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Record of an unknown species of *Aylacostoma* (Gastropoda: Hemisinidae) evidenced by molecular data

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ABSTRACT. Gastropods belonging to the Hemisinidae family are distributed in the Neotropical region, mainly in Central and South America, with *Aylacostoma* being a native genus of South America, as documented in the Paraná River Basin. The Ivaí River is one of the last rivers in the state of Paraná free of dams along its main channel. The future construction of hydroelectric dams could lead to the extinction of populations, including those of *Aylacostoma*, as occurred with the construction of the Yacyretá reservoir (in the Argentina–Paraguay region). The morphological similarity between species of the genus and the limited number of specialized taxonomists may result in an underestimation of the diversity of these organisms in the region. Molecular techniques have become an invaluable tool for the characterization of gastropod populations. The main objective of this study was to provide a record of an unknown species of *Aylacostoma* found in the Ivaí River, as indicated by molecular data. The *COI* mitochondrial region of five specimens was sequenced and subsequently compared with other Hemisinidae and Thiaridae species available in GenBank. The genetic distances observed for *Aylacostoma* sp. were 5.4% with *A. chloroticum* and 6% with *A. brunneum*, indicating that it is a distinct species from those available in GenBank.

Keywords: Brazil; freshwater; gastropods; Ivaí River; mitochondrial.

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Introduction

Aquatic gastropods of the Hemisinidae Fischer & Crosse, 1891 family are characterized by their distribution in the Neotropical region and are considered medium-sized to large. They can also be distinguished by an elongate-ovate to ovate-conical shell, a corneous operculum with a paucispiral shape. In addition, they have a fringed mantle edge and share many characteristics with Thiaridae Gill, 1871 (1823). Hemisinidae species are distributed mainly in Central and South America (Glaubrecht & Neiber, 2019). In South America, the native genus *Aylacostoma* Spix, 1827 comprises 33 species distributed throughout Brazil (Simone, 2006). In the Paraná River Basin, four endemic species of the genus *Aylacostoma* have been recorded, with a majority of specimens found in the lower Paraná River region between Paraguay and Argentina. These include *Aylacostoma chloroticum* Hylton Scott, 1954, *Aylacostoma brunneum* Vogler & Peso, 2014, *Aylacostoma guaraniticum* (Hylton Scott, 1953), and *Aylacostoma stigmaticum* Hylton Scott, 1954 (Peso et al., 2013a, 2013b; Vogler, 2012; Vogler et al., 2014), of which the last two species are considered extinct (Peso et al., 2013a, 2013b; Vogler, 2013; Vogler et al., 2014). Additionally, records of *A. chloroticum* have been documented in the floodplain region of the upper Paraná River in Brazil (Onaca et al., 2019) and in the lower Ivaí region (Passere et al., 2022).

The Ivaí River is the second largest body of water in the Paraná River Basin, with a total drainage area of 36,646 km² and a length of 798 km from its source to where it flows into the Paraná River (Parolin et al., 2010). Furthermore, the Ivaí River Basin is renowned for its rich biodiversity, which encompasses a vast array of fish species (Frota et al., 2016). Moreover, this river represents one of the last undammed and free-flowing rivers in the Paraná River Basin, playing an important role in biodiversity preservation and conservation projects, such as the Pró Ivaí/Piquiri project (Affonso et al., 2015).

As a consequence of the increasing investment in the construction of small hydroelectric plants in the region, some species may be more affected, potentially leading to the local extinction of populations. This phenomenon has already been observed in the case of *A. chloroticum*, *A. brunneum*, *A. guaraniticum*, and *A. stigmaticum* in the Argentina–Paraguay region (Peso et al., 2013a, 2013b; Vogler, 2012; Vogler et al., 2014). The lack of studies for neglected or difficult-to-identify groups precludes the accurate delineation of their area of occurrence, which in turn hinders an accurate assessment of their conservation status (Cardoso et al., 2011).

Given the morphological similarity between Hemisinidae and Thiaridae species and the lack of taxonomists with expertise in these groups, the use of molecular techniques has become a valuable tool for characterizing the populations of these gastropods (Vogler et al., 2014). Prior molecular studies have underscored the necessity of developing a more comprehensive genetic database, given the restricted availability of gene sequences for comparison in existing databases and the still-unknown genetic information of these species (Passere et al., 2022; Vogler, 2012; Vogler et al., 2014).

Therefore, the main objective of this study was to document an unknown species of the genus *Aylacostoma* discovered in the Ivaí River, and to include the new molecular data in genetic databases.

