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Integrative diagnosis and review of the conservation status of a Near Threatened species of the "*Psalidodon scabripinnis* complex"

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The integration between morphological and barcode datasets allowed the discovery of new populations of *Psalidodon cremnobates*, expanding the distribution of this species to the Mampituba River drainage, municipality of Cambará do Sul, Rio Grande do Sul, Brazil and letting a review of the classification of extinction risk. DNA barcoding for these populations and other of the "*Psalidodon scabripinnis* complex" living in swift currents further supports the recognition of *Psalidodon brachypterygium* (new combination), *P. cremnobates* and *P. laticeps* as distinctive species, regardless of the lack of discrete and diagnosable ranges in body counts and measurements among them. Barcode DNA, in this sense, helps in recognizing monophyletic lineages and determining the boundaries between species with similar morphologies. The barcode divergence recommended to recognize species is also discussed.

Keywords: Barcode, Biodiversity, Headwater streams, *Psalidodon*, Taxonomy.

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A integração entre conjuntos de dados morfológicos e de código de barras permitiu a descoberta de novas populações de *Psalidodon cremnobates*, ampliando a distribuição desta espécie para a bacia do rio Mampituba, município de Cambará do Sul, Rio Grande do Sul, Brasil, com a revisão da classificação de seu risco de extinção. O código de barras de DNA para essas populações e outras populações do "complexo *Psalidodon scabripinnis*" que vivem em correntes rápidas corrobora o reconhecimento de *Psalidodon brachypterygium* (nova combinação), *P. cremnobates* e *P. laticeps* como espécies distintas, independentemente da falta de intervalos discretos e diagnósticos em dados morfométricos e merísticos entre elas. O DNA do código de barras, nesse sentido, auxilia no reconhecimento de linhagens monofiléticas e na determinação dos limites entre espécies com morfologias semelhantes. A divergência do código de barras recomendada para reconhecimento de espécies também é discutida.

Palavras-chave: Biodiversidade, Código de barras, *Psalidodon*, Riachos de cabeceira, Taxonomia.

INTRODUCTION

Low ray count in the anal fin, massive head, elongated body, deeper, and heavier in the middle of the pectorals is a body shape common to species that live in fast currents (Bertaco, Malabarba, 2001). Based on the difficulty of searching for characters to diagnose characid species that present this generalized pattern, Bertaco, Lucena (2006) gather and list fifteen of them as part of the so-called "*Psalidodon scabripinnis* species complex". This list includes three species of characids from southern Brazil: *Psalidodon laticeps* (Cope, 1894) from the coastal river drainages of Uruguay and Brazil to the south of the State of Paraná, and from the Uruguai River drainage (Bertaco, Lucena, 2010); *Astyanax brachypterygium* Bertaco & Malabarba, 2001, from the upper tributaries of the Jacuí River and Uruguai River drainages and *Psalidodon cremnobates* (Bertaco & Malabarba, 2001), from the upper tributaries of the Jacuí River and Maquiné River drainages.

As is usual in these *Astyanax*-like forms that live in swift currents, the three species are not diagnosable among themselves by discrete variations in fin ray or scale counts, or even by discrete variations in body proportions. *Psalidodon laticeps* was diagnosed mainly by the oval-shaped and horizontally elongated humeral spot, rather than the vertically elongated humeral spot as observed in the other two species (Bertaco, Lucena, 2010). *Astyanax brachypterygium* and *Psalidodon cremnobates*, however, were diagnosed from each other (Bertaco, Malabarba, 2001) by overlapping but statistically significant differences in anal fin ray counts (iii–v, 12–16, median = 14 in *A. brachypterygium*, *versus* iii–v, 14–18, median = 16 in *P. cremnobates*), proportions of orbital diameter and interorbital width in relation to head length and proportion of caudal peduncle depth in relation to standard length.

Here we investigated the identity of a population of characid fish that inhabit rapids in the Mampituba River drainage, which has a low anal fin ray count and a body shape typical of *Astyanax*-like forms that live in fast-flowing streams. The Mampituba is a coastal river that flows directly into the South Atlantic Ocean and is located on the border of the states of Rio Grande do Sul and Santa Catarina (SEMA, 2024). According to an analysis of the DPSIR socio-economic and environmental indicators matrix (Driving Force, Pressure, State, Impact and Response), Mampituba River drainage is classified in a regular situation (Porto *el al*., 2019). The main anthropic activities on the basis are agriculture, silviculture, and livestock farming, covering an area of 22.4%, 3.0% and 40.0% of the drainage, respectively (Porto *el al*., 2019). This drainage, together with the Tramandaí and Araranguá river drainages, make up one of the world's freshwater ecoregions proposed by Abell *et al*. (2008) based on the distribution and endemism of fish species.

The use of COI sequences has been proved to be efficient to recognize species among more than 70 species previously referred to *Astyanax* Baird & Girard, 1854 (including species now referred to *Psalidodon* Eigenmann, 1911), with 12% of fail in the dataset considered (Rossini *et al*., 2016). The analysis of mini barcode sequences of COI combined with morphological data have also allowed to recover the identity of early described species based on historical DNA techniques of long preserved type-specimens as exemplified in *Tetragonopterus taeniatus* Jenyns, 1842 (currently *Deuterodon taeniatus*) by Silva *et al*. (2019). The resurrection of some species of the *Astyanax bimaculatus* group that are currently in the synonym of *A. lacustris* Lütken, 1875 has been recently suggested by Gavazonni *et al*. (2024) using integrative taxonomy considering barcode DNA and morphology.

In order to address the lack of discrete morphological data to adequately identify the species found in the Mampituba River drainage, we provide and test the efficiency of cytochrome c oxidase subunit 1 (COI) gene sequences to identify this population, as well as to diagnose the three species of the so called "*Psalidodon scabripinnis* complex" (*A. brachypterygium*, *P. cremnobates* and *P. laticeps*) that occurs in southern Brazil.

