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Cryptic speciation in populations of the genus *Aphyocharax* (Characiformes: Characidae) from eastern Amazon coastal river drainages and surroundings revealed by single locus species delimitation methods

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Recent studies in eastern Amazon coastal drainages and their surroundings have revealed new fish species that sometimes exhibit little morphological differentiation (cryptic species). Thus, we used a DNA-based species delimitation approach to test if populations showing the morphotype and typical character states of the *Aphyocharax avary* holotype correspond either to *A. avary* or *A. brevicaudatus*, two known species from the region, or if they form independent lineages, indicating cryptic speciation. WP and GMYC analyses recovered five lineages (species) in the ingroup, while a bPTP analysis delimited three lineages. ABGD analyses produced two possible results: one corroborating the WP and GMYC methods and another corroborating the bPTP method. All methods indicate undescribed cryptic species in the region and show variation from at least 1 to 4 species in the ingroup, depending on the approach, corroborating previous studies, and revealing this region as a possible hotspot for discovering undescribed fish species.

Keywords: ABGD, Aphyocharacinae, bPTP, Cryptic speciation, GMYC.

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Estudos recentes nas drenagens costeiras da Amazônia oriental e seus arredores revelaram novas espécies de peixes que às vezes exibem pouca diferenciação morfológica (espécies crípticas). Assim, usamos uma abordagem de delimitação de espécies baseada em DNA para testar se as populações que apresentam o morfotipo e os estados de caráter típicos do holótipo *Aphyocharax avary* correspondem a *A. avary* ou *A. brevicaudatus*, duas espécies conhecidas da região, ou se formam linhagens independentes, indicando especiação críptica. As análises de WP e GMYC recuperaram cinco linhagens (espécies) no grupo interno, enquanto uma análise de bPTP delimitou três linhagens. As análises ABGD produziram dois resultados possíveis: um corroborando os métodos WP e GMYC e outro corroborando o método bPTP. Todos os métodos indicam espécies crípticas não descritas na região e apresentam variação de pelo menos uma a quatro espécies no grupo interno, dependendo da abordagem, corroborando estudos anteriores, e revelando esta região como um possível "hotspot" para descoberta de espécies de peixes não descritas.

Palavras-chave: ABGD, Aphyocharacinae, bPTP, Especiação críptica, GMYC.

INTRODUCTION

Speciation is not always accompanied by changes in morphology (= cryptic speciation), making it difficult to identify species using superficial morphological differentiation (Bickford *et al*., 2006; Adams *et al*., 2014; Ottoni *et al*., 2019). However, DNA-based tools help to identify cryptic speciation events and have been increasingly used in species descriptions and diagnoses (Goldstein, DeSalle, 2010; Pante *et al*., 2015; Souza *et al*., 2018; Ottoni *et al*., 2019; Ochoa *et al*., 2020; Souza *et al*., 2020). In this case, DNA lineages that diverged from an ancestral branch and evolved independently, as observed in differentiated species, must be named according to specific nomenclature codes used by the scientific community.

Aphyocharax Günther, 1868 (Aphyocharacinae) is a monophyletic, small-sized fish genus that comprises 12 valid species (Tagliacollo *et al*., 2012; Betancur-R. *et al*., 2018; Mirande, 2018; Brito *et al*., 2019) distributed in the Orinoco, Amazonas and La Plata river basins, as well as in coastal rivers that drain the Guiana Shield (Tagliacollo *et al*., 2012; Brito *et al*., 2018, 2019). *Aphyocharax brevicaudatus* Brito, Guimarães, Carvalho-Costa & Ottoni, 2019 is the most recently described species (Brito *et al*., 2019) that inhabits the Maracaçumé River basin, an eastern Amazon costal drainage in the state of Maranhão, in northeastern Brazil. The phylogenetic status of *Aphyocharax* is well established, but its internal relationships are not fully resolved (*e.g*., Tagliacollo *et al*., 2012; Brito *et al*., 2019). There are at least four putative species in the genus that are not yet described (see Buckup *et al*., 2007; Brito *et al*., 2019), and the identification and taxonomic status of several populations and species are still unclear (Lima *et al*., 2013; Ohara *et al*., 2017; Brito *et al*., 2018, 2019).

In the last decades, most publications about *Aphyocharax* have focused on ecology or distribution (*e.g*., Gonçalves *et al*., 2005; Corrêa *et al*., 2009; Terán *et al*., 2016), cytogenetic characterization (*e.g*., Souza *et al*., 1995) or phylogeny (*e.g*., Tagliacollo

et al., 2012). However, few studies have focused on the species-level taxonomy of *Aphyocharax*, such as the single taxonomic revision of the genus (an unpublished thesis, Souza-Lima, 2003), some species descriptions (*e.g*., Taphorn, Thomerson, 1991; Willink *et al*., 2003; Brito *et al*., 2019), and some taxonomic works (*e.g*., Souza-Lima, 2003; Brito *et al*., 2018). Moreover, the taxonomic status of some *Aphyocharax* species is questionable and problematic. The imprecise definition of the type locality of several species, old, and change, to and brief morphological descriptions and diagnoses, and confusing taxonomic histories contribute to the problematic taxonomy of this group (Brito *et al*., 2018).

Previous studies indicate the occurrence of several populations of *Aphyocharax* in the coastal basins of the eastern Amazon and surroundings at the state limits of Pará and Maranhão (see Souza-Lima, 2003; Barros *et al*., 2011; Guimarães *et al*., 2020a). These populations have the morphotype and typical character states of the holotype of *Aphyocharax avary* Fowler, 1913, as described and discussed by Brito *et al*. (2018). However, *A. avary* is a species with an imprecise type locality in the Madeira River drainage of the Amazon River basin, with vague distribution records (Brito *et al*., 2018; Dagosta, de Pinna, 2019), and requires a comprehensive taxonomic revision.

