



Population genomics of *Gymnogeophagus labiatus* and *G. lacustris* reinforces the role of hydrographic isolation between evolutionary lineages (Cichlidae: Geophagini)

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Gymnogeophagus labiatus and *G. lacustris* represent a pair of sister taxa distributed in the Patos Lagoon (ELP) and Tramandaí–Mampituba (ETM) ecoregions in Southern Brazil and Uruguay. While *G. lacustris* was traditionally considered endemic to the ETM, *G. labiatus* has been assigned to both ecoregions, being distinguished from *G. lacustris* by its hypertrophied lips, which represent an adaptation for foraging in rocky environments, and by the variation in coloration. A recent study using mtDNA and morphological data challenged this interpretation and suggested that *G. labiatus* is exclusive of ELP, and that all individuals in ETM should be considered *G. lacustris*. In this work we used genome-wide ddRADseq markers to evaluate the evolutionary relationships between these species. The results corroborated early findings that each ecoregion harbors an independent evolutionary lineage, and that all individuals in ETM correspond to *G. lacustris*. Our results do not show significant genetic structure between riverine and lacustrine populations in ETM. However, we found evidence for secondary contact between *G. labiatus* and the riverine population of *G. lacustris*, suggesting that the hypertrophied lips in both groups may have a common genetic background, and may indicate an instance of adaptive introgression.

Keywords: Adaptive introgression, ddRADseq, Ecological divergence, Phylogeography, Morphological convergence.

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Gymnogeophagus labiatus e *G. lacustris* representam um par de espécies irmãs distribuídas nas ecorregiões da Laguna dos Patos (ELP) e Tramandaí-Mampituba (ETM), no sul do Brasil e no Uruguai. Enquanto *G. lacustris* tem sido considerada endêmica da ETM, *G. labiatus* tem sido associada a ambas ecorregiões, sendo distinta de *G. lacustris* por seus lábios hipertrofiados, que representam uma adaptação para o forrageio em ambientes rochosos, além de variação na coloração. Um estudo recente que usou dados morfológicos e de mtDNA questionou essa interpretação, sugerindo que *G. labiatus* seria exclusivo da ELP, e que todos os indivíduos na ETM deveriam ser considerados *G. lacustris*. Nesse trabalho, usamos marcadores genômicos ddRADseq para avaliar a relação evolutiva entre essas espécies. Os resultados corroboraram achados anteriores de que cada ecorregião abriga uma linhagem evolutiva independente, e que todos os indivíduos na ETM correspondem a *G. lacustris*. Nossos resultados não evidenciam uma estrutura genética significativa entre as populações lacustres e fluviais na ETM. Entretanto, encontramos evidências de um contato secundário entre *G. labiatus* e as populações fluviais de *G. lacustris*, sugerindo que os lábios hipertrofiados em ambos os grupos podem ter uma base genética comum, podendo indicar um exemplo de introgressão adaptativa.

Palavras-chave: Convergência morfológica, Divergência ecológica, ddRADseq, Filogeografia, Introgressão adaptativa.

INTRODUCTION

Assigning individuals into species' categories is still one of the most debated issues in the biological sciences. One of the main reasons is the lack of a single concept that could define "species" and delimit these taxa objectively, consistently, and in a biologically meaningful way for all living beings (Hey, 2001; De Queiroz, 2007; Zachos, 2018). In the case of freshwater fishes, while the geographical isolation imposed by different drainages constitutes obvious barriers to gene flow that might align with different evolutionary lineages (or species), shared morphological features may indicate that the same species occurs in different isolated drainages. However, one must be aware that morphological convergence and phenotypic plasticity can represent challenges to the proper identification of taxonomic species, as the former makes similar (but convergent) morphologies to be present in different drainages, and the latter results in different morphologies within the same genealogical lineage (see Figueiredo *et al.*, 2021). These issues may be even more complicated in recent timescales, because it is difficult to distinguish between phenotypic plasticity and incipient speciation in ecological gradients, originating the so-called gray zones of speciation (De Queiroz, 2007; Roux *et al.*, 2016)

Cichlidae is one of the most diverse vertebrate families in the world, famous for being extremely diverse in terms of body shape and coloration pattern, presenting extraordinary cases of evolutionary parallelism in ecological morphologies. Convergent phenotypic evolution is generally interpreted as a strong indication of adaptive evolution

(Burriss, 2015). Parallelism has already been documented in the main adaptive radiations of African and Neotropical cichlids (Henning, Meyer, 2014) and involves features with a known adaptive function, such as hypertrophic lips (which facilitate larval foraging in rocky crevices) and traits under sexual selection (e.g., body coloration; Urban *et al.*, 2022) and lip size itself (Machado-Schiaffino *et al.*, 2014). Interestingly, it has been shown that lip size evolution in cichlids depends on either genetic (Masonick *et al.*, 2022) and environmental components (Machado-Schiaffino *et al.*, 2014).

There are currently 1,747 valid species in this family, 563 of which belong to the Cichlinae subfamily of Neotropical fish (Fricke *et al.*, 2024). The tribe Geophagini, which forms a clade within Cichlinae, represents approximately 18 genera and 250 species distributed across South America and southern Panama (López-Fernández *et al.*, 2010). Among these, *Gymnogeophagus* Miranda Ribeiro, 1918 contains 20 valid species (Turcati *et al.*, 2018; Alonso *et al.*, 2019) whose synapomorphies are the presence of a forward-directed spine at the top of the first dorsal pterygiophore and the lack of supraneurals, unique among Neotropical cichlids (Reis, Malabarba, 1988). Representatives of this genus are distributed in Uruguay, Paraguay, Argentina, and in the Brazilian states of Santa Catarina and Rio Grande do Sul (Malabarba *et al.*, 2015; Loureiro *et al.*, 2016; Casciotta *et al.*, 2017), occurring in a series of distinct environments, with varying degrees of ecological specialization.

