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DECIFRANDO A RELAÇÃO EVOLUTIVA ENTRE *Gymnogeophagus labiatus* (Hensel, 1870) E *Gymnogeophagus lacustris* Reis & Malabarba 1988 (CICHLIDAE: GEOPHAGINI)

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Nota à banca

O programa de Pós-graduação em Biologia Animal estabelece como norma, em dissertações em forma de artigo, a presença de uma introdução geral com uma revisão bibliográfica sobre o tema, e os objetivos do trabalho, assim como uma seção final com as conclusões alcançadas.

A dissertação contém um artigo já submetido à revista *Neotropical Ichthyology*. As normas para submissão da revista podem ser encontradas no link: http://www.scielo.br/revistas/ni/iinstruc.htm

Sumário

RESUMO

A “classificação” de indivíduos em espécie ainda é um dos assuntos mais debatidos nas Ciências Biológicas. O gênero *Gymnogeophagus* Miranda Ribeiro, 1918 de ciclídeos neotropicais, contém 18 espécies válidas, sendo as duas espécies, foco deste estudo, reconhecidas há longo tempo como espécies irmãs. *Gymnogeophagus labiatus*, que ocupa rios de fundo rochoso nas bacias da laguna dos Patos (BHP) e do rio Tramandaí (BHT), e *G. lacustris*, que ocupa lagoas costeiras de fundo arenoso na BHT somente. Recentemente um estudo baseado em DNA *barcode*, com o gene mitocondrial Citocromo Oxidase I (COI), não conseguiu separar essas duas espécies na BHT, apontando diferenças genéticas consideráveis entre indivíduos de *G. labiatus* da BHP e BHT. Uma hipótese é que *G. lacustris* divergiu de *G. labiatus* há pouco tempo, de tal forma que o COI, um marcador genético conservado, é incapaz de separar entre estas duas espécies (tal que a classificação taxonômica atual é adequada para o grupo). Alternativamente, *G. labiatus* e *G. lacustris* da BHT podem constituir, de fato, em uma população coesa, igualmente diferenciada das populações na BHP (tal que as diferenças entre bacias hidrográficas distintas refletem melhor a história do grupo). Nesse estudo, foram utilizados dados morfológicos e moleculares para avaliar as duas hipóteses mencionadas acima. Os indivíduos analisados foram provenientes da Coleção Científica do Laboratório de Ictiologia da UFRGS. Foram encontrados três clados mitocondriais: o primeiro composto por haplótipos distribuídos entre *G. labiatus* e *G. lacustris* da BHT, sendo dois desses compartilhados entre espécies. Os outros dois clados ocorram em indivíduos de *G. labiatus* da BHP, sendo um deles restrito à sub-bacia do Camaquã (SBC), onde foram encontrados indivíduos dos dois clados. As análises morfológicas agruparam os indivíduos da BHT separadamente da BHP, especialmente em relação ao tamanho da nadadeira peitoral, comprimento do focinho e padrão de coloração quando vivo. A área do lábio, considerada a principal medida diagnóstica para as duas espécies, apresentou plasticidade acentuada tanto na BHP como na BHT, questionando sua utilidade taxonômica. No geral, os resultados corroboram a estreita relação evolutiva entre as populações da BHT, sugerindo que os indivíduos que ocorrem nos rios da BHT devem ser interpretados como ecomorfos de *G. lacustris* com lábios hipertrofiados. Assim, os resultados sugerem que as populações da BHT são todas pertencentes a *G. lacustris*, enquanto *G. labiatus* é restrita à BHP.

Palavras-chave: Análise morfométrica, DNA mitocondrial, *Gymnogeophagus*, ciclídeos neotropicais.

ABSTRACT

Classifying individuals in "species" is among the most disputed issues in Biological Sciences. *Gymnogeophagus* Miranda Ribeiro 1918, a genus of Neotropical cichlids, contains 18 valid species, with the two species studied herein long hypothesized as sister species: *G. labiatus*, which occupies rocky bottom rivers in the Patos Lagoon (BHP) and Tramandaí river (BHT) drainages, and *G. lacustris*, which inhabits sandy bottom coastal lagoons in BHT. Recently, a DNA barcode study based on mitochondrial Cytochrome Oxidase I (COI) was unable to discriminate between these species in BHT but pointed to considerable genetic differences between *G. labiatus* individuals in BHP and BHT. One hypothesis is that *G. lacustris* diverged from *G. labiatus* recently, such that COI, a conserved genetic marker, is unable to discriminate between these species (such that the current taxonomic classification is adequate for this group). Alternatively, *G. labiatus* and *G. lacustris* in BHT may indeed constitute a cohesive population, equally differentiated from the population in the BHP (such that differences between distinct hydrographic basins provide a better portrait of the group's history). In this study, we used morphological and molecular data to evaluate the two hypotheses mentioned above. Analyzed individuals are housed in the scientific collection of UFRGS Ichthyology Laboratory. We found three mitochondrial clades: the first containing haplotypes distributed among *G. labiatus* and *G. lacustris* from BHT, with two haplotypes shared between species. The other two clades occurred in *G. labiatus* individuals from BHP, one of which being restricted to the Camaquã river sub-basin (CRS), where we found individuals from both clades. Morphological analyses grouped individuals from the BHT separately from the BHP, especially concerning the length of the pectoral fin, snout length and color in life. Lip area, considered as the main diagnostic trait between species, showed high plasticity within both the BHP and BHT, questioning its taxonomic usefulness. Overall, our results corroborate the close evolutionary relationship between species in BHT, suggesting that individuals occurring in BHT rivers must be interpreted as *G. lacustris* ecomorphs showing hypertrophic lips. Thus, our results suggest that all populations in BHT belong to *G. lacustris*, while *G. labiatus* is restricted to the BHP.

Keywords: Morphological analyses, Mitochondrial DNA, *Gymnogeophagus*, Neotropical cichlids

INTRODUÇÃO GERAL

Conceitos de Espécies e sua delimitação

A classificação de indivíduos na categoria de "espécie" é, ainda, um dos assuntos mais debatidos nas Ciências Biológicas. Atualmente existem um pouco mais de 34 conceitos de espécies (Zachos, 2016). Um dos principais problemas é a não existência de um conceito único com o qual seja possível, simultaneamente, definir a categoria de espécies e de delimitar esses táxons de uma maneira objetiva, consistente e biologicamente significativa em todos os seres vivos (Zachos, 2018).

Conceitos de espécies não apenas definem o que é uma espécie, mas, também esclarecem o que é especiação. Mas afinal, o que é uma espécie? Um dos conceitos de espécie mais populares é o Conceito Biológico de Espécie, que afirma que as espécies são grupos de organismos que não se cruzam com outros grupos desse tipo (Mayr, 1963). Essa definição se concentra no isolamento reprodutivo para explicar a causa da divergência entre grupos que culmina no reconhecimento de grupos distintos. Alternativamente, a divergência inferida através de análises filogenéticas pode ser considerada como o principal critério de delimitação entre espécies (no Conceito Filogenético de Espécie ver Wheeler, 1999). De modo ainda mais geral, podemos considerar conceitos de espécie como o Conceito Evolutivo de Espécie (Wiley, 1978), ou o Conceito Unificado de Espécie, que afirmam que espécies são linhagens evolutivas independentes (De Queiroz, 2007). Estes diferentes conceitos evidenciam o fato de que a discordância entre conceitos de espécie pode ser muito mais sobre o método de diagnosticar espécies do que sobre a ênfase em um determinado momento do processo de especiação em si (Hey, 2001; De Queiroz, 2007).

Na prática, no entanto, a delimitação entre espécies muitas vezes depende do reconhecimento de diferenças morfológicas, em que os indivíduos são categorizados por semelhanças de tamanho, forma e cor. Na maioria dos casos essa definição funciona e é altamente aplicável, principalmente para espécies de ciclídeos, que tipicamente podem ser classificadas pela morfologia externa, pelo padrão de coloração (Burress, 2015), e/ou por comportamentos de um determinado grupo taxonômico (Kocher, 2004). Embora a classificação morfológica possa ser feita sem nenhum pressuposto evolutivo, a diferenciação morfológica pode ser compreendida como uma etapa do processo de divergência entre linhagens, permitindo que dados morfológicos sejam inseridos naturalmente em um sistema de taxonomia interativa que usa várias fontes de dados (morfológicos, genéticos, comportamentais) para tomar decisões taxonômicas acerca de um determinado processo evolutivo (Carew *et al*., 2005; Yeates *et al.*, 2011)

Gymnogeophagus labiatus **(Hensel, 1870) e** ***G. lacustris*** **Reis & Malabarba 1988: o sistema biológico de estudo**

Cichlidae é uma das famílias de vertebrados mais diversas do mundo. Os ciclídeos são famosos por serem extremamente diversos não só quanto à forma do corpo, mas também quanto ao padrão de coloração, apresentando casos extraordinários de paralelismo evolucionário em morfologias ecológicas. Evolução fenotípica convergente é geralmente interpretada como uma forte indicação de evolução adaptativa (Burress, 2015). O paralelismo já foi documentado nas principais radiações adaptativas de ciclídeos africanos e neotropicais (Henning & Meyer, 2014) e envolve características com função adaptativa conhecida, como lábios hipertróficos (que facilitam o forrageamento de larvas em fendas rochosas) e características sob seleção sexual (por exemplo, coloração do corpo). Atualmente existem 1706 espécies válidas para essa família, sendo 569 dessas pertencentes à subfamília Cichlinae, de peixes Neotropicais (Eschmeyer *et al.*, 2018). A tribo Geophagini, que compõe um clado dentro de Cichlinae, é representada por aproximadamente 18 gêneros e 250 espécies que estão distribuídos entre a América do Sul e o sul do Panamá (López-Fernández *et al*., 2010). Dentre esses gêneros está *Gymnogeophagus* Miranda Ribeiro, 1918 que contém 18 espécies válidas atualmente (Eschmeyer *et al.*, 2018), e apresenta como característica sinapomórfica, um espinho dirigido para a frente no topo do primeiro pterigióforo dorsal, única entre os ciclídeos neotropicais (Reis & Malabarba, 1988). Os representantes desse gênero distribuem-se entre os estados brasileiros de Santa Catarina e Rio Grande do Sul, no Uruguai, Paraguai e Argentina (Eschmeyer, 2012), ocorrendo em uma série de ambientes distintos, com diferentes graus de especialização ecológica.

A Planície Costeira do Rio Grande do Sul (PCRS) foi formada a partir de dois diferentes sistemas deposicionais, sendo um deles um sistema de leques aluviais, e, o outro, sistemas do tipo Laguna-Barreira (Villwock *et al*., 1986). Sistemas do tipo Laguna-Barreira são caracterizados por um pico transgressivo seguido de um evento regressivo que resulta na formação da barreira de relevo. No caso da PCRS, quatro sistemas Laguna-Barreira ocorreram ao longo dos últimos 400 mil anos atrás (ka), sendo o sistema I o mais antigo e o sistema IV o mais recente com ~5 ka (Tomazelli & Villwock, 2000). resultando em duas ecorregiões de água doce: Tramandaí-Mampituba, no litoral norte, e Laguna dos Patos, ao sul (Abell *et al.*, 2008).

A região do litoral norte do estado do Rio Grande do Sul possui uma extensa área recoberta com corpos de água de diversos tipos (lagoas de água doce, lagunas, estuários e rios) que compõem a bacia hidrográfica do rio Tramandaí (BHT), a qual possui uma riqueza considerável de peixes, com alguns endemismos (Langeani *et al.*, 2009). Essa região pode ser dividida em duas sub-regiões principais, os rios e arroios da encosta da Serra Geral e os rios e lagoas da Planície Costeira propriamente dita. Os ambientes de água doce da Serra Geral ocupam vales profundos erodidos em derrames basálticos de cerca de 133 milhões de anos (Renne *et al.*, 1992). Na BHT, os ambientes de água doce da PCRS compreendem a foz do rio Maquiné na lagoa dos Quadros, a foz do rio Três Forquilhas na lagoa Itapeva, uma série de lagoas interligadas desde a lagoa Itapeva ao Norte até a lagoa da Cerquinha ao sul, o rio Tramandaí entre a lagoa dos Quadros e lagoa Tramandaí, lagoas isoladas, as áreas de banhados e os campos de inundação temporária. (Malabarba & Isaia, 1992). Aproximadamente um quarto das espécies de peixes de água doce ocorrentes no Rio Grande do Sul são encontradas na BHT, a menor do estado (Bertaco et al., 2016). Essa, essa riqueza de espécies pode ser relacionada à diversidade de ambientes disponíveis bem como à origem histórica distinta de seus componentes (Malabarba *et al.*, 2013). A bacia hidrográfica da Laguna dos Patos (BHP), onde ocorre cerca da metade das espécies de peixes de água doce do Rio Grande do Sul (Bertaco et al., 2016), é uma grande drenagem fluindo para o Oceano Atlântico, é formada por um complexo de sub-bacias, que abrangem a própria laguna, a lagoa Mirim, os tributários que desaguam diretamente na laguna, como o rio Camaquã, o lago Guaíba, o rio Jacuí e seus afluentes, irrigando uma porção considerável do Rio Grande do Sul, assim como o nordeste da República do Uruguai (Becker *et al.*, 2013).

