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CASCUDINHOS DO SUL DO BRASIL: SISTEMÁTICA, ENDEMISMO E RELAÇÕES USANDO NOVAS ABORDAGENS (LORICARIIDAE: HYPOPTOPOMATINAE)

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PÓS-GRADUAÇÃO - *STRICTO SENSU*

Pontifícia Universidade Católica
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Ms. Maria Laura de Souza Delapieve

Orientador: Dr. Roberto E. Reis

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PORTO ALEGRE – RS – BRASIL

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Tese apresentada como requisito para obtenção do grau de Doutor pelo Programa de Pós-Graduação em Ecologia e Evolução da Biodiversidade da Escola de Ciências da Saúde e da Vida da Pontifícia Universidade Católica do Rio Grande do Sul.

Aprovada em: 30 de março de 2020.

BANCA EXAMINADORA:

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Prof. Dr. Roberto Esser dos Reis – PUCRS – Orientador

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Prof. Dr. Nelson Jurandi Rosa Fagundes – UFRGS

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Prof. Dr. Sandro Luis Bonatto - PUCRS

Porto Alegre

2020

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Este trabalho é parte integrante dos requerimentos necessários à obtenção do título de Doutor em Ecologia e Evolução da Biodiversidade, e como tal, não deve ser vista como uma publicação no senso do Código Internacional de Nomenclatura Zoológica (artigo 9) (apesar de disponível publicamente sem restrições) e, portanto, quaisquer atos nomenclaturais nela contidos tornam-se sem efeito para os princípios de prioridade e homonímia. Desta forma, quaisquer informações inéditas, opiniões e hipóteses, bem como nomes novos, não estão disponíveis na literatura zoológica. Pessoas interessadas devem estar cientes de que referências públicas ao conteúdo deste estudo, na sua presente forma, somente devem ser feitas com aprovação prévia do autor.

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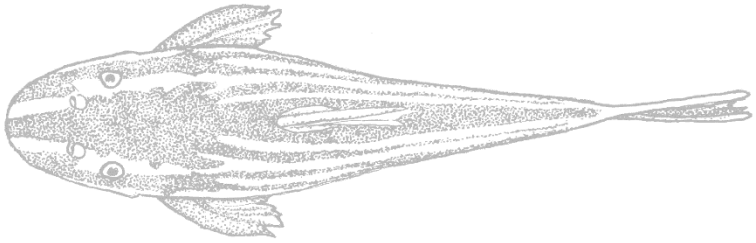
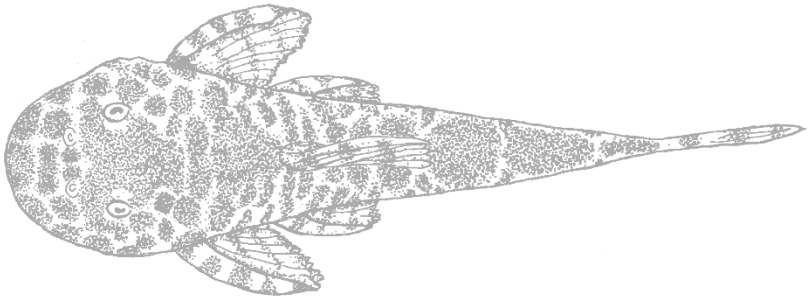
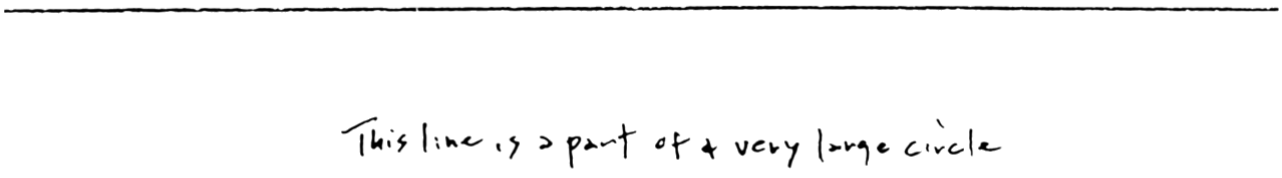


Epactionotus bilineatus



Eurycheilichthys pantherinus



Yoko Ono, 1996 (Blue Room Event)

Sumário

Ao meu pai Antonio (in memorian), minha mãe Maria Cristina, Pedro, Bruno e Marco Antonio.

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Resumo. *Epactionotus* e *Eurycheilichthys* são dois gêneros de cascudinhos com distribuição restrita ao sul da região Neotropical. Ambos foram foco de um trabalho realizado por Reis & Schaefer (1998), porém a descoberta e descrição de novas populações e espécies ao longo dos últimos 20 anos, resultou no desenvolvimento deste estudo visando incluir as novas espécies, estabelecer padrões filogenéticos e entender estruturação nas bacias em que os dois gêneros estão distribuídos. As espécies de *Epactionotus* habitam as porções rochosas de rios em uma limitada área geográfica da costa do Atlântico ao sul do Brasil. Cada uma das suas três espécies é endêmica a uma única drenagem (exceto por *E. bilineatus*), estando isoladas umas das outras por sistemas de lagoas costeiras ou pelo Oceano Atlântico. *Epactionotus bilineatus* é proveniente dos rios Maquiné e Três Forquilhas, ambos tributários do sistema do Rio Tramandaí, enquanto que *E. itaimbezinho* é endêmico do rio Mampituba e *E. gracilis*, do rio Araranguá. Recentemente, novas populações foram descobertas em drenagens costeiras adjacentes, mais especificamente, nos rios Urussanga, Tubarão, d'Una e Biguaçu. Um estudo integrativo de delimitação de espécies usando dados moleculares (citocromo oxidase subunidade I - COI) e morfologia (dados morfométricos e merísticos) foi aplicado visando avaliar o reconhecimento de espécies de populações isoladas. À luz dos novos dados, o gênero é rediagnosticado, o status das espécies/populações de *Epactionotus* é reavaliado e a validade das três espécies previamente reconhecidas foi corroborada. Quanto as novas populações, os dados suportam apenas aquela proveniente do rio Biguaçu, em Santa Catarina, como uma nova espécie. Os dados de distribuição e moleculares sugerem não apenas que as paleodrenagens tenham agido como barreiras, podendo explicar a distribuição das espécies, como também que a forte estruturação genética por bacia pode estar relacionada com especificidade de habitat. Quanto a *Eurycheilichthys*, o gênero compreende nove espécies endêmicas e restritamente distribuídas em apenas duas drenagens ao sul da região Neotropical. O gênero é mais comumente conhecido pelas espécies *E. pantherinus*, proveniente do alto rio Uruguai, e *E. limulus*, do alto rio Jacuí. As demais, e recentemente descritas, espécies do gênero, entretanto, são todas endêmicas e distribuídas em regiões de altitude da bacia Taquari-Antas, um tributário do baixo rio Jacuí. Esta diversidade, juntamente com seu endemismo, torna *Eurycheilichthys* um importante estudo de caso para entender biologia evolutiva. Análises filogenômicas foram realizadas e estruturação genética foi estimada comparando polimorfismos raros e comuns a partir de dados genômicos criados usando protocolo *ddRADseq* para 65 indivíduos das nove espécies, visando elucidar as relações entre as espécies e fornecer um componente de tempo de diversificação. As análises suportam *Eurycheilichthys* como um gênero monofilético, formado por dois clados com suporte absoluto e indicam duas linhagens no Taquari-Antas com divergência recente. Exceto por *E. luisae*, todas as espécies foram reconhecidas como sendo monofiléticas. Os menores padrões de estruturação encontrados no clado leste, sugerem que a maior diversidade encontrada no Taquari-Antas possa estar relacionada a um cenário bastante dinâmico, com a possibilidade de diversos eventos de captura de cabeceira.