In the Tramandaí-Mampituba Ecoregion (Ecorregião Tramandaí-Mampituba, in Portuguese, henceforth ETM) (Abell *et al.*, 2008) Reis, Malabarba (1988) recognized two species with distinct ecological habits: *Gymnogeophagus labiatus* (Hensel, 1870), and *Gymnogeophagus lacustris* Reis & Malabarba, 1988. ETM has an extensive area covered with diverse types of water bodies (freshwater lakes, lagoons, estuaries, and rivers) that can be divided into two main sub-regions: the rivers and streams of the Serra Geral slope, and the rivers and lagoons of the Coastal Plain of Rio Grande do Sul (PCRS). While the former sub-region occupies deep valleys eroded by basaltic flows dating back some 133 million years ago (mya) (Renne *et al.*, 1992), the latter was formed over the last 400,000 years (Villwock *et al.*, 1984; Tomazelli, Villwock, 2000) and includes the mouth of rio Maquiné at lagoa dos Quadros, the mouth of rio Três Forquilhas at lagoa Itapeva, a series of interconnected lagoons, rio Tramandaí, isolated lakes, wetlands and temporary flood fields (Malabarba, Isaia, 1992). Most lakes in ETM are younger than the Last-Glacial Maximum and found its current configuration only in the Holocene (Tomazelli, Villwock, 2000). The high biological diversity of species observed in the ETM corresponds to around a quarter of the freshwater fish species found in the entire state of Rio Grande do Sul. This high species' richness is possibly related to the diversity of environments available as well as the distinct historical origin of its components (Langeani *et al.*, 2009; Malabarba *et al.*, 2013).

On the other hand, in the adjacent Patos Lagoon Ecoregion (Ecorregião Laguna dos Patos, in Portuguese, henceforth ELP) (Abell *et al.*, 2008), there is only *G. labiatus* (Reis, Malabarba, 1988). The ELP represents a much larger (compared to ETM) drainage flowing into the Atlantic Ocean, composed by a complex of 10 river basins in Brazil plus the tributaries of laguna Mirim (or Merín) drainage in Uruguay. This complex includes laguna dos Patos itself, the tributaries that flow directly into the lagoon, such as rio Camaquã, lago Guaíba, rio Jacuí and its tributaries, and laguna Mirim, irrigating a considerable portion of Rio Grande do Sul, as well as the northeast of Uruguay (Becker *et al.*, 2013).

Both species have marked sexual dimorphism, and before the reproductive season the males develop a gibbosity, also known as nuptial (or nuchal) hump, in the anterior region of the head, used for display during the reproductive period or as a fat reserve for the period of caring for the eggs and fry (Lowe-McConnell, 1999). Among the characters described in the literature to differentiate the species, the hypertrophied lip of *G. labiatus* stands out. Another major difference refers to the coloration characteristics. *G. lacustris* has a few bright blue spots on the cheek, usually aligned with the series of infraorbital bones, the lip of mature males in the nuptial period is markedly dark orange and the anal fin sometimes has rounded spots. *G. labiatus* also has bright blue spots on the cheeks, but in greater quantity than those observed in *G. lacustris*, often aligned in the infraorbital series, and most of the scales on the flank with bright blue spots (Reis, Malabarba, 1988). *Gymnogeophagus gymnogenys* (Hensel, 1870) is the only other mouth brooder species of the genus that occurs sympatrically with both *G. labiatus* and *G. lacustris*. It is not, however, closely related to the clade formed by those two species. Instead, it is deeply inserted in a large clade containing all the 15 remaining mouth brooder species of the genus (Řičan *et al.*, 2019).

Recently, morphological and genetic data have questioned how accurately the current taxonomy reflects the evolutionary history of *G. labiatus* and *G. lacustris* (Figueiredo *et al.*, 2021). An analysis using mitochondrial (mtDNA) markers was unable to differentiate between *G. lacustris* and *G. labiatus* from ETM, although both were distinct from *G. labiatus* from ELP. The authors also observed extensive haplotype sharing within the ETM and dated the separation between *G. labiatus* and *G. lacustris* in the ETM as no older than the last glacial maximum, while the separation between *G. labiatus* ETM and ELP populations dated back to the Pliocene (~3.6 mya). Morphological data showed significant differences between *G. labiatus*, *G. labiatus* ETM and *G. labiatus* ELP, although no feature was able to differentiate these three lineages simultaneously. Two potentially adaptively relevant traits showed important differences among groups. Lip size was significantly larger for *G. labiatus* (in both ELP and ETM), while color pattern was significantly different between ELP and ETM (both *G. labiatus* and *G. lacustris*). However, lip size variation within ELP (where only *G. labiatus* occurs) was comparable to that seen in ETM as a whole (where, traditionally, both species occur). This study concluded that ecoregions reflect the evolutionary history of these lineages more accurately, and that the ETM populations should be considered a single taxonomic species, due to the importance of color pattern in sexual selection and subsequent reproductive isolation, while differences in lip morphology would be better understood as phenotypic plasticity (Fig. 1; Figueiredo *et al.*, 2021).

However, mtDNA has a limited power in distinguishing independent genealogical units. First, because of its non-recombining nature, mtDNA represents a single locus, such that its specific genealogical structure may be due to chance alone (*i.e.*, random genetic drift), introgression, or other evolutionary processes that are not representative of the genealogical structure of these terminals at the genome level (Boussau, Scornavacca, 2020). Second, Figueiredo *et al.* (2021) have shown that riverine and lacustrine populations in ETM have significant differences in lip morphology. Even if they interpret mtDNA data as suggesting that all individuals from ETM are *G. lacustris*, an alternative hypothesis is that riverine and lacustrine populations constitute different genealogical units (species), but that mtDNA did not have enough resolution

to discriminate between them because the lake system in ETM is very recent compared to mtDNA mutation rate (Hickerson *et al.*, 2006).

In the present study, we use a much larger, genome-wide data to test the speciation scenario proposed by Figueiredo *et al.* (2021) and to investigate the relationship between the riverine (thick lip) and lacustrine (thin lip) ecomorphs in ETM. More specifically, we ask first, do ETM and ELP represent different evolutionary lineages (corresponding to *G. lacustris* and *G. labiatus*, respectively, as defined by Figueiredo *et al.*)? Second, do riverine and lacustrine ecomorphs in ETM correspond to distinct genealogical units? Third, does genome-wide data support the notion that lip-size variation only reflects phenotypic plasticity?

