1758), commonly known as the “goldfish”, is an ornamental freshwater fish, largely cultured throughout the world. Due to its cosmopolitan distribution caused by accidental anthropogenic activities, this species is considered an IAS (Copp et al. 2005; Iriarte et al. 2005; Savini et al. 2010; Papavlasopoulou et al. 2014). Because of the management practices such as handling, crowding, transport and poor water quality that provoke stress to fish, several ornamental fish species—such as goldfish—are rendered susceptible to a variety of parasites (Paredes-Trujillo et al. 2016; Smit et al. 2017; Soler-Jiménez et al. 2017). Furthermore, the transmission of parasite species from ornamental fishes to wild freshwater fishes has been recorded in Mexico (Salgado-Maldonado and Rubio-Godoy 2014). The state of Yucatán is the region with the second

highest ornamental fish production in Mexico (Ramírez-Martínez et al. 2010; SAGARPA 2015). However, there are still no published data on the helminth parasites of *C. auratus* from Yucatán, and only five Mexican political regions present helminthological records published for the goldfish, i.e., Durango, Estado de México, Guanajuato, Hidalgo and Morelos (Flores-Crespo and Flores-Crespo 2003; Salgado-Maldonado 2006; Caspeta-Mandujano et al. 2009; Pérez-Ponce de León et al. 2007, 2010). These records originate from established feral populations and aquaculture facilities, rather than from pet shop fish samples based on molecular taxonomic identification.

Molecular markers are a practical tool to identify IAS and their associated emergent diseases, caused by parasite organisms from different regions of the world (Mattiucci et al. 2017; Sures et al. 2017). For example, nuclear 28S rDNA and the mitochondrial cytochrome *c* oxidase subunit I (COI) gene fragments were used for phylogenetic-based identification of heterophyid parasites for the diagnosis of trematode parasites in human and animal infections (Arya et al. 2016; Dao et al. 2016; Le et al. 2017).

To explore the introduction of IAS, in a parasite ecology context, from ornamental fishes and supporting the development of the sanitary aspects of aquaculture in Mexico (Vidal-Martínez 2012; FAO 2016a, b; Soler-Jiménez et al. 2017), the aims of this study were: 1) to search for *H. pumilio* from *C. auratus* collected from pet shops from Mérida City, Yucatán, Mexico (MCY); 2) to apply molecular phylogenetic tools for species identification, and measure the intraspecific genetic differentiation of *H. pumilio*; 3) to provide the infection parameters proposed by Bush et al. (1997) (i.e., prevalence (percent infected) and mean infection intensity (mean number of parasites per infected fish)) from pet shops.

## Materials and methods

### *Collection of hosts and metacercariae from pet shops*

As part of a study to explore the diversity of parasites of the freshwater aquarium fish *C. auratus* (VM Vidal-Martínez, *unpublished data*), between October and April 2015–2016, 210 specimens of this host were examined to detect the presence of the metacercaria of *H. pumilio*. Twenty-one goldfish were sampled from each of ten pet shops (Table 1). Fishes were taken alive to the laboratory of Aquatic Pathology in the Centro de Investigación y de Estudios Avanzados (CINVESTAV), Unidad Mérida, euthanised via cranial pithing and examined individually for ecto and endohelminths. The intestines, eyes, gall bladder, liver, spleen, swim and urinary bladders, as well body cavities and musculature were examined under a stereomicroscope in separate Petri dishes with 0.65% saline. The metacercariae were excysted before being preserved for molecular and morphological analyses. Metacercaria specimens were fixed in both 4% hot

**Table 1.** Pet shops studied for *Carassius auratus*, intermediate host of *Haplorchis pumilio* from Mérida City, Yucatán, Mexico. PS# = Pet shop number; NF = Total number of fishes *C. auratus* examined for this parasitological study per pet shop; NP = Total number of parasites *H. pumilio* collected per pet shop; % = Prevalence; MI = Mean intensity.

