A molecular phylogenetic appraisal of the acanthostomines *Acanthostomum* and *Timoniella* and their position within Cryptogonimidae (Trematoda: Opisthorchioidea) (#20015)

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A molecular phylogenetic appraisal of the acanthostomines *Acanthostomum* and *Timoniella* and their position within Cryptogonimidae (Trematoda: Opisthorchioidea)

Andres Martínez Aquino Corresp., [1] , **Victor M Vidal-Martinez** [2] , **Leopoldina Aguirre-Macedo** Corresp. [2]

[1] Recursos del Mar, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Merida

[2] Recursos del Mar, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Merida, Merida, Yucatan, Mexico

Corresponding Authors: Andres Martínez Aquino, Leopoldina Aguirre-Macedo
Email address: maandres_@hotmail.com, leopoldina.aguirre@cinvestav.mx

The phylogenetic position of three taxa from two trematode genera, belonging to the subfamily Acanthostominae (Opisthorchioidea: Cryptogonimidae), were analysed using 28S ribosomal DNA and internal transcribed spacers (ITS1–5.8S–ITS2). Bayesian inference and Maximum likelihood analyses of combined 28S rDNA and ITS1 + 5.8S + ITS2 sequences indicated the monophyly of the genus *Acanthostomum* (*A.* cf. *americanum* and *A. burminis*) and paraphyly of the genera *Acanthostomum* and *Timoniella* acanthostomines. These phylogenetic relationships were consistent analysing 28S by itself and the concatenated 28S + ITS1 + 5.8S + ITS2 sequences. Based on molecular phylogenetic analyses, the subfamily Acanthostominae is therefore a paraphyletic taxon, in contrast with previous classifications based on morphological data. Phylogenetic patterns of host specificity inferred from adult stages of other cryptogonimid taxa are also well-supported. However, analyses using additional genera and species are necessary to support the phylogenetic inferences from this study. Our molecular phylogenetic reconstruction linked two larval stages of *A.* cf. *americanum* cercariae and metacercariae. Here, we present the evolutionary and ecological implications of parasitic infections in freshwater and brackish environments.

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Andrés Martínez-Aquino, Víctor M. Vidal-Martínez, M. Leopoldina Aguirre-Macedo*

Laboratorio de Patología Acuática, Departamento de Recursos del Mar, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Mérida, Cordemex, Carretera Antigua a Progreso Km. 6, Mérida, Yucatán, 97310, México

*Corresponding author: leopoldina.aguirre@cinvestav.mx

Abstract

The phylogenetic position of three taxa from two trematode genera, belonging to the subfamily Acanthostominae (Opisthorchioidea: Cryptogonimidae), were analysed using 28S ribosomal DNA and internal transcribed spacers (ITS1–5.8S–ITS2). Bayesian inference and Maximum likelihood analyses of combined 28S rDNA and ITS1 + 5.8S + ITS2 sequences indicated the monophyly of the genus *Acanthostomum* (*A.* cf. *americanum* and *A. burminis*) and paraphyly of the genera *Acanthostomum* and *Timoniella* acanthostomines. These phylogenetic relationships were consistent analysing 28S by itself and the concatenated 28S + ITS1 + 5.8S + ITS2 sequences. Based on molecular phylogenetic analyses, the subfamily Acanthostominae is therefore a paraphyletic taxon, in contrast with previous classifications based on morphological data. Phylogenetic patterns of host specificity inferred from adult stages of other cryptogonimid taxa are also well-supported. However, analyses using additional genera and species are necessary to support the phylogenetic inferences from this study. Our molecular phylogenetic reconstruction linked two larval stages of *A.* cf. *americanum* cercariae and metacercariae. Here, we present the evolutionary and ecological implications of parasitic infections in freshwater and brackish environments.

Introduction

The Cryptogonimidae Ward, 1917, is a speciose family (≥ 370 species), consisting of 93 genera associated with the intestine or pyloric caeca of marine and freshwater teleosts, reptiles and occasionally amphibians around the world (Miller and Cribb 2008a, 2013; Miller et al. 2009, 2010a, b; Cribb and Gibson 2010; Tkach and Bush 2010; Fernandes et al. 2013). Since taxonomic identification based on morphological characters is complex (i.e., it is based on combinations of characters), the taxonomic classification of species within Cryptogonimidae (e.g., at the subfamily level) has been reworked several times (Miller and Cribb 2008a). Taxonomic schemes of subfamilies can also be detected based on ecological factors and host preference. For example, studies based on phylogenetic approaches infer hierarchical-taxonomic patterns between cryptogonimid species associated with specific marine fish hosts (e.g., *Retrovarium* spp. that are associated with perciform marine fishes), or cryptogonimid genera associated with reptile taxa (e.g., the subfamily Acanthostominae Looss, 1899) (Brooks 1980; Miller and Cribb 2007a, 2008a). In particular, the Acanthostominae was inferred based on morphology, phylogeny and biogeographical and host-parasite association patterns (Brooks 1980; Brooks and Holcman 1993). The criteria for the subfamily Acanthostominae, as recognized by Brook and Holcman (1993), was based on six characters: 1) terminal oral sucker; 2) body armed with single row of spines; 3) preacetabular pit; 4) genital pore not in preacetabular pit; 5) seminal vesicle coiled posteriorly; and 6) sucker-like gonotyl. Based on these criteria, the acanthostomine trematodes include five genera: *Timoniella* Rebecq, 1960; *Proctocaecum* Baught, 1957; *Gymnatrema* Morozov, 1955; *Caimanicola* Freitas and Lent, 1938; and *Acanthostomum* Looss, 1899 (Brooks 2004). Nevertheless, Miller and Cribb (2008a) were not convinced by the morphological characteristics that were used to justify subfamily-level