Palavras-chave: Cascudos; Delimitação de espécies; Distância genética; ddRADseq; Filogenia; Gene COI; Peixes neotropicais; Taxonomia iterativa.

Title: Cascudinhos from Southern Brazil: systematics, endemism and relationships using new approaches (Loricariidae: Hypoptopomatinae).

Abstract. *Epactionotus* and *Eurycheilichthys* are two genera of small catfishes with restricted distribution through the Southern Neotropical region. The two genera were the subject of a study provided by Reis & Schaefer (1998). With the discovery and description of new populations and species over the last 20 years, a new study was developed aiming at these two groups. *Epactionotus* species are known for inhabiting the rocky-bottom stretches of rivers in a limited geographic area along the Atlantic coast of southern Brazil. Distribution of each of the three species is endemic to single river drainage (except for *E. bilineatus*), being isolated from each other by the coastal lacustrine systems or the Atlantic Ocean. *Epactionotus bilineatus* is known from the rivers Maquiné and Três Forquilhas, both tributaries of the Tramandaí River System, while *E. itaimbezinho* is endemic to the Mampituba River drainage, and *E. gracilis* to the Araranguá River drainage. Most recently, new populations were revealed in other Atlantic coastal drainages of southern Brazil, more specifically, in the Urussanga, Tubarão, d'Una, and Biguaçu river drainages. Integrative species delimitation using molecular data (cytochrome oxidase subunit I - COI) and morphology (morphometrics and meristics) was applied in order to evaluate species recognition in isolated populations. In light of new data, the genus is re-diagnosed, the status of *Epactionotus* species/population is reevaluated, and the formerly described species are recognized. As for the newly discovered populations, the data strongly support only the population from the Biguaçu River drainage, in Santa Catarina State, as a new species and an independent lineage. Molecular and distributional data suggest that not only palaeodrainage connectivity can explain species distribution, but also, that strong per basin genetic structure may be related with species habitat specificity.As for *Eurycheilichthys*, the genus comprises nine species endemic and restrictedly distributed through two river basins in Southern Neotropical Region. The genus is better known by *E. pantherinus*, from the upper Uruguay River basin and *E. limulus*, from the upper reaches of the Jacuí River basin. The seven additional, and recently described, species of *Eurycheilichthys*, however, are all distributed through higher altitudes of the Taquari-Antas River basin, a tributary to the lower Jacuí River. Its diversity and endemism make *Eurycheilichthys* an important focal group for studying and understanding evolutionary biology. Phylogenomic analysis were carried out and interspecific genetic structure comparing rare and common polymorphisms were estimated from genomic data created for 65 individuals of the nine species using ddRADseq protocol, aiming to elucidate the relationships between the species and to provide a time divergence component. Analyses support *Eurycheilichthys* as a monophyletic genus comprising two species-inclusive clades, with absolute support and suggest two and very recently diverged lineages on the Taquari-Antas species. Except for *Eurycheilichthys luisae*, all remaining species were recovered as monophyletic. The more diverse lineages on the Taquari-Antas when compared to Uruguay and upper Jacuí River basins suggest a more dynamic landscape with several headwater capture events.

Keywords: Cascudos; COI gene; ddRADseq; Genetic distance; Iterative taxonomy; Neotropical fish; Phylogeny; Species delimitation.

Apresentação. *Epactionotus* Reis & Schaefer 1998 e *Eurycheilichthys* (Reis & Schaefer 1992) são dois gêneros de cascudinhos endêmicos e restritamente distribuídos ao sul da região Neotropical. Os dois gêneros foram foco de um trabalho realizado por Reis & Schaefer (1998). Contudo, a partir da descoberta e da descrição de novas populações e espécies ao longo dos últimos 20 anos, um novo estudo foi desenvolvido visando incluir as novas espécies, estabelecer padrões filogenéticos e entender estruturação nas bacias.

No seu endemismo, *Epactionotus bilineatus* foi descrito como sendo restrito aos rios Maquiné e Três Forquilhas, no nordeste do Rio Grande do Sul, *E. itaimbezinho* endêmico do rio Mampituba, no limite entre Rio Grande do Sul e Santa Catarina, e *E. gracilis* endêmico da bacia do rio Araranguá no sul de Santa Catarina. Desde o trabalho apresentado por Reis & Schaefer (1998), não houve descrições de espécies novas para o gênero. Porém, novas populações de *Epactionotus* foram recentemente descobertas em drenagens adjacentes ao norte da distribuição previamente conhecida para o grupo, ampliando-se, assim, a distribuição de *Epactionotus* para as drenagens dos rios Urussanga, Tubarão, d'Una e Biguaçu.

Quanto a *Eurycheilichthys*, o gênero compreende um grupo de peixes endêmicos do sul do Brasil, nos estados do Rio Grande do Sul e de Santa Catarina, e de Missiones, na Argentina, sendo sua distribuição restrita às cabeceiras do rio Uruguai e seus afluentes e à porção alta de afluentes do rio Jacuí, da drenagem da Laguna dos Patos.

O gênero é mais comumente conhecido pelas espécies *Eurycheilichthys pantherinus*, proveniente do alto rio Uruguai, e *E. limulus*, do alto rio Jacuí. Entretanto, possui outras sete espécies recentemente descritas por Reis (2017), sendo todas endêmicas e distribuídas em regiões de altitude do Taquari-Antas, um tributário do baixo rio Jacuí. Considerando-se a distribuição das espécies do Taquari-Antas, *Eurycheilichthys apocremnus* e *E. castaneus* estão geograficamente limitadas a apenas alguns riachos localizados em porções à oeste da bacia, enquanto que *E. coryphaenus*, *E. planus* e *E. vacariensis* estão restritas a porções à leste. As espécies *E. luisae* e *E. paucidens* são distribuídas ao longo de toda a bacia, podendo ser encontradas em pontos relativamente distantes uns dos outros e ocorrendo em simpatria com outras espécies do gênero. Esta diversidade, juntamente com seu endemismo, torna *Eurycheilichthys* um importante estudo de caso para entender biologia evolutiva.

À luz dessas informações, a presente tese tem por objetivo revisitar os dois gêneros endêmicos do sul da região Neotropical estudados por Reis & Schaefer (1998), *Epactionotus* e *Eurycheilichthys*. Através da geração de novos dados e utilização de novas abordagens, visou-se compreender os padrões de relação e endemismo e inferir possíveis processos envolvidos na diversidade de ambos. O Capítulo I, intitulado "Species delimitation in a restrictedly distributed group of cascudinhos (Loricariidae: *Epactionotus*) supports drainage basin endemism in coastal rivers of south Brazil", apresenta um manuscrito a ser submetido à revista *Journal of Fish Biology*. Trata-se de um estudo de delimitação de espécies usando novos dados moleculares (COI) e

morfológicos (morfometria e merística). As espécies e novas populações de *Epactionotus* pertencentes às drenagens isoladas do sul do Brasil são analisadas e reavaliadas, o gênero é diagnosticado e, com base nos novos dados, apenas a população do rio Biguaçu é considerada uma espécie nova e linhagem independente. A descrição desta nova espécie é apresentada e comentários a respeito dos possíveis processos envolvidos na distribuição do gênero são feitos.