Gymnogeophagus labiatus habita as regiões das Bacias da laguna dos Patos e do rio Tramandaí (Reis & Malabarba, 1988). Ocorre em ambientes lóticos com água transparente, em pequenos rios e riachos com fundo pedregoso. Na Bacia Hidrográfica do Rio Tramandaí, essa espécie tem registro de ocorrência nos rios Maquiné e Três Forquilhas (Malabarba *et al.*, 2013). A espécie é onívora, alimentando-se de restos de plantas e insetos aquáticos (Selmo, 2010). Já *G. lacustris* ocorre nas lagoas costeiras da bacia do rio Tramandaí, sendo endêmico dessa região (Reis & Malabarba, 1988). A espécie ocorre preferencialmente em ambientes de fundo arenoso sem vegetação ou com pouca vegetação submersa ou emergente (Malabarba *et al*., 2013). *Gymnogeophagus lacustris* é insetívora, alimentando-se de larvas de insetos e crustáceos (Hartz, Junior, & Formehl, 1998). Ambas as espécies possuem dimorfismo sexual acentuado, sendo que antes da época reprodutiva os machos desenvolvem uma gibosidade (corcova) na região anterior da cabeça, utilizada para exibição durante o período de corte ou como reserva de gordura para o período de cuidado dos ovos e dos alevinos (Lowe-McConnell, 1999). Dentre os caracteres descritos na literatura para diferenciação das espécies pode-se destacar o lábio hipertrofiado apresentado por *G. labiatus*. Outra diferença importante se refere às características de coloração. *Gymnogeophagus lacustris* apresenta poucos pontos azuis brilhantes na bochecha, geralmente alinhados na série de ossos infra-orbitais, o lábio dos machos maduros em período nupcial é marcadamente laranja-escuro e a barbatana anal às vezes apresenta pontos arredondados. *Gymnogeophagus labiatus* também apresenta pontos azuis brilhantes nas bochechas, mas em maior quantidade do que os observados em *G. lacustris*, muitas vezes alinhados na série infra-orbital e a maioria das escamas no flanco com pontos azuis brilhantes (Reis & Malabarba, 1988).

Recentemente, uma análise utilizando o gene mitocondrial Citocromo Oxidase I (COI), gene utilizado como um método de identificação e descobrimento de espécies (DNA *Barcode*), não foi capaz de diferenciar populações de *G. labiatus* e *G. lacustris* da BHT, embora tenha havido diferenças entre essas populações e *G. labiatus* da BHP (L. R. Malabarba e colaboradores, dados não publicados). Esses resultados sugerem uma separação genética mais relevante por bacia hidrográfica do que por categoria taxonômica. Duas hipóteses podem ser levantadas para explicar esses achados. Ou *G. lacustris* representa o resultado de um evento de especiação recente nas lagoas da PCRS devido à ocupação de habitats geologicamente recentes, ou as espécies da BHT constituem uma única linhagem evolutiva cujas diferenças morfológicas (relevantes para taxonomia) são mais bem interpretadas como uma plasticidade fenotípica associada a hábitos ecológicos distintos.

Objetivo Geral

O objetivo geral do trabalho é compreender melhor a história evolutiva de *G. labiatus* e *G. lacustris* para testar hipóteses de diferenciação entre essas duas espécies.

Objetivos Específicos

- Caracterizar a variação genética, em nível de mtDNA, para indivíduos de *G. labiatus* e *G. lacustris* oriundos de diferentes populações geográficas.

- Caracterizar a variação morfológica de indivíduos de *G. labiatus* e *G. lacustris* oriundos de diferentes populações geográficas.

- Testar o grau de diferenciação molecular e morfológica para as espécies reconhecidas tradicionalmente e para os grupos geográficos amostrados.

ARTIGO

Hydrography rather than lip morphology better explains the evolutionary relationship between *Gymnogeophagus labiatus* and *G. lacustris* in Southern Brazil (Cichlidae: Geophagini)

Artigo submetido ao periódico *Neotropical Ichthyology*

Hydrography rather than lip morphology better explains the evolutionary relationship between *Gymnogeophagus labiatus* and *G. lacustris* in Southern Brazil (Cichlidae: Geophagini)

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Abstract

Gymnogeophagus labiatus and *G. lacustris* have been long recognized as sister species exhibiting different ecological requirements. *Gymnogeophagus labiatus* occurs in rock bottom rivers in the hydrographic basins of Patos lagoon (HBP) and Tramandaí river (HBT), while *G. lacustris* is exclusive from sand bottom coastal lagoons of the HBT. In this study, we used molecular markers, morphological measurements and data from nuptial male coloration to investigate the evolutionary relationship between these species in each hydrographic basin. We found, for all data sets, a closer relationship between *G. labiatus* and *G. lacustris* from the HBT than between *G. labiatus* populations from HBT and HBP. In particular, lip area had a large intraspecific plasticity, being uninformative to diagnose *G. lacustris* from *G. labiatus*. Molecular clock-based estimates suggest a recent divergence between species in the HBT (17,000 years ago), but not between *G. labiatus* from HBP and HBT (3.6 millions of years ago). Finally, we also found a divergent *G. labiatus* genetic lineage from the Camaquã river, in the HBP. These results provide convincing evidence for the recognition of a single species from HBT, in which lip morphology polymorphism characterizes ecotypes of *G. lacustris*, whereas *G. labiatus* should include specimens from the HBP only.

Keywords: Ecological divergence, Iterative taxonomy, Mitochondrial DNA, Phenotypic plasticity, Tramandaí-Mampituba ecoregion

Resumo

Gymnogeophagus labiatus e *G. lacustris* vêm sendo consideradas espécies irmãs que possuem diferentes exigências ecológicas. *Gymnogeophagus labiatus* ocorre em rios de fundo de pedra nas bacias hidrográficas do da Laguna dos Patos (HBP) e do rio Tramandaí (HBT), enquanto *G. lacustris* é exclusivo da HBT, ocorrendo em lagoas costeiras de fundo de arenoso. Nesse estudo, foram usados marcadores moleculares, medidas morfológicas e dados sobre a coloração nupcial em machos para investigar a relação evolutiva entre estas espécies em cada bacia hidrográfica. Para todos os conjuntos de dados foi observada uma relação mais próxima entre *G. labiatus* e *G. lacustris* da HBT do que entre as populações de *G. labiatus* da HBP e HBT. Em particular, a área do lábio teve uma grande plasticidade intraespecífica, não sendo informativa para diagnosticar *G. lacustris* de *G. labiatus*. Estimativas baseadas no relógio molecular sugeriram uma divergência recente entre as espécies da HBT (17.000 anos atrás), mas não entre as populações de *G. labiatus* da HBP e HBT (3,6 milhões de anos atrás). Finalmente, também foi encontrada uma linhagem genética de *G. labiatus* divergente no rio Camaquã, na HBP. Esses resultados fornecem evidências convincentes para o reconhecimento de uma única espécie na HBT, na qual o polimorfismo na morfologia labial caracteriza ecótipos de *G. lacustris*, enquanto *G. labiatus* deve incluir apenas os espécimes da HBP.

Palavras-chave: Divergência ecológica, Taxonomia integrativa, DNA mitocondrial, Plasticidade fenotípica, Ecorregião Tramandaí-Mampituba

Running Head: Ecological divergence in *Gymnogeophagus lacustris*

Introduction

Cichlids comprise 1727 valid species showing a wide morphological diversity in the shape of the body and also in its color pattern (Fricke *et al.*, 2020). Many morphological features contributing to such diversity evolved repeatedly in the adaptive radiations of both African and Neotropical cichlids, including characters with known adaptive roles, such as hypertrophic lips, which facilitate foraging in rocky slit, and body color, which has an important role in sexual selection (Henning, Meyer, 2014; Meier *et al.*, 2017). The extensive parallelism at the morphological level and the relatively frequent hybridization events make species delimitation a difficult task in cichlids (Meier *et al.*, 2017; Salzburger, 2018).

About one third of all species valid for the family (569 species) belong to the Cichlinae, exclusive from the Neotropics (Fricke *et al.*, 2020), approximately half of which are represented by the Geophagini, a well-supported clade within Cichlinae with approximately 18 genera and 250 species distributed in South America and southern Panama (López-Fernández *et al.*, 2010). *Gymnogeophagus* Miranda Ribeiro, 1918 contains 19 valid species (Turcati *et al.* 2018; Alonso *et al.*, 2019) showing two synapomorphies: a spine directed forward on top of the first dorsal pterygiophore (unique among Neotropical cichlids), and the absence of supraneurals (Reis, Malabarba, 1988). Representatives of this kind are distributed among the Paraná, Paraguay and Uruguay basins and in small coastal drainages of Uruguay and southern Brazil, with the exception of *G. balzanii*, which also occurs in the Guaporé river, in the Amazon drainage (Malabarba *et al.*, 2015; Loureiro *et al.*, 2016; Casciotta *et al.*, 2017).

Gymnogeophagus labiatus (Hensel, 1870) inhabits rivers in the hydrographic basins of Patos lagoon (HBP) and Tramandaí river (HBT) (Reis, Malabarba, 1988), with additional records from the Mampituba river, an isolated coastal drainage (Malabarba *et al.*, 2013). HBP, as well as HBT (together with the Mampituba river drainage) represent distinct ecoregions for freshwater fishes (Abell *et al.*, 2008), but both comprise a considerable ecological heterogeneity. HBP is a large drainage flowing into the Atlantic Ocean formed by a complex of sub-basins, which encompass the lagoon itself, the Mirim lagoon, the tributaries that flow directly into the lagoon, such as the Camaquã and Jacuí rivers and its tributaries (Becker *et al.*, 2013). Similarly, HBT can be divided into two main ecological subregions (Malabarba, Isaia 1992), the ancient rivers and streams that flow in the Serra Geral slopes, where the Maquiné and Três Forquilhas rivers are the major water courses, and, the lagoons of the Coastal Plain that formed in the last 16,000 years following recurrent episodes of Laguna-Barreira marine transgressions (Schwarzbold, Schäfer 1984; Villwock, 1984; Tomazelli, Villwock, 2000). *Gymnogeophagus labiatus* has been recorded in both basins, associated with lotic environments with transparent water, in rock bottom streams (Reis, Malabarba, 1988) in detriment of the more lentic environments. In contrast, *G. lacustris* Reis & Malabarba, 1988 is restricted to sandy bottom coastal lagoons in the HBT (Reis, Malabarba, 1988), where it shows a preference for environments with little submerged or emergent vegetation or with no vegetation at all (Malabarba *et al.*, 2013). Both species are oral incubators and have marked sexual dimorphism. In the reproductive season, males develop a hump in the anterior region of the head, used for display during the reproductive period or as an energy reserve during the period of parental care (Lowe-McConnell, 1999). These species have been differentiated by the hypertrophied lip presented by *G. labiatus* (Reis, Malabarba, 1988), which likely represents an adaptation for foraging in rocky environments (Elmer *et al*., 2010; Burress *et al*., 2013).

The ecological differences between these species, but their contiguous distribution in the HBT are compatible with different evolutionary hypotheses. For example, one possibility is that *G. lacustris* represents a relatively recent speciation event from *G. labiatus* populations occurring in the slopes of the Serra Geral that colonized and adapted to a lentic environment. In this case, *G. lacustris* and *G. labiatus* populations from HBT must represent recognizable separate lineages (Wiley, 1978; De Queiroz, 2007; Malabarba *et al*., 2020), but HBT populations from both species would be more related and *G. labiatus* paraphyletic in relation to *G. lacustris*. Alternatively, HBT populations could, in fact, constitute a single cohesive evolutionary lineage, so that the morphological differences between species should be better interpreted as ontogenetic and ecological differences between populations. In this case, *G. labiatus* and *G. lacustris* populations from the HBT should be not recognizable as independent evolutionary lineages. Finally, determining the evolutionary relationship between *G. labiatus* populations from HBP and HBT is important to evaluate the impact of hydrographic structure over the genetic and morphological structure in these species. Here, we used genetic markers, morphological measurements and color pattern to discriminate among these alternatives, which may have important taxonomic implications.