53 divisions in Cryptogonimidae because several subfamilies were separated by few and trivial
54 characters. Miller and Cribb (2008a) also recognized that the phylogenetic analyses of
55 acanthostomines by Brooks (1980) could be used to infer intergeneric relationships between
56 cryptogonimids.
57 To explore the diversity of helminth parasite fauna from aquatic invertebrate and
58 vertebrate hosts in Mexico (Vidal-Martínez et al. 2001; Aguirre-Macedo et al. 2017), we
59 collected specimens of cryptogonimid metacercariae presumed to be of the subfamily
60 Acanthostominae: *Acanthostomum americanum* (= *Atrophecaecum astorquii*), Pérez-Vigueras,
61 1956, and *Timoniella* (= *Pelaezia*) *loossi* Pérez-Vigueras, 1956, from the Ria Celestun Biosphere
62 Reserve, Yucatan Peninsula, Mexico (based on Moravec 2001; Vidal-Martínez et al. 2001;
63 Brooks 2004; Miller and Cribb 2008a). These metacercariae were collected from the euryhaline
64 fish *Cichlasoma urophthalmus* (Günter, 1862) (Perciformes: Cichlidae) from the Yaxaá water
65 spring (20° 53’ 12.57” N; 90° 20’ 58.86” W), located in the Celestun tropical lagoon (Fig. 1).
66 We also collected cercariae presumed to be of the Cryptogonimidae from the aquatic gastropod
67 *Pyrgophorus coronatus* (Pfeiffer, 1840) (Gastropoda: Hybrobiidae) (see Scholz et al. 2000), at
68 the same location, to test for possible life-cycle links between the cercariae and metacercariae
69 with molecular data. To examine the systematic framework of representative species of our
70 specimens of cercariae and metacercariae, we carried out a phylogenetic reconstruction based on
71 molecular markers, i.e., 28S ribosomal DNA and the internal transcribed spacers (ITS1–5.8S–
72 ITS2). Additionally, to investigate the monophyly of the taxa included in Cryptogonimidae at the
73 subfamily level, we included sequences from additional species of two sister taxa, i.e.,
74 Opisthorchiidae and Heterophyidae (Trematoda: Opisthorchioidea) (Thaenkham et al. 2011,
75 2012). To do this, we used sequence data for the 28S and ITS1–5.8S–ITS2 rDNA markers

(available through GenBank) of species belonging to these two families. Based on the results of the molecular phylogenetic analyses, the systematic position of the acanthostomines ~~genus~~ *Acanthosthomum* and *Timoniella* ~~was~~ evaluated, with a brief discussion of the taxonomic implications for the subfamily Acanthostominae, and phylogenetic evidence to support the different intergeneric relationships among Cryptogonimidae is provided.

Material and methods

Collection of hosts and trematode parasites

As part of our ongoing study in the Celestun lagoon (Sosa-Medina et al. 2015), in March 2016 we collected 223 hydrobiid snails of *P. coronatus* from two localities: Baldiocera spring (20° 54' 6.29" N; 90° 20' 26.46" W) (156 snails) and Yaxaá spring (67 snails) (the two springs are approximately 1,400 metres apart). The snails were collected using strainers, placed separately into glass tubes and maintained in artificial light in the laboratory to stimulate the emergence of cercariae. After 2–3 days, portions of the snails were removed from their shells by dissection under a stereomicroscope. The only representatives of Cyptogonimidae (3 cercariae) were collected from a single *P. coronatus* from Yaxaá spring. As for representatives of other families, of the 156 *P. coronatus* examined from Baldiocera spring, we observed two cercaria of *Ascocotyle* (*Phagicola*) *nana* Ransom, 1920 (Heterophyidae) in each of two individual snails; and one metacercaria of *Crassicutis cichlasomae* Manter, 1936 (Apocreadiidae) from one snail. Both larvae were previously recorded from *P. coronatus* (Scholz et al. 2000). Of the 67 *P. coronatus* examined from Yaxaá spring, the only cercariae observed belonged to the aforementioned cryptogonimids. We also sampled specimens of other adult cryptogonimids, e.g., *Oligogonotylus mayae* Razo-Mendivil et al. 2008, from the cichlid fish *C. urophthalmus*. The protocols for host dissection, examination, collection and preservation, and the morphological