According to Guimarães *et al*. (2018a) the river systems in northeastern Brazil, particularly the river basins of the occidental portion (including the region between Rio Gurupi and the Parnaíba basin), exhibit a diversified but poorly explored freshwater fish fauna that is interpreted in different ways in studies based on the distribution patterns of species (*e.g*., Hubert, Renno, 2006; Abell *et al*., 2008; Abreu *et al*., 2019). This region is herein termed "eastern Amazon coastal river basins". Recent studies exemplify the great diversification between coastal basins of Maranhão State, as well as the lack of knowledge about the fish in these areas (*e.g*., Guimarães *et al*., 2018a,b; Abreu *et al*., 2019; Brito *et al*., 2019; Guimarães *et al*., 2019; Abreu *et al*., 2020; Guimarães *et al*., 2020a).

In the river drainages of the state of Maranhão (Itapecuru, Mearim, Munim, and Tocantins River draineages), there are several populations with superficial morphology similar to *A. avary*. In this context, we combined newly generated mitochondrial sequences with those generated by Oliveira *et al*. (2011) and Tagliacollo *et al*. (2012) to study species diversity and delimit species boundaries within *Aphyocharax* from several coastal basins in the eastern Amazon coastal river basins and surroundings.

MATERIAL AND METHODS

Study area. The study was carried out in the coastal basins and drainages in the state of Maranhão, including a region between the Gurupi and Parnaíba river basins, in addition to the Tocantins River basin in the state (Fig. 1). The study area consists of 10 hydrographic basins, seven of which are state owned (Mearim, Itapecuru, Munim, Turiaçú, Maracaçumé, Preguiças and Periá basins) and three are federally owned (Parnaíba, Tocantins, Gurupi). Together, these occupy an area of 113,068.15 km^2 (34.6% of the state of Maranhão) that is subdivided into three Brazilian hydrographic regions (Tocantins-Araguaia, Western Northeast Atlantic and Parnaíba) (MMA, 2006; NUGEO, 2016).

Maranhão is the westernmost state in the Northeast Region of Brazil and borders the eastern side of Pará State in the North Region of the country. It has an area of about 320,000 km^2 and occupies about 3.9% of Brazil (Rebêlo *et al*., 2003). The state harbors three of the main Brazilian biomes, and transition ecotone areas between Amazonian tropical forests and open shrubby and dry forests of Cerrado and Caatinga. Therefore, this region is considered very important for ecological services and biodiversity conservation (Guimarães *et al*., 2018a).

Ethical statement, specimen collection and preservation. Specimens selected for molecular data analysis (Tab. 1) were fixed and preserved in absolute ethanol.