PS#	NF	NP	%	MI
1	21	0	–	–
2	21	17	10	8.50
3	21	0	–	–
4	21	3	10	1.50
5	21	25	52	2.27
6	21	7	14	2.33
7	21	15	43	1.67
8	21	4	19	1
9	21	0	–	–
10	21	1	5	1

formalin for morphological identification and 100% ethanol for molecular study. Metacercaria specimens were stained with Mayer's paracarmine and mounted as permanent slides using Canada balsam. Metacercariae were identified based on morphological criteria following Scholz et al. (2001), Vidal-Martínez et al. (2001), Umadevi and Madhavi (2006) and Díaz et al. (2008). Specimens of unencysted metacercariae were measured, and drawings were made with the aid of a drawing tube attached to an Olympus BX50 microscope. Morphological attributes were measured and presented in micrometres ( $\mu\text{m}$ ) as ranges followed by the means in parentheses. Microphotographs of both encysted and unencysted metacercariae were obtained (Supplementary data Figures S1A and S1B). Specimens collected for morphological analysis were deposited as voucher specimens in the Colección Helmintológica del CINVESTAV-Unidad Mérida (CHCM), with voucher numbers 551–554.

#### *Species identification based on molecular analyses*

Metacercariae fixed in 100% ethanol were digested with Proteinase K, and DNA extraction was performed using the DNeasy blood and tissue extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The 28S ribosomal gene region was amplified by Polymerase Chain Reaction (PCR) (Saiki et al. 1988) using 28sl fwd (5'-AAC AGT GCG TGA AAC CGC TC-3') (Palumbi 1996) and LO rev (5'-GCT ATC CTG AG (AG) GAA ACT TCG-3') (Tkach et al. 2000). The primers JB3 fwd (5'-TTT TTT GGG CAT CCT GAG GTT TAT-3') (Morgan and Blair 1998) and CO1R tremata rev (5'-CAA CAA ATC ATG ATG CAA AAG G-3') (Miura et al. 2005) were used for the COI fragment. We also obtained sequences of COI fragment from an adult specimen of *Crassicutis cichlasomae* Manter, 1936 (Apocreadiidae), from a sample previously collected (Martínez-Aquino et al. 2017) to use as an outgroup taxon for the phylogenetic analyses in this study, based on its previously established sister group relationship of Ophisthorchioidea (Bray et al. 2009; Fraija-Fernández et al. 2015). The PCR reactions were prepared using the Green

GoTaq Master Mix (Promega) and the cycling was carried out in an Axygen Maxygen thermocycler under the following conditions: for COI, an initial denaturing step of three min at 95 °C, followed by 35 cycles of 95 °C for one min, 50 °C for one min, and 72 °C for one min, and a final extension step at 72 °C for ten min; for 28S, an initial denaturing step of two min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for two min, and a final extension step at 72 °C for five min. The PCR products were visualized by electrophoresis in 1% agarose gel using TAE 1X buffer and observed under UV light using the QIAxcel® Advanced System. The purification and sequencing of the PCR products were carried out by Genewiz, South Plainfield, NJ, USA (<https://www.genewiz.com/>).

To obtain the bi-directional consensus sequences of *H. pumilio* and *C. cichlasomae*, we edited the chromatograms of the forward and reverse sequences and assembled them using the platform Geneious Pro v5.1.7 (Drummond et al. 2010). The 28S and COI sequences that were generated during this study were aligned with sequences of heterophyids and opisthorchiids following the dataset of Martínez-Aquino et al. (2017), using an interface available in MAFFT v.7.263 (Katoh and Standley 2016), an “auto” strategy and a gap-opening penalty of 1.53 with Geneious Pro, and a final edition by eye in the same platform.

The Gblocks Website v.0.91b (Castresana 2000; Talavera and Castresana 2007) was used to detect ambiguously aligned hypervariable regions in the 28S dataset, which were excluded from posterior analyses in MrBayes v.3.2.3 (Ronquist et al. 2012) (see Miller and Cribb 2009; Martínez-Aquino et al. 2017). To evaluate the utility of the molecular markers chosen for phylogenetic analyses at the taxonomic level intended (family level for the complete-outgroup dataset and genus level for the heterophyd/opisthorchiid dataset), we tested the nucleotide composition homogeneity within each data alignment (28S and COI matrix data), using chi-squared metric provided in the program TreePuzzle 5.3.rc (Schmidt et al. 2002).

The software Partition Finder v.1.1.1 (Lanfear et al. 2012, 2014) was used to select evolution models through the Bayesian Information Criterion (BIC) (Schwarz 1978) for each dataset separately (28S and COI). The nucleotide substitution model that best fit 28S was TVM+I+G (Posada 2003). The COI dataset was partitioned into first-, second- and third-codon positions with the appropriate nucleotide substitution model implemented for each codon position (TrN+G for the first (Tamura and Nei 1993); K81uf+G for the second (Kimura 1981); and GTR+G for the third codon position (Lanave et al. 1984)). Genetic divergence estimations for the 28S fragment of *H. pumilio* sequences, generated in this study plus those available on GenBank, were performed using *p*-value with variance estimation, with the bootstrap method (500 replicates) and with a uniform nucleotide substitution (transitions + transversions) rate, in MEGA v.7.0 (Kumar et al. 2016).

