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Molecular characterization and identification of digenean larval stages in *Aylacostoma chloroticum* (Prosobranchia: Thiaridae) from a neotropical floodplain

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Abstract

Digeneans (Trematoda: Digenea) are endoparasites that present a complex life cycle, generally involving an intermediate invertebrate host and a vertebrate host. There is limited information about which species of molluscs may act as intermediate hosts in the upper Paraná River floodplain (UPRF), where *Aylacostoma chloroticum* can be considered a potential candidate. The study of digeneans in this region is important because some of these parasites are potentially zoonotic, and, therefore, are relevant to public health. However, the correct identification of these organisms during the larval stages is difficult because of the lack of morphologically diagnostic characteristics. The objective of this study was to identify and molecularly characterize the larval stages of digeneans found in *A. chloroticum* in the UPRF, using the mitochondrial marker of subunit I of cytochrome c oxidase and the 28S nuclear marker. The molluscs were examined in the laboratory and three morphotypes of cercariae were found. DNA was extracted from the specimens obtained and was then amplified and sequenced. The morphotypes exhibited high genetic similarities with *Pseudosellacotyla*, *Paralecithodendrium* and *Philophthalmus*, indicating that these organisms belong to these genera. This is the first record of larval stages of these genera in molluscs collected in the UPRF.

Introduction

Digeneans have a complex life cycle, involving a definitive vertebrate host and at least one first intermediate host (usually an invertebrate), in which the larval stages are found. Sometimes, digeneans may present a second intermediate host (Combes *et al.*, 2002; Rosser *et al.*, 2016). Although the life cycle of digeneans has been commonly studied, the larval stages of numerous taxa are still unknown and their identification remains difficult due to the small number of morphologically diagnostic characteristics (Scholz *et al.*, 2000).

Digeneans are among the most common parasites identified in fish in the upper Paraná River floodplain (UPRF) (Chapell, 1995; Takemoto *et al.*, 2009), a region important to biodiversity conservation because it maintains highly heterogeneous habitats and species richness (Filho & Ibarras, 2005), consequently enabling an interesting diversity of parasites (Souza *et al.*, 2008; Takemoto *et al.*, 2009). Currently, only *Biomphalaria peregrina* (Orbiny, 1835) has been identified as a potential invertebrate host for digeneans in the UPRF (Souza *et al.*, 2008), however, the low prevalence of parasitism indicates that other mollusc species could be acting as intermediate hosts for these organisms.

Because other mollusc species inhabit the UPRF, *Aylacostoma chloroticum* (Scott, 1954) is an interesting potential host candidate for digeneans because it has been registered as an intermediate host of *Pseudosellacotyla lutzi* (Freitas, 1941), *Neocladocystis intestinalis* (Vaz, 1932), *Stephanoprora aylacostoma* and the *Echinostoma* group (Quintana & Núñez, 2008, 2014, 2016; Pinto & Melo, 2013a) and it is native to this environment. This mollusc is currently considered an endangered species according to the IUCN Red List of Threatened Species (Mansur, 2000), and in South America it is part of a conservation breeding programme called the *Aylacostoma* Project (Vogler *et al.*, 2014). In addition, it is the only species of this genus in the UPRF, a region impacted by the construction of dams in the Paraná River basin and by the introduction of species, including molluscs (Takeda *et al.*, 2004).

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Table 1. 28S sequences used in this study.

