



**UNIVERSIDADE ESTADUAL DO MARANHÃO
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE
E BIOTECNOLOGIA REDE BIONORTE**



**SISTEMÁTICA DE *LEPORINUS* (CHARACIFORMES,
ANOSTOMIDAE) A PARTIR DE FERRAMENTAS MOLECULARES E
MORFOLÓGICA**

MARIA HISTELLE SOUSA DO NASCIMENTO

Caxias - MA

JUNHO/2024

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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Rede BIONORTE, na Universidade Estadual do Maranhão-UEMA, como requisito para a obtenção do Título de Doutor em Biodiversidade e Biotecnologia.

Orientador(a): Prof. Dra. Maria Claudene Barros

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DEDICATÓRIA

À minha família, que acreditou nos meus sonhos e nas oportunidades... papai e mamãe (Sr. Francisco e Sra. Nazaré), aos meus irmãos, minhas irmãs, sobrinhos e sobrinhas, cunhados (as) e aos meus saudosos avós: Joaquim e Benedita, José e Maria Gonçalves (*in memoriam*).

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“Costumávamos pensar que o nosso futuro estava nas estrelas. Agora sabemos que está nos nossos genes”

(James Watson)

NASCIMENTO, Maria Histelle Sousa do. **Sistemática de *Leporinus* (Characiformes, Anostomidae) a partir de ferramentas moleculares e morfológica.** 2024. 89 f. Tese (Doutorado em Biodiversidade e Biotecnologia) – Universidade Estadual do Maranhão, Caxias, 2024.

RESUMO

O gênero *Leporinus* inclui cerca de 80 espécies válidas, agrupando o maior número de espécies dentro da ordem Characiformes e sendo o mais diversificado dentro da família Anostomidae. As espécies encontram-se amplamente distribuídas em bacias hidrográficas da região Neotropical, porém é fato a dificuldade na compreensão da biodiversidade do gênero *Leporinus*, isto é, do limite específico, pois os padrões morfológicos são bastante similares entre as espécies deste gênero, o mesmo ocorrendo com as características fenotípicas observadas em indivíduos juvenis e adultos. Neste estudo utilizou-se sequências do genoma mitocondrial e nuclear e dados morfológicos para identificar e fornecer as relações de parentescos das espécies de *Leporinus* contribuindo assim com informações que deverão ser usadas em plano de manejo e conservação das espécies. O estudo integrativo usando dados morfológicos e moleculares possibilitou o registro de *Megaleporinus macrocephalus* nas bacias hidrográficas maranhenses, representando uma importante extensão da sua distribuição que hoje é confirmada na bacia do rio Itapecuru e Mearim. A análise de 157 sequências de *Leporinus* para gene COI das bacias dos rios Itapecuru, Mearim, Turiacu, Pericumã, Peria, Preguicas, Parnaíba e Tocantins identificou *L. maculatus*, *L. unitaeniatus*, *L. affinis* e *L. piau* (do rio Parnaíba) como agrupamentos distintos corroborados por métodos de delimitação das espécies, os quais revelaram a presença de quatro MOTUs distintas correspondendo a cada uma destas espécies. O método bPTP restringiu *L. venerei* a uma única MOTU apontando para a ocorrência pela primeira vez desta espécie em rios do Maranhão. Ainda foi evidenciado a separação de *L. cf. friderici* em dois clados e a subsequente formação de diferentes MOTUs, sendo, portanto, consistente com a polifilia desta espécie indicando a existência de diversidade críptica, bem como a configuração de *L. cf. friderici* e *L. piau* em dois clados suportando a conclusão de que os espécimes de *L. piau* do Maranhão foram identificados erroneamente. Na investigação da diversidade críptica em *L. piau* das bacias hidrográficas do Maranhão e Piauí, usando marcadores mitocondriais e nuclear foram observados dois grupos bem suportados, em que os espécimes dos rios Itapecuru, Mearim, Turiacu e Pericumã foram atribuídos a um clado sem nome, representando uma diversificação relativamente antiga e provavelmente escondendo uma diversidade críptica. O segundo grupo incluiu os espécimes dos rios Parnaíba e Mearim, denominado *L. cf. friderici sensu stricto*, um grupo que se diversificou relativamente recente e mais estreitamente relacionado a *L. friderici* da localidade tipo. Os resultados confirmaram a existência de um cenário complexo de

diversidade críptica no gênero *Leporinus* e as inconsistências taxonômicas encontradas neste grupo de peixes.

Palavras-chaves: Biodiversidade; Delimitação de espécie; Identificação integrativa; Piau.

NASCIMENTO, Maria Histelle Sousa do. **Sistemática de *Leporinus* (Characiformes, Anostomidae) a partir de ferramentas moleculares e morfológica**. 2024. 89 f. Tese (Doutorado em Biodiversidade e Biotecnologia) – Universidade Estadual do Maranhão, Caxias, 2024.

ABSTRACT

The genus *Leporinus* includes around 80 valid species, grouping the largest number of species within the Characiformes order and being the most diverse within the Anostomidae family. The species are widely distributed in river basins in the Neotropical region, but it is a fact that it is difficult to understand the biodiversity of the genus *Leporinus*, i.e. the specific limit, because the morphological patterns are very similar between the species of this genus, as are the phenotypic characteristics observed in juvenile and adult individuals. This study used mitochondrial and nuclear genome sequences and morphological data to identify and provide the kinship relationships of the *Leporinus* species, thus contributing information that should be used in the species' management and conservation plans. The integrative study using morphological and molecular data made it possible to record *Megaleporinus macrocephalus* in the Maranhão river basins, representing an important extension of its distribution, which is now confirmed in the Itapecuru and Mearim river basins. The analysis of 157 *Leporinus* COI gene sequences from the Itapecuru, Mearim, Turiacu, Pericumã, Peria, Preguicas, Parnaíba and Tocantins river basins identified *L. maculatus*, *L. unitaeniatus*, *L. affinis* and *L. piau* (from the Parnaíba river) as distinct groupings corroborated by species delimitation methods, which revealed the presence of four distinct MOTUs corresponding to each of these species. The bPTP method restricted *L. venerei* to a single MOTU, pointing to the first occurrence of this species in rivers in Maranhão. There was also evidence of the separation of *L. cf. friderici* into two clades and the subsequent formation of different MOTUs, which is therefore consistent with the polyphyly of this species indicating the existence of cryptic diversity, as well as the configuration of *L. cf. friderici* and *L. piau* into two clades supporting the conclusion that the specimens of *L. piau* from Maranhão were misidentified. In the investigation of cryptic diversity in *L. piau* from the Maranhão and Piauí river basins, using mitochondrial and nuclear markers, two well-supported groups were observed, in which specimens from the Itapecuru, Mearim, Turiacu and Pericumã rivers were assigned to an unnamed clade, representing relatively ancient diversification and probably hiding cryptic diversity. The second group included specimens from the Parnaíba and Mearim rivers, called *L. cf. friderici* sensu stricto, a group that diversified relatively recently and is more closely related to *L. friderici* from the type locality. The results confirmed the existence of a complex scenario of cryptic diversity in the *Leporinus* genus and the taxonomic inconsistencies found in this group of fish.

Keywords: Biodiversity; Fish; Integrative identification; Piau; Species delimitation.

APRESENTAÇÃO

A tese está estruturada em uma introdução geral, referencial teórico e dois capítulos, onde no primeiro apresenta-se o artigo publicado revista PeerJ intitulado “The DNA barcode reveals cryptic diversity and a new record for the genus *Leporinus* (Characiformes, Anostomidae) in the hydrographic basins of central northern Brazil”. O segundo capítulo refere-se ao artigo publicado na revista Journal of Fish Biology com título “Exploring Hidden Diversity: Molecular Insights into the *Leporinus* Species of the Rivers of the Brazilian States of Maranhão and Piauí”. Na seção apêndice encontra-se artigo de coautoria publicado na revista Brazilian Journal of Biology com título “New records of the occurrence of *Megaleporinus macrocephalus* (Garavello and Britski, 1988) (Characiformes, Anostomidae) from the basins of the Itapecuru and Mearim rivers in Maranhão, northeastern Brazil”.

Esta tese foi desenvolvida no complexo GENBIMOL que compreende os laboratórios de Genética e de Biologia Molecular da Universidade Estadual do Maranhão, campus Caxias. A pesquisa com os peixes do gênero *Leporinus* é resultante de projetos fomentados pelo Banco do Nordeste do Brasil S/A (aviso: ETENE/FUNDECI 01/2004) e Fundação de Amparo à Pesquisa e o Desenvolvimento Científico e Tecnológico do Maranhão - FAPEMA (Editais: Nº 30/2010; Nº 001/2013; Nº 004/2018; Nº 002/2018; Nº 002/2022). Teve aprovação da Comissão de Controle para Tratamento Ético de Animais, Universidade Estadual do Maranhão (Protocolo Nº 47/2022) e Comissão de Ética no Uso de Animais do Instituto Nacional de Pesquisas da Amazônia (Nº 006/2021), SEI 01280.000116/2021-45. As coletas foram autorizadas pelo Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis e Sistema de Autorização e Informação em Biodiversidade por meio de licenças (IBAMA 02012.004159/2006; ICMBio/MMA 42119-1/2013; ICMBio/MMA 46367-1/2015; ICMBio/MMA 83138-1/2022 e ICMBio/MMA 73790-6/2022).

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1 INTRODUÇÃO GERAL

Os peixes de água doce da região Neotropical apresentam uma significativa diversidade ultrapassando 8.000 espécies, com destaque para bacia amazônica que compreende cerca de 2.400 espécies de água doce conhecidas. No entanto, existem muitas incertezas taxonômicas levando a uma diversidade subestimada (PEREIRA *et al.*, 2013; REIS *et al.*, 2016; JÉZÉQUEL *et al.*, 2020). Dentre os peixes de água doce neotropicais, a ordem Characiformes representa mais de 30% das espécies conhecidas e Anostomidae é uma das famílias mais ricas em espécies, ocorrendo em todas as grandes bacias hidrográficas da América do Sul (REIS *et al.*, 2003).

A família Anostomidae compreende 16 gêneros e aproximadamente 147 espécies válidas encontrando-se amplamente distribuída desde o Sul da América Central até o Norte da Argentina (SANTOS, JEGU e LIMA 1996; NELSON, 2006; BUCKUP *et al.*, 2007; ESCHMEYER e FONG, 2014; RAMIREZ *et al.*, 2016; BRITSKI e BIRINDELLI, 2019; RAMIREZ *et al.*, 2020; FRICKE *et al.*, 2024). Na última década esta família tem sido objeto de interesse de muitos estudos, resultando na criação de dois novos gêneros e na descrição de 11 espécies (FRICKE *et al.*, 2024). Apesar das recorrentes revisões taxonômicas em peixes da família Anostomidae na América do Sul, esta família ainda permanece com o *status* de um grupo muito complexo (VARI e WILLIAMS, 1987; SIDLAUSKAS e SANTOS, 2005; MAUTARI e MENEZES, 2006; BRITSKI e BIRINDELLI, 2008; SIDLAUSKAS *et al.*, 2011; BRITSKI *et al.*, 2012; BIRINDELLI *et al.*, 2012; BIRINDELLI e BRITSKI, 2013; BURNS *et al.*, 2014; ASSEGA e BIRINDELLI, 2019).

Dentre os gêneros da família Anostomidae, *Leporinus* é o mais especioso com cerca de 80 espécies nominais válidas (FRICKE *et al.*, 2024; TOLEDO-PIZA *et al.*, 2024). Segundo Géry (1977), constitui um dos gêneros que agrupa maior número de espécies dentro da ordem Characiformes, ocorrendo desde a América Central ao Sul da América do Sul. As espécies de *Leporinus* possuem uma grande diversidade em forma, posição da boca, número de dentes e padrão de colorido. Britski e Garavello (1978) dividiu o gênero em três categorias considerando o padrão de colorido: barras transversais, manchas e linhas longitudinais.

O monofiletismo do gênero é controverso, resultando em várias tentativas de subdividi-lo em outros gêneros ou subgêneros (BRITSKI e BIRINDELLI, 2008; SIDLAUSKAS *et al.*, 2011; BRITSKI *et al.*, 2012; BIRINDELLI e BRITSKI, 2013; BURNS *et al.*, 2014; RAMIREZ *et al.*, 2016; RAMIREZ *et al.*, 2017a), no entanto, sem grandes sucessos. Do ponto de vista citogenético, *Leporinus* tem sido bastante estudado (GALETTI JR *et al.*, 1981; GALETTI JR *et al.*, 1991; GALETTI JR *et al.*, 1995; KOEHLER *et al.*, 1997a, 1997b), as espécies apresentam um número de cromossomo conservado $2n=54$ e a presença de

apenas um par de cromossomos com regiões organizadoras de nucléolos (NORs), embora para seis espécies até então classificadas dentro deste gênero, *Leporinus conirostris*, *Leporinus piavussu*, *Leporinus macrocephalus*, *Leporinus obtusidens*, *Leporinus reinhardti* e *Leporinus trifasciatus* a existência de um sistema de cromossomos sexuais ZZ/ZW descrito as diferenciavam das demais espécies. Galetti Jr et al. (1995) propuseram que a presença do sistema ZW em seis espécies de *Leporinus*, configuraria uma sinapomorfia e que essas espécies formariam um grupo monofilético, hipótese esta que é corroborada por caracteres morfológicos. Ramirez et al. (2017a) usando dados moleculares descreveu um novo gênero denominado *Megaleporinus*, no qual realocou espécies de dois gêneros de Anostomidae, *Leporinus* e *Hypomasticus*, com cromossomos sexuais ZZ/ZW constituindo um grupo monofilético.

Atualmente para as espécies que compõem o gênero *Leporinus* verifica-se divergências entre dados moleculares e morfológicos, com baixos índices de divergência genética entre espécies, sugerindo assim a presença de espécies crípticas, e possivelmente por isso, inúmeras espécies vêm sendo designadas como *Leporinus* sp. Estudos realizados por Fraga et al. (2014) e Nascimento et al. (2016) em rios maranhenses evidenciaram uma diferenciação genética em *Leporinus piau* Fowler, 1941 apontando para ocorrência de no mínimo duas linhagens, pois foi revelado um alto índice de similaridade genética entre *L. piau* e *Leporinus* cf. *friderici*, advertindo para a necessidade do uso de outras ferramentas para discriminar as espécies.

Dentre os diversos acordos firmados na Conferência das Nações Unidas sobre Meio Ambiente e Desenvolvimento, ocorrida no Rio de Janeiro e assinada por 168 países, incluindo o Brasil, está a importância da conservação da biodiversidade (SECRETARIAT OF THE CONVENTION ON BIOLOGICAL DIVERSITY, 1992). Essa preocupação de associar estudos sobre ictiofauna com a conservação da biodiversidade vai ao encontro da Agenda 2030 para o desenvolvimento sustentável, publicada pela Organização das Nações Unidas (ONU) em 2015 e que tem como 15º Objetivo de Desenvolvimento Sustentável (ODS 15): “Proteger, restaurar e promover o uso sustentável dos ecossistemas terrestres, gerir de forma sustentável as florestas, combater a desertificação, travar e reverter a degradação dos solos e travar a perda da biodiversidade”. Dentre as 12 metas do ODS 15, a meta 15.1 estabelece que, “Até 2020, assegurar a conservação, recuperação e uso sustentável de ecossistemas terrestres e de água doce interiores e seus serviços, em especial florestas, zonas úmidas, montanhas e terras áridas, em conformidade com as obrigações decorrentes dos acordos internacionais”, e a meta 15.5 “Tomar medidas urgentes e significativas para reduzir a degradação de habitat naturais, deter a perda de biodiversidade e, até 2020, proteger e evitar a extinção de espécies ameaçadas” (NAÇÕES UNIDAS BRASIL, 2023). Embora o prazo para o estabelecimento de tais metas tenha expirado, os esforços para frear a perda da biodiversidade são permanentes e em constante

atualização. No entanto, a conservação para vários grupos existente só será efetivamente bem-sucedida quando questões taxonômicas de grupos problemáticos, como *Leporinus*, forem esclarecidas.

O grande número de espécies no gênero *Leporinus*, reflete uma alta complexidade em relação a identificação e descrição de novas espécies, uma vez que a plasticidade fenotípica (principalmente do indivíduo juvenil para o adulto) e os caracteres utilizados na identificação morfológica de espécies pode levar a identificações errôneas. Deste modo, uma alternativa a catalogação é a utilização de dados morfológicos e citogenéticos atrelados a genética molecular, com ênfase nas relações filogenéticas usando marcadores mitocondriais e nucleares para que as topologias obtidas reflitam a história evolutiva das espécies, e ainda, a identificação molecular via DNA barcode.

As contribuições geradas neste estudo são de extrema relevância, pois, fornecerão informações sobre diversidade das espécies de *Leporinus* em importantes bacias hidrográficas maranhenses. Neste sentido, as hipóteses norteadoras consistiram em corroborar a parafilía do grupo, pois há evidências que o gênero *Leporinus* constitua um grupo parafilético. Ainda hipotetizamos que, os resultados obtidos forneçam códigos de barras de DNA específico para cada espécie, ocorrendo o *barcoding gap* entre as distâncias intraespecíficas e interespecífica, bem como sinalizem a ocorrência de novos registros de espécies para as bacias hidrográficas maranhenses, uma vez que estudos com *Leporinus* pra essa região ainda são escassos.

A presente pesquisa tem como objetivo geral utilizar sequências dos genomas mitocondrial, nuclear e dados morfológicos para identificar e fornecer as relações de parentescos das espécies de *Leporinus* e assim buscar solucionar as incertezas taxonômicas para este gênero contribuindo com informações que deverão ser usadas em plano de manejo e conservação das espécies. Ainda, utilizando ferramentas de taxonomia integrativa avaliar a diversidade de espécies objetivando (i) Diferenciar a partir de dados morfológicos as espécies de *Leporinus*; (ii) Identificar as espécies do gênero *Leporinus* baseado em DNA *barcode* e (iii) Reconstruir as relações filogenéticas do gênero *Leporinus* com base nos genes mitocondriais e nucleares.

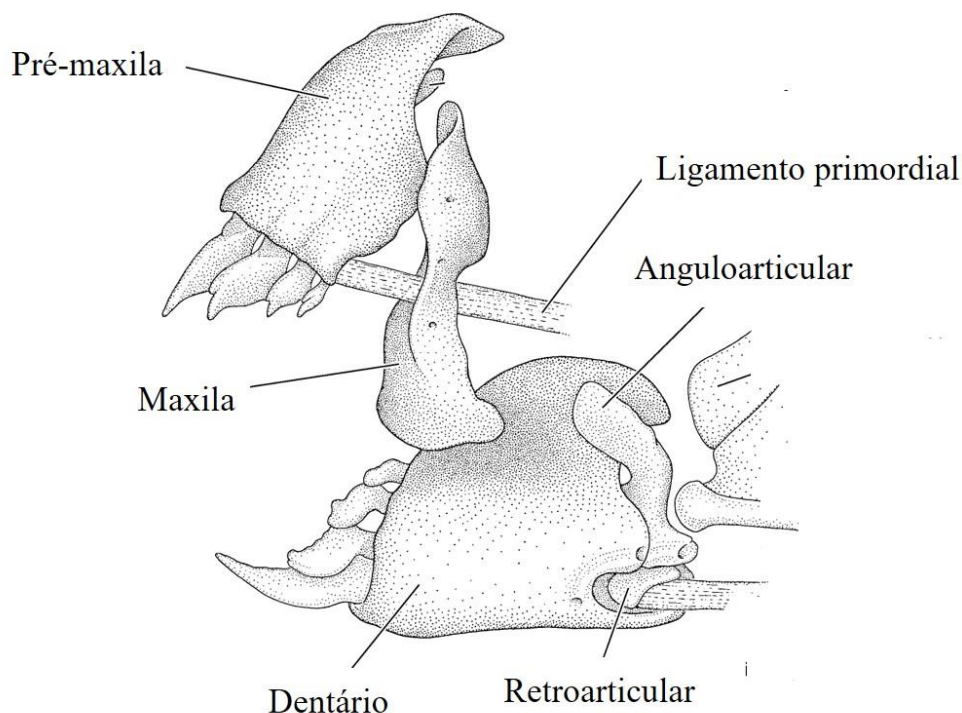
2 REVISÃO BIBLIOGRÁFICA

2.1 FAMÍLIA ANOSTOMIDAE

Os peixes da família Anostomidae são facilmente reconhecíveis pelo corpo fusiforme, variando de 10 cm (espécies pequenas dos sistemas do Rio Orinoco e a Amazônia) a 80 cm (espécies grandes dos tributários dos rios Paraná e do Pantanal) com 3 ou 4 dentes grandes no pré-maxilar e dentário (maxilar sem dentes), escamas grandes e pouco numerosas (32 a 46 escamas perfuradas na linha lateral na maioria das espécies), nadadeira anal curta com menos de 10 raios ramificados (GÉRY, 1977; GARAVELLO e BRITSKI, 2003). Apresentam grande variação na posição da boca, como também, no padrão de colorido, composto desde manchas redondas no flanco a faixas longitudinais, ou ainda barras verticais (GARAVELLO e BRITSKI, 1987). Essas duas características são fortemente influenciadas pela ontogenia sendo possível encontrar formas diferenciadas de posição da boca e padrão de coloração durante o desenvolvimento de uma mesma espécie (GARAVELLO, 1979; BIRINDELLI e BRITSKI, 2009).

Os primeiros anostomídeos foram descritos por Linnaeus (LINNAEUS, 1758) e incluídos em um grupo denominado Anostomina. Com o passar dos anos e a ocorrência de revisões taxonômicas, Regan (1911) propôs a criação da família Anostomidae, na qual incluiu grupos que conhecemos como Anostomidae, Chilodontidae, Curimatidae e Prochilodontidae. Atualmente, a família Anostomidae é representada por 147 espécies válidas que estão distribuídas em 16 gêneros (FRICKE *et al.*, 2024).