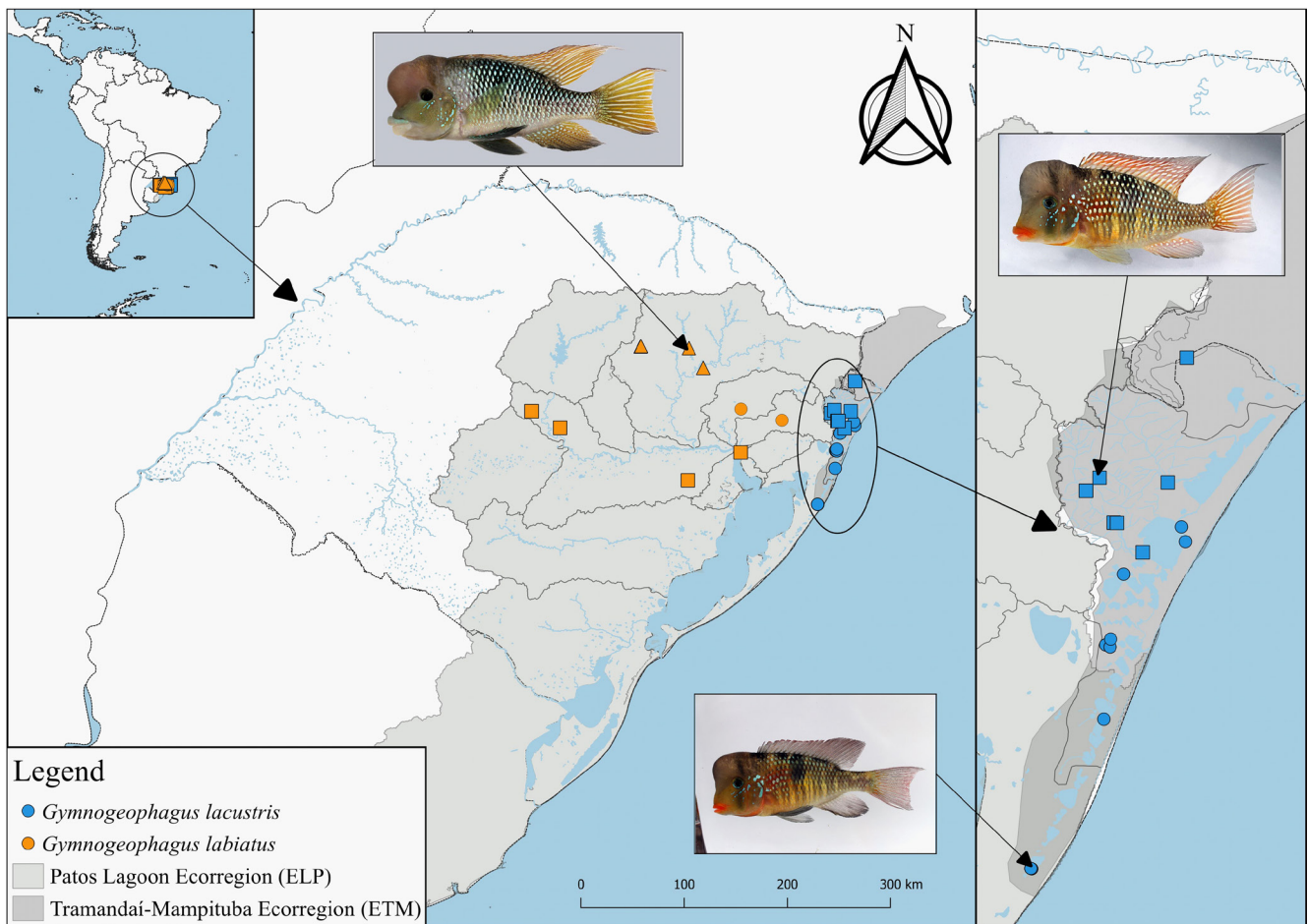


FIGURE | 1 | Map with the distribution of the individuals used in the present study. The hydrographic basins corresponding to ELP and ETM are indicated in different shades of gray. The gray lines represent the watershed between basins or sub-basins in the study area. Sample sites for *Gymnogeophagus labiatus* are shown in orange (TA – triangles; DC – squares; SC – circles). Sample sites for *G. lacustris* are shown in blue (ETMR – squares; ETML – circles) Species’ distributions according to Figueiredo *et al.* (2021).

MATERIAL AND METHODS

Sampling and genomic data. All individuals used in the study were deposited in the scientific collection of the Laboratory of Ichthyology, Departamento de Zoologia, Universidade Federal do Rio Grande do Sul, Porto Alegre (UFRGS). Taxonomic identification of all specimens was based on their external morphology (Reis, Malabarba, 1988) and mitochondrial data (Figueiredo *et al.*, 2021). In other words, all individuals from ETM were considered *G. lacustris*, and all individuals from ELP were considered *G. labiatus*. These ecoregions thus represent the two major units we tested for genome-wide genealogical distinctiveness. Within ELP, we defined distinct (genetic) populations based on the results of the genetic structure analyses (Tab. S1), which were then used in downstream analyses. Within ETM, for which the overall genetic structure was much lower (see Results), we considered individuals coming from riverine (ETMR) and lacustrine (ETML) environments as distinct (ecological) populations, as this was also consistent with the morphological difference in lip size between them (Tab. S1). Overall, we used 60 individuals (44 *G. lacustris* and 16 *G. labiatus*) for the genomic analysis (Fig. 1; Tabs. 1, S1).

TABLE 1 | Individuals and locations of *Gymnogeophagus labiatus* and *G. lacustris* included in the present study. ¹Population labels in Ecorregião Tramandaí-Mampituba (ETM) correspond to ecological populations (*i.e.*, riverine (ETMR) or lacustrine (ETML) sites), while in Ecorregião Laguna dos Patos (ELP), population labels indicate genetic populations as defined in genetic structure analyses. ²Raw sequence data is provided for all individuals as SRA, but only those passing the filters for coverage/missing were included in the datasets analyzed in this study. *These individuals have originally been recognized as *G. labiatus* according to Reis, Malabarba (1988), but see Figueiredo *et al.* (2021) and the present study for a discussion of their taxonomic status. Populations: DC = Depressão Central, SC = Sinos-Caí, TA = Taquari-Antas.