Species	Location	GenBank accession number	Reference
Paralecithodendrium chilostomum	Ukraine	AF151920	Tkach *et al.* (2003)
Paralecithodendrium longiforme	Ukraine	AF151921	Tkach *et al.* (2003)
Paralecithodendrium hurkovaae	Ukraine	AF151922	Tkach *et al.* (2003)
Paralecithodendrium parvouterus	Spain	AY220617	Tkach *et al.* (2003)
Paralecithodendrium sp.	England	JF784196	Lord *et al.* (2012)
Cercaria 09 Morphotype 1	Brazil	MK629697	This study
Cercaria 04 Morphotype 1	Brazil	MK629698	This study
Pycnoporus megacotyle	Ukraine	AF151917	Tkach *et al.* (2003)
Lecithodendrium linstowi	Ukraine	AF151919	Tkach *et al.* (2003)
Pseudosellacotyla lutzi	Brazil	MH368357	Pantoja *et al.* (2018)
Cercaria 08 Morphotype 2	Brazil	MK629699	This study
Cercaria 03 Morphotype 2	Brazil	MK629700	This study
Acanthostomum burminis	Sri Lanka	KC489791	Jayawardena *et al.* (2013)
Siphoderina virga	Australia	EU571262	Miller & Cribb (2008)
Philophthalmus sp.	Iberian Peninsula	KX672819	Heneberg *et al.* (2018)
Philophthalmus sp.	Iberian Peninsula	KX672818	Heneberg *et al.* (2018)
Philophthalmus gralli	USA	JQ246434	Church *et al.* (2013)
Philophthalmus gralli	Peru	JQ627832	Literák *et al.* (2013)
Cercaria 06 Morphotype 3	Brazil	MK629701	This study
Cercaria 05 Morphotype 3	Brazil	MK629702	This study
Cloacitrema michiganensis	USA	KT956948	Tkach *et al.* (2016)
Parorchis acanthus	USA	KT956949	Tkach *et al.* (2016)
Schistosoma japonicum	Philippines	Z46504	Littlewood & Johnston (1995)

Table 2. COI sequences used in this study.

Species	Location	GenBank accession number	Reference
Philophthalmus gralli	Peru	JQ675731	Literák *et al.* (2013)
Philophthalmus gralli	Peru	JX113673	Literák *et al.* (2013)
Philophthalmus lacrymosus	Iberian Peninsula	KX925599	Heneberg *et al.* (2018)
Philophthalmus lacrymosus	Iberian Peninsula	KX925600	Heneberg *et al.* (2018)
Philophthalmus lucipetus	Iberian Peninsula	KX925571	Heneberg *et al.* (2018)
Philophthalmus lucipetus	Iberian Peninsula	KX925572	Heneberg *et al.* (2018)
Philophthalmus sp.	Iberian Peninsula	KX672820	Heneberg *et al.* (2018)
Philophthalmus sp.	Iran	JN621324	–
Philophthalmus sp.	New Zealand	FJ765485	Leung *et al.* (2010)
Cercaria 1 Morphotype 3	Brazil	MK629696	This study
Cercaria 2 Morphotype 3	Brazil	MK629695	This study
Schistosoma japonicum	China	EU340360	–

According to Abdul-Salam & Al-Khedery (1992), the populations of molluscs can determine the species of digeneans present in birds and fish in a certain region and changes in the malacological community may alter the life cycle of these parasites; therefore, infections caused by trematode larvae can be used as bioindicators of environmental quality (Souza *et al.*, 2008). However, the identification of larval stages is difficult, so the use of molecular techniques is important for the correct

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Table 3. Mean measurements (min–max) (in μm) of cercariae obtained from *Aylacostoma chloroticum*.

		Morphotype 1 ($n = 8$)	Morphotype 2 ($n = 5$)	Morphotype 3 ($n = 7$)
Body	Length	105 (82–129)	238 (197–256)	487 (420–553)
	Width	59 (51–78)	71 (62–81)	157 (145–171)
Tail	Length	81 (51–135)	312 (208–342)	259 (206–318)
	Width	28 (23–34)	37 (36–40)	46 (43–53)

identification of Platyhelminthes (Locke *et al*., 2015). Therefore, molecular markers are commonly used and the fragment of the mitochondrial gene of subunit I of cytochrome *c* oxidase (COI) and the 28S nuclear ribosomal gene are both helpful for identification at a specific level because they represent conserved regions present in interesting variations among species of this group (Vilas *et al*., 2005; Moszczynska *et al*., 2009; Steenkiste *et al*., 2015).