Dentre os gêneros de Anostomidae, *Leporinus*, foi descrito por Agassiz (SPIX e AGASSIZ, 1829) e nomeado em referência aos fortes dentes incisivos de seus representantes (Figura 1) com *Leporinus novemfasciatus* Spix e Agassiz, 1829 como a primeira espécie do gênero, que mais tarde foi sinonimizada como *Leporinus fasciatus* (BLOCH, 1794). Britski (1976), ao examinar a coleção de peixes do Instituto Nacional de Pesquisas da Amazônia (INPA) coletadas no rio Ariapuana, encontrou espécimes incomuns de Anostomidae, cuja parte inferior da boca acabou por ser um caráter muito diferente de outras espécies de *Leporinus*. A referida espécie foi descrita pelo mesmo autor em 1976 como *Leporinus pachychelius*, que para a época era classificada dentro do subgênero *Hypomasticus* (BORODIN, 1929), 40 anos mais tarde *Hypomasticus* adquiriu o status de gênero (SIDLAUSKAS e VARI, 2008).

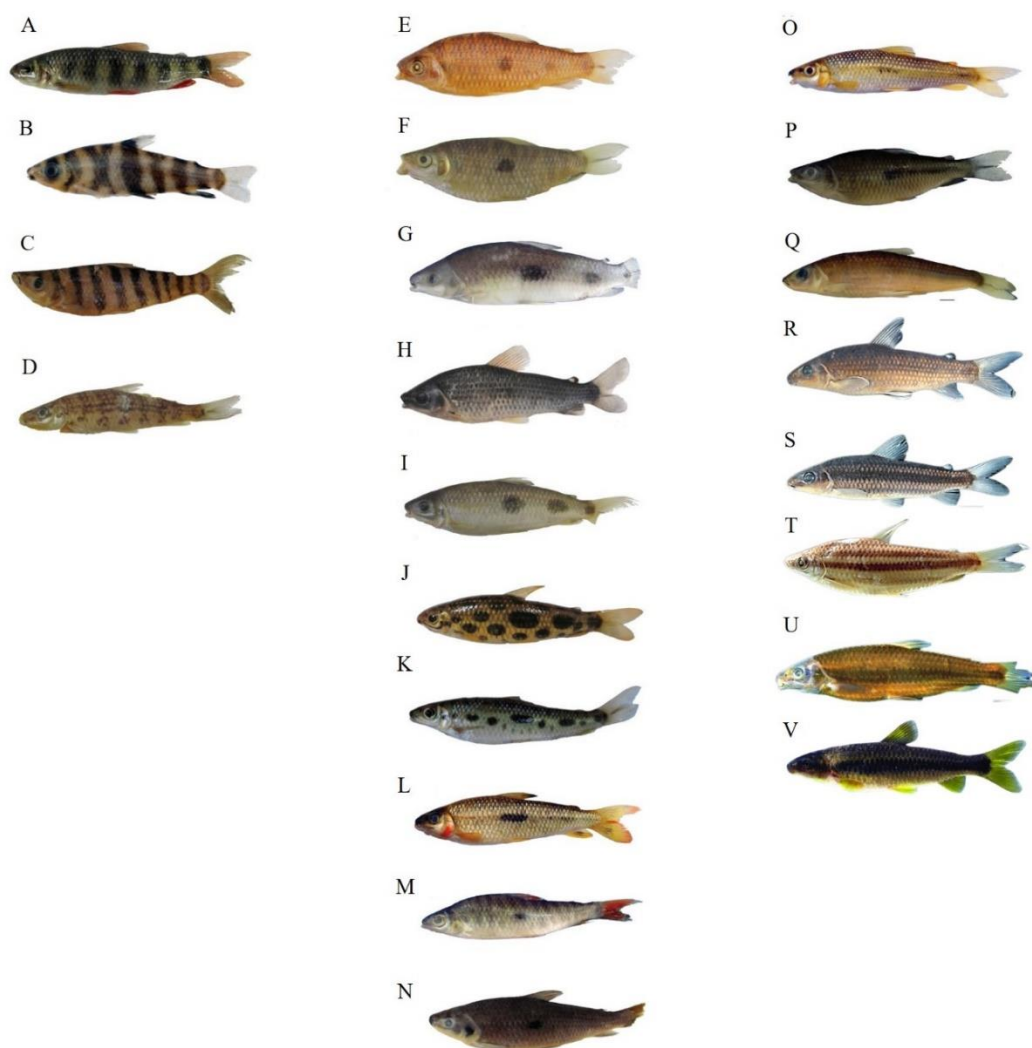


Fonte: Imagem adaptada de Sidlauskas e Vari (2008).

Figura 1. Vista lateral dos maxilares superior e inferior esquerdos de *Leporinus fasciatus* USNM 225991, 87,8 mm SL dissecado.

Leporinus é considerado o gênero com maior riqueza dentre os anostomídeos, compreendendo 80 espécies nominais válidas (FRICKE *et al.*, 2024; TOLEDO-PIZA *et al.*, 2024). Seus representantes apresentam uma grande variabilidade de caracteres fenotípicos, variando desde tamanho, posição da boca e padrão de coloração. Segundo Birindelli e Britski (2013), o padrão de cor pode ser usado para subdividir as espécies de *Leporinus* em três grupos: o primeiro grupo formado por aquelas que possuem entre seis e 14 bandas transversais no corpo, o segundo com uma ou várias manchas escuras no corpo e o terceiro grupo inclui aquelas espécies que apresentam uma ou mais listras longitudinais escuras como ilustra a figura 2. Contudo, estudos moleculares demonstraram que esses padrões na cor não representam grupos monofiléticos (RAMIREZ *et al.*, 2016; RAMIREZ *et al.*, 2017a).

No longo histórico de consideráveis revisões taxonômicas, nas quais os anostomídeos foram submetidos, as tentativas de elucidar as classificações foram muito importantes para se seguir uma linha de estudos, tanto para a família Anostomidae quanto para os gêneros. As principais revisões no nível de gênero ocorreram em *Abramites* (VARI e WILLIAMS, 1987), *Laemolyta* (MAUTARI e MENEZES, 2006), *Pseudanos* (SIDLAUSKAS e SANTOS, 2005; BIRINDELLI *et al.*, 2012), *Anostomoides* (ASSEGA e BIRINDELLI, 2019) e *Leporinus* (BRITSKI e BIRINDELLI, 2008; SIDLAUSKAS *et al.*, 2011; BRITSKI *et al.*, 2012; BIRINDELLI e BRITSKI, 2013; BURNS *et al.*, 2014; RAMIREZ *et al.*, 2016).



Fonte: Fotos de A-Q por P. Venere e J. Ramirez; R-T por J. Birindelli e H. Britski; U-V por J. Birindelli, H. Britski e J. Garavello.

Figura 2. Espécies de *Leporinus* segundo subdivisão por padrão de coloração. A-D: Espécies com seis a 14 bandas transversais no corpo; E-N: Padrão de coloração com uma ou várias manchas escuras no corpo; O-V: Espécies com uma ou mais listras longitudinais escuras. **A:** *Leporinus octofasciatus* Steindachner 1915; **B:** *Leporinus desmotes* Fowler 1914 ; **C:** *Leporinus fasciatus* (BLOCH, 1794) ; **D:** *Leporinus reticulatus* Britski e Garavello 1993; **E:** *Leporinus venerei* Britski e Birindelli 2008; **F:** *Leporinus lacustris* Amaral Campos 1945; **G:** *Leporinus* cf. *parae*; **H:** *Leporinus* sp. 4; **I:** *Leporinus* cf. *friderici*; **J:** *L.* sp.; **K:** *Leporinus* sp.; **L:** *Leporinus geminis* Garavello e Santos 2009; **M:** *Leporinus* cf. *niceforoi*; **N:** *Leporinus amazonicus* Santos e Zuanon 2008; **O:** *Leporinus unitaeniatus* Garavello e Santos 2009; **P:** *Leporinus agassizi* Steindachner 1876; **Q:** *Leporinus brunneus* Myers 1950; **R:** *Leporinus microphysus* Birindelli e Britski 2013; **S:** *Leporinus tristriatus* Birindelli e Britski 2013; **T:** *Leporinus striatus* Kner 1858; **U:** *Leporinus melanopleura* Günther 1864; **V:** *Leporinus melanopleurodes* Birindelli, Britski e Garavello 2013.

2.2 GÊNERO *LEPORINUS*

As diversas tentativas de subdividir gênero *Leporinus* em novos gêneros ou subgêneros não tem sido uma tarefa muito exitosa (GARAVELLO e BRITSKI, 2003). Por exemplo, Fowler (1914) descreveu o subgênero *Myocharax* baseado na espécie *Leporinus desmotes* Fowler, 1914 da Guiana. O subgênero *Leporinops* foi descrito a partir de *Leporinus moralesi* Fowler, 1942 como espécie tipo, no qual também foi incluído a espécie *Leporinus jamesi* Garman, 1929 (GÉRY, 1960). No entanto, a partir de dados osteológicos, Sidlauskas e

Vari (2008) não encontraram evidências para reconhecer *Myocharax* e *Leporinops* como grupos válidos.

O subgênero *Hypomasticus* (BORODIN, 1929) foi descrito pelo caráter peculiar da boca, a qual é totalmente voltada para baixo e com dentes não serrilhados. *Hypomasticus mormyrops* (STEINDACHNER, 1875) foi designado como espécie tipo e *Hypomasticus garmani* (BORODIN, 1929) foi incluído nesse subgênero (BORODIN, 1929), no entanto esta última espécie atualmente está classificada dentro do novo gênero *Megaleporinus* Ramirez, Birindelli e Galletti, 2017 (RAMIREZ *et al.*, 2017a). Quando Sidlauskas e Vari (2008) elevaram *Hypomasticus* ao status de gênero incluíram no mesmo cinco espécies até então classificadas dentro de *Leporinus*, a saber: *Hypomasticus despaxi* (PUYO, 1943), *Hypomasticus julii* (SANTOS, JÉGU e LIMA, 1996), *Hypomasticus megalepis* (GÜNTHER, 1863), *Hypomasticus pachycheilus* (BRITSKI, 1976) e *Hypomasticus thayeri* (BORODIN, 1929). Na última década, duas novas espécies foram descritas para o gênero *Hypomasticus* (BIRINDELLI *et al.*, 2013; BIRINDELLI *et al.*, 2020), no entanto, os autores reforçam que os limites do gênero ainda são incertos, sendo impossível alocar com precisão algumas espécies dentro de *Leporinus* ou de *Hypomasticus* com base no conhecimento atual (BIRINDELLI e BRITSKI, 2009).

Estudos filogenéticos a partir de abordagens morfológicas e moleculares caracterizaram *Leporinus* como um gênero parafilético (SIDLAUSKAS e VARI, 2008; RAMIREZ *et al.*, 2016). Sidlauskas e Vari, (2008) estabeleceram a reconstrução filogenética com 152 variáveis osteológicas confirmando Anostomidae como um grupo monofilético, mas não o gênero *Leporinus*. O que foi corroborado mais tarde por Ramires *et al.* (2016), usando marcadores moleculares nucleares e mitocondriais que ao tentarem provar a monofilia de *Leporinus*, tiveram a hipótese rejeitada, confirmando a parafilia do grupo.

O gênero *Megaleporinus* foi descrito por Ramirez *et al.* (2017a) para incluir 16 linhagens, correspondendo a 10 espécies nominais, previamente reconhecidas em *Leporinus* e *Hypomasticus*, e como tentativa de recuperação da monofilia de *Leporinus* (RAMIREZ *et al.*, 2017a). A descrição desse novo gênero foi suportada por dados citogenéticos, moleculares e morfológicos, caracterizando-se por ter um sistema único de cromossomos sexuais ZZ/ZW (GALETTI JR *et al.*, 1995), enquanto a maioria das espécies de *Leporinus* conhecidas pela citogenética não possuem estes cromossomos sexuais (GALETTI JR *et al.*, 1981, 1991). A monofilia do gênero *Megaleporinus* ainda é bem suportada por marcadores mitocondriais e nucleares, que o identificaram como o grupo irmão de *Abramites* (RAMIREZ *et al.*, 2017a). Quanto à morfologia caracteriza-se por suas espécies apresentarem um tamanho relativamente grande, três dentes unicúspides em cada osso pré-maxilar e dentário e um padrão de cor

composto por uma a quatro manchas mediolaterais variando de um a quatro (RAMIREZ *et al.*, 2017a).

Nos últimos anos, estudos moleculares mostraram que existe uma diversidade oculta ao nível de espécie dentro da família Anostomidae, como é o caso dos gêneros *Laemolyta* (RAMIREZ e GALETTI JR, 2015), *Megaleporinus* (RAMIREZ *et al.*, 2017b) e *Leporinus* (SILVA-SANTOS *et al.*, 2018). No estudo de Silva-Santos *et al.* (2018) a partir de dados moleculares foi sugerido que a espécie *Leporinus friderici* Block (1794) representaria um complexo de espécies, do qual apenas *L. friderici* dos rios do Suriname e Guiana Francesa deve ser reconhecido como espécie-tipo (SIDLAUSKAS e VARI, 2012) e aos demais morfotipo ocorrendo dentro do Escudo Brasileiro foi recomendado a adoção da nomenclatura provisória de *L. cf. friderici* (SILVA-SANTOS *et al.*, 2018). Essa diversidade pode representar uma situação típica de especiação recente formando grupos estritamente relacionados revelando incertezas taxonômicas típicas (RAMIREZ *et al.*, 2017b).

A identificação de espécies de peixes neotropicais avançou significativamente com o estabelecimento de abordagens moleculares (PEREIRA *et al.*, 2011, 2013; ROSSINI *et al.*, 2016; MACHADO *et al.*, 2018) e análises integrativas de dados morfológicos e moleculares (MELO *et al.*, 2016; RAMIREZ *et al.*, 2016; SILVA *et al.*, 2016; CALEGARI *et al.*, 2019). Essas abordagens têm sido úteis na resolução de incertezas taxonômicas (BRITSKI *et al.*, 2019), na detecção de diversidade oculta (CARVALHO *et al.*, 2012; PIRES *et al.*, 2017; RAMIREZ *et al.*, 2017a), bem como na descoberta de novas espécies e linhagens crípticas (GOMES *et al.*, 2015; RAMIREZ e GALETTI JR, 2015; MELO *et al.*, 2016; RAMIREZ *et al.*, 2016; PUGEDO *et al.*, 2016; SILVA-SANTOS *et al.*, 2018). Porém, estudos utilizando dados moleculares ou integrando morfologia e molecular em anostomídeos ainda são incipientes, visto a grande problemática taxonômica evidenciada do grupo, o cenário é ainda mais preocupante quando volta-se para os anostomídeos de bacias do Maranhão (FRAGA *et al.*, 2014; NASCIMENTO *et al.*, 2016).

Atualmente, para o Maranhão são descritas as ocorrências das espécies: *Leporinus affinis* (GÜNTHER, 1864) para bacia do Rio Itapecuru (ABREU *et al.*, 2019); *L. friderici* distribuídos nas bacias dos rios Itapecuru, Mearim, Maracaçumé, Munim, Peria e Parnaíba (PIORSKI *et al.*, 1998; SOARES, 2005; RAMOS *et al.*, 2014; MELO *et al.*, 2016; ABREU *et al.*, 2019; BRITO *et al.*, 2019; BRITO *et al.*, 2020; GUIMARÃES *et al.*, 2021a, 2021b, 2021c; NASCIMENTO *et al.*, 2023); *L. piau* nas bacias dos rios Itapecuru, Mearim, Turiaçu e Parnaíba (BARROS *et al.*, 2011; RAMOS *et al.*, 2014; RIBEIRO *et al.*, 2014; FRAGA *et al.*, 2014; NASCIMENTO *et al.*, 2016; ASSEGA e BIRINDELLI, 2019; ABREU *et al.*, 2019; NASCIMENTO *et al.*, 2023); bem como a ocorrência de *Megaleporinus macrocephalus*

(GARAVELLO e BRITSKI, 1988) para as bacias dos rios Itapecuru e Mearim (GUIMARÃES *et al.*, 2020c; ALMEIDA *et al.*, 2021).

Em um cenário em que a identificação baseada em morfologia, por si só, não é suficiente, o uso de informações moleculares pode ser útil para delimitar espécies e indicar a potencial diversidade oculta (COSTA-SILVA *et al.*, 2015; SILVA-SANTOS *et al.*, 2018), bem como para a construção de filogenias robustas como observado nos estudos de Oliveira *et al.* (2011) e Ramirez *et al.* (2016; 2017b). Portanto, estudos que integram diferentes abordagens, como o apresentado aqui, possibilitam uma melhor resolução das complexidades taxonômicas permitindo o reconhecimento de espécies de importância pesqueira sob um nome comum, informação esta muito importante em tempos em que muitas espécies estão se extinguindo rapidamente.

2.3 MARCADORES MOLECULARES

Abordagens utilizando marcadores moleculares têm sido cada vez mais adotadas em estudos de peixes do gênero *Leporinus*, tanto a partir da análise de marcadores mitocondriais, quanto nucleares ou ambas combinadas (CHIARI; SODRÉ 1999; MARTINS *et al.*, 2003; CALCAGNOTTO *et al.*, 2005; SIDLAUSKAS; VARI, 2012; FRAGA *et al.*, 2014; RAMIREZ; GALETTI JR, 2015; RAMIREZ *et al.*, 2016; NASCIMENTO *et al.*, 2016; SILVA-SANTOS *et al.*, 2018).

O genoma animal está distribuído no núcleo e nas mitocôndrias. O genoma nuclear apresenta uma extrema complexidade organizacional e um tamanho de ordem de bilhões de pares de bases nitrogenadas, enquanto o genoma mitocondrial apresenta alguns poucos genes codificados por algumas dezenas de pares de bases. A maior parte das investigações com informações de DNA em animais utiliza genes de genoma mitocondrial e isso se deve ao fato de que esse genoma apresenta uma série de particularidades importantes, como sua herança quase que exclusivamente materna e sua presença nos organismos em número haplóide, o que torna raro os eventos de recombinação (MEYER, 1993; ROCHA-OLIVARES *et al.*, 1999; LOVEJOY e DE ARAUJO, 2000; TSIGENOPOULOS e BERREBI, 2000).

Entre os marcadores moleculares do DNA mitocondrial destaca-se o gene Citocromo Oxidase Subunidade I (COI) bastante utilizados nas investigações moleculares. Em 2004 foi proposta a utilização de um fragmento deste gene como um sistema de identificação global (HERBET *et al.*, 2004; HUBERT *et al.*, 2008; WARD *et al.*, 2008) popularizado mundialmente como técnica de DNA *barcoding* ou simplesmente, “código de barras de DNA”. O gene COI é uma subunidade do complexo Citocromo Oxidase, que faz parte da cadeia transportadora de elétrons e tem sido utilizado com o objetivo de identificar espécies, possibilitar o estudo

comparativo entre espécimes de posição taxonômica desconhecida, aumentar a descoberta de novas espécies e como ferramenta auxiliar em estudos filogenéticos e catalogação da biodiversidade, constituindo uma ferramenta poderosa nos estudos de peixes neotropicais (ROSA e PAIVA, 2009).

Outro gene bastante utilizado em investigações moleculares é o rRNA 16S, um gene estrutural que faz parte da grande subunidade ribossomal do DNA mitocondrial, assim como o rRNA 12S (ROMANO e PALUMBI, 1996). As sequências do rRNA 16S são razoavelmente conservadas, e tem se mostrado como um bom marcador na diferenciação de peixes, como também em estudos comparativos intergenéricos e interespecíficos (SANTOS *et al.*, 2003; CALCAGNOTTO *et al.*, 2005; FRAGA *et al.*, 2007; FRAGA *et al.*, 2014). O uso desse gene tem sido utilizado com sucesso em estudos filogenéticos de diversos grupos de vertebrados, como em peixes, pois existe uma considerável variação em algumas espécies (CALCAGNOTTO *et al.*, 2005).

A exemplo dos genes COI e rRNA 16S, o gene Citocromo *b* (Cyt *b*) também é um gene mitocondrial amplamente utilizado em estudos sistemáticos para resolver divergências em muitos níveis taxonômicos. Apesar da sua ampla utilização, provavelmente constitui o gene mitocondrial mais conhecido no que diz respeito a estrutura e função do seu produto protéico (ESPOSTI *et al.*, 1993). O gene Cyt *b* contém posições de códons de evolução lenta e rápida, assim como regiões mais conservadoras e outras mais variáveis, o que reflete na sua utilização em estudos com grupos que apresentam conflitos taxonômicos e em investigações filogenéticas (MEYER e WILSON, 1990; IRWIN *et al.*, 1991; NORMARK *et al.*, 1991; CANTATORE *et al.*, 1994; LYDEARD e ROE, 1997; KUMAZAWA e NISHIDA, 2000; RAMIREZ *et al.*, 2016; SILVA-SANTOS *et al.*, 2018); assim como em investigações com táxons que apresentam níveis de divergência recente (STURMBAUER e MEYER, 1992; ROCHA-OLIVARES *et al.*, 1999; KIRCHMAN *et al.*, 2000; LOVEJOY e DE ARAUJO, 2000).

O uso de genes nucleares na elaboração de filogenias é bastante útil quando o tempo de divergência dos grupos é elevado, uma vez que esses genes aprestam taxas evolutivas mais lentas (os genes são mais conservados), quando comparados aos mitocondriais, no entanto por apresentar extrema complexidade organizacional e um tamanho da ordem de bilhões de pares de bases pode refletir diretamente na baixa adesão nos estudos, quando comparado ao número daqueles que utilizam genes mitocondriais (STEPIEN e KOCHER, 1997; ORTÍ, 1997). Segundo Slowinski e Page (1999), em estudos moleculares o ideal seria combinar dados obtidos de DNA mitocondrial e nuclear, pois as histórias dos genes são independentes.