The phylogenetic trees were reconstructed for each gene separately using Bayesian Inference (BI) in MrBayes v.3.2.3 (Ronquist et al. 2012), using two parallel analyses of  $MC^3$  for  $20 \times 10^6$  generations each. Topologies were sampled every 1,000 generations and the average standard deviation of split frequencies was observed to be less than 0.01, as suggested by Ronquist et al. (2012). A majority consensus tree with branch lengths was reconstructed for the two runs after discarding the first 5,000 sampled trees. The robustness of the clades was assessed using Bayesian Posterior Probability (PP), where  $PP > 0.95$  was considered strongly supported. The Bayesian phylogenetic reconstructions were run through the CIPRES Science Gateway v.3.3 (Miller et al. 2010). The 28S and COI sequences were deposited in GenBank (accession numbers MN106786–MN106794).

## Results

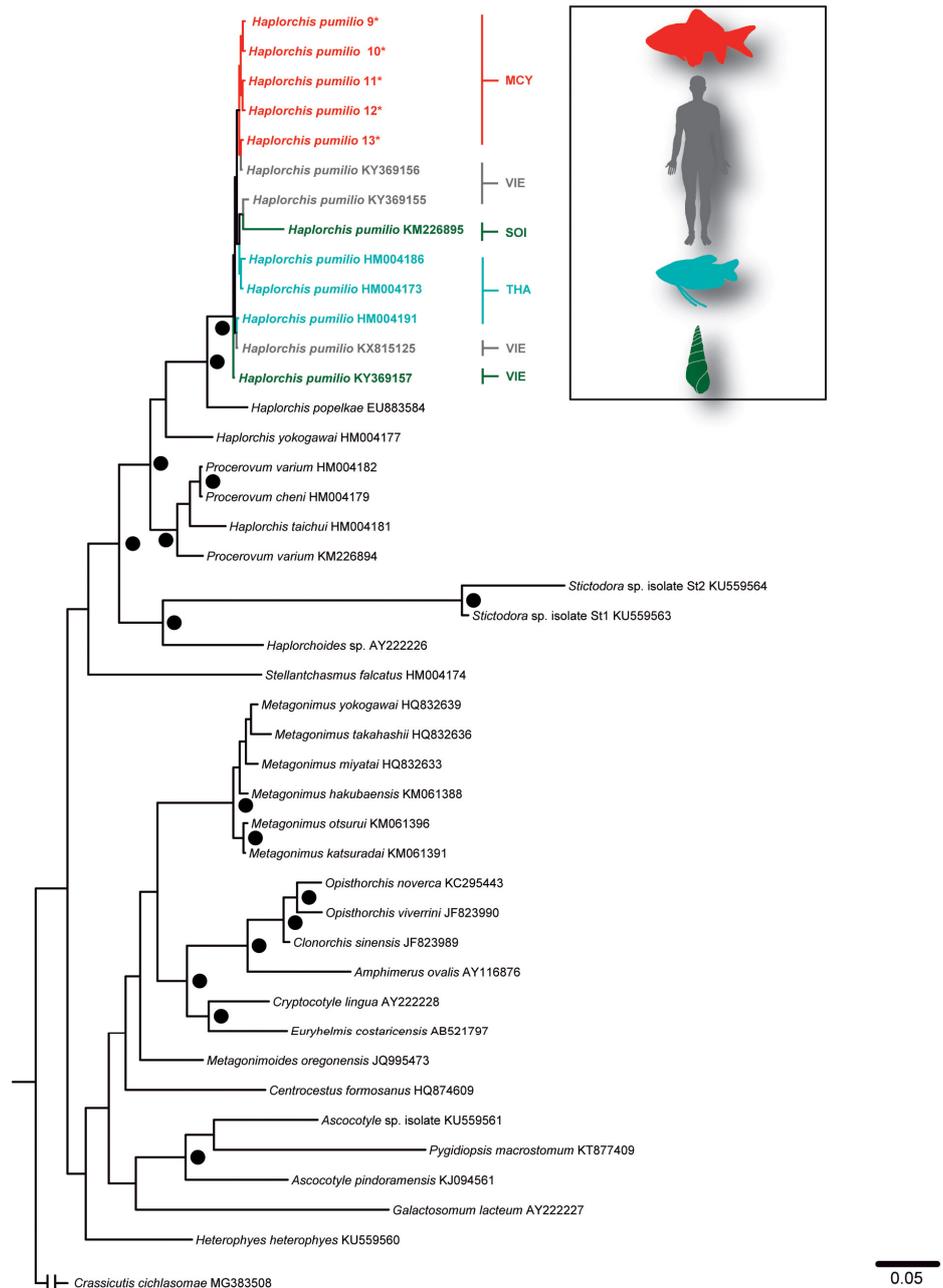
### *Parasitic infection parameters*

Seventy-two metacercariae of *H. pumilio* were recovered encysted in the caudal fins of 32 out of 210 *C. auratus* revised. The infected hosts came from seven out of ten pet shops. Prevalence and mean intensity for each pet shop are shown in Table 1. Pet shop number five had the highest prevalence of metacercariae recorded, accounting for about 52% of the