The identification of digenean species and their respective intermediate hosts in the UPRF are important considerations for public health policies. Some species are zoonotic (Fried *et al*., 2004; Pinto & Melo, 2013b), which is a concern for the riverside population and tourists. In addition, some species use fish as a second intermediate host, which causes damage to fish farming, making them susceptible to predation or increasing their mortality rate (Eiras *et al*., 2011). Therefore, the objective of this study was to characterize and identify digeneans parasitizing *A. chloroticum* using molecular tools.

Materials and methods

Sampling was performed during July 2017 and May 2018, in the upper Paraná River floodplain (Garças lagoon, 22°43′30.7″S, 53°13′11.6″W) in Batayporã city (Mato Grosso do Sul State, Brazil). The specimens were maintained alive in aquariums in the Laboratório de Ictioparasitologia of the NUPÉLIA (Núcleo de Pesquisa em Limnologia, Ictiologia e Aquicultura) and analysed during the study.

To identify the gastropods, the individuals were examined and identified morphologically with the support of researchers from the Laboratório de Zoobentos (NUPÉLIA) and identification was confirmed using the COI mitochondrial gene. Parasite specimens were obtained from the gastropods collected. Subsequently, the body of the gastropod was carefully removed from its shell to avoid damaging the specimen and was examined under a stereomicroscope. The digestive gland was specifically examined because this organ is generally an infection site. The digeneans obtained were identified by their morphology (Gibson *et al*., 2002; Pinto & Melo, 2013b) and isolated in 1.5 ml tubes containing Milli-Q water, according to morphotype.

Two specimens of parasites of each morphotype were used for DNA extraction, which was carried out using the ReliaPrep™ gDNA Tissue Miniprep System kit, following the manufacturer's instructions. The 28S gene was partially amplified using the U178 primer: 5′-GCACCCGCTGAAYTTAAG-3′ and the L1642 primer: 5′-CCAGCGCCATCCATTTTCA-3′ (Lockyer *et al*., 2003). The polymerase chain reaction (PCR) conditions comprised an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 56°C for 1 min, 72°C for 1 min and a final elongation cycle at 72°C for 5 min. The COI gene also was partially amplified using the Trem CoI: F 5′-TTTCGTTGGA TCATAAGCG-3′ and e Trem CoI: R 5′-GCAGCACTAAAT TTACGATCAAA-3′ primers (Bonett *et al*., 2011). The PCR conditions for the COI gene amplification comprised an initial denaturation at 94°C for 1 min, followed by ten cycles at 94°C for 30 s, 42°C for 40 s and 72°C for 1 min, then 30 cycles at 94°C for 30 s, 50°C for 40 s, 72°C for 1 min and a final elongation step at 72°C for 7 min. The amplicons were verified on 1% agarose gel and purified using polyethylene glycol. The sequencing reactions were prepared using BigDye™ Terminator 3.1. Cycle sequencing was performed following the manufacturer's instructions and the products were sent to the Complexo de Centrais de Apoio à Pesquisa (COMCAP) of the Universidade Estadual de Maringá for automated sequencing using an ABI3500 Applied Biosystems sequencer.

The sequences obtained were edited and aligned using BioEdit (Hall, 1999) and MEGA 7.0 (Kumar *et al*., 1999) software, respectively. Species identification of the parasites was performed by comparing the results with previous data available in GenBank using the BLAST tool implemented in MEGA 7.0. Therefore, the sequences with high similarity were selected for further analysis (tables 1 and 2). The Kimura-2-parameter (K2P) distance among the analysed species were also calculated using MEGA 7.0. The best nucleotide substitution model was selected using jModelTest 2 (Darriba *et al*., 2012) and maximum-likelihood gene trees were constructed using the RAxML Black Box (Kozlov *et al*., 2019). *Schistosoma japonicum* was used as an outgroup in both analyses because this species belongs to a family distant from those of the digeneans obtained in this study – Faustulidae, Lecithodendriidae and Philophthalmidae. The novel sequences were deposited in GenBank (MK629695–MK629702).

Results

Partial sequences of the COI gene obtained from the mollusc specimens used in this study showed 98% similarity with *A. chloroticum* sequences available in GenBank and a 0.7% genetic distance. A total of 26 specimens of *A. chloroticum* were analysed, of which 19 (73.08%) were parasitized. Three distinct digenean morphotypes were recorded in these molluscs, morphotype 1, 2 and 3. Each morphotype showed the following prevalence: morphotype 1 (31%), morphotype 2 (50%) and morphotype 3 (34%). The mean measurements of the morphotypes are presented in table 3.