study of parasitic specimens followed Vidal-Martínez et al. (2001). We also collected specimens of ~~other adult trematode~~ species ~~from the same fish host~~, *Crassicutis cichlasomae*~~. The apocreadiid~~ was used as an outgroup taxon for the phylogenetic analysis in this study, based on previously established sister group relationship of Ophisthorchioidae (Bray et al. 2009; Fraija-Fernandez et al. 2015). Trematodes were identified based on morphological criteria suggested by Vidal-Martínez et al. (2001), Miller and Cribb (2008a) and Razo-Mendivil et al. (2008, 2010). The identification to genus level for both *Timoniella* and *Acanthosthomum* is certain based on metacercariae morphology. Microphotographs of both taxa can be found in Supplementary information Fig. S1. However, identification to species level may be questioned, therefore we hereafter refer to the species as *T.* cf. *loossi* and *A.* cf. *americanum*. Several metacercarian~~s~~ and adult specimens collected for morphological analysis were deposited as voucher specimens [*T.* cf. *loossi* (No. 525), *A.* cf. *americanum* (No. 526), *C. cichlasomae* (No. 527) and *O. mayae* (No. 528)] in the Colección Helmintológica del CINVESTAV (CHCM), Departamento de Recursos del Mar, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Unidad Mérida, Yucatán, México. Acanthostomine cercariae were not deposited because each specimen was required for the molecular study. Comisión Nacional de Acuacultura y Pesca (PPF/DGOPA-070/16) issued the collecting permits.

DNA extraction, PCR amplification and sequencing

DNA was extracted from individual cercariae, metacercariae and adult trematodes. DNA extraction was performed using the DNAeasy blood and tissue extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. For the four trematode taxa, the 28S ribosomal gene region was amplified by Polymerase Chain Reaction (PCR) (Saiki 1988), using 28sl forward (5´-AAC AGT GCG TGA AAC CGC TC- 3´) (Palumbi et al 1996) and LO reverse

(5′-GCT ATC CTG AG(AG) GAA ACT TCG- 3′) (Tkach et al. 2000). The primers BD1 forward (5′ -GTC GTA ACA AGG TTT CCG TA- 3′) and BD2 reverse (5′-TAT GCT TAA ATT CAG CGG GT-3′) (Bowles et al., 1995) were used for ITS1–5.8S–ITS2. The reactions were prepared using the Green GoTaq Master Mix (Promega). This procedure was carried out using an Axygen Maxygen thermocycler. PCR cycling conditions by both molecular markers were as follows: an initial denaturing step of 5 min at 94 °C, followed by 35 cycles of 92 °C for 30 s, 55 °C for 45 s, and 72 °C for 90 s, and a final extension step at 72 °C for 10 min. The PCR products were analysed 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/).

Molecular data and phylogenetic reconstruction

To obtain the consensus sequences of the larvae and adults of *A.* cf. *americanum, T.* cf. *loossi*, *O. mayae* and *C. cichlasomae,* we assembled and edited the chromatograms of forward and reverse sequences using the Geneious Pro v5.1.7 platform (Drummond et al. 2010). The 28S, ITS1, 5.8S and ITS2 sequences that were generated during this study were aligned with sequences of cryptogonimid, heterophyid and opisthorchiid taxa obtained from GenBank (see GenBank accession numbers in Supplementary Table S1), using an interface available with 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 best partitioning scheme and substitution model for each molecular marker was selected by using the “greedy” search strategy in Partition Finder v.1.1.1 (Lanfear et al. 2011, 2014) and applying the Bayesian Information Criterion (BIC) (Schwarz 1978). The nucleotide substitution model that best fit the 28S data was

145 TVM + I + G (Posada 2003); for ITS1 and ITS2 it was TVMef + G (Posada 2003); and for 5.8S,
146 it was JC + G (Jukes and Cantor 1969). Hypervariable regions of 28S, ITS1 and ITS2 alignments
147 were excluded using the Gblocks Web Server (Castresana 2000; Talavera and Castresana 2007).

148 The datasets were analysed with Bayesian inference (BI) and Maximum likelihood (ML)
149 using the CIPRES Science Gateway v. 3.3 (Miller et al. 2010). The ML was conducted in
150 RaxML v. 8 (Stamatakis 2014) using the GTRCAT approximation as a model of nucleotide
151 substitution (Yang 1994, 1996; Stamatakis 2006). The BI was carried out with MrBayes v. 3.2.1
152 (Ronquist et al. 2012). The Bayesian phylogenetic trees were reconstructed for each gene
153 separately using two parallel analyses of Metropolis-Coupled Markov Chain Monte Carlo
154 (MCMC) for 20 x 10^6 generations each. Topologies were sampled every 1,000 generations and
155 the average standard deviation of split frequencies was observed until it reached < 0.01, as
156 suggested by Ronquist et al. (2012). A majority consensus tree with branch lengths was
157 reconstructed for the two runs after discarding the first 5,000 sampled trees. For both ML and BI
158 analyses, model parameters were independently optimized for each partition. Node support was
159 evaluated by non-parametric bootstrapping (Felsenstein 1985) with 1,000 replicates performed
160 with RAxML (ML) and BI by Posterior probabilities (PP), where bootstrap values ≥ 75% and PP
161 ≥ 0.95, were considered strongly supported.