MATERIAL AND METHODS

Morphological analyses. Specimens were counted and measured following Bertaco, Malabarba (2001). In order to standardize the data and avoid the influence of measurements taken by different people, the measurements of the the specimens under analysis from the Mampituba River drainage were taken by the same person (VAB) that measured the type-specimens of *Psalidodon cremnobates* and *Astyanax brachypterygium*. Institutional codes follow Sabaj (2023).

Meristic data that vary among species or populations compared herein do not have a normal distribution, which is expected for meristic data taken from fish populations. In this case we chose the Kruskal-Wallis analysis to test the null hypothesis that population samples are equal for two meristic data (branched anal-fin ray and lateral line scales). If this test indicated a significant difference among the populations, the pairwise multiple comparisons were done using the Dunn test. Violin graphs with boxplots were generated to display the distribution of the data. All tests and the violin plot were performed in the R environment v. 4.4.1 (R Development Core Team, 2024) and R Studio v. 2024.09.0+375 (R Studio Team, 2024).

Molecular analyses. We sequenced 670 base pairs of the COI gene from 31 individuals of *P. laticeps*, *A. brachypterygium*, *P. cremnobates* and four other species (Tab. **S1** with Genbank accession numbers). Twenty-one sequences from six species were obtained from Genbank. The species were selected based on their relationship with the target species (Terán *et al*., 2020) or their occurrence in the Mampituba River (Tab. **S1**). DNA was extracted from gill filaments, muscles or liver tissue of the samples, with "Phire Animal Tissue Direct PCR Kit" developed by Thermo Scientific® under commercial recommendations. The COI was amplified with the primer cocktail FishF1t1 and FishR1t1 (Ivanova *et al*., 2007). The PCR reactions were carried out in a reaction volume of 20 µL [10.3 µL of H20, 2 µL of 10× reaction buffer (Platinum®Taq), 0.6 µL of MgCl2 (50 mM), 2 µL of dNTPs (2 mM), 2 µL of each primer (2 µM), 0.1 µL (5 U) of Platinum® Taq (Invitrogen), and 1 µL of template DNA]. The PCR conditions were: an initial DNA denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, at 52°C for 40 s, and at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were purified using enzymatic method Exosap (25% exonuclease, 25% Shrimp Alkaline Phosphatase and 50% of deionized water), and sequencing was performed by Macrogen Inc, Seoul, South Korea and by Ludwig Biotec at Porto Alegre, RS, Brazil.

Sequences were aligned using Muscle (Edgar, 2004) in MEGA 11 software (Tamura *et al*., 2021) and alignments were visually inspected for any obvious misalignments and then corrected. The p-distance was estimated twice, one time using the default conditions and the second time considering the best molecular evolutionary model; both analyzes were done in MEGA 11 software (Tamura *et al*., 2021). To illustrate the relationship between the sequences, a Neighbor Joining tree was used, considering the parameters suggested by Hebert (Hebert *et al*., 2003) using Mega11 (Tamura *et al*., 2021). A haplotype network was constructed with DnaSP 6.12 (Rozas *et al*., 2017) and Network 5.0 softwares (Fluxus technology Ltd.). All work was performed at the Molecular Laboratory of Departamento de Zoologia (UFRGS, Porto Alegre, RS, Brazil), with separately ordered primers (Ishida *et al*., 2011).

Data availability. The COI sequences used for this study are openly available in Genbank, GenBank accessions PQ341685-PQ341716. A BOLD project, *i.e*., a library consisting of voucher ID, collection numbers, photographs, and sequence data, is available for the data within this article. Morphological identifications and sampling localities are available in Tab. **S1**.

RESULTS

Astyanax brachypterygium was recovered under Neighbor Joining analyses (Fig. 1A) as closely related to *Psalidodon cremnobates* and *P. laticeps*, as well as deeply inserted in a clade that includes *P. gymnodontus* Eigenmann, 1911, type-species of the genus *Psalidodon.* Therefore, we now refer to this species here as *Psalidodon brachypterygium* (new combination). See further comments on discussion.

Molecular analysis. Under Neighbor Joining analyses (Fig. 1A), the COI sequence of one specimen from Mampituba River (Perdizes stream) from Mampituba forms a clade with *Psalidodon cremnobates* from Tramandaí and Jacuí river drainages. This clade is the sister group of a clade composed of *Psalidodon brachypterygium* and *P. laticeps*. The