For the 28S gene, morphotype 1 shared high (95–96%) genetic similarity with *Paralecithodendrium parvouterus* (Bhaleraeo, 1926); morphotype 2 was most similar to *Pseudosellacotyla lutzi*, with 99–100% similarity; and morphotype 3 was most similar to *Philophthalmus gralii* (Mathis and Leger, 1910), with 98% similarity. The sequences used in the analyses are shown in table 1. The mean genetic distance between the different species of *Paralecithodendrium* was 6.6% and ranged from 3.9% to

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Table 4. Genetic distances resulting from the comparison of 28S gene sequences obtained from GenBank and morphotype 1, 2 and 3 cercariae of *Aylacostoma chloroticum*.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*Paralecithodendrium chilostomum*																					
2	*Paralecithodendrium longiforme*	0.040																				
3	*Paralecithodendrium hurkovaae*	0.067	0.058																			
4	*Paralecithodendrium parvouterus*	0.077	0.082	0.079																		
5	*Paralecithodendrium* sp.	0.070	0.069	0.039	0.079																	
6	Cercaria 09 Morphotype 1	0.074	0.084	0.079	0.033	0.079																
7	Cercaria 04 Morphotype 1	0.077	0.087	0.082	0.035	0.082	0.002															
8	*Pycnoporus megacotyle*	0.094	0.104	0.067	0.086	0.067	0.081	0.084														
9	*Lecithodendrium linstowi*	0.067	0.079	0.051	0.077	0.060	0.067	0.070	0.065													
10	*Pseudosellacotyla lutzi*	0.159	0.173	0.181	0.170	0.187	0.179	0.182	0.186	0.173												
11	Cercaria 08 Morphotype 2	0.159	0.173	0.181	0.170	0.187	0.179	0.182	0.186	0.173	0.000											
12	Cercaria 03 Morphotype 2	0.159	0.173	0.181	0.170	0.187	0.179	0.182	0.186	0.173	0.000	0.000										
13	*Acanthostomum burminis*	0.179	0.193	0.199	0.205	0.211	0.199	0.202	0.198	0.190	0.079	0.079	0.079									
14	*Siphoderina virga*	0.189	0.210	0.219	0.204	0.227	0.201	0.204	0.221	0.186	0.086	0.086	0.086	0.110								
15	*Philophthalmus* sp.	0.268	0.280	0.282	0.280	0.295	0.293	0.296	0.288	0.260	0.232	0.232	0.232	0.239	0.252							
16	*Philophthalmus* sp.	0.262	0.276	0.272	0.269	0.284	0.282	0.285	0.278	0.249	0.222	0.222	0.222	0.230	0.236	0.022						
17	*Philophthalmus gralli*	0.249	0.256	0.259	0.256	0.274	0.269	0.272	0.265	0.237	0.210	0.210	0.210	0.221	0.227	0.024	0.019					
18	*Philophthalmus gralli*	0.249	0.256	0.259	0.256	0.274	0.269	0.272	0.265	0.237	0.210	0.210	0.210	0.221	0.227	0.024	0.019	0.000				
19	Cercaria 06 Morphotype 3	0.255	0.270	0.272	0.263	0.281	0.275	0.279	0.271	0.243	0.222	0.222	0.222	0.236	0.239	0.024	0.024	0.017	0.017			
20	Cercaria 05 Morphotype 3	0.255	0.270	0.272	0.263	0.281	0.275	0.279	0.271	0.243	0.222	0.222	0.222	0.236	0.239	0.024	0.024	0.017	0.017	0.000		
21	*Cloacitrema michiganensis*	0.268	0.278	0.268	0.262	0.277	0.275	0.278	0.249	0.237	0.215	0.215	0.215	0.217	0.229	0.058	0.049	0.042	0.042	0.056	0.056	
22	*Parorchis acanthus*	0.269	0.283	0.272	0.257	0.271	0.276	0.279	0.265	0.244	0.215	0.215	0.215	0.245	0.236	0.080	0.075	0.067	0.067	0.072	0.072	0.063

