Neotropical Ichthyology

Original article

https://doi.org/10.1590/1982-0224-2023-0037

DNA Barcoding of *Pyrrhulina australis* (Characiformes: Lebiasinidae) reveals unexpected cryptic diversity in the group

Taina Barbosa de Souza[1], Daniela Cristina Ferreira[2], Hugmar Pains da Silva[3], André Luiz Netto-Ferreira[4] and Paulo Cesar Venere[2]

Correspondence:
Daniela Cristina Ferreira
ferreiradc@gmail.com

The family Lebiasinidae includes a number of miniature and medium-sized fish species that are endemic to the Neotropical region. *Pyrrhulina* is the second most speciose lebiasinid genus and it is also the one with the most taxonomic uncertainties. In this context, the present study focused on the *Pyrrhulina* morphospecies found in a number of different drainage basins in South America to test the alternative proposals on the arrangement of the taxonomic units found within what is assumed to be a single nominal species, *Pyrrhulina australis*, based on a DNA Barcoding approach. The results of the analyses indicate that *Pyrrhulina australis* is a species complex, with intraspecific (within-group) genetic distances of up to 3.74%, well above the Optimal Threshold of 1.79% defined in the present study. The species delimitation analyses revealed a surprising level of diversification among the morphospecies evaluated, in particular, in the clade that encompasses *Pyrrhulina australis* (from the Paraguay River) + *P.* cf. *rachoviana* (Lower Paraná River), *P.* aff. *australis* I (Araguaia River)/II (Paraguay River)/III (Upper Paraná River)/IV (Guaporé River), and *P. marilynae* (Teles Pires River), which were arranged in six distinct evolutionary lineages that align with the geographical distribution of the respective drainage basins.

Keywords: Cryptic species, Freshwater fish, Molecular identification, Small fishes, Species delimitation methods.

Submitted April 12, 2023
Accepted September 26, 2023
by Matt Kolmann
Epub November 13, 2023

Online version ISSN 1982-0224
Print version ISSN 1679-6225

Neotrop. Ichthyol.
vol. 21, no. 4, Maringá 2023

1 Programa de Pós Graduação em Ecologia e Conservação da Biodiversidade, Universidade Federal de Mato Grosso, Avenida Fernando Correa da Costa, 2367, Bairro Boa Esperança, 78060-900 Cuiabá, MT, Brazil. (TBS) taina.souzab1@gmail.com.

2 Laboratório de Genética e Citogenetica Animal, Instituto de Biociências, Universidade Federal de Mato Grosso, Av. Fernando Correa da Costa, 2367, Bairro Boa Esperança, 78060-900 Cuiabá, MT, Brazil. (DCF) ferreiradc@gmail.com (corresponding author), (PCV) paulo.venere@gmail.com.

3 Faculdade de Ciências Agrárias, Biológicas, Engenharia e Saúde, Universidade do Estado de Mato Grosso, Campus Universitário Professor Eugênio Carlos Stieler de Tangará da Serra, Avenida Inácio Bittencourt Cardoso, 6967 E, Bairro Jardim Aeroporto, 78301-532 Tangará da Serra, MT, Brazil. (HPS) painsbio@gmail.com.

4 Laboratório de Ictiologia, Departamento de Zoologia, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, 91501-970 Porto Alegre, RS, Brazil. (ALNF) alnferreira@gmail.com.

A família Lebiasinidae é endêmica da região Neotropical, composta por espécies de peixes em miniaturas e de médio porte. O gênero *Pyrrhulina* é o segundo maior em número de espécies e o grupo com mais incertezas taxonômicas dentre os representantes dessa família. Diante disso, o objetivo deste estudo foi avaliar morfoespécies provenientes de diferentes bacias de drenagens, a fim de testar as diferentes propostas de reconhecimento de unidades taxonômicas dentro do que se acreditava ser a espécie nominal *Pyrrhulina australis*, utilizando o princípio do DNA Barcoding. Os resultados mostraram que *Pyrrhulina australis* é um complexo de espécies, com distância intraespecífica dentro do grupo de 3,74%, valor acima do Threshold Ótimo de 1,79% definido para este estudo. As análises de delimitação de espécies indicam uma surpreendente diversidade dentro das morfoespécies analisadas, especialmente no clado que engloba *Pyrrhulina australis* (rio Paraguai) + *P.* cf. *rachoviana* (baixo rio Paraná), *P.* aff. *australis* I (rio Araguaia), II (rio Paraguai), III (alto rio Paraná), IV (rio Guaporé) e *P. marilynae* (rio Teles Pires), que apresentou seis linhagens evolutivas que podem estar relacionadas ao padrão de distribuição pelas bacias de drenagens.

Palavras-chave: Delimitação de espécies, Espécies crípticas, Identificação molecular, Peixes de água doce, Peixes de pequeno porte.

INTRODUCTION

The Lebiasinidae (Characiformes) is endemic to the Neotropical region, and is composed of approximately 75 valid species (Van der Laan, Fricke, 2023). The lebiasinids are miniature to medium-sized fish that have fusiform bodies and are known popularly as pencilfish or, in Brazil, as "pirrulinas" or "charutinhos" (Géry, 1977; Malabarba, Malabarba, 2020). Based on a molecular analysis, Melo *et al.* (2022) estimated that the family experienced a notable diversification event during the Cenozoic era, approximately 30 million years ago, which gave rise to the present-day lineages. The six valid genera in two subfamilies, Lebiasininae and the Pyrrhulininae, with the latter being the more speciose, comprising 47 species distributed among four genera (Weitzman, Weitzman, 2003; Netto-Ferreira *et al*., 2011; Van der Laan, Fricke, 2023): *Nannostomus* Günther, 1872 (20 species), *Copella* Myers, 1956 (six species), *Copeina* Fowler, 1906 (two species), and *Pyrrhulina* Valenciennes, 1847 (19 species).

The 19 valid species (Fricke *et al*., 2023) of *Pyrrhulina* are distributed in the Amazon and Araguaia-Tocantins basins, as well as in the Paraguay-Paraná-La Plata hydrographic network, and the Laguna dos Patos and Tramandaí River systems in southern South America (Weitzman, Weitzman, 2003; Venere, Garutti, 2011; Bertaco *et al*., 2016; Dagosta, de Pinna, 2019). The genus houses most of the current taxonomic uncertainties within the Lebisinidae (Netto-Ferreira, Marinho, 2013), including a range of inconsistencies in the descriptions and diagnosis of the species of this group (Géry, 1977), which are difficult to distinguish on the basis of their morphological traits. This has led to the incorrect identification of many species in the past (Vieira, Netto-Ferreira, 2019).

In the first study based on genomic data, Ferreira *et al.* (2022) were able to determine the phylogenetic relationships among *Pyrrhulina australis* Eigenmann & Kennedy, 1903, *P. marilynae* Netto-Ferreira & Marinho, 2013, *P. obermulleri* Myers, 1926, *P. brevis* Steindachner, 1876, and *P. semifasciata* Steindachner, 1876, with a high level of genetic divergence among the species, which correlated with the geographic distribution of the taxa among the respective drainage basins. This was an important initial step toward understanding of the phylogenetic relationships among the species of the group, given the general lack of data available for species of *Pyrrhulina*. Although *Pyrrhulina* was under-represented in the aforementioned study, the close relationship between *P. australis* and *P. marilynae* obtained by the authors agree with the relationships proposed by Netto-Ferreira, Marinho (2013), based on morphological data, placing them within the "*Pyrrhulina australis* group" with *P. rachoviana* Myers, 1926 and *P. vittata* Regan, 1912. Except for *P. vittata*, the taxonomic limits among these species have been the subject of considerable debate by systematic biologists, and represent a major challenge for the definition of the taxonomy of the group. *Pyrrhulina australis* is a broadly-distributed species that is found between Argentina and French Guiana, in the Amazon (including the Araguaia-Tocantins system) and Paraguay-Paraná-La Plata basins, and adjacent coastal drainages (Weitzman, Weitzman, 2003). The species was reviewed by Zarske, Géry (2004), who synonymized *P. macrolepis* Ahl & Schindler, 1937 and *P. rachoviana* with *P. australis*. Netto-Ferreira, Marinho (2013) considered the synonymization of *P. rachoviana* to be erroneous. However, Zarske (2016) subsequentially confirmed the validity of *P. rachoviana*, and argued that *P. marilynae* would be a synonym of *P. rachoviana* instead. That conclusion was based on Zarske's hypothesis that the type locality of *P. rachoviana* to be in the Amazon basin, contradicting Myers (1926) , who suggested the type specimens of the species as originating from Rosário, Argentina.

The precise identification of species becomes imperative and challenging, especially when dealing with the vast diversity of neotropical ichthyofauna, estimated at around 9,000 fish species (Reis *et al*., 2016). However, despite this extensive diversity, many natural stocks are facing local or global extinction processes before even being known to science (Manel *et al*., 2020). In this context, molecular tools such as DNA barcoding can provide valuable insights for the resolution of complex taxonomic questions, in particular, the delimitation of species that are difficult to diagnose based solely on their morphological traits, and this approach has been widely used in phylogenetic studies of an enormous diversity of organisms (see *e.g*., Hebert *et al*., 2003; Pereira *et al*., 2013; Díaz *et al*., 2016; Machado *et al*., 2017; Costa-Silva *et al*., 2018; Arruda *et al*., 2019; Ramirez *et al*., 2020).

Given this scenario, the present study aimed to delimitate the species of the *Pyrrhulina australis* group, *sensu* Netto-Ferreira, Marinho (2013), based on the application of DNA barcoding. In addition, the present data allowed to objectively evaluate the different taxonomic proposals for the *Pyrrhulina australis* group – *i.e*., a single species (Zarske, Géry, 2004), two species (Zarske, 2016) or three (Netto-Ferreira, Marinho, 2013) – and to test the validity of the species that make up the group proposed by Netto-Ferreira, Marinho (2013), based on the morphological evidence.