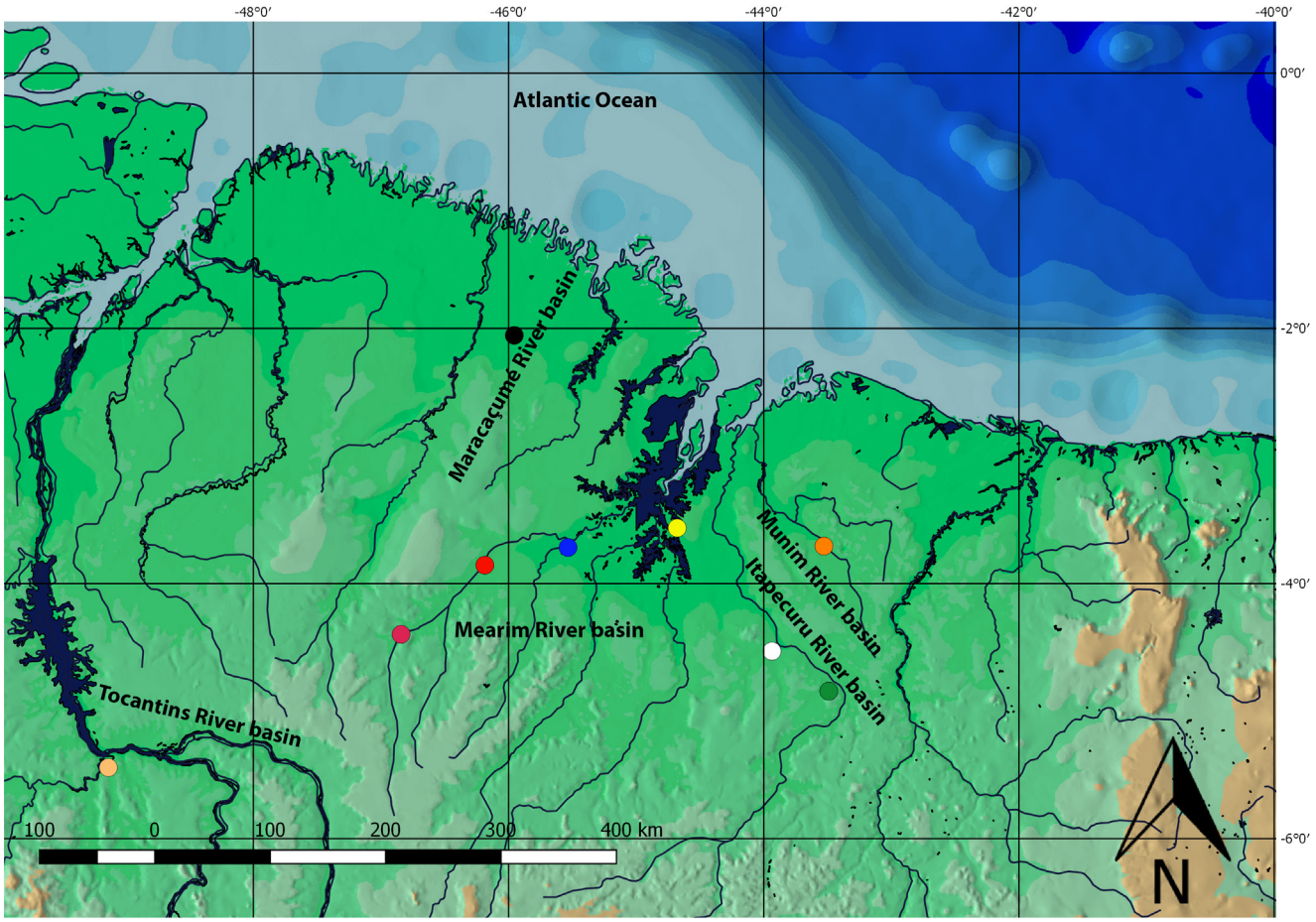

FIGURE 1 | Map of the sampling localities of *Aphyocharax* specimens in eastern Amazon coastal river drainages and surroundings in Brazil. Black circle = *Aphyocharax brevicaudatus*, Rio Maracaçumé/Maracaçumé/MA; orange circle = *Aphyocharax* sp. "Munim", Riacho Fundo/Rio Munim/Chapadinha/MA; white circle = *Aphyocharax* sp. (Itapecuru), Riacho Primavera/Rio Itapecuru/Caxias/MA; green circle = *Aphyocharax* sp. (Itapecuru), Rio Saco/Rio Itapecuru/Codó/MA; yellow circle = *Aphyocharax* sp. (Mearim), Bacia 464/Rio Mearim/Arari/MA; blue circle = *Aphyocharax* sp. (Mearim), Rio Zutíua/Rio Pindaré/Rio Mearim/Pindaré-Mirim/MA; red circle = *Aphyocharax* sp. (Mearim), Rio Zutíua/Rio Pindaré/Rio Mearim/Pindaré-Mirim/MA; pink circle = *Aphyocharax* sp. (Mearim), Rio Pindaré/Rio Mearim/Bom Jesus das Selvas/MA; salmon-pink circle = *Aphyocharax avary*, Rio Sororó/Rio Tocantins/Marabá/PA.

DNA extraction, amplification, and sequencing. DNA extraction was carried out with a Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's protocol. The DNA quality was evaluated using 0.8% agarose gel electrophoresis stained with GelRed (Biotium). The DNA was stored at -20 °C until further procedures. The partial Cytochrome B mitochondrial gene (CytB) was amplified using standard PCR (polymerase chain reaction) and primers developed by Kocher *et al*. (1989) (L14841 5' – AAATCAAAGCATAACACTGAAGATG – 3') and Irwin *et al*. (1991) (H15915 5' – CCAATTTGCATGGATGTCTTCTCGG – 3').

Amplification reactions were performed at a total volume of 15 µl comprising 10× buffer, 1.5 mM $MgCl_2$, 400 µM dNTP, 0.2 µM of each primer, 1 U of Taq Polymerase (Invitrogen), 100 ηg of DNA template and ultrapure water. The amplification program consisted of a denaturation of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 46–48 °C for 45 s, and 72 °C for 80 s, and an extension phase of 5 min at 72 °C. Amplicons were visualized using 1% agarose gel electrophoresis stained with GelRed (Biotium) and purified with Illustra GFX PCR DNA and a Gel Purification Kit (GE Healthcare). Samples were sequenced using both forward and reverse primers with a BigDye Terminator 3.1 Cycle Sequencing Kit in an ABI 3730 DNA Analyzer (Thermo Fisher Scientific).

Data analyses. The dataset included the partial CytB (678 base pairs, bp) and sequences from other species of *Aphyocharax* and allied genera from the National Center for Biotechnology Information (NCBI) databases (Tab. 1). Sequences were aligned using ClustalW (Chenna *et al*., 2003) and translated into amino acid residues using the program MEGA 7 (Kumar *et al*., 2016) to test if the sequences came from NUMTs (nuclear mitochondrial DNA sequences), in which case premature stop codons or indels are expected. The best-fit evolutionary model (GTR+I+G) was selected using the Akaike information criterion (AIC) and jModelTest 2.1.7 (Darriba *et al*., 2012), and used in all analyses, except for Automatic Barcode Gap Discovery (ABGD).

Species concept, species delimitation, and diagnoses. The unified species concept was adopted, considering that "species are (segments of) separately evolving metapopulation lineages" (de Queiroz, 2005, 2007). According to this concept, species are treated as hypothetical units that could be tested (detected) by applying distinct criteria (*i.e*., species delimitation methods), allowing for any method to independently provide evidence about species limits and identities (de Queiroz, 2005, 2007). Four distinct and independent single locus species delimitation methods relying on different operational criteria for species delimitation were implemented: ABGD, Automatic Barcode Gap Discovery (Puillandre *et al*., 2012); WP, a tree-based method proposed by Wiens, Penkrot (2002) (following Sites, Marshall, 2003); and two coalescent-based species delimitation methods termed bPTP, the Bayesian implementation of the Poisson tree processes (Zhang *et al*., 2013), and GMYC, the General Mixed Yule Coalescent method, single-threshold version (Fujisawa, Barraclough, 2013). All species delimitation methods were performed using the Cytb sequences, which is widely used for single locus species delimitation approaches (Avise, 2000).

TABLE 1 | List of species, specimens and their respective GenBank sequence accession numbers. Sequences made available by this study are in bold.