parasites in all stores, while in pet shop number ten, each individual was infected with at least one metacercaria and in pet shops one, three and nine, no *H. pumilio* were found. The total number of individuals of *H. pumilio* per host varied from one to 14.

### *Species identification based on molecular analysis*

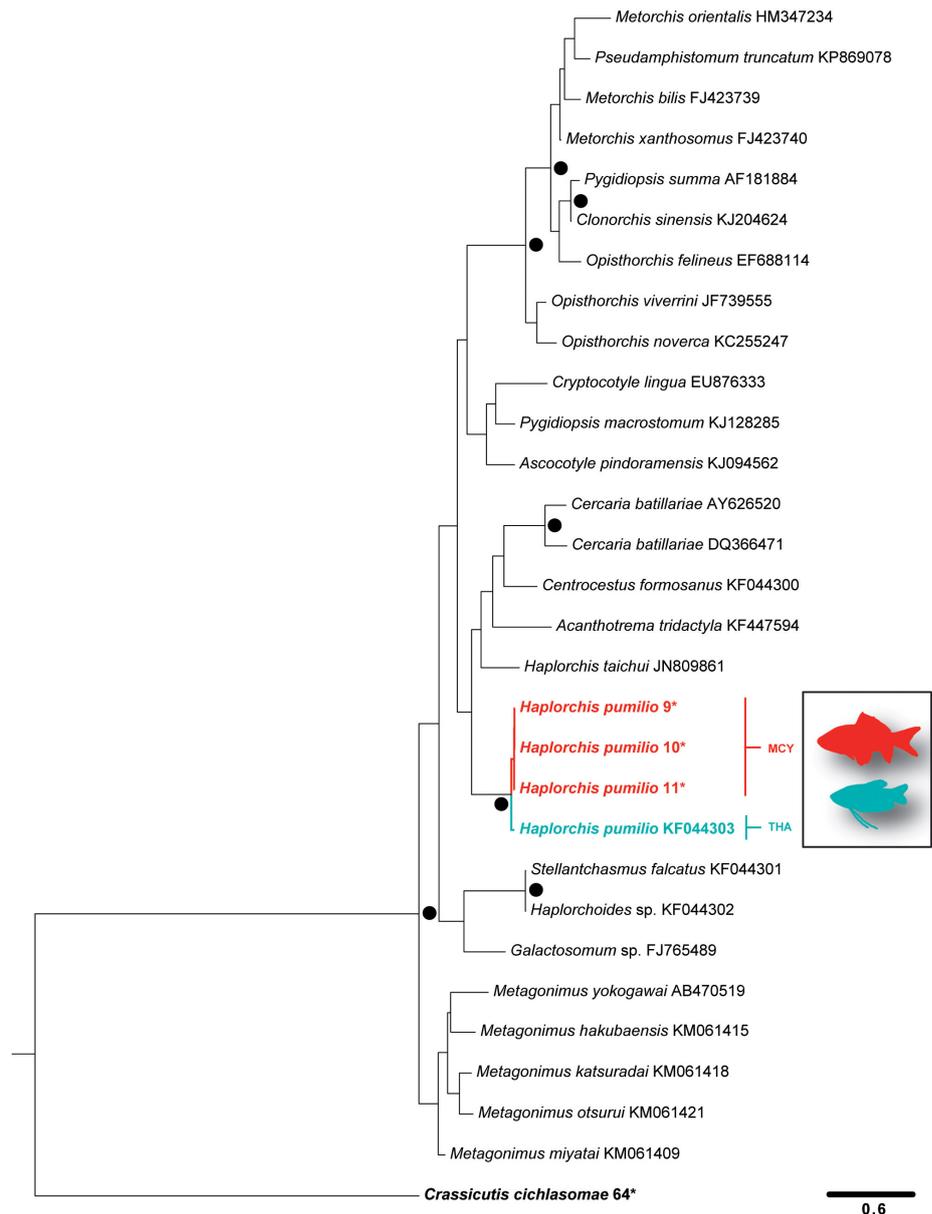
A total of eight assembled sequences were obtained: five for 28S from five individual metacercariae of *H. pumilio*, and only three of the same five individuals for COI (see Supplementary data Tables S1 and S2). The 28S ribosomal sequence fragment for three individuals consisted of 790, 875, 898, and for two individuals of 899 base-pairs (bp) and did not show genetic divergence. Nucleotide sequence variation in the 28S alignment from Heterophyidae/Opisthorchiidae (excluding the outgroup taxon) included 779 conserved sites, 457 variable sites, 304 parsimony-informative sites, and 153 singleton sites. The COI dataset consisted of one sequence of 366 bp and two sequences of 381 bp and did not show genetic divergence. Nucleotide sequence variation (excluding the outgroup taxa) for each partition from COI (first, second and third codon positions) was 77/108/5 conserved, 50/19/122 variable, 36/7/115 parsimony-informative and 14/12/7 single sites, respectively.