Material and methods

Sampling and preparation of biological material

The gastropods were collected during two periods: in February 2021 in the lower Ivaí River, Paraná, Brazil, near the municipality of Doutor Camargo (23°35'12.3"S 52°17'27.7"W) (*n* = 3), and in February 2023 in the same river near the municipality of Engenheiro Beltrão, in the district of Ivailândia (23°39'23.7"S 52°12'36.5"W) (*n* = 2). The gastropods were collected using landing nets and manual capture, stored in plastic bags with water and transported to the Ichthyoparasitology Laboratory (*Laboratório de Ictioparasitologia*) of *Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura* (Nupélia/UEM), where they were kept alive in aquariums until screening. All the specimens were measured and photographed before screening for parasitological analysis, where the shells were broken. Tissue from the foot region of the gastropods was stored in 1.5 mL microtubes containing absolute ethanol for later DNA extraction. The collection of biological material was authorized by the *Sistema de Autorização e Informação em Biodiversidade* (Biodiversity Information and Authorization System – SISBio 76413 and 85597).

Molecular characterization

The DNA from the five gastropod specimens was extracted using the Wizard® Genomic DNA Purification Kit (Promega®), in accordance with the instructions provided by the manufacturer. Following extraction, the DNA was quantified using a NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific®). For the polymerase chain reaction (PCR), a ProFlex™ 3 x 32-well PCR System thermocycler from Applied Biosystems® was used. The reaction was conducted with 1 U of *Taq* DNA polymerase (5 U μL^{-1}, Invitrogen), Tris-KCL (20 mM Tris-HCl pH 8.4 with 50 mM KCl), 1.87 mM $MgCl_2$, 0.1 mM of each dNTP, 4 μM of each primer, template DNA (10 ng) and Milli-Q water for a final volume of 25 μL.

Specific primers for the mitochondrial region of cytochrome *c* oxidase I (*COI*) were used to amplify the gastropod region of interest. The primers were LCO1490 (5′-GGTCAACAAATCATAAAGATATTG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′), as described by Folmer et al. (1994). The PCR conditions consisted of an initial denaturation at 94°C for three minutes; five cycles of 30 seconds at 94°C, 30 seconds at 45°C, and one minute at 72°C; 30 cycles of 30 seconds at 94°C, one minute at 48°C, and one minute at 72°C; and a final extension at 72°C for 10 minutes.

The amplicons were analyzed on a 1% agarose gel, and the size of the fragments was estimated using a marker of known molecular weight (100 bp DNA Ladder, Invitrogen 0.5 μg μL^{-1}). The products obtained from the DNA amplification were purified in accordance with the protocol described by Rosenthal et al. (1993). Sequencing was conducted on an Applied Biosystems® AB 3500 Genetic Analyzer. Access to the genetic heritage of the gastropods was granted by the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (National System for the Management of Genetic Heritage and Associated Traditional Knowledge – SisGen A00CC4C).

The DNA sequences were manually edited using the BioEdit program (Hall, 1999). Following the editing process, the sequences were aligned using the MEGA7 program (Kumar et al., 2016), with the Clustal W algorithm (Thompson et al., 1994). Sequences of the *COI* region of members of the Hemisinidae and Thiaridae families (*A. chloroticum*, *A. brunneum*, *Melanoides tuberculata* (Müller, 1774), *Tarebia granifera* (Lamarck, 1816), *Thiara aspera* (Lesson, 1831), *Thiara australis* (Lea & Lea, 1851), *Thiara amarula* (Linnaeus, 1758), *Thiara scabra* (Müller, 1774) and *Thiara winteri* Busch, 1842) available in the GenBank database were added to the analyses (information on the sequences added to the analyses can be found in Supplementary Material 1). Sequences shorter than 600 bp were excluded to ensure the integrity of the final alignment. The BLASTn tool (Altschul et al., 1990; Johnson et al., 2008) was employed to facilitate a comparison of the sequences obtained in the present study with those that have been previously deposited in GenBank. The nucleotide substitution model was selected using the MEGA7 program, which was also employed to calculate the Kimura 2-parameter (K2P) genetic distance. The DnaSP v6 program (Rozas et al., 2017) was used to characterize the haplotypes. A phylogenetic reconstruction was conducted using the Maximum Likelihood method with 1,000 bootstrap resamplings, as implemented in the MEGA7 program. The sequences of *Doryssa* sp. (JQ966087) and *Pachychilus laevissimus* (Sowerby, 1824) (JQ966088) were included as outgroups in the analyses. All the sequences obtained in this study have been deposited in GenBank (PQ074122–PQ074126).