162 **Results**

163 **DNA sequences and dataset analyses**

164 In total, 36 bi-directional 28S and ITS1-5.8S-ITS2 sequences were obtained from three
165 individual cercariae and three individual metacercariae from *A.* cf. *americanum*, as well as three
166 individual metacercariae from *T.* cf. *loossi*, *O. mayae* (one adult specimen), and *C. cichlasomae*

(one adult specimen, outgroup) (Table 1). The 28S rDNA sequence fragment consisted of 881 base-pairs (bp) for the cercariae and metacercariae of *A.* cf. *americanum*; 880 bp in *T.* cf. *loossi*, 871 bp in *O. mayae*, and 870 bp in those of *C. cichlasomae*. The 28S sequences of cercariae and metacercariae of *A.* cf. *americanum* from *P. coronatus* were identical, while the sequences of *T.* cf. *loossi* showed a divergence of 0.03%. Nucleotide sequence variation in the 28S alignment from cryptogonimids (excluding the outgroup taxon) from 28S included 722 conserved sites, 537 variable sites, 403 parsimony-informative sites, and 134 singleton sites. The sequence fragments for the ITS1 nuclear marker were between 709 and 781 bp in length for *A.* cf. *americanum*; and were 805 bp in *T.* cf. *loossi*, 613 bp in *O. mayae*, and 424 bp in *C. cichlasomae*. The 5.8S nuclear marker was composed of 160 bp in *A.* cf. *americanum*, *T.* cf. *loossi*, *O. mayae* and *C. cichlasomae.* The length of the ITS2 nuclear marker ranged from 259 bp to 277 bp in *A.* cf. *americanum* and from 268 bp to 277 bp in *T.* cf. *loossi*; 260 bp in *O. mayae*, and 295 bp in *C. cichlasomae*. The ITS1 and ITS2 sequences of *A.* cf. *americanum* displayed 4% and 0.7% divergence, respectively, and those from *T.* cf. *loossi* displayed 0.9% divergence and 100% pairwise identity; the 5.8S sequences were identical. Nucleotide sequence variation (excluding the outgroup taxa) for ITS1, 5.8S and ITS2 were 62/69/50 conserved, 406/92/212 variable, 341/36/184 parsimony-informative, and 65/56/28 singleton sites, respectively.

Phylogenetic reconstructions

We inferred the phylogenetic relationships of Cryptogonimidae, based on the BI and ML analyses, from the following two datasets. The 28S gene dataset contained 92 terminals belonging to 81 species, and the combined dataset (28S + ITS1 + 5.8S + ITS2) contained 294 sequences belonging to 81 taxa concatenated (all sequences available from GenBank, see Supplementary Table S1). The phylogenetic trees constructed from the 28S and the concatenated

datasets (28S + ITS1 + 5.8S + ITS2), based on BI and ML analyses, were broadly congruent. For example, all clades with high nodal support values (PP ≥ 0.95 and bootstrap ≥ 75%) and analysed with the concatenated and 28S datasets were recovered with both BI and ML (Fig. 2; Supplementary Figs. S2–4). Only three high nodal support values (PP ≥ 0.95) from three clades were identified with BI [i.e., (*Gynichthys diadikidnus*, *Neoparacryptogonimus ovatus*); (*Metagonimus takahashii, M. yokogawai*); and (*Haplorchis yokogawai* (*Haplorchis popelkae, Haplorchis pumilio*))], while only one high nodal support value (bootstrap ≥ 75%) for one clade was identified with ML [i.e., (*Haplorchoides* sp. (*Stictodora* sp. isolate St1, *Stictodora* sp. isolate St2)) (Fig. 2). Conversely, only one difference was observed between the topology of the phylogenetic trees obtained from the 28S and concatenated datasets with BI and ML. Namely, only the phylogenetic tree obtained from the ML analysis of the 28S sequence dataset contained a polyphyletic group (without nodal support value), i.e., *Siphodera vinaledwardsii*, *Gynichthys diakidnus*, *Chelediadema marjoriae*, *Caecincola parvulus*, and *Tabascotrema verai* (Supplementary Fig. S3). In all trees, acanthostomines form a paraphyletic group, with high nodal support values (PP ≥ 0.95), with *Acanthostomum* and *Timoniella* not clustering together. Lastly, based on all trees, the family Cryptogonimidae appears to have arisen from a paraphyletic Heterophyidae/Opisthorchiidae group.

The phylogenetic relationships among Cryptogonimidae at the generic level had high support (PP ≥ 0.95) and in several cases, the clades with high nodal support values were coherent with their geographic distribution and their association with the host group that they parasitize. For example, the genera *Siphoderina*, *Belusca*, *Varialvus*, *Caulanus* and *Latuterus* form a monophyletic group (Clade I) (Fig. 2), with geographical distribution associated with the Indo-Pacific region (I-P); at the same time, the genera *Belusca* and *Varialvus* [the latter with

distribution only in the Indo-West Pacific (IW-P)], *Caulanus*, and *Latuterus* ~~genera~~ are associated with hosts of the marine fish families Haemulidae and Lutjanidae. Furthermore, *Retrovarium* spp. is associated with Lutjanidae and Haemulidae from the IW-P (Fig. 2). Geographical and host congruence was also found in cryptogonimid groups from North and Central America, although the clade did not receive high nodal support values in either analysis.