O gene nuclear α -tropomiosina (TROP) é responsável pela produção da proteína Tropomiosina que em conjunto a Troponina medeiam a regulação da contração dos músculos

esqueléticos por meio dos íons Ca^{2+} (ALBERTS, *et al.* 2006). Alguns estudos filogenéticos têm sido realizados mostrando a utilidade e eficácia deste gene em diversos grupos de peixes (ORTÍ e MEYER, 1996; LOVEJOY e COLLETE, 2001; HARDMAN, 2004; NEAR *et al.*, 2004; MOYER *et al.*, 2004; QUENOUILLE *et al.*, 2004; CALCAGNOTTO *et al.* 2005).

2.4 REDE HIDROLÓGICA DO ESTADO DO MARANHÃO

O Maranhão, apesar de conter uma importante rede hidrológica localizada em uma área de transição entre o semiárido nordestino e a floresta amazônica, tem sua fauna piscícola pouco estudada. Os estudos realizados com os peixes maranhenses são em sua maioria inventários, sendo ainda escassos estudos sobre sistemática, variações geográficas e biogeografia integrandos dados moleculares aos morfológicos, principalmente para os grupos de ampla distribuição, como os *Leporinus*.

O Núcleo Geoambiental da Universidade Estadual do Maranhão - NuGeo/UEMA, em 2006, delimitou no estado do Maranhão 12 bacias hidrográficas. Divisão semelhante a aquela definida por meio do Decreto Estadual nº 27.845/2011, do Governo do Maranhão, em que no seu Art. 5º estabelece a divisão do estado em 12 bacias hidrográficas. Sendo assim, o Estado do Maranhão é constituído de nove bacias hidrográficas de domínio estadual e três de domínio federal. As bacias de domínio estadual são representadas pelos Sistemas hidrográficos estaduais das Ilhas Maranhenses e do Litoral Ocidental, bem como as bacias do Mearim, Itapecuru, Munim, Turiaçu, Maracaçumé, Preguiças e Peria. De domínio federal tem-se as bacias do Parnaíba, a leste; do Tocantins, a sudoeste; e Gurupi, a noroeste.

Entre as bacias de domínio estadual, também designada por bacias genuinamente maranhenses, enfocaremos nas bacias hidrográficas dos rios Mearim, Itapecuru, Pericumã, Turiaçu, Preguiças e Peria. Para as bacias de domínio federal destacaremos as bacias hidrográficas dos rios Parnaíba e Tocantins (Figura 3).

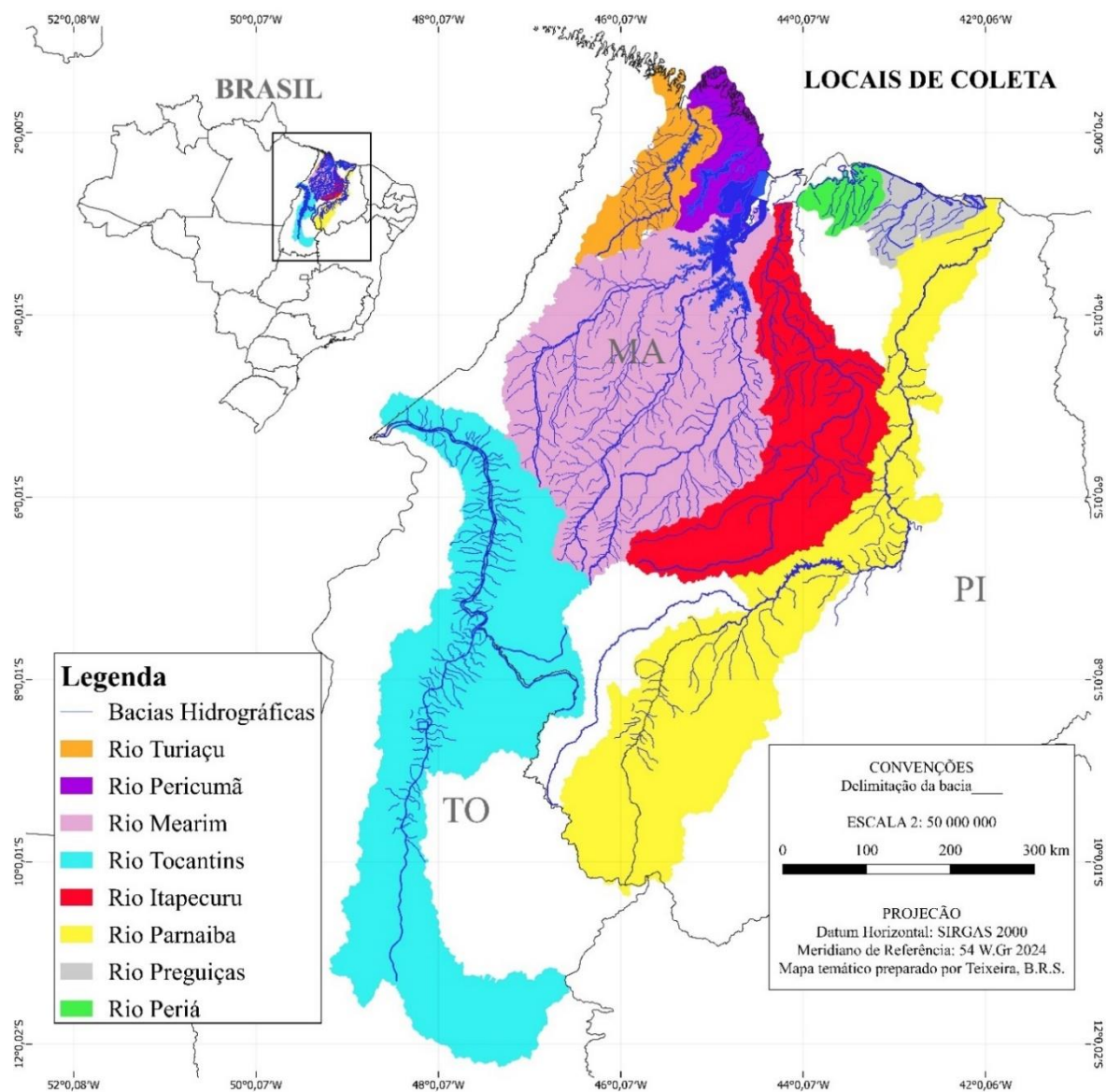


Figura 3. Mapa evidenciando as bacias hidrográficas dos rios Itapecuru, Mearim, Peria, Preguicas, Turiaçu, Pericumã (Maranhão), Tocantins (Tocantins) e Parnaíba (Piauí), locais de coleta das amostras de *Leporinus*.

A bacia do rio Mearim possui uma área de aproximadamente 96.000 Km² e nasce na confluência das serras Negra, Menina e Cruzeiras desembocando na Baía de São Marcos, na altura da ilha do Caranguejo (STELLA, 2011). Esta bacia tem o rio Pindaré como principal afluente, nasce nas elevações que formam o divisor entre as bacias Mearim e Tocantins, com aproximadamente 686 Km², sendo navegável no trecho compreendido entre a sua foz no Km 41 do rio Mearim até a foz do rio Buriticupu no Km 456 (STELLA, 2011).

A bacia do rio Itapecuru apresenta uma área de 52.972,1 Km² extensão, que percorre desde sua nascente, nas serras da Cruieira, Itapecuru e Alpercatas até sua desembocadura na baía do Arraial, a sul da ilha de São Luís/MA, caracterizando-se como a segunda maior bacia hidrográfica do estado do Maranhão (DE ALCÂNTARA, 2004).

Os rios Itapecuru, Mearim e Pindaré, formam um grupo de bacias com topografia similar desaguardo no golfo maranhense (Mearim/Pindaré na Baía de São Marcos e Itapecuru na Baía de São José), sendo que em seus trechos inferiores, exibem padrão diferenciado com formação de meandros e lagos exclusivo na bacia do Mearim. A evolução geomorfológica do Golfo Maranhense resultou de flutuações marinhas durante o Plioceno e Pleistoceno delineando o padrão atual de desembocadura dos rios da região central do Maranhão (AB'SÁBER, 1960).

A bacia do rio Pericumã está localizada na Microrregião da Baixada Maranhense é uma das bacias hidrográficas secundárias do Estado do Maranhão, com aproximadamente 10.800 km², sua nascente está situada na Lagoa da Traíra e deságua na baía de Cumã, entre os municípios de Guimarães e Alcântara (PINHEIRO, 2000; BRASIL, 2006).

A bacia hidrográfica do rio Turiaçu possui uma área de 14.149,87 km², representando cerca de 4,26% da área do Estado, cujo leito principal é o rio Turiaçu. Sua nascente está localizada nas vertentes da Serra do Tiracambu, a partir desse ponto percorre 720 km de extensão em direção à baía de Turiaçu, entre os municípios de Turiaçu e Bacuri. Durante este percurso, o rio Turiaçu recebe a contribuição dos rios Paraná e Caxias pela margem esquerda e, inúmeros igarapés pela margem direita (MARANHÃO, 2016; MARTINS e OLIVEIRA, 2011).

As bacias dos rios Preguiças e Peria situadas na porção Nordeste do Estado, pertencem à região dos Lençóis Maranhenses e são pouco amostradas em termos de sua ictiofauna, apresentando pouca ou nenhuma informação, principalmente no que diz respeito aos aspectos genéticos (BRITO *et al.*, 2019). A bacia Hidrográfica do Rio Preguiças possui uma área total de 6.750,91 km² e constituída por três rios: o rio Preguiças, que é o rio principal e tem a maior extensão; o rio Negro e o rio Cangatã. O rio Preguiças, também chamado de rio Grande, nasce no município de Santana do Maranhão percorrendo quase 135 km de extensão até chegar à sua foz, no Oceano Atlântico no município de Barreirinhas (MARANHÃO, 2002). A bacia hidrográfica do rio Peria apresenta a menor área hidrográfica do Estado com um total de 5.395,37 km², representando cerca de 1,62% da área total do Maranhão. Limitando-se com as bacias do Munim, Preguiças e com o Oceano Atlântico, e tem como seus principais afluentes os rios Bacaba e Ribeira.

A bacia hidrográfica do rio Parnaíba possui uma área de 342.988 Km², nasce nos contrafortes da Chapada das Mangabeiras, sul do Maranhão, percorrendo cerca de 1450 km até sua desembocadura no Oceano Atlântico (STELLA, 2011). Esta bacia se consagra como a segunda maior bacia hidrográfica do nordeste brasileiro com ampla diversidade ictiofaunística, tem regime perene e drena cerca de 275 municípios localizados nos estados do Piauí, Ceará e

Maranhão (ROSA *et al.*, 2003; RAMOS *et al.*, 2014; MINISTÉRIO DO MEIO AMBIENTE, 2006).

A bacia do rio Tocantins faz parte da região hidrográfica do Tocantins-Araguaia que cobre uma área de 921.921 km² e abrange parte dos estados do Maranhão (3,8%), Tocantins (34,2%), Pará (20,8%), Goiás (26,8%), Mato Grosso (14,3%) e Distrito Federal (0,1%), o equivalente a aproximadamente 10,8% do território brasileiro (MINISTÉRIO DO MEIO AMBIENTE, 2006). Esta região hidrográfica possui configuração alongada no sentido longitudinal, seguindo as diretrizes dos dois importantes eixos fluviais – os rios Tocantins e Araguaia – unindo-se no extremo setentrional da bacia, formando o baixo Tocantins, que desemboca no rio Pará, pertencente ao estuário do rio Amazonas (MARTINS e OLIVEIRA, 2011). O rio Tocantins, por sua vez, tem sua nascente no planalto de Goiás, sendo formado pelos rios das Almas e Maranhão, e possui a extensão total aproximada de 1.960 km até a sua foz no Oceano Atlântico (AGÊNCIA NACIONAL DE ÁGUAS, 2013).

3 RESULTADOS

3.1 CAPÍTULO I

Artigo publicado na revista PeerJ: **THE DNA BARCODE REVEALS CRYPTIC DIVERSITY AND A NEW RECORD FOR THE GENUS *LEPORINUS* (CHARACIFORMES, ANOSTOMIDAE) IN THE HYDROGRAPHIC BASINS OF CENTRAL NORTHERN BRAZIL**



The DNA barcode reveals cryptic diversity and a new record for the genus *Leporinus* (Characiformes, Anostomidae) in the hydrographic basins of central northern Brazil

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ABSTRACT

Leporinus is one of the most speciose genera of the order Characiformes, with 81 valid species distributed throughout much of Central and South America. The considerable diversity of this genus has generated extensive debate on its classification and internal arrangement. In the present study, we investigated the species diversity of the genus *Leporinus* in central northern Brazil, and conclude that six valid species—*Leporinus maculatus*, *Leporinus unitaeniatus*, *Leporinus affinis*, *Leporinus venerei*, *Leporinus* cf. *friderici*, and *Leporinus piau*—are found in the hydrographic basins of the Brazilian states of Maranhão, Piauí, and Tocantins. We analyzed 182 sequences of the Cytochrome Oxidase subunit I gene, of which, 157 were obtained from *Leporinus* specimens collected from the basins of the Itapeturu, Mearim, Turiaçu, Pericumã, Peria, Preguiças, Parnaíba, and Tocantins rivers. The species delimitation analyses, based on the ABGD, ASAP, mPTP, bPTP, and GMYC methods, revealed the presence of four distinct molecular operational taxonomic units (MOTUs), identified as *L. maculatus*, *L. unitaeniatus*, *L. affinis*, and *L. piau* (from the Parnaíba River). The bPTP method restricted *L. venerei* to a single MOTU, and confirmed the occurrence of this species in the rivers of Maranhão for the first time. The separation of *L. cf. friderici* into two clades and the subsequent formation of different operational taxonomic units was consistent with polyphyly in this species, which indicates the existence of cryptic diversity. The arrangement of *L. cf. friderici* and *L. piau* in two different clades supports the conclusion that the *L. piau* specimens from Maranhão were misidentified, based on their morphological traits, reflecting the taxonomic inconsistencies that exist among morphologically similar species. Overall, then, the species delimitation methods employed in the present study indicated the presence of six MOTUs—*L. maculatus*, *L. unitaeniatus*, *L. affinis*, *L. cf. friderici*, *L. venerei*, and *L. piau*. In the case of two other MOTUs identified in the present study, one (*L. venerei*) is a new record for the state of

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Maranhão, and we believe that the other represents a population of *L. piau* from the basin of the Parnaíba River.

Subjects Aquaculture, Fisheries and Fish Science, Genetics, Molecular Biology, Zoology, Freshwater Biology

Keywords Molecular identification, Freshwater fish, Leporinus, Neotropical biodiversity, Systematics

INTRODUCTION

The family Anostomidae is a prominent group of Neotropical fish that includes 15 genera and approximately 151 valid species ([Ramirez et al., 2016](#); [Britski & Birindelli, 2019](#); [Ramirez et al., 2020](#)). The most speciose genus is *Leporinus*, which has approximately 81 valid nominal species ([Fricke, Eschmeyer & Laan, 2021](#)). [Géry \(1977\)](#) concluded that *Leporinus* is one of the most diverse genera of the order Characiformes, which is distributed between Central America and southern South America.

The considerable diversity found in the genus *Leporinus* has led to numerous attempts to classify its species and determine its internal arrangement. A number of studies have proposed subdivisions based on the position of the mouth, and the shape and arrangement of the teeth ([Borodin, 1929](#); [Myers, 1950](#); [Garavello, 1979](#)). [Britski & Garavello \(1978\)](#) divided the genus into three groups based on coloration patterns, that is, banding, spots, and longitudinal lines, although these proposals have been contradicted by more comprehensive studies, such as those of [Sidlauskas & Vari \(2008\)](#) and [Ramirez et al. \(2016\)](#). In their cytogenetic study, [Galetti, Lima & Venere \(1995\)](#) confirmed the existence of a well-defined ZZ/ZW sex chromosome system in six *Leporinus* species. These authors proposed that the presence of the ZW system represents a synapomorphy, and that the six species with this system form a monophyletic group. This conclusion is reinforced by morphological traits, such as coloration patterns, relatively large body sizes, and the number of teeth, as confirmed by [Ramirez et al. \(2016\)](#), which led to the allocation of this group to a new genus, *Megaleporinus*, by [Ramirez, Birindelli & Galetti \(2017\)](#).

Using osteological markers, [Sidlauskas & Vari \(2008\)](#) evaluated the phylogenetic relationships of the anostomids, and concluded that this family is monophyletic, although they were unable to confirm the monophyly of the genus *Leporinus*. [Ramirez et al. \(2016\)](#) used nuclear and mitochondrial molecular markers to confirm the paraphyly of the genus *Leporinus*, and concluded that the recuperation of the monophyly of the group would depend on further taxonomic reviews, including the creation of new genera and the description of new species.

Traditional taxonomic approaches have been essential for the delimitation of anostomid species based on morphological traits, although this does not necessarily resolve some natural groups, given that morphologically similar species may be assigned to the same nominal taxon ([Bickford et al., 2007](#)). Deciphering and defining cryptic diversity accurately is fundamental to the understanding of the ecological, biogeographic, and evolutionary

patterns of a group of organisms, in addition to its other biological features ([Kress et al., 2015](#)).

[Hebert et al. \(2003\)](#) proposed the use of a DNA barcode, based on a standard sequence of the mitochondrial Cytochrome Oxidase subunit I (COI) gene, as the basis for a global species identification system. This approach has been widely-used for the identification of species and the resolution of cryptic diversity within genera and, in particular, in species complexes. A species complex consists of a group of closely-related taxa that have typically undergone recent speciation, which means that their taxonomic differences are still incipient, as observed in the case of the *Leporinus* cf. *friderici* species complex, in which [Silva-Santos et al. \(2018\)](#) confirmed the presence of eight distinct Molecular Operational Taxonomic Units (MOTUs) arranged in three clades.

Leporinus is not only one of the most diverse fish genera, but its species also play an important ecological role in many freshwater ecosystems, as well as having considerable economic and social importance for local fisheries. Given this, we compiled a dataset of the mitochondrial COI gene of 182,179 *Leporinus* specimens, which included specimens from the hydrographic basins of the Brazilian state of Maranhão to verify the potential intrageneric diversity of this genus, *i.e.*, the presence of different putative species for the study region.

Here we present the diversity of *Leporinus* from hydrographic basins of central northern Brazil. We used integrative taxonomy tools to assess the species diversity of *Leporinus* based on (i) morphological identification from external characters, (ii) morphological identification from dentary characters, and (iii) molecular identification from COI gene fragment.

MATERIAL AND METHODS

Sampling

The present study was based on the analysis of a total of 185 sequences, of which 182 were of *Leporinus* species, with the other three representing the outgroup. The vast majority (157) of these 182 *Leporinus* sequences were collected during the present study, being extracted from specimens collected from basins in the Brazilian states of Maranhão (Itapecuru, Mearim, Turiaçu, Pericumã, and Periá rivers), Piauí (Parnaíba River), and Tocantins, that is, the Tocantins River ([Fig. 1](#) and [Table S1](#)). The other 25 sequences were obtained from GenBank ([Table S2](#)).

The samples from the rivers of Maranhão, Piauí and Tocantins were obtained during extensive fieldwork, which has been ongoing since 2006. This research was authorized by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) through license 02012.004159/2006, and licenses ICMBio/MMA 42119-1/2013, ICMBio/MMA 46367-1/2015, ICMBio/MMA 83138-1/2022, ICMBio/MMA 73790-6/2022 issued by the Chico Mendes Institute for Biodiversity Conservation.

After collection, the specimens were taken to the Genetics and Molecular Biology Laboratory (GENBIMOL) of the Advanced Studies Center of Maranhão State University (CESC/UEMA), where they photographed and registered using a coding system. Samples

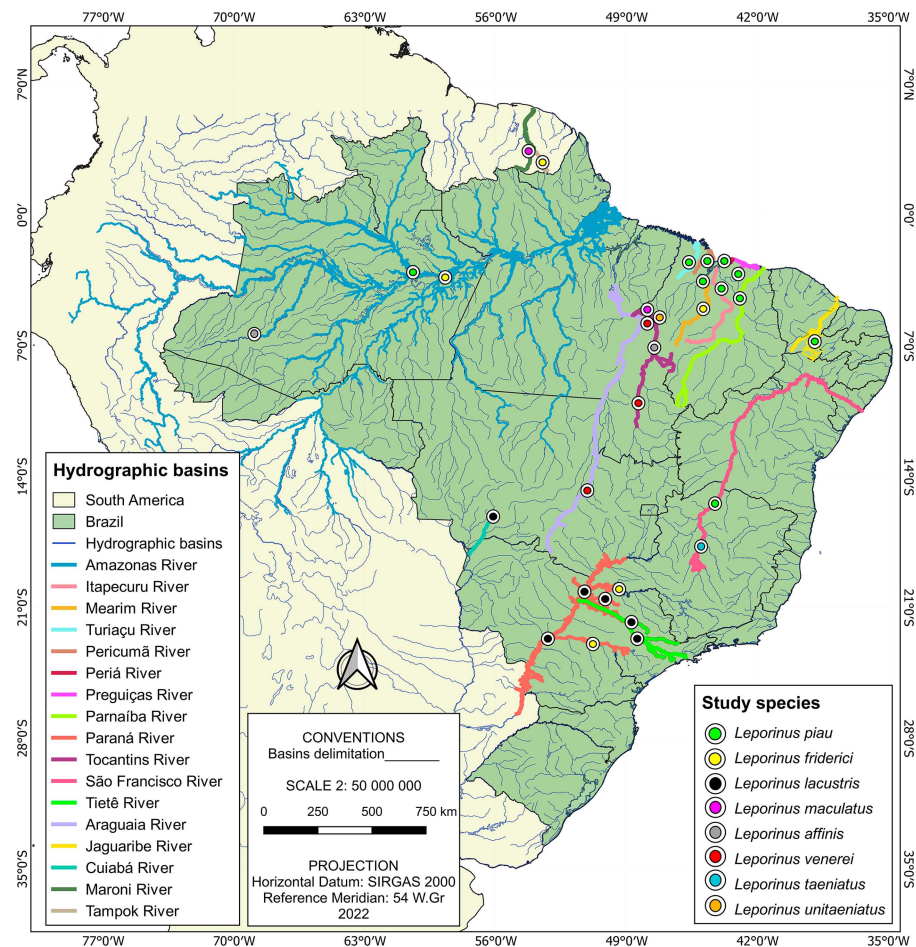


Figure 1 Sample localities. Each data point indicates the location where *Leporinus* samples were collected.

