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Low genetic variability in *Prochilodus lacustris* and *Prochilodus nigricans* from river basins of northern and northeastern Brazil

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ABSTRACT

The genus *Prochilodus* is an important fish resource. The various species within this genus are morphologically very similar, making reliable taxonomic identification difficult and creating uncertainty regarding the exact status of the different forms. This study aimed to evaluate the genetic differentiation between two species of *Prochilodus* belonging to the same lineage from the Maranhão and Amazon River basins based on mitochondrial DNA markers (16S rRNA, COI, Cyt b) and nuclear (TROP) DNA sequences. A total of 151 samples were collected from the Maranhão and Amazon rivers, followed by DNA extraction, polymerase chain reaction, sequencing, and data analysis, with the gene fragments analyzed separately and concatenated. The results revealed a strong genetic similarity between the species, with low genetic divergence values (0 to 0.35%) for the genes under study.

Keywords: Maranhão, Neotropical region, Taxonomic, Migratory fishes, Hydrographic basin.

Evidência de baixa variabilidade genética em *Prochilodus lacustris* e *Prochilodus nigricans* nas bacias hidrográficas do Atlântico nordeste ocidental

RESUMO

O gênero *Prochilodus* é um importante recurso pesqueiro. As várias espécies desse gênero são morfologicamente muito semelhantes, dificultando a identificação taxonômica confiável e criando incerteza quanto ao *status* exato das diferentes formas. O objetivo deste estudo foi investigar duas espécies de *Prochilodus* pertencentes à mesma linhagem nas bacias dos rios Maranhão e Amazonas com base em sequências de DNA mitocondrial (16S rRNA, COI, Cyt b) e nuclear (TROP). O total de 151 amostras foi coletada dos rios Maranhão e Amazonas, coleta seguida de extração de DNA, reação em cadeia da polimerase, sequenciamento e análise de dados, com os fragmentos de genes analisados separadamente e concatenados. Os resultados revelaram forte similaridade genética entre as espécies, com baixos valores de divergência genética (0 a 0,35%) para os genes em estudo.

Palavras-chave: Maranhão, Região neotropical, Taxonômica, Peixes migratórios, Bacia hidrográfica.

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INTRODUCTION

The family Prochilodontidae are widely distributed in the freshwater environments of the Neotropical region (Taphorn, 1992; Turner et al., 2004). There are three prochilodontid genera (*Ichthyoelephas*, *Semaprochilodus*, and *Prochilodus*) and around 20 valid species (Castro & Vari, 2004). The genus *Prochilodus* is the most speciose, with 13 representatives (Castro & Vari, 2004).

The species *Prochilodus lacustris* (Steindachner, 1907) is endemic to the hydrographic basins of the Parnaíba, Mearim, and Tocantins rivers in northern Brazil (Cardoso et al., 2019; Garavello et al., 2010; Piorski et al., 2007; Piorski, 2010; Ramos et al., 2014), while *Prochilodus nigricans* (Spix & Agassiz, 1829) is endemic to the Amazon and Tocantins basins (Castro & Vari, 2004; Machado et al., 2017; Queiroz et al., 2013). These distributions have since been extended to other Maranhão basins, with *P. lacustris* found in the Itapecuru, Mearim, Turiaçu, and Pericumã river basins (Abreu et al., 2019; Barros et al., 2011; Limeira-Filho et al., 2023; Nascimento et al., 2016), and *P. nigricans* found in the Itapecuru, Mearim, and Turiaçu basins (Abreu et al., 2019). Both species represent significant fishery resources in their respective local areas.

The species *P. lacustris* can be distinguished from their congeners by traits such as scales with poorly defined cruciform subdivisions and a greater number of horizontal rows of scales between the origin of the pelvic fin and the lateral line (Castro & Vari, 2004). *Prochilodus nigricans* is characterized by dark, though inconspicuous, stripes on the back, while the caudal, dorsal, and anal fins exhibit several alternating dark and light spots (Mota & Ruffino, 1997). Consequently, the two species can only be distinguished by their striping patterns and meristic parameters (Castro & Vari, 2004). Traditional taxonomic approaches have relied on morphological characteristics for the delimitation of prochilodontid species, but this does not necessarily resolve the classification of some natural groups, as morphologically similar species can be assigned to the same nominal taxon (Bickford et al., 2007). Deciphering and defining the enigmatic diversity within a group of organisms has been challenging, being necessary the use of additional biological tools (Kress et al., 2015).

Given the limited morphological differentiation, molecular markers have indicated that these species form one of the problematic species complexes (Ferreira et al., 2016; Frable et al., 2016; Melo et al., 2016; Melo et al., 2018; Sales et al., 2018; Turner et al., 2004). Studies utilizing chromosomal and molecular markers have provided valuable information for the separation or union of closely related species and can often clarify the evolutionary history of populations, including problematic species. Melo et al. (2018) demonstrated low genetic divergence among *Prochilodus* lineages, high levels of genetic variability, and low levels of population structure, noting that one contributing factor is the migratory behavior of the species, which facilitates extensive gene flow between distinct populations (Godinho & Kynard, 2006).

Lopes et al. (2020) questioned the monophyly of *P. nigricans*, pointing out the existence of two mitochondrial lineages in the Amazon River basin. One lineage includes specimens of *P. nigricans* from the lowlands of Western Amazonia, while the second is considered a species complex encompassing what is described as *P. nigricans* from the highlands of Eastern Amazonia (Araguaia River, Upper and Middle Tapajos), *P. britskii* from Apiacá (Upper Tapajos), *P. brevis* from northeastern Brazil (the states of Ceará and Rio Grande do Norte), *P. lacustris* from the Parnaíba River, and *P. rubrotaeniatus* from the Upper Orinoco and Upper Essequibo basins. In other words, within this complex lineage, there are two distinct taxonomic units.