Discussion

The phylogenetic trees obtained from BI and ML analyses, inferred from the 28S and concatenated dataset, identified the phylogenetic position of the acanthostomines *A.* cf. *americanum* and *T.* cf. *loossi,* and illustrate differents intergeneric relationships among cryptogonimids (see below). Phylogenetic analyses show that the Heterophyidae and Opisthorchiidae are paraphyletic as previously reported (Thaenkham et al. 2011, 2012; Fraija-Fernández et al. 2015; Stoyanov et al. 2015; Borges et al. 2016). The family Cryptogonimidae appears to have arisen from the paraphyletic Heterophyidae/Opistorchiidae. This phylogenetic inference is based on a dataset of 51 taxa of Cryptogonimidae that included 24 genera. At present, the family Cryptogonimidae includes 93 genera (Cribb and Gibson 2012). It's indicate that we analysed almost 40% (38.75%) of recorded genera of Cryptogonimidae. Therefore, the phylogenetic inference of Cryptogonimidae has appropriate taxonomical representation, but it is necessary to complete it.

Based on the phylogenetic position of *A.* cf. *americanus*, *A. burminis* (which formed a separate single clade) and *T.* cf. *loossi* (independent lineage), we find that the subfamily Acanthostominae is paraphyletic. Therefore, the monophyly proposed for the subfamily Acanthostominae based on morphological analyses (i.e., Brooks 1980, 2004; Brooks and Caira 1982; Brooks and Holcman 1993) does not appear to be valid. These data support the proposed

invalidity of the subfamily-level division of Acanthostominae into Cryptogonimidae, suggested by Miller and Cribb (2008a). Therefore, it is necessary to include more acanthostomine taxa (i.e., *Proctocaecum*, *Gymnatrema, Caimanicola*) in future studies to determine their phylogenetic position and test their monophyly.

Based on the phylogenetic positions of *Acanthostomum* spp. and *T. loossi* in this study, we postulate a probable host-specificity pattern at a supra-specific level. The adult trematodes *A. burminis*, *A. americanum* and *T. loossi* are associated with freshwater diapsid sauropsids, i.e., *Xenochrophis piscator* (Schneider, 1799) (snake) (Reptilia: Colubridae) and *Crocodylus moreletii* Duméril & Bibron, 1851 (crocodile) (Reptilia: Crocodylidae) (Moravec 2001; Jayawardena et al. 2013; Sosa-Medina et al. 2015). The molecular evidence that links the two larval stages of *A. americanum* to the freshwater environment (from their intermediate hosts: snail and fish) and their later development as adults in freshwater crocodiles, may reflect an ecological preference to a freshwater environment. More specifically, the first larval stage (i.e., cercaria) of *A.* cf. *americanum* is restricted to freshwater environments due to the intermediate host snail's intolerance to brackish water (Scholz et al. 2000). The trematode's intermediate and definitive vertebrate hosts (*C. urophthalmus* and *C. moreletii*) are both tolerant to brackish water and can move between the two aquatic environments (Platt et al. 2010; Miller et al. 2009); however, the freshwater environment is essential to completing the trematode's life cycle. This assertion is supported by taxonomic records of metacercariae of *A.* cf. *americanum* being only from freshwater fishes of the families Characidae, Cichlidae, Clupeidae and Poeciliidae (Sosa-Medina et al. 2015; Salgado-Maldonado 2006).

Our phylogenetic trees indicated that the Acanthostominae was a freshwater group that was sister to the remaining marine cryptogonimids (supporting the sister-group relation found by

Stoyanov et al. 2015) (Fig. 2). If the acanthostomine taxa are truly sister to the remaining Cyrptogonomidae, there would be a strong argument for the hypothesis that the cryptogonimids originated in a freshwater environment and later diversified and colonized brackish and marine environments. The transition from a freshwater environment to a brackish environment to a marine environment is an evolutionary process also inferred for other platyhelminth groups (e.g., Torchin et al. 2002; Boeger et al. 2003; Van Steenkiste et al. 2013). Future studies may test the hypothesis regarding the colonization from freshwater to marine environments (e.g., Waters and Wallis 2001; Grosholz 2002; Lee and Gelembiuk 2008). The identification of the link between the cercariae and metacercariae of *A.* cf. *americanum* may represent a step in the understanding of the evolutionary strategies employed within different aquatic environments and the potential repercussions on food webs (e.g., Shoop 1988; Dobson et al. 2006; Poulin 2006).

It is noteworthy that the hydrobiid snail *P. coronatus* is highly susceptible to trematode infection having been reported to harbour 12 trematode species, e.g., *Genarchella astyanactis* Watson 1976; *Echinochasmus leopoldinae* Scholz et al. 1996; *Echinochasmus macrocaudatus* Ditrich et al. 1996; *Saccocoelioides* ? *sogandoresi* Lumsden 1963; *Crassicutis cichlasomae* Manter 1936; Homalometridae gen. sp.; *Oligogonotylus manteri* Watson 1976; *A.* (*Phagicola*) *nana* Ransom 1920; *Ascocotyle* (*Ascocotyle*) sp.; Xiphidiocercaria type 1, Xiphidiocercaria type 2 and Xiphidiocercaria type 3 (Scholz et al. 2000). The record of *A.* cf. *americanum* in *P. coronatus* is a new cercaria record for this snail. However, unfortunately, we did not collect sufficient cercariae of *A.* cf. *americanum* to describe their morphology.