Full-size [DOI: 10.7717/peerj.15184/fig-1](https://doi.org/10.7717/peerj.15184/fig-1)

of muscle tissue were extracted from the specimens for the genetic analyses. The specimens were then fixed in 10% formaldehyde and conserved in 70% alcohol, before being sent to the Museum of Zoology at Londrina State University (MZUEL) in Londrina, Paraná, Brazil, for morphological identification and cataloguing. The study of wild animals was approved by the Regulatory Committee for the Ethical Treatment of Animals of Maranhão State University (protocol 47/2022) and by the Committee for the Ethical Use of Animals of the National Institute for Amazonian Research, registered under protocol number 006/2021, SEI 01280.000116/2021-45.

The total DNA was extracted using the Wizard Genomic DNA Purification kit from Promega, following the maker's instructions. The genomic region was isolated and amplified by Polymerase Chain Reaction (PCR), using the universal primers COI FishF1 5'-TCAACCAACCACAAAGACATTGCCAC-3' and COI FishR1 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3', described by [Ward et al. \(2005\)](#). The samples

were sequenced by the *Sanger, Nicklen & Coulson (1977)* method, using the Big Dye kit in an ABI Prism™ 3500 automatic sequencer (Applied Biosystems, EUA).

The sequences were aligned and edited in the Clustal W (*Thompson, Higgins & Gibson, 1994*) application of the Bioedit 7.2.5 program (*Hall, 1999*). All newly generated sequences (175) were deposited in GenBank under accession numbers [OP781850–OP781884](#), [OP782222–OP782283](#), [OP782350–OP7882375](#) and [OP782385–OP782418](#) (Table S1). The haplotypes were delineated in DnaSP 5.1 (*Librado & Rozas, 2009*). The mean genetic distances and the Maximum Likelihood (ML) tree were obtained in MEGA X (*Kumar et al., 2018*), using the Kimura 2-Parameter and Hasegawa-Kishino-Yano (HKY) models, respectively, with the trees being reconstructed using 1,000 bootstrap replicates.

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*), which is available at CIPRES Science Gateway v3.3 (*Miller, Pfeiffer & Schwartz, 2010*), using the Hasegawa-Kishino-Yano (HKY+G+I) algorithm. The BI tree was generated in BEAST v.1.10.4 (*Drummond et al., 2012*; *Suchard et al., 2018*), using the relaxed lognormal clock (*Drummond et al., 2006*) and the birth-death speciation model (*Gernhard, 2008*).

This analysis was based on 40,000,000 generations with the log files being verified in Tracer v1.6 (*Rambaut et al., 2014*) to evaluate convergence and the most adequate burn-in, with the convergence being considered adequate when the Effective Sample Size (ESS) was over 200. The trees generated in BEAST were summarized in TreeAnnotator v.10.4 (*Suchard et al., 2018*) to obtain the consensus tree, which was then visualized and edited in Fig Tree v1.4.2 (*Rambaut, 2014*) and the Inkscape image editing system. Clades with a bootstrap percentage of at least 85% or posterior probability of at least 0.95 were considered to be well supported.

The delimitation analyses of the MOTUs of the COI gene were run using the following models: the Automatic Barcode Gap Discovery (ABGD), Assemble Species by Automatic Partitioning (ASAP), Poisson Tree Process (PTP), and the Generalized Mixed Yule Coalescent (GMYC) model. The ABGD test (*Puillandre et al., 2012*) was run in <https://bioinfo.mnhn.fr/abi/public/abgd/> using the dataset of aligned sequences, while the ASAP test (*Puillandre, Brouillet & Achaz, 2020*) was implemented in <https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html> using the matrix of genetic distances, extracted using MEGA X, as the input. The PTP (*Zhang et al., 2013*) was run on the web server <https://species.h-its.org/>. In this case, the input was the Maximum Likelihood phylogenetic tree produced in RaxML v.8.29 (*Stamatakis, 2014*), which is available in the CIPRES Science Gateway v3.3 (*Miller, Pfeiffer & Schwartz, 2010*). The GMYC (*Fujisawa & Barraclough, 2013*) was based on the ultrametric consensus tree constructed in BEAST v1.10.1, which was processed in the Ape (*Paradis & Schliep, 2019*), Splits (*Ezard, Fujisawa & Barraclough, 2009*), Paran (*Dinno, 2009*), and Mass (*Venables & Ripley, 2002*) packages available in the R v. 4.1.0 software (*R Core Team, 2021*).

RESULTS

The present study focused on 182 sequences of the COI gene of *Leporinus*, each consisting of 620 base pairs (bps). The phylogenetic trees generated by the ML and BI analyses were

highly congruent and well-supported at both the intra- and interspecific levels (Figs. 2–4), except in the case of *Leporinus piau*, which grouped with either *Leporinus* cf. *friderici* or *Leporinus venerei*. The ABGD analysis delimited 12 MOTUs, while the ASAP defined 15, the mPTP and bPTP each delimited nine, and the GMYC, six MOTUs (Fig. 2).

The results of the five delimitation methods applied in the present study had three species in common—*L. maculatus*, *L. unitaeniatus*, and *L. affinis*—as well as differentiating two specimens (PALEP01 and PALEP09) from the basin of the Parnaíba River in a distinct molecular taxonomic unit, which indicates the occurrence of a fourth species, which we believe to be *L. piau*.

In the present study, the five delimitation methods had three species in common—*L. maculatus*, *L. unitaeniatus*, and *L. affinis*—and differentiated specimens from the basin of the Parnaíba River in a distinct molecular taxonomic unit, which is more basal than the other *Leporinus* species, and groups with the the *Megaleporinus* species that were previously assigned to *Leporinus*.

Clade VI (Fig. 3) was strongly supported, and includes *L. venerei*, *L. lacustris*, *L. piau*, and *L. cf. friderici*, with *L. piau* occurring in Maranhão, in the Mearim, Itapecuru, Pericumã, Turiaçu, Preguiças and Peria basins. In this case, the clade was formed by *L. venerei* from the Tocantins basin, *L. lacustris* from the basin of the Paraná River, *L. piau* from the Jaguaribe, Itapecuru, Mearim, Pericumã, Turiaçu, Preguiças and Peria basins, and *L. cf. friderici* from the Amazon and Mearim basins, which all share a single molecular taxonomic unit. Only the bPTP analysis separated *L. venerei* from *L. lacustris*, *L. piau*, and *L. cf. friderici*, which together formed a single MOTU in the ABGD, ASAP, mPTP, and GMYC models (Fig. 2).

The BI and ML analyses identified the formation of subclades within clade VI (Figs. 2 and 3), in which the *L. piau* from Maranhão, in the Itapecuru, Turiaçu, Mearim, and Peria basins, grouped with *L. venerei* from the Tocantins basin, with genetic distances ranging from only 0.16% to 1.54% (Table S3). Other *L. lacustris* and *L. piau* subclades were identified in the Jaguaribe basin, where the genetic distances ranged from 0.0% to 3.5% (Table S3). The *L. piau* subclade from Maranhão, found in the Itapecuru, Mearim, Pericumã, and Turiaçu basins, grouped with *L. cf. friderici* from the Mearim (Maranhão) and Amazon basins (Amazonas state), with genetic distances of between 0.16% and 5.88% (Table S3). All three groups were supported by significant posterior probability (BI) and bootstrap(ML) values (Figs. 2 and 3).

Leporinus cf. *friderici*, whose type locality is the basin of the Tampok River in French Guiana, formed a group together with *L. piau* from the basins of the São Francisco, Amazon, and Mearim rivers, an arrangement found in both the species delimitation models and the BI and ML trees. In the ABGD, ASAP mPTP, and bPTP delimitation models, however, *L. cf. friderici* was differentiated in its own operational unit (Fig. 2).

The genetic distance matrix derived from the molecular taxonomic units revealed relatively high values for both the intra- and inter-MOTU distances. The highest mean intra-MOTU distance was 5.9%, in *L. maculatus*, while the lowest mean was 0.4%, in *L. unitaeniatus*, whereas the mean inter-MOTU distances ranged from 7.8% to 17.4%. The MOTUs formed by *L. venerei*, *L. lacustris*, *L. piau*, and *L. cf. friderici* were separated by a mean genetic distance of 2.2% (Table 1). In this context, it is important to note the genetic

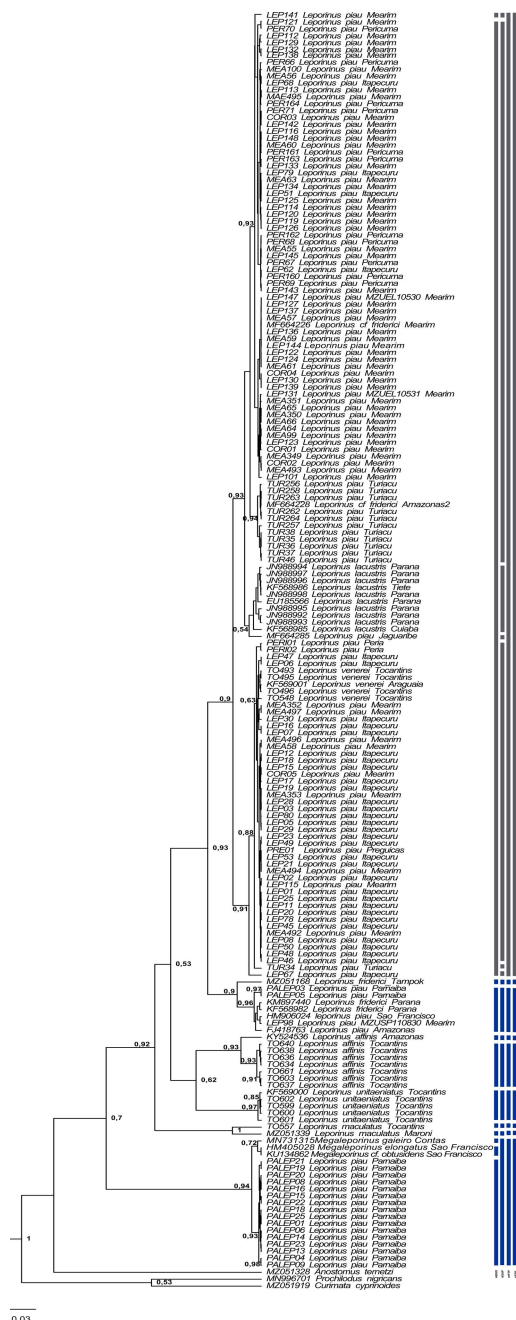


Figure 2 Bayesian Inference tree showing the arrangement of the MOTUs of the *Leporinus* species analyzed in the present study. This arrangement was obtained using the ABGD, ASAP, mPTP, bPTP, and GMYC species delimitation approaches for the analysis of the mitochondrial COI gene, based on the Hasegawa-Kishino-Yano (HKY+G+I) algorithm, generated in BEAST. The species delimited by the specific estimates are shown by the vertical bars, with the color representing the current status of the species. The blue bars correspond to valid species, while the gray bars indicate the species delimited differently from the current classification.

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Full-size DOI: [10.7717/peerj.15184/fig-3](https://doi.org/10.7717/peerj.15184/fig-3)

distance of 7.8% between *L. piau* (MOTU 1) and *L. cf. friderici* (MOTU 2), which may be the result of an error in the identification of the species of one of the groups.

Given the levels of congruence identified in the different delimitation analyses applied in the present study, the ASAP method appeared to be the most effective interpretation, in biological terms, of the dataset considered here, given that it identified 10 MOTUs, which distinguished four of the seven nominal species, including *L. venerei*, in a distinct MOTU. This confirmed the occurrence of this species the Itapecuru, Mearim, Turiaçu, Preguiças and Peria basins, which constitutes the first record of *L. venerei* in the Brazilian state of Maranhão.

DISCUSSION

An adequate taxonomic assessment is fundamental for the success of many types of biological research, and DNA data have provided additional insights for the resolution of taxonomic questions in many groups of organisms, including elements of the megadiverse Neotropical fish fauna, such as the anostomids. The COI barcode proved to be an extremely valuable tool for the identification and separation of the species assessed in the present study, based on the analysis of genetic distances and species delimitation, which identified evidence of the potential presence of more than one taxon in some nominal species.

In many previous studies of DNA barcoding and molecular diversity, the number of species or lineages delimited by the analysis has tended to exceed the number of nominal taxa or even the morphospecies analyzed ([Carvalho et al., 2018](#)). A similar tendency was observed here, in addition to the opposite pattern, given that, in some species delimitation analyses, more than one valid species was allocated to the same MOTU, as in the case of *L. venerei*, *L. lacustris*, *L. cf. friderici*, and *L. piau*.

In the present study, the *L. venerei*, *L. lacustris*, *L. cf. friderici*, and *L. piau* specimens were assigned to a single molecular taxonomic unit by the ABGD, ASAP, mPTP, and GMYC methods, reflecting their similar morphological characteristics, such as their coloration pattern, dental formula, and meristic parameters ([Table 2](#)), although the intra-MOTU analyses revealed mean genetic distances of 2.7% ([Table 1](#)), ranging from 0.0% to 6.11% ([Table S3](#)). This is consistent with the current classification of the valid nominal species (BI and ML analyses: [Figs. 2–4](#); [Table 1](#)). All the species of clade VI shared the same morphological pattern, which is considered to be diagnostic of *L. friderici*, such as the number of spots along the lateral line (1–3) and the 4/4 dental formula, except for *L. venerei*, which has four teeth in the pre-maxilla and three in the dentary row. *Leporinus lacustris* and *L. venerei* are highly similar morphologically, given their relatively tall body, terminal mouth, long, dark anal fin, and three spots on the lateral line ([Britski & Birindelli, 2008](#); [Silva-Santos et al., 2018](#)). *Leporinus piau* presents the D-type coloration pattern described by [Garavello \(1979\)](#), which consists of three well-defined black spots on the lateral line, and four teeth in both rows, with a dental formula of 4/4. The *L. venerei*, *L. lacustris*, *L. cf. friderici*, and *L. piau* MOTU was subdivided into three subclades (*i*, *ii*, *iii* and *iv*—[Fig. 3](#)). Subclade *i* includes *L. piau* from Maranhão and *L. venerei* from the Tocantins basin, while subclade *ii* has *L. cf. friderici* from the Amazon and *L. piau* from the Turiaçu

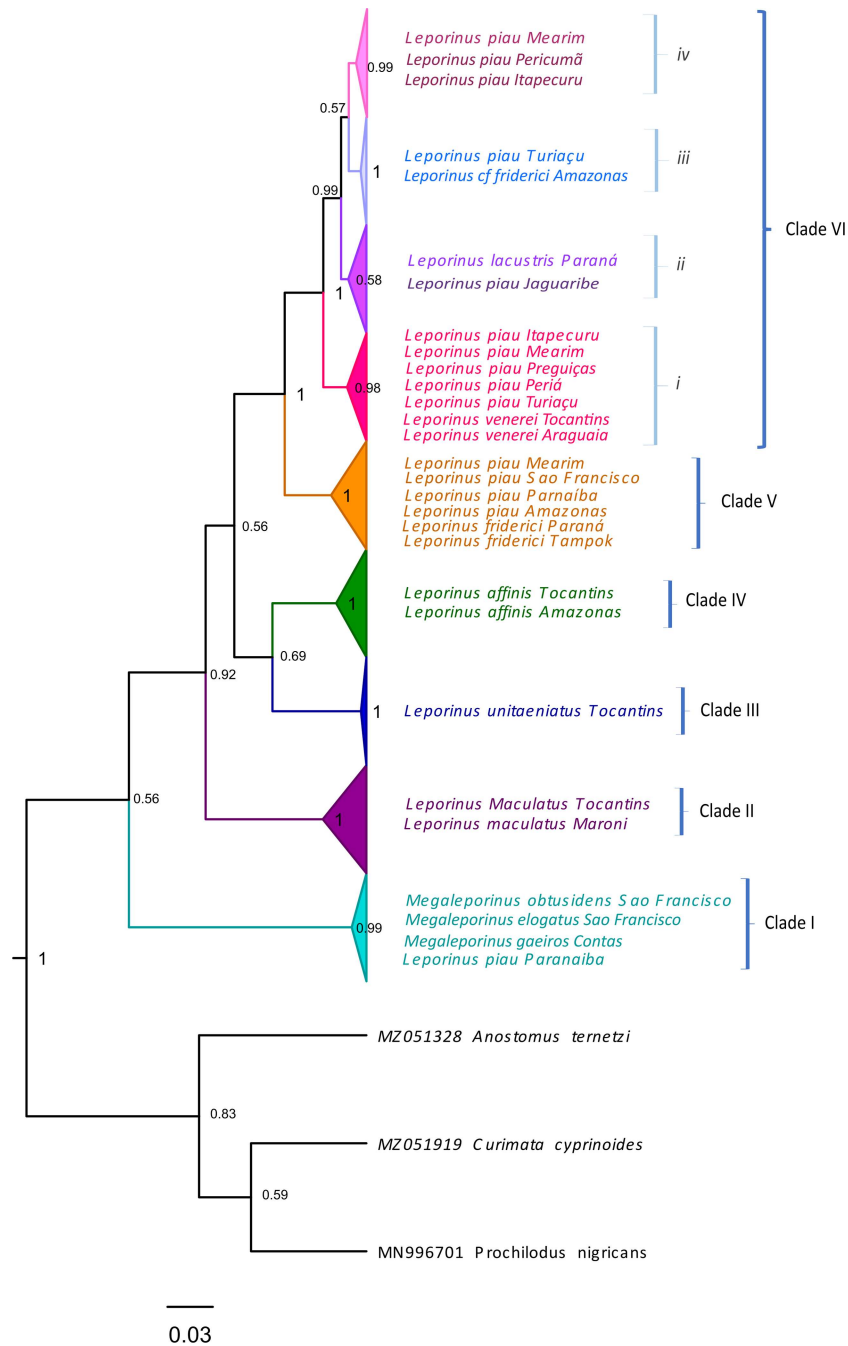


Figure 4 Collapsed Bayesian inference tree of the MOTUs of the *Leporinus* species. Collapsed Bayesian inference tree showing the arrangement of the MOTUs of the *Leporinus* species based on 185 samples of the mitochondrial COI gene analyzed using the Hasegawa-Kishino-Yano (HKY+G+I) algorithm, applied in BEAST. The groups were defined by observing the congruence between the MOTUs generated in the species delimitation analyses based on the ABGD, ASAP, mPTP, bPTP, and GMYC methods.

Full-size [DOI: 10.7717/peerj.15184/fig-4](https://doi.org/10.7717/peerj.15184/fig-4)

Table 1 Mean genetic distance of the *Leporinus* and *Megaleporinus*. Mean genetic distance, based on the Kimura 2-Parameter algorithm, generated by MEGA X for the MOTUs defined by the ABGD, ASAP, mPTP, bPTP, and GMYC analyses.

MOTU	Genetic Distance					
	1	2	3	4	5	6
1. <i>Leporinus lacustris</i> + <i>Leporinus venerei</i> + <i>Leporinus piau</i> + <i>Leporinus cf friderici</i>	2.7					
2. <i>Leporinus piau</i> + <i>Leporinus friderici</i>	7.91	2.2				
3. <i>Leporinus maculatus</i>	10.89	10.58	5.9			
4. <i>Leporinus affinis</i>	12.61	11.26	11.05	1.8		
5. <i>Leporinus unitaeniatus</i>	13.04	13.49	11.97	11.27	0.4	
6. <i>Leporinus piau</i>	15.49	16.24	15.64	17.45	14.53	0.5

basins. Subclade *iii* groups *L. lacustris* and *L. piau* from the Jaguaribe basin, and subclade *iv* groups *L. piau* from Maranhão and *L. cf. friderici* from the Mearim. The composition of subclade *i* (*L. piau* and *L. venerei*—Fig. 3), together with the diagnostic morphological features of the species, indicates that the specimens from the basins of Maranhão identified as *L. piau* may in fact be *L. venerei*, which would be the first record of this species from this Brazilian state.

The *L. venerei*, *L. lacustris*, *L. cf. friderici*, and *L. piau* MOTU was subdivided into three subclades (*i*, *ii*, *iii* and *iv*—Fig. 3). Subclade *i* includes *L. piau* from Maranhão and *L. venerei* from the Tocantins basin, while subclade *ii* has *L. cf. friderici* from the Amazon and *L. piau* from the Turiaçu basins. Subclade *iii* groups *L. lacustris* and *L. piau* from the Jaguaribe basin, and subclade *iv* groups *L. piau* from Maranhão and *L. cf. friderici* from the Mearim. The composition of subclade *i* (*L. piau* and *L. venerei*—Fig. 3), together with the diagnostic morphological features of the species, indicates that the specimens from the basins of Maranhão identified as *L. piau* may in fact be *L. venerei*, which would be the first record of this species from this Brazilian state.

One other clade, formed by *L. friderici* from French Guiana and Paraná with *L. piau* from the Mearim, São Francisco, Parnaíba, and Amazon basins, is also well supported (Fig. 3). This raises two important points: (1) the clear polyphyly of *L. friderici* and *L. piau*, which, in the latter case implies a possible error of identification based on the type specimen, and (2) the existence of cryptic diversity in the genus *Leporinus*, in particular in *L. friderici*. Silva-Santos et al. (2018) concluded that the samples identified morphologically as *L. friderici* are in fact a polyphyletic group, given that the specimens collected from the basins of the Brazilian Shield are different from those of *L. friderici* from the type locality. The polyphyly of *L. cf. friderici* was also confirmed in the present study, which is consistent with Silva-Santos et al. (2018), in which a species complex is revealed by the genetic differentiation of the populations present in distinct hydrographic basins. In this case, individuals identified consistently as *L. cf. friderici* may not in fact be conspecific with *L. friderici* from the type locality, that is, they represent different species. Ramirez, Birindelli & Galetti (2017) confirmed the presence of cryptic diversity in this taxon, which may represent a typical scenario of recent diversification, when closely-related taxa may be

Table 2 Meristic traits of the adult *Leporinus* species. Meristic traits of the adult *Leporinus* used to identify the samples analyzed in the present study, following Garavello (1979), Garavello (1989), Garavello & Santos (2007), Britski, Sato & Rosa (1984), and Britski & Birindelli (2008).