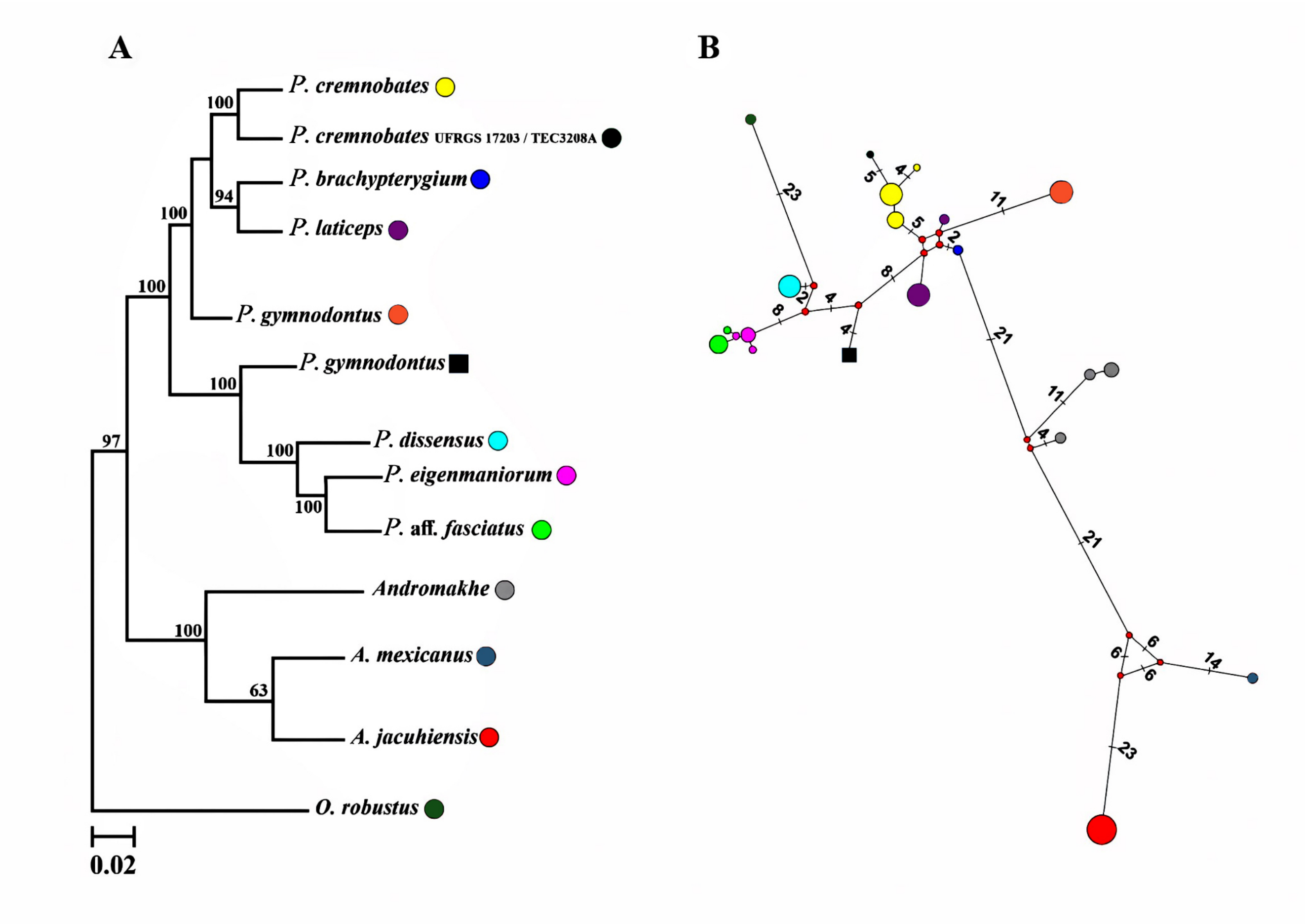

FIGURE 1 | Genetic analyses of *Astyanax*, *Psalidodon*, *Andromakhe* Terán, Benitez & Mirande, 2020, and *Oligosarcus* Günther, 1864 genera based on COI gene. The yellow circles represent *Astyanax cremnobates* from Jacuí River drainage; the black circle represents *A. cremnobates* from Mampituba River drainage; the blue circles represent *A. brachypterygium* from Laguna dos Patos and the purple circles are *A. laticeps* from Tramandaí river drainage. **A.** Neighbor-joining tree of 54 sequences (Tab. S1) using genetic distance, with the branch lengths equivalent to the number of base substitutions per site. Bootstrap values (1000 replications) are indicated next to the branches. **B.** Haplotype network of the 11 taxa. Numbers in each branch refer to the number of mutational steps between haplotypes; branches with no number represent only one mutational step; size of the haplotypes increases according with the number of specimens that are part of it (*i.e.*, higher sizes = more specimens). *Astyanax jacuhiensis* (following Gavazzoni *et al.*, 2024) and *A. mexicanus* (De Filippi, 1853).

clade composed by *P. brachypterygium*, *P. cremnobates*, and *P. laticeps* is closer to species of the genus *Psalidodon* than with species of the genus *Astyanax* (Fig. 1A). The NJ result was the same in both analyses, considering default conditions for barcode analyses (Hebert *et al*., 2003) and the best molecular evolutionary model (Kimura 2 parameters with gamma).

The lowest p-distance (Tab. **S2**) of the sequence from Mampituba River specimen was 0.024 with *P. cremnobates* from Tramandaí River drainage, 0.026 with *P. cremnobates* from Jacuí River drainage, 0.029 with *P. laticeps* and 0.030 with *P. brachypterygium*. The highest was 0.17 with *Astyanax jacuhiensis* (Cope, 1894) (following Gavazzoni *et al*., 2024). The network analysis (Fig. 1B) shows the haplotype from Mampituba has fewer mutational steps with *P. cremnobates* haplotypes than with other *Astyanax* and *Psalidodon* species. *Psalidodon cremnobates*, *P. brachypterygium* and *P. laticeps* haplotypes showed fewer mutational steps with *Psalidodon* species than with *Astyanax* species.

Morphological analysis. A total of 124 specimens of *Psalidodon cremnobates* collected at Preá and Perdizes streams, tributaries of the Mampituba River, within the Parque Nacional de Aparados da Serra located in the municipality of Cambará do Sul, Rio Grande do Sul, Brazil (Fig. 2), were examined. These specimens easily differ from *P. laticeps* by showing a vertical humeral spot, instead of a horizontally elongated and oval humeral spot (Fig. 3).