Results

The gastropods were previously identified according to their morphology as belonging to *Aylacostoma*, referred to as *Aylacostoma* sp. in this study (Figure 1). The total length measurements obtained for the analyzed specimens are presented in Table 1, along with a comparison to other species within the genus. The total length of the five specimens of *Aylacostoma* sp. ranged from 20 to 36 mm, with an average total length of 29.20 mm, which is greater than the observed for *A. chloroticum*, *A. brunneum*, and *A. stigmaticum*.

Figure 1. Morphotype of *Aylacostoma* sp., where the bar indicates a scale of 1 centimeter.

Table 1. Total length (min–max) and average total length, in mm, of *Aylacostoma* sp. from the Ivaí River and other species of the genus.

Species	Total length (min-max)	Average total length	Reference
Aylacostoma sp.	20-36	29.20	This study
Aylacostoma chloroticum	20.90-41.59	28.28	Vogler et al. (2014)
Aylacostoma brunneum	13.30-27.89	21.81	Vogler et al. (2014)
Aylacostoma guaraniticum	33-36	34.50	Hylton Scott (1953)
Aylacostoma stigmaticum	23-28	25.50	Hylton Scott (1954)

Five *COI* sequences with 654 bp were obtained and, following editing and alignment with the other sequences in the database, the final alignment showed 604 bp. The five sequenced specimens exhibited similarity values ranging from 94.58 to 95.17% with *A. chloroticum* sequences and 94.26% similarity with *A. brunneum*, thereby indicating that it is a distinct species from those available in GenBank.

The sequences obtained for the unknown species of *Aylacostoma* exhibited a single haplotype. The nucleotide substitution model selected by the MEGA7 program was T92 + G + I. With regard to K2P distances, the values observed between *Aylacostoma* sp. and *A. chloroticum* were 5.40%, and with *A. brunneum* they were 6% (Table 2). Furthermore, the distance values ranged from 15.50 to 21.80% when compared to the Thiaridae species deposited in GenBank.

Table 2. K2P interspecific genetic distance matrix calculated from the *COI* sequences of the Hemisinidae and Thiaridae families.

	1	2	3	4	5	6	7	8	9
1 *Aylacostoma* sp.									
2 *Aylacostoma chloroticum*	0.054								
3 *Aylacostoma brunneum*	0.060	0.043							
4 *Thiara winteri*	0.166	0.153	0.172						
5 *Melanoides tuberculata*	0.176	0.169	0.183	0.167					
6 *Thiara aspera*	0.186	0.180	0.189	0.150	0.172				
7 *Thiara australis*	0.189	0.178	0.174	0.162	0.162	0.157			
8 *Thiara amarula*	0.189	0.177	0.188	0.147	0.181	0.187	0.194		
9 *Thiara scabra*	0.193	0.185	0.196	0.145	0.126	0.151	0.167	0.170	
10 *Tarebia granifera*	0.204	0.195	0.193	0.171	0.171	0.167	0.140	0.204	0.160

In the gene tree presented in Figure 2, we observed that *Aylacostoma* sp. was situated as a sister group to the clade formed by *A. chloroticum* and *A. brunneum*. Collectively, these groups formed a sister clade to the Thiaridae species available in GenBank.