Species	Body	Coloration	Number of scales around the peduncle	Dental formula	Number of scales in the lateral line	Fin coloration
<i>L. affinis</i> Günther (1864)	Pre-dorsal somewhat convex; dorsal inclined slightly between the dorsal and adipose fins, and concave between the adipose and caudal fins.	Body yellowish, with 7 dark transversal stripes on the body and 3–4 on the head.	16	4/4	*	Peitoral and pelvic fins light yellow; all other fins hyaline.
<i>L. friderici</i> Bloch (1794)	Body tall and robust; large, with Standard Length (SL) of ca. 40 cm; body height 26–30% of SL, head length 27–29% of SL; mouth terminal.	Body brownish chestnut, with 2–4 dark spots, rounded or oval, on the lateral line.	16	4/4	38 to 40	Anal fin dark gray; all other fins yellowish-gray.
<i>L. lacustris</i> Campos (1945)	Body elongated, with a maximum standard length of 20 cm; mouth terminal; incisors truncated.	2–3 dark, rounded, mediolateral spots on the dorsal fin, the first larger and more conspicuous.	16	4/4	33 to 36	All fins yellowish, except the adipose and anal fins, which are darkened.
<i>L. maculatus</i> Müller and Troschel (1844)	Body small, with Standard Length (SL) of ca. 10 cm; body height 22–26% of SL, head length 23–25% of SL; mouth subterminal.	Body with 4 black, rounded spots connected by 3 transversal stripes, which cross the lateral line.	16	4/4	39 to 40	*
<i>L. piau</i>	Body relatively tall.	Body with 3 black spots on the flank, which are elongated horizontally.	16	4/4	35 to 37	*
<i>L. unitaeniatus</i> Garavello & Santos (2007)	Body elongated and fusiform; small, with maximum standard length of 12 cm; relatively low body (23% of the standard length); mouth subterminal.	Body yellowish, with a conspicuous black longitudinal streak running along the lateral line; 11–13 dark chestnut transversal stripes separated from the lateral line by two rows of scales.	16	4/4	40 to 44	Hyaline.

(continued on next page)

Table 2 (continued)

Species	Body	Coloration	Number of scales around the peduncle	Dental formula	Number of scales in the lateral line	Fin coloration
<i>L. veneri</i> <i>Britski & Birindelli</i> (2008)	Body tall; mouth terminal; anal fin long and dark.	3 small, dark spots on the lateral line, of which, the last 2, in particular, the last, are typically faded.	16	4/3	36 to 37	*

Notes.

*Data not available.

poorly-distinguished morphologically, creating predictable taxonomic uncertainties, such as those observed in the populations of *L. friderici*.

In the present study, the relationship found among *L. piau*, *L. friderici*, and *L. cf. friderici* (Figs. 2–4) alludes to a possible taxonomic inconsistency derived from *Fowler's* (1941) description of *L. piau*, as well as the geographic origin of the specimen analyzed in the present study, which was from the São Francisco basin. *Fowler* (1941) described *Leporinus piau* based on a type specimen from the Salgado River in the Jaguaribe basin of the Brazilian state of Ceará, but included a paratype from the Jatobá River, in the São Francisco basin, which led to the subsequent identification of most *Leporinus* specimens from the São Francisco River as *L. piau* (*Garavello & Britski, 2003; Carvalho et al., 2011*). However, *Silva-Santos et al. (2018)*, who analyzed nuclear and mitochondrial genetic markers, including COI, noted that the *Leporinus* specimens from the São Francisco basin represent a species distinct from *L. piau* from the type locality in the Jaguaribe basin. Clearly, *Fowler's* (1941) inclusion of a paratype from a distinct hydrographic basin have contributed fundamentally to the taxonomic uncertainties surrounding *L. piau*.

In the present study, *L. maculatus*, *L. unitaeniatus*, and *L. affinis* are valid nominal species, which presented considerable congruence between the traditional and molecular taxonomies. These three species constitute distinct MOTUs, which reflect their arrangement in different clades (BI and ML analyses: Figs. 2–4). All these species present easily distinguished diagnostic traits, such as the numerous spots dotting the body of *L. maculatus*, the single longitudinal stripe of *L. unitaeniatus*, and the lateral bands with no subdivisions observed in *L. affinis* (*Britski & Garavello, 2005; Sidlauskas & Vari, 2012*).

The samples from the Parnaíba basin identified here as *L. piau* and defined as a single MOTU by all the species delimitation models were grouped in a single clade with a genetic distance of 0.5%. These samples were delimited clearly as a more basal species separate from all the others, with evidence that they had been wrongly identified, and are in fact representatives of the genus *Megaleporinus*. This genus was described recently by *Ramirez, Birindelli & Galetti (2017)*, based on a combined morphological, chromosomal, and molecular approach, which assigned the large-bodied *Leporinus* to a monophyletic clade, which was denominated *Megaleporinus*. In the present study, these samples were delimited clearly as a single, basal species well separated from all the others, although a more detailed analysis would be necessary to better determine their taxonomic status.

The samples from the basins of Maranhão and Piauí, together with those from the Tocantins River collected for the present study revealed the cryptic diversity found in *Leporinus*, given that the specimens from the basins of the Itapecuru, Mearim, Pericumã, Turiaçu, Peria, and Preguiças rivers in Maranhão, and the Parnaíba River in Piauí were identified as *L. piau* based on their morphological traits. The study of the DNA barcode and the analytical tools employed here confirmed that *L. friderici* likely constitutes a polyphyletic species complex, leading to the frequent misidentification of specimens as *L. piau*. It will only be possible to resolve this scenario definitively with a systematic re-evaluation of the specimens collected from the hydrographic basins of the states of Maranhão and Piauí.

In the specific case of subclade *i* (Fig. 3; Tables S3-S4), which groups *L. piau* from Maranhão with *L. venerei* from the Tocantins basin, the most parsimonious interpretation of the results of this analysis, together with the diagnostic traits of the two species, would be to consider them to be a single taxon, that is, *L. venerei*. This would thus be the first record of *L. venerei* from the basins of the Itapecuru, Mearim, Turiaçu, and Peria rivers, in the state of Maranhão.

Prior to the present study, three *Leporinus* species were considered to be present in the hydrographic basins of the Brazilian state of Maranhão—*L. affinis*, in the Itapecuru basin (Abreu et al., 2019), *L. friderici* in the Itapecuru, Mearim, Maracaçumé, Munim, Peria, and Parnaíba basins (Piorski et al., 1998; Soares, 2005; Ramos, Ramos & Ramos, 2014; Melo et al., 2016; Abreu et al., 2019; Brito et al., 2019; Brito et al., 2020; Guimarães et al., 2021a; Guimarães et al., 2021b; Guimarães et al., 2021c), and *L. piau* in the basins of the Itapecuru, Mearim, Turiaçu, and Parnaíba rivers (Barros, Fraga & Birindelli, 2011; Ramos, Ramos & Ramos, 2014; Ribeiro et al., 2014; Assega & Birindelli, 2019; Abreu et al., 2019). Based on analyses of molecular data, however, Fraga et al. (2014) and Nascimento et al. (2016) found evidence of two distinct lineages in the *L. piau* group from the Itapecuru basin, while in the present study, *L. piau* was assigned to three different clades, being associated strongly with *L. cf. friderici* in two clades and with *L. venerei* in one. This leads us to conclude that *L. piau* is, in fact, absent from the basins of Maranhão, which are instead populated by *L. cf. friderici* and *L. venerei*, with the latter being recorded in Maranhão for the first time. This restricts *L. piau* to the basin of the Parnaíba River.

CONCLUSIONS

The molecular analyses presented here, including the different species delimitation approaches, identified the presence of four *Leporinus* species in the hydrographic basins of central northern Brazil—*L. maculatus*, *L. unitaenitus*, *L. affinis*, and *L. venerei*. However, the species delimitation analyses also assigned *L. cf. friderici* and *L. piau* to two different molecular operational units, which leads us to believe that an additional species, morphologically indistinguishable from *L. cf. friderici*, may be present. The analyses also revealed a distinct group of two of the specimens, which indicates emphatically the presence of *L. piau* in the basin of the Parnaíba River, which indicates the presence of a total of six nominal species in the hydrographic basins of central northern Brazil. The confirmation of the presence of *L. venerei* in the Itapecuru, Mearim, Turiaçu, Preguiças and Peria basins

represents a new record for the state of Maranhão, amplifying the known distribution of this species in Brazil.

ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Maria Histelle Sousa Nascimento conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Deborah Gaído Aragão performed the experiments, prepared figures and/or tables, and approved the final draft.
- Jordânia Leticia Nascimento Silva performed the experiments, prepared figures and/or tables, and approved the final draft.
- Renato Correia Lima performed the experiments, prepared figures and/or tables, and approved the final draft.
- José Luis Olivan Birindelli performed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Elmary Costa Fraga conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Maria Claudene Barros conceived and designed the experiments, analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (*i.e.*, approving body and any reference numbers):

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Data Availability

The following information was supplied regarding data availability:

The data is available at NCBI GenBank: [OP781850](#) to [OP781884](#), [OP782222](#) to [OP782283](#), [OP782350](#) to [OP7882375](#) and [OP782385](#) to [OP782418](#).

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3.2 CAPÍTULO II

Artigo publicado na revista Journal of Fish Biology: **EXPLORING HIDDEN DIVERSITY: MOLECULAR INSIGHTS INTO THE *LEPORINUS* SPECIES OF THE RIVERS OF THE BRAZILIAN STATES OF MARANHÃO AND PIAUÍ**

REGULAR ARTICLE

Exploring hidden diversity: Molecular insights into the *Leporinus* species of the rivers of the Brazilian states of Maranhão and Piauí

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Abstract

The present study delved into the world of hidden diversity by examining specimens identified as *Leporinus piau* from the river basins of the northern Brazilian states of Maranhão and Piauí. Using genetic analyses that combined data from three mitochondrial markers and one nuclear marker, the study identified two well-supported groups, reinforcing the findings of previous publications. The first group, found in samples from the Itapecuru, Mearim, Turiaçu, and Pericumã basins, in Maranhão, appears to represent a relatively ancient diversification and the possibility of concealed cryptic diversity. The second group, comprising specimens from the Parnaíba (Piauí) and Mearim (Maranhão) basins, appears to have resulted from a more recent process of diversification and has a close relationship with *Leporinus friderici* from the type locality. Our findings not only confirm the existence of a complex scenario of cryptic diversity in the genus *Leporinus* from the study basins but also underscore the taxonomic inconsistencies within this group of fish. This study offers a comprehensive analysis of the species diversity of the Maranhão and Piauí basins, which are critical regions for the conservation of Amazonian fish, providing valuable insights for the sustainable management and conservation of these fish.

KEYWORDS

cryptic diversity, morphologically similar, species delimitation methods, taxonomic inconsistencies

1 | INTRODUCTION

The fish genus *Leporinus* Agassiz 1829 (Spix & Agassiz, 1829) of the family Anostomidae is considered to be one of the most speciose genera of the order Characiformes, which is distributed between Central America and southern South America, and the Amazonian basin holds most of this variability; the isolated coastal rivers of the Guianas, the São Francisco basin, and other coastal rivers of northeastern Brazil are less represented (Garavello & Britski, 2003; Santos & Zuanon, 2008). The species of this genus present a close anatomical similarity, which infers a widely distributed natural group (Garavello, 1979).

Morphological and molecular studies indicate that this genus is not monophyletic (Ramirez et al., 2016; Sidlauskas & Vari, 2008), which highlights the need for a thorough taxonomic review of the species of this group. For example, in a study that integrated morphological, chromosomal, and molecular data, Ramirez, Birindelli, Carvalho, et al. (2017) described a new genus, *Megaleporinus* (Ramirez et al., 2017), which assigns the large-bodied *Leporinus* species to a distinct, monophyletic clade.

Leporinus friderici (Bloch, 1794) is one of the most amply distributed species of the genus and has been found in the majority of the hydrographic basins of South America (Garavello et al., 1992). Given

its broad distribution, many different studies have investigated the characteristics of this species over the years and have found considerable variation in the morphological and genetic patterns of the different *L. friderici* populations (Garavello et al., 1992; Géry et al., 1987; Nascimento et al., 2023; Renno et al., 1990; Renno et al., 1991; Sidlauskas & Vari, 2012; Silva-Santos et al., 2018). The sum of this evidence indicates that the *L. friderici* morphotype does in fact represent a species complex, with only the *L. friderici* specimens from the rivers of Suriname and French Guiana being recognized as members of the type species (Sidlauskas & Vari, 2012). This led to the application of the provisional nomenclature *Leporinus cf. friderici* to designate the specimens that deviate from the type species (Silva-Santos et al., 2018).

In their molecular study, Silva-Santos et al. (2018) identified two principal scenarios within the *L. cf. friderici* complex. The first scenario is an *L. cf. friderici* sensu stricto group or clade, which may in fact represent a true species complex, formed by recent diversification in the basins of the Brazilian Shield. This would have resulted in genetic structuring throughout this hydrographic network, which is probably related to the geomorphological processes that established the present-day hydrographic layout. The authors nevertheless conclude that the taxonomic status of these populations is still unclear and requires further, more detailed research. The second scenario identified by Silva-Santos et al. (2018) refers to an unnamed group or clade, which encompasses the relatively ancient diversification of this taxon, and includes as-yet undescribed cryptic species currently designated *L. cf. friderici* (Figure 1a).

Nascimento et al. (2023) recently published a rearrangement of the taxa, in which the *L. friderici*/*L. cf. friderici* and *Leporinus piau* Fowler (1941) populations of the basins of the Itapecuru, Mearim, Pericumã, and Parnaíba rivers form two distinct groups—one group that includes *L. friderici* from the basin of the Tampok River and *L. piau* from the basins of the Mearim and Parnaíba rivers, and a second group that includes *L. piau* from the basins of Maranhão, together with *L. cf. friderici* from the Amazon basin (Figure 1b). The presence of *L. piau* in both groups indicates that the specimens of this species collected in central Maranhão and Piauí were identified erroneously, based on their morphological characteristics, which reflects the taxonomic inconsistencies that persist among the morphologically similar species of this genus. A relatively high level of genetic divergence, of up to 10.7%, was also found between these two groups.

The present study tested two hypotheses based on the analysis of mitochondrial and nuclear DNA sequences: (i) the *L. cf. friderici*/*L. friderici* and *L. piau* group identified by Nascimento et al. (2023) is in fact the *L. friderici* sensu stricto clade proposed by Silva-Santos et al. (2018), and (ii) the second group of *L. cf. friderici* and *L. piau* corresponds to the unnamed clade.

Given the clear taxonomic instability of *Leporinus*, we sampled the species *L. piau* in five basins, namely those of the Itapecuru, Mearim, Pericumã, and Turiaçu rivers in the Brazilian state of Maranhão and the Parnaíba basin in the state of Piauí. The study revealed the same taxonomic scenario in Maranhão as that found in South America as a whole by Silva-Santos et al. (2018). This similarity indicates that

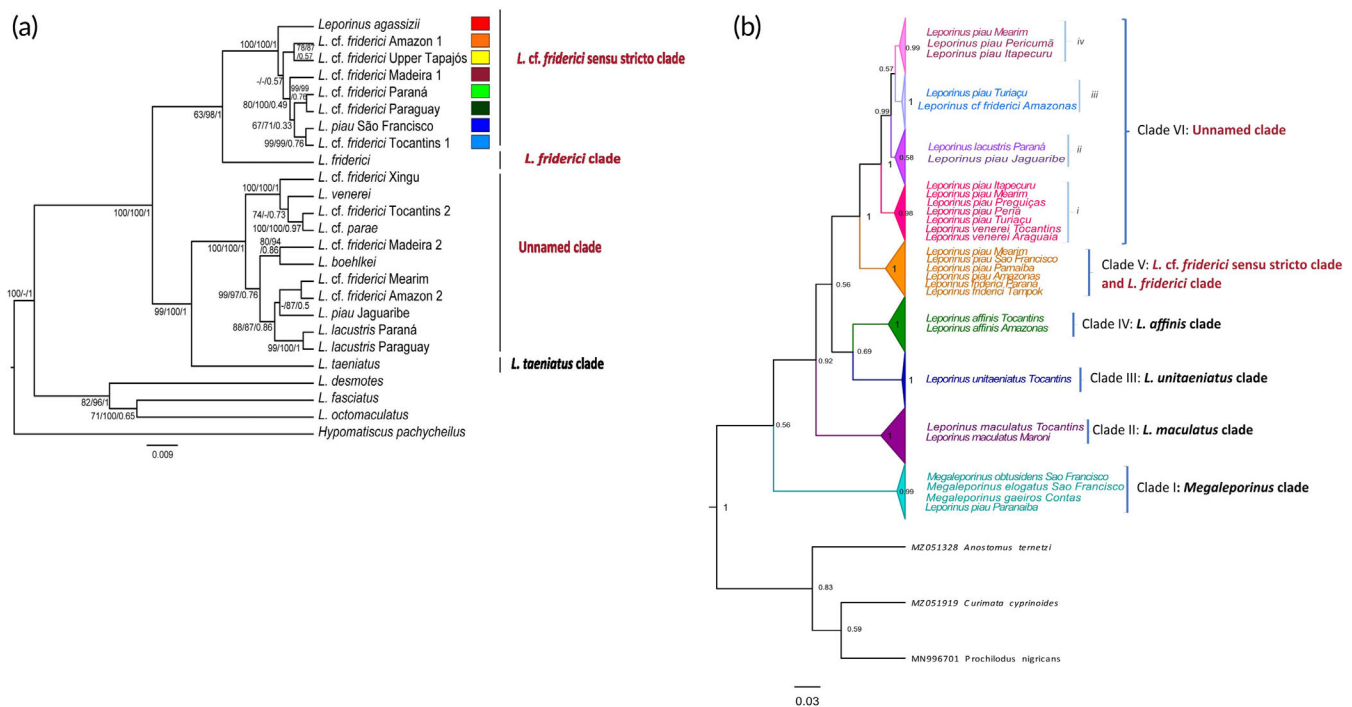


FIGURE 1 (a) Species tree, showing the phylogenetic relationships of *Leporinus cf. friderici*, adapted from Silva-Santos et al. (2018).

(b) Collapsed tree showing the arrangement of the MOTUs (molecular operational taxonomic units) of the *Leporinus piau* and *L. cf. friderici* species, adapted from Nascimento et al. (2023).

Maranhão is an ideal geographic model for the analysis of the genetic diversity of this genus.

2 | MATERIALS AND METHODS

A total of 77 *L. piau* specimens were collected from the Itapecuru, Mearim, Turiaçu, Pericumã, and Parnaíba rivers (Figure 2; Table S1). Twenty-seven sequences were obtained from GenBank, namely 25 of *Leporinus* species and 2 species of other characiform genera, which were included as the out-group (Table S2). The collection of the specimens analysed in the present study was authorized by IBAMA (license 02012.004159/2006) and SISBIO (licenses 42119-2/2013, 46367-1/2014, 83138-1/2022). The experimental procedures were approved by the Maranhão State University (UEMA) Committee for the Ethical Treatment of Animals (protocol 47/2022).

Once collected, the specimens were taken to the Molecular Biology and Genetics Laboratory (GENBIMOL) at the UEMA Caxias campus, where they were photographed and each identified with an individual code. Samples of muscle tissue were extracted from these specimens for genetic analyses. The specimens were fixed in 10% formaldehyde and preserved in 70% alcohol. The prepared specimens

were deposited in the Zoology Museum of Londrina State University (MZUEL) in Paraná, Brazil, for cataloging by specialists.

2.1 | Molecular analyses

The DNA of the samples was extracted, amplified, and sequenced in the GENBIMOL. The total DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). The partial gene sequences were isolated and amplified by PCR using specific primers for the 16S rRNA (ribosomal RNA) (Palumbi et al., 1991) and cytochrome c oxidase subunit I (COI) genes (Ward et al., 2005), as well as the L14725L (Smith & Patton, 1993) and MVZ16 (Santos et al., 2003) primers for the cytochrome b (cyt b) gene, and the TROPF (Calcagnotto et al., 2005) and TROPB (Santos, 2007) primers for the α -Tropomyosin (TROP) gene (Table S3). The TROPF primers are located in the region of intron 3 and the TROPB primers in exon 5 (Table S3). The COI sequences used in this study are the same as those obtained by Nascimento et al. (2023).