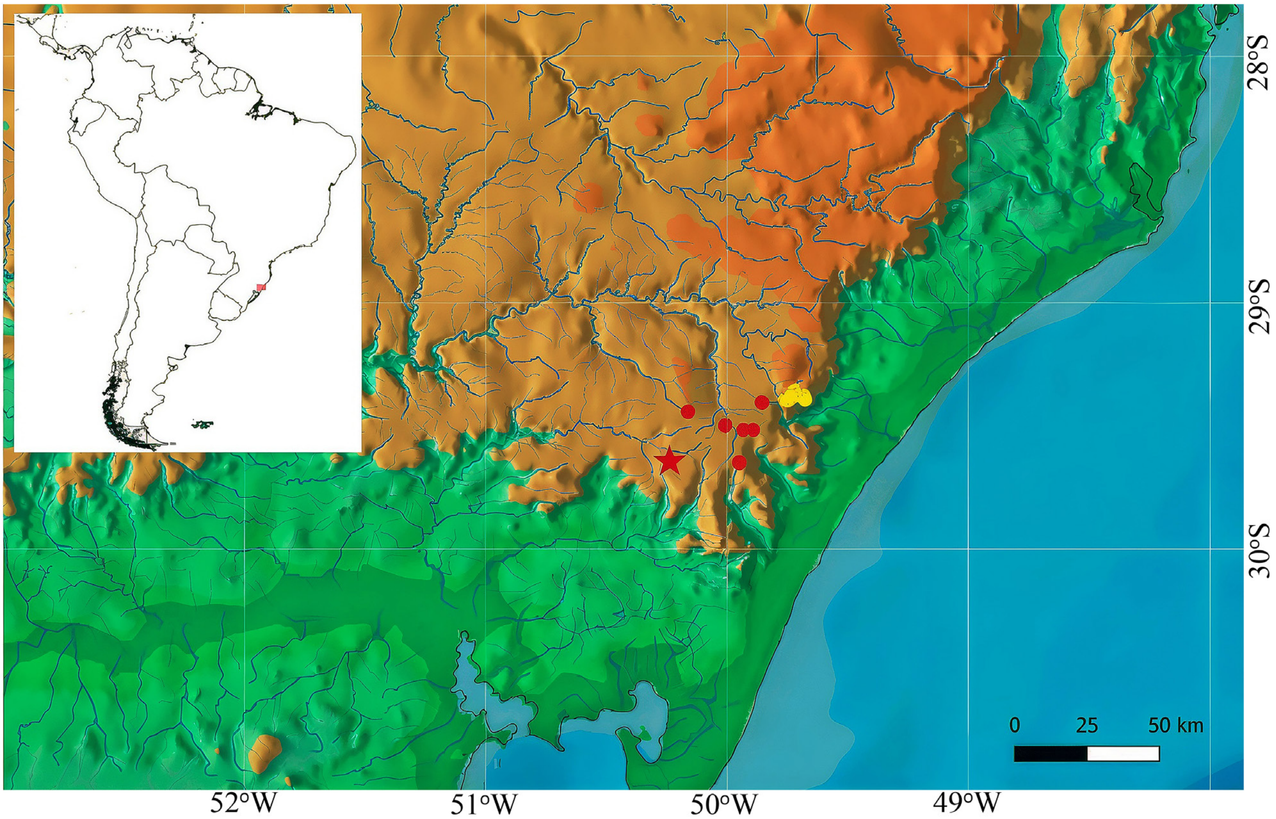

FIGURE 2 | Distribution of *Psalidodon cremnobates*. Red star corresponds to the type-locality from the Jacuí River drainage. Red and blue circles represent the localities of collection of the paratypes from the Jacuí and Maquiné river drainages, respectively. Yellow circles represent the new records from Mampituba River drainage. Orange represents the upland plateau located on Serra Geral and green represents the lowland areas of the river valleys and coastal plain.