O Capítulo II, intitulado "Phylogenomics of the narrowly endemic genus *Eurycheilichthys* (Siluriformes: Loricariidae): a history of recent and rapid radiation in Southern Neotropical Freshwaters", consiste em um manuscrito a ser submetido à revista *Evolution*. Neste estudo, dados genômicos foram gerados a partir do método de *ddRADseq*, visando inferir as relações entre as espécies de *Eurycheilichthys*, apresentar uma estimativa de tempo de divergência e avaliar a estrutura dessas espécies. As análises indicam *Eurycheilichthys* como sendo um gênero monofilético e sugerem duas linhagens principais e extremamente recentes nas espécies do rio Taquari-Antas. Todas as espécies, exceto *E. luisae*, foram reconhecidas como monofiléticas e uma discussão acerca destes resultados e da distribuição das espécies é apresentada. As normas de submissão para ambas as revistas são fornecidas no final deste documento.

Capítulo I

Species delimitation in a restrictedly distributed group of cascudinhos (Loricariidae: *Epactionotus*) supports drainage basin endemism in coastal rivers of south Brazil.

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Running headline: Species delimitation in *Epactionotus*

Abstract

Species of *Epactionotus* are known for inhabiting the rocky-bottom stretches of rivers in a limited geographic area along the Atlantic coast of southern Brazil. Each of the three known species is endemic to single coastal river drainage (two neighbor drainages for *E. bilineatus*), which are isolated from each other by the coastal lacustrine systems or the Atlantic Ocean. *Epactionotus bilineatus* is from Maquiné and Três Forquilhas river basins, both tributaries of the Tramandaí River system, while *E. itaimbezinho* is endemic to the Mampituba River drainage, and *E. gracilis* to the Araranguá River drainage. Recent fieldwork in the Atlantic coastal drainages of southern Brazil revealed new populations in the Urussanga, Tubarão, d'Una, and Biguaçu rivers. Iterative species delimitation using molecular data (cytochrome oxidase subunit I - COI) and morphology (morphometrics and meristics) was applied in order to evaluate species recognition of isolated populations. In light of new data, the genus was re-diagnosed; the status of *Epactionotus* species/population was reevaluated, and the formerly described species were recognized, in addition to interspecific population structure. As for the newly discovered populations, both morphological and molecular data strongly support the population from the Biguaçu River drainage, in Santa Catarina State, as a new species. Molecular and distributional data suggest that not only palaeodrainage connectivity can explain species distribution, but also, that strong per basin genetic structure may be related with species habitat specificity.

Keywords: cascudinhos; coastal Brazilian drainages; COI gene; genetic distance; iterative taxonomy; Neotropical fish.

Introduction

Species of the cascudinho *Epactionotus* Reis and Schaefer 1998 were originally described to a limited geographic area along the Atlantic coast of southern Brazil. River drainages included in this region are part of the Tramandai-Mampituba Freshwater Ecoregion of the World (Abell *et al*., 2008 – FEOW 335), and records of this genus, until recently, were exclusive to this freshwater ecoregion. This ecoregion contains a large number of endemic species (Albert *et al*., 2011; Ferrer *et al*., 2015; Malabarba and Isaia, 1992; Reis and Schaefer, 1998) and have a relatively well-known species diversity (Bertaco *et al*., 2016). This area is a stage for recent studies testing phylogeographic questions associated with Pleistocene sea-level changes and ecologically mediated dispersal and species delimitation based on both morphological and molecular data (Angrizani and Malabarba, 2018; Hirschmann *et al*., 2015, 2017; Thomaz *et al*., 2015, 2017).

Species of *Epactionotus* are restricted to the rocky-bottom stretches of rivers, inhabiting fast-flowing waters and each of its three species is endemic to a single river drainage (except for *E. bilineatus*), which are isolated from each other by the Atlantic Ocean or coastal lacustrine systems (Figures 1 and S1). More specifically, *Epactionotus bilineatus* (Figures 2 and 3) is known from the rivers Maquiné and Três Forquilhas, both tributaries of the Tramandaí River system, while *E. itaimbezinho* (Figure 4) is endemic to the Mampituba River drainage, and *E. gracilis* (Figure 5) to the Araranguá River drainage (Malabarba *et al*., 2013; Reis and Schaefer, 1998).

The genus was morphologically diagnosed from other Hypoptopomatinae (Reis and Schafer, 1998; Schaefer, 1998) by several apomorphic features such as the posteriorly displaced dorsal fin, the absence of a fleshy flap in the dorsal portion of first pelvic-fin ray in males, and the possession of dentary and premaxillary accessory teeth.

Recent phylogenetic studies contrast to some degree regarding the position of *Epactionotus* within Hypoptopomatinae (Chiachio *et al*., 2008; Gauger and Buckup, 2005; Roxo *et al*., 2019) bur concur at pointing a sister group relationship with *Eurycheilichthys* (Cramer *et al*., 2008, 2011; Roxo *et al*., 2014).

Recent fieldwork in the Atlantic coastal drainages of southern Brazil revealed new populations north to the previously known area in the Urussanga, Tubarão, d'Una, and Biguaçu river drainages (Figures 1 and 6-9; Figure S1). In the light of new data, the status of *Epactionotus* species/population was reevaluated in these isolated drainages of southern Brazil and a new species is described from the Biguaçu River drainage, in Santa Catarina State.

Material and Methods

Morphological procedures and terminology

Morphological measurements were made with digital calipers point-to-point under a stereo microscope on the left side of specimens. Morphometric measurements were treated as percents of standard length (SL), except for subunits of the head, which were treated as percents of head length (HL). Counts performed on rays, vertebrae, teeth, and dermal plates were also conducted under the stereo microscope and the latter followed the serial homology and terminology proposed by Schaefer (1997). Morphological measurements and counts followed the descriptions made by Pereira *et al*. (2007) and include most of the modifications suggested by Calegari *et al*. (2011, 2014) and Lippert *et al*. (2014). Vertebral counts include all vertebral centra, including the five centra that comprise the Weberian apparatus, and the caudal complex centrum (PU1 + U1) counted as a single element.

The morphological data, except the number of vertebrae, were statistically analyzed comparing the populations and species of *Epactionotus* by drainages. Counts were analyzed with ANOVA (Analysis of Variance), aiming to compare the different means between groups and tested with Tukey's Test to determine which counts are significantly different between groups using PAST v. 3.12 (Palaeontological Statistics, Hammer *et al*., 2001). Based on Tukey's Pairwise results, box plots were also created with PAST.

Before statistically analyzing morphometric data, the VARSEDIG algorithm (Chuctaya *et al*., 2018; Faustino-Fuster *et al*., 2019; Guisande *et al*., 2016; Leigh and Bryant, 2015) was used to identify measurements that could significantly express sexual dimorphism in *Epactionotus*. Linear regression was then built to represent the morphometric character found to discriminate males and females. After excluding the measurement associated with sexual dimorphism (*i.e.* pelvic-fin unbranched ray width), statistical analyzes were conducted and all remaining morphometric variables were standardized according to Aitchison (1982) log-ratio transformation in order to adjust for size variation. The Aitchison-transformed data was then used in both principal component analysis (PCA) and linear discriminant analysis (LDA), also performed with PAST, to search for general patterns of variation among specimens (Leal and Sant'Anna, 2006) and assess between-groups patterns of body shape variation, respectively.

Individuals were diagnosed as *Epactionotus* based on the posterior displacement of the dorsal fin, lack of expanded fleshy flap on the dorsal surface of first pelvic-fin ray of males, possession of accessory oral teeth, and by the presence of two longitudinal light stripe markings on the dorsal surface of the head and trunk. Additionally, diagnostic osteological characters as the neural spine of seventh vertebra contacting

unpaired predorsal plate anterior to the nuchal plate, dorsal-fin proximal radial contacting the eighth vertebra, and the absence of the connecting bone were checked from cleared and double-stained specimens prepared according to a modification of the procedure described by Taylor and Van Dyke (1985).