Species	Ecoregion	Population ¹	Drainage	Localities	Sample size ²	
<i>G. lacustris</i>	ETM	ETML	Lagoa Bacopari	1	4	2
<i>G. lacustris</i>	ETM	ETML	Lagoa Corvina	1	7	1
<i>G. lacustris</i>	ETM	ETML	Lagoa dos Quadros	3	3	2
<i>G. lacustris</i>	ETM	ETML	Lagoa da Fortaleza	1	1	1
<i>G. lacustris</i>	ETM	ETML	Lagoa Emboaba	1	4	2
<i>G. lacustris</i>	ETM	ETML	Lagoa Emboabinha	1	1	1
<i>G. lacustris</i>	ETM	ETML	Lagoa da Pinguela	2	6	4
<i>G. lacustris</i> *	ETM	ETMR	Rio Maquiné	6	13	10
<i>G. lacustris</i> *	ETM	ETMR	Rio Três Forquilhas	1	2	1
<i>G. lacustris</i> *	ETM	ETMR	Rio Mampituba	2	3	1
<i>G. labiatus</i>	ELP	DC	Bacia do baixo rio Jacuí	2	3	2
<i>G. labiatus</i>	ELP	DC	Bacia do alto rio Jacuí	1	1	1
<i>G. labiatus</i>	ELP	DC	Bacia do lago Guaíba	2	3	3
<i>G. labiatus</i>	ELP	TA	Bacia do rio Taquari-Antas	3	5	5
<i>G. labiatus</i>	ELP	SC	Bacia do rio dos Sinos	1	2	2
<i>G. labiatus</i>	ELP	SC	Bacia do rio Caí	1	2	2

ELP includes all the interconnected rivers and lagoons that drain into the laguna dos Patos estuary. ETM includes all the interconnected lakes that ultimately drain into rio Tramandaí and can be further divided into two subsystems of interconnected lakes (Schwarzbold, Schäfer, 1984): one north of laguna Tramandaí (formed by rio Tramandaí itself, lagoa Itapeva, lagoa dos Quadros, and a set of small lakes in the municipality of Osório), and one to the south (including a series of interconnected lakes that reach lagoa da Porteira). We also included individuals sampled from isolated coastal lakes (lagoa Bacopari and lagoa Corvina) that are currently mapped to ELP (Abell *et al.*, 2008) to understand the origin of their fish fauna. Based on the results considering the whole sample, these individuals were later included in the ETM dataset (see Results).

For molecular analyses, genomic DNA was obtained using a method adapted from Doyle, Doyle (1987) based on cetyltrimethylammonium bromide (CTAB), and extractions were stored at -20°C . After verification of genomic DNA concentrations, DNA quality was quantified with the Qubit™ 3 fluorometer. Subsequently, the libraries were constructed using only samples with concentrations $\geq 30\text{ng}/\mu\text{l}$. Two RADSeq libraries were constructed following the double digestion protocol of Peterson *et al.* (2012). Each library contains 30 individuals of both species of *Gymnogeophagus*. Succinctly, DNA was double digested with two restriction enzymes (*SphI* and *MluCI*), followed by a PCR ligation and amplification step, where unique barcodes (5 bp) and Illumina adapters were added to the digested DNA so that individuals could be grouped. The PCR products were cleaned, and fragments between 200–300 bp were selected (target size: 250 bp) from the pooled DNA after agarose gel electrophoresis by slicing the gel and recovering the DNA using magnetic beads. Libraries were sequenced on an Illumina HiSeq 2500, targeting single-end 100 bp reads. Sequencing generated approximately 140 million total reads per library that passed initial quality control at the sequencing facility.

We used the same filtering steps to create three SNP datasets, one for ETM, one for ELP and a general dataset including individuals from both ecoregions. Initially, raw reads were de-multiplexed and filtered using the *process_radtags.pl* script in STACKS v. 2.64 (Catchen *et al.*, 2011, 2013) using the default configurations, and reads with Phred < 33 were discarded. We also used STACKS to assemble putative loci from demultiplexed reads. Next, we used USTACKS to reassemble the reads from each individual, which allows the detection of SNPs in each locus. We used a minimum depth coverage of three to create a stack ($-m\ 3$), allowing a maximum distance of two nucleotides among stacks ($-M\ 2$). The Removal ($-r$) and Deleveraging ($-d$) algorithms, were used to remove overrepresented stacks and resolve over merged loci, respectively, with the equal model type bounded ($--model_type$), and an error bounded for ϵ of 0.1 ($--bound_high$) (Catchen *et al.*, 2013). From the sample processed by USTACKS a catalog of genomic sequences (*i.e.*, consensus homozygous loci) was constructed in CSTACKS, creating a set of consensus loci by merging the alleles. Finally, the loci for all individuals were matched to the assembled contigs using SSTACKS by searching the set of stacks (constructed by USTACKS) against the catalog (created by CSTACKS). The search was run under default options, allowing two mismatches between individuals ($-n\ 2$).

From SSTACKS the POPULATIONS module was run for the three datasets under loose parameters ($-r\ 0\ -p\ 2\ -m\ 5\ --min_maf\ 0\ --max_obs_het\ 0.5$), creating a .vcf output with all SNPs per locus. Loci in the upper 95% quantile of the genetic diversity (*theta*)

distribution were flagged as either potential sequencing artefacts or assembly errors and removed (Thomaz *et al.*, 2017). In addition, the number of SNPs per sequence position was plotted and the 10 positions at the 3' end with an inflated number of SNPs (compared to other positions) were manually trimmed. A *whitelist* was created identifying the selected SNPs, and the POPULATIONS program was run again, exporting the new filtered vcf file.

The program PLINK 2 (Purcell *et al.*, 2007) was then used to test the effect of missing data on the number of retained SNPs for each dataset. The full dataset was checked for missingness per SNP (--geno) and individual (--mind). Among the tested levels of missing data, all other analyses were performed with a dataset containing a maximum of 25% missing data per unlinked SNP and 10% per individual. These parameters were chosen to minimize the number of excluded individuals and maximize the number of SNPs retained. A final run of the POPULATIONS module was executed *blacklisting* loci with exceeding values for missing data. The --write-single-snp flag was used to take only one SNP per loci to avoid retaining loci in obvious linkage disequilibrium (LD). We generated three vcf files: one with all individuals (I), from which the other two datasets were derived, with individuals from ETM (II) or ELP (III) exclusively. The vcf file containing all retained SNPs can be found in <https://10.5281/zenodo.11477978>. Raw sequencing data is available from NCBI as Sequence Read Archive (SRA) associated with BioSamples SAMN44088444 – SAMN44088503.