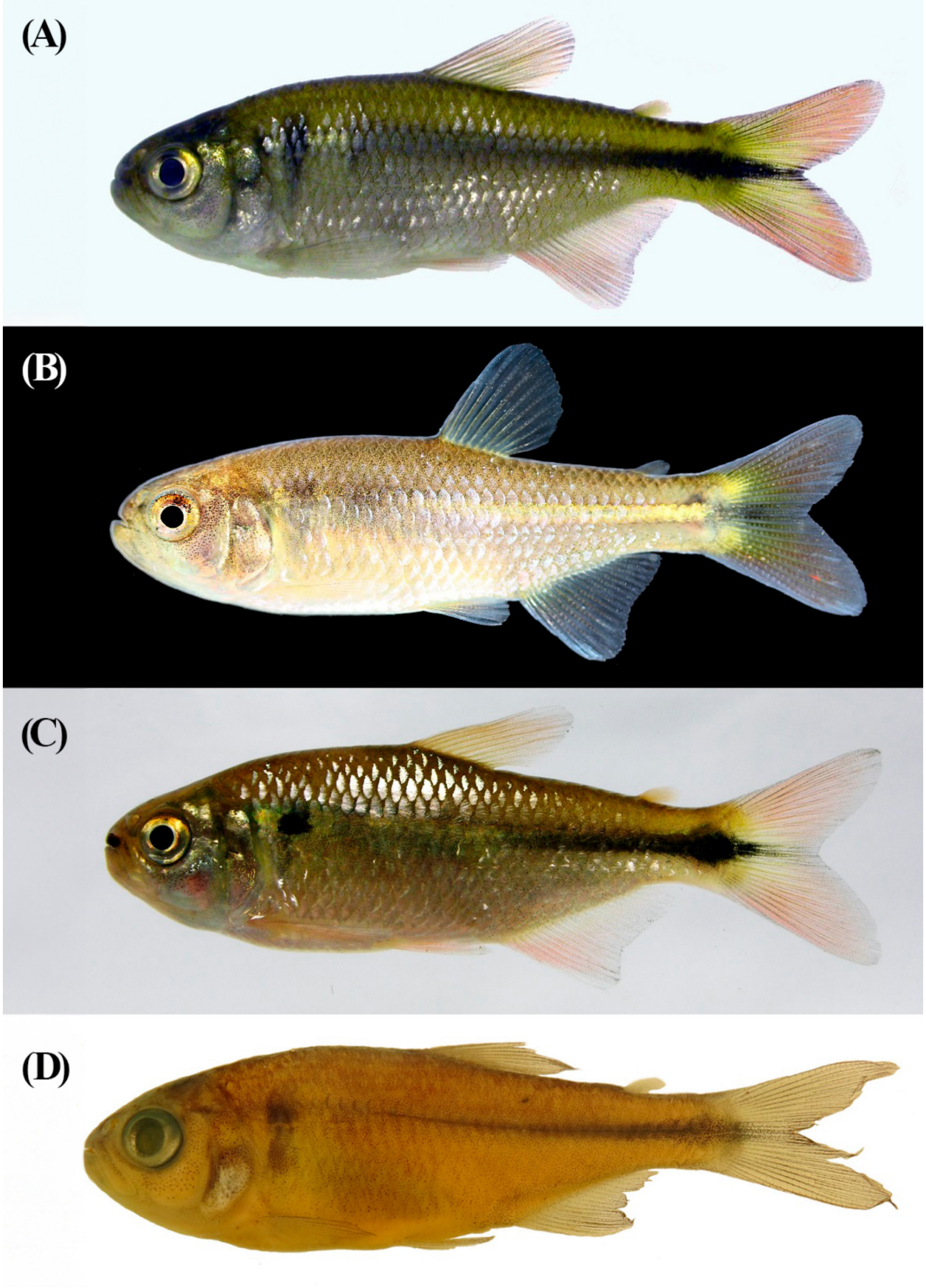

FIGURE 3 | Characid fish species of the "*Psalidodon scabripinnis* complex" from Rio Grande do Sul State, Southern Brazil. **A.** *Psalidodon cremnobates*, UFRGS 8229, 45.4 mm SL, arroio Pinto, Jacuí River drainage, São Francisco de Paula, Rio Grande do Sul, Brazil, 29°23'04"S 50°32'04"W. **B.** *P. brachypterygium*, UFRGS 21849, 33.2 mm SL, creek tributary to rio Pelotas, Uruguai River drainage, São José dos Ausentes, Rio Grande do Sul, Brazil, 28°40'26"S 49°58'00"W. **C.** *P. laticeps*, UFRGS 8828, 58.5 mm SL, Amola Faca River, Maquiné River drainage, Maquiné, Rio Grande do Sul, Brazil, 29°32'19"S 50°14'46"W. **D.** *P. cremnobates*, UFRGS 17203, 48.2 mm SL, Perdizes stream, Cambará do Sul, Mampituba River drainage, Rio Grande do Sul, Brazil, 29°08'28"S 50°04'55"W.

Meristic data of Mampituba River population are summarized in Tab. 1 and compared to data from the type-series of *P. cremnobates* and *P. brachypterygium*. Likewise Bertaco, Malabarba (2001), we found a unique count that differs between the two species. The Mampituba River population showed a number of branched anal-fin rays that ranges from 13 to 17, that is intermediate between the range described for *P. cremnobates* (14 to 18) and that described for *P. brachypterygium* (12 to 16). The median and the mode of the count of anal-fin rays in the Mampituba River population (median = 16, mode = 16), however, equals that of *P. cremnobates* (median = 16, mode = 16) and differ from that of *P. brachypterygium* (median = 14, mode = 14). The count of anal-fin rays of the three populations of *P. cremnobates* and the single population of *P. brachypterygium* was submitted to a the Kruskal-Wallis analysis and the null hypothesis was rejected, showing that there is a significant difference ($X^2_{(3)}$= 70.14; $p < 0.001$). The Dunn test comparing each pair of populations showed that the *P. brachypterygium* population differed from all three populations of *P. cremnobates* when we compared the branched anal-fin ray, and that these three populations do not differ among themselves (Tab. 2; Fig. 4). The counts of lateral line scales differed *P. brachypterygium* population only from *P. cremnobates* population of the Jacuí drainage, and the Jacuí drainage's population differs from Tramandaí and Mampituba populations (Tab. 3; Fig. 4).

TABLE 1 | Meristic data of *Psalidodon cremnobates* from Mampituba River drainage. UFRGS 16286 (10 of 22), 16290 (2 of 4), 16294 (4 of 8), 17203 (4 of 7), 17205 (3), 17212 (3 of 14). N = 26 specimens.

	Minimum	Maximum	Mode
Unbranched anal-fin rays	3	4	3
Branched anal-fin rays	13	17	16
Unbranched dorsal-fin rays	2	2	2
Branched dorsal-fin rays	9	9	9
Unbranched pelvic-fin rays	1	1	1
Branched pelvic-fin rays	7	7	7
Unbranched pectoral-fin rays	1	1	1
Branched pectoral-fin rays	11	13	12
Total caudal-fin rays	19	19	19
Lateral line scales	35	37	36
Scale rows between dorsal-fin origin and lateral line	6	6	6
Scale rows between lateral line and pelvic-fin origin	5	5	5
Predorsal scales	11	12	11
Scale rows around caudal peduncle	14	16	14
Scale sheath along anal-fin base	3	6	4
Maxilla teeth	1	2	1

TABLE 2 | Results of Dunn parwise multiple comparisons test for branched anal-fin ray and lateral line scales of the *Psalidodon brachypterygium* and *P. cremnobates* drainage populations.

Species	Branched anal-fin ray		Lateral line scales	
	Statistic	*p*	Statistic	*p*
P. brachypterygium (Uruguai) x *P. cremnobates* (Jacuí)	8.13	< 0.05	3.01	< 0.05
P. brachypterygium (Uruguai) x *P. cremnobates* (Mampituba)	5.04	< 0.05	-0.42	> 0.05
P. brachypterygium (Uruguai) x *P. cremnobates* (Tramandaí)	5.00	< 0.05	2.58	> 0.05
P. cremnobates (Jacuí) x *P. cremnobates* (Mampituba)	-1.81	> 0.05	-3.02	< 0.05
P. cremnobates (Jacuí) x *P. cremnobates* (Tramandaí)	-1.68	> 0.05	0.13	> 0.05
P. cremnobates (Mampituba) x *P. cremnobates* (Tramandaí)	0.08	> 0.05	2.69	< 0.05

TABLE 3 | Median and inter-quartile range (iqr) for branched anal-fin ray and lateral line scales of the *Psalidodon brachypterygium* and *P. cremnobates* drainage populations. N = number of specimens.