The uncertainties surrounding this scenario reinforce the need for further research using molecular tools for more reliable species delimitation. The mitochondrial markers most commonly used for phylogenetic studies include the Cytochrome c Oxidase Subunit I (COI) gene (Hebert et al., 2003; Hubert et al., 2008), the 16S rRNA gene (Palumbi et al., 1996), and the Cytochrome b (Cyt *b*) gene (Farias et al., 2001; Fraga et al., 2007; Santos et al., 2003). The nuclear Alpha-Tropomyosin gene (TROP) is also widely used in this type of research (Friesen et al., 1999). The combination of these molecular markers provides a powerful tool for verifying patterns of genetic diversity and identifying species.

This study aimed to evaluate the genetic differentiation between *P. lacustris* and *P. nigricans* in the hydrographic basins of Maranhão (Itapecuru, Pindaré, Turiaçu, Pericumã, and Mearim rivers) and Amazonas state (Xingu and Tapajós rivers), using mitochondrial and nuclear markers. The research sought to investigate whether these localities belong to the same lineage, as suggested by Lopes et al. (2020), elucidating persistent taxonomic uncertainties. After all, the precise identification of these species is a fundamental step for the proper management and successful conservation of fishery resources in both regions.

MATERIAL AND METHODS

Sampling

A total of 151 specimens of *Prochilodus* were collected under the authorization of the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) and the Chico Mendes Institute for Biodiversity and Conservation (ICMBIO)

(No.02012.004159/2006—ItapecuruRiver,ICMBIONo.46367-1—Pindaré River, Turiaçu River, Pericumã River, ICMBIO—MMA No. 42.119-2—Mearim River, ICMBio No. 81592-1—Munim River), including 125 samples of *P. lacustris*/*P. nigricans* from the Maranhão basins under protocol no. 47/2020.

This study was submitted to the Ethics Committee of the Universidade Estadual do Maranhão (UEMA), under protocol no. 47/2022, which included 26 samples of *P. nigricans* from the Tapajós River and the Xingu River (Amazon basin).

These samples were deposited in the Molecular Biology Laboratory in the Molecular Biology Genetics complex (GENBIMOL) at the Caxias Higher Education Center, UEMA. A total of 124 sequences were obtained and deposited on the National Center for Biotechnology Information (NCBI) platform for the 16S rRNA gene (PP916553-PP919955), 130 for the COI gene (PP917855-PP916185), 94 for the Cyt *b* gene (PP938653-PP938725), and 101 sequences for the TROP gene (BankIt2842185-Bankit2842194) (Fig. 1 and Table 1).

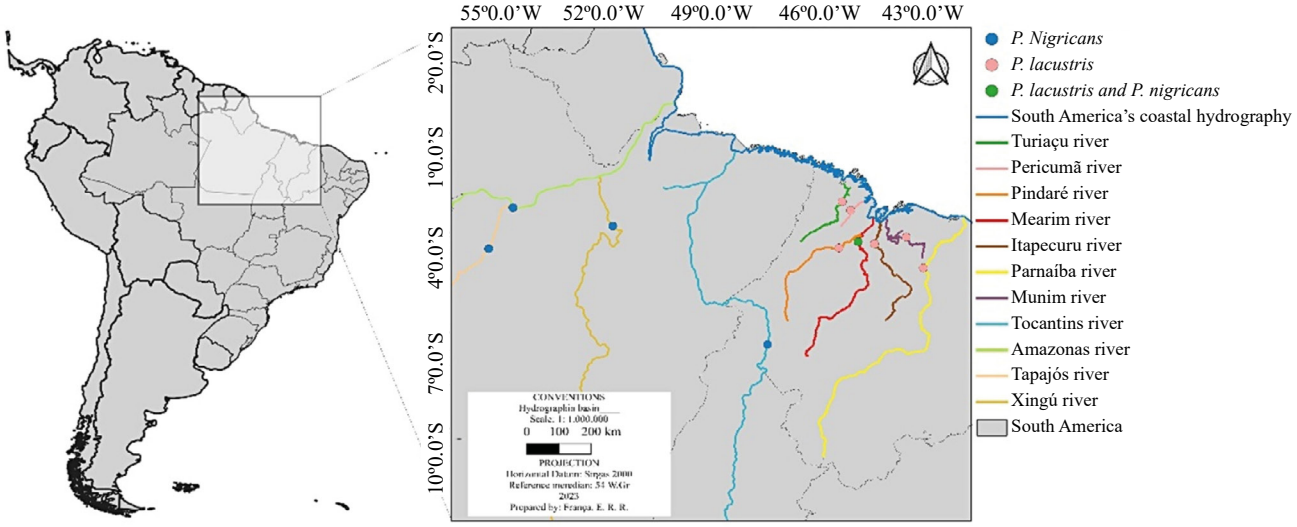

Figure 1. Sample localities, each data point indicates the place where the *Prochilodus* samples were collected.

Table 1. Species of *Prochilodus lacustris* and *Prochilodus nigricans* collected in Brazilian river.