The PCRs were run in a final volume of 25 μ L containing 4 μ L of deoxynucleotide triphosphate (1.25 M), 2.5 μ L of 10 $\times$ buffer, 0.5 μ L of $MgCl_2$ (25 mM), 0.25 μ L of the forward and reverse primers at 200 nM,

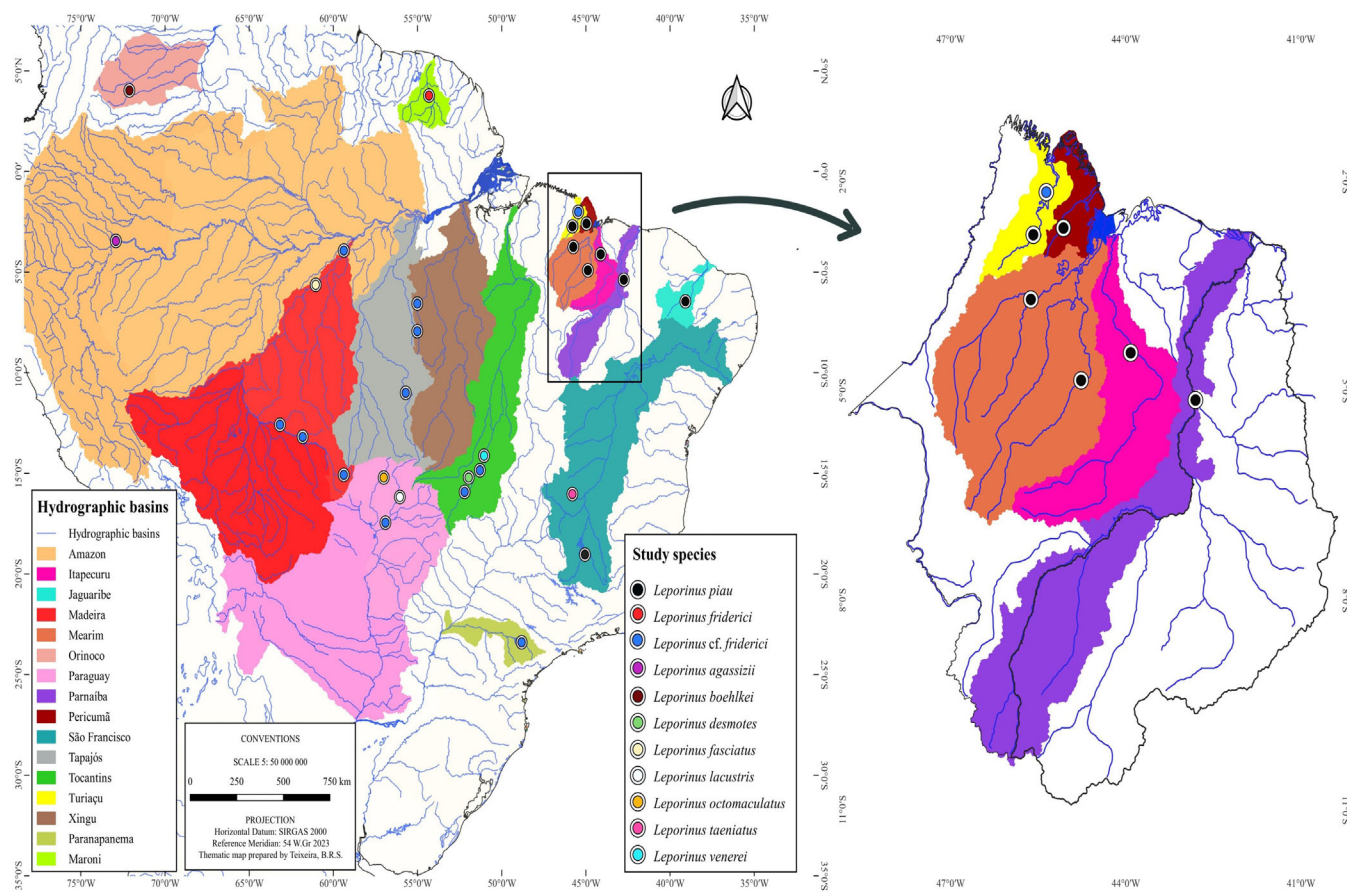


FIGURE 2 *Leporinus* specimens analysed in the present study. The dots (localities) are color coded by species, and each hydrographic basin included in the present study is highlighted using a different color.

1 μL of the DNA sample at 50 ng, and 0.2 μL of 0.02 U/ μL of Taq DNA polymerase. The amplification cycle began with an initial denaturation for 5 min at 95°C; a cycle of denaturation, annealing, and extension specific to each pair of primers (Table S3); and a final extension of 5 min at 72°C. The positive PCR products were purified using an Exo-Sap IT Kit and sequenced using the Sanger et al. (1977) method using the BigDye Terminator 3.1 Kit, with the products being processed in an automated ABI 3500 capillary system (Life Technologies).

2.2 | Genetic data analyses

The sequences were aligned and edited using the CLUSTAL-W package (Thompson et al., 1994) of Bioedit 7.2.5 (Hall, 1999), where the gaps, insertions, and deletions were identified and corrected manually. The haplotypes were delineated using DnaSP 5.1 (Librado & Rozas, 2009), based on the sequences obtained from the basins of Maranhão and Piauí, with an unrooted haplotype network being compiled in NETWORK 10.2 using the median-joining network algorithm (Bandelt et al., 1999). The mitochondrial and nuclear gene sequences were concatenated using SeaView 4 (Gouy et al., 2010) for phylogenetic analysis.

The maximum likelihood (ML) and Bayesian inference (BI) phylogenetic trees were constructed through the partitioning of the dataset in PartitionFinder2 (Lanfear et al., 2017). The ML analyses were run in RAxML, version 8.2.10 (Stamatakis, 2014), with 1000 rapid bootstrap replicates and the best scores being determined using the GTRGAMMA model, run in the CIPRES Science Gateway (Miller et al., 2010).

The partitioned BI analyses were run in BEAST, version 1.10.4 (Drummond et al., 2012; Suchard et al., 2018), using the GTR + I, K81UF + G, and TIM + I + G nucleotide substitution models, which were selected following the Bayesian criterion in JModeltest 2 (Darriba et al., 2012) for the 16S rRNA (GTR + I), COI (K81UF + G), cyt *b* (K81UF + G), and TROP (TIM + I + G) genes. A relaxed clock with lognormal distribution and the birth-death speciation process were applied to all the partitions. This analysis was based on 150 million generations, with the parameters logged every 5000 generations and the log files being verified in Tracer version 1.6 (Rambaut et al., 2014) to evaluate the convergence and the most adequate interval for the burn-in, with the first 10% of the trees discarded as burn-in. Convergence was considered to be adequate when the parameters presented an effective sample size of at least 200. The consensus tree was generated in BEAST, summarized in TreeAnnotator version 10.4 (Suchard et al., 2018), and visualized and edited in FigTree version 1.4.2 (Rambaut, 2014).

The mean genetic distances between the clades identified in the phylogenetic trees were obtained based on p-distance model using MEGA 11 (Tamura et al., 2021).

2.3 | Species delimitation analyses

Species delimitation based on the molecular data was run in two steps, with no out-group. In the first step, exploration or candidate

species discovery methods were used without attributing a priori information on the existence of potential species. Three distinct approaches were used here—the automatic barcode gap discovery (ABGD) for the data on a single gene (rRNA 16S, COI, cyt *b*, or TROP, see Supporting Information S1), the Poisson tree process (PTP), and the generalized mixed Yule coalescent (GMYC) approach for the concatenated mitochondrial sequences (rRNA 16S + COI + cyt *b*). The second step was based on the Bayesian phylogenetics and phylogeography (BPP) species validation test, using the concatenated mitochondrial and nuclear data (rRNA16S + COI + cyt *b* + TROP).

The ABGD test (Puillandre et al., 2012) was run in an online tool (<https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>) using the dataset of the aligned sequences with the default settings, P (prior limit of intraspecific diversity) ranging between 0.001 and 0.1; X (gap widths) = 1 using the K80 (Kimura) and JC69 (Jukes-Cantor) models. The PTP (Zhang et al., 2013) was run on the web server <https://species.h-its.org/>. In this case, the input was the ML phylogenetic tree produced in RAxML, version 8.2.9 (Stamatakis, 2014), which is available in the CIPRES Science Gateway, version 3.3 (Miller et al., 2010). The GMYC (Fujisawa & Barraclough, 2013) was based on the ultrametric consensus tree built in BEAST, version 1.8, which was submitted to the Ape (Paradis & Schliep, 2019), Splits (Ezard & Purvis, 2009), Paran (Dinno & Dinno, 2018), and Mass (Venables & Ripley, 2002) packages available in R, version 4.1.0 (Venables & Smith, 2021).

A Bayesian multilocus species delimitation analysis was conducted using the BPP programme, version 4.7.0 (Rannala & Yang, 2003; Yang, 2015; Yang & Rannala, 2010). The BPP is a Bayesian Markov chain Monte Carlo (MCMC) method for analysing DNA sequence alignments under the multispecies coalescent (MSC) model, which can accurately assign individuals at the species level without setting determined distance thresholds in advance and thus provides significant improvements for species delimitation analyses (Yang, 2015; Yang & Rannala, 2017).

The population size (θ) and divergence times (τ) were attributed to the gamma priors $\theta \sim G(2, 2000)$ and $\theta \sim G(2, 1000)$, respectively. We also investigated the influence of different gamma priors on the posterior probabilities, although the results were robust in terms of the attribution of the priors.

The number of species and the allocation of individuals to the different species were determined by analysis A11 of the BPP. We also ran analysis A01, which encompasses the heterogeneity of the gene trees and uses the nearest neighbor interchange or subtree pruning and regrafting algorithms to infer the topology of the species tree based on coalescence. In comparison with more traditional phylogenetic analysis, the MSC models the coalescence process along the branches, in both the extant and ancestral species. This method can thus be considered to provide an incomplete ordination of the lineages, due to the ancestral polymorphism and the conflicts between the gene and species trees.

Following the A11 and A01 analyses, we ran the A00 analysis to outline the posterior theta distribution. Each rjMCMC was run for 100,000 generations, with a burn-in of 8000 generations and a sampling frequency of 2. In this analysis, the posterior probabilities of at

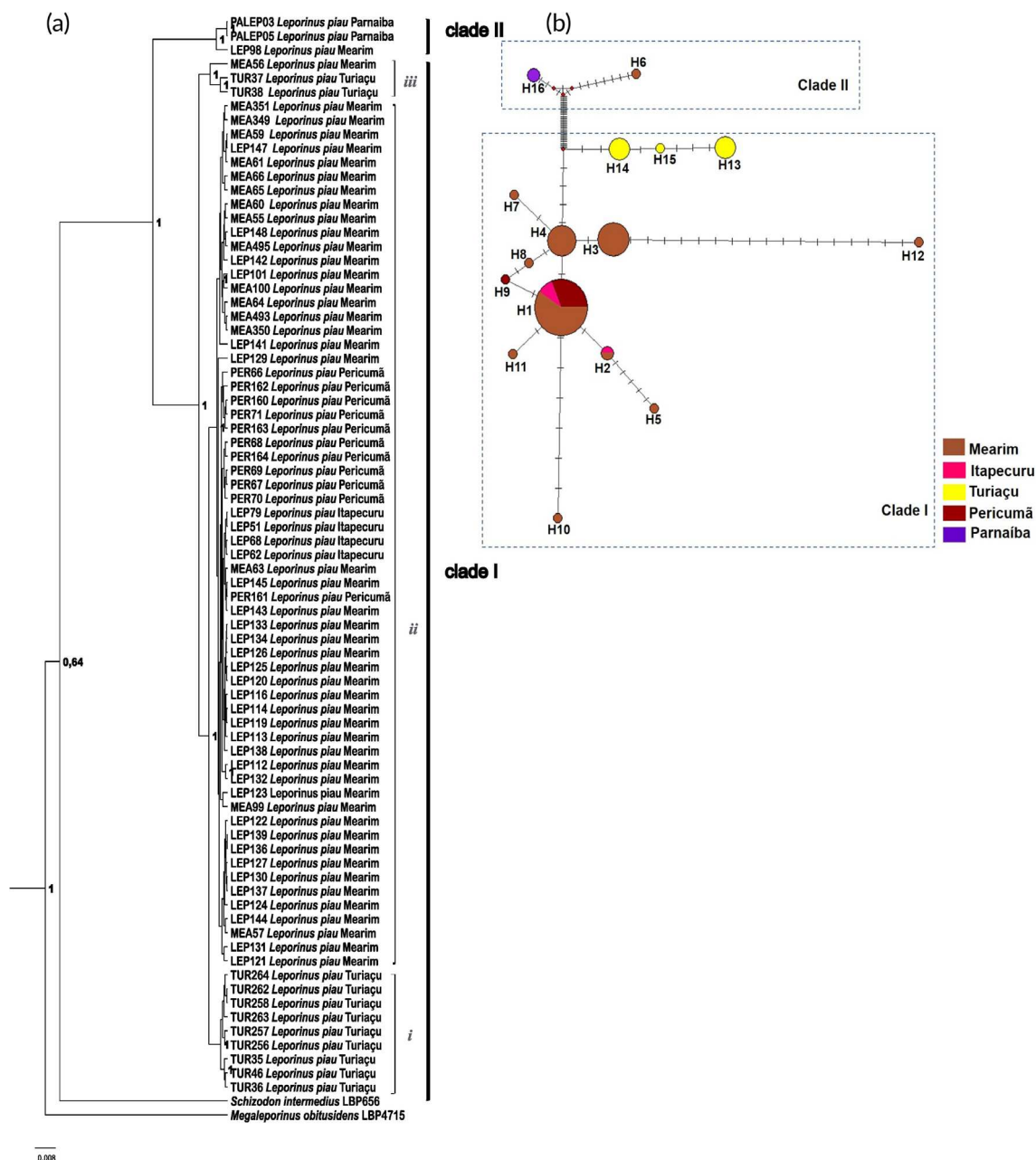


FIGURE 3 (a) Bayesian Inference phylogenetic tree for the *Leporinus* specimens from the hydrographic basins of the Brazilian states of Maranhão and Piauí. This tree was generated using the four concatenated molecular markers sequenced in the present study (COI, rRNA 16S, cyt *b*, and TROP). (b) Haplotype network derived from the *Leporinus* sequences. Each circle represents a haplotype, whose size is proportional to its frequency, whereas the bars on the lines that connect the haplotypes indicate the number of mutations that separate them. The red circles indicate haplotypes that are missing or were not sampled. The collecting localities are color coded, as shown in the legend.

least 0.95 were considered to be strongly supported, those between 0.90 and 0.95 were considered to have moderate support, and those <0.90 were classified as weakly supported.

The A00 analysis provides an estimate of the parameters of the MSC model (species delimitation = 0, species tree = 0), which generates the posterior distribution of the divergence times (θ) and population sizes (τ) of the species in the MSC model when the phylogeny of the species is fixed, with the parameters being a product of the time and mutation rates. The A01 analysis estimates the

species tree (species delimitation = 0, species tree = 1) based on the assumption that the attribution and delimitation of the species are fixed. Based on the MSC model, the A01 analysis considers the polymorphism of the ancestral species and the conflicts between the species tree and the genetic trees. The A11 analysis provides a combined delimitation of the species and estimates the species trees (species delimitation = 1, species tree = 1), with the algorithm assessing different species delimitation models and phylogenies.

TABLE 1 Mean inter- and intra-clade genetic distances in *Leporinus*.

(a)									
% Genetic distance									
Clade			Clade I				Clade II		
Clade I			0.68						
Clade II			5.36				0.55		
(b)									
% Genetic distance									
Clade			<i>Leporinus cf. friderici sensu stricto</i>		<i>L. friderici</i>		Unnamed		
							<i>i</i>	<i>ii</i>	<i>iii</i>
<i>L. cf. friderici sensu stricto</i>			2.17						
<i>L. friderici</i>			4.97		n/c				
Unnamed	<i>i</i>	7.11	6.78		2.48				
	<i>ii</i>	6.85	6.98		3.63	2.00			
	<i>iii</i>	7.19	6.98		3.18	3.82	n/c		
	<i>iv</i>	7.33	7.06		2.95	3.16	2.41	n/c	
	<i>v</i>	6.70	7.35		3.15	2.95	2.39	1.98	0.31
	<i>vi</i>	6.75	7.44		3.20	3.26	2.74	1.90	1.17

Note: (a) Genetic distances between and within (bold values) the *Leporinus* clades from the basins of Maranhão and Piauí based on the concatenated sequences of the COI, rRNA 16S, cyt *b*, and TROP genes; (b) genetic distances between the *Leporinus* clades from Maranhão and Piauí, and the GenBank sequences, based on the concatenated sequences of the COI, rRNA 16S, cyt *b*, and TROP genes. The presence of n/c in the results denotes cases in which it was not possible to estimate evolutionary distances.

Abbreviation: COI, cytochrome c oxidase subunit I.

3 | RESULTS

The ML and BI trees generated from the multilocus sequences obtained from the Maranhão and Piauí basins reveal two extremely well-supported groups (posterior probability = 1, bootstrap = 100), identified here as clades I and II (Figure 3a; Figure S1), which correspond to Nascimento et al.'s (2023) clades VI and V, respectively. Clade I grouped the *L. piau* specimens from the basins of the Maranhão river, whereas clade II included those from the basins of the Parnaíba and Mearim rivers (Figure 3a). Three subgroups can be observed within clade I; however, the first includes specimens only from the Turiaçu basin; the second comprises specimens from the Mearim, Itapecuru, and Pericumã basins; and the third includes specimens from the Turiaçu and Mearim basins. The haplotype network (Figure 3b) further supported the differentiation of clades I and II, as well as the separation of the Turiaçu group and the differentiation of haplotype H12 from the Mearim basin, which may represent a distinct group. The mean genetic divergence between clades I and II was 5.36% (Table 1a).

When the GenBank *Leporinus* sequences were added to the analyses, the BI and ML trees revealed that the samples collected in the present study encompassed four distinct subclades differentiated by the colouration of the branches (Figure 4) and distributed in two superclades. The first group that includes our samples is the *L. cf. friderici* clade from the Amazon, Madeira, Tapajós, Tocantins, Paraná, and Paraguay basins, together with *L. piau* from the Parnaíba

(PALEP03 and 05), Mearim (LEP98), and São Francisco basins, on the branches highlighted in blue (Figure 4) with a mean distance of 2.17%. We adopted the nomenclature of Silva-Santos et al. (2018) for this group, *L. cf. friderici sensu stricto* (Figure 4; Figure S2) and associated with this group; we have a clade formed only by *L. friderici* from the basins of Suriname (Figure 4; Figure S2), which was designated as the *L. friderici* clade, following Silva-Santos et al. (2018). The mean genetic divergence between the *L. friderici* and *L. cf. friderici sensu stricto* clades was 4.97% (Table 1b).

The clade highlighted in green includes *L. piau* from the Turiaçu (TUR37 and 38) and Mearim (MEA56) basins, which was grouped with a posterior probability of 0.88 with *Leporinus venerei* Britski and Birindelli (2008) from the Tocantins basin, *L. cf. friderici* from the Tocantins and Xingu basins, and *Leporinus trimaculatus* Garavello and Santos (1992) from the Amazon basin (Figure 4), with a mean divergence of 2%. The samples from Maranhão form a subclade here, revealing their presumed differentiation from the other species. The majority of the *L. piau* samples from the Turiaçu basin was grouped with *L. cf. friderici* from the Amazon and Madeira basins, with a posterior probability of 1, on the branches in orange (Figure 4). The fourth clade (magenta) was formed by the remaining *L. piau* samples from the basins of Maranhão (Mearim, Itapecuru, and Pericumã) obtained in the present study, together with the GenBank *L. cf. friderici* sequence from the Mearim basin (Figure 4), with a mean genetic divergence of 0.39% (Table 1b).

All these clades, with the exception of the *L. cf. friderici sensu stricto* clade and *L. friderici* from the type locality, form an unnamed

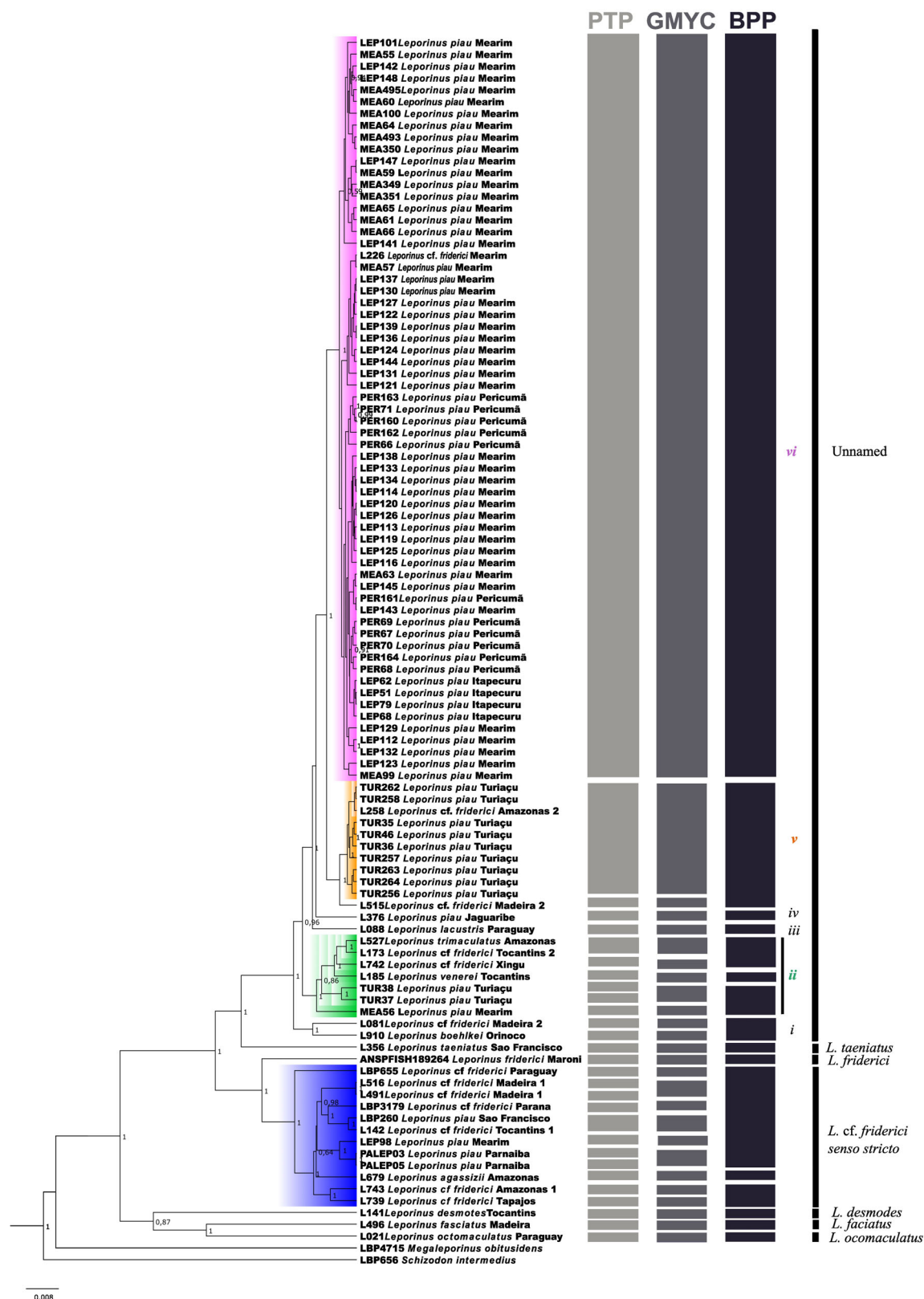


FIGURE 4 Bayesian Inference phylogenetic tree for the *Leporinus* specimens from the basins of the Brazilian states of Maranhão and Piauí, together with the GenBank sequences from other basins, based on the concatenated sequences of the mitochondrial and nuclear genes. The numbers above the branches are the posterior probabilities for the Bayesian Inference. The species identified using the different species delimitation methods are shown by color-coded bars.

superclade, following the nomenclature adopted by Silva-Santos et al. (2018), together with *L. piau* from the Jaguaribe basin, *Leporinus lacustris* Amaral Campos (1945) from the Paraguay basin, and *Leporinus boehlkei* Garavello (1988) from the basin of the Orinoco River, with absolute support, that is, a posterior probability of 1. To facilitate the understanding of this configuration, all the unnamed groups of this superclade have been designated a lowercase Roman numeral, from *i* to *vi* (Figure 4).