MATERIAL AND METHODS

Study area and sample collection. The samples of the nominal species of *Pyrrhulina australis*, *P. marilynae*, and *P. obermulleri* were collected in the different drainage basins (Paraguay, Paraná, Araguaia-Tocantins, Tapajós, Guaporé, Madeira, and Maroni) in South America (Fig. 1). Additional sequences were obtained from GenBank and the BOLD systems database (Tab. 1). Samples of *P. obermulleri*, *P. spilota* Weitzman, 1960, and *P. filamentosa* Valenciennes, 1847 (Tab. 1) were included as outgroups in relation to the *P. australis* group. The species *Nannostomus marginatus* Eigenmann, 1909 (BOLD registration numbers GBMND24648-21, GBMND24649-21), was included as an outgroup to root the tree in the analysis performed with MrBayes. The species were identified based on the morphological traits (Fig. 2) described by Zarske, Géry (2004) and Netto-Ferreira, Marinho (2013) or the probable distribution of the taxa, estimated from the type locality, in the case of *P. rachoviana* (Weitzman, Weitzman, 2003).

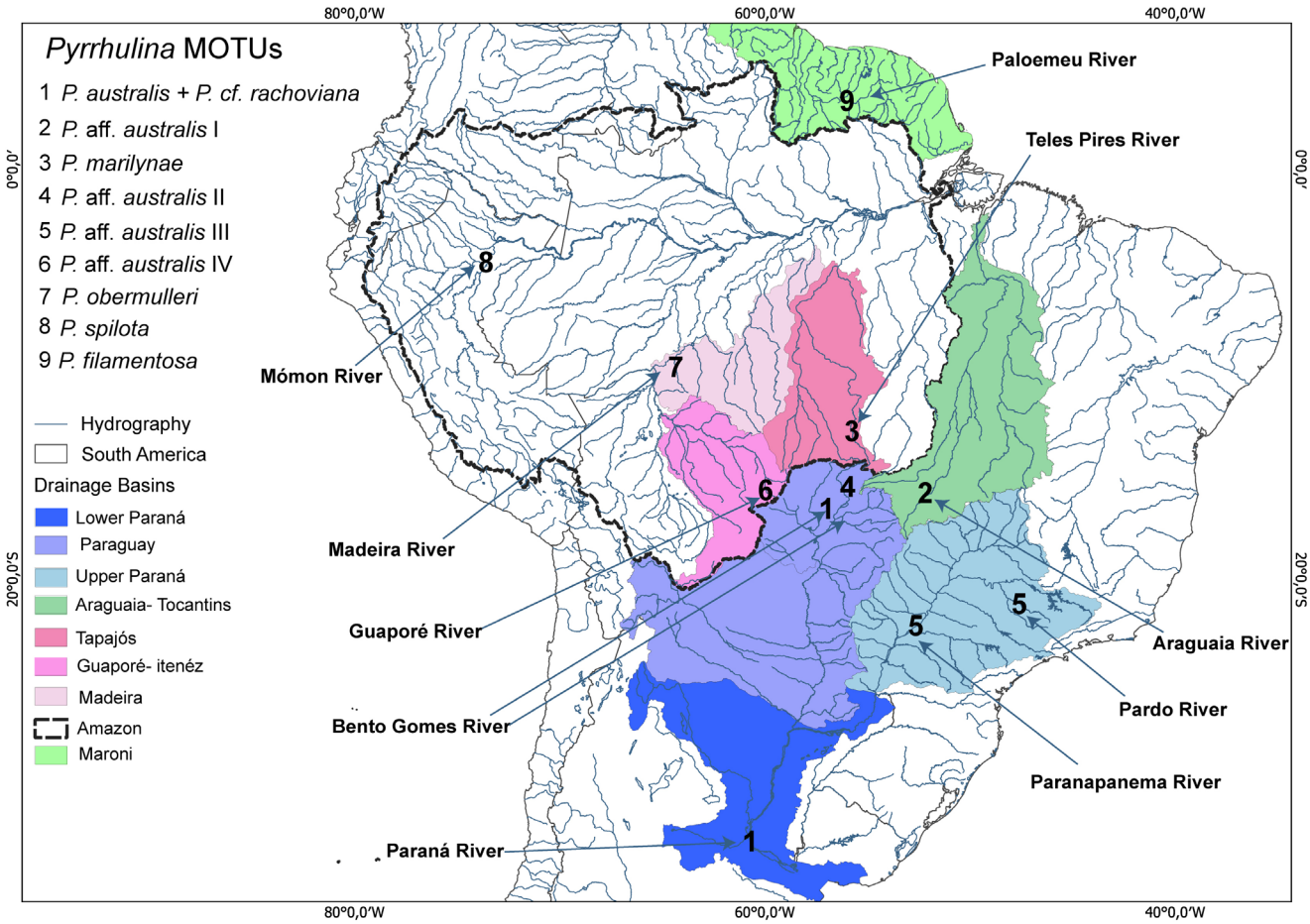

FIGURE 1 | Geographic distribution of the *Pyrrhulina* morphospecies and MOTUs throughout South American river basins.

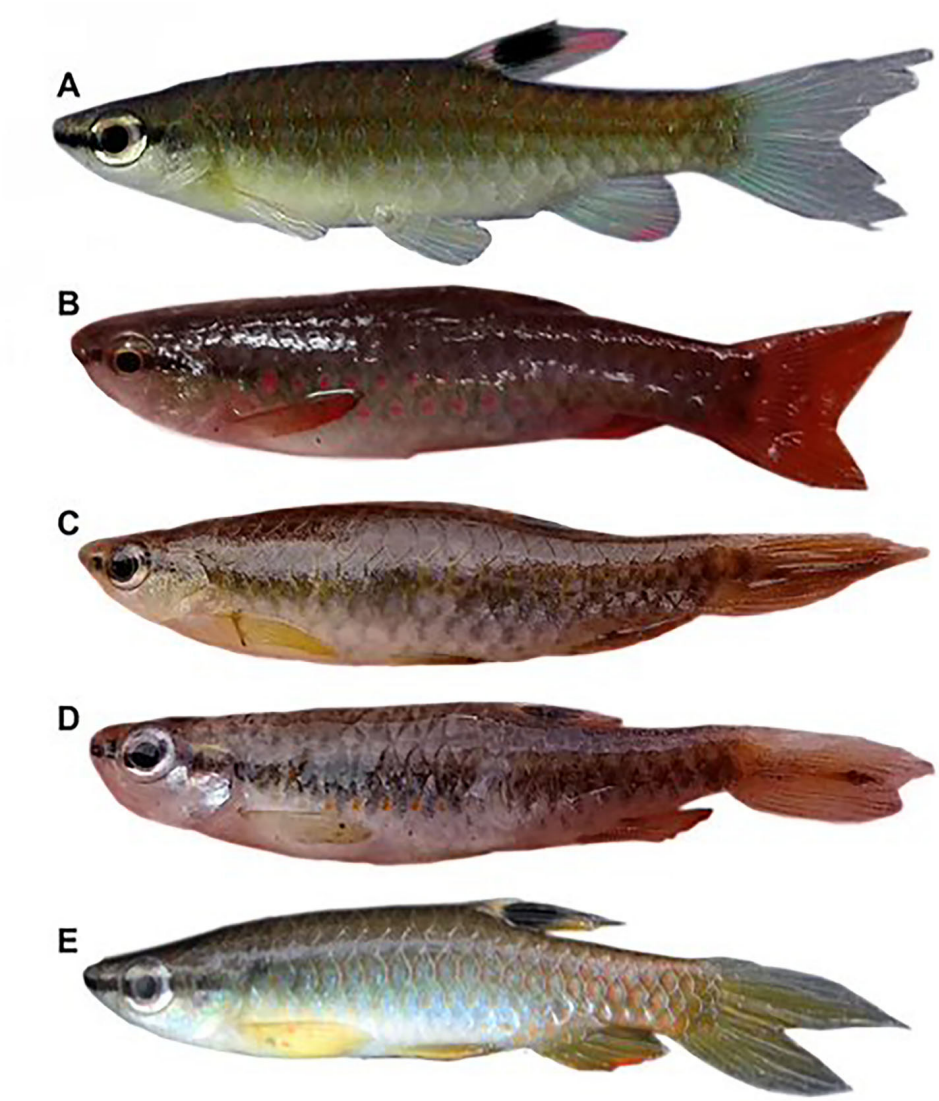

FIGURE 2 | Morphospecies considered in this study: **A.** *Pyrrhulina* aff. *australis* I (Araguaia, modified from Venere, Garuti, 2011); **B.** *P. obermulleri* (Madeira); **C.** *P.* aff. *australis* IV (Guaporé); **D.** *P. marilynae* (Teles Pires) and **E.** *P. australis* (Pantanal, Paraguay basin). *Pyrrhulina spilota* and *P. filamentosa* are not shown here because the sequences were obtained from the BOLD systems database, and no physical specimens were collected in the field.

The voucher specimens were deposited in the ichthyological collection of the Universidade Federal do Mato Grosso, Cuiabá (CPUFMT 7757–7762), and the tissue samples are stored in the Laboratório de Genética e Citogenética Animal (LABGEN) of the Instituto de Biociências of the UFMT. The gene sequences were deposited in the BOLD systems database, under the accession numbers shown in Tab. 1.

TABLE 1 | Voucher specimen information of the *Pyrrhulina* specimens analyzed in the present study, including the collecting locality, geographic coordinates, and BOLD accession numbers. The sequences of the specimens marked with an asterisk were obtained from the BOLD. Brazilian states: MT = Mato Grosso; PR = Paraná; RO = Rondônia; SP = São Paulo.