Watersheds	Taxon	Sample No.	Voucher	Coordinate
Itapecuru River	*P. lacustris*	26	MZUSP 104603	03°31'39" S 44°24'19" W
Mearim River	*P. lacustris*/*P. nigricans*	42	MZUSP634/COFAUMA300	3°27'54.3" S 44°52'11.4" W
Pindaré River	*P. lacustris*	10	MZUEL 17383	3°39'14.36" S 45°25' W
Pericumã River	*P. lacustris*	06	MZUEL 17355	02°31'17" S 45°04'57" W
Turiaçu River	*P. lacustris*	09	MZUEL 17428	02°15'49" S 45°19'24" W
Parnaíba River	*P. lacustris*	18	-	04°15'24" S 43°00'46" W
Munim River	*P. lacustris*	06	-	03°19'35.6"S 43°29'54.6"W
Tocantins River	*P. nigricans*	08	-	06°33'38" S 47°27'04" W
Xingu River	*P.nigricans*	03	-	02°59'35" S 51° 51'39" W
Amazonas River	*P. nigricans*	17	-	02°26'34" S54°42'28" W
Tapajós River	*P. nigricans*	06	-	03°40'39" S 55°23'48" W
Total		151		
Sequence Codes Genbank				
Genbank COI		130	PP917855-PP916185	
Genbank 16 rRNA		124	PP916553-PP919955	
Genbank Cyt *b*		94	PP938653-PP938725	
Genbank TROP		101	BankIt2842185-Bankit2842194	

The samples were collected using fishing gear such as trawls, gillnets, and cast net with varying mesh sizes (10 to 200 mm). The specimens collected during this study were euthanized by immersion in freezing water (Ashley, 2007), placed in individually labeled plastic bags, and transported on ice to the GENBIMOL at the Caxias Higher Education Center at UEMA. The fish were labeled and photographed, and muscle tissue samples were extracted for analysis. These samples were preserved in 90% alcohol and stored at -20°C. The specimens were then fixed in 10% formalin and conserved in 70% alcohol. Identification of the specimens was conducted using basic references (Britski et al., 1999; Piorski et al., 2007; Santos et al., 2004; Soares, 2005) and was confirmed by a specialist. Voucher specimens were deposited in the Museum of Zoology of the Universidade de São Paulo (MZUSP 104576) and Universidade Estadual de Londrina.

Extraction and amplification of the DNA

Total DNA was extracted using the Promega Wizard Genomic DNA Purification Kit, following the manufacturer's instructions. The four molecular markers (16S rRNA, COI, Cyt *b*, and TROP) were amplified using polymerase chain reaction (PCR) with specific primers for each gene (Table 2).

Table 2. Sequences and references of the primers used in the amplification of mitochondrial and nuclear fragments.

Primers	Sequences
rRNA 16S Palumbi et al. (1991).	16SL1: 5'GCCTCGCCTGTTTACCAAAAAC 3' 16SH2: 5'CCGGTCTGAACTCAGATCACGT 3'
Cytochrome c oxidase subunit I Ward et al. (2005)	COIF1:5'TCAACCAACCACAAAGACATTGCCAC 3' COIR1:5'TAGACTTCTGGGTGGCCAAAGAATCA 3'
Citocromo *b* Santos et al. (2003) Smith e Patton (1993)	L14725- 5'CGAAACTAATGACTTGAAAAACCACCC CGTTG 3' MVZ16-5'AAATAGGAARTATCAYTCTGGTTTRAT 3'
TROP Friesen et al. (1999)	5'GAGTTGGATCGGGCTCAGGAGCG3' 5'CGGTCGGCCTCTTCACAATGTGCTT3'

The PCRs were run in a final volume of 25 µL, which contained 2µL of the DNA (25ng/µL), 4 µL of total dNTP mix (1.25 mM), 2.5µL of 10x buffer solution, 0.5µL of $MgCl_2$ solution (50 mM), 0.25µL of each primer (200µM), 0.2µL of Taq DNA polymerase (5U/µL, Invitrogen), and purified water to complete the final reaction volume. Samples were amplified using a specific cycle, as shown in Table 3.

Table 3. Description of the cycles of each mitochondrial and nuclear region amplified in polymerase chain reaction.

Primers	Initial denaturation	Denaturation	Ringing	Extension	Final extension
rRNA 16S	95°C/2 min	94°C/30 s	50°C/1 min	72°C/2 min	72°C/7 min
COI	95°C/2 min	94°C/1 min	50°C/30 s	72°C/1 min	72°C/7 min
Cyt B	95°C/2 min	94°C/30 s	51°C/1 min	72°C/2 min	72°C/7 min
TROP	95°C/2 min	95°C/30s	60°C/30 s	72°C/45 s	72°C/7 min

The PCR products were purified with EXoSAP-IT, following the manufacturer's recommendations, and sequenced using a Big Dye kit with Sanger et al.'s (1977) dideoxy nucleotide method. The products were precipitated and analyzed in an ABI Prism™ 3500 automatic sequencer (Applied Biosystems, United States of America).

Data analysis

The obtained sequences were manually edited and aligned using BioEdit software (Hall, 1999), in which chromatograms were visually inspected to assemble forward and reverse contigs and generate consensus sequences. Base-calling quality was assessed via Phred scores, and low-quality terminal regions

(Q < 20) were trimmed to ensure sequence accuracy. To rule out the presence of nuclear and mitochondrial pseudogenes (NUMTs), the gene sequences were translated based on the vertebrate mitochondrial genetic code in MEGA (Kumar et al., 2018) fragments exhibiting premature stop codons, frameshifts, or anomalous indels were promptly discarded. Finally, the four markers were concatenated in SeaView4 (Gouy et al., 2010), and mean genetic distances were calculated in MEGA X under the Kimura 2-Parameter (K2P) substitution model.

The optimum evolutionary model for the construction of the Bayesian inference (BI) and maximum likelihood (ML) trees was generated in JModelTest2 (Darriba et al., 2012). The BI tree was generated in BEAST v.1.10.4 (Drummond et al., 2012; Suchard et al., 2018), using the Hasegawa-Kishino-Yano (HKY) algorithm, with a strict clock and Yule speciation process as prior. The analysis was run for 40,000,000 generations. The log files were then checked in Tracer v1.6 (Rambaut et al., 2014) to evaluate convergence and determine appropriate burn-in parameters. Convergence was considered adequate when the effective sample size (ESS) was at least 200. The tree generated in BEAST was summarized in TreeAnnotator v.1.10.4 (Suchard et al., 2018) to obtain the consensus tree, which was then visualized and edited in FigTree v1.4.2 (Rambaut, 2014) and the Inkscape image-editing software.