The genetic divergence between these six groups was high, ranging from 1.17% to 3.82% (Table 1b).

The species delimitation analyses used here produced similar arrangements (Figure 4; Supporting Information S1). The exploration and candidate species discovery methods employed here (Figure 4), using only the mitochondrial data, revealed 29 (PTP) and 26 (GMYC) molecular operational taxonomic units (MOTU). The results of both these analyses made biological sense in the context of our dataset, given that the valid nominal species were allocated to distinct MOTUs (Figure 4).

In the candidate species validation approach, using both mitochondrial and nuclear data, the BPP separated the samples in 16 MOTUs, once again, with the valid nominal species being allocated to distinct MOTUs and the *L. cf. friderici* sensu stricto clade being defined as a single MOTU. In addition, group *ii* of the unnamed superclade is divided into three MOTUs: the first corresponds to the samples from the Turiaçu (TUR37 and 38) and Mearim (MEA56) basins, the second contains only *L. venerei*, and the third includes *L. cf. friderici* from the Tocantins and Xingu basins together with *L. trimaculatus* from the Amazon basin (Figure 4). This separation in distinct MOTUs is consistent with the differentiation of the samples from Maranhão, in terms of both their formation of a subgroup and the mean genetic divergence of 2% observed among the samples of group *ii* (Table 1b).

For the unnamed clade, once again, the distance-based species delimitation methods were the most effective for the distribution of the valid nominal taxa in distinct MOTUs *L. venerei*, *L. trimaculatus*, *L. boehlkei*, *L. lacustris*, and *L. piau* from the type locality.

4 | DISCUSSION

The correct identification of species is an essential prerequisite for any biological research and in particular for successful conservation practices (Li et al., 2018). Traditional taxonomic approaches depend on the interpretation of morphological characteristics and rely fundamentally on the knowledge of specialists in the fields of taxonomy and systematics (Bingpeng et al., 2018; Nelson et al., 2016; Xu et al., 2021). However, individual fish may vary enormously in their body shape and size, number of rays and scales, and colouration (Strauss & Bond, 1990; Zhang & Hanner, 2012), whereas some natural groups may present little noticeable morphological variation, leading to their inclusion in a single nominal taxon (Bickford et al., 2007). In addition, sister species may often share morphological traits, leading to identification errors in taxonomic studies (Li et al., 2018). These inherent limitations of the traditional taxonomic systems reinforce the

need for a new, more reliable approach to the identification of fish species, in which DNA sequences provide a more objective and reliable tool for the delimitation of species, when used systematically in conjunction with morphological traits (Steinke et al., 2009).

Species with an ample geographic distribution and a long history of taxonomic controversy, such as those of the genus *Leporinus*, may often conceal cryptic diversity, given that the identification of species based on morphological traits may not contemplate the full intraspecific variation in the taxon, especially considering that the morphological characteristics of an organism may not necessarily be altered by the speciation process, especially in the case of recent events (Fraga et al., 2014; Garavello et al., 1992; Géry et al., 1987; Nascimento et al., 2023; Ramirez et al., 2016; Renno et al., 1990; Renno et al., 1991; Sidlauskas & Vari, 2012; Silva-Santos et al., 2018). The reliable identification of cryptic diversity has profound implications for the advancement of our understanding of species and their ecological and biogeographic features, as well as evolutionary patterns, through the integrated analysis of morphological and molecular data, which has proven to be the most effective approach (Kress et al., 2015).

In the present study, the set of specimens identified initially as *L. piau*, which occur in the basins of Maranhão and Piauí, were grouped together with *L. cf. friderici*, in a well-supported arrangement, rather than with *L. friderici*, in either the sensu stricto or the unnamed clade (Figure 4), which indicates that they are genetically distinct from the *L. friderici* from Suriname (Maroni basin). This is consistent with the study of Sidlauskas and Vari (2012), who found that the *L. friderici* populations of Suriname and French Guiana are genetically distant from the Brazilian populations, which also supports the provisional identification of the Brazilian specimens as *L. cf. friderici*. As observed by Silva-Santos et al. (2018), *L. friderici* from Suriname formed a clade distinct from those of the other specimens (Figure 4), which reinforces the hypothesis presented here, that is, that the *L. cf. friderici* and *L. piau* specimens collected here are not conspecific with *L. friderici* from the type locality, which further reinforces the conclusion that *L. piau* from Maranhão and Piauí is nominally *L. cf. friderici*, or at least part of the specimens, given that the other part makes up the unnamed clade and are subdivided into three groups (groups *ii*, *v*, and *vi* of the unnamed superclade; Figure 4).

The sum of the evidence thus indicates that the *L. piau* denomination was adopted erroneously for the samples from the Maranhão and Piauí basins, given that the morphology of *L. piau* is consistent with that of *L. friderici*, which is increasingly being considered to be a species complex (Nascimento et al., 2023; Ramirez et al., 2016; Sidlauskas & Vari, 2012; Silva-Santos et al., 2018). The results of the present study indicate the presence of two groups, one formed by the specimens of the Mearim and Parnaíba basins, and the other by the *L. cf. friderici* specimens from the other basins that represent the *L. cf. friderici* clade (sensu stricto), given that, as found by Silva-Santos et al. (2018), this clade is also associated with the *L. friderici* clade from the type locality. The samples of these two clades share a number of morphological traits, such as the presence of three dark, elongated spots along the lateral line, a 4/4 dental formula, and certain meristic parameters (Britski & Birindelli, 2008; Garavello & Britski, 1987; Silva-

Santos et al., 2018). These common characteristics have generated persistent inconsistencies in the taxonomy of this group, which has led to the constant revision and reevaluation of the fish species of the genus *Leporinus*.

The second group matched with the unnamed clade identified by Silva-Santos et al. (2018), which is made up of a number of different *Leporinus* species, including *L. piau* from the type locality (Jaguaribe), which indicates that this species may occur in the basins of Maranhão; its more basal position in comparison with the *L. piau* from the Turiaçu, Pericumã, Mearim, and Itapecuru basins means that this arrangement provides an excellent baseline for a more detailed investigation of the possible differentiation of the populations from the different basins, especially as the samples from the Mearim, Pericumã, and Itapecuru basins share more genetic similarities than those of *L. piau* from the Turiaçu basin, whose biodiversity has, historically, been seen as having greater affinities with that of the Amazon basin (Abreu et al., 2019).

The majority of the samples identified as *L. piau* in the unnamed clade (Figure 4), together with the *L. cf. friderici* specimens from the Mearim, Tocantins, Turiaçu, Xingu, and Madeira basins, and *L. piau* from the Jaguaribe basin indicate a relatively ancient diversification process. This is consistent with the findings of Silva-Santos et al. (2018), who grouped these taxa with four other nominal species, *L. boehlkei*, *L. trimaculatus*, *L. lacustris*, and *L. venerei*. These authors nevertheless alert that this group may still contain cryptic species identified as *L. cf. friderici*. In addition to corroborating the findings of Silva-Santos et al. (2018), the results of the present study show that the specimens that make up the unnamed clade form subgroups in the basins of Maranhão, as seen in the case of subgroup *i* in Figure 3a. The formation of these subgroups is likely the result of the size of the sample employed here, which emphasizes the need for a sample of adequate size to ensure the most conclusive analysis of the data.

The *L. cf. friderici* sensu stricto clade encompassed specimens of *L. cf. friderici* from different basins, together with *L. piau* from the Mearim and Parnaíba basins, which reinforces the hypothesis that the *L. piau* samples from this group are, in fact, *L. cf. friderici* (Figure 3). Silva-Santos et al. (2018) obtained similar results, concluding that this group represents a typical scenario of recent diversification from the Suriname population of *L. friderici* (the type species), which appears as the basal group, forming a strictly related group that is nevertheless composed of potentially cryptic species, revealing the taxonomic uncertainties that are inherent to this type of diversification process (Ramirez, Birindelli, Carvalho, et al., 2017).

One of the principal taxonomic problems of Brazilian *Leporinus* is related to the general lack of morphological differentiation among the species, as observed in the case of *L. piau* and *L. friderici*, which became even more problematic when Fowler (1941) selected the Salgado River, in the Jaguaribe basin, as the type locality of *L. piau*, and included a paratype from the Jatobá River, in the basin of the São Francisco River, which favored the recurrent collection of records of *L. piau* that were genetically distinct from *L. piau* from the type locality. Given their significant genetic differences in comparison with *L. piau* from the type locality and the fact that their morphotype aligns

with that of *L. cf. friderici*, the specimens from the São Francisco basin identified as *L. piau* are, in fact, a different species, which has been designated *L. cf. friderici* (Carvalho et al., 2011; Garavello & Britski, 2003; Ramirez et al., 2016; Silva-Santos et al., 2018).

The complex scenario reported by Silva-Santos et al. (2018) in their analysis of *L. cf. friderici* specimens from nine basins that originate on the Brazilian Shield (the Mearim, Amazon, Madeira, Tapajós, Xingu, Tocantins, São Francisco, Paraguay, and Paraná rivers), which differentiated two distinct groups, was also observed here in the hydrographic basins of Maranhão (Itapecuru, Mearim, Pericumã, and Turiaçu) and Piauí (Parnaíba). The sharing of genetic stocks between basins was also apparent, as in the case of the Itapecuru, Mearim, and Pericumã basins, which shared haplotypes, although the Turiaçu basin had distinct and unique haplotypes. The samples from the Parnaíba River in Piauí were also different from those of the rivers of Maranhão, once again, with a unique haplotype (Figure 3b).

The sharing of haplotypes among the Maranhão basins has been confirmed in previous studies, as well as the greater distance of these fish in comparison with those from the Parnaíba basin, in Piauí (Carvalho-Costa et al., 2011; Hubert & Renno, 2006). In Maranhão, this close relationship is found primarily in the fish of the order Characiformes, which form an isolated and divergent clade, with a considerable number of endemic species, in comparison with the fish of other orders (Hubert & Renno, 2006). This interaction may have been supported by the temporary connections arising during past marine regressions, which linked rivers through their estuaries, favoring dispersal between basins that had previously been isolated (Abreu et al., 2019; Vail & Hardenbol, 1979). By contrast, the subsequent increase in sea level likely fragmented populations in the basins that had previously been interlinked, supporting allopatric differentiation (Cardoso & Montoya-Burgos, 2009), as observed in the present study.

The group of samples that includes the Turiaçu basin and rivers of the Amazon basin, which had significant support (Figure 4), can be accounted for by the occurrence of dispersal across the coast during periods of marine regression. In a study of the fish of the genus *Pseudoplatystoma* Bleeker, 1862, Carvalho-Costa et al. (2011) found that the specimens from the Turiaçu basin were more closely related, in evolutionary terms, to those of the Amazon basin, in comparison with the other basins of Maranhão, and concluded that the Turiaçu population originated more recently. In a study of the biogeography of the coastal basins of Maranhão, Abreu et al. (2019) included sequences from Turiaçu, which invariably formed a separate clade that was normally closer to the Amazonian fish than those of Maranhão, apparently reflecting vicariant events (Hubert et al., 2007).

5 | CONCLUSIONS

The molecular delimitation methods applied in the present study showed that the *Leporinus* specimens from the basins of the Parnaíba and Mearim rivers that formed the *L. cf. friderici* sensu stricto clade should be designated as the *L. cf. friderici* morphotype. The *L. piau* specimens from the Itapecuru, Mearim, Turiaçu, and Pericumã rivers

that were assigned to the unnamed clade should be designated provisionally as *L. cf. piau*. This is because the cryptic diversity of this clade does not exclude the presence of *L. piau* from the basins of Maranhão, given that the *L. piau* from the type locality is also in this clade. This supports the conclusion that the *L. piau* may be included in the species of the unnamed clade under the denomination of *L. cf. friderici*.

The results of the present study of the Maranhão and Piauí basins confirmed the hypothesis that the *L. cf. friderici/L. piau* group that we designated here corresponds to *L. cf. friderici* sensu stricto and that the *L. cf. friderici* and *L. piau* groups correspond to the unnamed group. In summary, our analyses substantiate the existence of a complex scenario of cryptic diversity within the *Leporinus* genus, underscore the taxonomic inconsistencies within this fish group, and highlight the potential of the hydrographic basins of Maranhão to act as a geographic model for the study of this genus.

AUTHOR CONTRIBUTIONS

Data generation: Maria Histelle Sousa Nascimento, Elmary Fraga, José Luis Olivan Birindelli, and Maria Claudene Barros; data analysis: Maria Histelle Sousa Nascimento, Elmary Fraga, José Luis Olivan Birindelli, and Maria Claudene Barros; manuscript preparation: Maria Histelle Sousa Nascimento, José Luis Olivan Birindelli, Elmary Fraga, and Maria Claudene Barros; writing, reviewing, and editing: Maria Histelle Sousa Nascimento, Elmary Fraga, and Maria Claudene Barros; supervision: Maria Claudene Barros; funding acquisition: Elmary Fraga and Maria Claudene Barros.

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4 DISCUSSÃO INTEGRADORA

Estudos de identificação e filogenéticos empregando sequências parciais ou totais do DNA em peixes são uma realidade bastante atual, o que pode ser confirmado pelo grande número de trabalhos recentemente publicados com essa abordagem (PEREIRA *et al.*, 2011; CARVALHO *et al.*, 2012; PEREIRA *et al.*, 2013; GOMES *et al.*, 2015; RAMIREZ e GALETTI JR, 2015; MELO B *et al.*, 2016; MELO F *et al.*, 2016; PUGEDO *et al.*, 2016; RAMIREZ *et al.*, 2016; ROSSINI *et al.*, 2016; SILVA *et al.*, 2016; PIRES *et al.*, 2017; RAMIREZ *et al.*, 2017a; MACHADO *et al.*, 2018; SILVA-SANTOS *et al.*, 2018; CALEGARI *et al.*, 2019; BRITSKI *et al.*, 2019; RAMIREZ *et al.*, 2020; GARAVELLO *et al.*, 2021; LIMEIRA-FILHO *et al.*, 2023; entre outros). O uso de genes nucleares é bastante útil quando o tempo de divergência dos grupos é antigo, uma vez que esses genes evoluem muito mais lentamente do que os genes mitocondriais. Quando juntos, estudos com genes mitocondriais e nucleares, apresentam grande poder de resolução de problemas enfrentados pela taxonomia tradicional, como na separação de espécies crípticas e identificação de espécies com grande plasticidade fenotípica (STEPIEN e KOCHER, 1997; ORTÍ e KOCHER, 1997; HEBERT, *et al.*, 2003).

Os dados apresentados nos dois capítulos e apêndices constituíram uma ferramenta útil para identificar e separar a maioria das espécies de anostomídeos aqui apresentadas. Por exemplo, o uso de dados morfológicos integrados a informações moleculares possibilitou a identificação de *M. macrocephalus* permitindo a inferência de que este estudo constituiu o primeiro registro desta espécie para as bacias do Mearim e Itapecuru, ampliando a distribuição geográfica desta espécie para a região Nordeste, uma vez que dados da literatura apontavam sua ocorrência para as bacias das regiões Sul, Sudeste e Centro-Oeste do Brasil (GARAVELLO e BRITSKI, 1988; ALVES *et al.*, 2007; GRAÇA e PAVANELLI 2007; LANGEANI *et al.*, 2007; GOMES *et al.*, 2015; BERTACO *et al.*, 2016). Por tratar-se de uma espécie de peixe comercialmente importante (SOARES *et al.*, 2000; ZEINAD e PRADO, 2012), a ocorrência *M. macrocephalus* nas bacias do Mearim e Itapecuru é provavelmente resultante da introdução de espécies exóticas na piscicultura maranhense, onde espécimes escapam dos tanques, por transbordamento, para lagos e rios durante a estação chuvosa (ver artigo em apêndice).

A Convenção sobre a Diversidade Biológica (CDB), Decisão VI-23, (SECRETARIAT OF THE CONVENTION ON BIOLOGICAL DIVERSITY, 2005) define espécies exóticas como: “uma espécie, subespécie ou táxon de classificação inferior que ocorre fora da sua área de distribuição natural passada ou presente; inclui qualquer parte, como gametas, sementes, ovos ou propágulos que possam sobreviver e reproduzir-se subsequentemente”. A CDB

também define espécie exótica invasora como “uma espécie exótica cuja introdução e/ou dispersão ameaça a diversidade biológica”; e ainda conceitua introdução de espécies como: “movimento por ação humana, direta ou indireta, de uma espécie exótica para fora de sua área de distribuição natural (passada ou presente). Esse movimento pode ocorrer dentro de um país ou entre países ou áreas além da jurisdição nacional”. Assim, a introdução de *M. macrocephalus* nos bacias dos rios Itapecuru e Mearim, por escape de indivíduos de criações, deve ser melhor monitorada, uma vez que populações viáveis sejam estabelecidas nos rios maranhenses, esta espécie poderá representar um problema a ictiofauna nativa. Estudos apontam a introdução de espécies exóticas como um dos principais motivos de perda de biodiversidade mundial, causando alterações nos processos e interações ecológicas, como predação, e ainda competição por alimento, espaços e sítios de reprodução, resultando na diminuição da riqueza da ictiofauna nativa e a homogeneização da biota aquática (LEÃO *et al.*, 2011; LATINI *et al.*, 2016).

A análise do código de barras de DNA nos deu indícios por meio de análises de distância genética e métodos de delimitação de espécies, da ocorrência de mais de uma espécie de *Leporinus* dentro de espécies nominais válidas e subdividas em MOTUs distintas, apoiada pela distância genética, bem como a ampliação da distribuição geográfica de uma espécie nominal para bacias maranhenses.

Em relação ao limiar de diferenciação intraespecífico recomendado na literatura, para a gênero *Leporinus* de bacias hidrográficas do Maranhão e Piauí podemos inferir que o uso de 2% não é adequado, uma vez que esse percentual não foi suficiente para separar as espécies entre bacias diferentes. No entanto, pode ser aplicado quando for necessário avaliar algumas espécies provenientes de mesma bacia. O valor tradicional de limiar na separação espécies de peixes continentais, mediante uso do código de barras de DNA ou DNA *barcoding* (2%) deve ser feito com bastante cautela, uma vez que algumas espécies possuem mínimas interespecíficas menores a esse valor. Como alternativa, Hebert *et al.* (2003) propõe a adoção de 10 vezes o valor da média intraespecífica, para grupos que possuem valores de distância mínima interespecíficas inferiores a esse limiar. Ainda foi proposto como limiar de delimitação, o menor valor entre duas espécies taxonomicamente válidas (FEITOSA, SANTOS e BIRINDELLI, 2011). Esses resultados demonstram que o uso de valores “fechados” como limiar para separar espécies podem levar a informações subestimadas acerca da biodiversidade, representando assim um risco para o reconhecimento de espécies que não se enquadrem nesse perfil.

Nas análises moleculares empregadas no presente estudo, incluindo as diferentes abordagens de delimitação de espécies de locus único (COI) foi identificada a presença de quatro espécies de *Leporinus*, sendo elas: *L. maculatus*, *L. unitaenitus*, *L. affinis* e *L. venerei*.

A confirmação da presença de *L. venerei* nas bacias do Itapecuru, Mearim, Turiaçu, Preguiças e Periaá representa um novo registro para o estado do Maranhão e ampliou a distribuição conhecida desta espécie no Brasil. Esses dados já eram sinalizados em estudos anteriores quando Fraga et al. (2014) e Nascimento et al. (2016) evidenciaram a formação de dois subclado em *L. piau* do Maranhão, sugerindo tratar-se de linhagens distintas, resultado este que o presente estudo revelou como agrupamentos que na verdade correspondem as espécies *L. venerei* e *L. cf. friderici*.

A problemática taxonômica envolvendo *L. piau*, *L. cf. friderici* e *L. venerei* está diretamente relacionada a elevada semelhança dos caracteres morfológicos diagnósticos que estas espécies compartilham, dificultando o reconhecimento de *L. venerei* nos rios maranhenses ao longo dos anos, no entanto através de dados moleculares gerados em nosso estudo foi possível apontar para ocorrência de *L. venerei* em quatro bacias maranhenses representando o primeiro registro desta espécie para o estado do Maranhão.