Patterns of specific associations (e.g., codivergence (Page 2003; Martínez-Aquino 2016)) between other cryptogonimids were also revealed in our analyses; e.g., we detected a monophyletic group (Clade I) that included *Belusca*, *Caulanus*, *Latuterus*, *Siphoderina* and

282 *Varialvus* with a geographical distribution associated with the I-P (Miller and Cribb 2007a,
283 2008b; Miller et al. 2010b) (Fig. 2). Based on the diversity of genera in this clade, possible
284 taxonomic implications include the erection a new taxonomic hierarchy at the subfamily level.
285 Future studies based on morphological evidence may support or reject this taxonomic inference.
286 Alternatively, a host specificity pattern at the supra-specific level (marine fishes of the
287 Lutjanidae and Haemulidae families from IW-P) is also supported in *Euryakaina* spp. and
288 *Retrovarium* spp., as previously recorded (Miller et al. 2007b, 2010a, 2011). Presently, more
289 than 50 cryptogonimid taxa have been recorded from fishes belonging to the Lutjanidae and
290 Haemulidae of the IW-P (Miller and Cribb 2007b; Cribb et al. 2016). These specific associations
291 of cryptogonimids with fish from the IP and the IW-P, can be observed in the phylogenetic
292 topology revealed in this study; e.g., the genera *Beluesca*, *Varialvus*, *Caulanus*, *Latuterus*,
293 *Siphomutabilus*, *Metadena*, *Chelediadema*, and *Gynichthys* (Fig. 2) (Miller and Cribb 2007c,
294 2009, 2013; Miller et al. 2010a,b, 2011; Overstreet et al. 2009). Another possible case of host
295 specificity is *Adlardia novaecaledoniae* with Nemipteridae from the IW-P (Miller et al. 2009).
296 Future taxonomical studies of cyryptogonomid trematodes from marine fishes from other parts of
297 the world will shed more light on host-specificity patterns (e.g., Barger 2010; Montoya-Mendoza
298 et al. 2014).

299 Additionally, several non-acanthostomine cryptogonimid clades associated with the
300 freshwater environment are specialist parasites of particular families of freshwater fishes from
301 North and Central America; e.g., *Caecincola parvulus* is associated with Centrarchidae from
302 North America (NA), and *Tabascotrema verai*, *O. mayae* and *O. manteri* are associated with
303 Cichlidae from Central America (CA) (Choudhury et al. 2016). Even though these groups did not
304 have valid nodal support in this study (Fig. 2), it is important to mention three points. First, the

freshwater cryptogonimids appear to arise from among the marine taxa. Second, *C. parvulus* and *Oligogonotylus* spp. occur in freshwater fishes as both adults and metacercariae (Stoyanov et al. 2015; Choudhury et al. 2016). If new data reveal that species with a two-host life-cycle are monophyletic, the adaptation to the short life span of the fish host should be considered a derived state of the general life-cycle of cryptogonimids (Stoyanov et al. 2015; Lefebvre and Poulin 2005). Third, considering that centrarchids and cichlids are both members of Percomorpha and have marine affinities, Choudhury et al. (2016) suggest testing the hypothesis that a close relationship exists between Middle-American cryptogonimids of cichlids and cryptogonimids of North American centrarchids. The phylogenetic relationship we found between cryptogonimids of cichlids and centrarchids would supports this hypothesis. However, recent records of *C. parvulus* from other freshwater fish families should also be considered (McAllister et al. 2015, 2016).

Studies of cryptogonimids (and trematodes in general) are negatively impacted by the lack of taxonomical records of helminth parasites of freshwater and marine fishes of different regions (Scholz and Choudhury 2014; Cribb et al. 2016; Vidal-Martínez et al. 2016), as well as the lack of knowledge concerning intermediate and definitive host life cycles (Cribb and Bray 2011; Blasco-Acosta and Poulin 2017). This has led to a reduction in postulated evolutionary hypotheses on the diversification patterns of parasites. However, the development of phylogenetic hypotheses, as presented, provide a modern framework in parasite evolutionary ecology (e.g., Littlewood 2011; Gómez Nichols 2013; Poulin et al. 2016).

Acknowledgments

Thanks to staff of the laboratory of Patología Acuática: Clara Vivas Rodríguez, Gregory Arjona-Torres, Ana L. May-Tec, Francisco Puc Itzá, Nadia Herrera Castillo, Jhonny G. García-Teh,

Arturo Centeno-Chale, Germán López-Guerra, Daniel Aguirre-Ayala and Efraín Sarabia from CINVESTAV-IPN, Unidad Mérida, México. We are grateful to Abril Gamboa and José García Maldonado for their technical assistance in the molecular lab. We also thank Dr. Fadia Sara Ceccarelli who reviewed the first draft of this manuscript and made very useful suggestions of the phylogenetic analyses that we present in this contribution. The manuscript greatly benefited from comments of two anonymous referees.

We thank the American Journal Experts for editing the English text. Financial support of AM-A at the laboratory of Aquatic Pathology at CINVESTAV–IPN came from Grant No. 201441–SENER–CONACYT.

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Figure 1(on next page)

Map of the study area, Yaxaá spring, Celestun coastal lagoon, Yucatan, Mexico.