The ML tree from the concatenated data was generated in the PhyML 3.0 program (Guindon et al., 2010), using the general time reversible (GTR) algorithm, with support values estimated using 1,000 bootstrap replicates. For the separate genes (COI, 16S rRNA, Cyt *b*, and TROP), the neighbor-joining (NJ) and ML methods were used in the MEGA X program (Kumar et al., 2018), both employing the Kimura-2 parameter nucleotide substitution evolutionary model with 1,000 bootstrap replicates. Both trees were visualized and edited in FigTree v1.4.2 (Rambaut, 2014) and the Inkscape image editing software. Clades with a bootstrap percentage of at least 85% or a posterior probability of at least 0.95 were considered to be well supported.

Molecular operational taxonomic units (MOTUs) delimitation analyses for the COI gene were conducted using the following models: automatic barcode gap discovery (ABGD), assemble species by automatic partitioning (ASAP), Poisson tree process (PTP), and generalized model yule coalescent (GMYC). The ABGD test (Puillandre et al., 2012) was run on https://bioinfo.mnhn.fr/abi/public/abgd/ using the bank of aligned sequences and the Kimura-2-parameter (K2P) algorithm. The ASAP test (Puillandre et al., 2020) was conducted on https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html using a genetic distance matrix created in MEGA X. The PTP (Zhang et al., 2013) was run on the web server https://species.h-its.org/, using the ML phylogenetic tree generated by RaxML v.8.29 (Stamatakis, 2014) as input. This program is available in CIPRES Science Gateway v3.3 (Miller et al., 2010). The GMYC (Fujisawa & Barraclough, 2013) was performed using the ultrametric consensus tree built in BEAST v1.8 software, which was submitted to the APE (Paradis & Schliep, 2019), SPLITS (Ezard et al., 2009), Paran (Dinno, 2009), and Mass (Venables & Ripley, 2002) packages available in R v.4.1.0 software (Venables & Smith, 2021).

GenBank sequences of the 16S rRNA, COI, and Cyt *b* genes of *Semaprochilodus varii* MZ051939/KX087058/KX086859 (Melo et al., 2016; Papa et al., 2021) and *Semaprochilodus taeniurus* KX086752/KX087052/KX086855 (Melo et al., 2016) were used as the outgroup for the mitochondrial analyses. For the TROP gene, *Leporinus piau* (EU181682) (Santos, 2007) was used to root the tree, as no sequences close to the *Prochilodus* genus were found for the gene, and *Prochilodus lineatus* (AY817275) (Calcagnotto et al., 2005) was obtained from GenBank to root the trees.

Population analyses were performed using DNAsp 6.1 software (Rozas et al., 2018). Relationships among the haplotypes were inferred from an unrooted haplotype network obtained from NETWORK 5.0.1.1 (http://www.fluxus-engineering.com) using the median-joining method (Bandelt et al., 1999).

RESULTS

In this study, 124 sequences of the 16S rRNA gene were obtained, 139 for the COI gene, 94 for the Cyt *b* gene, and 101 sequences for the TROP. It was also possible to obtain 40 concatenated sequences. Haplotypic and nucleotide diversity varied considerably between the different markers (Table 4).

The phylogenetic analyses of the haplotypes (Table 5), based on the three alternative approaches (BI, ML, and NJ), all produced trees with a similar topology, that is, with a single cluster, with all the sequences of *P. lacustris* from Maranhão being grouped in a single clade with *P. nigricans* from the Amazon basin (Figs. 2, 3 and 4). The species delimitation analyses of the COI gene identified a single MOTUs based on the ABGD, ASAP, PTP, and GMYC models, which is consistent with a single group observed in the tree topology (Fig. 4).

The genetic distance matrix for the genes analyzed revealed that the average divergence between the species ranged from 0 to 0.35%, this value being below 1% when compared to those from the Amazon, with average intraspecific divergence of 0% (Table 6).

Table 4. Genetic variation parameters in the sample populations of *Prochilodus nigricans* and *Prochilodus lacustris*. The number of sequences analyzed (N), informative sites for parcimonia (Pi), number of polymorphic sites (S), number of haplotypes (H), haplotypic diversity (Hd), base pairs (Pb) and nucleotide diversity (π) are given for each marker.

Genes	N	*P. lacustris*	*P. nigricans*	Pb	H	Pi	S	Molecular diversity index	
								Hd	π
rRNA 16S	124	110	14	557	7	4	6	0.226	0.0053
COI	136	121	15	625	7	6	6	0.418	0.0081
Cyt *b*	94	78	16	790	7	10	10	0.546	0.0017
Trop	101	86	15	290	3	3	2	0.096	0.0003
Concatenated	40	31	9	2,262	4	5	22	0.780	0.0101

Table 5. Haplotypes based on variation in COI, rRNA16S, Cyt *b*, and TROP genes in sample populations of *Prochilodus lacustris* and *Prochilodus nigricans* from the Maranhão and Amazon basins.