Institutional abbreviations are those listed at http://www.asih.org/sites/default/files/2019-04/Sabaj_2019_ASIH_Symbolic_Codes_v7.1.pdf (Sabaj, 2019), except for UNICTIO that stands for Coleção de Peixes da Universidade do Vale do Rio dos Sinos (UNISINOS).

Distribution map

The distribution map was created using the QGIS software (v. 3.8), with shape and raster files from databases of IBGE (Instituto Brasileiro de Geografia e Estatística: http://mapas.ibge.gov.br/bases-e-referenciais), and ANA (Agência Nacional de Águas: http://www.snirh.gov.br/hidroweb), and followed the tutorial provided by Calegari *et al*. (2016). Species distribution data includes all records from the original publication and available material in the collections of MCN, MCP, MZUEL, UFRGS, and UNICTIO.

Molecular data and alignment

Tissue sample vouchers include material deposited in the collections of MCP, UFRGS, and UNICTIO. Muscles samples were removed from specimens, conserved in 99.8% ethanol and stored in -20°C freezers. From the ethanol-preserved samples, total genomic DNA was extracted using the DNeasy Blood and Tissue extraction kits (Qiagen, Valencia, CA, USA) following the manufacturer's protocol for animal tissues. The extractions from 23 individuals of *Epactionotus* (Table S1) were stored at -20ºC and partial sequences of the mitochondrial cytochrome oxidase C subunit I (COI) gene were

amplified using the primers COI L6252-Asn (5'- AAG GCG GGG AAA GCC CCG GCA G -3') and H7271-COXI (5'- TCC TAT GTA GCC GAA TGG TTC TTT T -3') (Melo *et al*., 2011). Polymerase Chain Reaction (PCR) was performed in a solution with a total volume of 25 µl: 2 µl of DNA template, 14.5 µl of PCR Master Mix (Invitrogen), 1.25 µl of each primer, and 6 µl of nuclease-free water to complete the total volume. Some samples were amplified using 1.2 µl of $MgCl^{+2}$ and a lower amount of water (4.8 µl).

The PCR amplifications consisted of a modified protocol from Melo *et al*. (2011), using Master Mix manufacturer's instructions. Amplification was conducted with an initial denaturation step (4 min at 94ºC) followed by 40 cycles of chain denaturation (30s at 95ºC), annealing (20s at 48ºC and 46ºC each), and nucleotide extension (60s at 72ºC). After the cycles, the final extension step was performed at 72ºC for 10 minutes. The PCR products were identified by electrophoresis in a 1% agarose gel, and successful DNA amplifications were sent to Function Bioscences (USA) for further purification and sequencing.

Newly generated sequences were edited and forward and reverse reads were assembled and visualized using Geneious v. 8.1. Under default parameters, all sequences were aligned with the algorithm Muscle (Edgar, 2004) also in Geneious. Three different datasets (alignments) were analyzed, one containing the newly sequenced specimens of *Epactionotus*, another containing additional COI sequences from five different individuals of *Epactionotus* available on GenBank provided by Cramer *et al*. (2008, 2011), and an additional alignment containing 12 sequences representing eight species of *Eurycheilichthys* also from Cramer *et al*., 2008, 2011 (Table S1). Ideally, there would be no distinction between newly sequenced *Epactionotus* and those found in GenBank, but available data present a few base pairs

with “N’s” and IUPAC codes for ambiguous nucleotide suggesting that prior sequenced material quality was not optimal and therefore may add some noise to the analyses.

Calculation of genetic distances within and among species was performed with software MEGA v. 7.0.26. (Kumar *et al*., 2016) under Kimura 2-parameter + G + I model (Kimura, 1980), the best-fit substitution model selected for the data set according to Bayesian Information Criterion (BIC).

Phylogenetic and time-divergence coalescence analyses

Alignment of the mitochondrial gene COI was partitioned by codon position, and the best model of nucleotide substitution and partition schemes were evaluated using PartitionFinder v. 2.1.1 (Lanfear *et al*., 2016) under the Bayesian Information Criterion (BIC). Phylogenetic relationships between haplotypes were inferred in BEAST v. 2.5 (Bouckaert *et al*., 2019) using a strict molecular clock and Yule process tree prior. MCMC analyses ran for 10 million generations and a single best tree was saved every 10 thousand generations. Run stabilization (EES > 200) was checked using Tracer v. 1.7 (Rambaut *et al*., 2018). The first 10% runs were discarded as burn-in and the remaining trees summarized using Maximum Clade Credibility Tree in TreeAnnotator 2.5. The gene COI was analyzed assuming an evolutionary rate of 0.01/site/Myr following mutation rates previously proposed to mitochondrial markers in fishes (Bermingham *et al*., 1997). For evaluating genetic data under coalescence-based approach the GMYC method (Generalized Mixed Yule-Coalescent; Fujisawa and Barraclough, 2013) was applied using the ultrametric tree obtained in BEAST. For the GMYC analyses the package “splits” (Species Limits by Threshold Statistics; Ezard *et al*., 2009) (http://rforge.r-project.org/projects/splits) was used in the program R version 3.0.0 (R

Core Team, 2013). GenBank accession numbers upon submission will be available in Table S1.

Results

Morphological analyses

Measurements and counts obtained for species/populations from each of the eight drainages are presented in Tables 1-4. When comparing the different means of meristic data between groups, the ANOVA disclosed statistically significant variation for: number of both right and left premaxillary teeth (f= 6.435, *p*= 3.07E-06 and f= 6.078, *p*=6.64E-06, respectively), number of both right and left dentary teeth (f= 8.331, *p*=5.91E-08 and f=6.28, *p*=4.28E-06, respectively), number of plates in median lateral series (f=5.99, *p*= 8.03E-06), number of plates in dorsal series (f=8.02, *p*=1.11E-07), number of plates in mid-ventral series (f=8.276, *p*=6.61E-08), number of plates in ventral series (f=9.534, *p*=5.50E-09), number of unpaired predorsal plates (f=12.28, *p*=3.47E-11), and number of both right and left abdominal plates (f=18.36, *p*=3.67E-18 and f=22.2, *p*=2.91E-21, respectively). As for the number of plates in mid-dorsal series, plates between anal and caudal-fin series, plates at both dorsal and anal-fin bases, predorsal plates, number of medium abdominal plates, and number of caudal-fin rays the analysis found no statistically significant variance whatsoever. The box plots of the significantly variant meristic data and results of Tukey's Pairwise are shown in Figure 10.

Morphometric variables not associated with sexual dimorphism (after Aitchison, 1986, log-ratio transformation) were used in principal components analysis (PCA) and linear discriminant analysis (LDA). When analyzing the general patterns of variation among specimens, plots of factor scores of principal component 1 *vs*. 2 grouped

specimens into four clusters partially overlapping each other (Figure 11). The specimens from Biguaçu (*Epactionotus* BI) and d'Una (*Epactionotus* DU) form two overlapping clusters that are well separated from all other populations, having low loadings on PC1. Individuals from Maquiné (part of *E. bilineatus*) and Urussanga (*Epactionotus* UR) form two clusters well separated from each other, but with both clouds slightly overlapping with specimens from Tubarão drainage (*Epactionotus* TB). Remaining specimens from Três Forquilhas (other part of *E. bilineatus*), Mampituba (*E. itaimbezinho*), Araranguá (*E. gracilis*), and Tubarão (*Epactionotus* TB), are grouped together. The first two components (PC1 and PC2) represent a variance of 24.3% and 16.9%, respectively. Measurements with heavier loadings on PC1 were caudal-peduncle width (0.47), body width (0.29), caudal-peduncle length (-0.25), and predorsal length (-0.23). On PC2 heavier loadings were caudal-peduncle width (0.59), pectoral-pelvic-fins distance (0.24), suborbital depth (-0.40), and dorsal-fin base length (-0.26).