Material and Methods

Sampling and laboratory methods. All individuals used in the study are deposited in the scientific collection of the Laboratory of Ichthyology, Department of Zoology, Federal University of Rio Grande do Sul. The taxonomic identification of all specimens was based on their external morphology (Reis, Malabarba, 1988). We used 78 individuals (60 *G. labiatus* and 18 *G. lacustris*) for the morphological analysis, and tissue samples from 61 individuals (36 *G. labiatus* and 25 *G. lacustris*) for the genetic analysis (Tab. 1, Fig. 1).

Specimens were obtained from the laguna dos Patos and from Tramandaí-Mampituba Ecoregions (Abell *et al*. (2008). The first includes all interconnected rivers and lagoons draining to the laguna dos Patos estuary. The second includes all interconnected lagoons draining to the laguna de Tramandaí estuary. This system of lagoons can be further divided into two subsystems of interconnected lagoons: one to the north of the laguna de Tramandaí, formed by the rio Tramandaí itself, lagoa Itapeva, lagoa dos Quadros, and a set of small lagoons in the municipality of Osório, and the second, to the south, including a string of interconnected lagoons reaching lagoa da Porteira (Schwarzbold, Schäfer 1984). We also included individuals sampled from isolated coastal lagoons (lagoa Bacopari and lagoa Corvina) that are presently mapped to the laguna dos Patos ecoregion (Abell *et al*., 2008) in order to understand the origin of its fish fauna.

TABLE 1

FIGURE 1

For the molecular analysis genomic DNA, was obtained using the CTAB method adapted from Doyle, Doyle (1987). Two mitochondrial DNA (mtDNA) markers: Cytochrome B (*cytB*) and Control Region (*D-loop*) were amplified using the polymerase chain reaction (PCR) technique and specific primers (Palumbi *et al.*, 2002; Sivasundar *et al.*, 2001). The amplification reactions were prepared with 0.4 mM dNTP, 1.5 mM MgCl2, 0.5 µM of each primer, 1U Taq Polymerase and 40ng of genomic DNA. Amplification conditions for *cytB* consisted of 94ºC for 5' and 10 cycles of 94ºC for 1', 55ºC (−0.5ºC/cycle) for 1' and 72ºC for 1'30", followed by 30 cycles of 94ºC for 1', 50ºC for 1' and 72ºC for 1'30", with a final extension of 72ºC for 5'. For *D-loop*, amplification conditions were 94ºC for 5' and 10 cycles of 94ºC for 1', 70ºC (−0.5ºC/cycle) for 1' and 72ºC for 1'30", followed by 30 cycles of 94ºC for 1', 65ºC for 1' and 72ºC for 1'30", with a final extension of 72ºC for 5'. The success of the amplification was checked on a 1% agarose gel stained with GelRedTM (Biotium). The PCR products were enzymatically purified with Exonuclease I and Alkaline Phosphatase (ExoSAP) and sequenced by the Sanger method (Ludwig Biotec, Porto Alegre, Brazil). DNA sequencing was carried out in both directions (forward and reverse) for *cytB*, and only in the forward direction for the *D-loop* due to the presence of a repetitive region close to the annealing site of the reverse primer that caused poor reading quality. DNA sequencing was repeated from independent PCR amplifications whenever necessary to resolve ambiguities. We used Geneious 11.1.2 program (http://www.geneious.com/) to check the quality of the chromatograms and to assemble the consensus sequence for all individuals. The sequences were automatically aligned using ClustalW (Thompson *et al.*, 1994) and edited in BioEdit (Hall, 1999). Sequences produced in the present study are available from the GenBank (*cytB*: MZ667483 - MZ667543; *D-loop*: MZ667544 - MZ667609).

Molecular data analysis. All analyses were performed with the concatenated *cytB* and *D-loop* mitochondrial markers. We retrieved from the GenBank *cytB* and *D-loop* sequences (GU736952 and MG581478, respectively) for *G. setequedas* Reis, Malabarba & Pavanelli 1992, which was used as an outgroup. The list of distinct haplotypes was generated in the DnaSP 5.10.01 software (Librado, Rozas, 2009). The evolutionary relationship between haplotypes was estimated by the *median-joining* method (Bandelt *et al.*, 1999) in the Network program (http://www.fluxus-engineering.com/) and by the Bayesian phylogenetic framework implemented in the package BEAST2 (Bouckaert *et al.*, 2014). The best evolutionary model for each marker was estimated in Partition Finder

(Lanfear *et al.*, 2012) based on the Bayesian information criterion (BIC) (Sullivan, Joyce, 2005), which indicated four different substitution models: one for each *cytB* codon position (K80, HKY; TrN+G) and another for *D-loop* (HKY+I+G). For both markers, we assumed a strict molecular clock model, which is generally well justified for analysis between closely related species (Li, Drummond, 2012). We assumed an evolutionary rate of 0.0024/site/million years (0.0019 - 0.0029/s/My) for *cytB*, which was inferred for Neotropical cichlids using a dataset with *cytB* and *COI* markers and fossil data to calibrate the phylogeny (Tougard *et al.*, 2017). For *D-loop*, we assumed a lognormal prior distribution (M=0.0, S=2.0), so that the posterior for the *D-loop* rate can be estimated relative to the *cytB* rate. We used 100.000.000 steps in the Markov Chain Monte Carlo (MCMC) sampling every 1.000 steps and discarding the first 10.000.000 samples as burnin. We checked for convergence and sampling sufficiency in Tracer 1.7 (Rambaut *et al.*, 2018) ensuring an effective sample size (ESS) >200 for all parameters.

The evolutionary relationship among populations was estimated using the STACEY module (Jones, 2017) implemented in BEAST2 (Bouckaert *et al.*, 2014). In this strategy, individuals are classified into *N* “minimal clusters” *a priori*, and the number of potential species (ranging from one to *N*) is estimated based on the depth of coalescence of the genetic lineages within and between potential species, which is controlled by parameters collapse height (ε) and collapse weight (ω). In other words, the minimal clusters may be merged but not split to form potential species (Jones *et al.*, 2015). Smaller values for ε are more sensitive to recent divergences but may inflate the number of potential species. The parameter ω controls the number of potential species and can be used as a proxy for prior taxonomic knowledge (Matos-Maraví *et al.*, 2019). We assumed minimal clusters of individuals: *G. setequedas*, *G. lacustris*, *G. labiatus* HBT, *G. labiatus* HBP, and *G. labiatus* CSB (from the Camaquã river drainage), which showed a very divergent haplotype (see Results). We used different values for ε between 0.001 and 0.00001 to evaluate its impact over species delimitation. The prior for ω was set using a beta distribution between [0,1], with an initial value of 0.5. Priors for the Yule birth-death model were set to the default distributions. Priors for population size parameters were changed according to the program manual. For *popPriorScale* we used a lognormal prior distribution (M=-4.0, S=2.0, respectively), while for the parameter *popPriorInvGamma* we used a mixture of four gamma distributions (α=1.0 β=1.0). We used a strict clock model and an arbitrary evolutionary rate for *cytB*, which served as a reference for estimating the evolutionary rate for the *D-loop* from a lognormal prior distribution (M=0.0, S=2.0). We used 100.000.000 steps in the Markov Chain Monte Carlo (MCMC) sampling every 1.000 steps and discarding the first 10.000.000 steps as burnin. As before, we checked for convergence and sampling sufficiency in Tracer 1.7 (Rambaut *et al.*, 2018) ensuring an effective sample size (ESS) >200 for all parameters.

We used the Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) to measure the degree of genetic structure among groups based on Φ-statistics. For this analysis, we assumed four populations: *G. labiatus* HBP, *G. labiatus* CSB *G. labiatus* HBT and *G. lacustris* (from HBT). We also estimated pairwise population differentiation based on Φ-statistics. All calculations were performed in Arlequin 3.5 (Excoffier, Lischer, 2010) using 10,000 permutations to assess statistical significance.

Morphological data analysis. For the morphological analyzes we use the linear measurements and counts following Malabarba *et al.*, (2015), which are represented in Fig. 2a. The measurements were taken using a pachymeter with a precision of 0.05 mm, and the counts were made under the stereomicroscope. For measuring the lip area we took standardized photographs of all individuals in lateral view and measured the lip area using the software ImageJ (Abramoff *et al.*, 2004) (Fig. 2b). All linear measurements and photographs were taken from the specimens' left side.

FIGURE 2

For the morphological analyses, three populations were considered: *G. labiatus* HBP, *G. labiatus* HBT and *G. lacustris*. *G. labiatus* CSB was not considered because only one individual was sampled. To correct for allometry effects, all linear measurements were divided by the standard body length, while the lip area was divided by the total body area. All measurements were also transformed using logarithms to normalize the scale of variation among variables. All analyzes were performed considering all measurements or only with linear measurements, to check the role of lip area in the morphological differentiation among populations. A principal component analysis (PCA) was performed to estimate the degree of morphological variation present in the sample, and population differentiation was tested using a non-parametric multivariate analysis of variance (PERMANOVA) from the Mahalanobis distance using 9999 replications for the *post-hoc* pairwise test and considering Bonferroni's correction for multiple tests. The degree of differentiation between populations was also estimated using a Discriminant Analysis or

Canonical Variable Analysis (CVA). We used a Kuskal-Wallis test to check for interpopulation differences for the three most informative morphological variables in CVA. All statistical tests were performed using the Past3.2 program (Hammer *et al.*, 2001). Photographs of live specimens were analyzed to assess the color pattern of these groups.

Results

Considering both *cytB* and *D-loop* markers, we obtained a total alignment of 1367bp (773bp, 594bp, respectively), with 92 variable sites distributed among 32 haplotypes for a global haplotype diversity of 0.884 +/- 0.030. We found 36 sites separating HBP and HBT populations, irrespective of the taxonomic affiliation of the specimens. Samples from Bacopari and Corvina isolated lagoons grouped with HBT populations. As shown in the haplotype network (Fig. 3), *G. labiatus* and *G. lacustris* had 10 closely related haplotypes in the HBT, two of which are shared between species, while the remaining haplotypes were observed in a single individual (seven *G. lacustris* and one *G. labiatus*). Concerning the HBP, one individual identified as *G. labiatus* sampled in the Camaquã Sub-Basin (CSB) showed a very divergent haplotype, sister to the clade formed by HBT and the remaining HBP haplotypes, which was considered as a different genetic population in the remaining analyses (Fig. 4). Curiously, in other specimens from the Camaquã river basin, but for which we only obtained DNA sequence for one of the two markers, we found a lineage belonging to the canonical HBP clade, indicating that both mitochondrial linages occur in this drainage (Fig. S1, available only in the online version). The AMOVA corroborated the large genetic structure among populations, which accounted for nearly ~90% of the total variation (Φ_{ST}=0.909, P<0.0001). Pairwise Φ_{ST} values ranged between -0.034 and 0.997 (Tab. 2), with non-significant values between the two species in HBT and for the comparisons involving CSB due to the small sample size for this population.

FIGURE 3

TABLE 2

FIGURE 4

The population tree (Fig. 5) evidenced a very recent relationship between species in the HBT (~17,000 years, 95% CI 1,000 – 53,000 years). However, the divergence between the HBP and HBT clades dated ~ 3.6 Ma (95% CI 1.1 Ma - 6.0 Ma, while the divergence between the clade formed by these populations and the CSB lineage dated ~ 4.7 Ma (95% CI 2.8 Ma - 7.4 Ma). Under a coalescent criterion, The estimated number of putative species suggested, with high support, that *G. labiatus* HBT and *G. lacustris* belong to the same evolutionary lineage, while the other genetic populations of *G. labiatus* would constitute independent evolutionary lineages. These results were consistent regardless of the specific value of ε (Tab. 3).

FIGURE 5

TABLE 3

Because the individual showing the CSB lineage had a small body size (69.74mm), and to avoid having a group with a single individual in the morphological analysis, only individuals sampled in HBT and HBP (except CSB) were included and assigned to one out of three populations: *G. labiatus* HBP, *G. labiatus* HBT and *G. lacustris*. Scale counts showed low variation among populations and, for this reason, were excluded from further analysis. All measures are shown in Tab. 4. The PCA did not show a strong separation among populations, either or not considering lip area in addition to linear measurements (Fig. 6). Considering linear measurements only, PC1 accounted for 39.75% while PC2 accounted for 19.83% of the total variance. Considering lip area, PC1 accounted for 81.37% while PC2 accounted for 6.69% of the total variance. In addition to lip area, other variables with heavy loadings in the PCA were the length of the pectoral fin, the size of the snout and the height of the body. PERMANOVA showed significant differences among populations, regardless of whether or not the lip area was included in the analysis (F=3.359, P=0.0001; F=3.643, P=0.0001, respectively), with all pairs being statistically different from each other in both analyses (P<0.01 for all pairs).