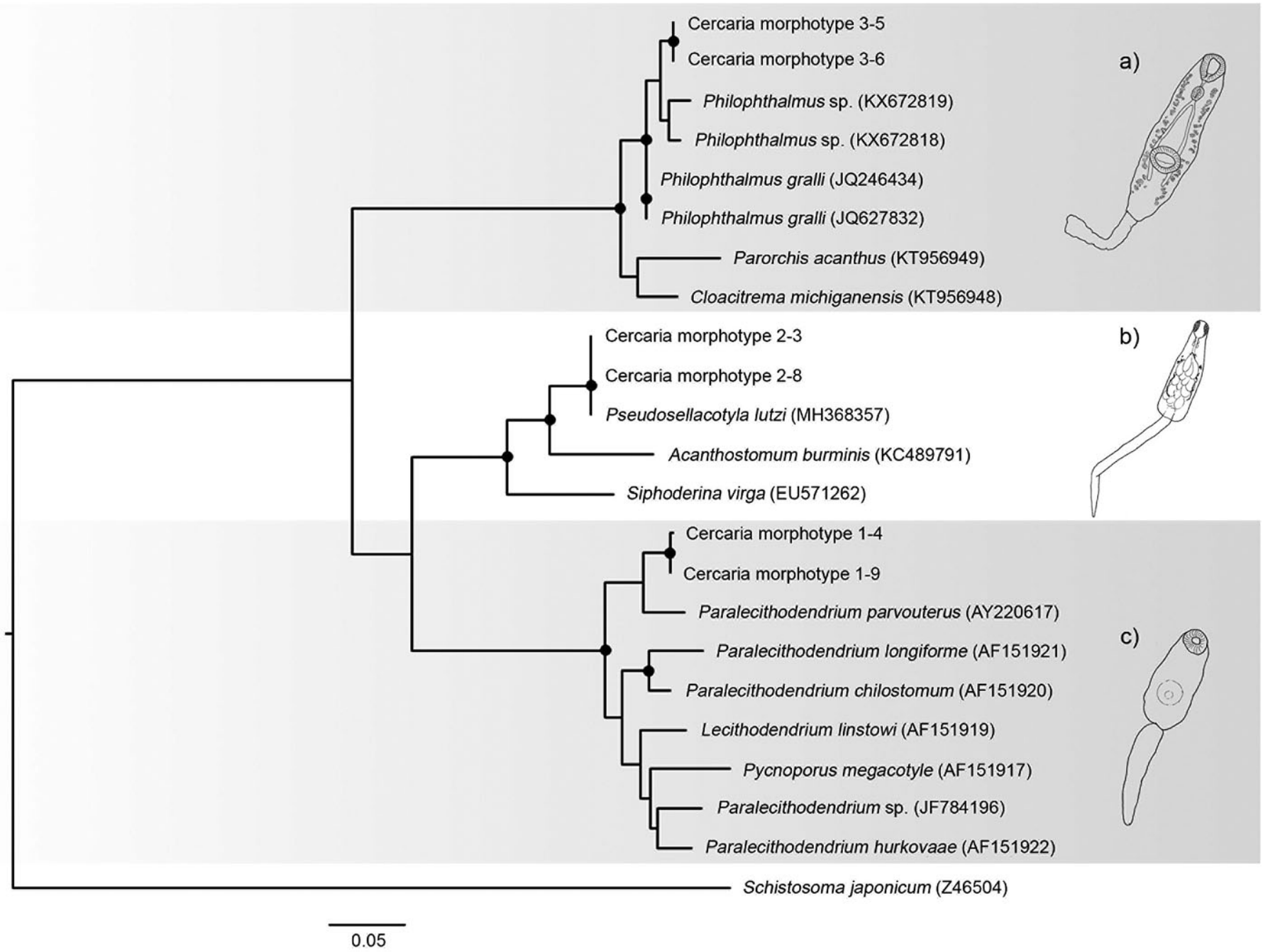

Fig. 1. Maximum-likelihood gene tree constructed using the 28S gene sequences analysed in this study. *Schistosoma japonicum* was used as an outgroup. Support values above 85 are represented by circles. (a) Cercaria morphotype 3 obtained in this study; (b) cercaria morphotype 2 obtained in this study; (c) cercaria morphotype 1 obtained in this study. Scale bars indicates the mean number of nucleotide substitutions per site.