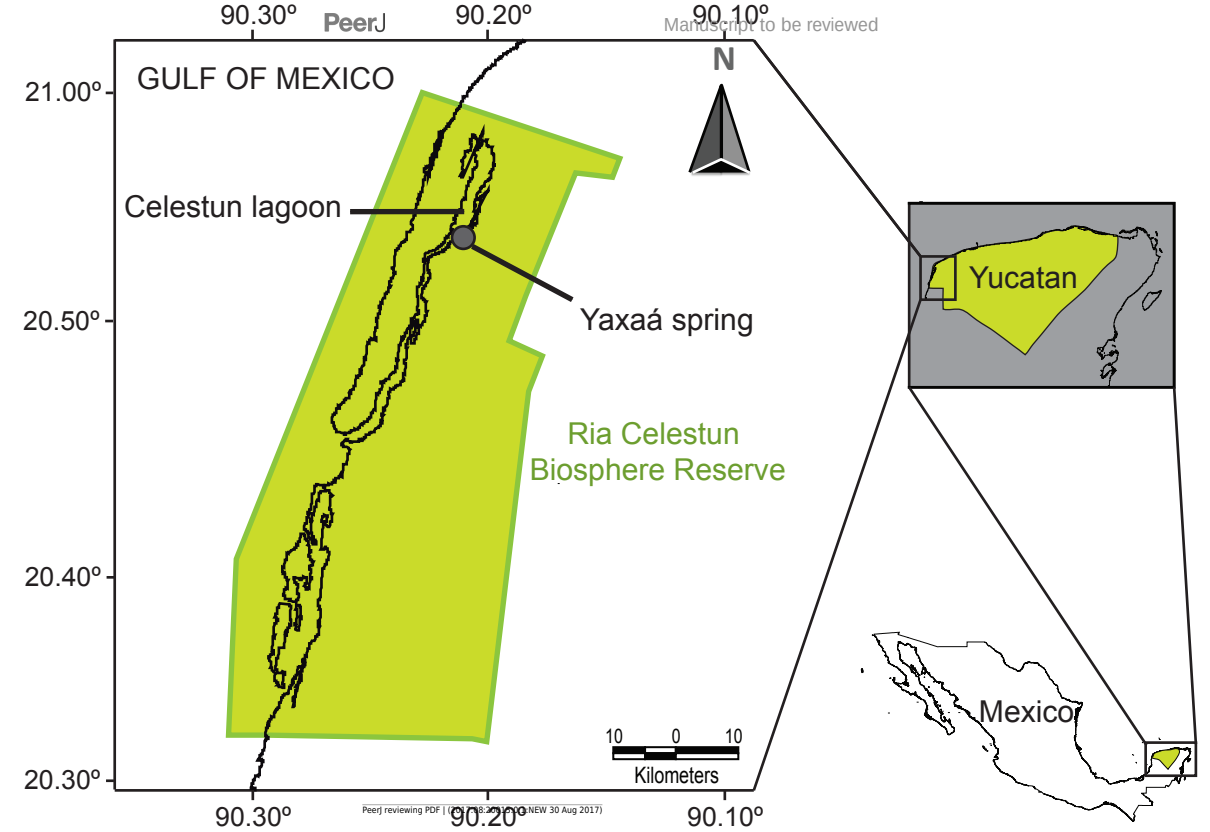

Figure 2(on next page)

Phylogenetic tree obtained from Bayesian inference analysis of the concatenated data (28S + ITS1 + 5.8S + ITS2) of species of the Cryptogonimidae.

The scale bar represents the number of nucleotide substitutions per site. Codes following taxon names are cross-referenced in Table 1 and Supplementary Table S1. Filled black circles above and white circles below the branches represent Bayesian posterior probability ≥ 0.95 and Maximum likelihood bootstrap support values ≥ 75%, respectively. Diffused green = Freshwater environment; Diffused green-yellow = Brackish environment; Diffused blue = Marine environment; IH = Intermediate host; DH = Definitive host; ? = Intermediate host unknown. I-P = Indo-Pacific; IW-P = Indo-west Pacific; CA = Central America; GM = Gulf of Mexico; NA = North America; EA = Eastern Atlantic; Se-A & SL = South-eastern Asia and Sri Lanka. The black snail outline corresponds to *Pyrgophorus coronatus*. The black fish outline corresponds to *Cichlasoma urophthalmus*. The black crocodile outline corresponds to *Crocodylus moreletii.* The black fishes outline on the remaining Cryptogonomidae refer to host specificity at family (ies) recording to species, species groups or genus (black line) of cryptogonomids. The animals´ silhouettes were modified from Ditrich et al. (1997) (snail); Gray (1830) (snake); Nelson (2006) (fishes), and Sánchez-Herrera et al. (2011) (crocodile). The cryptogonomid taxa without black fish outline are not specific to one host. See text for more details.