TABLE 4

FIGURE 6

The CVA showed high discrimination among groups, with 89.74% and 87.18% of correct classifications, either considering or not lip area, respectively (Tab. 5). Considering each measure individually, no morphological character was able to differentiate among the three populations simultaneously. However, while the size of the pectoral fin and the size of the snout discriminated populations from HBP or HBT (Fig. 7), lip area and body height differentiated between *G. lacustris* and *G. labiatus* (Fig. 8).

TABLE 5

FIGURE 7

FIGURE 8

The analysis of the color pattern of live reproductive males and females corroborated the differences between hydrographic basins (HBP *vs*. HBT), but do not support the differentiation between *G. labiatus* HBT and *G. lacustris*. All analyzed populations from HBT with or without developed lips showed a similar color pattern (Fig. 9). The lips of fully developed males (nuptials) from HBT populations have a vibrant orange to reddish lip color, regardless of lip size (specimens G vs. I in Fig. 9)). The upper lip had almost always a more vibrant orange hue than the lower lip. Orange pigmentation can be also observed in females (Fig. 9B and D), even though this was not as intense as observed in males.

FIGURE 9

In HBP populations, in contrast, fully developed males (nuptials) have yellow to light orange lips, usually not very distinct from the coloration of nearby areas of head (Fig. 10). The body scales of nuptial males have blueish iridescent marks nearly all over the lateral of body in HBP populations, but are mostly absent in the lateral portion of the belly, behind pectoral fin, and above pelvic fin in HBT populations, making vertical black bars on body clearly visible in that body portion through translucent scales. Another difference is that in HBT populations the iris is mostly blueish in the middle and lower portions, and yellowish in the upper portion, while in HBP populations the iris is mostly black with yellowish marks. The branchiostegal membrane in HBT populations has a conspicuous orange color, being yellowish in HBP populations. No distinguishable color pattern was observed between the fins of nuptial males of HPT and HBP populations. Dorsal fin is reddish showing white or light blue marks arranged its stripes. Anal fin is reddish proximally and dark gray distally, with white or light blue dots, mostly distributed near anal fin base. The caudal fin is brownish to red, with interradial membranes white or slightly blue. Some specimens of the isolated lagoons of the HBT showed no colored fins, but these specimens coexist with colored specimens denotating a local variation (Fig. 9, specimens I, J, respectively).

FIGURE 10

Discussion

Under a broad evolutionary perspective, one can evoke the unified species concept (USC, or general lineage concept (GLC)) and define species as "independent evolutionary lineages" (Wiley, 1978; De Queiroz, 2007). The key issue, therefore, is how do we recognize such independent lineages, given that different sets of data (morphological, genetic, behavioral) can be used to inform about "evolutionary independency" (Hey, 2001; De Queiroz, 2007). For genetic data, it is becoming increasingly common to use objective methods that integrate a set of gene trees to infer, based on some probabilistic model, which sets of individuals or populations can be included in the same evolutionary lineage (*e.g.* Yang, 2015; Jones, 2017). However, it is still controversial how this "objectivity" would impact the traditional description based on morphological characters (see, for example, Leaché, Fujita, 2010; Bauer *et al.*, 2010; Fujita, Leaché, 2010) for a discussion on this issue). Although morphological data can be used in taxonomy without any evolutionary assumption, morphological differentiation can be understood as a step (or stage) in the divergence process between evolutionary lineages (Hey, 2001, De Queiroz, 2007), allowing this kind of data to be fully integrated into an iterative system that uses several data sources (morphological, genetic, behavioral) to test evolutionary distinctness and make taxonomic decisions based on the evolutionary process (Carew *et al.*, 2005; Yeates *et al.*, 2011). If we assume species are separately evolving metapopulation lineages that can be assessed by multiple operational criteria, final decision on the recognition of one or multiple species must be based on the demonstration that two metapopulations constitute or not distinct lineages, and not based on the choice of a preferred operational criteria (Malabarba *et al.* 2020). Our results strongly indicate that the current taxonomy of

G. labiatus and *G. lacustris* do not represent adequately evolutionary lineages in these species. From the genetic point of view, *G. labiatus* presents a paraphyletic set of mitochondrial lineages distributed in three major clades related the major hydrographic basin (or subbasin in the case of CSB). The deep divergence between CSB and the remaining lineages indicate that it could represent a new candidate species. Unfortunately, we were not able to include individuals carrying this lineage in the morphological analyses. Since we detected both the CSB and HBP clades in the Camaquã river, future studies should use genomic and morphological data to perform a thorough comparison between individuals carrying either mtDNA lineage to clarify the evolutionary meaning of such divergent lineage.

A second candidate species would be represented by the HBP clade, which is genetically and morphologically differentiated from the remaining *G. labiatus* from HBT. Finally, a third candidate species would be represented by the HBT clade, which included all individuals from this basin irrespective of its taxonomic affiliation. We found even shared haplotypes between *G. labiatus* HBT and *G. lacustris*. However, even if the analysis suggested that the two HBT populations constitute a single candidate species from a coalescent point of view, we detected significant morphological differences between *G. labiatus* HBT and *G. lacustris*. Thus, these two coalescent candidate species represent three statistically different morphologies. These results could be reconciled under a scenario of recent speciation in which natural selection facilitates the morphological divergence between *G. lacustris* and *G. labiatus*, with a high probability of ancestral polymorphism being shared between species (Jamie, Meier, 2020). The questions that arise, therefore, are: is there morphological evidence to support the differentiation of *G. lacustris* as an independent taxon from *G. labiatus* HBT? Is there morphological justification to consider *G. labiatus* HBP and HBT as a single, cohesive, evolutionary unit?

No single measurement was sufficient to discriminate, simultaneously, among the three groups analyzed for morphology (*G. labiatus* HBP, *G. labiatus* HBT, and *G. lacustris*). As expected by its taxonomic description (Reis, Malabarba, 1988), lip area was the main trait differentiating *G. labiatus* from *G. lacustris*, followed by body height. Other measurements with high loadings in the PCA, instead, (pectoral fin size and snout size) showed a significant difference between HBP (*G. labiatus*) and HBT populations (*G. labiatus* HBT + *G. lacustris*), which was corroborated by the analysis of the color pattern in vivo of nuptial males.

In deciding if separate lineages consist of separate species or structured populations, Malabarba *et al*. (2020) proposed a perspective based on the life history of the taxa – on the traits that can be more prone to differentiation. Cichlids are very diverse with regard to body color and pigmentation, this characteristic has been implicated, in some groups of the family, in female mate choice (Seehausen, van Alphen, 1999) and mimicry (Boileau *et al.*, 2015). The relationship with sexual selection is especially important given its role in evolutionary isolation between emerging lineages. It has been shown that color diversity among cichlids, especially in relation to male nuptial color, is the result of strong sexual and natural selection (Barson *et al.*, 2007; Kocher, 2004; Seehausen *et al.*, 1999). Indeed, the relationship between sexual selection and speciation led some authors to propose that this is probably one of the main factors that drive diversification in cichlids (Kocher, 2004; Wagner *et al.*, 2012). In this context, the difference in color shown by nuptial males from HBT vs HBP can be interpreted as a strong character for the separation between these groups into independent evolutionary lineages. It must be acknowledged that this character is challenging given the impossibility to assess it in museum specimens for which no photographs has been taken from the live individual. However, other measurements (pectoral fin size and snout size) which are available from museum specimens were effective in evidencing the same differences between HBT and HBP.

The development of hypertrophied lips, which has been consistently linked to the specialization in foraging oriented towards rock cracks (Baumgarten *et al.*, 2015), emerged independently in all major cichlid clades (Burress, 2014). Even though hypertrophied lips have been implicated in cases of incipient speciation (Elmer *et al.*, 2010; Colombo *et al.*, 2013; Manousaki *et al.*, 2013), it has also been shown in *Amphilophus labiatus* (Günther, 1864) that there is phenotypic plasticity for lip size, and that this plasticity may have been selected for in this thick lip species, representing a foraging adaptation to different environments and allowing the emergence of thin lip morphs in lentic environments (Machado-Schiaffino *et al.*, 2014). Similarly, *G. lacustris* and *G. labiatus* HBT could be interpreted as ecomorphs, with the difference in lip area between reflecting the phenotypic plasticity of an evolutionary lineage adapted to both lotic (in rock bottom rivers) and lentic (in sandy bottom lagoons) environments. It must be noted that the co-occurrence of these two morphs has been recorded at least in one location (Fig. 9, specimens G and H).

Our results do not refute completely the hypothesis that variation in lip area could indicate a very recent or ongoing speciation between *G. lacustris* and *G. labiatus* HBT. In fact, recent speciation would be expected considering the recent (~5,000 years) geological history of the lagoon complex occupied by *G. lacustris* (Tomazelli, Villwock, 2000), which is in line with the estimated genetic divergence between populations (~17,000 years). Moreover, we used only a single genetic marker (mtDNA), which may result in low power to detect independent "coalescent species" compared to genomic loci (Bernardi, 2013; Nadeau, Kawakami, 2018). However, considering the phenotypic plasticity exhibited by other cichlid species showing hypertrophied lips (Machado-Schiaffino *et al.*, 2014), considering the morphological variation in other characters such as the length of the pectoral fin and the length of the snout, considering the distinct color pattern of nuptial males, and considering the degree of genetic divergence between HBT and HBP, we consider that both populations of the Tramandaí river basin should belong to the same taxonomic group (*G. lacustris*). Thus, *G. labiatus* occur only at the Patos lagoon basin while *G. lacustris* would be restricted to the Tramandaí river basin. Future studies including more individuals in the morphological analyses and considering genomic data will be necessary to clarify the evolutionary relationship between HBT populations, as well as characterize the degree of genetic structure among HBP populations, with special emphasis in the individuals whose mitochondrial lineages belong to the CSB clade.

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Tables legends

Tab. 1. Individuals and locations included in the present study. [1]Sample size for the molecular markers consider individuals characterized for both *cytB* and *D-loop*. [2]Located in the Camaquã river sub-basin.

Tab. 2. Genetic structure (Φ_{ST}) between populations. Values in bold values were statistically significant (P<0.05)

Tab. 3. Delimitation of candidate species under a coalescent criterion. N species = number of candidate species; PP = Posterior probability for a specific arrangement, considering the number of species and which populations make up each group. The letters assigned to each population correspond to all candidate species suggested in the analysis. For a *collapse height* value of 0.001, three alternative arrangements were contained within the 95% credibility range. Note that in all arrangements *G. labiatus* HBT and *G. lacustris* were assigned as the same candidate species.

Tab. 4. Morphological measurements for the analyzed specimens. Min = minimum observed value; Max = maximum observed value; SD = standard deviation; *Values relative to Standard length; **Values relative to Body area; E1 = Scales in a longitudinal series; DL = Scales between the origin of the dorsal fin and the upper part of the lateral line; AL = Scales between the origin of the anal fin and the top of the lateral line.

Tab. 5. Confusion matrix for the CVA of morphological data.

Figure legends

Fig. 1. Map with the distribution of all individuals used in the present study. The hydrographic basins of the Patos Lagoon (HBP) and the Tramandaí River (HBT), as well as the Camaquã Sub-Basin (CSB), are indicated on the map. The gray lines represent the watershed between basins or sub-basins in the study area. The red dots correspond to *G. lacustris*, the green to *G. labiatus* HBT, the yellow to *G. labiatus* HBP and the orange to *G. labiatus* CSB.

Fig. 2. Measurements and counts applied to specimens used in the present study, according to Malabarba *et al*. (2015). 1: Standard length; 2: Body depth; 3: Head length; 4: Dorsal-fin base length; 5: Pectoral-fin length; 6: Caudal peduncle depth; 7: Caudal peduncle length; 8: Eye diameter; 9: Interorbital width; 10: Upper jaw length; 11: Pre-orbital length; 12: Snout length; E1: Scales in a longitudinal series; DL: Scales between the origin of the dorsal fin and the upper part of the lateral line; UL: Scales in the upper portion of the lateral line; LL: Scales in the lower portion of the lateral line; AL: Scales between the origin of the anal fin and the top of the lateral line. Measurements of the lip area applied to the specimens used in the present study, modified by Buckup, Reis, (1985). SL: upper lip area; IL: lower lip area; TL: total lip area; BA: body area.