Phylogenetic relationships were inferred from two datasets; i.e., 28S and COI sequence matrices separately. The 28S dataset contained 43 sequences (representing 31 taxa), and the COI dataset contained 29 sequences



**Figure 1.** Phylogenetic tree obtained through Bayesian inference for the 28S rRNA dataset. The scale bar represents the number of nucleotide substitutions per site. GenBank accession numbers of the new sequences of *Haplorchis pumilio* are indicating with asterisk (\*). Filled circles below branches and at the nodes represent Bayesian Posterior Probability  $\geq 0.95$ . MCY = Mérida City Yucatán, Mexico; SOI = South of India; THA = Thailand; VIE = Vietnam. The red fish outline represents *Carassius auratus*. The grey human outline represents *Homo sapiens*. The blue fish outline represents *Trichogaster trichopterus*. The green snail outline represents *Melanoides tuberculata*.

(representing 25 taxa). Figures 1 and 2 show the phylogenetic topologies resulting from the 28S and COI datasets analysed, respectively. In the 28S tree, the sequences generated in this study form a monophyletic group with high nodal support values (PP  $\geq 0.95$ ) (Figure 1), together with the eight *H. pumilio* specimens previously identified from freshwater aquarium fishes, *Trichogaster trichopterus* (Pallas) (metacercariae sequences: HM004173, HM004186, HM004191), from Thailand (Thaenkham et al. 2010); cercariae



**Figure 2.** Phylogenetic tree obtained through Bayesian inference for the COI dataset. The scale bar represents the number of nucleotide substitutions per site. GenBank accession numbers of the new sequences are indicating with asterisk (\*). Filled circles below branches and at the nodes represent Bayesian Posterior Probability  $\geq 0.95$ . Colored fish outlines correspond to those mentioned in Figure 1.

from the IAS snail, *M. tuberculata* (cercariae sequences: KY369157, KM226895) (Arya et al. 2016; Le et al. 2017) from Vietnam and South India, and from humans from Vietnam (adult sequences: KY369155, KY369156, KX815125) (Le et al. 2017). The 28S and COI trees show that the species of *Haplorchis* genus are paraphyletic (Figures 1, 2). In the COI tree all the sequences generated in this study are similar to *H. pumilio* identified from *T. trichopterus* (metacercaria sequence: KF044303), from Thailand, and form a monophyletic group. However, a sister clade for *H. pumilio* cannot be defined, based on a lack of nodal support (Figure 2). In contrast, the phylogenetic tree constructed from 28S dataset, based on high nodal support

**Table 2.** Distance matrix of uncorrected p-distances among of the sequences of 28S from Genbank and this study (\*) (values in percentage). Genbank numbers according to their geographic distribution and host groups (as in the Figures 1, 2). 1–3 = Thailand (*Haplorchis pumilio* from *Trichogaster trichopterus*); 4–6: Vietnam (*H. pumilio* from humans); 7–8: South of India (*H. pumilio* from *Melanoides tuberculata*); 9–12: Mérida City, Yucatán, Mexico (*H. pumilio* from *Carassius auratus*).

