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A new species of genus *Psalidodon* Eigenmann, 1911 related to the *P. paranae* complex (Characiformes Characidae) from Upper Paranaíba river basin, Brazil, supported by genetic and morphometric data

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ABSTRACT The genus *Psalidodon* Eigenmann, 1911 (Characiformes Characidae) is a fish group with great diversity, expressed at the chromosomal, genetic and taxonomic level. The genus is marked by events of allopatric and vicariant evolution, by the formation of complexes of species and by wide geographical distribution. Both in these fish and other organisms, the association of studies with molecular markers and geometric morphometric techniques are useful in delimiting significantly evolutionary units (ESU). In this work, we performed maximum likelihood estimates (MLE) from mitochondrial Cyt b gene sequences and canonical variables (CVA) from 13 landmarks in eight populations of *P.* aff. *paranae* Eigenmann, 1914. The analysis of Maximum likelihood resulted in the structuring of populations in two different clades, one of which was composed only of individuals from a small population inhabiting a stream with approximately two km of length, demonstrating their clear distinction from the other populations. The analysis of canonical variation demonstrated the complete structuring of this population, and the position of each clade in the morpho-space was congruent with the topography observed in the MLE. Based on the results found, the existence of a new endemic species of the genus *Psalidodon* is evident.

KEY WORDS Endemism; geometric morphometry; mtDNA; vicariance.

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INTRODUCTION

The genus *Psalidodon* Eigenmann, 1911 (Characiformes Characidae) is part of the former genus *Astyanax* S.F. Baird et Girard, 1854, that is widespread in the Neotropicalis widespread of Neotropical ichthyofauna, with model species in developmental and evolutionary studies (Jeffery, 2001, 2008; Borowski, 2009). Such former genus contains at last 170 species (Eschmeyer et al., 2020), meanwhile *Psalidodon* 38 species so far, inhabiting almost all Brazilian rivers and constituting a significant fraction of the fish studied at the chromosomal and genetic level (Pasa & Kavalco, 2007).

Recently, Terán et al. (2020) moved species of

Astyanax to other six genera. More specifically, while the species complexes *A. fasciatus* (Cuvier, 1819) and *A. scabripinnis* Jenyns, 1842 were relocated to *Psalidodon*, and the *Astyanax* species from the coastal river basins of Brazil, like *A. giton* Eigenmann, 1908, were relocated to *Deuterodon* Eigenmann, 1907, the ones from the complex *A. bimaculatus* (Linnaeus, 1758) and the North American species remain in the *Astyanax* genus.

Psalidodon paranae (Eigenmann, 1914) is a small fish species inhabiting headwaters of rivers and streams from the Upper Parana River basin, belonging to a larger species complex group, formerly subspecies of *P. scabripinnis* (Eigenmann, 1927).

Moreira-Filho & Bertollo (1991) proposed that *P. scabripinnis* should be a group of cryptic species, based on cytogenetic and morphometric characters in populations of the upper Paraná and São Francisco river basins, where they are widely distributed (Bertaco & Lucena, 2006). However, one can even consider *P. paranae* as a group of cryptic species that occur in the Upper Paraná River Basin, because of different diploid number and several distinct karyomorphs (2n = 50, 2n = 48 and 2n = 46) (see Pasa & Kavalco, 2007 for a review).

Despite morphometric and chromosomal features, several studies in genus *Psalidodon* and other cryptic fish species used mitochondrial sequences to elucidate their distinctiveness. Rocha et al. (2019) used morphometrics and cytochrome b (cyt b) sequence to demonstrate the distinctiveness between *P. paranae* and *P. rivularis* Lütken, 1875, both formerly subspecies of *P. scabripinnis* from Upper Parana and São Francisco river basins, respectively. Kumar et al. (2017) also used cytB to show three mitochondrial lineages with high genetic variation and haplotypic diversity in *Ompok bimaculatus* Bloch, 1974. Using the same tool, Zhu et al. (2016) found moderate to high levels of genetic differentiation in 11 populations of *Scomber japonicus* Houttuyn, 1782, with two of them well structured, showing high diversity besides the other nine with low ones.