Table 5. Genetic distances resulting from the comparison of COI gene sequences obtained from GenBank and morphotype 3 cercariae of *Aylacostoma chloroticum*.

		1	2	3	4	5	6	7	8	9	10
1	*Philophthalmus gralli*										
2	*Philophthalmus gralli*	0.000									
3	*Philophthalmus lacrymosus*	0.153	0.153								
4	*Philophthalmus lacrymosus*	0.154	0.154	0.001							
5	*Philophthalmus lucipetus*	0.151	0.151	0.162	0.164						
6	*Philophthalmus lucipetus*	0.147	0.147	0.159	0.161	0.003					
7	*Philophthalmus* sp.	0.149	0.149	0.161	0.162	0.001	0.001				
8	*Philophthalmus* sp.	0.017	0.017	0.160	0.162	0.162	0.159	0.160			
9	*Philophthalmus* sp.	0.150	0.150	0.168	0.169	0.121	0.121	0.120	0.156		
10	Cercaria 01 Morphotype 3	0.138	0.138	0.168	0.168	0.138	0.135	0.137	0.143	0.151	
11	Cercaria 02 Morphotype 3	0.135	0.135	0.165	0.165	0.135	0.132	0.134	0.140	0.148	0.004

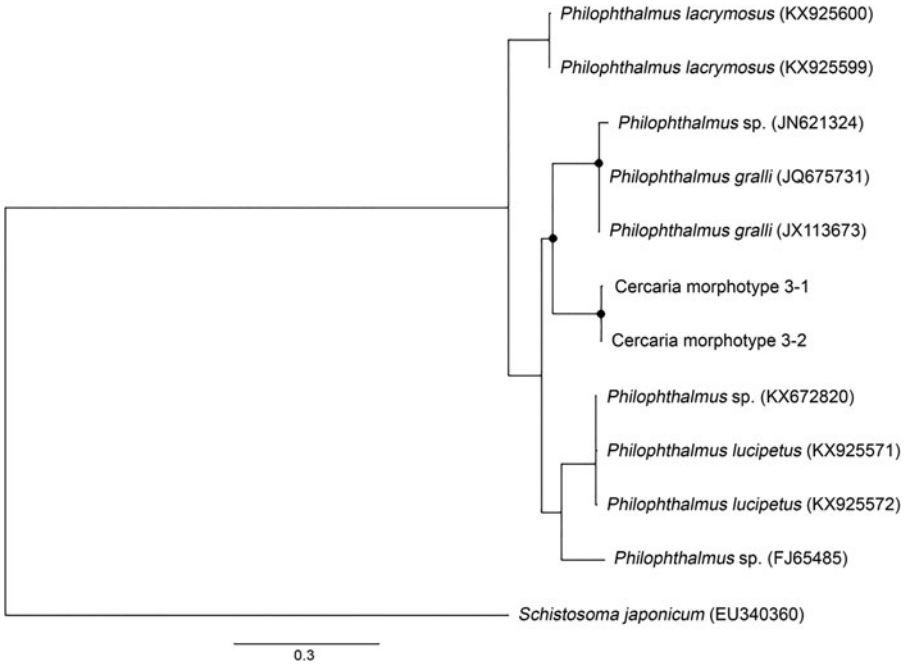

Fig. 2. Maximum-likelihood gene tree constructed using the COI gene sequences of *Philophthalmus* species used in this study. *Schistosoma japonicum* was used as an outgroup. Support values above 85 are represented by circles. Scale bars indicates the mean number of nucleotide substitutions per site.