Species/Meristic data	N	Median	iqr
Branched anal-fin ray			
P. brachypterygium (Uruguai)	43	14	0
P. cremnobates (Jacuí)	50	16	0
P. cremnobates (Mampituba)	26	16	1
P. cremnobates (Tramandaí)	24	15	1
Lateral line scales			
P. brachypterygium (Uruguai)	43	36	1
P. cremnobates (Jacuí)	50	36	1
P. cremnobates (Mampituba)	26	36	1
P. cremnobates (Tramandaí)	24	36.5	1

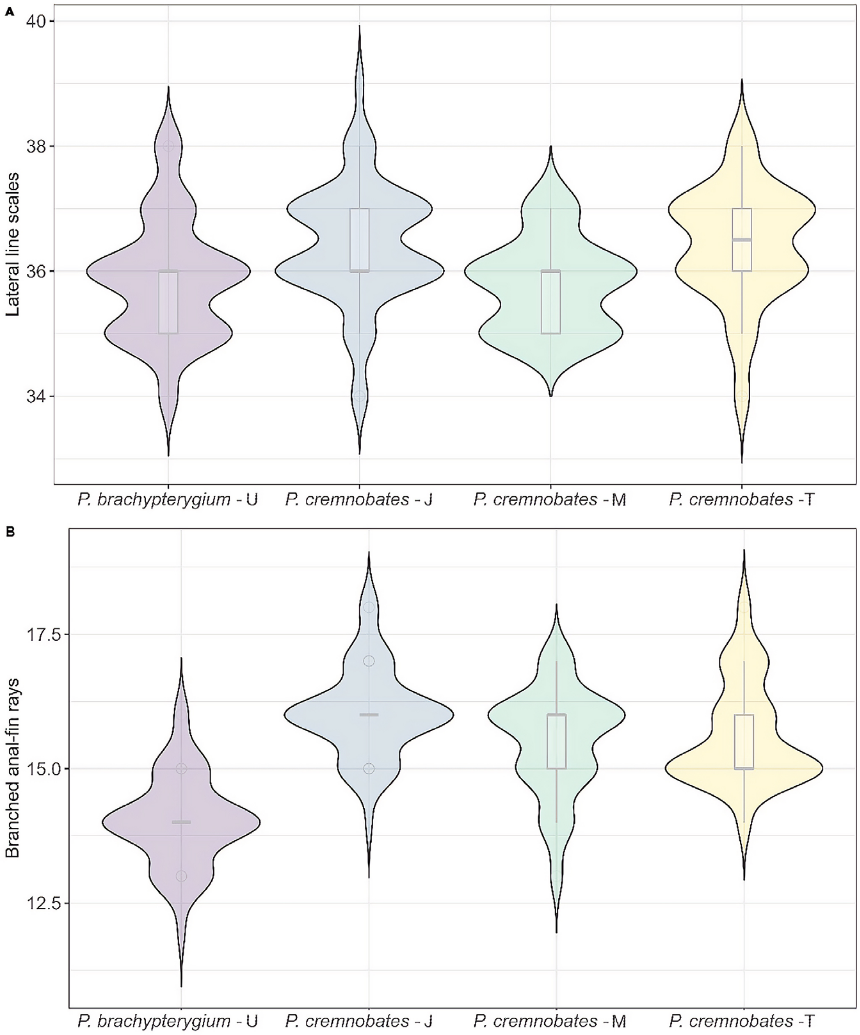

FIGURE 4 | The violin plots and boxplots of the two meristic data (A= lateral line scales and B = branched anal-fin ray) for the *Psalidodon brachypterygium* of the rio Uruguai (U) and *P. cremnobates* of the Jacuí (J), Mampituba (M), and Tramandaí (T) drainages. The violin plot shows the density distribution of meristic data for each population; the central line in the box represents the median, the whiskers show the distribution outside the central quartiles (the lowest/highest value within 1.5 times inter-quartile range from the box), and the circle are outliners.

Morphometric data further corroborates that specimens from Mampituba River belong to *P. cremnobates* (Tab. **S3**). Among measurements used to distinguish the two species by Bertaco, Malabarba (2001), specimens from Mampituba River have a shallower caudal peduncle depth (10.1–12.1% in SL, mean = 11.2) with similar range and mean to that observed in *P. cremnobates* (10.9–12.6%, mean = 11.7), and different from that of *P. brachypterygium* (13.7–15.3%, mean = 14.3). The larger orbital diameter (30.2–35.4% of head length, mean = 33.1) also fits with *P. cremnobates* (32.2–35.0%, mean = 33.7) and not with *P. brachypterygium* (26.3–31.2%, mean = 29.9). We were not able, however, to classify the specimens from Mampituba River based on the interorbital width, once they showed intermediate range and mean (26.1–31.9% of head length, mean = 29.3), comparatively to those observed in *P. cremnobates* (25.7–27.7%, mean = 26.6) and in *P. brachypterygium* (29.6–33.6%, mean = 31.4).