Species - consensus MOTU	Locality	Basin/River	Number of the sampled specimens	Lat	Long	Sequence id.	Voucher CPUFMT	BOLD systems accession number
Pyrrhulina aff. *australis* IV - MOTU 6	Pontes e Lacerda, MT, Brazil	Amazon (Guaporé)	7	15°21'30.24"S	59°15'10.56"W	91	7762	PYR001-22
						92		PYR002-22
						93		PYR003-22
						94		PYR004-22
						95		PYR005-22
						96		PYR006-22
						97		PYR007-22
Pyrrhulina obermulleri- MOTU 7	Porto Velho, RO, Brazil	Amazon (Madeira)	14	09°02'36.31"S	64°36'34.99"W	76	7760	PYR008-22
						77		PYR009-22
						78		PYR010-22
						79		PYR011-22
						80		PYR012-22
						81		PYR013-22
						82		PYR014-22
						83		PYR015-22
						84		PYR016-22
						85		PYR017-22
						86		PYR018-22
						87		PYR019-22
						88		PYR020-22
						90		PYR022-22
Pyrrhulina marilynae- MOTU 3	Sorriso, MT, Brazil	Amazon (Teles Pires)	1	12°57'17.68"S	55°44'37.82"W	75	7759	PYR023-22
Pyrrhulina aff. *australis* I- MOTU 2	Barra do Garças, MT, Brazil	Araguaia, Tocantins (Araguaia)	7	15°46'43.17"S	52°16'36.48"W	7021	7758	PYR024-22
						7022		PYR025-22
						7023		PYR026-22
						7024		PYR027-22
						7025		PYR028-22
						7026		PYR029-22
						7027		PYR030-22
Pyrrhulina australis- MOTU 1	Poconé, MT, Brazil	Paraguay (Bento Gomes)	5	16°09'07.39"S	56°37'02.85"W	98	7757	PYR031-22
						99		PYR032-22
						100		PYR033-22
						101		PYR034-22
						102		PYR035-22
Pyrrhulina cf. *rachoviana**- MOTU 1	Victoria, Argentina	Lower Paraná (Paraná)	2	32°53'13.20"S	60°40'08.40"W	315	-	LARI315-13
						316		LARI316-13
Pyrrhulina aff. *australis* III*- MOTU 5	Cravinhos, SP, Brazil	Upper Paraná (Pardo)	2	21°22'39.00"S	47°39'33.12"W	26301	-	FUPR018-09
						26302		FUPR019-09
Pyrrhulina aff. *australis* III*- MOTU 5	Marilena, PR, Brazil	Upper Paraná (Paranapanema)	3	22°40'24.24"S	53°05'08.88"W	32189	-	FUPR020-09
						32190		FUPR021-09
						32191		FUPR022-09
*Pyrrhulina filamentosa**- MOTU 9	Sipaliwini, Suriname	Maroni (Paloemeu)	3	03°10'41.99"N	55°25'09.12"W	976	-	GBOL976-16
						1032		GBOL1032-16
						1033		GBOL1033-16
*Pyrrhulina spilota**- MOTU 8	Puchana, Peru	Amazon (Mómon)	2	03°41'24.00"S	73°16'12.00"W	24650	-	GBMND24650-21
						24651		GBMND24651-21

Extraction, amplification, and sequencing of the DNA. The DNA was extracted using the saline extraction protocol of Aljanabi, Martinez (1997). The COI gene was amplified using the primers COI FISH F1 and FISH R1 described by Ward *et al*. (2005). The reagents and cycling conditions were the same as those described by Arruda *et al.* (2019).

The amplicons of the COI gene were purified and sequenced by Biotecnologia Pesquisa e Inovação (BPI, https://bpibiotecnologia.com.br). The samples were purified using a magnetic bead kit of the AMpure XP type, and sequenced with a BigDye® Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems), following the manufacturer's protocols. The samples were sequenced automatically by capillary electrophoresis in an ABI3730xl Genetic Analyzer (Applied Biosystems).

Data analysis. The raw sequences were edited and the presence of gaps was verified in Geneious® 7.1.3 (Kearse *et al*., 2012) while the sequences were aligned in Mega v. 11 (Tamura *et al*., 2021) using the ClustalW algorithm (Thompson *et al*., 2003). The aligned sequences were inspected in MEGA for the identification of stop codons, pseudogenes, and deletions and insertions. The sequences were tested in DAMBE7 (Xia *et al*., 2003) to determine the saturation of nucleotide substitutions.

The mean intraspecific and interspecific genetic distances between the morphospecies and the consensus MOTUs were calculated in Mega v. 11 using the Kimura-2-Parameter (K2P) model (Kimura, 1980), following Hebert *et al*. (2004), who employed the minimum interspecific and the maximum intraspecific distances identified in Excel, using the Minimum and Maximum functions. The Molecular Operational Taxonomic Units (MOTUs) were identified in the JMotu software (Jones *et al*., 2011) based on the Optimum Threshold (OT). The OT was calculated in the SPIDER (*SPecies IDentify and Evolutions in R*, Brown *et al*., 2012) package using the "LocalMinima" function in the R environment v. 3.6.3 (https://www.r-project.org; R Development Core Team, 2016). The groups formed by the Assemble Species by Automatic Partitioning software (ASAP, Puillandre *et al*., 2021) were estimated at the site https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html using the K2P substitution model, with all other parameters at the default values. The partition was selected based on the second-highest significant score that was closest to that of the OT generated by SPIDER.

The best evolutionary model (HKY+G) for the coalescence analyses was identified in JModeltest2 v. 2.1.6, implemented on the CIPRES platform (Miller *et al*., 2010). The Maximum Likelihood *Poisson Tree Processes* (PTP) analysis was based on a Bayesian ultrametric tree generated in MrBayes (version available at CIPRES), using the HKY substitution model, gamma rated, Markov Chain Monte Carlo (MCMC) of 10,000,000 generations, sump burn-in 9.001, with *Nannostomus marginatus*, as the outgroup.

This non-ultrametric tree was used in the analysis of the PTP model at the site https://species.h-its.org/ (Zhang *et al*., 2013), with the following parameters: rooted, 40,000 MCMC generations, with the outgroup omitted from the analysis. All other configurations were the default values. The Bayesian ultrametric input tree used for the Generalized Mixed Yule Coalescence (GMYC) method was constructed in BEAST2 (version available at CIPRES) with the following parameters: HKY+G model, gamma shape, relaxed molecular clock with lognormal distribution, and the birth-death speciation model, which was run three times independently, initiated using random trees,

each with 50 million generations, based on the Markov Chain Monte Carlo (MCMC), with 25% of the topologies being discarded as burn-in during each run. The results of the three runs were combined in LogCombiner v. 2. Effective Sample Size (ESS > 200) was verified in Tracer v. 1.6. The three files with the ".tree" extension were combined in Treeannotator v. 1.8 (available at CIPRES), visualized in FigTree v. 1.4, and exported with a NEWICK final extension. This final tree was used in the GMYC analysis, which was run in the SPLITs (SPecies LImits by Threshold Statistics; Monaghan *et al*., 2009) package in the R environment v. 3.6.3, using a single threshold model.

The definition of Consensus MOTUs was established by assessing the congruence among the previously mentioned delimitation methods, specifically through the agreement between two or more methods. However, in groups where the congruence between the analyses was not observed, we opted to employ the Optimal Threshold in the formation.

RESULTS

A total of 46 COI sequences were obtained for analysis, including 34 sequences obtained for the present study, and 12 retrieved from the BOLD systems database. Following edition and alignment, the sequences were 628 base pairs long, of which 127 were variable sites. No insertions, deletions or stop codons were observed, which indicate that the fragments used in the analysis were of adequate quality. The Index of nucleotide substitution (Iss) also indicated the lack of base saturation, given that the observed value ($R^2 = 0.1168$) was lower than Iss.c ($R^2 = 0.7358$), which also supported the quality of the data for the species delimitation analyses.

The Bayesian Inference analysis grouped all the representatives of the *Pyrrhulina australis* species group in a monophyletic clade, sister to ((*P. filamentosa*, *P. spilota*) *P. obermulleri*)). The integrated analysis based on the different species delimitation approaches recognized six, well-supported (> 95%) monophyletic lineages within the *P. australis* species group (Fig. 3), with a mean intraspecific distance within the nominal species of 3.74%. These findings indicate that *Pyrrhulina australis* is a species complex with five independent lineages: MOTU 1 – *P. australis* + *P.* cf. *rachoviana* (Lower Paraná and Paraguay rivers); MOTU 2 – *Pyrrhulina* aff. *australis* I (Araguaia River); MOTU 4 – *P.* aff. *australis* II (Paraguay River); MOTU 5 – *P.* aff. *australis* III (Upper Paraná River), and MOTU 6 – *Pyrrhulina* aff. *australis* IV (Guaporé River). The other MOTUs (Fig. 3) correspond to *Pyrrhulina marilynae* (MOTU 3 – Teles Pires River), *P. obermulleri* (MOTU 7 – Madeira River), *P. spilota* (MOTU 8 – Mómon River), and *P. filamentosa* (MOTU 9 – Paloemeu River).

In the genetic distance analyses, the OT approach indicated the existence of seven MOTUs, whereas the ASAP indicated nine. The OT was 0.0179 (1.79%), and grouped all the individuals identified *a priori* as *P. australis* (Paraguay and Upper Paraná basins), *P.* cf. *rachoviana* (Lower Paraná), and the *P. marilynae* morphospecies (Teles Pires River), except for specimen 99, which was collected from the Paraguay basin and identified as *P. australis*. In this analysis, MOTUs 2 (*P.* aff. *australis* I from the Araguaia River) and 6 (*P.* aff. *australis* IV from the Guaporé River) were distinct from the *P. australis* group. The ASAP, in turn, defined nine MOTUs, subdividing *P. australis* into six MOTUs: *P.*