Species	Catalog number	Genbank accession	Locality (Country/River/River drainage/Municipality/State)
Aphyocharacidium bolivianum	LBP 9055	HQ289710	Brazil/Arara/RO
Aphyocharax anisitsi	LBP 4750	JQ820081	Brazil
Aphyocharax anisitsi	LBP 3764	HQ289581	Brazil/Rio Negro/Aquidauana/MS
Aphyocharax avary	CICCAA02344.1	MK409660	Brazil/Rio Sororó/Rio Tocantins/Marabá/PA
Aphyocharax avary	**CICCAA 02344.2**	**MZ558446**	Brazil/Rio Sororó/Rio Tocantins/Marabá/PA
Aphyocharax avary	CICCAA02344.3	MK409661	Brazil/Rio Sororó/Rio Tocantins/Marabá/PA
Aphyocharax brevicaudatus (female)	CICCAA 02306	MK409668	Brazil/Rio Maracaçumé/Maracaçumé/MA
Aphyocharax brevicaudatus (male)	CICCAA 02308	MK409669	Brazil/Rio Maracaçumé/Maracaçumé/MA
Aphyocharax brevicaudatus (male)	CICCAA 02310	MK409670	Brazil/Rio Maracaçumé/Maracaçumé/MA
Aphyocharax dentatus	LBP 5112	JQ820082	Brazil/Rio Paraguai/Cáceres/MT
Aphyocharax dentatus	LBP 20	JQ820083	Brazil/Rio Paraguai/Rio Miranda/Corumbá/MT
Aphyocharax cf. *erythrurus*	LBP 15819	JQ820076	Venezuela
Aphyocharax cf. *erythrurus*	LBP 15820	JQ820077	Venezuela
Aphyocharax nattereri	LBP 3786	JQ820070	Brazil/Rio Paraguai/lagoa marginal do Rio Negro/Aquidauana/MS
Aphyocharax nattereri	LBP 3734	JQ820071	Brazil
Aphyocharax pusillus	LBP 23546	JQ820078	Brazil
Aphyocharax pusillus	LBP 4046	HQ289590	Brazil/Rio Moa/Cruzeiro do Sul/AC
Aphyocharax rathbuni	LBP 7608	JQ820079	Brazil/La Plata basin /Lagoa marginal do rio Cuiabá/Barão de Melgaço/MT
Aphyocharax rathbuni	LBP 8457	JQ820080	Brazil
Aphyocharax sp.	LBP 1587	HQ289533	Brazil/Rio das Garças/Rio Araguaia/Barra do Garças/MT
Aphyocharax sp.	LBP 2480	JQ820084	Brazil/Rio Araguaia/Aragarças/GO
***Aphyocharax* sp. "Tapajós"**	**CICCAA 04851.1**	**MZ558447**	Brazil/Lago Papucu/Rio Tapajós/Santarém/PA
***Aphyocharax* sp. "Tapajós"**	**CICCAA 04851.3**	**MZ558448**	Brazil/Lago Papucu/Rio Tapajós/Santarém/PA
***Aphyocharax* sp. "Tapajós"**	**CICCAA 04851.4**	**MZ558449**	Brazil/Lago Papucu/Rio Tapajós/Santarém/PA
***Aphyocharax* sp. "Tapajós"**	**CICCAA 04851.6**	**MZ558450**	Brazil/Lago Papucu/Rio Tapajós/Santarém/PA
***Aphyocharax* sp. "Solimões"**	**CICCAA 04836.1**	**MZ558451**	Brazil/Ilha Cuera/Rio Solimões/Tefé/AM
***Aphyocharax* sp. "Solimões"**	**CICCAA 04836.3**	**MZ558452**	Brazil/Ilha Cuera/Rio Solimões/Tefé/AM
***Aphyocharax* sp. "Solimões"**	**CICCAA 04840.1**	**MZ558453**	Brazil/Lago Amanã/Rio Solimões/Maraã/AM
***Aphyocharax* sp. "Solimões"**	**CICCAA 04840.2**	**MZ5584534**	Brazil/Lago Amanã/Rio Solimões/Maraã/AM
***Aphyocharax* sp. "Solimões"**	**CICCAA 04840.3**	**MZ5584535**	Brazil/Lago Amanã/Rio Solimões/Maraã/AM
***Aphyocharax* sp. "Mearim"**	**CICCAA 02107.1**	**MZ558436**	Brazil/Bacia 464/Rio Mearim/Arari/MA
***Aphyocharax* sp. "Mearim"**	**CICCAA 02107.2**	**MZ558437**	Brazil/Bacia 464/Rio Mearim/Arari/MA
***Aphyocharax* sp. "Mearim"**	**CICCAA 02107.3**	**MZ558438**	Brazil/Bacia 464/Rio Mearim/Arari/MA
***Aphyocharax* sp. "Mearim"**	**CICCAA 02107.4**	**MZ558439**	Brazil/Bacia 464/Rio Mearim/Arari/MA

TABLE 1 | (Continued)

Species	Catalog number	Genbank accession	Locality (Country/River/River drainage/Municipality/State)
***Aphyocharax* sp. "Mearim"**	**CICCAA 02107.5**	**MZ558440**	Brazil/Bacia 464/Rio Mearim/Arari/MA
***Aphyocharax* sp. "Pindaré"**	**CICCAA 02320**	**MZ558441**	Brazil/Igarapé Jenipapo/Rio Pindaré/Rio Mearim/Alto Alegre do Pindaré/MA
***Aphyocharax* sp. "Pindaré"**	**CICCAA 02323**	**MZ558442**	Brazil/Rio Zutíua/Rio Pindaré/Rio Mearim/Pindaré-Mirim/MA
***Aphyocharax* sp. "Pindaré"**	**CICCAA 02033**	**MK409665**	Brazil/Rio Pindaré/Rio Mearim/Bom Jesus das Selvas/MA
***Aphyocharax* sp. "Itapecuru"**	**CICCAA 02034**	**MK409666**	Brazil/Riacho Primavera/Rio Itapecuru/Caxias/MA
***Aphyocharax* sp. "Itapecuru"**	**CICCAA 02357**	**MK409667**	Brazil/Riacho Primavera/Rio Itapecuru/Caxias/MA
***Aphyocharax* sp. "Itapecuru"**	**CICCAA 02315.1**	**MZ558443**	Brazil/Rio Saco/Rio Itapecuru/Codó/MA
***Aphyocharax* sp. "Itapecuru"**	**CICCAA 02315.2**	**MZ558444**	Brazil/Rio Saco/Rio Itapecuru/Codó/MA
***Aphyocharax* sp. "Itapecuru"**	**CICCAA 02316**	**MZ558445**	Brazil/Rio Saco/Rio Itapecuru/Codó/MA
***Aphyocharax* sp. "Munim"**	**CICCAA02345.1**	MK409662	Brazil/Riacho Fundo/Rio Munim/Chapadinha/MA
***Aphyocharax* sp. "Munim"**	**CICCAA02345.2**	MK409663	Brazil/Riacho Fundo/Rio Munim/Chapadinha/MA
***Aphyocharax* sp. "Munim"**	**CICCAA02345.3**	MK409664	Brazil/Riacho Fundo/Rio Munim/Chapadinha/MA
Prionobrama paraguayensis	LBP 3230	JQ820073	Brazil/Lagoa marginal/Rio Cuiabazinho/Nobre/MT
Prionobrama paraguayensis	LBP 3230	JQ820072	Brazil/Lagoa marginal/Rio Cuiabazinho/Nobre/MT
Prionobrama filigera	LBP 4139	JQ820075	Brazil/Rio Juruá/Rio Moa/Mâncio Lima/AC
Prionobrama filigera	LBP 4139	JQ820074	Brazil/Rio Juruá/Rio Moa/Mâncio Lima/AC
Leptagoniates steindachneri	LBP 4137	HQ289600	Brazil/ Rio Moa/Mâncio Lima/AC
Paragoniates alburnus	LBP 9208	HQ289712	Venezuela/Rio Manapire/Cabruta/Guárico
Phenagoniates macrolepis	LBP 6105	HQ289678	Venezuela/Rio Apon Medio/Machiques de Perijá/Zulia
Xenagoniates bondi	LBP 3074	HQ289563	Venezuela/Rio Orinoco/Caicara del Orinoco/Bolivar