Fig. 3. Network of mitochondrial haplotypes considering the concatenated *D-loop* and *CytB* regions. The size of the circles is proportional to the number of individuals present in each haplotype. The colors represent the analyzed populations, according to the legend. Small circles represent medium vectors, and the number of traits or values associated with each branch corresponds to the mutational distance between haplotypes.

Fig. 4. mtDNA genealogy for the individuals analyzed. The values above the nodes correspond to the posterior probability of each clade. Only the values for the clades discussed in the text were presented. The colors represent the analyzed populations, according to the legend.

Fig. 5. Evolutionary relationship between populations based on mitochondrial haplotypes. The x-axis scale corresponds to millions of years. The values above the nodes correspond to the posterior probability of each clade. The bar associated with each node represents the

95% credibility interval for the date of the common ancestor. The colors represent the analyzed populations, according to the legend.

Fig. 6. Principal Component Analysis (PCA) for morphological data (A) disregarding the lip area; (B) considering the lip area. *G. lacustris* is represented in red, *G. labiatus* HBT is represented in green and *G. labiatus* HBP is represented in yellow.

Fig. 7. Kruskal-Wallis box-plot and test for (A) the length of the pectoral fin (Hc=32.18, P=1.03E-8) and (B) the muzzle length (Hc=8.47, P=0.014). The significance values for the peer-to-peer post-hoc tests are presented directly in the figure.

Fig. 8. Kruskal-Wallis box-plot and test for (A) the lip area (Hc=29.19, P=4.59E-7) and (B) the body height (Hc=30.78, P=2.07E-7). The significance values for the peer-to-peer post-hoc tests are presented directly in the figure.

Fig. 9. Color pattern in the HBT. A – male (UFRGS 26300) and B – female (UFRGS 20302), rio Três Forquilhas. C – male (UFRGS 26311) and D – female (UFRGS 26311), lagoa Itapeva. E – male (UFRGS 26265) and F – female (not catalogued), lagoa dos Quadros. G – male (UFRGS 21101) and H – male (UFRGS 26264), rio Maquiné. I – male (UFRGS 16751) and J – male (UFRGS 16751), lagoa Bacopari. Specimens A, B, and G come from rivers draining the mountains (green dots) with rock bottom sharing hypertrophied lips and have been referred to as *G. labiatus* HBT. Specimens C-F, H-J, come from sand bottom environments (red dots) and have been referred to as *G. lacustris*. Note that specimen H, with undeveloped lips, was collected syntopically with a specimen with hypertrophied lips (G). The color pattern of specimens from the currently isolated lagoa Bacopari in the southernmost distribution of this species varies from plain dorsal, anal and caudal fins (I) to white striped or dotted fins (J).

Fig. 10. Color pattern in the HBP. *Gymnogeophagus labiatus*. A – male (117 mm SL; uncatalogued, lago Guaíba near the mouth of arroio Celupa) and B – female (104 mm SL; uncatalogued, lago Guaíba, Barra do Ribeiro). C - male (UFRGS 26425) and D – female (UFRGS 26425), Camaquã Sub-Basin.

Supporting Information

S1. Network of mitochondrial haplotypes for *D-loop* (A) and *CytB* (B).

Table 1. Individuals and locations included in the present study. [1]Sample size for the molecular markers consider individuals characterized for both *cytB* and *D-loop*. [2]Located in the Camaquã river sub-basin.

Voucher	**Species**	**Basin**	**Sample size**		**Coordinates**	**Locality**
			Molecular[1]	**Morphology**		
3904	*G. labiatus*	Patos	-	2	31°37’00'” S, 53°41’00” W	Bagé, RS
5924	*G. labiatus*	Patos	-	1	28°32’24” S, 51°33’36” W	Guaporé, RS
6381	*G. labiatus*	Patos	-	4	28°57’40” S, 51°45’17” W	Cotiporã, RS
6402	*G. labiatus*	Patos	-	1	28°53’04” S, 51°47’28” W	Guaporé, RS
6409	*G. labiatus*	Patos	-	2	28°56’23” S, 51°46’47” W	Dois Lajeados, RS
6464	*G. labiatus*	Patos	-	1	28°56’01” S ,51°28’01” W	Vila Flores, RS
6596	*G. labiatus*	Patos	-	1	29°33’54” S, 53°17’09” W	Agudo, RS
6606	*G. labiatus*	Patos	-	1	31°32’59” S, 53°46’17” W	Candiota, RS
6962	*G. labiatus*	Patos	-	2	-	Rio Carreiro, RS
8402	*G. labiatus*	Patos	-	2	31°43’10” S, 52°53’59” W	Pedro Osório, RS
8800	*G. labiatus*	Patos	-	1	29°22’08” S, 52°03’30” W	Lageado, RS
8807	*G. labiatus*	Patos	-	1	29°19’21” S, 52°14’03” W	Lageado, RS
9978	*G. labiatus*	Patos	-	3	28°56’15” S, 51°27’54” W	Veranópolis, RS
10099	*G. labiatus*	Patos	-	1	28°57’47” S, 51°45’43” W	Dois Lajeados, RS
10746	*G. labiatus*	Patos	1	1	30°06’02” S, 51°41’40” W	Eldorado do Sul, RS
14174	*G. labiatus*	Patos	-	4	29°17’35” S, 52°03’44” W	Travesseiro, RS
14317	*G. labiatus*	Patos	-	2	29°15’44” S, 52°08’50” W	Marques de Souza, RS
19659	*G. labiatus*	Patos	-	2	29°20’47” S, 50°42’04” W	Canela, RS

20360	*G. labiatus*	Patos	4	1	29°32’56” S, 53°27’50” W	Faxinal do Soturno, RS
20396	*G. labiatus*	Patos	1	-	29°22’12” S, 52°07’01” W	Forquetinha, RS
20407	*G. labiatus*	Patos	2	-	29°43’31” S, 53°09’39” W	Paraíso do Sul, RS
22378	*G. labiatus*	Patos	2	2	30°23’00” S, 51°26’00” W	Barra do Ribeiro, RS
22379	*G. labiatus*	Patos	8	3	29°59’15” S, 51°14’24” W	Eldorado do Sul, RS
22380	*G. labiatus*	Patos	-	1	30°17’53” S, 51°41’03” W	Barão do Triunfo, RS
22524	*G. labiatus*	Patos	-	2	28°42’12” S, 51°50’57” W	Serafina Corrêa, RS
22719	*G. labiatus*	Patos	-	1	29°02’08” S, 51°05’16” W	São Marcos, RS
23018	*G. labiatus*	Patos	4	6	30°17’00” S, 51°48’00” W	Barra do Ribeiro, RS
22132	*G. labiatus*	Patos[2]	1	-	30°57’35” S, 53°28’52” W	Caçapava do Sul, RS
17753	*G. labiatus*	Tramandaí	3	2	29°34’13” S, 50°16’49” W	Maquiné, RS
17761	*G. labiatus*	Tramandaí	2	5	29°32’56” S, 50°04’13” W	Três Forquilhas, RS
18279	*G. labiatus*	Tramandaí	-	1	29°43’42” S, 50°08’06” W	Maquiné, RS
18437	*G. labiatus*	Tramandaí	1	-	29°34’14” S, 50°16’49” W	Maquiné, RS
18457	*G. labiatus*	Tramandaí	1	-	29°40’09” S, 50°04’59” W	Maquiné, RS
18464	*G. labiatus*	Tramandaí	2	-	29°40’08” S, 50°12’24” W	Maquiné, RS
19594	*G. labiatus*	Tramandaí	1	-	29°32’13” S, 50°14’45” W	Barra do Ouro, RS
21101	*G. labiatus*	Tramandaí	3	2	29°39’07” S, 50°12’34” W	Maquiné, RS
21912	*G. labiatus*	Tramandaí	-	2	29°13’44” S 50°01’18” W	Praia Grande, SC
3885	*G. lacustris*	Tramandaí	-	1	29°42’00” S, 50°05’59” W	Capão da Canoa, RS
3894	*G. lacustris*	Tramandaí	-	2	29°22’60” S, 49°49’59” W	Torres, RS
10751	*G. lacustris*	Tramandaí	6	-	30°32’26” S, 50°25’12” W	Mostardas, RS
16751	*G. lacustris*	Tramandaí	-	6	30°32’22” S, 50°25’18” W	Mostardas, RS

16915	*G. lacustris*	Tramandaí	-	1	29°35’56” S, 49°58’44” W	Terra de Areia, RS
17246	*G. lacustris*	Tramandaí	6	1	30°32’22” S, 50°25’18” W	Mostardas, RS
17345	*G. lacustris*	Tramandaí	-	2	29°36’01” S, 49°58’53” W	Terra de Areia, RS
17481	*G. lacustris*	Tramandaí	1	1	30°09’23” S, 50°14’04” W	Cidreira, RS
18273	*G. lacustris*	Tramandaí	-	1	29°39’07” S, 50°12’33” W	Maquiné, RS
18405	*G. lacustris*	Tramandaí	-	1	29°45’44” S, 50°05’02” W	Capão da Canoa, RS
18406	*G. lacustris*	Tramandaí	-	2	30°32’26” S, 50°25’16” W	Mostardas, RS
19081	*G. lacustris*	Tramandaí	4	-	29°57’56” S, 50°13’46” W	Osório, RS
19511	*G. lacustris*	Tramandaí	4	-	29°47’03” S, 50°11’02” W	Osório, RS
19570	*G. lacustris*	Tramandaí	3	-	29°58’16” S, 50°13’07” W	Osório, RS
19586	*G. lacustris*	Tramandaí	1	-	29°57’04” S, 50°13’02” W	Osório, RS

Table 2. Genetic structure (Φ_{ST}) between populations. Values in bold values were statistically significant (P<0.05)

Population	***G. labiatus* CSB**	***G. labiatus* HBP**	***G. labiatus* HBT**	***G. lacustris***
G. labiatus CSB	-			
G. labiatus HBP	0.857	-		
G. labiatus HBT	0.997	**0.895**	-	
G. lacustris	0.988	**0.915**	-0.034	-

Table 3. Delimitation of candidate species under a coalescent criterion. N species = number of candidate species; PP = Posterior probability for a specific arrangement, considering the number of species and which populations make up each group. The letters assigned to each population correspond to all candidate species suggested in the analysis. For a *collapse height* value of 0.001, three alternative arrangements were contained within the 95% credibility range. Note that in all arrangements *G. labiatus* HBT and *G. lacustris* were assigned as the same candidate species.

Collapse Height	**N species**	***PP***	**Populations**				
			***G. labiatus* CSB**	***G. labiatus* HBP**	***G. labiatus* HBT**	***G. lacustris***	***G. setequedas***
0.00001	4	0.995	A	B	C	C	D
0.0001	4	0.962	A	B	C	C	D
0.001	2	0.387	A	A	A	A	B
	3	0.276	A	B	B	B	C
	4	0.199	A	B	C	C	D

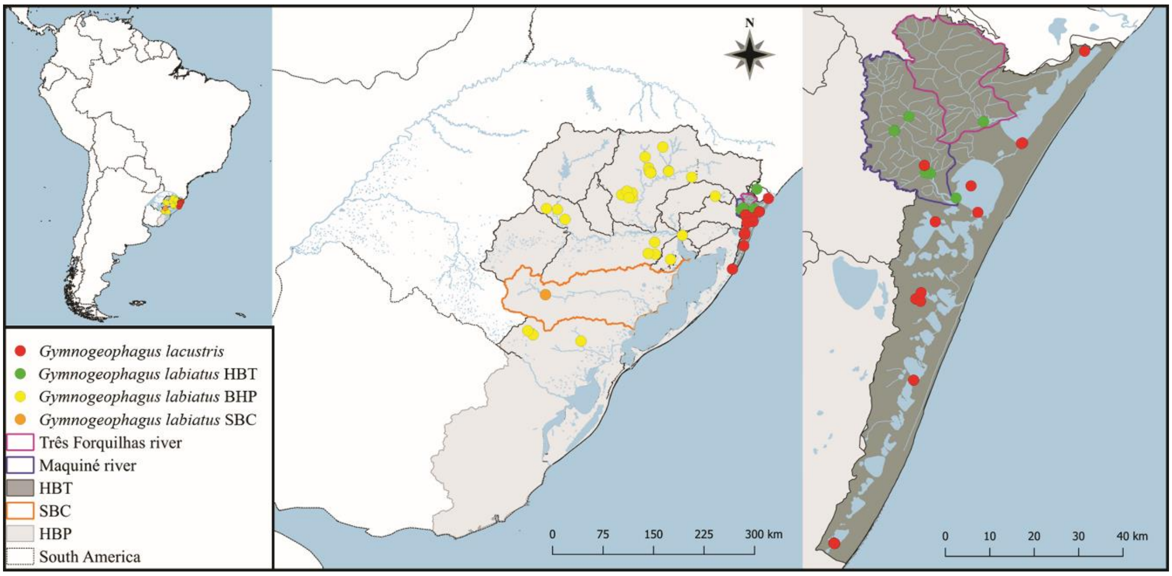
Table 4. Morphological measurements for the analyzed specimens. Min = minimum observed value; Max = maximum observed value; SD = standard deviation; *Values relative to Standard length; **Values relative to Body area; E1 = Scales in a longitudinal series; DL = Scales between the origin of the dorsal fin and the upper part of the lateral line; AL = Scales between the origin of the anal fin and the top of the lateral line.