	1	2	3	4	5	6	7	8	9*	10*	11*	12*
1. HM004173												
2. HM004186	0.19											
3. HM004191	0.00	0.19										
4. KX815125	0.00	0.19	0.00									
5. KY369155	0.00	0.19	0.00	0.00								
6. KY369156	0.00	0.19	0.00	0.00	0.00							
7. KY369157	0.00	0.19	0.00	0.00	0.00	0.00						
8. KM226895	2.72	2.92	2.72	2.72	2.72	2.72	2.72					
9*	0.00	0.19	0.00	0.00	0.00	0.00	0.00	2.72				
10*	0.00	0.19	0.00	0.00	0.00	0.00	0.00	2.72	0.00			
11*	0.00	0.19	0.00	0.00	0.00	0.00	0.00	2.72	0.00	0.00		
12*	0.00	0.19	0.00	0.00	0.00	0.00	0.00	2.72	0.00	0.00	0.00	
13*	0.00	0.19	0.00	0.00	0.00	0.00	0.00	2.72	0.00	0.00	0.00	0.00

values (PP  $\geq$  0.95), detected *H. popelkae* as sister group of *H. pumilio* (Snyder and Tkach 2009) (Figure 1). The intraspecific genetic distance values based on the 28S sequences of *H. pumilio* used in this study, were 0.19% between HM004173 with HM004186 (both from Thailand), 2.72% between HM004173 (from Thailand) and KM226895 (from South of India), while HM004186 (from Thailand) and KM226895 (from South of India) show distance values of 0.19% and 2.72%, respectively compared to the other sequences (Table 2).

#### *Morphological description of the metacercariae of Haplorchis pumilio from Carassius auratus*

Description based on five fixed, stained and mounted unencysted metacercariae of *H. pumilio* from the base of the caudal fin of *C. auratus* from MCY, Mexico. Cyst oval and small. Body oval to elongated, with rounded ends, 294.5 (260–325) long and 129.5 (107.5–150) wide, width represents 43.9% (38.1%–48.1%) with respect to body length. Tegument covered by fine spines in all its extension, more conspicuous in anterior third, decreasing in size to the posterior end. Oral sucker large, spherical, subterminal, 43 (35–57.5) long and 52.5 (42.5–60) wide. Ventral sucker smaller than oral sucker, postecuatorial, slightly muscular, forming part of the ventrogenital complex, with numerous bar-like sclerites, forming an incomplete circle, 22 (20–25) long and 25 (22.5–27.5) wide, 173.9 (145.0–187.5) from anterior body end. Sucker ratio 1:500 (430–580). Prepharynx short, 31 (12.5–62.5) long and 7 (5–7.5) wide. Pharynx spherical, muscular, 31 (27.5–35) long and 26 (20–30) wide. Oesophagus long, 45 (27.5–65) long and 8 (7.5–10) wide. Intestinal caeca short, thick, extending to anterior border of the testicle, with discoidal corpuscles inside, 95.5 (70–125) long and 13.5 (10–17.5) wide. Testicle single, oval, postacetabular, 34 (25–40) long and 54 (52.5–55) long. Ovary spherical, located between

ventral sucker and testicle, 28.7 (22.5–40) long and 30.6 (17.5–35) wide. Excretory vesicle cup shaped, occupying posttesticular zone, 63.5 (55–80) long and 90 (70–102.5) wide. Excretory pore terminal (see Figure S2).

## Discussion

*Haplorchis pumilio* is recorded for the first time from pet shops in MCY as a parasite of *C. auratus* and we present the first molecular sequences for this parasite from Mexican pet shops. The phylogenetic evidence obtained from the 28S dataset shows that all members of *H. pumilio* included in the present study form a well-supported clade together with their sister group, *H. popelkae* Snyder and Tkach 2009, a trematode which as an adult parasitizes turtles (Snyder and Tkach 2009). Furthermore, the relationships found in this study between *H. yokogawai* (Katsuta, 1932) Chen, 1936, *H. pumilio* and *H. popelkae* are in agreement with the phylogenetic relationship discovered in previous analyses with the same gene and with similar taxa (Martínez-Aquino et al. 2017; Hernández-Orts et al. 2019).