O compartilhamento de agrupamentos e MOTUs entre *L. piau* e *L. cf. friderici*, bem como a evidência de elevadas distancias genéticas dentro das amostras que compõem esses dois táxons, tanto nas análises de locus único como multilocus, reforçam que tanto *L. piau* das bacias do Maranhão e Piauí, quanto *L. cf. friderici* de diferentes bacias brasileiras não constituem duas entidades taxonômicas, ambas possivelmente tratam-se um complexo de espécie, como já havia sido observado para *L. cf. friderici* (RAMIREZ, BIRINDELLI e GALETTI JR, 2017; SILVA-SANTOS *et al.*, 2018).

Para *L. piau* do Maranhão, no entanto, pode-se notar uma maior similaridade entre bacias que apresentam semelhantes característica hidrológicas resultantes dos processos biogeográficos responsáveis pelos padrões de dispersão e interação entre a biota pretérita, como é o caso das amostras proveniente das bacias do Mearim, Itapecuru e Pericumã. Tais bacias apresentam pequenas conexões entre si através de lagoas marginais e pequenos rios permitindo fluxo gênico entre as populações dessas drenagens (PIORSKI, 2010; ABREU, 2013). Essas três bacias (Mearim, Itapecuru e Pericumã) estão situadas na Região Hidrográfica Atlântico Nordeste Oriental (RHANO), e dentro dessa região Dagosta e Pinna (2017) reconhece três áreas de endemismo, onde Mearim/Itapecuru aparecem como área endêmica única, o que favorece a maior similaridade genética entre os peixes dessas duas bacias. Por outro lado, a formação de um subagrupamento para *L. piau* da bacia do Turiaçu junto a *L. piau* da bacia amazônica reforça as observações de Abreu (2020) de que as espécies de peixes do Turiaçu são mais proximamente relacionadas aos peixes da bacia amazônica que as bacias maranhenses. Essa proximidade com os peixes amazônicos pode ser derivada de eventos de vicariância e o isolamento e diferenciação da ictiofauna Turiaçu pode ter resultado de reconstruções de

paleodrenagens, bem como pelo surgimento da Serra do Tiracambu (COSTA *et al.*, 1996; SOARES *et al.*, 2011).

5 CONCLUSÃO

Os resultados obtidos neste estudo demonstram a ocorrência e separação de *L. maculatus*, *L. unitaenitus*, *L. affinis* e *L. venerei* em táxons distintos. Além de diferencia-las, conseguimos registrar pela primeira vez a ocorrência de *L. venerei* em quatro bacias maranhenses: Itapecuru, Mearim, Peria e Preguicas. Dessa forma, adicionar dados genéticos ao estudo de *Leporinus* pode garantir que a identificação das espécies seja mais precisa.

O registro de *Megaleporinus macrocephalus*, uma espécie exótica, nas bacias hidrográficas maranhenses, representa uma extensão da sua distribuição que hoje é confirmada na bacia do rio Itapecuru e Mearim, evidenciando para a necessidade do monitoramento dos mecanismos de interações estabelecidas entre *M. macrocephalus* e as espécies nativas, melhorando a capacidade de previsão sobre os impactos e consequências que a presença desta espécie nos rios maranhenses pode provocar, para assim estabelecer medidas preventivas de controle.

Os marcadores mitocondriais e nucleares evidenciam a separação de espécimes de *L. piau* das bacias dos rios Parnaiba e Mearim que formaram o clado *L. cf. friderici* sensu stricto, sendo assim devem ser denominados como o morfotipo *L. cf. friderici*, e que provavelmente tais espécimes foram identificados erroneamente, tornando claro a importância do uso de dados moleculares aliados a taxonomia tradicional.

Os espécimes de *L. piau* dos rios Itapecuru, Mearim, Turiaçu e Pericumã que foram atribuídos ao clado “sem nome” devem ser denominados provisoriamente como *L. cf. piau*, pois a diversidade críptica observada neste clado não exclui categoricamente a presença de *L. piau* das bacias do Maranhão, uma vez que o *L. piau* da bacia do Rio Jaguaribe também compõe este agrupamento.

Os espécimes de *L. piau* do Maranhão que constituíram o clado “sem nome” tanto para os estudos de locus único quanto multilocus revelam diferenciação genética entre as bacias, com evidente separação da bacia do Turiaçu em clado mais geneticamente relacionado com espécimes da bacia Amazônica, bem como mostra uma relação mais estreita entre os *Leporinus* do Mearim e Itapecuru.

A partir dos resultados obtidos podemos inferir que os diferentes agrupamentos de *L. cf. friderici/L. piau* formados nas análises de locus único e multilocus confirmam a hipótese de que ocorre os dois agrupamentos denominados respectivamente de *L. cf. friderici* sensu stricto e “sem nome” refletindo um cenário complexo na diversidade de *Leporinus* que ocorrem nos rios do estado do Maranhão e Piauí, o que evidencia o potencial das bacias hidrográficas maranhenses como modelo geográfico para o estudo do gênero.

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APÊNDICE

Original Article

New records of the occurrence of *Megaleporinus macrocephalus* (Garavello & Britski, 1988) (Characiformes, Anostomidae) from the basins of the Itapecuru and Mearim rivers in Maranhão, Northeastern Brazil

Novos registros da ocorrência de *Megaleporinus macrocephalus* (Garavello & Britski, 1988) (Characiformes, Anostomidae) nas bacias dos rios Itapecuru e Mearim no Maranhão, Nordeste, Brasil

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Abstract

The “piaussu”, *Megaleporinus macrocephalus* is an anostomatid fish species native to the basin of the Paraguay River, in the Pantanal biome of western Brazil. However, this species has now been recorded in a number of other drainages, including those of the upper Paraná, Uruguay, Jacuí, Doce, Mucuri, and Paraíba do Sul rivers. This study presents two new records of the occurrence of *M. macrocephalus*, in the basins of the Itapecuru and Mearim rivers in the state of Maranhão, in the Brazilian Northeast. The piaussu is a large-bodied fish of commercial interest that is widely raised on fish farms, and its occurrence in the Itapecuru and Mearim rivers is likely the result of individuals escaping from fish tanks when they overflow during the rainy season. Morphological analyses and sequences of the Cytochrome Oxidase Subunit I (COI) gene confirmed the taxonomic identification of the specimens as *M. macrocephalus*. The COI sequences were 99.66% similar to those of *M. macrocephalus* deposited in the BOLD Systems database. These records extend the known distribution of *M. macrocephalus* to the basins of the Itapecuru and Mearim rivers in the Brazilian Northeast, highlighting a new case of introduction of exotic fish species into Brazilian river basins.

Keywords: freshwater, Ichthyofauna, taxonomy, biological invasion, COI.

Resumo

Megaleporinus macrocephalus é uma espécie de peixe anostomatídeo nativa da bacia do rio Paraguai, no bioma Pantanal do oeste do Brasil. No entanto, essa espécie já foi registrada em várias outras drenagens, incluindo as dos rios Alto Paraná, Uruguai, Jacuí, Doce, Mucuri e Paraíba do Sul. Este estudo apresenta dois novos registros da ocorrência de *M. macrocephalus*, nas bacias dos rios Itapecuru e Mearim, no estado do Maranhão, no nordeste brasileiro. O piaussu é um peixe de grande porte, de interesse comercial, amplamente criado em pisciculturas, e sua ocorrência nos rios Itapecuru e Mearim é provavelmente o resultado de indivíduos que escapam dos tanques quando transbordam durante a estação chuvosa. Análises morfológicas e sequências do gene da subunidade I do citocromo oxidase (COI) confirmaram a identificação taxonômica dos espécimes como *M. macrocephalus*. As sequências de COI foram 99,66% semelhantes às de *M. macrocephalus* depositadas no banco de dados BOLD Systems. Esses registros estendem a conhecida distribuição de *M. macrocephalus* às bacias dos rios Itapecuru e Mearim, no nordeste brasileiro, destacando um novo caso de introdução de espécies exóticas de peixes nas bacias hidrográficas brasileiras.

Palavras-chave: água doce, Ictiofauna, taxonomia, invasão biológica, COI.

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1. Introduction

The family Anostomidae is currently composed of 149 valid species, distributed in 15 genera (Fricke et al., 2020). Species of the family are endemic to the Neotropical region, and are widely distributed from north of Colombia to La Plata River, in Argentina (Garavello and Britski, 2003). The greatest diversity of anostomatids is found in the Amazon basin, where 91 species have been recorded to date (Dagosta and Pinna, 2019). The genus *Leporinus* Agassiz, 1829, is the most species-rich of Anostomidae (Burns et al., 2017).

Garavello and Britski (1988) described *Leporinus macrocephalus* based on 15 specimens collected in the Paraguay River basin in Brazil. The species was recently transferred to *Megaleporinus*, a genus described in 2017 by Ramirez et al. (2017), based on morphological, molecular, and cytogenetic data, including a unique system of ZZ/ZW sex chromosomes.

The fish of the genus *Megaleporinus* are relatively large in size (adult standard length typically over 35 cm), and are diagnosed among anostomids by having the following combination of exclusive features: premaxilla with three unicuspid teeth; dentary with three unicuspid teeth; and body with one to four dark midlateral blotches (Ramirez et al., 2017).

Megaleporinus macrocephalus (Garavello and Britski, 1988), known in Brazil as the “piaussu”, “piavuçu” or “piau-açu”, is an omnivorous fish that feeds on small fruit and seeds, as well as small fish and crabs (Peruca et al., 2000; Navarro et al., 2006). *M. macrocephalus* is distributed

in the Paraguay River in Argentina, Brazil, Bolivia and Paraguay (Garavello and Britski, 1988; Britski et al., 1999; Fricke, 2019). However, individuals escaped from fish farms have colonized a number of other South American basins, including that of the upper Paraná River (Graça and Pavanelli, 2007; Langeani et al., 2007), the Jacuí and Uruguay Rivers in the Brazilian state of Rio Grande do Sul (Bertaco et al., 2016), the Mucuri River (Gomes et al., 2015), and the Doce and Paraíba do Sul rivers (Alves et al., 2007). The piaussu is commercially important for sports fishing (Zeinad and Prado, 2012) and is widely raised on fish farms to supply markets in southern and central Brazil (Soares et al., 2000; IBGE, 2014).

The present study provides the first report of the occurrence of *M. macrocephalus* in northeastern Brazil, more specifically in the Itapecuru and Mearim rivers, in the state of Maranhão. The Mearim is the state's largest hydrographic basin, and plays an important role in the economy and subsistence of the region's riverside populations (Maranhão, 2011). These records are the first evidence of the occurrence of *M. macrocephalus* in the Itapecuru and Mearim Rivers, extending the limits of the known geographic distribution of the species.

2. Material and Methods

Specimens of *M. macrocephalus* were collected in the basins of the Itapecuru and Mearim rivers, in the state of Maranhão, Northeastern Brazil (Figure 1). The Mearim

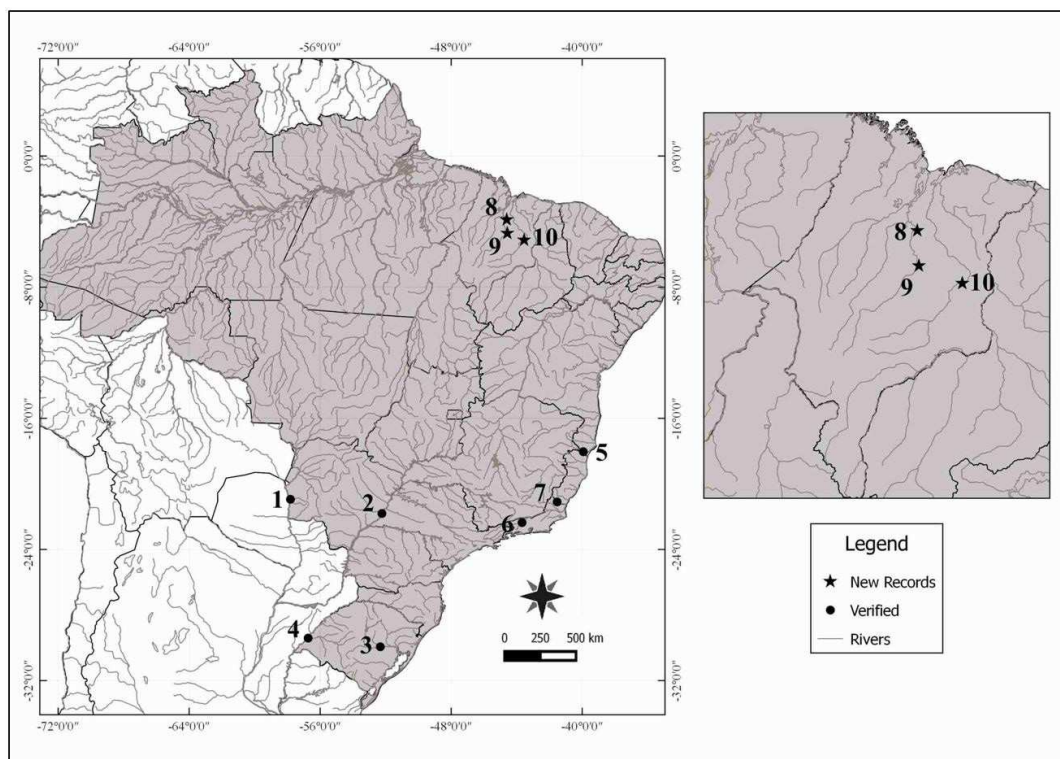


Figure 1. Geographic distribution of *Megaleporinus macrocephalus* in Brazil. The area of the new registrations for the Itapecuru rivers in the municipality of Pé da Serra and Mearim in the locality Laje dos Currais, in São Mateus, and in the city of Pedreiras in the Northeast Region.

River has a total extension of 832.18 km, and has a number of tributaries, in particular the Pindaré River, which joins the Mearim only 20 km from the mouth of the Grajaú River, a second important tributary of the Mearim River. The Itapecuru River is located wholly within the state of Maranhão, and has three distinct stretches – the upper river (from the source to the municipality of Colinas), the middle river (from Colinas to the municipality of Caxias), and the lower Itapecuru, from Caxias to the mouth, in São José Bay (Alcântara, 2004). The Itapecuru River, which is 852.71 km long, has eight major tributaries on its right margin, in particular the Pirapemas and Itapecuruzinho Rivers, and the Seco, do Ouro, Gameleira, Cachimbo, and Guariba streams (UEMA, 2016).

The specimens were collected using gill, drag, and cast nets. The voucher specimens were taken to the Laboratory of Genetics and Molecular Biology (GENBIMOL) at the Center for Higher Studies at Maranhão State University (CESC/UEMA), fixed in 10% formalin, and conserved in 70% alcohol. The prepared specimens were sent to the Zoology Museum at the State University of Londrina (MZUEL), Paraná, Brazil, for morphological identification and cataloguing (voucher MZUEL: 15357 – Mearim river) e (voucher MZUEL: 20221 – Itapecuru river).

The collection of specimens was authorized by the Brazilian Federal Institute for the Environment and Renewable Natural Resources (IBAMA) through licence number 02012.004159/2006, and ICMBio/MMA licence 42119-2, from the Chico Mendes Institute for Biodiversity Conservation.

Measurements were taken following Britski and Garavello (1978) and Birindelli et al. (2013). Morphometric data were obtained with a digital caliper with accuracy of 0.01 mm. Counts of scales and rays were taken according to Birindelli et al. (2013).

Samples of muscle tissue were extracted from the specimens for the genetic analyses. The total DNA was extracted using the Promega Wizard Genomic DNA purification kit, following the manufacturer's instructions. The Cytochrome Oxidase Subunit I (COI) was then amplified by Polymerase Chain Reaction, using the universal primers COI FishF1: 5' -TCAACCAACCACAAAGACATTGCCAC - 3'; and COI FishR1: 5' - TAGACTTCTGGGTGGCCAAAGAATCA - 3', as described by Ward et al. (2005). The samples were sequenced by Sanger et al. (1977) dideoxyterminal method, using a Big Dye kit in an ABI Prism™ 3500 automatic sequencer (Applied Biosystems, USA). The sequences were edited and aligned in BIOEDIT 7.0 (Hall, 1999), and plotted in the BOLD Systems v4 platform (BOLD, 2020) to evaluate their similarity with existing sequences (Ratnasingham and Hebert, 2007).

3. Results

On the Mearim River, one *M. macrocephalus* specimen was collected on the lower course of the river at Laje dos Currais, in the municipality of São Mateus, during the flood season, while two specimen was obtained on the middle course of the river in the town of Pedreiras during the dry (low water) season. The *M. macrocephalus* specimen was

collected at the locality of Pé da Serra in the municipality of Caxias during the dry season.

The four *M. macrocephalus* specimens (Table 1) present a characteristic coloration pattern, with a darker superior half of the body, three large black spots on each flank, and dark lines between the rows of scales (Ramirez et al., 2017). The body is robust, and the mouth terminal, with three teeth in the pre-maxillary bone and three teeth in

Table 1. Morphological data of *Megaleporinus macrocephalus*.

Characters	MZUEL 20221	MZUEL 15357
	Itapecuru	Mearim
Standard Length (mm)	387.1	175.2
Percentages of standard length		
Predorsal distance	41.10	43.83
Dorsal-fin origin to adipose-fin origin	41.37	37.76
Prepelvic distance	43.40	47.62
Body depth	28.14	28.06
Caudal-peduncle length	11.41	12.50
Caudal-peduncle depth	16.93	15.45
Anal-fin lobe length	10.59	10.04
Head length	22.32	27.26
Percentages of head length		
Preopercle length	75.81	64.68
Snout length	35.78	29.45
Head depth	96.92	85.41
Eye diameter	13.33	23.51
Bony interorbital	58.88	54.53
Meristics		
Lateral-line scales	43	42
Scale rows between lateral line and dorsal-fin origin	7	7
Scale rows between lateral line and anal-fin origin	6	6
Scale rows around caudal peduncle	16	16
Predorsal scales	13	13
Dorsal-fin unbranched rays	2	2
Dorsal-fin branched rays	10	10
Pectoral-fin branched rays	14	15
Pelvic-fin branched rays	8	8
Anal-fin unbranched rays	3	3
Anal-fin branched rays	8	8
Upper caudal-fin branched rays	9	9
Upper caudal-fin branched rays	8	8
Teeth on left premaxilla	3	3
Teeth on left dentary	3	3



Figure 2. *Megaleporinus macrocephalus*, MZUEL 20221, 387.10 mm SL.

Table 2. Similarity percentage obtained by comparing the sequences of the COI gene on the BOLDSystems platform (The Barcode of Life Data System) for the *M. macrocephalus* species from the Itapecuru and Mearim rivers, Maranhão, Brazil, identified morphologically and which were confirmed with the molecular data.

Identificação			
Código	N	Taxonômica	Molecular: Bold
MEA 831	1	<i>M. macrocephalus</i>	<i>M. macrocephalus</i> (99.66%)
MEA 835	1	<i>M. macrocephalus</i>	<i>M. macrocephalus</i> (99.66%)
MEA 836	1	<i>M. macrocephalus</i>	<i>M. macrocephalus</i> (99.66%)
LEP 158	1	<i>M. macrocephalus</i>	<i>M. macrocephalus</i> (99.66%)

the dentary bone (Figure 2). The specimens have 42–43 scales in the lateral line, seven rows of scales between the lateral line and the origin of the dorsal fin, six rows of scales between the lateral line and the origin of the pelvic fin, and 16 rows of scales around the caudal peduncle. These characteristics, together with the morphometry of the specimens, are fully consistent with the descriptions of *M. macrocephalus* published Garavello and Britski (1988) and Britski et al. (1999), and leave no doubt with regard to the identity of the taxon.

Four COI sequences were obtained, one from the specimen collected from the Itapecuru River, and three from the specimens obtained from the basin of the Mearim River. Comparisons with the sequences available in the BOLDSystems database confirmed the morphological identification of the specimens. The COI sequences obtained from the specimens collected in the present study were more than 99% similar to that of *M. macrocephalus* from the basin of the Paraná River (Table 2). This value is well within the 2% threshold for the identification of fish species by DNA barcoding (Ward et al., 2009; April et al., 2011; Carvalho et al., 2011; Mabragaña et al., 2011; Castro Paz et al., 2014).

4. Discussion

No records of *M. macrocephalus* species were obtained during previous studies in the Itapecuru and Mearim rivers (Soares, 2005; Barros et al., 2011; Fraga et al., 2012; Nascimento et al., 2016; Abreu et al., 2019). It seems likely that the species was exhaust accidentally during the rainy season, when the tanks of local fish farms overflow and become connected with the tributaries of the two basins.

The species *M. macrocephalus* is used in fish pisciculture, larger and has economic importance for fishing in some regions, in particular in the Pantanal Mato-grossense (Navarro et al., 2007). *M. macrocephalus* is well-suited to fish pisciculture, which has led to its accidental introduction into a number of different Brazilian river basins (Godinho and Godinho, 2003; Barbosa and Soares, 2009; Vieira, 2010).

It is interesting to note that *M. macrocephalus* has not been recorded in recent surveys of other, nearby basins, in the Brazilian Northeast, including the Munim and Parnaíba in Maranhão/Piauí (Matavelli et al., 2015), the Parnaíba in Maranhão/Piauí (Ramos et al., 2014), and the São Francisco, in Bahia (Carvalho et al., 2011).

The records of *M. macrocephalus* presented here represent an important extension of the known distribution of the species, whose occurrence is now confirmed in the Itapecuru and Mearim rivers of Maranhão, in the Brazilian Northeast. It is nevertheless premature to confirm that *M. macrocephalus* has established viable populations in these rivers, and what effects its presence may have on the resident species. However, the introduction or transfer of exotic fish species into a river basin, whether intentional or otherwise, almost always has negative implications for the resident native species (Pelicice et al., 2014), and any invasion of this type requires carefully-planned management strategies to guarantee the long-term integrity of the local biota.

5. Conclusion

The combined analysis of morphological and molecular analysis confirmed the occurrence of *M. macrocephalus* in the Itapecuru and Mearim Rivers, state of Maranhão.

This substantially amplifies the known distribution of this species to the Brazilian Northeast.

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