	***G. labiatus* HBP (N=48)**				***G. labiatus* HBT (N=12)**				***G. lacustris* (N=18)**			
	Min	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
Linear Measurements (mm)												
Standard length (SL)	80.9	180.2	111.4	-	80.3	133.5	100.6	-	80.43	146.2	100.8	-
Body depth*	31.7	39.3	36.1	1.57	34.8	39.6	37.1	1.62	37.0	41.0	39.0	1.18
Head length*	28.6	38.1	33.9	1.78	32.5	38.0	34.6	1.65	32.0	35.8	33.9	0.92
Dorsal-fin base length*	47.7	56.5	52.3	2.02	48.1	54.9	51.5	2.01	51.0	57.1	52.9	1.49
Pectoral-fin length*	24.0	34.4	27.7	2.31	27.7	36.4	31.0	2.32	25.4	35.2	32.0	2.51
Caudal peduncle depth*	11.6	13.8	12.7	0.50	12.1	13.6	12.8	0.42	12.7	14.2	13.4	0.39
Caudal peduncle length*	16.3	21.2	18.7	1.20	17.4	20.4	18.9	1.08	16.6	21.1	18.7	1.24
Eye diameter*	5.7	9.7	7.4	0.82	6.8	9.6	8.2	1.17	6.9	10.2	8.6	0.92
Interorbital width*	8.1	12.5	9.9	1.06	9.0	12.4	10.7	1.11	8.9	12.8	10.1	1.06
Upper jaw length*	6.2	11.1	8.3	1.11	5.9	8.9	7.8	0.89	4.8	8.6	7.0	0.94
Pre-orbital length*	9.2	14.5	12.0	1.24	10.6	15.0	12.4	1.33	10.8	15.6	13.1	1.45
Snout length *	11.6	19.6	14.9	1.85	10.9	19.0	14.0	2.17	11.3	15.6	13.5	1.22
Area (cm^2)												
Body area	8,09	56,77	20,07	-	10,78	34,80	18,94	-	12,31	41,90	19,77	-
Upper lip area**	0,3	2,5	1,2	0,58	0,5	1,3	0,9	0,24	0,2	0,7	0,4	0,12
Lower lip area**	0,5	3,2	1,6	0,70	0,7	1,3	1,1	0,20	0,4	0,8	0,6	0,12
Total lip area**	1,0	5,6	2,7	1,25	1,3	2,6	1,9	0,42	0,6	1,4	1,0	0,21
Counts												

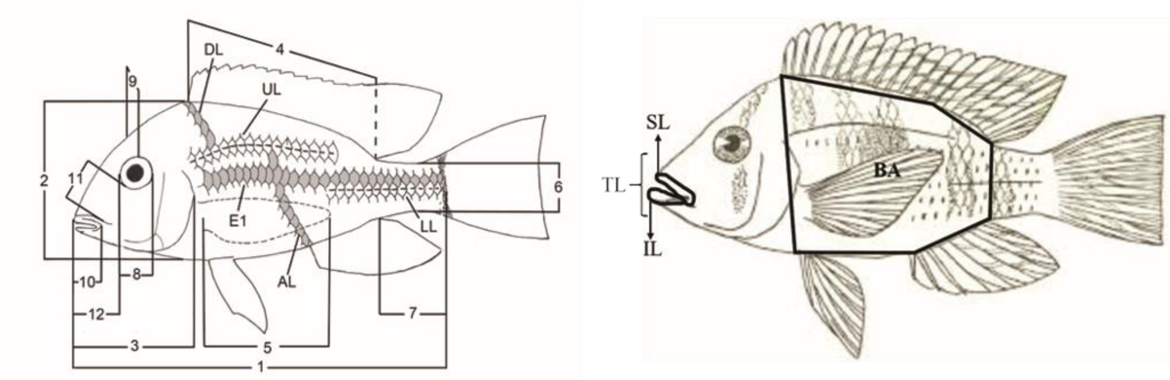
Upper lateral line	25	30	27.4	1.03	26	28	27.0	0.60	26	28	26.9	0.73
Lower lateral line	11	15	12.4	0.98	10	14	11.8	1.22	10	13	11.7	0.84
E1	8	11	9.3	0.66	9	11	9.3	0.62	8	11	9.0	0.77
DL	16	20	18.2	0.93	16	20	17.8	1.11	17	19	18.0	0.69
AL	4	6	4.6	0.54	4	5	4.6	0.51	4	5	4.7	0.46
Spines	12	15	13.7	0.69	12	14	12.9	0.51	12	14	12.8	0.55
Rays	8	12	10.1	0.92	10	12	11.0	0.60	10	12	10.8	0.71

Table 5. Confusion matrix for the CVA of morphological data.

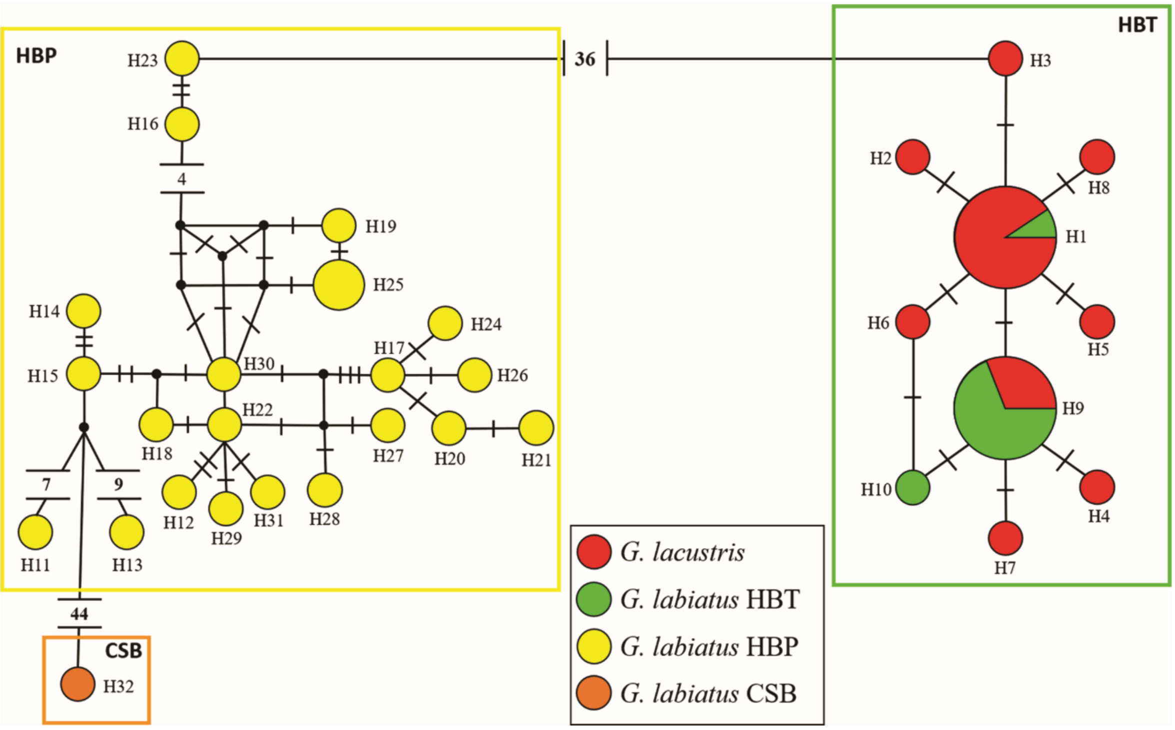
Original population	**Inferred population**			
	***G. labiatus* HBP**	***G. labiatus* HBT**	***G. lacustris***	**Total**
	Linear measurements + lip area			
G. labiatus HBP	42	5	1	48
G. labiatus HBT	0	11	1	12
G. lacustris	1	0	17	18
Total	43	16	19	78
	Linear measurements only			
G. labiatus HBP	41	5	2	48
G. labiatus HBT	1	10	1	12
G. lacustris	1	0	17	18
Total	43	15	20	78



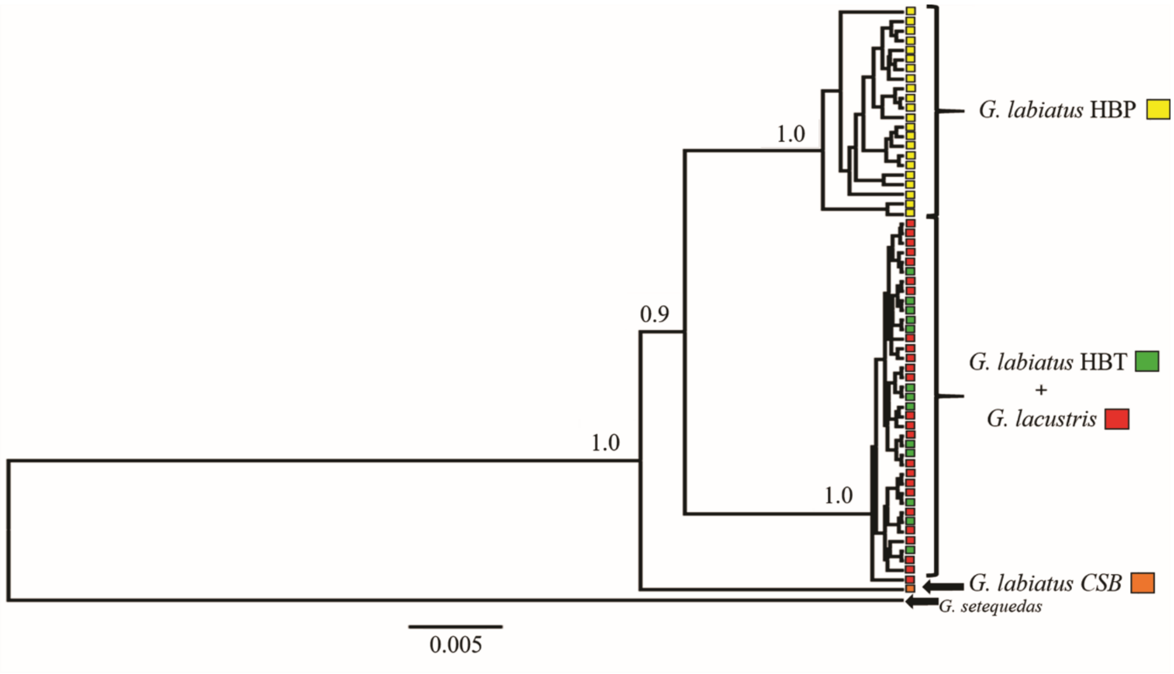
Map with the distribution of all individuals used in the present study. The hydrographic basins of the Patos Lagoon (HBP) and the Tramandaí River (HBT), as well as the Camaquã Sub-Basin (CSB), are indicated on the map. The gray lines represent the watershed between basins or sub-basins in the study area. The red dots correspond to *G. lacustris*, the green to *G. labiatus* HBT, the yellow to *G. labiatus* HBP and the orange to *G. labiatus* CSB.