Material examined. All from Brazil: Rio Grande do Sul State. *Psalidodon brachypterygium*: **Uruguai River drainage.** MCP 8627, 1 paratype (c&s), 36.1 mm SL, rio Tourinhos, Bom Jesus, *ca.* 28°42'S 50°08'W, 30 Sep 1980, C. A. Lucena. MCP 11661, 5 paratypes, 31.8–55.5 mm SL, headwaters of arroio Lageado Bonito, Bom Jesus, *ca.* 28°38'S 50°33'W, 4 May 1985, C. A. Lucena, L. R. Malabarba & R. E. Reis; MCP 14367, 24 paratypes, 39.2–50.9 mm SL, rio Manoel Leão, Bom Jesus, *ca.* 28°44'S 50°02'W, 14 Jan 1989, C. A. Lucena, E. H. L. Pereira & P. V. Azevedo; MCP 14391, 26 (5 c&s), 39.4–58.3 mm SL; MCP 22296, 10 paratypes, 24.5–43.7 mm SL, tributary of rio Manoel Leão, São José dos Ausentes, *ca.* 28°43'S 50°00'W, 17 Dec 1998, R. E. Reis, A. R. Cardoso, P. A. Buckup & F. Melo; MCP 26094, holotype, 40.8 mm SL, arroio Água Branca, Bom Jesus, *ca.* 28°36'S 50°24'W, 15 Jan 1989, C. A. Lucena, E. H. L. Pereira & P. V. Azevedo. MZUSP 62713, 20, 37.2–59.5 mm SL. NMW 94544, 20, 37.7–55.1 mm SL. UFRGS 4950, 20, 39.6–58.5 mm SL; UFRGS 21849, 1, 33.2 mm SL, creek tributary to rio Pelotas, São José dos Ausentes, 28°40'26"S 49°58'00"W, 29 Apr 2016, L. R. Malabarba, J. Ferrer, T. P. Carvalho, R. Agrizani & E. Wendt. USNM 364303, 20, 36.3–62.5 mm SL; paratypes and same data of holotype. *Psalidodon cremnobates*: **Jacuí River drainage.** MCP 11142, 25 (5 c&s), 40.7–71.5 mm SL; MCP 11650, 34 paratypes (2 c&s), 13.5–81.9 mm SL, arroio Camisa, Cambará do Sul, *ca.* 29°11'S 50°12'W, 1 May 1985, C. A. Lucena, L. R. Malabarba & R. E. Reis; MCP 21101, 12 paratypes, 31.6–59.9 mm SL, rio Contendas, Tainhas, *ca.* 29°17'S 50°14'W, 23 Sep 1997, W. Bruschi & G. Vinciprova. MCP 26093, holotype, 43.8 mm SL, tributary of rio Santa Cruz, São Francisco de Paula, *ca.* 29°23'S 50°32'W, 16 May 1987, R. E. Reis, L. A. Bergman & P. V. Azevedo. MCP 22295, 78 paratypes, 22.1–57.6 mm SL, tributary of rio Tainhas, Tainhas, *ca.* 29°16'S 50°20'W, 16 Dec 1998, R. E. Reis, A. R. Cardoso, P. A. Buckup & F. Melo. MCP 22297, 9 paratypes, 25.2–45.9 mm SL, headwaters of rio Lageado Grande, *ca.* 29°13'S 50°28'W, 16 Dec 1998, R. E. Reis, A. R. Cardoso, P. A. Buckup & F. Melo. MCP 25660, 15 paratypes, 30.7–41.4 mm SL, rio Contendas, São Francisco de Paula, *ca.* 29°17'S 50°16'W, 20 Mar 2000, W. Bruschi. MZUSP 62712, 20, 37.5–64.9 mm SL; NMW 94543, 20, 35.7–49.4 mm SL; UFRGS 4949, 20, 37.2–54.1 mm SL; USNM 364302, 20, 36.8–57.6 mm SL; paratypes and same data as holotype. UFRGS 8229, 1, 45.4 mm SL, arroio Pinto, São Francisco de Paula, 29º23'04"S 50º32'04"W, 11 Sep 2009, J. A. Anza, J. Ferrer, L. R. Malabarba & G. Neves. **Mampituba River drainage:** UFRGS 16282, 30, 24.5–45.4 mm SL, tributary of arroio Preá, 29º10'22"S 50º 06'11"W, 10 May 2012, J. Ferrer, T. Guimarães, M. Severo & L. Santos. UFRGS 16283, 2, 25.1–49.8 mm SL, Quebrada Funda, tributary of arroio Perdizes, 29º10'22"S 50º02'43"W, 12 May 2012, J. Ferrer, T. Guimarães, M. Severo, L. Santos, P. Lehmann & A. Souza. UFRGS 16286, 22, 28.6–63.6 mm SL, arroio Preá, 29º09'49"S 50º05'21"W, 12 May 2012, T. Guimarães, L. Santos, P. Lehmann & A. Souza. UFRGS 16290, 4, 33.7–59.3 mm SL, UFRGS 16294, 8, 35.5–48.4 mm SL, arroio Perdizes, 29º09'25"S 50º03'03"W, 12 May 2012, J. Ferrer, T. Guimarães, M. Severo, L. Santos, P. Lehmann & A. Souza. UFRGS 16291, 5, 21.5–47.5 mm SL, tributary of arroio Preá, 29º10'21"S 50º06'43"W, 10 May 2012, J. Ferrer, T. Guimarães, M. Severo, L. Santos & A. Souza. UFRGS 16292, 23, 27.5–54.5 mm SL, floodplain that drains to arroio Preá, 29º10'27"S 50º07'00"W, 10 May 2012, J. Ferrer, T. Guimarães, M. Severo & L. Santos. UFRGS 16293, 2, 29.1–33.5 mm SL, tributary of arroio Preá, 29º10'22"S 50º07'00"W, 10 May 2012, J. Ferrer, T. Guimarães, M. Severo, L. Santos. UFRGS 16296, 1, 48.5 mm SL, tributary of arroio Preá, 29º09'49"S 50º05'51"W, 10 May 2012, J. Ferrer, T. Guimarães, M. Severo & L. Santos. UFRGS 17203, 8, 28.1–49.2 mm SL, stream that drains to arroio Perdizes, 29º08'28"S 50º04'55"W, 11 Oct 2012, A. Fregonezi, F. G. Becker & T. Guimarães. UFRGS 17205, 3, 45.0–63.2 mm SL, arroio Perdizes, 29º10'21"S 50º02'41"W, 11 Oct 2012, A. Fregonezi, F. G. Becker & T. Guimarães. UFRGS 17211, 2, 20.5–40.5 mm SL, arroio Perdizes, 29º09'28"S 50º04'47"W, 10 Oct 2012, A. Fregonezi, F. G. Becker & T. Guimarães. UFRGS 17212, 14, 28.7–51.2 mm SL, arroio Preá, 29º09'47"S 50º05'50"W, 11 Oct 2012, A. Fregonezi, F. G. Becker & T. Guimarães. *Psalidodon laticeps*: **Maquiné River drainage:** UFRGS 8828, 1, 58.5 mm SL, Amola Faca River, Maquiné, 29°32'19"S 50°14'46"W, 6 Jan 2007, L. R. Malabarba, J. Ferrer & C. B. Fialho.