In this way, we aim to demonstrate a new*Psalidodon* species based on morphometric and Cyt b mitochondrial sequences. We sampled this species only isolated in a small stream used for water supply, endangered by an anthropic disturbance menace.

MATERIAL AND METHODS

We sampled eight populations of *Psalidodon* aff. *paranae* from Upper Paraná river hydrographic system, Paranaíba river Basin (Fig. 1, Table 1). After sampling, we brought the living specimens to the laboratory, euthanized them according to the technical standards of CONCEA - National Council for Control of Animal Experimentation of Brazil and CEUA/UFV - Animal Use Ethics Committee/Federal University of Viçosa (760/2018). We performed the sampling with licenses provided by SISBIO - Biodiversity Authorization and Information System (1938128) and SISGEN - National System for the Management of Genetic Heritage and Associated Traditional Knowledge (A9FE946). After that, we deposited the samples under vouchers numbers in the Ichthyology Collection of the Federal University of Viçosa, Campus Rio Paranaíba (supplement). We checked their species identities according to their morphological diagnostics (Oliveira, 2017).

We extracted the total genomic DNA from liver and heart samples according to the manufacturer instructions (Invitrogen PureLink DNA extraction and purification kit). We used the primers H16460 (CGAYCTTCGGATTACAAGAC) and GluDG.L (TGACCTGAARAACCAYCGTT) (Perdices et al., 2002) to amplify the cytochrome oxidase b gene by PCR (according to Prioli et al., 2002) and sequenced the samples with a private company (Myleus, Belo Horizonte, MG, Brazil). Sequences was deposited on GenBank, accession number MK756216 to MK756259.

We visualize the obtained sequences, align them with ClustalW v1.6 (Thompson et al., 1994) and calculate the genetic distances in MEGA X (Chernomor et al., 2016; Kumar et al., 2018), with *P. rivularis* specimens as outgroup. To reconstruct the Maximum Likelihood phylogram, we used the software MEGA X (Kumar et al., 2018), with the substitution model HKY (Hasegawa et al., 1985) according to the test of models in the same software.

We photographed every sampled individual with a Sony Cybershot camera (14.1 megapixels), and we prepare the files with TPSUtil 1.64 (Rohlf, 2013). We delimited thirteen anatomic landmarks with TPSDig2 2.32 (Rohlf, 2015) with 1 cm of scale factor to include the objects in an X/Y coor-

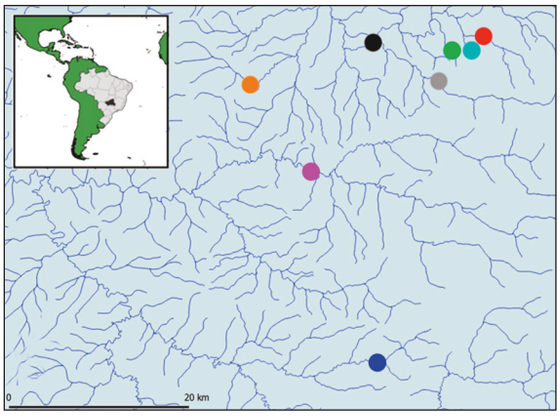

Figure 1. Distribution of samples in the Paranaíba river basin: Água Grande stream (red); Rita stream (light blue); Lava-pés stream (green); Olhos d'Água stream (gray); Paranaíba river (black); São João river (pink); Fora river (orange) and Quilombo river (dark blue).