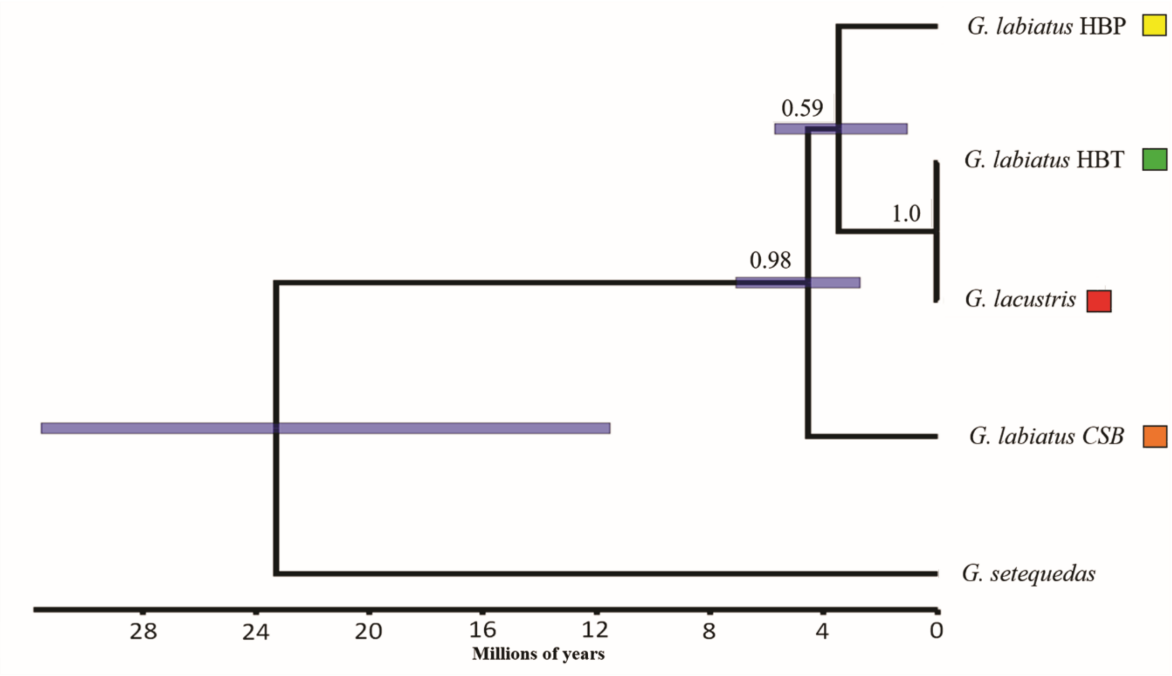
Measurements and counts applied to specimens used in the present study, according to Malabarba *et al*. (2015). 1: Standard length; 2: Body depth; 3: Head length; 4: Dorsal-fin base length; 5: Pectoral-fin length; 6: Caudal peduncle depth; 7: Caudal peduncle length; 8: Eye diameter; 9: Interorbital width; 10: Upper jaw length; 11: Pre-orbital length; 12: Snout length; E1: Scales in a longitudinal series; DL: Scales between the origin of the dorsal fin and the upper part of the lateral line; UL: Scales in the upper portion of the lateral line; LL: Scales in the lower portion of the lateral line; AL: Scales between the origin of the anal fin and the top of the lateral line. Measurements of the lip area applied to the specimens used in the present study, modified by Buckup, Reis, (1985). SL: upper lip area; IL: lower lip area; TL: total lip area; BA: body area.



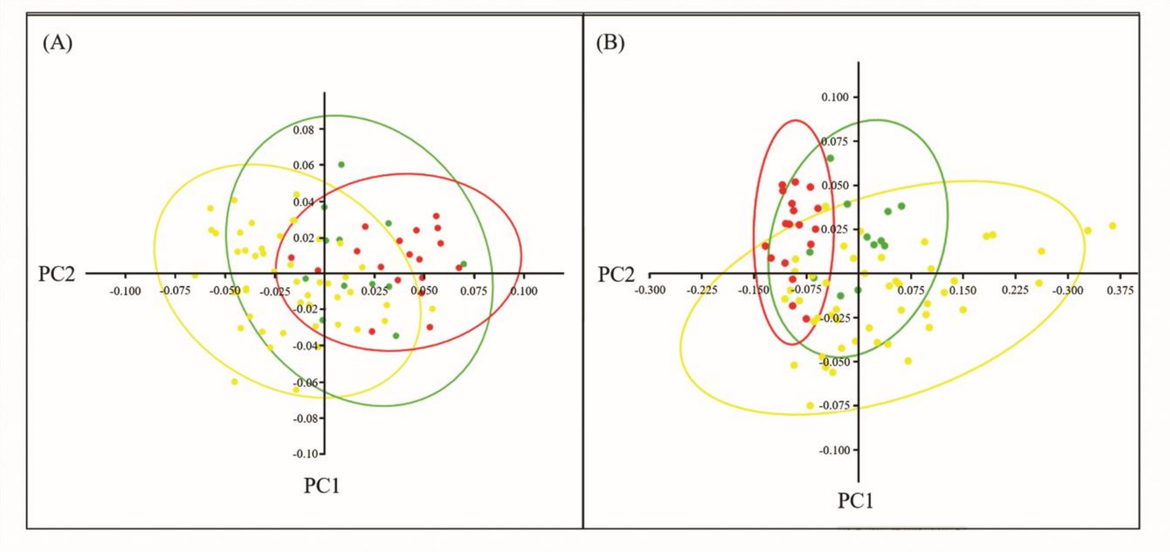
Network of mitochondrial haplotypes considering the concatenated *D-loop* and *CytB* regions. The size of the circles is proportional to the number of individuals present in each haplotype. The colors represent the analyzed populations, according to the legend. Small circles represent medium vectors, and the number of traits or values associated with each branch corresponds to the mutational distance between haplotypes.



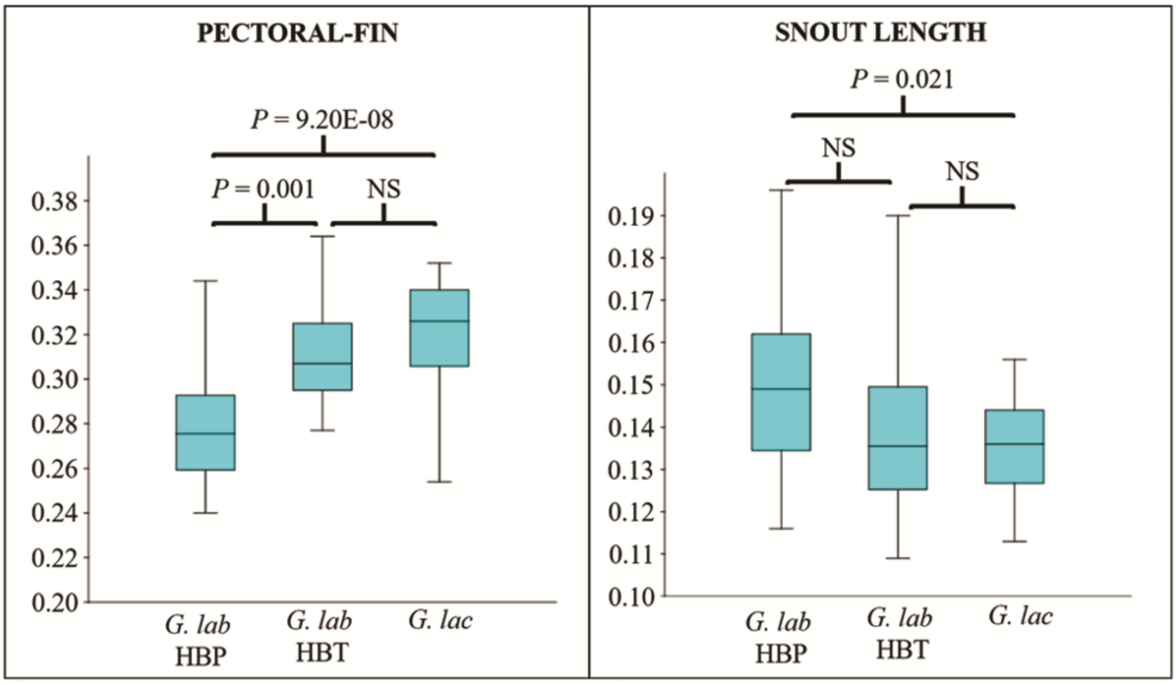
mtDNA genealogy for the individuals analyzed. The values above the nodes correspond to the posterior probability of each clade. Only the values for the clades discussed in the text were presented. The colors represent the analyzed populations, according to the legend.



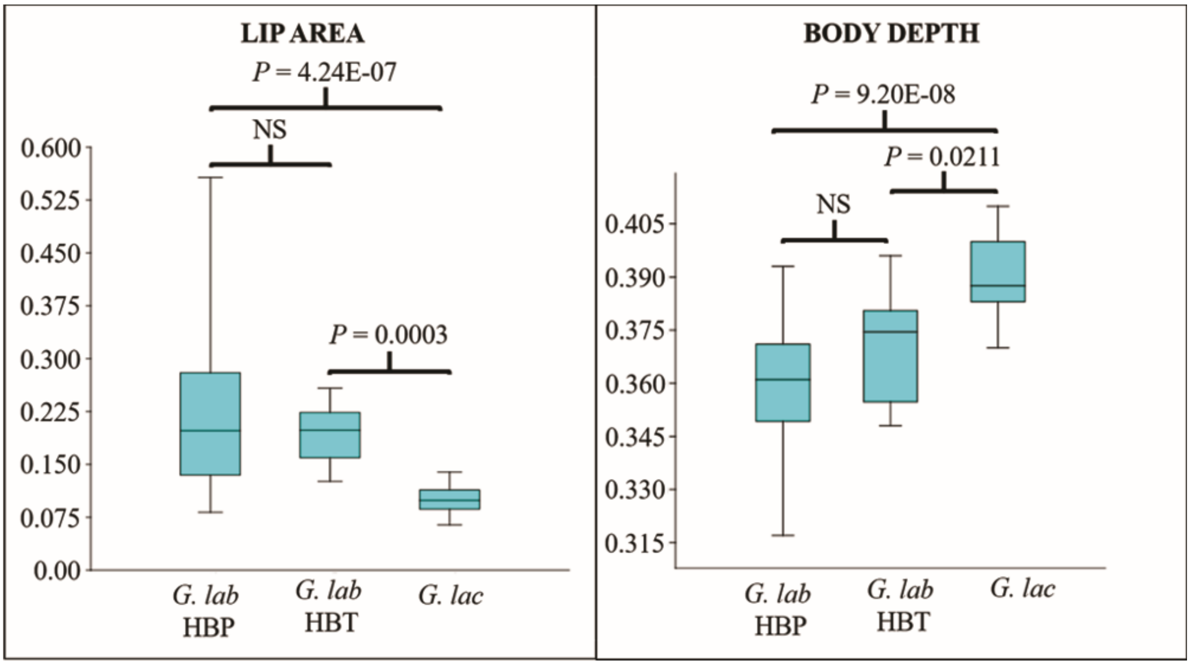
Evolutionary relationship between populations based on mitochondrial haplotypes. The x-axis scale corresponds to millions of years. The values above the nodes correspond to the posterior probability of each clade. The bar associated with each node represents the 95% credibility interval for the date of the common ancestor. The colors represent the analyzed populations, according to the legend.



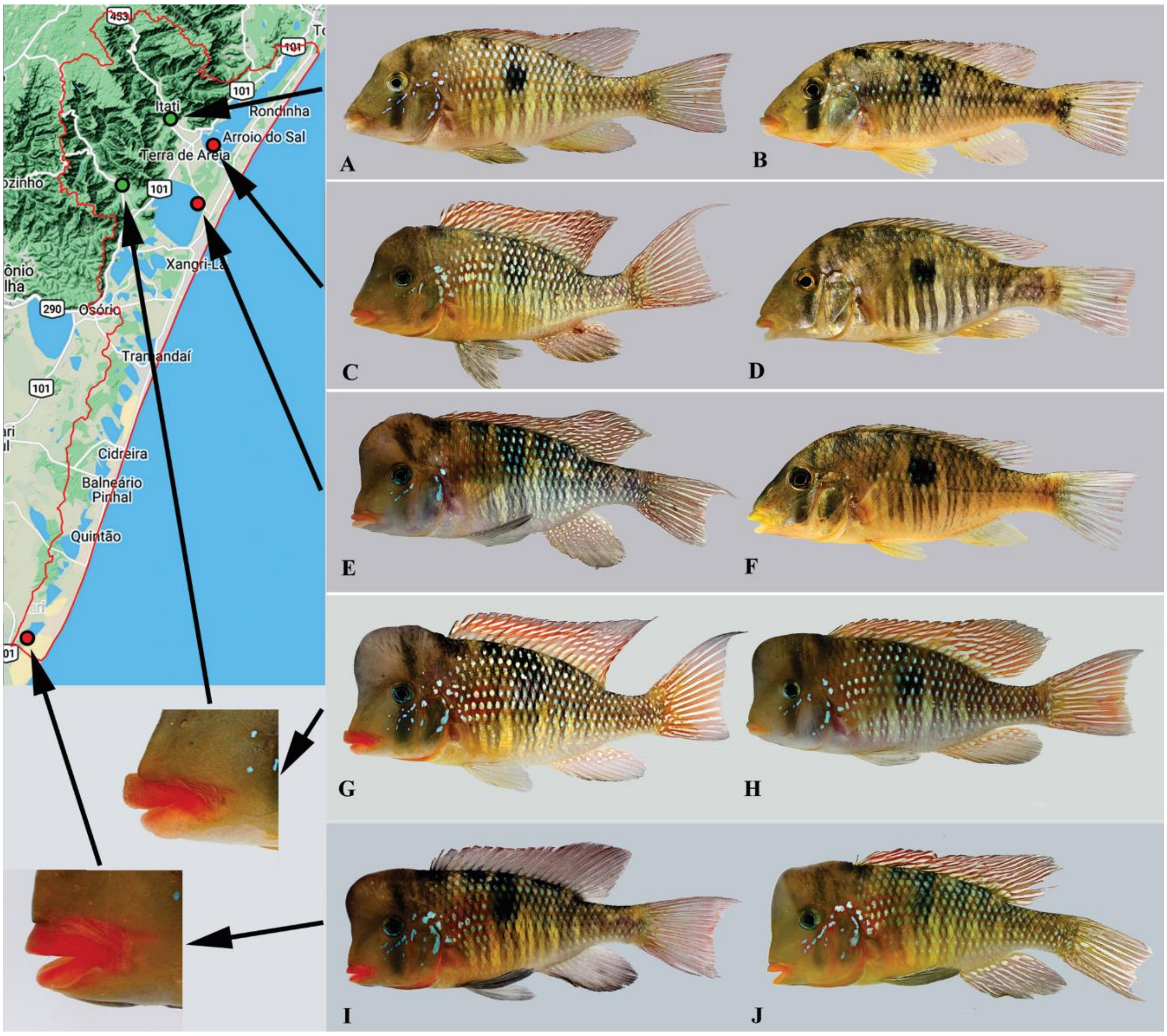
Principal Component Analysis (PCA) for morphological data (A) disregarding the lip area; (B) considering the lip area. *G. lacustris* is represented in red, *G. labiatus* HBT is represented in green and *G. labiatus* HBP is represented in yellow.