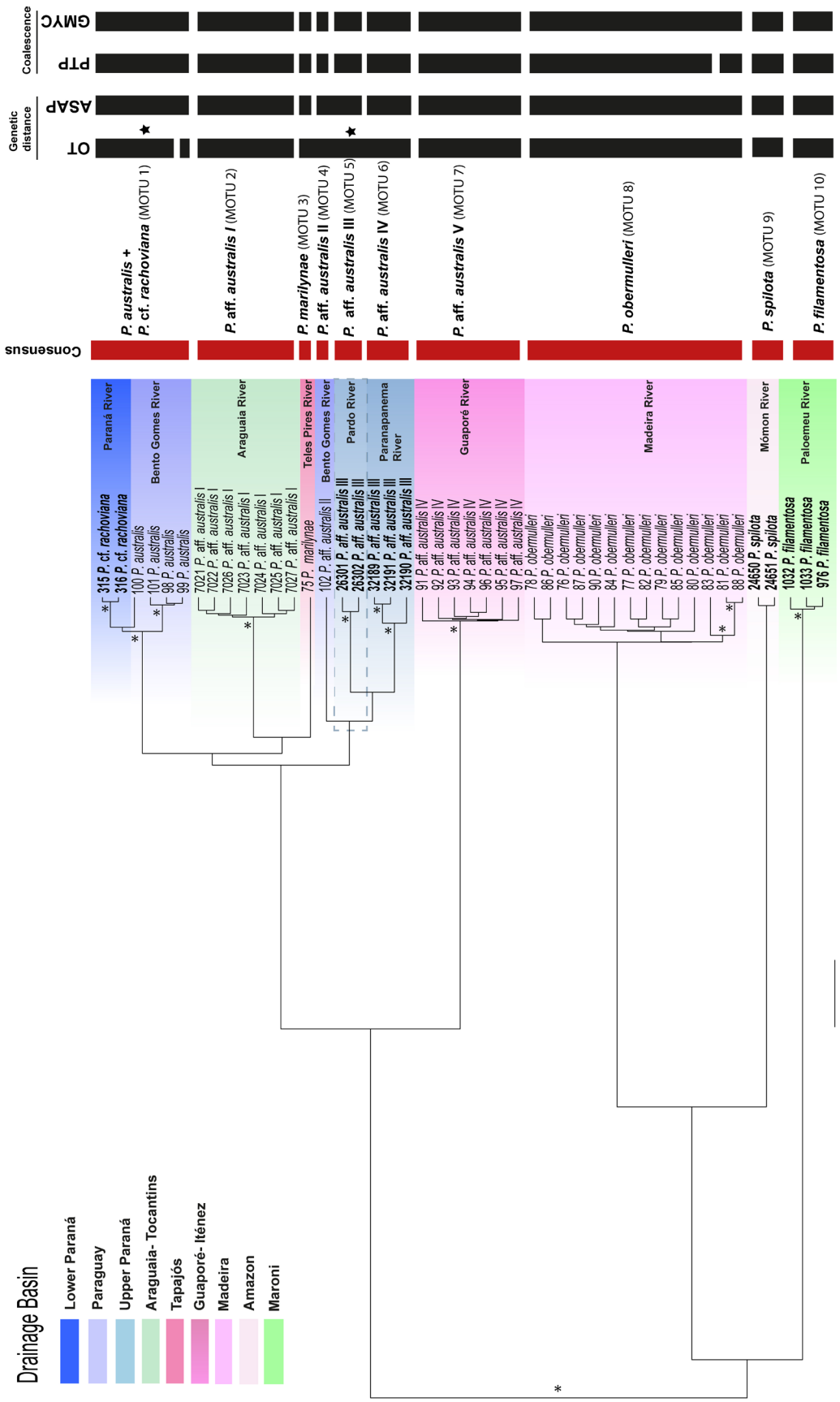

FIGURE 3 | Dendrogram of the *Pyrrhulina* species based on a Bayesisan Inference analysis of the COI sequences obtained in the present study. The red bars represent the consensus MOTUs, defined according to the congruity between the results of the species delimitation methods applied in the present study. The black bars represent the Molecular Operational Units (MOTUs) formed by the different species delimitation methods: Optimal Threshold (OT); Assemble Species by Automatic Partitioning (ASAP); Poisson Tree Processes (PTP) and Generalized Mixed Yule Coalescence (GMYC). Bars marked with a star represent the same MOTU under the OT analysis. The sequence codes in bold script indicate the samples obtained from the BOLD systems database.

australis + *P.* cf. *rachoviana* (Lower Paraná and Paraguay rivers); *P* aff. *australis* I (Araguaia River); *P. marilynae* (Teles Pires River); *P.* aff. *australis* II (Paraguay River, individual 102), *P.* aff. *australis* III (Parapanema and Pardo rivers), and *P.* aff. *australis* IV (Guaporé River).

The results of the coalescence analyses and the PTP revealed the presence of 11 MOTUs, with high levels of support for each of the groups. Individuals 81 and 88 (*P. obermulleri*) were assigned to the same MOTU, separate from the other individual of this species. The analysis also divided *P. australis* into seven MOTUs, as in the arrangement of the ASAP, albeit with three MOTUs for the individuals representing *P.* aff. *australis* II (Paraguay River), *P.* aff. *australis* III (Upper Paraná and Pardo rivers), and *P.* aff. *australis* III (Upper Paraná and Paranapanema rivers).

The arrangement defined by the GMYC analysis based on the Maximum Likelihood model (L = 322.998) was significantly (p = 0.005) different from the null model (L0 = 314.3342). While the arrangement defined by the GMYC was very similar to the configuration of the MOTUs established by the PTP, in this analysis, the nominal species *P. obermulleri* forms only a single MOTU. These analyses indicate the existence of 10 distinct entities, with a confidence interval of 10–12. However, eight of these entities are groups formed by more than one sequence, while the other two – *P. marilynae* (MOTU 3, Teles Pires River) and *P.* aff. *australis* II (MOTU 4, Paraguay River) – are singletons.

The mean intra- and interspecific genetic distances between the nominal species and the consensus MOTUs are shown in Tab. 2. The maximum intraspecific and minimum interspecific distances between the consensus MOTUs and the nominal species are shown in Tab. 3. These results are presented graphically in the plots of the genetic distances between the nominal species (Fig. 4) and the consensus MOTUs (Fig. 5).

The mean intraspecific genetic distance within the *Pyrrhulina australis* species complex

TABLE 2 | Mean K2P interspecific genetic distances obtained for the *Pyrrhulina* morphospecies or consensus MOTUs identified in the present study. The values in bold script in the diagonal are the mean within-group genetic distances (%).

Morphospecies	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1. *Pyrrhulina australis*	**3.74**				-	-	-	-	-
2. *Pyrrhulina spilota*	8.95	**0.00**			-	-	-	-	-
3. *Pyrrhulina obermulleri*	8.99	9.15	**0.00**		-	-	-	-	-
4. *Pyrrhulina filamentosa*	11.67	15.01	14.48	**0.00**	-	-	-	-	-
Consensus MOTU	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1. *Pyrrhulina* aff. *australis* IV (MOTU 6, Guaporé River)	**0.00**								
2. *Pyrrhulina spilota* (MOTU 8, Mómon River)	8.31	**0.00**							
3. *Pyrrhulina obermulleri* (MOTU 7, Madeira River)	7.88	9.16	**0.00**						
4. *Pyrrhulina australis* + *Pyrrhulina* cf. *rachoviana* (MOTU 1, Paraguay and Lower Paraná)	7.02	9.68	9.13	**0.00**					
5. *Pyrrhulina* aff. *australis* II (MOTU 4, Bento Gomes River)	5.70	8.31	8.92	1.87	**0.00**				
6. *Pyrrhulina* aff. *australis* I (MOTU 2, Araguaia River)	6.78	9.24	9.86	3.20	1.95	**0.00**			
7. *Pyrrhulina australis* III (MOTU 5, Upper Paraná)	6.27	8.81	9.19	2.53	1.42	2.71	**1.09**		
8. *Pyrrhulina marilynae* (MOTU 3, Teles Pires River)	5.35	8.54	8.92	2.17	0.96	2.28	1.88	**0.00**	
9. *Pyrrhulina filamentosa* (MOTU 9, Paloemeu River)	12.94	15.02	14.48	11.24	10.98	11.56	10.97	10.40	**0.00**

TABLE 3 | Maximum intraspecific and minimum interspecific K2P genetic distances between the *Pyrrhulina* morphospecies and consensus MOTUs identified in the present study (%).

Morphospecies	Maximum intraspecific distance (%)	Minimum interspecific distance (%)
Pyrrhulina australis	7.32	7.64
Pyrrhulina obermulleri	0.48	7.64
Pyrrhulina spilota	0.00	8.30
Pyrrhulina filamentosa	0.00	10.40
Consensus MOTU		
Pyrrhulina aff. *australis* IV (MOTU 6, Guaporé River)	0.00	5.35
Pyrrhulina spilota (MOTU 8, Mómon River)	0.00	8.31
Pyrrhulina obermulleri (MOTU 7, Madeira River)	0.00	7.64
Pyrrhulina australis + *Pyrrhulina* cf. *rachoviana* (MOTU 1, Paraguay and Lower Paraná)	0.64	1.61
Pyrrhulina aff. *australis* I (MOTU 2, Araguaia River)	0.00	1.95
Pyrrhulina aff. *australis* III (MOTU 5, Upper Paraná)	1.94	0.48
Pyrrhulina. aff. *australis* II (MOTU 4, Paraguai)	0.00	0.48
Pyrrhulina marilynae (MOTU 3, Teles Pires River)	0.00	0.96
Pyrrhulina filamentosa (MOTU 9, Paloemeu River)	0.00	10.40

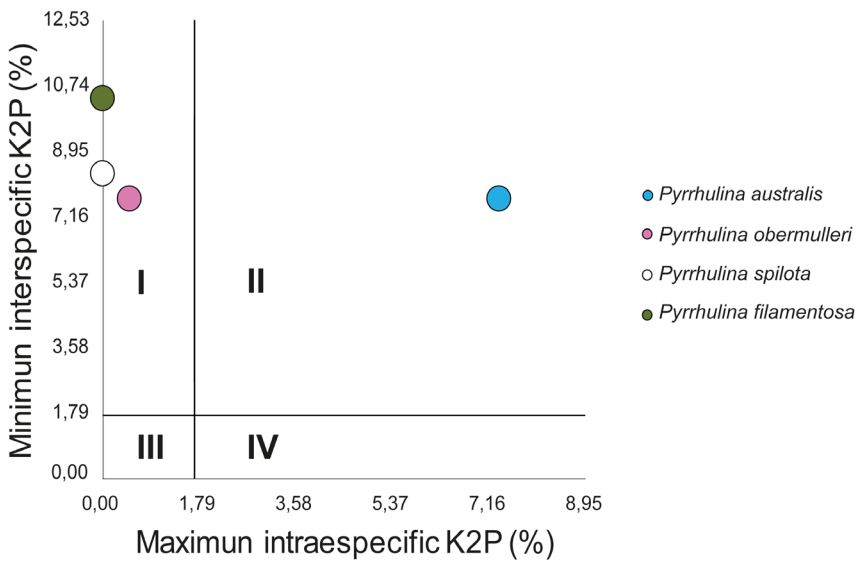

FIGURE 4 | Quadrant plot showing the maximum K2P intraspecific distances and the maximum K2P interspecific in percentages for the nominal *Pyrrhulina* species analyzed in the present study. The lines indicate the threshold (1.79%) between the intra- and interspecific distances. The morphology of the species in quadrant I is consistent with the molecular identification. The species in quadrant II probably have cryptic forms. Species present in quadrant III are likely the result of recent divergence, hybridization or synonimization, while quadrant IV represents a lack of correspondence between the morphological and molecular identifications.