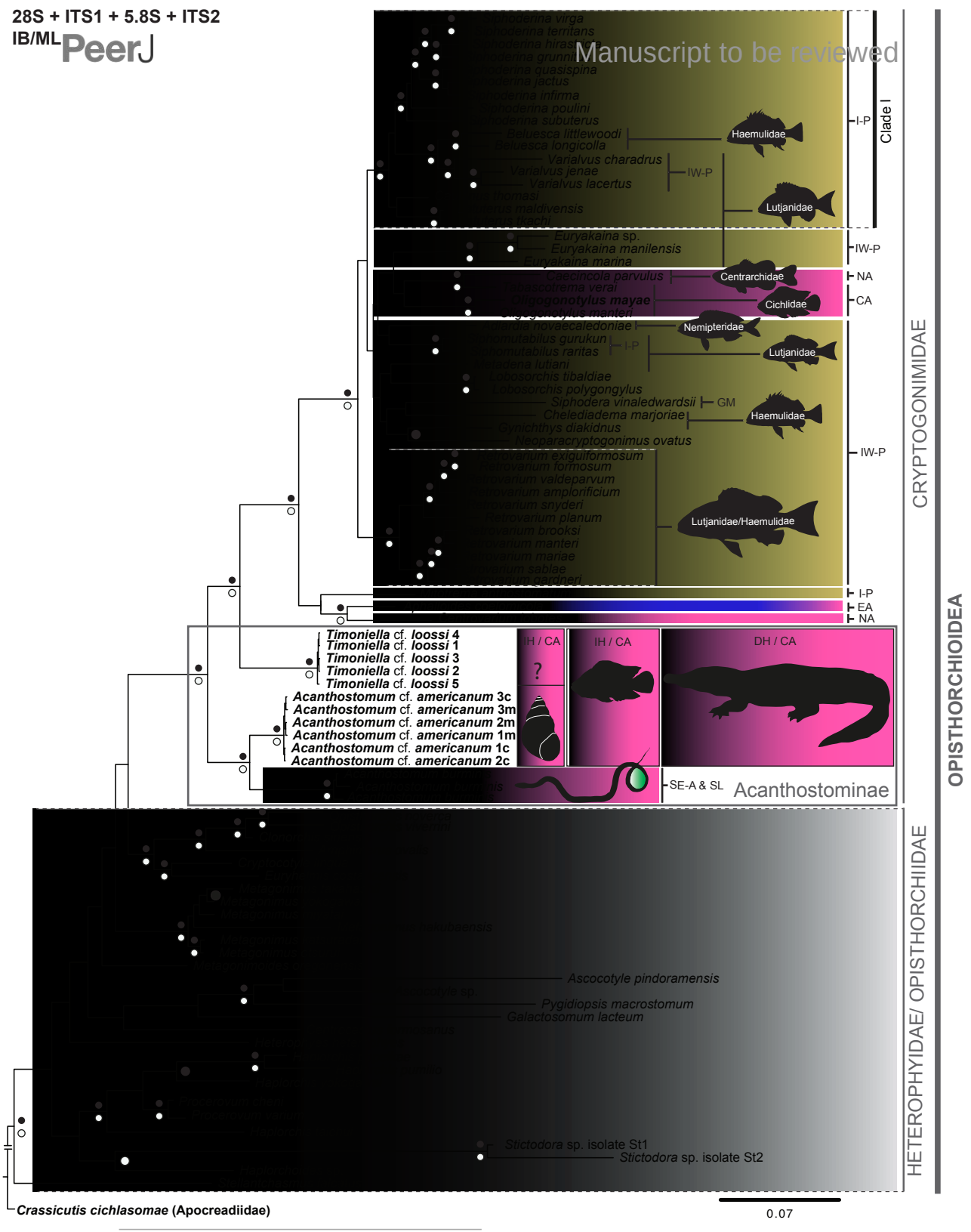

Table 1(on next page)

GenBank accession numbers for cryptogonimid species sequences newly generated for this study.

Codes used for each cryptogonimid sequenced are as shown in the terminal taxa names of Figure 2 and Supplementary figures S3–4.

Table 1. GenBank accession numbers for cryptogonimid species sequences newly generated for this study. Codes used for each cryptogonimid sequenced are as shown in the terminal taxa names of Figure 1 and Supplementary Figures S2–4.

Name	Code	Life cycle stage	GenBank Accession 28S	GenBank Accession ITS1-5.8S-ITS2
Timoniella cf. *loossi*	1	Metacercarie	XXXXX	XXXXX
Timoniella cf. loossi	2	Metacercarie	XXXXX	XXXXX
Timoniella cf. *loossi*	3	Metacercarie	XXXXX	XXXXX
Timoniella cf. *loossi*	4	Metacercarie	XXXXX	XXXXX
Timoniella cf. *loossi*	5	Metacercarie	XXXXX	XXXXX
Acanthostomum cf. *americanum*	1c	Cercarie	XXXXX	XXXXX
Acanthostomum cf. *americanum*	2c	Cercarie	XXXXX	XXXXX
Acanthostomum cf. *americanum*	3c	Cercarie	XXXXX	XXXXX
Acanthostomum cf. *americanum*	1m	Metacercarie	XXXXX	XXXXX
Acanthostomum cf. *americanum*	2m	Metacercarie	XXXXX	XXXXX
Acanthostomum cf. *americanum*	3m	Metacercarie	XXXXX	XXXXX
Oligogonotylus mayae		Adult	XXXXX	XXXXX
Crassicutis cichlasomae		Adult	XXXXX	XXXXX