Haplotype	COI								
	Number of individuals recorded in the population								
	MEA	PIN	PARNA	TUR	ITA	TOC	PER	MUN	AMAZ
H1	37	16	8	6	24	-	1	6	5
H2	-	-	8	-	-	-	-	-	-
H3	3	-	-	-	-		3	-	-
H4	-	-	-	-	-	9	-	-	-
H5	-	-	-	-	-	-	-	-	-
H6	-	-	-	-	-	-	-	-	7
H7	-	-	-	-	-	-	-	-	3
All	40	16	16	6	24	9	4	6	15
Haplotype	**16S rRna**								
	Number of individuals recorded in the population								
	MEA	PIN	PARNA	TUR	ITA	TOC	PER	AMAZ	
H1	31	18	7	8	18	8	6	13	
H2	-	-	-	-	1	-	-	-	
H3	-	-	-	-	1	-	-	-	
H4	-	-	1	-	-	-	-	-	
H5	-	-	5	-	-	-	-	-	
H6	2	-	-	-	-	-	2	-	
H7	3	-	-	-	-	-	-	-	
All	36	18	13	8	20	8	8	13	
Haplotype	**Ctyb**								
	Number of individuals recorded in the population								
	MEA	PIN	PARNA	TUR	ITA	TOC	PER	AMAZ	
H1	22	10	-	8	9	1	3	8	
H2	-	-	-	-	-	3	-	-	
H3	-	-	-	-	-	2	-	-	
H4	-	-	-	-	-	1	-	-	
H5	-	-	13	-	3	-	-	-	
H6	3	-	-	-	-	-	-	-	
H7	-	-	-	-	-	-	-	8	
All	25	10	13	8	12	7	3	16	
Haplotype	**TROP**								
	Number of individuals recorded in the population								
	MEA	PIN	PARNA	TUR	ITA	TOC	PER	AMAZ	
H1	16	10	15	8	18	9	5	15	
H2	3	-	-	-	-	-	-	-	
H3	-	-	-	-	-	2	-	-	
All	19	10	15	8	18	11	5	15	

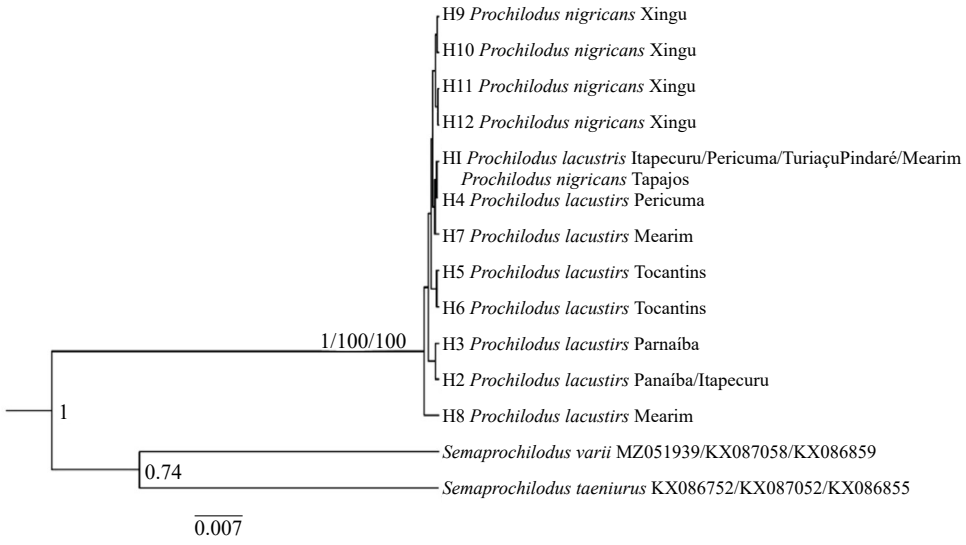

Figure 2. Concatenated haplotype tree (COI, 16S rRNA, Cyt b and TROP) obtained using the Bayesian inference (BI), maximum likelihood (ML), and neighbor-joining (NJ) methods, using the general time reversible model for the ML and NJ models and the Hasegawa-Kishino-Yano for BI.