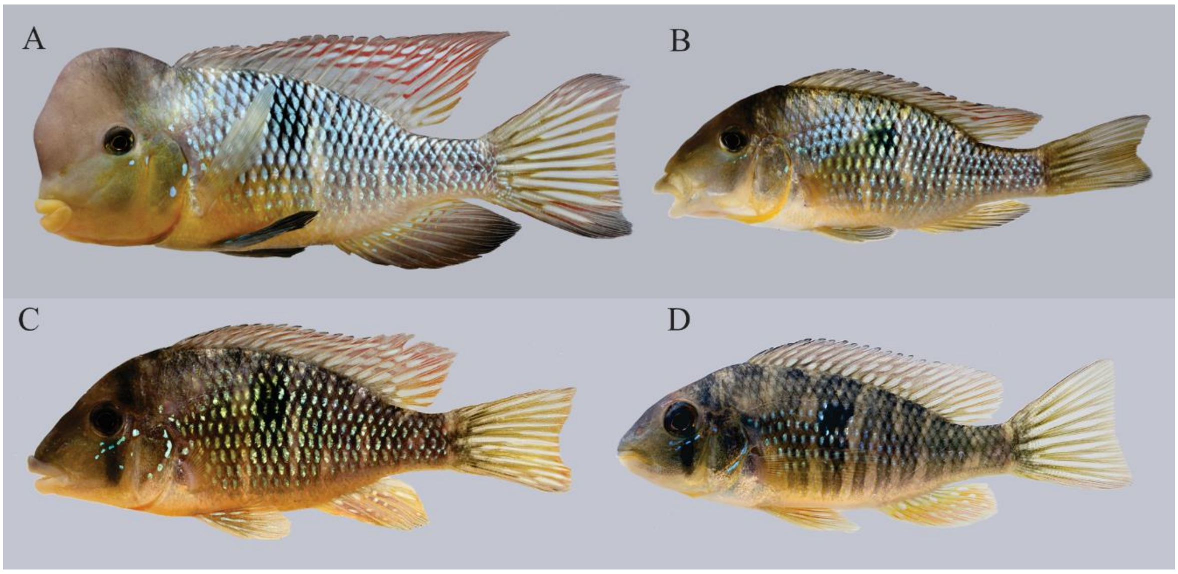
Kruskal-Wallis box-plot and test for (A) the length of the pectoral fin (Hc=32.18, P=1.03E-8) and (B) the muzzle length (Hc=8.47, P=0.014). The significance values for the peer-to-peer post-hoc tests are presented directly in the figure.



Kruskal-Wallis box-plot and test for (A) the lip area (Hc=29.19, P=4.59E-7) and (B) the body height (Hc=30.78, P=2.07E-7). The significance values for the peer-to-peer post-hoc tests are presented directly in the figure.



Color pattern in the HBT. A – male (UFRGS 26300) and B – female (UFRGS 20302), rio Três Forquilhas. C – male (UFRGS 26311) and D – female (UFRGS 26311), lagoa Itapeva. E – male (UFRGS 26265) and F – female (not catalogued), lagoa dos Quadros. G – male (UFRGS 21101) and H – male (UFRGS 26264), rio Maquiné. I – male (UFRGS 16751) and J – male (UFRGS 16751), lagoa Bacopari. Specimens A, B, and G, come from rivers draining the mountains (green dots) with rock bottom sharing hypertrophied lips and have been referred to as *G. labiatus* HBT. Specimens C-F, H-J, come from sand bottom environments (red dots) and have been referred to as *G. lacustris*. Note that specimen H, with undeveloped lips, was collected syntopically with a specimen with hypertrophied lips (G). The color pattern of specimens from the currently isolated lagoa Bacopari in the southernmost distribution of this species varies from plain dorsal, anal and caudal fins (I) to white striped or dotted fins (J).



Color pattern in the HBP. *Gymnogeophagus labiatus*. A – male (117 mm SL; uncatalogued, lago Guaíba near the mouth of arroio Celupa) and B – female (104 mm SL; uncatalogued, lago Guaíba, Barra do Ribeiro). C - male (UFRGS 26425) and D – female (UFRGS 26425), Camaquã Sub-Basin.

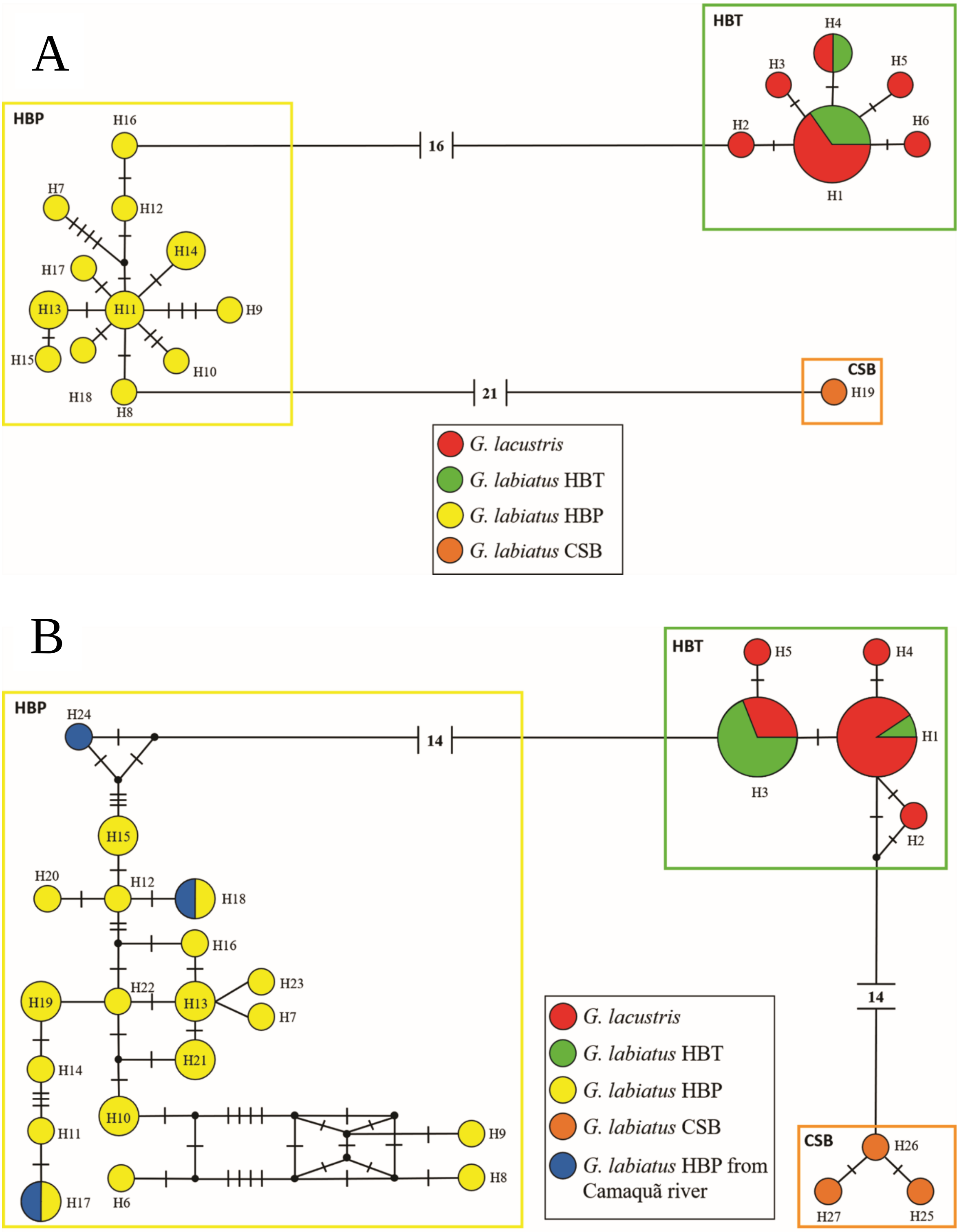


Fig. S1. Network of mitochondrial haplotypes for *D-loop* (A) and *CytB* (B).

CONCLUSÕES FINAIS

A dissertação ora apresentada analisou a história evolutiva e relações entre as populações de duas espécies parapátricas: *Gymnogeophagus labiatus* e *G. lacustris,* que, na PCRS ocorrem em regiões geograficamente próximas, mas com características morfoecológicas distintas. As análises moleculares indicaram uma forte estruturação genética, que foi muito mais pronunciada considerando a relação entre bacias hidrográficas do que considerando a taxonomia atual para o grupo. Em outras palavras, as duas espécies presentes na BHT foram muito mais próximas geneticamente do que quando comparada às populações de *G. labiatus* de outras bacias. Viu-se também uma profunda estrutura genealógica dentro da BHP, com um indivíduo da SBC apresentando um haplótipo mitocondrial divergente, e irmão de todos os demais presentes tanto na BHP quanto na BHT. As análises temporais sugerem uma diversificação antiga na BHP e uma divergência muito recente na BHT.

Embora o padrão reportado acima pudesse sugerir uma especiação recente na BHT, em acordo com a taxonomia atual para o grupo, as análises morfológicas não parecem apoiar de maneira inequívoca essa interpretação. Há diferenças morfológicas claras entre *G. labiatus* BHT e BHP, especialmente associadas a diferenças no comprimento da nadadeira peitoral e do focinho. Diferenças essas que são compartilhadas entre *G. labiatus* BHT e *G. lacustris*. De fato, a área do lábio é o principal caráter que separa as espécies conforme a classificação taxonômica atual. Porém, nossos resultados demonstram que há uma enorme plasticidade quanto ao tamanho do lábio em ambas as bacias, sugerindo que essa variação, a qual está intimamente ligada com hábitos de forrageio e características físicas do local de ocorrência dos indivíduos (cursos d'água com fundo pedregoso), não representa um bom caractere diagnóstico para separação dos grupos. O padrão encontrado na BHT, nesse caso, seria semelhante àquele descrito para *Amphilophus labiatus* na América Central (Machado-Schiaffino *et al.*, 2014).

Embora trabalhos que analisem mais indivíduos, mais marcadores genéticos e um maior número de sub-bacias dentro da BHP sejam necessários para elucidar as relações evolutivas entre as populações dessa bacia - e em especial no significado da linhagem mitocondrial divergente encontrada na SBC, nossos resultados parecem indicar claramente que, enquanto *G. labiatus* e *G. lacustris* são de fato espécies distintas, todas as populações da BHT deveriam ser consideradas como parte da variação de *G. lacustris*, enquanto *G. labiatus* seria uma espécie restrita à BHP. Além das diferenças morfométricas revisadas anteriormente, uma das principais diferenças entre essas espécies são os padrões de coloração, os quais funcionam como forte mecanismo de seleção sexual, e, possivelmente, de isolamento reprodutivo entre essas espécies.

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