Wiens and Penkrot analysis (WP). WP is based on the direct inspection of haplotype trees generated by a phylogenetic analysis with at least two individuals (haplotypes) of each focal species as terminals. In this method, the term "exclusive" is used instead of monophyletic since the term monophyly is considered inapplicable below the species level (Wiens, Penkrot, 2002). Clustered haplotypes with a concordant geographic distribution that form mutual and well supported clades (exclusive lineages) are strong evidence for species discrimination (absence of gene flow with other lineages). When haplotypes from the same locality fail to cluster together, there is potential evidence for gene flow with other populations (Wiens, Penkrot, 2002). Statistical support for the haplotypic tree was assessed by the posterior probability, with about 0.95 or higher considered significant (Alfaro, Holder, 2006). When only one haplotype (specimen) from one putative population was available, the species delimitation was based on the exclusivity of the sister clade of this single haplotype supported by significant values (Wiens, Penkrot, 2002). In addition, the method recognizes non-exclusive lineages as species since their sister clades are exclusive and supported by significant values (Wiens, Penkrot, 2002).

A Bayesian inference (BI) phylogenetic tree was estimated with the software MrBayes 3.2 (Ronquist *et al*., 2012) to reconstruct the evolutionary relationships among terminals using the general time reversible (GTR+I+G) evolutionary model. The BI analysis was conducted with the following parameters: two independent Markov chain Monte Carlo (MCMC) runs of two chains each for 10 million generations, with a tree sampling frequency every 1,000 generations. The convergence of the MCMC chains and the proper burn-in value were assessed by evaluating the stationary phase of the chains using Tracer v. 1.6 (Rambaut *et al*., 2014). After removing the first 25% of the samples (burn-in), the final consensus tree and its posterior probabilities were generated with the remaining tree samples.

The ingroup included species and populations with the morphotype and typical characters of *A. avary* in the eastern Amazon coastal river drainages and surroundings: *Aphyocharax brevicaudatus* (from the Maracaçumé River basin), *A. avary* (from the Tocantins River basin), *Aphyocharax* sp. "Itapecuru" (from the Itapecuru River basin), *Aphyocharax* sp. "Mearim" (from the Mearim River basin), and *Aphyocharax* sp. "Munim" (from the Munim River basin) (colored haplotypes in Fig. 2; Tab. 1). As the outgroup, we used sequences of *Aphyocharacidium bolivianum* Géry, 1973, *Leptagoniates steindachneri* Boulenger, 1887, *Paragoniates alburnus* Steindachner, 1876, *Phenagoniates macrolepis* (Meek & Hildebrand, 1913), *Prionobrama filigera* (Cope, 1870), *Prionobrama paraguayensis* (Eigenmann, 1914), *Xenagoniates bondi* Myers, 1942, and other species and populations of *Aphyocharax* (excluding those of the ingroup) (uncolored haplotypes in Fig. 2; Tab. 1).

General Mixed Yule Coalescent (GMYC). The GMYC is a single locus coalescent-based species delimitation approach that relies on branch lengths to establish a threshold between speciation and coalescent processes (Fujisawa, Barraclough, 2013). Here we applied the single-threshold version of the method, which usually outperforms the multiple-threshold version (Fujisawa, Barraclough, 2013). A new dataset was created for this analysis, including only the ingroup (see WP section) and the clade with *Aphyocharax* cf. *erythrurus*, *A. pusillus*, *Aphyocharax* sp. "Solimões" + *Aphyocharax* sp. "Tapajós" (see Tab. 1) as the outgroup, which has the geographically closest populations to the ingroup. This new dataset was reduced to include only unique haplotypes using DAMBE5 (Xia, 2013) and the requirements of this method.

The input ultrametric phylogenetic tree was made in BEAST v.1.8.4 (Drummond *et al*., 2012) with the following parameters: an uncorrelated relaxed clock with lognormal distribution, a Yule process as the tree prior with 10 million generations and sampling frequency of 1000. The GMYC analysis was performed using the Exelixis Lab's server (https://species.h-its.org/gmyc/). We also performed a GMYC test analysis with the same parameters but included all the available species of *Aphyocharax* (see Fig. **S1**).

Bayesian implementation of the Poisson tree processes (bPTP). The bPTP is another single locus coalescent-based species delimitation method, but it differs from other similar approaches, such as GMYC, since an ultrametric tree is not needed (not relying on branch lengths to delimit species), thus avoiding errors and computer intensive processes (Zhang *et al*., 2013). The method assumes that more molecular variability (number of nucleotide substitutions) is expected between haplotypes from

different species than within a species (Zhang *et al*., 2013), establishing a threshold between speciation and coalescent processes. The reduced dataset for performing the bPTP was the same used in the GMYC, following the requirements of this method. The input phylogenetic tree was estimated with the software Mrbayes 3.2 (Ronquist *et al*., 2012) following the same parameters used in the WP. The bPTP analysis was performed using the Exelixis Lab's web server (http://species.h-its.org/ptp/), following the default parameters except for a 20% burn-in. *Aphyocharax* cf. *erythrurus* was chosen as the outgroup since it is the most geographically distant species from the ingroup in this reduced dataset. We also performed a bPTP test analysis with the same parameters but included all the available species of *Aphyocharax* (see Fig. **S2**).