Collection points	Geographical coordinates	Morphometry	*Cyt b*
Água Grande stream	19.177967"S 46.22613"W	9	3
Lava-Pés stream	19.191615"S 46.257387"W	10	3
São João river	19.308256"S 46.397117"W	8	6
Paranaíba river	19.182286"S 46.332794"W	10	10
Rita stream	19.187714"S 46.236178"W	7	6
Fora river	19.2232"S 46.45889"W	10	3
Quilombo river	19.49413"S 46.33163"W	10	3
Olhos d'Água stream	19.21285"S 46.27091"W	11	3

Table 1. Collection points, geographical coordinates and number of individuals used in each analysis.

dinates system (Figs. 2, 3). In MorphoJ 1.06 (Klingenberg, 2011), we made the Generalized Procrustes Analysis (GPA) in order to remove the size, rotation and orientation effects from the sample. We also searched for outliers and checked the normal distribution. We used two criteria as classifiers: collect point and clade (clade 1 = Lava-pés stream, Olhos d'Água stream, Fora river, Paranaíba river and Água Grande stream; clade 2 = Quilombo river and São João river; and clade 3 = Rita stream; according to ML phylogram). We verified the homoscedasticity of data with Procrustes ANOVA.

To minimize the effects of allometry on the sample, we performed a multivariate regression of the Procrustes coordinates vs centroid size. For a discriminatory analysis between populations and clades, we carried out the canonical variation analysis (CVA), and included it in both analyzes a permutation test for null hypothesis for the average of equal groups, using 10,000 permutation rounds.

RESULTS AND DISCUSSION

All individuals were identified *a priori* as *P. paranae* according to description available. We did not found any diagnostic characters distinguishing any of populations.

The Maximum Likelihood shows a structuring of the 38 individuals into two principal clades, excluding the outgroup, *P. rivularis* (Fig. 4). One of them is composed only with individuals from Rita stream, indicating a clear genetic differentiation between this population and the others. Such differentiation is corroborated by the genetic distance between population, meanwhile a low genetic distance is present within (Table 2).

The values of the Procrustes ANOVA test rejected the null hypothesis both in the analysis between populations (SS = 0.0393, MS = 0.0003, df = 154, F = 3.87, p <0.0001) and between clades (SS = 0.0181, MS = 0.0004 , df = 44, F = 5.59, p <0.0001).

In the analysis of canonical variation, it was possible to observe that the population from the Rita stream was structured along CV2 (responsible for 24.9% of the variation), whereas in CV1 (responsible for 30.9% of the variation), it was possible to observe a tendency to structure the populations of Água Grande stream and Fora river (Fig. 4). The permutation test rejected the null hypothesis (p <0.05) among all populations, with the exception of Olhos d'Água stream and São João river, and Paranaíba and São João rivers.

In the analysis of canonical variation between clades, it was possible to observe a clear structuring of clade 3 (Parque das Exposições stream) in relation to clades 1 and 2 along CV1, responsible for 66.2% of the variation. Clades 1 and 2 showed a tendency to structure themselves along CV2, responsible for 33.8% of the variation (Fig. 5). The permutation test rejected the null hypothesis (p <0.01) among all clades.

Through the Wireframes corresponding to the

ends of each axis of the CVA responsible for structuring the Rita stream or Clade 3 (Figs. 5, 6), it was possible to observe that the main morphological difference between this population and the others is the shorter caudal peduncle.

Using morphometric and genetic information, we demonstrate that the *Psalidodon* population from the Rita stream represents, in reality, a new species, inhabiting a stream approximately two km in length, surrounded by populations of *P. paranae*, which we describe below.

Psalidodon rioparanaibanus n. sp. (Fig. 3)
http://zoobank.org/urn:lsid:zoobank.org:104718CE-F240-4832-89F2-76A9DB879E83

EXAMINED MATERIAL. Holotype (LaGEEvo 4011 - L12), Rita stream, Rio Paranaíba, Minas Gerais, Brazil, 19.187714"S 46.236178"W, 21 Aug 2017, I.B. da Silva & M.A. da Silva. Paratypes (LaGEEvo 4236, 4277, 4279, 4280, 4281, XXX1, XXX2 - L13), da Rita stream, Rio Paranaíba, Minas Gerais, Brazil, 19.187714"S 46.236178"W, 23 Aug 2018, I.H.R. Oliveira, I.B. da Silva, G.K. Leles, R.L. Oliveira, T. Castanho.