At the moment, the genus *Haplorchis* consists of ten species including three species considered human pathogenic parasites, i.e., *H. taichui* (Nishigori, 1924), *H. pumilio* and *H. yokogawai* (Chai et al. 2009; Snyder and Tkach 2009). In previous molecular phylogenetic studies, the species included in the *Haplorchis* genus were found to be non-monophyletic (Le et al. 2017; Martínez-Aquino et al. 2017). Based on the 28S dataset used in this study, the three species of *Haplorchis* that associate as adult parasites with humans do not form a monophyletic group either, which suggests that the genus *Haplorchis* will either require revision in the future, or the non-monophyly could be explained by evolutionary mechanisms such as horizontal transfer. Such co-phylogenetic hypotheses can be tested using bioinformatic tools (Page 2003; Charleston and Libeskind-Hadas 2014; Martínez-Aquino 2016), but to be effective, they must include sequences from more *Haplorchis* species from distinct developmental stages and different types of hosts. Finally, based on 28S phylogenetic topology, *H. taichui* is included in a clade with representatives of the genus *Procerovum*. However, the taxonomic identity of the sequences from *Procerovum varium* Onji and Nishio 1916 (i.e., HM004182 (Thaenkham et al. 2010) and KM226894 (Arya et al. 2016)), must be revised because they do not form a monophyletic group.

In the COI topology, the clade of *H. pumilio* from sequences generated in this study plus previously published sequences (Chontanarith et al. 2014) is supported, but it was not possible to determine the sister group based on nodal support values. However, the sequences of *H. pumilio* used in this study correspond to metacercariae associated with two ornamental freshwater fish species from two families without phylogenetic affinities, i.e., *C. auratus* (Cypriniformes: Cyprinidae) and *T. trichopterus* (Actinopterygii: Osphronemidae) (Nelson 2006; Froese and Pauly 2017; but also see Scholz

et al. 2001; Huston et al. 2014). In this context, and based on the findings from the 28S topology, the generalist patterns of *H. pumilio* (metacercaria stage) are supported and a secondary effect of parasitic infection, due to the establishment of ornamental fishes as IAS, can be inferred. The process of parasitic infection from ornamental fishes to wildlife fishes has been recorded for other trematode parasites, e.g., *Ascocotyle tenuicollis* Price, 1935 and *Centrocestus formosanus* (Nishigori, 1924) (Scholz and Salgado-Maldonado 2000; Shoaibi-Omrani et al. 2010; Pinto and Melo 2012; Mehrdana et al. 2014). Therefore, we can speculate that the infection of aquatic and terrestrial vertebrates (including humans) by the ingestion of fishes parasitized by the metacercariae of *H. pumilio* (Chai and Jung 2017) may be caused by either 1) infected ornamental fishes (i.e., *C. auratus*) accidentally freed into the natural hydrological systems, or 2) wildlife fishes infected by contact with species of IAS snails (e.g., *M. tuberculata* or *T. granifera*) parasitized by the cercaria of *H. pumilio*. For these reasons, in several cases the presence/infection of *H. pumilio* was indicated as a potential emergent disease at regional level (Hung et al. 2013; Li et al. 2013; Arya et al. 2016). The first larval stage of *H. pumilio* is recorded in the IAS *M. tuberculata* (e.g., Huston et al. 2014; Krailas et al. 2014), a snail whose shells were also noted as being found in the same pet shops studied here (Chan-Martin, *pers. obs.*). Additionally, in a different study, *C. formosanus* was found infecting *C. auratus* from the same pet shops (Vidal-Martínez, *pers. comm.*), as recorded by Huston et al. (2014) and McDermott et al. (2014). The presence of the snail *M. tuberculata* in the same pet shops is important to correlate the presence of the trematode species, i.e., *C. formosanus* and *H. pumilio* (Aguilar-Aguilar et al. 2009; Krailas et al. 2014). In this context, the complex of IAS observed in this study (i.e., *M. tuberculata*, *H. pumilio* and *C. auratus*) probably includes a higher number of IAS than reported here. Furthermore, *H. pumilio* cercariae were also recorded from other snail species (also categorized as IAS from the aquarium industry), the quilted melania *T. granifera* (Umadevi and Madhavi 2006; Tolley-Jordan and Owen 2008), and other species of *Melanoides* snails (e.g., *Melanoides jugicostis* Hanley and Theobald 1876) (Dechruksa et al. 2017). In fact, we may be witnessing two biological invasion processes, 1) co-introduced parasite, an alien parasite species (i.e., *H. pumilio*) that has been transported into a new area with an alien host species (i.e., *C. auratus*) and 2) co-invasive parasite, a co-introduced parasite species that has infected native host species in the new range (Lymbery et al. 2014). If the populations of these IASs (i.e., *C. auratus*, *H. pumilio* and probably the snails *M. tuberculata*, *M. jugicostis* and *Tarebia granifera*) continue to be accidentally introduced in many parts of the world, the populations of these IASs will keep expanding. At the moment, the natural process of expansion–contraction of the wildlife populations of *H. pumilio* is unknown (from all developmental stages); therefore, we cannot infer how and when their

populations can or cannot be a major risk. However, we know that *C. auratus* is an intermediate hosts of *H. pumilio*, and that both IASs are present in pet shops in Mérida City, Yucatán, Mexico.