When evaluating patterns of body shape variation between groups defined by drainage basin populations, the LDA recognized seven distinct clusters, with an overlap between Araranguá (*E. gracilis*) and Mampituba (*E. itaimbezinho*) and with one point of contact shared between part of *E. bilineatus* (from Três Forquilhas) and *E. itaimbezinho*, and *Epactionotus* BI and *Epactionotus* DU, respectively (Figure 12). The percentage of separation obtained for each discriminant function (from LD1-LD4) was 45.8%, 26.1%, 12.6%, and 9.5%, respectively. The loadings for discriminant function LD1 indicate caudal-peduncle length (0.01), predorsal length (0.009), first pelvic-fin unbranched ray length (-0.09), and caudal-peduncle width (-0.01) as the more significant measurements. As for LD2 heavier loadings were standard length (0.41), internareal width (0.39), suborbital depth (-0.01), and dorsal-fin base length (-0.009).

Phylogenetic and time-divergence analyses

The mitochondrial gene COI was sequenced for 23 individuals of *Epactionotus*. The combined data resulted in a matrix of 731 base pairs (bp). The best-fit model of nucleotide evolution estimated by PartitionFinder partitioned by codon position in each data set examined in this study is shown in Table S2. Analyses indicate two species-inclusive clades of *Epactionotus*, one of those with weak support (PP =0.44; Figure 13, Figures S2-S4). One of these clades contains *E. itaimbezinho* and *E. gracilis* (including individual from Urussanga), both species being reciprocally monophyletic to each other. In the other clade *E. bilineatus* is composed by two reciprocally monophyletic highly supported groups representing populations of *E. bilineatus* in the Maquiné and Três Forquilhas river drainages, respectively (PP=1.0; Figure 13). *Epactionotus bilineatus* is sister to a group formed by the *Epactionotus* BI and the population of *Epactionotus* TB (and *Epactionotus* DU in the analyses including all *Epactionotus* sequences; Figure S3). The *Epactionotus* radiation (first split within the genus) is dated to the Pleistocene (1.54 Ma, 95% confidence intervals 1.92-1.15 Ma; Figure 13). Splits between recognized species dated from 1.38 Ma to 0.74 Ma. Events of divergence between allopatric populations of *E. bilineatus* are dated to Maquiné and Três Forquilhas river drainages (0.96 Ma; 95% intervals 1.31-0.65 Ma).

Coalescence and genetic distance

Results of the GMYC analyses vary depending on the dataset used, being more conservative with the matrix containing the outgroup *Eurycheilichthys* (four clusters, one single entity) when compared to the analyses where only *Epactionotus* specimens were examined (5-6 clusters and 3-4 single entities; Table 5; Figure 13; Figures S2-S4). Clusters in the analyses containing *Eurycheilichthys* correspond to morphologically

delimited species (e.g. *E. bilineatus*, *E. gracilis*, and *E. itaimbezinho*) with the exception of the clustering of *Epactionotus* BI with samples of *Epactionotus* TU and *Epactionotus* DU. Analyses of only *Epactionotus* sequences (excluding *Eurycheilichthys*) support less conservative species delimitation and suggest species clusters for most drainages as for example the separation between Maquiné and Três Forquilhas populations in *E. bilineatus* (Table 5; Figure 13; Figures S2-S4). Comparison between Yule and Coalescent constant population size tree priors have little influence on species recognition by the GMYC approach but some influence in tree topology (Figures S2-S4).

According to Bayesian Information Criterion (BIC = 3585.76) the best nucleotide model selected for the genetic distance analysis was K2+G+I (Table S3). Distance values within drainages (Table 6) ranged from 0.00% (within individuals of *Epactionotus* from Biguaçu) to 0.78% (within *E. bilineatus* from the Três Forquilhas drainage). As for between drainages, distance values varied from 1.2% and 1.5% (between populations of *Epactionotus* TU and *Epactionotus* DU, and between *E. bilineatus* from Três Forquilhas and Maquiné, respectively), to 4.07% (between *Epactionotus* BI and *Epactionotus* UR).

Species description

***Epactionotus* (BI), new species**

Figure 9, Tables 2 and 4

Holotype: UFRGS 28220, female, 35.4 mm SL, Brazil, Santa Catarina, Antônio Carlos, Rachadel River and a small tributary, Biguaçu Drainage, inside property of Mr. Paulo

Lopes at locality of Guiomar, 27°29'44"S, 48°46'57" W, 2 Aug 2015, T. P. Carvalho, F. Carvalho, and A. Thomaz.

Paratypes: UFRGS 20926 (11, 32.7-39.0 mm SL + 3 fixed in alcohol for tissue samples) and MCP uncataloged (4, 36-37.9 mm SL+ 2 c&s, 35.4-36.1 mm SL, collected with holotype. UFRGS 22913 (9, 30.8-37.8 mm SL+ 8 fixed in alcohol), Brazil, Santa Catarina, Antonio Carlos, Rachadel River, N of Rachadel, 27°28'22.8"S 48°48'00.8"W, 30 May 2017, J. Ferrer, L. Donin, N. Pio, and T. P. Carvalho.

Diagnosis

Epactionotus BI is distinguished from *Epactionotus bilineatus*, *E. itaimbezinho*, and *E. gracilis* by having the posterior region of abdomen naked, devoid of any embedded platelets between pelvic fins and anal tube (*vs*. at least one small platelet between pelvic fins and anal tube), a shallower caudal peduncle (7.7-9.3% *vs*. 9.2-10.8%, 9.9-11.2%, 9.8-10.9 % SL, respectively). Additionally, it can be distinguished from *E. bilineatus* and *E. itaimbezinho* by having comparatively narrower light stripes on head, predorsal region, and dorsal surface of the trunk (*vs*. broader light stripe markings), narrower body (cleithral width 19-20.8% *vs*. 23.1-26.1% and 22.2-23.8% SL, respectively) and a shorter pectoral-fin spine (15.5-18.8% *vs*. 20.2-23.1%, 19.1-21.6% SL, respectively). From *E. itaimbezinho* and *E. gracilis* it is also diagnosed by having the chromatophores of first thickened rays of dorsal, pectoral, and pelvic fins evenly arranged and distributed, leaving fin rays plain and dusky (*vs*. chromatophores arranged in series of 5-6 small dots), and by the ventral lobe of caudal-fin completely dark brown (*vs*. ventral lobe of caudal-fin dark brown with hyaline spot in the middle portion of the interradial membrane between two most ventral rays – Figure 14). From *Epactionotus bilineatus* it

is also differentiated by a narrower head (59.5-66.6% *vs*. 70.2-77.2% HL), smaller interorbital distance (34.5-38.6% *vs*. 38.4-42.3% HL), shorter pectoral-fin length (19.0-22.3% *vs*. 22.3-26.1% SL), and shorter first pelvic-fin unbranched ray length (13.6-16.5% *vs*. 16.3-19.4% SL). From the populations of *Epactionotus* UR and *Epactionotus* TU the new species can be distinguished by a narrower body (cleithral width 19.0-20.8% *vs*. 20.6-22.8% and 22.1-24.3% SL, respectively). From the populations of *Epactionotus* UR and of *Epactionotus* DU it can be diagnosed by the ventral lobe of caudal-fin completely dark brown (*vs*. ventral lobe of caudal-fin dark brown with hyaline spot in the middle portion of the interradial membrane between two most ventral rays – Figure 14). Additionally, from population of *Epactionotus* TU it is distinguished by a shorter pectoral-fin spine (15.5-18.8% *vs*. 19.3-20.7% SL), shorter pectoral-fin length (19.0-22.3% *vs*. 22.3-23.5% SL), shallower caudal peduncle (7.7-9.3% *vs*. 9.3-10.7 % SL), narrower head (59.5-66.6% *vs*. 67.1-71.6% SL), and smaller interorbital distance (34.5-38.6% *vs*. 39.1-41.2% HL).