DESCRIPTION OF HOLOTYPE. A vertically elongated humeral spot, the body more robust in the anterior part, short snout smaller than the diameter of the orbit, darker black sideband at the base of the caudal peduncle and close to the humeral spot, complete lateral line, dorsal fin with 11 rays being nine-branched, caudal fin with 17 rays being 14 branched, eight rows of scales above the lateral line and six below, 33 scales perforated along the lateral line.

VARIABILITY. Dorsal fin with 10–11 rays with 8–9 branched, anal fin with 16–19 rays with 14–18 branched, 7–9 rows of scales above the lateral line and 4–6 below, 33–36 perforated scales along the sideline.

ETYMOLOGY. Specific name in allusion to the municipality of Rio Paranaíba where the type series was discovered.

DISTRIBUTION AND BIOLOGY. *Psalidodon rioparanaibanus* n. sp. is endemic to the da Rita stream, a tributary of the Paranaíba river, belonging to the Upper Paraná River basin.

REMARKS. Conserved environments have more stable environmental characteristics, which favours the establishment, adaptation and development of a population (Frankham et al., 2002). This pattern can occur in the case of *P. rioparanaibanus* n. sp., which has a clear distinction from the *P. paranae* analyzed populations and represents a significantly evolutionary unit (ESU). The location of this point is in a green area within the city of Rio Paranaíba, which does not suffer constant human interventions that hinder the conservation of the place since the water supply of Rio Paranaíba municipality depends on this stream. Also, the stream is isolated from other by a small waterfall (about 2 meters high), followed by a long steep slope through a pasture. The isolation of the population meant that the

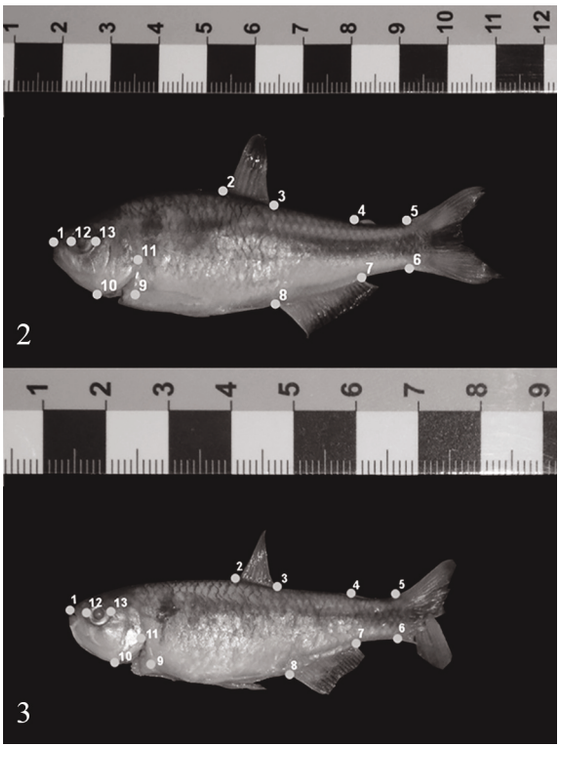

Figure 2. *Psalidodon paranae* specimen demonstrating the landmarks used in the analysis of geometric morphometry: 1 - snout tip; 2 - anterior insertion of the dorsal fin; 3 - posterior insertion of the dorsal fin; 4 - anterior insertion of the adipose fin; 5 - insertion of the first ray in the dorsal margin of the caudal fin; 6 - insertion of the first ray in the ventral margin of the caudal fin; 7 - posterior insertion of the anal fin; 8 - anterior insertion of the anal fin; 9 - insertion of the pectoral fin; 10 - ventral limit of the opercular opening; 11 - end of the opercular curve; 12 - anterior margin of the orbit and 13 - posterior margin of the orbit. Figure 3. *Psalidodon rioparanaibanus* n. sp.

genetic differences accumulated over time were more evident when compared to the other populations analyzed in this work (Table 2).

Along with genetic divergence, *P. rioparanaibanus* n. sp. differed from *P. paranae* in all morphometric analysis (Figs. 5, 6), with the p-value associated with discriminatory tests following the observed distinction of this population on the score charts. Geographic isolation can contribute not only to genetic divergence but also to morphology since some of the fastest responses presented by populations to environmental pressures are related to changes in the morphology of individuals (Streelman & Danley, 2003).