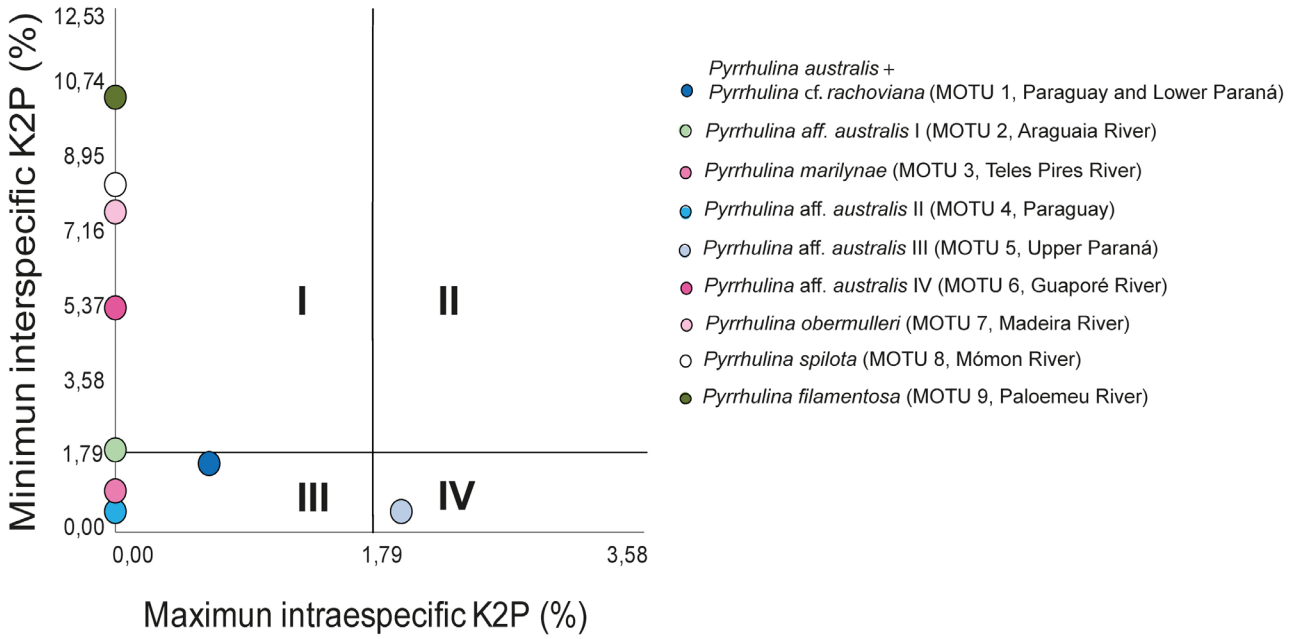

FIGURE 5 | Quadrant plot showing the maximum K2P intraspecific distances and the maximum K2P interspecific in percentages for the MOTUs of *Pyrrhulina* identified in the present study. The lines indicate the threshold (1.79%) between the intra- and interspecific distances. The morphology of the species in quadrant I is consistent with the molecular identification. The species in quadrant II probably have cryptic forms. Species present in quadrant III are likely the result of recent divergence, hybridization or synonimization, while quadrant IV represents a lack of correspondence between the morphological and molecular identifications.

is 3.74%, which is higher than the threshold of 1.79% (Tab. 2), with a maximum value of 7.32% (Tab. 3). A mean intraspecific distance of 1.09% (Tab. 2) was recorded in MOTU 5 (*P.* aff. *australis* III, Upper Paraná), with a maximum distance of 1.94% (Tab. 3). Additionally, the lowest minimum interspecific distance of 0.48% (Tab. 3) was found in MOTU 4 (*P.* aff. *australis* I, Paraguay) and MOTU 5. No significant divergence was found within the other groups of morphospecies and MOTUs.

The greatest mean distance between nominal species was 15.01%, recorded between *P. filamentosa* and *P. spilota*. The lowest mean interspecific distance was 8.95%, recorded between the *P. australis* group and *P. spilota* (Tab. 2). The lowest mean inter-MOTU genetic distance was 0.96%, observed between *P. marilynae* (MOTU 3) and *P.* aff. *australis* II (MOTU 4, Paraguay River; Tab. 2). The greatest interspecific distance between nominal species (10.40%) was observed in *P. filamentosa*, while the lowest value (7.64%) was recorded in *P. australis* and *P. obermulleri* (Tab. 3).

When the minimum interspecific and maximum intraspecific distances are compared among the morphospecies (Fig. 4), *P. australis* (*sensu lato*) is the only nominal species in quadrant II, which means that both distances are above the threshold of 1.79%, indicating the presence of cryptic species in this group. By contrast, MOTU 3 (*P. marilynae*, Teles Pires River) falls in quadrant III (Fig. 5), where both inter- and intraspecific distances are lower than 1.79%, which indicates that the species may have diverged recently. Similar conclusions apply to MOTUs 1 (*P. australis* + *P.* cf. *rachoviana*, Paraguay and Lower Paraná basins) and 2 (*P.* aff. *australis*, Paraguay River), which are also located

in quadrant III (Fig. 5). Only MOTU 5 (*P.* aff. *australis* IV – Upper Paraná basin) is found in quadrant IV, which implies that the morphological identification does not correspond to the molecular identification. Five MOTUs – 2 (*P.* aff. *australis* I, Araguaia River), 6 (*P.* aff. *australis* IV, Guaporé River), 7 (*P. obermulleri*, Madeira River), 8 (*P. spilota*, Mómon River), and 9 (*P. filamentosa*, Paloemeu River) – are located in quadrant I (Fig. 5). This indicates that the morphological and molecular analyses produced the same arrangement, given that the highest interspecific distances were over 1.79% and the lowest intraspecific distances were less than 1.79%.

DISCUSSION

The species delimitation analyses based on genetic distance (OT and ASAP) and coalescence (PTP and GMYC), together with the Bayesian phylogenetic analysis, which has well-supported clades, indicate the existence of nine distinct molecular clades in the dataset analyzed in the present study, with some variation and divergence among the results of the different delimitation methods. The congruence between these analyses is commonly employed in species delimitation based on a single locus (*e.g.*, Machado *et al*., 2018; Ramirez *et al*., 2020; Nogueira *et al*., 2021). Multiple approaches are necessary due to the computational limitations of each method commonly used for species delimitation (Carstens *et al*., 2013).

The clade composed of MOTUs 1 (*P. australis* + *P.* cf. *rachoviana*, Lower Paraná and Paraguay basins), 2 (*Pyrrhulina* aff. *australis* I, Araguaia River), 3 (*P. marilynae*, Teles Pires River), 4 (*P.* aff. *australis* II, Paraguay River), 5 (*P.* aff. *australis* III, Upper Paraná River), and 6 (*Pyrrhulina* aff. *australis* IV, Guaporé River), are distributed in six monophyletic lineages, with a maximum intraspecific distance of 7.32% within the nominal species *P. australis*, which is thus unlikely to represent a single, monophyletic species, but rather, a species complex.

The MOTU 1, which includes *P. australis* (from the Paraguay River) and *P.* cf. *rachoviana* (from the Lower Paraná basin) was a consensus arrangement in all the species delimitation methods used in the present study, which further highlights the debate on the relationship between these two species. While *P. rachoviana* was identified as a synonym of *P. australis* by Zarske, Géry (2004), these authors concluded that the type locality of *P. rachoviana* (Rosário, Argentina), was erroneously identified by Myers (1926), whereas Zarske (2016) suggested that the species did in fact originate from the Amazon basin. However, the contestation of Zarske, Géry (2001) and Zarske (2016) is not supported by any conclusive evidence, except for an inconspicuous line extending along the whole length of the body in the original description of Myers (1926). The only way to resolve this question would be to run a molecular analysis of the *P. rachoviana* type specimens and identify their closest relationship with specimens from a given drainage basin. As the type specimens of *P. rachoviana* were not available for analysis in the present study, it was decided to follow the original species description and consider Rosário in Argentina to be the type locality, contradicting more recent studies. The results of the present study do in fact indicate that the *Pyrrhulina* population from Rosário is part of the same MOTU as *P. australis*, which is consistent with the synonymization suggested by Zarske, Géry (2004) and contradicts Netto-Ferreira, Marinho (2013).

In the case of MOTU 3 (*P. marilynae*, Teles Pires River), the group is well defined by the different species delimitation methods, despite the low mean genetic distance, of 0.96% (Tab. 2), in comparison with *P.* aff. *australis* II (Paraguay River). This MOTU was well differentiated from MOTU 1 (*P. australis* + *P.* cf. *rachoviana*, Paraguay and Lower Paraná basins), however, with a mean distance of 2.17% between the two groups (Tab. 2), which is well above the OT. This further reinforces the close proximity of *P. australis* and *P. marilynae*, as demonstrated in the genomic study of Ferreira *et al.* (2022) and ratified by the findings of Moraes *et al.* (2021), who showed that the diploid chromosome number of *P. marilynae* from the Amazon basin (2n = 32) is the lowest of any *Pyrrhulina* species, given that all the others analyzed in the present study have diploid numbers of 2n = 40–42. This difference is the result of major structural chromosomal rearrangements, reinforced by the highly dynamic configuration of the repetitive DNA of these fish (Moraes *et al*., 2021). These findings contradict, once again, the synonymy between *P. rachoviana* and *P. marilynae* proposed by Zarske (2016), based on the comparison of the coloration patterns of specimens from the Lower Amazon, rather than the Lower Paraná basin. Given this, in addition to the analysis of the type specimens of *P. rachoviana* mentioned above, it would be necessary to examine specimens from the Amazonian populations described by Zarske (2016) for the definitive resolution of these taxonomic questions.

The allocation of the specimens from the Paraguay and Lower Paraná basins (*i.e*., MOTU 1 – *P. australis* and *P.* cf. *rachoviana*) in quadrant III of Fig. 5 indicates a possible recent divergence, hybridization, or synonymy, as discussed by Hebert *et al*. (2004). This allocation is supported by consistent interspecific genetic distance compared to specimens of the Upper Paraná basin (*i.e*., MOTU 5, *P.* aff. *australis* III), confirming that MOTU 1 and MOTU 5 represent distinct lineages. By contrast, the genetic distances between these individuals and those from the Upper Paraná basin (MOTU 5) reach 2.53%, which is consistent with the conclusions of Costa-Silva *et al*. (2018), *i.e*., that the historical isolation of the fish populations of the Upper Paraná basin has been upheld, with this scenario being reflected in the significant genetic distances between the different populations. It is important to note here that, while MOTU 5 (*P.* aff. *australis* IV from the Upper Paraná-Pardo and Paranapanema rivers) is located in quadrant IV (Fig. 5), this morphospecies was the only one allocated to quadrant III in Fig. 4, which indicates that the morphological identification of the specimens contradicts the molecular analysis (Hebert *et al*., 2004). This MOTU also has a mean intraspecific distance of 1.09% (Tab. 2), which may be related to the fact that this group includes individuals from the same basin, but from different rivers. In the present analysis, these groups were not considered to be distinct MOTUs due to the fact that their genetic distances were below the OT, although a more comprehensive sample of the population of the Upper Paraná basin would be necessary to provide a more conclusive interpretation of the relationships among the taxa.