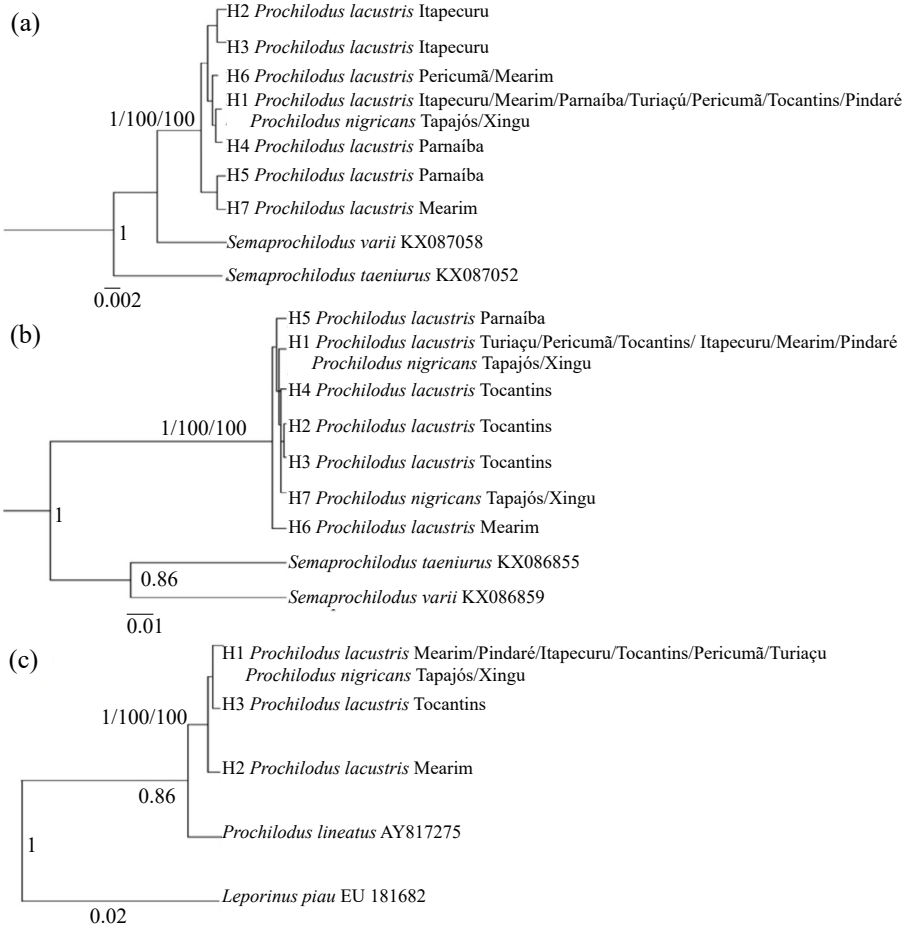

Figure 3. Haplotype trees for the (a) 16S rRNA, (b) Cyt b and (c) TROP genes obtained using the Bayesian inference (BI), maximum likelihood (ML) and neighbor-joining (NJ) methods using the Kimura-2 parameter model for the ML and NJ models and the Hasegawa-Kishino-Yano for (BI).

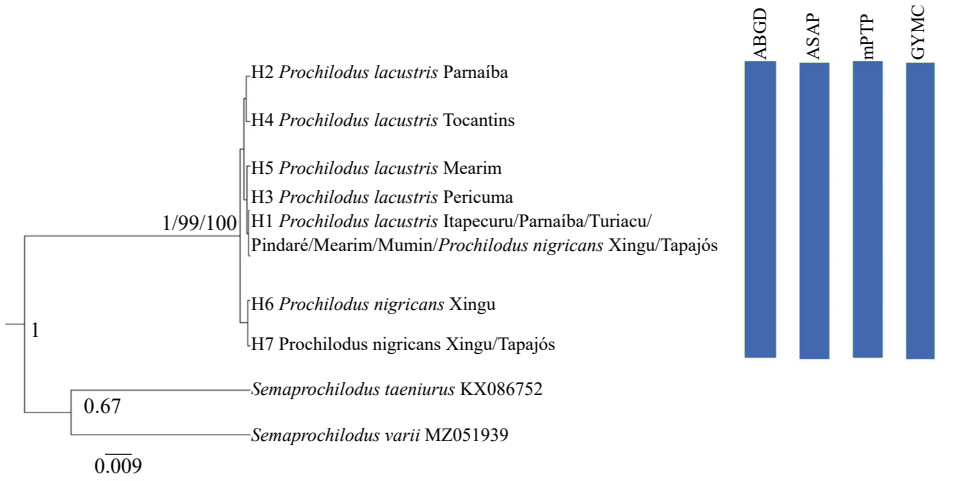

Figure 4. Haplotype tree and delimitation analysis for the COI gene obtained using the Bayesian inference (BI), maximum likelihood (ML) and neighbor-joining (NJ) methods using the general time reversible model for the ML and NJ models and the Hasegawa-Kishino-Yano for BI.

Table 6. Matrix of genetic distances between populations of *Prochilodus lacustris* and *P. nigricans* from the Maranhão and Amazon basins (Xingu and Tapajos), with values given as percentages.

Species	Averages of genetic divergence for the COI gene (%)									
	Populations	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Prochilodus lacustris	1. Itapecuru	0.00									
	2. Parnaíba	0.01	**0.00**								
	3. Pericumã	0.01	0.02	**0.00**							
	4. Tocantins	0.02	0.02	0.03	**0.00**						
	5. Turiaçu	0.00	0.01	0.01	0.02	**0.00**					
	6. Pindaré	0.00	0.01	0.01	0.02	0.00	**0.00**				
	7. Mearim	0.00	0.01	0.01	0.02	0.00	0.00	**0.00**			
	8. Munin	0.00	0.08	0.12	0.16	0.00	0.00	0.01	0.00		
Prochilodus nigricans	9. Tapajos	0.00	0.01	0.01	0.02	0.00	0.00	0.00	0.03	**0.00**	
	10. Xingu	0.02	0.03	0.03	0.04	0.02	0.02	0.02	0.21	0.02	0.00
Species	**Averages of genetic divergence for the gene rRNA 16s (%)**									
	Populations	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	
Prochilodus lacustris	1. Itapecuru	0.00									
	2. Parnaíba	0.11	**0.00**								
	3. Pericumã	0.12	0.17	**0.00**							
	4. Tocantins	0.03	0.08	0.09	**0.00**						
	5. Turiaçu	0.03	0.08	0.09	0.00	**0.00**					
	6. Pindaré	0.03	0.08	0.09	0.00	0.00	**0.00**				
	7. Mearim	0.07	0.11	0.12	0.04	0.04	0.04	**0.00**			
Prochilodus nigricans	8. Tapajos	0.03	0.08	0.09	0.00	0.00	0.00	0.04	**0.00**		
	9. Xingu	0.03	0.08	0.09	0.00	0.00	0.00	0.04	0.00	**0.00**	
Species	**Averages of genetic divergence for the gene Cyt b (%)**									
	Populations	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	
Prochilodus lacustris	1. Itapecuru	0.00									
	2. Parnaíba	0.19	**0.00**								
	3. Pericumã	0.06	0.25	**0.00**							
	4. Tocantins	0.32	0.51	0.25	**0.00**						
	5. Turiaçu	0.06	0.25	0.00	0.25	**0.00**					
	6. Pindaré	0.06	0.25	0.00	0.25	0.00	**0.00**				
	7. Mearim	0.15	0.32	0.09	0.35	0.09	0.09	0.00			
Prochilodus nigricans	8. Tapajos	0.10	0.29	0.03	0.24	0.03	0.03	0.12	**0.00**		
	9. Xingu	0.21	0.40	0.14	0.26	0.14	0.14	0.23	0.13	**0.00**	

Continue...