Genetic structure and phylogenetic relationships. We performed a principal component analysis (PCA) to evaluate the general genetic structure in the data. The analysis was made for all datasets using the plink2 software, where the eigenvalues files were generated. The PCA was plotted using ggplot2 package in R v. 4.3.1 (R Development Core Team, 2023). For each dataset, the most likely number of genetic clusters (*i.e.*, *K*-value) was estimated in ADMIXTURE (Alexander *et al.*, 2009) which uses large autosomal SNP genotype datasets to group individuals into genetic populations and provides, for each individual, an estimate of its ancestry coming from each of the *K* genetic clusters. We explored *K*-values ranging from one (a single panmictic population) to *N*, where *N* represents the number of populations in each dataset. The best *K*-value was selected based on a cross-validation procedure (--cv), where the best value of *K* had the lowest cross-validation error value.

We used SVDQUARTETS (Chifman, Kubatko, 2014) to infer the genealogical relationships among populations based on a coalescent population model. The NEXUS file containing the more inclusive SNP dataset was run in PAUP* v. 4.0a166 (Swofford, 2003). The tree resulting from the SVDQUARTETS analysis was rooted according to the mtDNA tree (Figueiredo *et al.*, 2021), assuming that *G. labiatus* and *G. lacustris* represent reciprocally monophyletic groups. The occurrence of gene flow among populations, which is indicated by negative and significant values for the *f*₃-statistic, was tested in the ADMIXTOOLS program (Maier, Patterson, 2024). Finally, basic genetic diversity statistics (*H*_O, *H*_E and *F*_{IS}), as well as pairwise *F*_{ST} values between populations were estimated with the DartT package (Gruber *et al.*, 2018) in R. Genetic diversity analyses considered rivers and lakes in ETM as distinct populations (ETMR and ETML, respectively), as these were considered different species until recently. In ELP, we used the results from genetic structure analysis to identify three populations: Taquari-Antas (TA), including samples from drainages associated with the the Taquari-

Antas drainage, Sinos-Caí (SC), including samples associated with drainages of rio dos Sinos and rio Caí, Depressão Central (DC), including samples from the upper and lower rio Jacuí system, and lago Guaíba.

RESULTS

Three basic data sets were created from the STACKS assemblies. The complete dataset (Dataset I) consisted of 41 individuals and 29,747 variant sites (SNPs). This dataset was used in all subsequent analyses including both ETM and ELP lineages (species). Individuals from ETM and isolated coastal lagoons (see Material and Methods) were included in Dataset II, which consisted of 26 individuals and 16,920 SNPs. Finally, the 15 individuals from ELP were included in Dataset III, containing 26,478 SNPs. These two latter datasets aimed at maximizing the power to detect genetic structure within each drainage (species), considering missing data levels for the relevant individuals in each case.

The PCA for Dataset I showed a clear separation between ETM and ELP individuals associated to PC1 (that contained 35.6% of the total variance), while PC2 (9.98% of the total variance) separated individuals from Taquari-Antas (TA) from other ELP drainages (Fig. 2A). For Dataset II, an initial analysis showed the individual from rio Mampituba very separated from the other samples in PC1 (Fig. S2) When this sample was excluded, PC1 and PC2 retained only 5.93% and 5.5% of the total variance, respectively, and showed no clear separation between ETMR and ETML, which would be expected based on the traditional taxonomic classification that assigned them to different species (Fig. 2B). Finally, Dataset III revealed a separation of individuals from TA associated with PC1 (18.4% of the total variation), while PC2 (10.4% of the total variation) separated individuals from SC apart from those coming from DC) (Fig. 2C).

ADMIXTURE identified $K = 2$ as the best K value for Dataset I. However, the difference in cross-validation error between $K = 2$ and $K = 3$ was very small (0.335 and 0.359, respectively). While for $K = 2$ there was a separation between ecoregions (*i.e.*, ETM *vs.* ELP), with $K = 3$ TA formed a distinct genetic cluster within ELP (Fig. 3A). The consideration of higher K -values resulted in additional genetic components associated to each river in ELP, consistent with a lack of further structure within ETM (Figs. S3, S4, S5). The SVDQUARTETS analysis of all 29,747 SNPs resulted in a fully resolved species tree in which ETM and ELP form reciprocally monophyletic groups with maximum support. Internal relationships were also fully resolved, showing, in ELP, a sister relationship between DC and SC regarding TA (Fig. 3B).

Genetic diversity was highest in SC, followed by DC, TA, ETMR and then ETML (Tab. 2). While F_{IS} values seem a bit larger in TA compared to other drainages, this could result from differences in genetic structure within each drainage rather than differences in inbreeding levels. As expected, pairwise F_{ST} values were higher among populations between ELP *vs.* ETM (which represent different species), ranging between 0.699 (TA x ETMR) and 0.730 (SC x ETML). Within each ecoregion, ELP had higher structure, with F_{ST} values ranging between 0.114 (TA x DC) and 0.287 (SC *vs.* DC), while ETM had the lowest value (0.002, ETML x ETMR) (Tab. 3). The ADMIXTOOLS f_3 -statistic indicated, with strong statistical support, secondary contact between ETMR and the other populations (Tab. 4).