This same divergence pattern has been found in the small-bodied species of the family Parodontidae from the La Plata basin, with mean genetic distances of 0.3% between the populations of the Uruguay and Paraguay basins, but 6.1% between these populations and those of the Upper Paraná basin (Bellafronte *et al*., 2013). Using the DNA barcode, Costa-Silva *et al*. (2018) compared the fish faunas of the Paraguay River and Upper Paraná basin, including *P. australis*. In the specific case of this morphospecies, the authors did not record any significant genetic distances (K2P) between the basins, although they

did conclude that it may be undergoing a process of speciation, based on the findings of the other analytical approaches employed in the study. These findings may be related to the historical context of the Upper Paraná basin, which is separated from rest of the basin by the Guaíra Falls, which formed an insurmountable barrier for almost all the local fish species. In the 1980s, the construction of the Itaipu dam at the Guaíra Falls had an enormous impact on local fish diversity, affecting both sedentary and migratory species (Agostinho *et al*., 2007; Díaz *et al*., 2016). Even so, the specimens from the Paraguay and Lower Paraná basins (MOTU 4) are not divided, given that, while they are from geographically distant areas. These areas are located within environments that are connected by the flood pulse of the Pantanal wetlands, which plays a major role in the local hydrological regime, and has a fundamental influence on the similarity of the fish faunas of the Paraguay-Paraná system (Quirós *et al*., 2007).

The divergence between the *P. australis* MOTUs may be related to the fact that small-bodied fish do not undertake major migrations, which leads to the accumulation of genetic diversity in isolated populations. This pattern of genetic divergence is typical of fish species of small size, such as *Piabina argentea* Reinhardt, 1867, in which Pereira *et al*. (2013) recorded high intraspecific genetic distances, with means of 2.0–5.6%, a finding that supported the separation of the species into at least six distinct evolutionary units. In a molecular analysis of samples of *Nannostomus eques* Steindachner, 1876 from the Amazon basin based on the mitochondrial D-Loop, Terencio *et al*. (2012) concluded that the populations were subdivided into two evolutionary units, separated by very high interspecific genetic distances, of between 5.5% and 8.3%. These findings confirm the hypothesis that the limited dispersal and geographic subdivision of the populations, which is typical of small-bodied species, facilitate the appearance of new species through geographic isolation (Terencio *et al*., 2012). This genetic diversity was also found in other *Nannostomus* species, with divergent genetic lineages being found in four of the nominal species, with a mean interspecific distance of 19% within this group, which obviously means that a threshold of 2% is inadequate for the diagnosis of this group of species (Benzaquem *et al*., 2015).

The results of the present study reinforce the importance of analytical tools that take the evolutionary history of the target groups into account for the interpretation of diversity patterns, with an Optimal Threshold (OT) of 1.79% being considered here. This OT value was below the standard limit of divergence established by Ward *et al*. (2009) for fishes, that is 2.1%, although many studies have found that an OT of less than 2% is effective for the delimitation of species (*e.g*., Bellafronte *et al*., 2013; Machado *et al*., 2017; Arruda *et al*., 2019). The mean intraspecific distance in the *Pyrrhulina australis* group (including *P. marilynae* and "*P. rachoviana*") was above this threshold (3.74%), which confirms the separation of this group into six genetic lineages. Species delimitation analyses of closely-related taxa based on COI sequences have also reported thresholds lower than the standard value, reaching less than 1% in some cases (inter-MOTU distances), which have been attributed in general to the recent divergence of the taxonomic units (Pereira *et al*., 2013; Ramirez, Galetti, 2015; Ramirez *et al*., 2017).

Previous taxonomic (Ardila-Rodríguez 1994, 1999, 2000, 2001, 2002, 2004, 2008a,b, 2010; Netto-Ferreira, 2010, 2012; Netto-Ferreira *et al*., 2011, 2013; Netto-Ferreira, Marinho, 2013; Vieira, Netto-Ferreira, 2019) and revisionary studies (Weitzman, 1966; Weitzman, Cobb, 1975; Marinho, Menezes, 2017) with lebiasinid genera have shown

the color pattern as the most relevant source of characters for recognizing species among congeners, as the aforementioned studies revealed meristic and morphometric characters to be well conserved among representatives of the family, being useful to distinguish very few species among their congeners. Likewise, within *Pyrrhulina* the pigmentation pattern is also and is commonly used in species diagnoses and identification keys (Zarske, Géry, 2004; Netto-Ferreira, Marinho, 2013; Marinho, Netto-Ferreira, 2014; Vieira, Netto-Ferreira, 2019). Although the external morphological examination of the representatives of the MOTUS, agreed with the characters proposed by Netto-Ferreira, Marinho (2013) for the *Pyrrhulina australis* species group, no conspicuous anatomical character allowed their prompt recognition, reinforcing the difficulty in obtaining unique diagnostic characters distinguishing closely related species. Instead, the color pattern variation among each MOTUs confirmed the importance of that source of characters for the systematics of the genus. The morphospecies *P.* aff. *australis* I (Araguaia River, Fig. 2A) differs from *P. australis* (Paraguay basin, Fig. 2E) by presenting reddish coloration on the dorsal and anal fins, as well as presenting a discrete, straight, slender stripe passing onto scales of lateral series 3 and 4, bordered by clearer stripes dorsal- and ventrally. *Pyrrhulina marilynae* (Teles Pires River, Fig. 2D) differs from *P. australis* (Paraguay River, Fig. 2E) by having a conspicuous dark stripe along the body, a characteristic pattern described for *P. marilynae*, and *Pyrrhulina zigzag* Zarske & Géry, 1997 (Zarske, Géry, 2004; Netto-Ferreira, Marinho, 2013; Vieira, Netto-Ferreira, 2019). Specimens of *P.* aff. *australis* IV (Fig. 2 D), present a dark blotch on the caudal fin that extends to the tip of the median caudal-fin rays, a pattern also observed in *P. marilynae* (Netto-Ferreira, Marinho, 2013) and other *Pyrrhulina*, not included in the present contribution, collected in the Amazon basin (Zarske, Géry, 2004). The specimens attributable to *P. rachoviana* (*sensu* Myers) and *Pyrrhulina* aff. *australis* IV also exhibit a distinct checkerboard pattern along the body (Figs. 2B–D), absent in the morphospecies from the Araguaia-Tocantins and Paraná-Paraguay basins (Figs. 2A, E). Besides the lack of the characters defining the *P. australis* species group (*sensu* Netto-Ferreira, Marinho, 2013), *P. obermulleri* can be distinguished from the members of that clade by the primary stripe extending posteriorly to the pectoral girdle; *P. filamentosa* differs from that species group by having a long, streamlined body, with the largest scale count among *Pyrrhulina* (25–28 scales on the lateral line series); and *P. spilota* can be readily recognized by the presence of a series of dark blotches on the flanks and anal-fin rays.

The MOTUs of *P. obermulleri*, *P. spilota*, and *P. filamentosa* were well-defined by the different species delimitation methods employed in the present study. These species are distributed allopatrically across the drainages of the Amazon and Maroni basins. *P. filamentosa* (Maroni basin), exhibits a high inter-specific distance of 15.01% from *P. spilota* (Amazon basin) and 14.48% from *P. obermulleri* (Madeira basin). The evolution of Neotropical ichthyofauna is closely linked to the evolutionary history of drainage basins (Reis *et al*., 2016), and the Maroni basin, situated in the Guiana Shield, represents an endemic zone with distinct geological formation compared to the Brazilian Shield and lowlands (Lundberg *et al*., 1998; Albert *et al*., 2011). These patterns favor allopatric speciation due to the interruption of gene flow between geographically separated populations, associated with limited dispersal capabilities of these species (Albert *et al*., 2011, 2020; Bernardi, 2013). Similar patterns of allopatric speciation were observed in the geographical distributions of MOTUs 1to 6 within the *P. australis* complex, across

the Paraná-Paraguay, Guaporé, Tapajós, and Araguaia-Tocantins river basins. These basins have a complex history of geographical, and the evolution of ichthyofauna has been influenced by vicariance events between the Amazonia and Paraguay basins approximately 30 Ma (Lundberg *et al*., 1998), as well as events involving the dispersal of Amazonian species into the Paraguay basin during the last 10 Ma (Lundberg *et al*., 1998; Carvalho, Albert, 2011). These events have contributed to the sharing of fauna among the different river basins (Carvalho, Albert, 2011; Dagosta, de Pinna, 2019). However, the gene tree was not capable, on its own, of testing adequately the phylogenetic relationships among the *Pyrrhulina* species, it is necessary to integrate the use of multiple molecular markers and morphological data (Oliveira *et al*., 2011; Roxo *et al*., 2017; Ramirez *et al*., 2020), which will demand the application of a more comprehensive approach for the definitive diagnosis of the relationships among the *Pyrrhulina* species.

Overall, then, the results of the analyses presented here indicate an unexpected level of diversity within the *Pyrrhulina australis* morphospecies, which contrasts with the findings of the previous studies of the diversity of this species. The "*P. australis*" morphospecies (*sensu lato*) was subdivided into six evolutionary lineages related systematically with the geographic distribution of the populations in the different drainage basins, and were well diagnosed, forming a clade that encompasses the *Pyrrhulina australis* + *P.* cf. *rachoviana*, *P.* aff. *australis* I/II/III/IV, and *P. marilynae* morphospecies. In this context, it would be most parsimonious to conclude that the geographic subdivisions of the populations are favoring speciation processes in this genus, including the detection of a possible species complex centered on the nominal species *P. australis*. In the specific case of the species identified as *P. australis* and *P.* aff. *australis*, previous studies have shown that, while they are highly similar in morphological terms and both have the same diploid chromosome number of 2n = 40, they do present divergences in certain classes of repetitive DNA, and can be considered to be two distinct evolutionary units of *P. australis* (Moraes *et al*., 2017).