Description

Measurements and counts in Tables 2 and 4. Body relatively slender and elongated. Dorsal profile of head and body slightly convex from snout tip to dorsal-fin origin; interorbital slightly elevated. Trunk profile mostly straight and slightly tapering from dorsal-fin origin to anteriormost procurrent caudal-fin ray. Body deepest at dorsal-fin origin and shallowest at posterior portion of caudal peduncle. Caudal peduncle ovoid to rounded in cross section, progressively compressed from anteriormost anal-fin ray to caudal-fin base. Greatest body width at cleithrum.

Anterior margin of snout rounded and head narrow in dorsal view. Snout with paired depressions anterior to nostrils; depression beginning close to snout tip. Eye

small, dorsolaterally positioned, iris operculum present. Fenestrae of compound pterotic increasing in size towards posterolateral margin of bone. Four to five (usually four) paired predorsal plates and one to three (usually two) unpaired predorsal plates anterior to square shaped nuchal plate. Odontodes on margin of snout slightly larger than remaining odontodes on head. Odontodes on ventral margin of snout distinctly enlarged. Posterior tip of parieto-supraoccipital without small tuft of enlarged odontodes. No other crests of odontodes on dorsal surface of head. Lips rounded and covered with globular papillae; small fleshy ridge posterior to dentary. Maxillary barbel short. Teeth slender, bifid, with blade-like larger medial cusp and smaller lateral cusp.

Accessory patch of unicuspid teeth on both premaxilla and dentary, located more internally into mouth and attached to dermal bone. Accessory teeth elongate, sharply pointed, directed posteroventrally (on premaxilla) and anteroventrally (on dentary).

Median series of lateral plates complete; some median lateral plates without lateral line canal; lateral line gap starting at vertical line through midpoint of dorsal fin. Odontodes on head and trunk pointed, uniform in size and shape and somewhat aligned; odontodes on trunk and caudal peduncle slightly larger. Odontodes on ventral surface of body smaller and evenly distributed, not arranged in lines. Body almost entirely covered by plates, except nostrils, area between lower lip and pectoral girdle, region overlying lateral opening of swimbladder capsule, most of abdomen, area around anus, and fin bases. Ventral portions of cleithrum and coracoid almost entirely exposed and supporting odontodes, except for small median region, especially of cleithrum, covered with skin. Abdomen with none to four (usually one) small, rounded to slightly laterally elongate lateral abdominal plates, located between posterior process of coracoids and pelvic-fin insertions; median and posterior region of abdomen between pelvic fins and

urogenital papilla naked, devoid of any plates or platelets embedded in skin or scattered odontodes. Total vertebrae 31, ribs 5, beginning on eighth or ninth vertebral centrum, in addition to large rib on sixth centrum.

Dorsal fin I,7 (one specimen with I,8), its origin at vertical line through middle of pelvic fin. Dorsal-fin spinelet short and slightly wider than dorsal-fin spine. Pectoral fin I,6, with large axillary slit in skin behind fin insertion. Serrae absent along mesial margin of pectoral-fin spine. Pectoral fin reaching to vertical line slightly posterior to insertion of pelvic-fin unbranched ray in males; reaching to midpoint of pelvic-fin unbranched ray in females. Pelvic fin i,5, with robust first ray shorter than branched rays. Skin flap absent on first unbranched pelvic-fin ray of males and females. First pelvic-fin unbranched ray slightly thicker in males than females (see sexual dimorphism section). Anal fin i,5; first anal-fin pterygiophore usually exposed in front of unbranched fin ray. Odontodes on pelvic-fin unbranched ray turned and strongly pointing mesially. Adipose fin absent. Caudal fin i,14,i (one specimens with i,13,i), forked, lower lobe equal to or slightly longer than upper lobe.

Coloration in alcohol

Ground color of dorsal surface of head and trunk medium to dark greyish brown, yellowish white and mostly unpigmented ventrally. Pair of longitudinal light cream stripes on each side of snout; stripes begin medially on tip of snout, passing laterally between nostrils and orbits on each side, and proceed backward, narrowing after orbit and terminating near posterior margin of compound pterotic. Second pair of longitudinal light stripes on each side of dorsal surface of body from predorsal region to near caudal peduncle. Lateral margins of head and trunk, especially head, below line from ventral margin of snout to posterior tip of opercular bone and tip of posterior

process of cleithrum lighter than dorsal portions of head, but with scattered small dark dots. Posterior tip of parieto-supraoccipital slightly unpigmented. First thickened rays of dorsal, pectoral, and pelvic fins with chromatophores equally arranged and distributed, leaving fin rays plain and dusky. Branched rays in these fins with similar color pattern. Dorsal and ventral borders of pectoral-fin slit densely pigmented with brownish black chromatophores forming dark blotches of irregular shape and size. Concentration of black chromatophores on ventral side of pectoral girdle, between posterior process of coracoid and origin of pectoral-fin spine. Few dots on leading anal-fin branched ray. Interradial membrane of all fins, except caudal-fin, unpigmented. Ventral lobe of caudal-fin completely dark brown; interradial membrane between five upper rays of caudal fin unpigmented, leaving dorsal lobe lighter towards posterior end.

Sexual dimorphism

Males have a small, conical urogenital papilla behind the anal tube, which is not present in females. Females have a longer pectoral fin than males (pectoral fin of females reaches to the midpoint of pelvic-fin unbranched ray *vs*. pectoral fin of males reaching to a vertical line slightly posterior to insertion of pelvic-fin unbranched ray). Finally, as identified by the VARSEDIG males of *Epactionotus,* including *E*. BI, have the first pelvic-fin unbranched ray slightly thicker than females (width of the first pelvic-fin unbranched ray of males 17.7-21.0%, mean 19.8%, *vs*. 13.4-17.6%, mean 15.4% of its length in females – Figures 15-17).

Habitat and ecological notes

Epactionotus BI inhabits medium to fast water in a creek about five meters wide and maximum of 0.5 m depth, with clear water running over sand, pebbles, and rocks

(Figure 18). The specimens were caught in the submersed marginal vegetation composed mostly by grasses.

Distribution

Epactionotus BI is so far known only from two localities in the Rachadel River, a tributary to the Biguaçu River drainage, in Santa Catarina State, South Brazil (Figures 1, 18 and S1).

Conservation status. *Epactionotus* BI is only known from two localities along the same stretch of the Rachadel River. This river basin along with its alluvial plain has suffered from deforestation, sand extraction, and transformation of its margins into agricultural land for vegetables farming. As its distribution is largely unknown and other conservation parameters cannot be accessed for the species, *Epactionotus* BI is provisionally categorized as Data Deficient according to the IUCN criteria and categories (IUCN Standards and Petitions Committee, 2019).

Discussion

When analyzing the species and populations of *Epactionotus*, all the diagnostic characters described by Reis and Schaefer (1998) added to the absence of the connecting bone, which has been considered as another independently derived diagnostic feature for *Epactionotus* (Calegari *et al*., 2011; Delapieve *et al*., 2017; Martins *et al*., 2014; Rodriguez *et al*., 2015), were herein observed. Therefore, in spite of new informations over the last 20 years, the combination of the diagnostic characters formerly given by Reis and Schaefer (1998) has proven to be useful to date.

Analyses of morphological data (ANOVA, PCA, and LDA) of previously known and new populations identified significant variation that coincide with geographical patterns. Likewise, most genetic distance values between drainages are above 2%, and some results of GMYC analyses support less conservative species delimitation and suggest species clusters for most drainages.