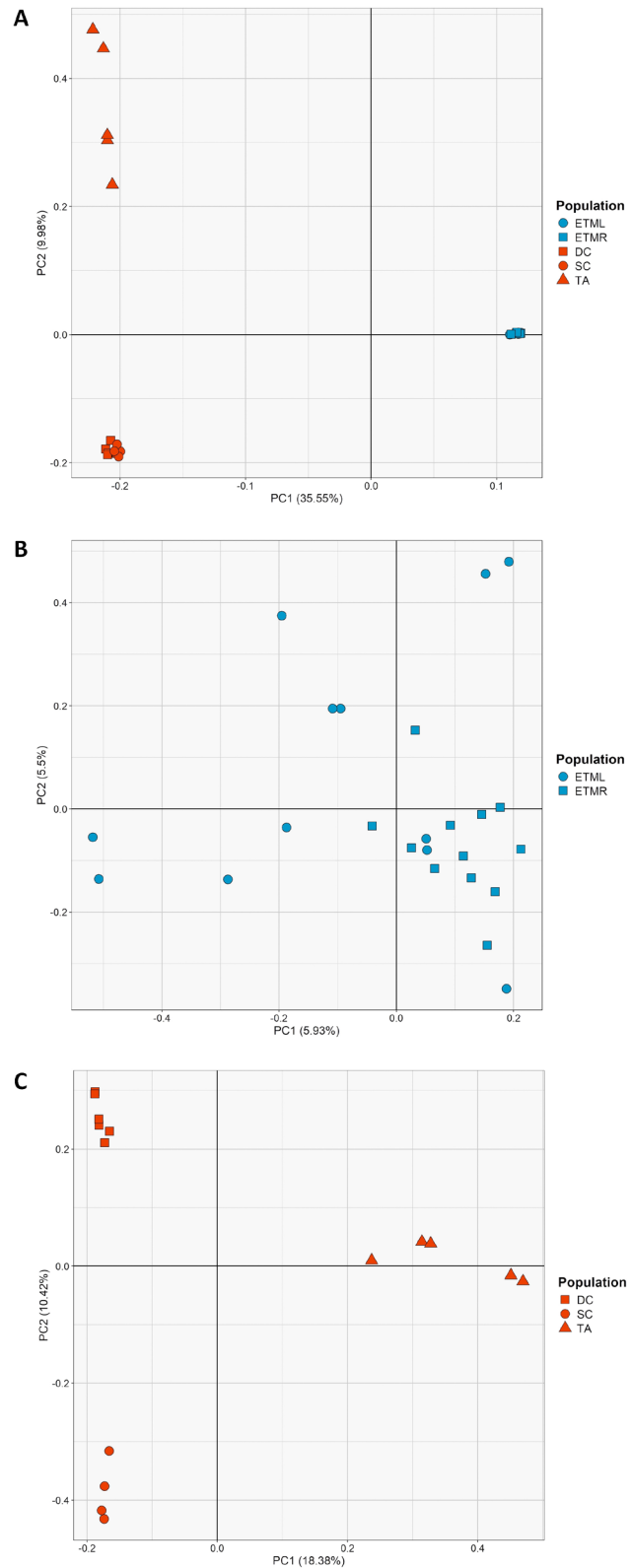


FIGURE 2 | PCA for all datasets. Individuals from *Gymnogeophagus labiatus* are shown in orange, while those from *G. lacustris* are shown in blue. **A.** Dataset I, including both species; **B.** Dataset II, including only *G. lacustris*; **C.** Dataset III, including only *G. labiatus*. See the meanings of the codes in Tab. 1.

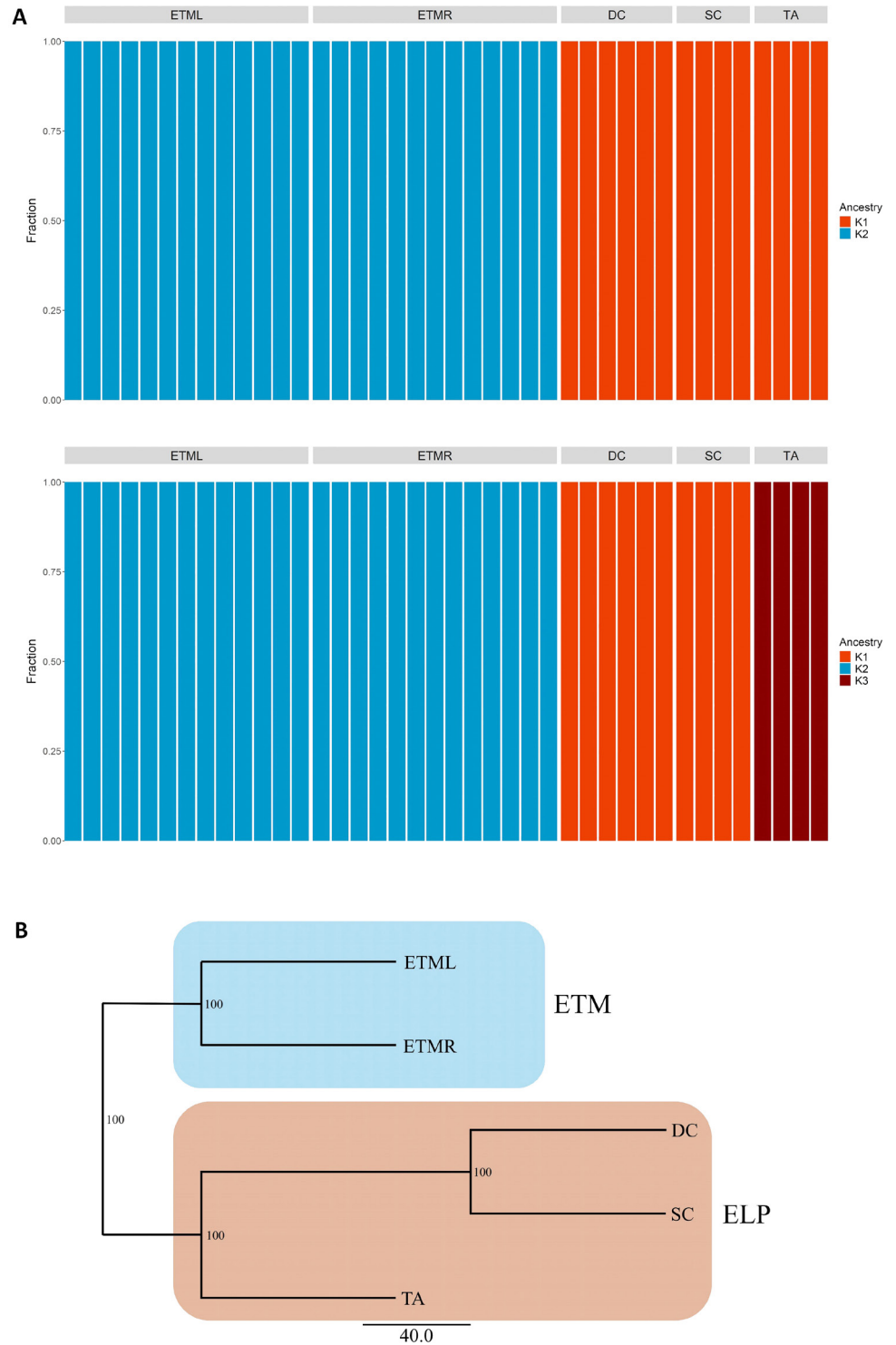


FIGURE 3 | A. Admixture analysis for Dataset I considering $K = 2$ (above) and $K = 3$ (below). Each column corresponds to the different individuals. The fraction of genetic ancestry in each of the K genetic clusters is represented in different colors. **B.** SVDQUARTETS tree based on a population coalescent model. Populations from the same ecoregions are highlighted in different colors. See the meanings of the codes in Tab. 1.