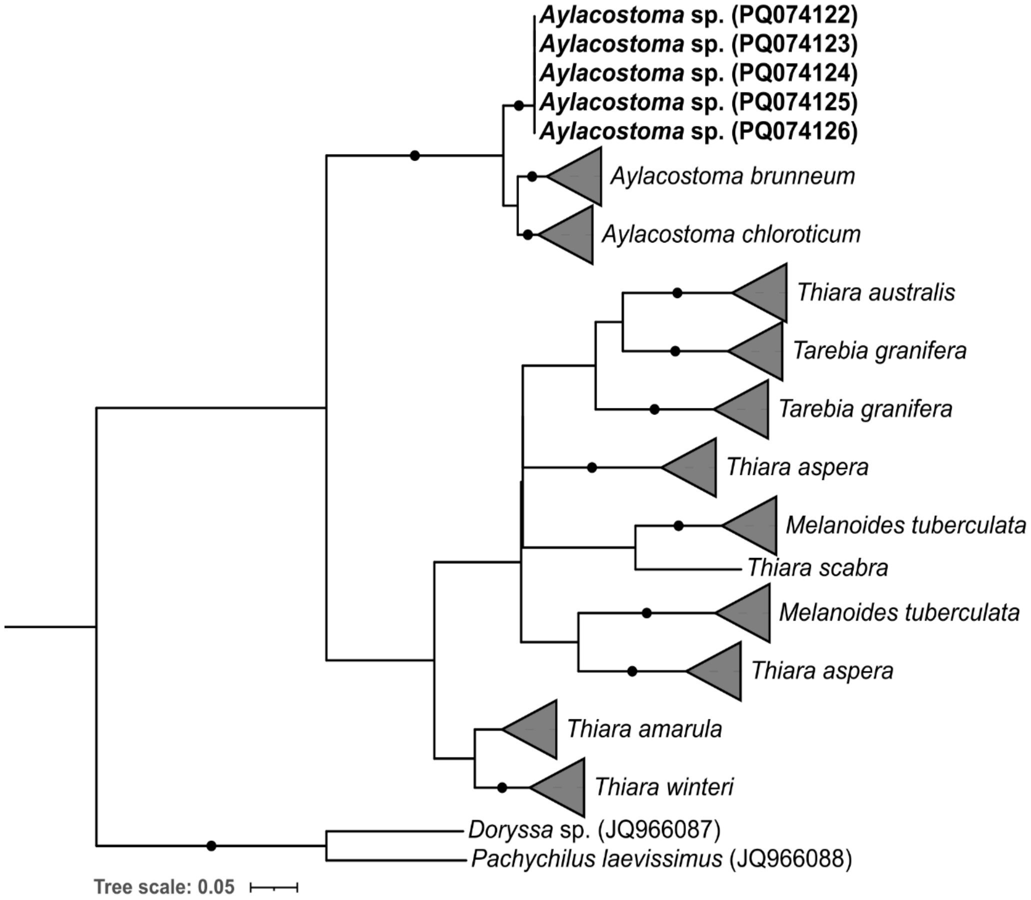

Figure 2. Gene tree constructed using the Maximum Likelihood method for the *COI* marker. The circles on the branches represent bootstrap values greater than 80%. The sequences in bold were obtained in this study.

Discussion

This study presents the first record of *Aylacostoma* sp. in the Ivaí River, based on the K2P genetic distance values observed between the analyzed specimens and the available genetic sequences in databases.

Though it is a different species from *A. chloroticum* and *A. brunneum* available in GenBank, it is not possible, however, to state that it is a new species of the genus, since genetic information for other species of *Aylacostoma* is absent and in this case, it may be a species already described, but without published molecular data.

As stated by Layton et al. (2014), when examining the barcode region of mollusks, genetic distance values greater than 2% indicate a potential threshold between different species, as observed in other groups (Hebert et al., 2003). The K2P genetic distance values obtained in this study for *Aylacostoma* sp. were 5.40% in relation to *A. chloroticum* and 6% in relation to *A. brunneum*, far exceeding this threshold. Additionally, the positioning of the analyzed specimens in the gene tree supports the hypothesis that these organisms from the Ivaí River belong to a different species of *Aylacostoma*.

The Ivaí River is one of the last rivers in the Paraná River Basin that does not have any dams along its main channel (Affonso et al., 2015), thus representing an important biodiversity refuge. The animals collected in this study were obtained from the same collection site as in previous studies, which recorded *A. chloroticum* (Passere et al., 2022). The differentiation of these organisms was made possible through the use of molecular tools, specifically the *COI* molecular marker, which proved to be an effective method for differentiating gastropod species in the region.

Species in the *Aylacostoma* genus exhibit considerable similarity in their biological characteristics. They occupy the same habitat, which is characterized by higher oxygen levels and a substrate of sand or gravel. Their diet consists of algae and macrophytes, and they undergo a similar life cycle. This is due to them being parthenogenetic, which results in the absence of males (Peso et al., 2013a, 2013b; Vogler, 2013; Vogler et al., 2014). These animals are highly susceptible to anthropogenic disturbances in their environment. The construction of a reservoir has been identified as a significant factor contributing to the extinction of at least three species in the Argentina–Paraguay region (Vogler et al, 2015). In addition to their biological aspects, these animals are extremely similar morphologically, which requires the expertise of a taxonomist who specializes in the group to distinguish the species without the aid of other tools (Vogler et al., 2014).

In light of the fact that the DNA sequences obtained in this study are distinct from any sequence deposited in GenBank and that there is a deficit of information regarding the various species of the genus, it is not feasible to assert that *Aylacostoma* sp. represents a novel species within the genus. Rather, it can be posited that it is a distinct species from those currently available in the genetic database and from those that have been previously identified in the Ivaí River.

Conclusion

In addition to providing the first record of *Aylacostoma* sp. in the Ivaí River, this study also expands taxonomic and genetic knowledge about *Aylacostoma* spp.. This underscores the necessity for continued research, including obtaining sequences of other species of the genus, in order to construct a more robust database that will facilitate the elucidation of gastropod diversity in the region and throughout South America.

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