Small and "closed" populations, such as that of the Rita stream, tend to face severe problems with loss of genetic diversity and increased inbreeding (Table 2). These factors together reduce the ability to respond to changes in the environment and adapt, making it difficult for a population to remain in a

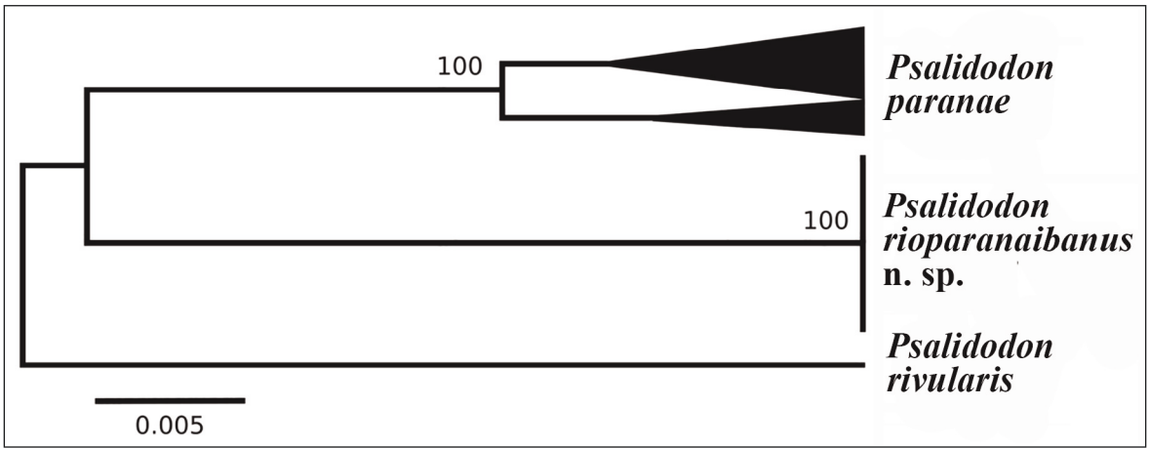

Figure 4. Phylogram resulting by (ML) Maximum Likelihood from the cyt b mitochondrial gene sequences of the specimens of *Psalidodon paranae*, *P. rioparanaibanus* n. sp. and *P. rivularis* as outgroup. The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model. The tree shown above is the one with the highest log likelihood (-1141.58). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 38 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 653 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1 – Olhos d'Água stream	**0.000**							
2 – Quilombo river	0.011	**0.000**						
3 – São João river	0.013	0.000	**0.001**					
4 – Rita stream	0.048	0.044	0.048	**0.000**				
5 – Lava-pés stream	0.002	0.010	0.011	0.046	**0.002**			
6 – Água Grande stream	0.004	0.012	0.013	0.049	0.003	**0.003**		
7 – Paranaiba river	0.003	0.011	0.011	0.048	0.001	0.002	**0.001**	
9 – Fora river	0.002	0.011	0.012	0.049	0.002	0.003	0.001	**0.001**

Table 2. Genetic distance between *Psalidodon paranae* populations. Diagonally and in bold, distance within populations.