TABLE 2 | Average observed (H_o), expected (H_e) heterozygosity, and the corresponding inbreeding coefficient (F_{IS}) for each population. See the meanings of the codes in Tab. 1.

Lineage	ETM		ELP		
	ETML	ETMR	DC	SC	TA
H_o	0.054	0.055	0.109	0.120	0.086
H_e	0.055	0.058	0.111	0.117	0.101
F_{IS}	0.061	0.09	0.112	0.110	0.246

TABLE 3 | Pairwise F_{ST} values. $P < 0.01$ for all values. See the meanings of the codes in Tab. 1.

Lineage	ETM		ELP		
	ETML	ETMR	DC	SC	TA
ETML	-	-	-	-	-
ETMR	0.002	-	-	-	-
DC	0.706	0.701	-	-	-
SC	0.730	0.724	0.287	-	-
TA	0.705	0.699	0.114	0.265	-

TABLE 4 | Test of population admixture based on the f_3 -statistic. Negative and significant z-scores indicate admixture between Pop1 and Pop2. P-values for positive z-scores were omitted, as they only indicate the amount of genetic drift for each population rather than admixture. See the meanings of the codes in Tab. 1.

Pop1	Pop2	Pop3	f_3	SE	z-score	P-value
ETMR	DC	ETML	-0.000769	0.000133	-5.78	7.27E-09
ETMR	SC	ETML	-0.00076	0.000133	-5.73	1.02E-08
ETMR	TA	ETML	-0.000702	0.000133	-5.27	1.33E-07
ETML	ETMR	TA	0.00084	0.000138	6.08	-
ETML	ETMR	SC	0.000898	0.000137	6.55	-
ETML	ETMR	DC	0.000906	0.000138	6.59	-
SC	DC	ETML	0.00549	0.000496	11.1	-
SC	DC	ETMR	0.0055	0.000495	11.1	-
SC	TA	DC	0.00873	0.000436	20	-
DC	SC	TA	0.0106	0.00042	25.3	-
DC	ETML	SC	0.0138	0.000538	25.7	-
DC	ETMR	SC	0.0138	0.000537	25.8	-
SC	TA	ETMR	0.0165	0.000691	23.9	-
SC	TA	ETML	0.0166	0.000691	24	-
DC	ETMR	TA	0.0216	0.000718	30.2	-
DC	ETML	TA	0.0217	0.000718	30.2	-
TA	DC	ETML	0.0315	0.00085	37.1	-
TA	DC	ETMR	0.0316	0.00085	37.2	-
TA	ETML	SC	0.0348	0.000868	40	-
TA	ETMR	SC	0.0348	0.000867	40.1	-
TA	DC	SC	0.0426	0.000809	52.7	-
ETMR	SC	TA	0.156	0.00183	85.6	-
ETML	SC	TA	0.158	0.00184	86	-
ETMR	TA	DC	0.16	0.00184	86.6	-
ETML	TA	DC	0.161	0.00185	87	-
ETMR	SC	DC	0.167	0.00186	89.8	-
ETML	SC	DC	0.169	0.00188	90.2	-
SC	ETML	ETMR	0.174	0.0019	91.2	-
DC	ETML	ETMR	0.182	0.00194	93.9	-
TA	ETML	ETMR	0.192	0.00198	96.9	-

DISCUSSION

If we take an evolutionary perspective to answer the question “What is a species?”, we could use the Unified Species Concept and state that species are independent evolutionary lineages (Wiley, 1978; De Queiroz, 2007). The key issue, therefore, is how do we recognize which evolutionary lineages are independent enough to be considered true “species”, and why do some data sets but not others support this distinction (Hey, 2001; De Queiroz, 2007). For genetic data, it has become relatively common to use “objective” methods that integrate a set of gene trees to compare, based on some probabilistic method, how alternative classification schemes fit multi-species genealogical models (e.g., Yang, 2015; Jones, 2017). On its turn, morphological differentiation shows another layer in the process of lineage divergence, which may be faster or slower than genealogical divergence, allowing its use as an independent source of evidence to make taxonomic decisions about a given evolutionary process (Carew *et al.*, 2005; Yeates *et al.*, 2011).

In this study, we corroborate the findings of Figueiredo *et al.* (2021) based on mtDNA and morphological data, and show that *Gymnogeophagus labiatus*, as originally defined in Reis, Malabarba (1988), represent a set of paraphyletic lineages in regard to *G. lacustris*. Consequently, from a coalescent point of view, genome-wide SNP data indicate that the two ETM morphotypes (with or without hypertrophied lips, previously considered as representative of *G. labiatus* and *G. lacustris*, respectively) constitute a single evolutionary lineage: *G. lacustris*, while the populations in ELP correspond to *G. labiatus*. This is not unexpected, as there are other sister species pairs that follow the ETM/ELP divide, such as *Diapoma itaimbe* (Malabarba & Weitzman, 2003) *vs.* *D. dicropotamicus* (Malabarba & Weitzman, 2003) (Ito *et al.*, 2022) and *Mimagoniates rheocharis* Menezes & Weitzman, 1990 *vs.* *M. inequalis* (Eigenmann, 1911) (Thomaz *et al.*, 2015). We also found a very distinct pattern of genetic diversity and structure between these two lineages. While *G. lacustris* has virtually no internal genetic structure and low diversity (but see below), *G. labiatus* is both more diverse and more structured, especially considering the Taquari-Antas population.

Figueiredo *et al.* (2021) revealed significant morphological differences among *G. labiatus* and the two ecomorphs of *G. lacustris*, although no measurement was sufficient to discriminate among the three groups simultaneously. As expected from their original taxonomic description (Reis, Malabarba, 1988), lip area was the main trait differentiating *G. labiatus* and the riverine ecomorph of *G. lacustris* from the lacustrine ecomorph. On the other hand, other measurements that showed significant morphological variation (pectoral fin size and snout size) discriminated between *G. labiatus* and *G. lacustris*. The analysis of live coloration further corroborated the differentiation according to river basins, the most striking differences being lip color, which was conspicuously reddish in individuals from *G. lacustris*, and the bright blue spots on the cheeks, which are more numerous in individuals from *G. labiatus*. The differences in coloration between river basins agree with the genomic data that indicates that distinct evolutionary units in each ecoregion. It has already been shown that color diversity among cichlids, especially in male nuptial coloration, is the result of strong sexual and natural selection (Seehausen *et al.*, 1999; Kocher, 2004; Barson *et al.*, 2007). Sexual selection associated with mate choice has a clear relationship with speciation, which has led many to propose that

sexual selection on nuptial coloration is probably one of the main factors driving the diversification of cichlids (Kocher, 2004; Wagner *et al.*, 2012) given its importance as a reproductive isolation mechanism.