Automatic Barcode Gap Discovery (ABGD). The ABGD is a barcode species delimitation method that aims to establish a minimum gap that probably corresponds to the threshold between interspecific and intraspecific processes (Puillandre *et al*., 2012). The major advantage of ABGD compared to the other barcode species delimitation methods is that the inference of the limit between interspecific and intraspecific processes (gap detection) is recursively applied to previously obtained groups to get finer partitions until there is no further partitioning, allowing a more refined search. Basically, the ABGD analysis indicates the number of groups (species) estimated relative to a large spectrum of p values (prior intraspecific values). For this, a 0.1 value assumes the maximum intraspecific variability, indicating that all sequences belong to only one species, and a 0.001 value assumes very low intraspecific variability, indicating that each distinct haplotype represents a different species. After running the ABGD, additional molecular, morphological, or ecological characters are needed to infer the correct number of species, following an integrative taxonomic perspective. The reduced dataset for performing the ABGD was the same used in the GMYC and bPTP. The analysis was conducted using the ABGD server website (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) following the default parameters.

RESULTS

Phylogenetic analysis. The topology of our phylogenetic analysis (Fig. 2) recovered *Aphyocharax* as monophyletic but supported by a low posterior probability value (0.88), with *A. nattereri* as sister to all other *Aphyocharax*. However, all the other lineages sister to *A. nattereri* formed a large clade supported with the maximum posterior probability value (1) (Fig. 2). Within this large clade, three other clades formed: 1– comprising *A. anisitsi*, *A. dentatus*, *A. rathbuni* and a species not identified to the species level, but with low node support (0.59); 2– comprising *Aphyocharax* cf. *erythrurus* and several haplotypes considered here as *A. pusillus*, supported by the maximum support value (1); and 3– comprising our ingroup (populations occurring in the eastern Amazon coastal river drainages and surroundings, *i.e*., *Aphyocharax brevicaudatus* [from the Maracaçumé River basin], *A. avary* [from the Tocantins River basin], *Aphyocharax* sp. "Itapecuru" [from the Itapecuru River basin], *Aphyocharax* sp. "Mearim" [from the Mearim River basin] and *Aphyocharax* sp. "Munim" [from the Munim River basin]) supported by the maximum support value (1). The last two clades are probably sister

groups; however, they are supported by low posterior probability (0.55) (Fig. 2).

Species delimitation. WP. The BI phylogenetic analysis delimited five species within our ingroup: 1– *Aphyocharax brevicaudatus* (yellow colored); 2– *A. avary* (grey colored); 3–*Aphyocharax* sp. "Itapecuru" (blue colored); 4– *Aphyocharax* sp. "Mearim" (red colored); and 5– *Aphyocharax* sp. "Munim" (green colored). The haplotypes of each species formed exclusive clades with the maximum posterior probability value (1) (Fig. 2).