Despite the results obtained in the present study, a definitive conclusion regarding the proposed synonymy of *P. australis* and *P. rachoviana* could not be provided, as we did not have access to the sequences of the type specimens of both species. The objective recognition of the relationships of the namebearing types with the MOTUs recognized herein are a necessary step to determine which of the lineages will be described as new species, helping to further restrict the confusion on the taxonomy of the genus. Considering that the plasticity of the aforementioned morphological/coloration features allowing the recognition of the morphospecies and would permit diagnosing the undescribed species, were not examined in detail, no nomenclatural act was taken on this occasion, and will be the subject of future investigation. The integration of new molecular markers, both mitochondrial and nuclear, is also necessary to better comprehend the evolutionary relationships within the group (Oliveira *et al*., 2011; Ramirez *et al*., 2020). Even so, the present study has made important advances in the understanding of the specific limits of the genus *Pyrrhulina*, and provides important insights that should help to resolve the taxonomic uncertainties of this fish group.

ACKNOWLEDGMENTS

Thanks are due to Dr. Carolina B. Machado (UFSC) for support during the analyses employed herein. Authors were financially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq process 421733/2017–9 INAU II and INPP, 446925/2014–43 to PCV; process 130650/2019–6 to TBS and 13834/2021–0 to ALNF), Fapemat Universal (Fundação de Amparo à Pesquisa do Mato Grosso; process 0336943/2017 to DCF). FAPERGS (Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul; call 14/2022 ARD/ARC to ALNF).

REFERENCES

- **Agostinho AA, Pelicice FM, Petry AC, Gomes LC, Júlio Jr. HF.** Fish diversity in the upper Paraná River basin: Habitats, fisheries, management and conservation. Aquat Ecosyst Health Manage. 2007; 10(2):174–86. https://doi.org/10.1080/14634980701341719
- **Albert JS, Reis RE.** Historical biogeography of Neotropical freshwater fishes. Berkeley, Los Angeles, London: University of California Press; 2011.
- **Albert JS, Tagliacollo VA, Dagosta F.** Diversification of Neotropical freshwater fishes. Annu Rev Ecol Evol Syst. 2020; 51:27–53. https://doi.org/10.1146/annurev-ecolsys-011620-031032
- **Aljanabi SM, Martinez I.** Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Res. 1997; 25(22):4692–93. https://doi.org/10.1093/nar/25.22.4692
- **Ardila-Rodríguez CA.** *Lebiasina floridablancaensis*, una nueva especie de pez para Colombia (Telesotei: Characiformes: Lebiasinidae). Rev Unimetro. 1994; 10(19):1–08.
- **Ardila-Rodríguez CA.** *Lebiasina provenzanoi*, una nueva especie de pez para Venezuela (Teleostei: Characiformes: Lebiasinidae). Rev Unimetro. 1999; 13(25–26):1–12.
- **Ardila-Rodríguez CA.** *Lebiasina yuruaniensis*, una nueva especie de pez para Venezuela (Teleostei: Characiformes: Lebiasinidae). Rev Unimetro. 2000; 12–13(25–26):1–16.
- **Ardila-Rodríguez CA.** *Lebiasina chucuriensis*, una nueva especie de pez para Colombia (Teleostei: Characiformes: Lebiasinidae). Rev Unimetro. 2001; 13(27–28):1–20.
- **Ardila-Rodríguez CA.** *Lebiasina nariñensis*, una nueva especie de pez para Colombia (Teleostei: Characiformes, Lebiasinidae). Dahlia. 2002; 5:11–18.
- **Ardila-Rodríguez CA.** *Lebiasina taphorni* (Pisces: Characiformes, Lebiasinidae), una nueva especie. Dahlia. 2004; 7:57–65.
- **Ardila-Rodríguez CA.** *Lebiasina colombia* (Characiformes: Lebiasinidae), nueva especie cuenca del Río Sinú, Colombia. Dahlia. 2008a; 10:27–32. Available from: https://carlosardilarodriguez.files.wordpress.com/2013/07/lebiasina-colombia.pdf
- **Ardila-Rodríguez CA.** *Lebiasina ortegai* (Characiformes: Lebiasinidae), nueva especie, sistema del Río Cauca, Colombia. Dahlia. 2008b; 10:17–25. Available from: https://carlosardilarodriguez.files.wordpress.com/2013/07/lebiasina-ortegai.pdf
- **Ardila-Rodríguez CA.** *Lebiasina chocoensis*, una nueva especie de pez para Colombia (Teleostei: Characiformes: Lebiasinidae: Lebiasininae). Peces Dep Chocó. 2010; 1:1–20.
- **Arruda PSS, Ferreira DC, Oliveira C, Venere PC.** DNA barcoding reveals high levels of divergence among mitochondrial lineages of *Brycon* (Characiformes, Bryconidae). Genes. 2019; 10(9):639. https://doi.org/10.3390/genes10090639
- **Bellafronte E, Mariguela TC, Pereira LHG, Oliveira C, Moreira-Filho O.** DNA barcode of Parodontidae species from the La Plata River basin - applying new data to clarify taxonomic problems. Neotrop Ichthyol. 2013; 11(3):497–506. https://doi.org/10.1590/S1679-62252013000300003

- **Benzaquem DC, Oliveira C, Batista JS, Zuanon J, Porto JIR.** DNA Barcoding in Pencilfishes (Lebiasinidae: *Nannostomus*) reveals cryptic diversity across the Brazilian Amazon. PLoS ONE. 2015; 10(2):e0112217. https://doi.org/10.1371/journal.pone.0112217
- **Bernardi G.** Speciation in fishes. Mol Ecol. 2013; 22(22):5487–502. https://doi.org/10.1111/mec.12494
- **Bertaco VA, Ferrer J, Carvalho FR, Malabarba LR.** Inventory of the freshwater fishes from a densely collected area in South America- a case study of the current knowledge of the Neotropical fish diversity. Zootaxa. 2016; 4138(3):401–40. http://doi.org/10.11646/zootaxa.4138.3.1
- **Carstens BC, Pelletier TA, Reid NM, Satler JD.** How to fail at species delimitation. Mol Ecol. 2013; 22(17):4369–83. https://doi.org/10.1111/mec.12413
- **Carvalho TP, Albert JS.** The Amazon-Paraguay divide. In: Albert JS, Reis RE, editors. Historical biogeography of Neotropical freshwater fishes. Berkeley, Los Angeles, London: University of California Press; 2011. p.119–36.
- **Costa-Silva GJ, Ashikaga FY, Dias CKS, Pereira LHG, Foresti F, Oliveira C.** DNA barcoding techniques used to identify the shared ichthyofauna between the Pantanal floodplain and Upper Paraná River. Mitochondrial DNA Part A. 2018; 29(7):1063–72. https://doi.org/10.1080/24701394.2017.1404046
- **Dagosta FCP, de Pinna M.** The fishes of the Amazon: Distribution and biogeographical patterns, with a comprehensive list of species. Bull Am Mus Nat Hist. 2019; (431):1–163. https://doi.org/10.1206/0003-0090.431.1.1
- **Díaz J, Villanova GV, Brancolini F, Pazo F, Posner VM, Grimberg A *et al*.** First DNA barcode reference library for the identification of South American freshwater fish from the lower Paraná River. PLoS ONE. 2016; 11(7):e0157419. https://doi.org/10.1371/journal.pone.0157419
- **Ferreira PHN, Souza FHS, Moraes RL, Perez MF, Sassi FMC, Viana PF *et al*.** The genetic differentiation of *Pyrrhulina* (Teleostei, Characiformes) species is likely influenced by both geographical distribution and chromosomal rearrangements. Front Genet. 2022; 13:869073. https://doi.org/10.3389/fgene.2022.869073
- **Fricke R, Eschmeyer WN, Van der Laan R.** Eschmeyer's catalog of fishes: genera, species, references [Internet]. San Francisco: California Academy of Science; 2023. Available from: http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp
- **Géry J.** Characoids of the World. TFH Publications, Neptune; 1977.
- **Hebert PDN, Ratnasingham S, Waard JR.** Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc B. 2003; 270:96–99. https://doi.org/10.1098/rsbl.2003.0025
- **Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM.** Identification of birds through DNA barcodes. PLoS Biol. 2004; 2(10):e312. https://doi.org/10.1371/journal.pbio.0020312
- **Jones M, Ghoorah A, Blaxter M.** JMOTU and taxonerator: Turning DNA barcode sequences into annotated operational taxonomic units. PLoS ONE. 2011; 6(4):e19259. https://doi.org/10.1371/journal.pone.0019259
- **Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S *et al*.** Geneious basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012; 28(12):1647–49. https://doi.org/10.1093/bioinformatics/bts199
- **Kimura M.** A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980; 16(2):111–20. https://doi.org/10.1007/bf01731581
- **Machado CB, Ishizuka TK, Freitas PD, Valiati VH, Galetti Jr. PM.** DNA barcoding reveals taxonomic uncertainty in *Salminus* (Characiformes). Syst Biodivers. 2017; 15(4):372–82. https://doi.org/10.1080/14772000.2016.1254390
- **Machado VN, Collins RA, Ota RP, Andrade MC, Farias IP, Hrbek T.** One thousand DNA barcodes of piranhas and pacus reveal geographic structure and unrecognised diversity in the Amazon. Sci Rep. 2018; 8:8387. https://doi.org/10.1038/s41598-018-26550-x