The results for lip area are at odds with those obtained for other morphological measurements and genetic (genomic and mtDNA) markers, which could call into question its usefulness in delimiting taxonomic groups. Hypertrophied lips are a rare phenotype, but they have arisen independently in all the main cichlid clades (Burruss, 2015). The development of hypertrophied lips may or may not be associated with a change in diet from the ancestral state, but it is consistently linked to specialization in foraging oriented towards rocky crevices (Baumgarten *et al.*, 2015). In addition, hypertrophied lips are also a polymorphism often associated with incipient species pairs (Elmer *et al.*, 2010; Colombo *et al.*, 2013; Manousaki *et al.*, 2013; Machado-Schiaffino *et al.*, 2014). Its trophic function seems to be associated with foraging in rocky crevices (Baumgarten *et al.*, 2015). The wide variety of habitats found in each river basin could explain the variation in lip size found within and between populations. This could be because both basins are made up of stony-bottomed rivers with lotic waters and strong currents, and sandy-bottomed lakes and lagoons with lentic waters. Thus, variation in lip size could be attributed to phenotypic plasticity, and individuals with hypertrophied lips could be considered as ecomorphs within their respective species (Figueiredo *et al.*, 2021). Phenotypic plasticity of lip size was reported in *Amphilophus labiatus* (Günther, 1864) from Lake Nicaragua, possibly playing an adaptive role in foraging (Machado-Schiaffino *et al.*, 2014). Alternatively, the different ecomorphs of *G. lacustris* could indicate incipient speciation, in which (adaptive) morphological divergence is faster than (neutral) genealogical divergence. Under this interpretation, these ecomorphs could be in the speciation “gray zone” (De Queiroz, 2007; Roux *et al.*, 2016). Recent speciation between riverine and lacustrine habitats in the Tramandaí-Mampituba ecoregion has been recently described for *Microglanis lucenai* Lehmann, Bartzén & Malabarba, 2024 and *M. cibela* Malabarba & Mahler, 1998 (Lehmann *et al.*, 2024).

Unexpectedly, we found evidence for a secondary contact between *G. lacustris* riverine (large-lipped) ecomorph) and all populations of *G. labiatus*. This suggests that large lips in these species may not represent convergence or phenotypic plasticity. Rather, they indicate a shared genetic background in both species, representing a possible case of adaptive introgression (Arnold, Kunte, 2017). The evolutionary history of cichlids is full of admixture and introgression events, many of which have been associated with adaptive radiations (Meier *et al.*, 2017; Svardal *et al.*, 2020; Singh *et al.*, 2022). Interestingly, genetic analysis based on RAD-Seq data suggests that the large lips in *Amphilophus labiatus* from Lakes Nicaragua and Managua also spread via adaptive introgression (Sowersby *et al.*, 2021). Given that all *G. labiatus* populations show evidence of contributing to the riverine ecomorph of *G. lacustris*, a possible scenario is that the secondary contact occurred after the divergence between *G. labiatus* and *G. lacustris*, but before the divergence among *G. labiatus* populations, which, based on the mtDNA scenario proposed by Figueiredo *et al.* (2021) correspond to about ~3.6 mya (95% CI 1.1 mya – 6.0 mya). Genetic introgression does not invalidate the finding that individuals in different ecoregions correspond to distinct species (*i.e.*, independent evolutionary lineages) but offers an alternative explanation for the hypertrophied lips shown by the riverine individuals of *G. lacustris*.

Concerning *G. lacustris*, further studies will be necessary to better understand the level of genetic structure in rio Mampituba populations, and the pattern of gene flow among riverine and lacustrine populations along the ecological gradient river/lagoon. Another fundamental question is whether the signal of introgression between ELP and ETMR is associated to lip morphology. Regarding *G. labiatus*, future work should focus on increasing geographic sampling to include the tributaries of laguna dos Patos south of rio Jacuí, and the tributaries of laguna Mirim. This would be important to understand the evolutionary meaning of the deeply divergent mtDNA lineages in rio Camaquã (a tributary of laguna dos Patos) identified by Figueiredo *et al.* (2021). In particular, the area around these lagunas shows a remarkable diversity of annual killifishes (*e.g.*, Garcez *et al.*, 2020; Volcan *et al.*, 2021), whose diversification may have started as early as ~2.7 mya (Fernandes *et al.*, 2021). However, while killifish ecology favors quick diversification (Garcez *et al.*, 2020), *G. labiatus* need proper riverine connections to, first, originate new populations, and, second, maintain gene flow among them. Differently from the northern part of its distribution, where different subdrainages are ultimately connected to lago Guaíba, southern drainages flew in isolation to the Atlantic Ocean until the emergence of laguna Mirim and laguna dos Patos (around 200,000 and 120,000 years ago, respectively) (Tomazelli, Villwock, 2000; Weschenfelder *et al.*, 2010). In this case, recent divergence times among southern drainages would reflect the effect of the hydrographic connections originated during the Middle/Late Pleistocene, while older divergence times might reflect colonization from river capture events.

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Pedro Ivo C. C. Figueiredo: Conceptualization, Data curation, Formal analysis, Methodology, Writing-original draft, Writing-review and editing.

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Luiz R. Malabarba: Conceptualization, Funding acquisition, Resources, Writing-review and editing.

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ETHICAL STATEMENT

All fishes used in this study were collected in accordance with Brazilian laws, under a scientific collection license to PICCF (ICMBio-MMA 77839-1). The Ichthyology Laboratory at UFRGS has federal permission to keep specimens and tissues in a public collection under our care. Fish collections by lab members were approved by the University's ethical committee (CEUA/UFRGS 24434).

COMPETING INTERESTS

The authors declare no competing interests.

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