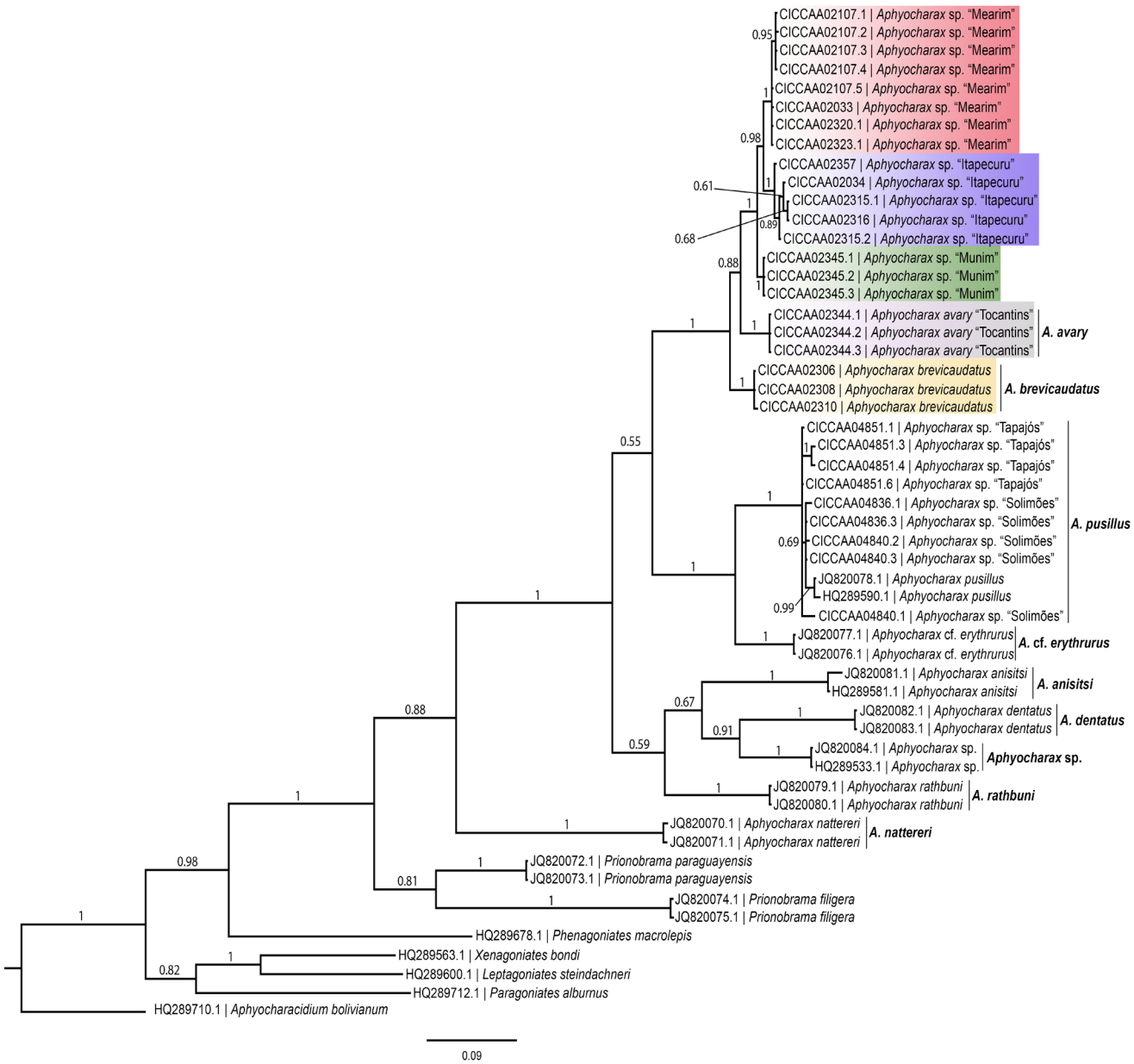

FIGURE 2 | Bayesian inference phylogenetic tree used in the WP species delimitation method. Numbers above and below branches are posterior probability values. The colored haplotypes indicate the ingroup. Each group of haplotypes in the ingroup delimitated by WP as an exclusive lineage (species) has a different coloration. Species delimitated in our ingroup by WP: 1– *Aphyocharax brevicaudatus* (yellow colored); 2– *A. avary* (grey colored); 3– *A.* sp. "Itapecuru" (blue colored); 4– *A.* sp. "Mearim" (red colored); and 5– *A.* sp. "Munim" (green colored).

GMYC and bPTP. Both single locus coalescent species delimitation methods delimited different lineages (species). The GMYC delimitated the following species: *Aphyocharax brevicaudatus*, *A. avary*, *Aphyocharax* sp. "Itapecuru," *Aphyocharax* sp. "Mearim," and *Aphyocharax* sp. "Munim" (Fig. 2). The bPTP delimitated the following species: *Aphyocharax brevicaudatus*, *A. avary*, and one single lineage including *Aphyocharax* sp. "Itapecuru" + *Aphyocharax* sp. "Mearim" + *Aphyocharax* sp. "Munim" (Fig. 3).

ABDG. This method had two possible results. Result 1 delimited three species within our ingroup: *Aphyocharax brevicaudatus*, *A. avary*, and one single lineage including *Aphyocharax* sp. "Itapecuru" + *Aphyocharax* sp. "Mearim" + *Aphyocharax* sp. "Munim" (Fig. 3). These same three groups (species) were delimited between p values ranging from 0.0077 and 0.0028. Result 2 delimited five species within our ingroup: *Aphyocharax brevicaudatus*, *A. avary*, *Aphyocharax* sp. "Itapecuru," *Aphyocharax* sp. "Mearim" and *Aphyocharax* sp. "Munim." Initially, result 2 delimited the same groups as result 1, but a "recursive partition" later delimited the five groups listed above (Fig. 3) with a p value of 0.0017.

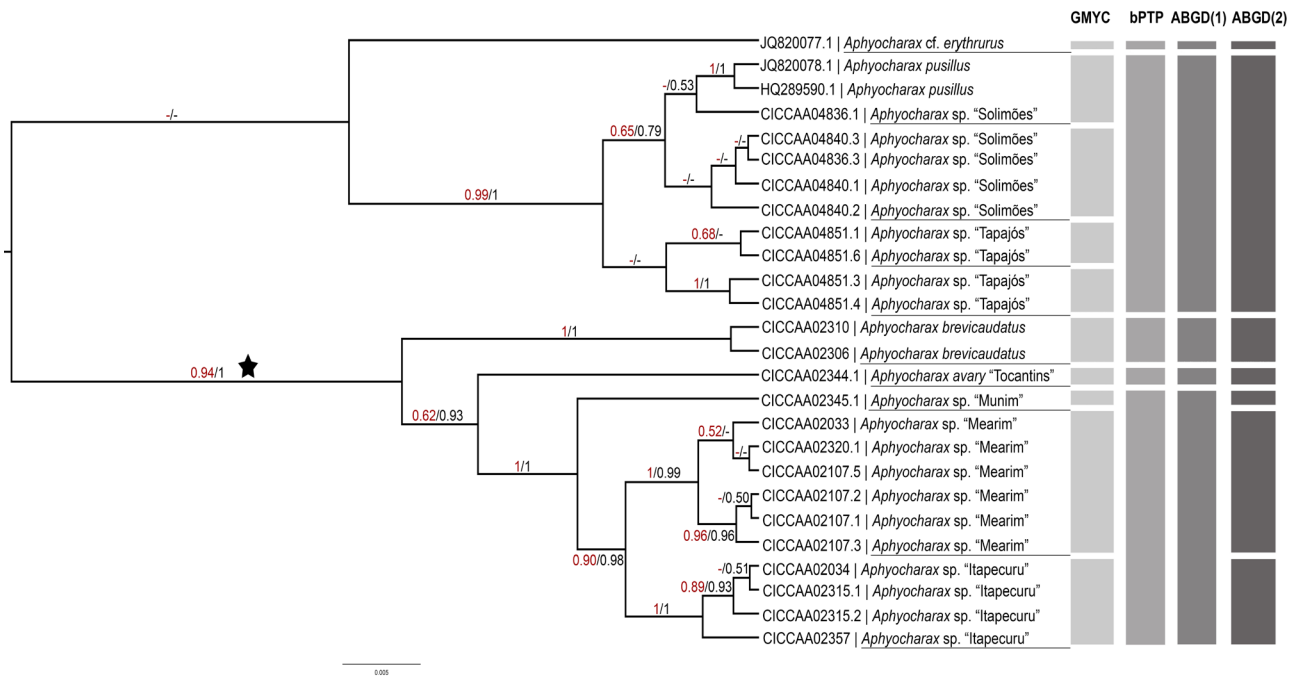

FIGURE 3 | Ultrametric tree (Bayesian inference) including unique haplotypes summarizing the results of the GMYC, bPTP and ABGD (results 1 and 2). Numbers above and below branches are posterior probability values. Red values: posterior probabilities calculated by the software Beast; black values: posterior probabilities calculated by the software Mr. Bayes. Values below 50 are indicated as "- ". The stars indicate the ingroup.

DISCUSSION

Besides our phylogenetic approach to delimit lineages using species delimitation methods, we can state the following about the *Aphyocharax* phylogeny. The topology of our phylogenetic analysis (Fig. 2) recovered *Aphyocharax* as monophyletic but supported by a low posterior probability value - pp (0.88), with *A. nattereri* as sister to all other *Aphyocharax* species. The reason for the low support is probably because the fast evolutionary rate of Cytochrome b (Cytb) is more suitable for species delimitation or to reconstruct the relationships of closely related species (Avise, 2000). However, besides *A. nattereri*, the other remaining OTU form a robust clade supported by the maximum posterior probability value.