In Mexico, *H. pumilio* has been recorded in wildlife from seven freshwater fish species included in three families (Cichlidae, Eleotridae and Poeciliidae) (Scholz et al. 2001), and all fish species are endemic to the Caribbean subregion (*sensu lato* Morrone 2006). In USA (south-west), based on experimental infection and examination of wild fishes, Huston et al. (2014) demonstrated that *H. pumilio* cercaria larva (originated from the snail *M. tuberculata*) can parasitize six native freshwater fish species (i.e., *Etheostoma fonticola* (Jordan and Gilbert), *Micropterus salmoides* (Lacepède), *Etheostoma grahami* (Girard), *Dionda diaboli* Hubbs and Brown, *Gambusia nobilis* Baird and Girard and *Cyprinodon elegans* Baird and Girard), included in five families (Centrarchidae, Cyprinidae, Cyprinodontidae, Percidae and Poeciliidae). In fact, *H. pumilio* presents a pattern of a generalist parasite, as it has the potential to infect native freshwater fishes from the Americas. In Latin America (LAC) (excluding Mexican records), *H. pumilio* has not been recorded in any freshwater fish species (Kohn et al. 2007; Pinto and Melo 2012; Fernandes et al. 2015). However, studies on the diversity of helminth parasites of aquatic vertebrates and invertebrates in LAC are scarce (Cribb and Bray 2011; Aguirre-Macedo et al. 2017; Luque et al. 2017). In other parts of the world (e.g., India, Vietnam and Laos), *H. pumilio* was recorded from various species of freshwater and brackish water fishes (e.g., *Acanthogobius* spp., *Ambassis buruensis* Bleeker, *Anabas* spp., *Astatotilapia desfontainesi* (Lacepède), *Barbus* spp., *Carassius* spp., *Cyprinus* spp., *Esomus longimana* (Lunel), *Gerris filamentosus* Cuvier, *Glossogobius giurus* (Hamilton), *Hampala macrolepidota* Kuhl and Van Hasselt, *Mugil capito* Cuvier, *Ophicephalus striatus* Bloch, *Puntius binotatus* (Valenciennes), *Therapon plumbeus* Kner, *Teuthis javus* Linnaeus and *Tilapia* spp.), included in three families (Cyprinidae, Siluridae and Cobitidae) (Chai and Jung 2017). Furthermore, *H. pumilio* causes human infections due to the ingestion of raw fish, and these infections are considered emerging infectious diseases (Dung et al. 2013; Chai and Jung 2017). Therefore, the introduction of ornamental fishes, such as *C. auratus*, are a risk to aquatic wildlife and to humans, since they come with parasites that get established in local hydrological systems, as described in detail by Scholz and Salgado-Maldonado (2000). These co-introduced parasites can have repercussions on human health. For example, in MCY it is common to eat local raw fish dishes (named “ceviche”); if the goldfish are released to natural hydrological systems, it is probable that also *H. pumilio* infect local fishes, and their posterior ingestion by humans can cause an emergence of infectious diseases. Therefore, a systematized management of the introduction of ornamental fauna at local level can be a solution to stop a possible global colonization of an ecological complex of IAS.

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### Supplementary material

The following supplementary material is available for this article:

**Appendix 1.** References to Table S1 and Table S2.

**Table S1.** List of analyzed taxa for 28S with their GenBank accession numbers (new sequences in bold font).

**Table S2.** List of analyzed taxa for COI with their GenBank accession numbers (new sequences in bold font).

**Figure S1.** Microphotographs of A) encysted metacercaria of *Haplorchis pumilio* from the caudal fin of *Carassius auratus* and B) unencysted metacercariae of *H. pumilio* from the caudal fin of *C. auratus*. Scale bars: 100 µm. Photo credit: Alberto J. Chan-Martin and Jhonny G. García-The.

**Figure S2.** Line drawing of encysted metacercaria of *Haplorchis pumilio* from the caudal fin of *Carassius auratus*. Scale bar: 100 µm.

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