Continuation.

Species	Averages of genetic divergence for the TROP (%)								
	Species	1	2	3	4	5	6	7	8	9
Prochilodus lacustris	1. Itapecuru	0.00								
	2. Parnaíba	0.00	**0.00**							
	3. Pericumã	0.14	0.14	**0.00**						
	4. Tocantins	0.06	0.06	0.20	**0.00**					
	5. Turiaçu	0.00	0.00	0.14	0.06	**0.00**				
	6. Pindaré	0.00	0.00	0.12	0.06	0.00	**0.00**			
	7. Mearim	0.05	0.05	0.19	0.12	0.05	0.06	**0.00**		
Prochilodus nigricans	8. Tapajos	0.00	0.00	0.14	0.06	0.00	0.00	0.05	**0.00**	
	9. Xingu	0.00	0.00	0.14	0.06	0.00	0.00	0.05	0.00	**0.00**

The haplotype network revealed, once again, the formation of a single grouping of the sequences of *P. nigricans* from the Amazon basin with *P. lacustris* from the watersheds of Maranhão. Most of the haplotypes were shared between populations and only a few mutational steps differentiated the haplotypes, in other words, with few differences between the samples collected, thus confirming the results obtained by the COI gene analysis, reaffirming the low difference between the species analyzed (Fig. 5).

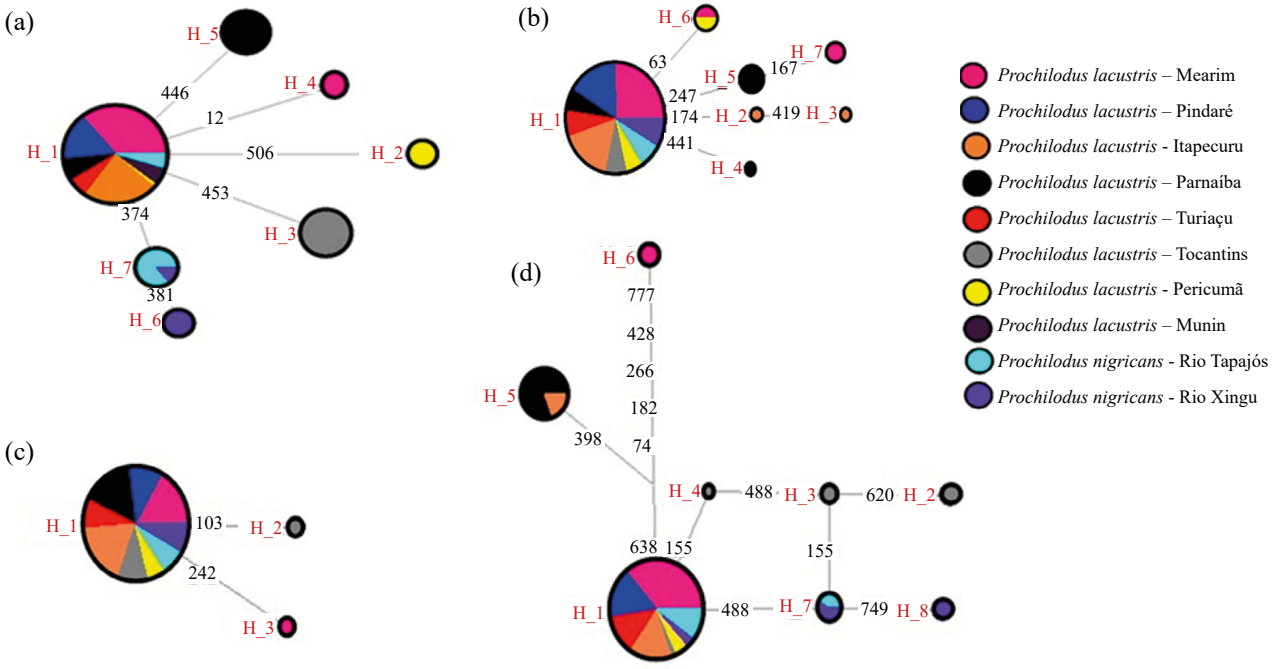

Figure 5. Haplotype network with genes (a) COI, (b) 16S rRNA, (c) TROP, and (d) Cyt b representing the dispersions of *Prochilodus lacustris* and *Prochilodus nigricans* for the Maranhão and Amazonas River basins (Xingu and Tapajos rivers).

DISCUSSION

The findings of the present study, based on the analysis of mitochondrial and nuclear genes, indicate low levels of genetic divergence between *P. lacustris* and *P. nigricans*. This finding is consistent with the typical genetic patterns observed in other species of this genus, as shown in several studies employing various molecular markers (Ferreira et al., 2016; Frable et al., 2016; Lopes et al., 2020; Melo et al., 2016; Melo et al., 2018; Sales et al., 2018; Turner et al., 2004).

The phylogenetic analyses of the COI gene indicated the

presence of only one haplogroup with a single MOTUs, based on results from the ABGD, ASAP, PTP, and GMYC models, which have been successfully used to delimit species in many previous studies (Fujisawa & Barraclough, 2013; Puillandre et al., 2012, 2020; Zhang et al., 2013), including fish taxa (Carvalho et al., 2019; da Silva et al., 2017; Guimarães, Lima et al., 2022; Guimarães, Rosso et al., 2022; Nogueira et al., 2021; Ochoa et al., 2020; Ramirez et al., 2017; Rossini et al., 2016; Silva-Santos et al., 2018; Souza et al., 2018).