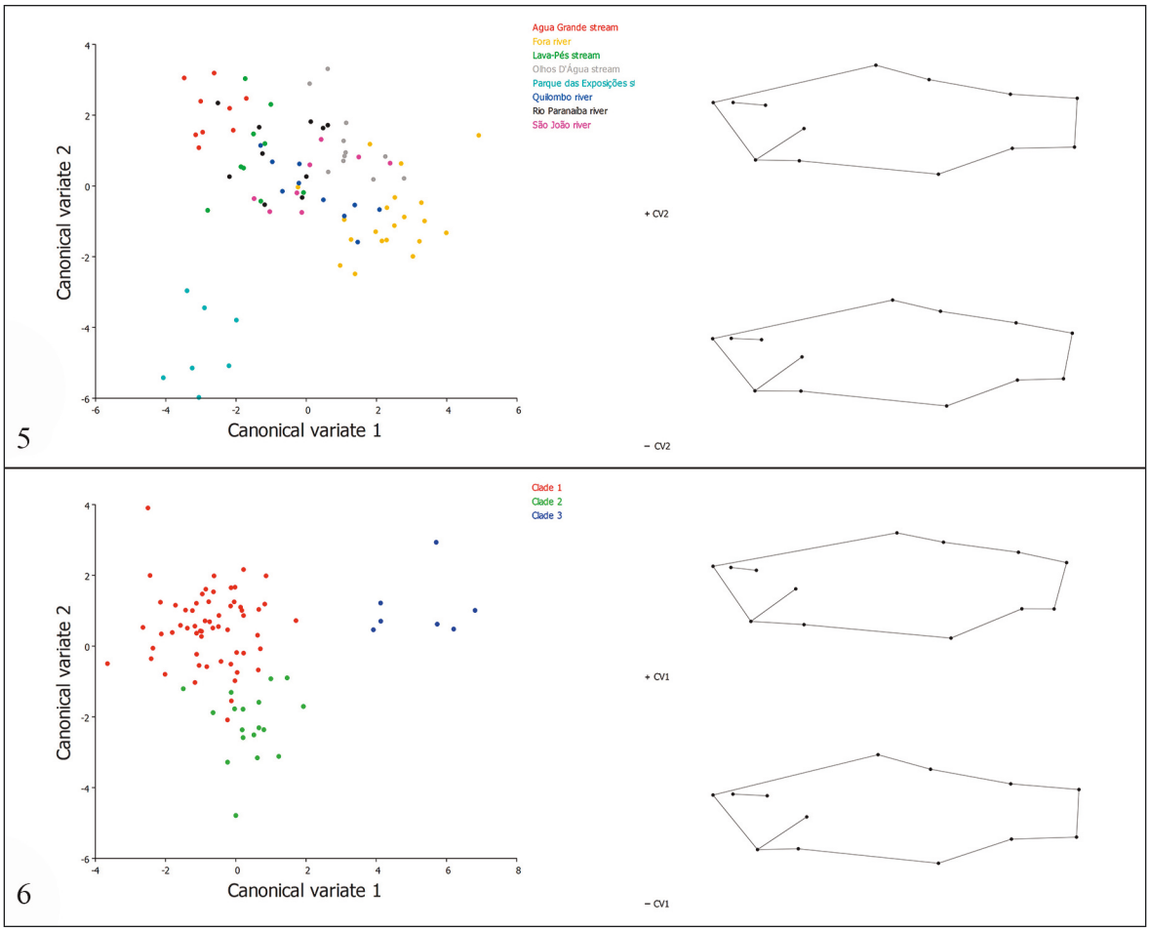

Figure 5. Morpho-space of CVA between collection points and Wireframes containing the shape found at the ends of CV2, responsible for structuring the Rita stream. Figure 6. Morpho-space of CVA between clades and Wireframes containing the shape found at the ends of CV1, responsible for structuring clade 3 (Rita stream) related to clades 1 and 2 (other populations).

given location (Hedrick & Kalinowski, 2000). Besides, genetic homogeneity between individuals caused by the high rate of inbreeding can lead to the elimination or drastic reduction of the population in the face of stochastic events that can happen in the environment, such as deforestation and reduction of the area for the permanence and adaptation of individuals. In small populations, the effects of genetic drift are also more significant, causing even greater impacts on their evolution (Frankham et al., 2002).

Along with this, there are issues related to habitat fragmentation and the consequently reducing migration, which directly influence the reduction of gene flow and increase isolation among populations. Over time, this generates a clear differentiation between populations of the same species, causing genetic and even morphological divergences (Allan & Castillo, 2007). Conservation and environmental protection practices can reduce the consequences generated by these problems.

From the data presented with genetic and morphometric evidence, we suggest that population belonging to the Rita stream constitutes a new species within the genus *Psalidodon*, here called *P. rioparanaibanus* n. sp., and must be formally recognized, receiving "endangered" status, due to their endemism.

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