The last clade also includes two smaller clades supported by low posterior probability values, which demonstrates that the gene used is not suitable for recovering older and the most basal branch and node relationships (Fig. 2). The first clade (pp = 0.59) includes *A. rathbuni*, *A. anisitsi*, a putative undescribed species (*Aphyocharax* sp.) and *A. dentatus*. The second clade (node support = 0.55) includes two other clades. Clade 1 comprises several haplotypes considered here as *A. pusillus* and *Aphyocharax* cf. *erythrurus* (with maximum node support). Clade 2 contains our ingroup (with maximum node support) that comprises species and populations occurring along the coastal river basins of the eastern Amazon region and surroundings: *Aphyocharax brevicaudatus* (from the Maracaçumé River basin), *A. avary* (from the Tocantins River basin), *Aphyocharax* sp. "Itapecuru" (from the Itapecuru River basin), *Aphyocharax* sp. "Mearim" (from the Mearim River basin) and *Aphyocharax* sp. "Munim" (from the Munim River basin) (colored haplotypes in Fig. 2; Tab. 1).

The species and populations of the latter clade (colored clade) have the morphotype and typical character states of the type material of *A. avary* (as described and discussed by Brito *et al*., 2018), a species with an imprecise type locality in the Madeira River drainage of the Amazon River basin (Brito *et al*., 2018). *Aphyocharax brevicaudatus* is easily distinguished from the populations of our ingroup by unambiguous character states (see Brito *et al*., 2019). It is the most basal taxon in the ingroup clade. *Aphyocharax avary* was recovered as the sister group of all the other lineages from the coastal river basins in the eastern Amazon region. This clade has a moderate node support value (0.88). The monophyly of the lineages from these coastal river basins is supported by the maximum node support value (Fig. 2). Within this latter group, the lineage *Aphyocharax* sp. "Munim" is the sister group to the clade *Aphyocharax* sp. "Itapecuru" and *Aphyocharax* sp. "Mearim." This last clade has a high node support value (0.98) (Fig. 2).

The WP method recovered five lineages (*i.e*., species) in our ingroup that are all supported by the maximum node support value: 1– *Aphyocharax brevicaudatus* (yellow colored); 2– *A. avary* (grey colored); 3– *Aphyocharax* sp. "Itapecuru" (blue colored); 4– *Aphyocharax* sp. "Mearim" (red colored); and 5– *Aphyocharax* sp. "Munim" (green colored) (Fig. 2). Three of these lineages are possible undescribed cryptic species. *Aphyocharax avary* from the Tocantins River basin might be another one due to its disjunct geographical distribution to the type locality of *A. avary*. However, resolving this issue depends on the inclusion of haplotypes from the type locality of *A. avary*. Therefore, this species delimitation method recovered at least three undescribed species.

The GMYC single-threshold version delimited the same lineages (*i.e*., species) as the

WP (Fig. 3) in our ingroup. On the other hand, the bPTP delimited three lineages in our ingroup: *Aphyocharax brevicaudatus*, *A. avary*, and a single lineage including *Aphyocharax* sp. "Itapecuru" + *Aphyocharax* sp. "Mearim" + *Aphyocharax* sp. "Munim" (Fig. 3). Thus, according to the bPTP, we have at least one undescribed cryptic species in the region. The situation for *Aphyocharax avary* from the Tocantins River is the same here compared to the WP and GMYC. The ABGD analyses produced two possible results: one corroborating the same result from the bPTP (ABGD result 1) in our ingroup, and another (ABGD result 2) corroborating the results from the WP and GMYC analyses (Fig. 2) in our ingroup. Therefore, the number of species depends on the method used, although all of them pointed to undescribed cryptic species in the region. Some studies argue that in some cases GMYC can lead to an overestimation of the number of species (*e.g.*, Talavera *et al.*, 2013; García-Melo *et al.*, 2019). Despite that in our study the GMYC analysis delimited the highest number of species, the results of this method were corroborated by the WP and ABGD result 2. Therefore, we cannot state that the GMYC analysis overestimated the number of delimited species in our study.

Although molecular taxonomy studies involving fishes from the coastal river basins of the Eastern Amazon region are still scarce, some studies have been published in recent years, especially for the Characidae, which corroborate that this region has endemic characid species (*e.g.*, Guimarães *et al.*, 2018b, 2019, 2020b; Brito *et al.*, 2019). Along with these studies, we show the usefulness of molecular approaches for revealing cryptic lineages (species) in the region and opportunities for new discoveries to enhance our knowledge of neotropical freshwater fishes.

Based on our data obtained here, it is clear that the Cytb gene is very effective at delimiting lineages (species) due to its rapid evolutionary rate. Another conclusion is that there is one or more cryptic undescribed species of *Aphyocharax*, depending on the operational criterium used to delimit species, in the coastal river basins of the Eastern Amazon region. This is not limited to *Aphyocharax*, since other published and unpublished studies have found similar results for other characin groups (*e.g.*, Guimarães *et al.*, 2018b, 2019, 2020) and Characidae genera (E. C. Guimarães, 2021, pers. comm.). If similar approaches are applied, the same results might be found for other freshwater fish groups in the studied region. Thus, further studies using molecular approaches in the study area are needed to unravel hidden species and accurately estimate the freshwater fish diversity of the region.

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Pâmella Silva de Brito: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing-original draft, Writing-review and editing.

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ETHICAL STATEMENTS

Individuals collected for this study were euthanized with a buffered solution of Tricaine methanesulfonate MS–222 (250 mg/L) for 10 min or more, until the opercular movements completely ceased, according to

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animal welfare laws and guidelines (Close *et al*., 1996, 1997; Leary *et al*., 2013). Sampling was authorized by IBAMA and SISBIO through the documents Nº 02001.007241/2004–37 and Nº 42415, respectively.

COMPETING INTERESTS

The authors declare no competing interests.

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