DISCUSSION

The samples collected at Mampituba proved to belong to *Psalidodon cremnobates* both in the morphological and molecular analyses, enlarging the distribution of this species to that drainage. *Psalidodon cremnobates* has been considered an endemic species from the "Campos de Altitude" (highland grasslands of southern Brazil), and has was found only in a small area at altitudes from 800 to 1.000 m a.s.l. in the headwaters of the Maquiné River and Laguna dos Patos drainages (Becker *et al*., 2023). Such a restricted distribution and the occurrence of *Oncorhynchus mykiss* (Walbaum, 1792), an exotic invasive species (Bertaco *et al*., 2023) lead the species to be considered Near Threatened (NT) in a regional list (RS, 2014) under criteria B1ab(iii). Our new records come from altitudes from 892 to 1,029 m a.s.l., but from different river drainage. The register of the species in Mampituba extends the geographic distribution of the species to the north from the known distribution. The additional locality is further important because it is inside of the protected area the Parque Nacional de Aparados da Serra, and near to another protected area the Parque Nacional da Serra Geral. The species has been recently reclassified as of Least Concern (LC) in the National list, based on new distribution records in the Maquiné and Jacuí river drainages and review of the extent of occurrence (EOO) criteria (ICMBio, 2024), but has not been reevaluated yet on the regional list. Our new records to the Mampituba River drainage corroborates ICMBio (2024) decision of changing the conservation status to Least Concern (LC), and allows us to recommend the same review of its status on the Rio Grande do Sul State regional list.

Integrative use of multiple operational criteria considering genes, morphology and color pattern showed it to be important in the recognition of three morphologically similar and closely related species, as well as of a new population of *P. cremnobates*. Based on these results, the use of a recommended 2% limit of genetic divergence (Hebert *et al*., 2003) to recognize different species deserves further discussion. We found genetic divergence between *A. brachypterygium* and *P. laticeps* ranges from 1.6 to 1.9%, while Mampituba River sample of *P. cremnobates* differ by 2.4% from Tramandaí River and 2.6% from Jacuí River samples of the same species. The three populations of *P. cremnobates*, however, constitute a single lineage, a unifying criteria for species recognition, and despite the p-distance values larger than 2% among the three populations, they lack additional criteria to split each population as a separate species. Species evolve in different rates (Melkikh, 2023) so the speciation process is occurring in different ways, printing (or not printing) differences on dataset in many different forms (Malabarba *et al*., 2021), varying among species (*e.g*., morphological differences and low genetic divergence or high genetic divergence and stable morphology). The use of a standard genetic distance to recognize species has not been supported in several studies (*e.g*., Pedraza-Marrón *et al*., 2019; Queiroz *et al*., 2020; Lehmann *et al*., 2024). Therefore, our dataset accurately points to the population of Mampituba as being *P. cremnobates* and validates the three species: *P. cremnobates*, *P. brachypterygium*, and *P. laticeps*.

Astyanax has long been considered non-monophyletic (Eigenmann, 1917; Rosen, 1972; Janovillo *et al*., 2010; Schmitter-Soto, 2016; Mirande, 2019). Recently, Terán *et al*. (2020) proposed a new hypothesis of the phylogenetic relationships of *Astyanax* species and proposed changes in the generic allocation of some species. They resurrected and expanded the genus *Psalidodon*, and identified the clade composed of *Astyanax cremnobates*, *A. laticeps*, and *A. serratus* Garavello & Sampaio, 2010 as the sister group to *Psalidodon*. More recently, Melo *et al*. (2024) transferred these three species to *Psalidodon*. None of the previous authors have examined *A. brachypterygium*. We found *A. brachypterygium* is closely related to *P. cremnobates* and *P. laticeps*, as well as included in the same clade with *P. gymnodontus*, type-species of the genus *Psalidodon.* In order to reflect these relationships and to maintain *Psalidodon* monophyletic, we now refer to this species here as *P. brachypterygium*.

Psalidodon gymnodontus was recovered as paraphyletic in our analysis. The samples are from two different locations in the Iguaçu River drainage, whose sequences were obtained on the NCBI platform. Differences found in the barcodes may suggest a possible species complex or an incorrect identification of one of the samples. Regardless of these facts, both clades of *P. gymnodontus* are closely related to other *Psalidodon* species (Fig. 1), which does not affect the results presented here. A taxonomic revision of *P. gymnodontus* shows a high polymorphism in morphological characters (Pavanelli, Oliveira, 2009), nevertheless molecular data was not included to compare the different morphotypes. An integrative taxonomic work is recommended to evaluate the genetic diversity, and also to help in the determination of the species boundaries.

The haplotype network showed that the sample from the Preá stream (Mampituba River) is more closely related to the *P. cremnobates* specimens from Maquiné (Tramandaí River drainage) than to the specimens from the Jacuí River drainage (Fig. 1). The Mampituba and Maquiné drainages are included in the Tramandai-Mampituba Freshwater Ecoregion (TMF) (Abell *et al*., 2008 – FEOW 335). The TMF ecoregion hosts many endemic species (Malabarba, Isaia, 1992; Reis, Schaefer, 1998; Albert *et al*., 2011; Ferrer *et al*., 2015; Delapieve *et al*., 2020) mainly due to shared geological historical changes. The region was highly impacted by changes in sea level in the Pleistocene that culminated in a division of areas and connections mainly to capture streams, modeling a pattern in the distribution of species that inhabit TMF (Hirschmann *et al*., 2015, 2017; Thomaz *et al*., 2015, 2017; Thomaz, Knowles, 2018).

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The authors declare no competing interests.

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