8.2%, with 7.1% genetic distance between these sequences and morphotype 1. The sequence obtained for morphotype 2 was identical to *Pseudosellacotyla lutzi* obtained from GenBank. In *Philophthalmus*, the mean distance between species was 1.8% and ranged from 1.7% to 2.4%, and 2.1% when *Philophthalmus* spp. are compared with morphotype 3. The genetic distance values between all sequences are shown in table 4. A maximum-likelihood tree was constructed from this data (fig. 1).

The COI region of morphotypes 1 and 2 were 77% and 78% similar, respectively. However, molecular identification using this gene was not conclusive when compared with previously published data, so the results were not used for maximum-likelihood tree construction. For morphotype 3, the sequences were 88% similar to *Philophthalmus gralli* and *P. lucipetus* (Rudolphi, 1819). The sequences were aligned with the sequences of different *Philophthalmus* species obtained from GenBank (table 2), with a mean distance of 15.4% between the species of this genus and 14.5% divergence from morphotype 3. The genetic distance values are shown in table 5. A maximum-likelihood tree was constructed using this data (fig. 2).

Discussion

This is the first molecular characterization and identification of digenean larvae obtained in an aquatic invertebrate host from the UPRF using the molecular markers 28S and cytochrome *c* oxidase. These results showed that *A. chloroticum* is an important intermediate host of digenean parasites in this region.

Based on the 28S gene partial sequence, the relationships observed in the gene tree (fig. 1) indicate that morphotypes 1 and 3 belong to *Paralecithodendrium* and *Philophthalmus*. However, morphotype 2 was identified as *Pseudosellacotyla*

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lutzi, previously recorded as parasitizing specimens of *Hoplias malabaricus* (Bloch, 1794) 'traíra' in Brazil (Pantoja *et al*., 2018), and using *A. chloroticum* as an intermediate host during its life cycle (Quintana & Núñez, 2014), which was validated by this study.

The COI gene provided conclusive information only for morphotype 3, indicating that this specimen belongs to *Philophthalmus*, a species that can use birds or mammals as definitive hosts. In Brazil, there are also records of *Philophthalmus gralli* parasitizing *Melanoides tuberculatus* (Müller, 1774), an invasive mollusc, but only under experimental conditions (Pinto & Melo, 2010, 2013a). In the UPRF, there are records of *Philophthalmus lachrymosus* (Braun, 1902) using Capybara, *Hydrochoerus hydrochaeris* (Linnaeus, 1766), as a definitive host, found in its vitreous humour (Souza *et al*., 2015). However, there have been no previous studies on the definitive host of this genus in the UPRF. *Paralecithodendrium* was also reported to use *M. tuberculatus* as a first intermediate host, insects as second intermediate hosts and bats as its definitive host (Fried *et al*., 2004; Santos & Gibson, 2015). These parasites are globally distributed, with human infections recorded in Asiatic countries, such as Indonesian and Thailand (Kumar, 1999).

In general, the molecular markers used in this study were useful for the identification of digenean parasites at the genus and species level, but the results also indicate that more than one molecular marker should be used, considering the difficulty in developing universal primers to these organisms (Moszczynska *et al*., 2009; Steenkiste *et al*., 2015). Another difficulty is the scarcity of DNA sequences in public databases. In addition, the difficulty in morphological identification of the parasites obtained in this study may be because these specimens represent previously undescribed digenean species, thereby necessitating new molecular and morphological studies in the future.

Monitoring the life cycle of the parasites, along with the identification of their larval stages, can help in the understanding of ecosystem dynamics and environmental quality because changes in the environment are reflected in the digenean species richness and in the prevalence of infection (Souza *et al*., 2008). Therefore, considering the high prevalence of parasitism (73.08%) in this gastropod, it is possible that this species is an important intermediate host in the life cycle of digenean and the maintenance of their populations is of great importance in terms of conservation and preservation of biodiversity. Finally, we suggest intensifying studies aimed at understanding the life cycles of digenean parasites using molecular tools because morphological identification is difficult during the initial stages of the life cycle and, perhaps more importantly, because two of the three digeneans found here present zoonotic potential.

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Conflicts of interest. None.

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