When considering variation between the populations of *Epactionotus bilineatus* from Maquiné and Três Forquilhas rivers, genetic values have minimum distances (1.5%) and meristic differences usually overlap with each other and are herein considered intraspecific variation. The newly discovered populations from the Urussanga, Tubarão, and d'Una rivers have variable support from either morphology or molecules to be recognized as independent evolutionary units.

The range extension of *Epactionotus* is expanded considerably northwards from its former northern limit in the Araranguá River basin (Reis and Schaefer, 1998), and is currently expanded to the Urussanga, Tubarão, d'Una, and Biguaçu river drainages. Previous authors (Reis and Schaefer, 1998; Abell *et al*., 2008) recognize the endemism of southern Brazilian coastal drainages. Causal association with this endemism and isolation of this fauna are often related to paleodrainage connection during marine regression on pleistocenic glacial periods (Thomaz *et al*., 2015; Thomaz and Knowles, 2018) or the presence of conspicuous mountainous barriers such as the Serra do Tabuleiro (Carvalho, 2007). Despite likely for the other basins, the presence of *Epactionotus* in the Biguaçu River drainage cannot be explained by any of these mechanisms. An often-cited model of fish dispersal within coastal basins, other than the paleodrainage connection by sea-level retreat, is the headwater river captures (Lima *et al*., 2017; Ribeiro, 2006). However, headwaters of the Biguaçu are not contiguous with those of the southward tributaries (e.g. Tubarão River drainage), and stepping-stone

dispersal via headwater river captures would preclude the absence of the genus in intervening drainages, such as the Cubatão Sul River drainage, or demand its extinction in that area (Figure 1 and S1).

Genetic data, and to some degree, morphology, support the uniqueness of each of the *Epactionotus* populations/species on isolated river drainages. Isolated river drainages have been extensively used as biogeographical units (Albert and Carvalho, 2011; Dagosta and de Pinna, 2017) and are often a primary hypothesis for species delimitation in freshwater fishes. Geographic distribution in freshwater fishes seems to be directly related to the position on the river network a fish occupies (Carvajal-Quintero *et al*., 2019). Species such as *Epactionotus* species that are ecologically associated with rapids on more upstream portions of the river network may be more susceptive to isolation and allopatric diversification and as a result have smaller distribution ranges. Another factor that may influence isolation and diversification is the use, or the lack thereof, of river connections on the palaeodranaiges during the sea-level retreats of the Pleistocene. *Epactionotus* lineages may not have used this lowland connection due to habitat specificities that created an ecological barrier of lowland habitat between these former palaeodrainages. This is also observed in the genetic signatures of other rapids dwelling headwater fishes in the region (Hirschmann *et al*., 2015). Therefore, analyses of diversification rates dependent on habitat types (Roxo *et al*., 2017) may reflect an association between population genetic differentiation and speciation rates (Harvey *et al*., 2017; Singhal *et al*., 2018).

Comparative material examined (all from Brazil)

Epactionotus bilineatus: MCN 12064, 3 alc, rio Pinheiros, Maquiné, Rio Grande do Sul (29°38'17"S 50°13'30"W). MCN 12080, 3 alc, rio Maquiné, Maquiné, Rio Grande do

Sul (29°39'07"S 50°12'32"W). MCP 18495, 52 alc, arroio Água Parada, tributary of the rio Maquiné, Maquiné, Rio Grande do Sul (approx. 29°40'S 50°12'W). MCP 19105, 7alc, arroio do Ouro, on BR-101 ca. 1 km west from Maquiné (29°39'58"S 50°10'59"W). MCP 21335, 15 alc, arroio Escangalhado, Maquiné, Rio Grande do Sul (29°34'05"S 50°17'15"W). MCP 26964, 2 alc, 2 tis, arroio Água Parada, Maquiné, Rio Grande do Sul (29°39'43"S 50°12'43"W). MCP 29116, 25 alc, 3 c&s, arroio Forqueta near mouth of a small tributary of the rio Maquiné, Barra do Ouro, Rio Grande do Sul (29°32’08”S 50°12’21”W). MCP 29119, 9 alc, 3 c&s, arroio Garapiá, ca 300m downstream from waterfall, tributary of rio Forqueta, Maquiné, Barra do Ouro, Rio Grande do Sul (29°30’26”S 50°14’39”W). UFRGS 3290, 1 alc, rio Maquiné, Maquiné, Rio Grande do Sul, (29°40’16”S 50°11’44”W). UFRGS 10649, 5 alc, rio Cerrito at Barra do Ouro, Barra do Ouro, Rio Grande do Sul (29°34'14"S 50°16'50"W). UFRGS 17817, 39 alc, Barra do Ouro on the road to Garapiá, Maquiné, Rio Grande do Sul (29°34’13.6”S 50°16’49.0”W). UFRGS 17967, 5 alc, rio Maquiné near camping ground of Maquiné, Maquiné, Rio Grande do Sul (29°38'53"S 50°13'04"W). UFRGS 20804, 6 alc, rio Escangalhado near Barra do Ouro, Barra do Ouro, Rio Grande do Sul (29°34'02"S 50°17'09"W). UFRGS 20943, 18 alc, rio Maquiné at bathing spot, Maquiné, Rio Grande do Sul (29°39'08"S 50°12'34"W). UFRGS 22210, 2 alc, arroio Água Parada at Barra do Ouro, Barra do Ouro, Rio Grande do Sul (29°40’19”S 50°12’12”W). UNICTIO 1406, 8 alc, 1 tis, rio Maquiné, Maquiné, Rio Grande do Sul (29°35'14.7"S 50°16'12.0"W). UNICTIO 1444, 1 alc, 1 tis, arroio Forqueta, Maquiné, Rio Grande do Sul (29°32'28.1"S 50°12'08.9"W). MCN 18573, 39 alc, rio Carvalho inside property of Dona Maria Luiza, São Francisco de Paula, Rio Grande do Sul (29°22'55"S 50°11'52"W). MCN 18598, 8 alc, arroio Bananeira, at bridge on road Rota do Sol, São Francisco de Paula, Rio Grande do Sul (29°25'17"S 50°09'56"W). MCN

18608, 19 alc, arroio Pinto at vicinal road to Rota do Sol, São Francisco de Paula (29°23'22"S 50°10'52"W). MCN 19405, 8 alc, rio Três Forquilhas, Terra de Areia, Rio Grande do Sul (29°32'29"S 50°01'54"W). MCN 19406, 8 alc, rio Três Forquilhas, Terra de Areia, Rio Grande do Sul (29°32'29"S 50°01'54"W). MCN 20068, 5 alc, arroio near to Linha Bernardes, Tramandaí (29°30'50.4"S 50°07'42.8"W). MCP 14806, paratypes, 4 alc, 1 c&s, rio Três Pinheiros, tributary of rio Três Forquilhas, 8 km NW of highway BR-101 towards Itati, Terra de Areia, Rio Grande do Sul (approx. 29°32'S, 50°06'W). MCP 23679, 40 alc, 1 tis, arroio do Padre ca 0.4 km upstream from church Arroio do Padre, Itati, Rio Grande do Sul (29°29'28"S 50°08'35"W). MCP 25277, 5 alc, rio Três Pinheiros, at bridge on road to Vila Itati, ca 7 km N of highway BR-101, Terra de Areia, Rio Grande do Sul (29°31’36”S 50°06’21”W). MCP 25311, 34 alc, stream on road between Terra de Areia and Vila Itati, ca 8 km N of highway BR-101, Vila Nova, Terra de Areia, Rio Grande do Sul (29°31’01”S 50°06’40”W). MCP 28978, 39 alc, arroio Japonês, between Três Forquilhas and Itati, Três Forquilhas, Rio Grande do Sul (approx. 29°32’S 50°05’W). MCP 29138, 14 alc, arroio Bananeira, tributary of rio Três Forquilhas, Itati, Rio Grande do Sul (29°27’22”S 50°11’13”W). MCP 29293, 29 alc, 3 c&s, arroio Bananeira, tributary of rio Três Forquilhas, Itati, Rio Grande do Sul (29°25’26”S 50°10’16”W). UFRGS 3257, 6 alc, rio Três Forquilhas near Três Forquilhas, Três Forquilhas, Rio Grande do Sul (29°31'60"S 50°04'60"W). UFRGS 6564, 22 alc, rio Três Forquilhas at Vila Boa União, Terra de Areia, Rio Grande do Sul (29°28’17”S 50°07’01”W). UFRGS 9128, 2 alc, rio Carvalho near road Rota do Sol, São Francisco de Paula, Rio Grande do Sul (29°22'55"S 50°11'52"W). UFRGS 12740, 6 alc, rio Três Forquilhas, Três Forquilhas, Rio Grande do Sul, (29°28'20.2"S 50°07'10.0"W). UFRGS 16506, 23 alc, mouth of arroio da Barra into arroio Bananeiras, Itati, Rio Grande do Sul (29°25'37"S 50°10'49"W). UFRGS 16538, 14 alc, arroio