The haplotype networks also revealed the existence of a single haplogroup, with haplotypes from all four markers being shared among the study populations. This finding does not support the differentiation of the two taxa, given the low divergence values (0–0.35%). Our study verified the lack of supported differentiation between the samples of *P. lacustris* from Maranhão (Itapecuru, Mearim, Pindaré, Parnaíba, Tocantins, Turiaçu, and Pericumã rivers) and the samples of *P. nigricans* from Amazonas (Tapajós and Xingu rivers). This conclusion is further reinforced by the analysis of multiple mitochondrial and nuclear genes to confirm the absence of differentiation. Lopes et al. (2020) and Melo et al. (2018) focused on sequences from the Tapajós, Araguaia, and Amazonas rivers, while our results also demonstrated high genetic similarities between *P. lacustris* and *P. nigricans* from the Xingu River.

The low divergence values recorded in the present study, alongside the high level of genetic similarity between the two taxa (*P. lacustris* and *P. nigricans*), suggest a low level of differentiation. Hebert et al. (2003) concluded that such scenarios may arise from the retention of ancestral polymorphism, recent speciation, or, in some cases, hybridization or introgression of mitochondrial DNA.

The distribution pattern and sharing of haplotypes found in our results align with the species' migratory behaviors, which allow them to travel kilometers between rivers to spawn during the rainy season (Godinho & Kynard, 2006). This mobility facilitates extensive gene flow between distant populations (Melo et al., 2016; Sivasundar et al., 2001). There is strong evidence that prochilodontid migrations have resulted in high genetic diversity and low levels of population structure (Ferreira et al., 2016; Machado et al., 2017; Rueda et al., 2013; Sivasundar et al., 2001). Stanley (1979) suggested that migration and gene flow directly influence the conserved morphological state in this genus, raising questions about the migration patterns that affect diversification without corresponding morphological changes. However, studies on the genetic divergence of freshwater fishes indicate that the genetic patterns currently observed are strongly influenced by historical migration events and gene flow among species (Fonseca et al., 2017; Mondin et al., 2018). Understanding how historical events impact genetic diversity is crucial for predicting the persistence of populations under future environmental changes (Pauls et al., 2013).

It is also noteworthy that fish in this family exhibit strong homogeneity and conservation of the morphological characteristics used to distinguish different species (Frable et al., 2016). Some species in the genus *Prochilodus* show little morphological differentiation, and are distinguished primarily by bands, meristic values, and the biogeographical drainage in which they are generally endemic (Castro & Vari, 2004). The species *P. lacustris* and *P. nigricans* are distinguished only by radial subdivision patterns on body scales, which include overlapping counts of scales, lateral lines, the number of lines on the horizontal scale below the lateral line, and the number of lines on the proportion scale (Castro & Vari, 2004).

The low levels of genetic divergence and haplotype sharing among populations in the rivers of Maranhão can be attributed to the state's extensive coastal plain geography. This geographical feature has likely facilitated hydrological and faunal exchanges across various basins through the capture of lateral tributaries and river mouths (Wilkinson et al., 2006). Historical events, including marine transgressions and regressions during the late Miocene and Pliocene, further influenced the composition of fish species in Maranhão (Abreu et al., 2019; Abreu et al., 2020; Albert et al., 2018; Lundberg et al., 1998; Soares Júnior et al., 2011), promoting similarities in fauna across basins (Abreu et al., 2019; Abreu et al., 2020). These factors explain the broad dispersal of *P. lacustris* beyond its endemic areas and underscore the connectivity among Maranhão's river systems. Studies of certain fish species have identified haplotype sharing between basins in Maranhão (Carvalho-Costa et al., 2011; Fraga et al., 2014), suggesting dispersal events possibly driven by climatological and geological phenomena (Hubert et al., 2007).

In summary, analyses based on molecular markers (16S rRNA, COI, Cyt *b*, and TROP) revealed no sufficient genetic divergence between *P. lacustris* populations from the Maranhão river basins and *P. nigricans* from the Amazon Basin to support their distinction as separate species. The results indicated the presence of a single molecular lineage with low genetic variability, shared by both *P. lacustris* and *P. nigricans.*

CONFLICT OF INTEREST

Nothing to declare.

DATA AVAILABILITY STATEMENT

The sequences included in the present study are available from GenBank (https://www.ncbi.nlm.nih.gov/genbank/), accessed numbers PP917855-PP916185, PP916553-PP919955, PP938653-PP938725, and BankIt2842185-Bankit2842194. The voucher specimens used for the taxonomic diagnoses are deposited at the Museum of Zoology of Universidade Estadual de Londrina, in Londrina, PR, Brazil. The remaining specimens are held in the Molecular Biology Laboratory of the GENBIMOL complex of the Caxias *campus* of UEMA. The codes used in this study are available from the corresponding author on reasonable request.

AUTHORS' CONTRIBUTIONS

Conceptualization: França, E.R.R., Barros, M.C., Fraga, E.C.; **Formal Analysis:** França, E.R.R., Silva, J.L.N., Silva, F.C., Nascimento, M.H.S., Correa, F., Barros, M.C., Fraga, E.C.; **Visualization:** França, E.R.R., Silva, J.L.N., Nascimento, M.H.S., Correa, F.; **Writing – original draft:** França, E.R.R.; **Writing – review & editing:** França, E.R.R., Silva, J.L.N., Nascimento, M.H.S., Correa, F., Barros, M.C., Fraga, E.C; **Final approval:** França, E.R.R.

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DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE TOOLS

The authors of this manuscript declare that no artificial intelligence (AI) tools, large language models, or AI-assisted technologies were used in the writing, translation, data analysis, linguistic revision, or conceptual design of any part of this work. All content presented was generated, analyzed, and reviewed exclusively through the intellectual and technical effort of the listed authors, ensuring the originality and academic integrity of the manuscript in accordance with current ethical guidelines.

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