- **Malabarba LR, Malabarba MC.** Phylogeny and classification of Neotropical fish. In: Baldisserotto B, Urbinati EC, Cyrino JEP, editors. Biology and physiology of freshwater Neotropical Fish. Academic Press: Massachusetts; 2020. p.1–17. https://doi.org/10.1016/B978-0-12-815872-2.00001-4
- **Manel S, Guerin PE, Mouillot D, Blanchet S, Velez L, Albouy C *et al*.** Global determinants of freshwater and marine fish genetic diversity. Nat Commun. 2020; 11(692). https://doi.org/10.1038/s41467-020-14409-7
- **Melo BF, Sidlauskas BL, Near TJ, Roxo FF, Ghezelayagh A, Ochoa LE *et al*.** Accelerated diversification explains the exceptional species richness of tropical Characoid fishes. Syst Biol. 2022; 71(1):78–92. https://doi.org/10.1093/sysbio/syab040
- **Miller MA, Pfeiffer W, Schwartz T.** Creating the CIPRES Science Gateway for inference of large phylogenetic trees [Internet]. New Orleans: Proceedings of the Gateway Computing Environments Workshop; 2010.
- **Monaghan MT, Wild R, Elliot M, Fujisawa T, Balke M, Inward DJG *et al*.** Accelerated species inventory on Madagascar using coalescent-based models of species delineation. Syst Biol. 2009; 58(3):298–311. https://doi.org/10.1093/sysbio/syp027
- **Moraes RLR, Bertollo LAC, Marinho MMF, Yano CF, Hatanaka T, Barby FF *et al*.** Evolutionary relationships and cytotaxonomy considerations in the genus *Pyrrhulina* (Characiformes, Lebiasinidae). Zebrafish. 2017; 14(6):536–46. https://doi.org/10.1089/zeb.2017.1465
- **Moraes RLR, Sassi FMC, Bertollo LAC, Marinho MMF, Viana PF, Feldberg E *et al*.** Tracking the evolutionary trends among small-size fishes of the genus *Pyrrhulina* (Characiforme, Lebiasinidae): New insights from a molecular cytogenetic Perspective. Front Genet. 2021; 12:769984. https://doi.org/10.3389/fgene.2021.769984
- **Myers GS.** Eine neue südamerikanische Characinidenart der Gattung *Pyrrhulina*. Blätter Aquar Terrarkde. 1926; 37(18):441–42.
- **Netto-Ferreira AL.** Revisão taxonômica e relações interespecíficas de Lebiasininae (Ostariophysi: Characiformes: Lebiasinidae). [PhD Thesis]. São Paulo: Universidade de São Paulo; 2010. Available from: https://doi.org/10.11606/T.41.2010.tde-02022011- 165808
- **Netto-Ferreira AL.** Three new species of *Lebiasina* (Characiformes: Lebiasinidae) from the Brazilian shield border at Serra do Cachimbo, Pará, Brazil. Neotrop Ichthyol. 2012; 10(3):487–98. https://doi.org/10.1590/S1679-62252012000300002
- **Netto-Ferreira AL, Marinho MMF.** New species of *Pyrrhulina* (Ostariophysi: Characiformes: Lebiasinidae) from the Brazilian shield, with comments on a putative monophyletic group of species in the genus. Zootaxa. 2013; 3664(3):369–76. https://doi.org/10.11646/zootaxa.3664.3.7
- **Netto-Ferreira AL, Oyakawa OT, Zuanon J, Nolasco JC.** *Lebiasina yepezi*, a new Lebiasininae (Characiformes: Lebiasinidae) from the Serra Parima-Tapirapecó mountains. Neotrop Ichthyol. 2011; 9(4):767–75. https://doi.org/10.1590/S1679-62252011000400008
- **Netto-Ferreira AL, Lopez-Fernandez H, Taphorn DC, Liverpool EA.** New species of *Lebiasina* (Ostariophysi: Characiformes: Lebiasinidae) from the upper Mazaruni River drainage, Guyana. Zootaxa. 2013; 3652(5):562–68. https://doi.org/10.11646/zootaxa.3652.5.5
- **Nogueira AF, Oliveira C, Langeani F, Netto-Ferreira AL.** Molecular species delimitation of the genera *Anodus*, *Argonectes*, *Bivibranchia* and *Micromischodus* (Ostariophysi: Characiformes). Neotrop Ichthyol. 2021; 19(4):e210005. https://doi.org/10.1590/1982-0224-2021-0005
- **Oliveira C, Avelino GS, Abe KT, Mariguela TC, Benine RC, Ortí G *et al*.** Phylogenetic relationships within the speciose family Characidae (Teleostei: Ostariophysi: Characiformes) based on multilocus analysis and extensive ingroup sampling. BMC Evol Biol. 2011; 11(275). https://doi.org/10.1186/1471-2148-11-275
- **Pereira LHG, Hanner R, Foresti F, Oliveira C.** Can DNA barcoding accurately discriminate megadiverse Neotropical freshwater fish fauna? BMC Genet. 2013; 14(20). https://doi.org/10.1186/1471-2156-14-20
- **Puillandre N, Brouillet S, Achaz G.** ASAP: Assemble Species by Automatic Partitioning. Mol Ecol Resour. 2021; 21(2):609–20. https://doi.org/10.1111/1755-0998.13281

- **Quirós R, Bechara JA, Resende EK.** Fish diversity and ecology, habitats and fisheries for the un-dammed riverine axis Paraguay-Parana-Rio de la Plata (Southern South America). Aquat Ecosyst Health Manage. 2007; 10(2):187–200. https://doi.org/10.1080/14634980701354761
- **R Development Core Team.** R: A language and environment for statistical computing [Internet]. Vienna: R Foundation for Statistical Computing; 2016. Available from: http://www.R-project.org/
- **Ramirez JL, Birindelli JL, Carvalho DC, Affonso PRAM, Venere PC, Ortega H *et al*.** Revealing hidden diversity of the underestimated Neotropical ichthyofauna: DNA barcoding in the recently described genus *Megaleporinus* (Characiformes: Anostomidae). Front Genet. 2017; 8:149. https://doi.org/10.3389/fgene.2017.00149
- **Ramirez JL, Galetti Jr. PM.** DNA barcode and evolutionary relationship within *Laemolyta* Cope 1872 (Characiformes: Anostomidae) through molecular analyses. Mol Phylogenet Evol. 2015; 93:77–82. https://doi.org/10.1016/j.ympev.2015.07.021
- **Ramirez JL, Santos CA, Machado CB, Oliveira AK, Garavello JC, Britski HA *et al*.** Molecular phylogeny and species delimitation of the genus *Schizodon* (Characiformes, Anostomidae). Mol Phylogenet Evol. 2020; 153:106959. https://doi.org/10.1016/j.ympev.2020.106959
- **Tamura K, Stecher G, Kumar S.** MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol. 2021; 38(7):3022–27. https://doi.org/10.1093/molbev/msab120
- **Terencio ML, Schneider CH, Porto JIR.** Molecular signature of the D-loop in the brown pencilfish *Nannostomus eques* (Characiformes, Lebiasinidae) reveals at least two evolutionary units in the Rio Negro basin, Brazil. J Fish Biol. 2012; 81(1):110–24. https://doi.org/10.1111/j.1095-8649.2012.03320.x
- **Thompson JD, Gibson TJ, Higgins DG.** Multiple sequence alignment using clustalW and clustalX. Curr Protoc Bioinf. 2003; (1):2–3. https://doi.org/10.1002/0471250953.bi0203s00
- **Venere PC, Garutti V.** Peixes do Cerrado: Parque Estadual da Serra Azul, rio Araguaia, MT. RiMa Editora, São Carlos: Fapemat; 2011.
- **Vieira LS, Netto-Ferreira AL.** New species of *Pyrrhulina* (Teleostei: Characiformes: Lebiasinidae) from the eastern Amazon, Pará, Brazil. Neotrop Ichthyol. 2019; 17(2):e190013. https://doi.org/10.1590/1982-0224-20190013
- **Ward RD.** DNA barcode divergence among species and genera of birds and fishes. Mol Ecol Resour. 2009; 9(4):1077–85. https://doi.org/10.1111/j.1755-0998.2009.02541.x
- **Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN.** DNA barcoding Australia's fish species. Phil Trans R Soc. B. 2005; 360:1847–57. http://doi.org/10.1098/rstb.2005.1716
- **Weitzman M, Weitzman SH.** Family Lebiasinidae. In: Reis RE, Kullander SO, Ferraris Jr. CJ, editors. Check list of the freshwater fishes of South and Central America. Porto Alegre: Edipucrs; 2003. p.241–51.
- **Weitzman SH.** Review of South American characid fishes of the subtribe Nannostomina. Proc U S Natl Mus.1966; 119(3538):1–56.
- **Weitzman SH, Cobb JS.** A revision of the South American fishes of the genus *Nannostomus* Günther (Family Lebiasinidae). Smithson Contrib Zool; 1975; (186):1–36. https://doi.org/10.5479/si.00810282.186
- **Xia X, Xie Z, Salemi M, Chen L, Wang Y.** An index of substitution saturation and its application. Mol Phylogenet Evol. 2003; 26(1):1–07. https://doi.org/10.1016/S1055-7903(02)00326-3
- **Zarske A, Géry J.** Zur Variabilität von *Pyrrhulina australis* Eigenmann & Kennedy, 1903 (Teleostei: Characiformes: Lebiasinidae). Zoologische Abhandlungen (Dresden). 2004; 64:39–54.
- **Zarske A.** Herkunft und Status von *Pyrrhulina rachoviana* Myers, 1926 (Teleostei, Characiformes, Lebiasinidae). Bull Fish Biol. 2016; 16(1–2):61–73.
- **Zhang J, Kapli P, Pavlidis P, Stamatakis A.** A general species delimitation method with applications to phylogenetic placements. Bioinformatics. 2013; 29(22):2869–76. https://doi.org/10.1093/bioinformatics/btt499

AUTHORS' CONTRIBUTION

Taina Barbosa de Souza: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing-original draft, Writing-review and editing.
Daniela Cristina Ferreira: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Visualization, Writing-review and editing.
Hugmar Pains da Silva: Conceptualization, Data curation, Methodology, Visualization, Writing-review and editing.
André Luiz Netto-Ferreira: Conceptualization, Data curation, Visualization, Writing-review and editing.
Paulo Cesar Venere: Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Visualization, Writing-review and editing.

ETHICAL STATEMENT

The fish sampling was supported by the license number 15226–2, provided by the Instituto Chico Mendes de Conservação de Biodiversidade (ICMBio), through the Sistema de Autorização e Informação em Biodiversidade (SISBIO).

COMPETING INTERESTS

The authors declare no competing interests.

HOW TO CITE THIS ARTICLE

- **Souza TB, Ferreira DC, Silva HP, Netto-Ferreira AL, Venere PC.** DNA Barcoding of *Pyrrhulina australis* (Characiformes: Lebiasinidae) reveals unexpected cryptic diversity in the group. Neotrop Ichthyol. 2023; 21(4):e230037. https://doi.org/10.1590/1982-0224-2023-0037

Neotropical Ichthyology

OPEN ACCESS

SBI
Official Journal of the
Sociedade Brasileira de Ictiologia