Carvalho tributary to rio Três Forquilhas on road Rota do Sol, Itati, Rio Grande do Sul (29°23’25”S 50°11’02”W). UFRGS 16545, 2 alc, rio da Boa União, tributary to rio Três Forquilhas at vicinal road to Rota do Sol, upstream Itati, Rio Grande do Sul (29°27'18"S 50°07'22"W). UFRGS 20747, 2 alc, arroio Bananeiras at vicinal road to Rota do Sol, Itati, Rio Grande do Sul (29°25'36"S 50°10'29"W). UFRGS 20827, 11 alc, creek tributary to rio Três Forquilhas on parallel road to Rota do Sul, Itati, Rio Grande do Sul (29°25'54.86"S 50°06'42.78"W). UFRGS 21392, 5 alc, rio do Padre, tributary to rio Três Forquilhas, Itati, Rio Grande do Sul (29°29'27.41"S 50°08'49.00"W).

***Epactionotus itaimbezinho*:** MCP 14708, paratypes, 12 alc, 3 c&s, rio Canoas, tributary of rio Mampituba, ca 8 km from Praia Grande towards Mãe dos Homens, Praia Grande, Santa Catarina (approx. 29°14'S, 50°01'W). MCP 23620, 19 alc, arroio Maia Coco in Vila Rosa ca 5 km NW of Praia Grande, Morrinhos do Sul, Santa Catarina (29°10’13”S 49°58’49”W). MCP 23683, 36 alc, rio Mangue between Morrinhos do Sul and Praia Grande, Morrinhos do Sul, Santa Catarina (29°14’55”S 49°55’30”W). MCP 29251, 15 alc, stream tributary to rio Mampituba towards Itaimbezinho Canion, Praia Grande, Santa Catarina (29°12’18”S 49°58’19”W). UFRGS 10833, 3 alc, stream tributary to rio Mampituba, Praia Grande, Santa Catarina (29°10’36”S 49°58’14”W). UFRGS 10849, 9 alc, arroio Molha Coco, tributary to rio Mampituba 0.6 km from Praia Grande at Vila Rosa, Praia Grande, Santa Catarina (29°10’09”S 49°58’56”W). UFRGS 12719, 3 alc, creek on road to Faxinalzinho Canion, Praia Grande, Rio Grande do Sul (29°11’54”S 49°57’57”W). UFRGS 23963, 1 alc, pool near rio Mampituba, Praia Grande, Santa Catarina (29°15'10"S 50°07'00"W). UNICTIO 1908, 1 of 5 alc, 3 tis, arroio Faxinalzinho, tributary to rio Mampituba, Praia Grande, Santa Catarina (29°14’57”S 50°07’17”W). UNICTIO 1993, 1of 2 alc, 1 tis, arroio Malacara, tributary to rio Mampituba, Praia Grande, Santa Catarina (29°10’07.2”S 49°58’17.7”W). UNICTIO

2123, 1 of 11 alc, 1 tis, arroio Cachoeira, tributary to rio Mampituba, Praia Grande, Santa Catarina (29°08’11.6”S 49°54’21.1”W). ***Epactionotus gracilis*:** MCP 20282, holotype, rio Jordão at Jordão Alto, Nova Veneza, Santa Catarina (approx. 28°36'S 49°29'W). MCP 11615, paratypes, 15 alc, 4 c&s, collected with holotype. MCP 19193, 2 alc, rio do Cedro on road from Meleiro to Forquilhinha, Meleiro, Santa Catarina (approx. 28°48'S 49°34'W). MCP 19198, 1 alc, rio Mãe Luzia, Forquilha, creek tributary of rio Araranguá, Treviso, Santa Catarina (28°27'40”S 49°30'04”W). MCP 23606, 5 alc, rio Morto ca 7 km N of Meleiro towards São Francisco, Meleiro, Santa Catarina (28°47’09”S 49°39’23”W). MCP 23638, 3 alc, rio Morto on road between Meleiro and São Francisco, ca 11 km N of Meleiro, Meleiro, Santa Catarina (28°45’00”S 49°39’29”W). MCP 53973, 3 alc, 1 tis, rio Amola Faca at bridge on road SC-285 between Turvo and Timbé do Sul, Timbé do Sul, Santa Catarina (28°50'25"S 49°48'02"W). MCN 4734, 4 alc, rio Jordão Baixo, tributary to rio Mãe Luzia, Siderópolis, Santa Catarina (28°35'13"S 49°29'20"W). UFRGS 261, 1 alc, rio Jordão at Jordão Baixo, Siderópolis, Santa Catarina (28°36'00.02"S 49°24'57.60"W). UFRGS 1861, 251 alc, rio Jordão at Jordão Baixo, Siderópolis, Santa Catarina, (approx. 28°36'S 49°25'W). UFRGS 6111, 60 alc, rio Mãe Luzia, Treviso, Santa Catarina (28°27’58” S 49°28’18”W). UFRGS 6214, 9 alc, rio Mãe Luzia at Mina Comim, Treviso, Santa Catarina. UFRGS 10863, 12 alc, rio do Salto at Parque Ecológico, Timbé do Sul, Santa Catarina (28°49'44"S 49°45'21"W). UFRGS 12544, 1 alc, rio Jordão Alto, Nova Veneza, Santa Catarina (28°39'29"S 49°32'36"W). UFRGS 15390, 9 alc, rio Mãe Luzia, Treviso, Santa Catarina (28°28'00"S 49°28'19"W). UFRGS 22945, 3 alc, stream next to Alto Jordão, Nova Veneza, Santa Catarina (28°35’02.2”S 49°32’31.2”W). UNICTIO 1866, 6 of 14 alc, 4 tis, stream on Road to Vila Artesanal, tributary to rio Araranguá, Jacinto Machado, Parque Nacional Aparados da Serra, Santa Catarina (29°01'47.8"S

49°54'04.4"W). UNICTIO 1882, 1 of 3 alc, 4 tis, arroio Pai José, tributary to rio Araranguá, Jacinto Machado, Santa Catarina (29°00'42.6"S 49°53'19.0"W). ***Epactionotus*** **Urussanga**: MCP 53836, 16 alc, 3 tis, creek tributary of rio Carvão Alto, Urussanga, Santa Catarina (28°30'02.7"S 49°23'10.0"W). UFRGS 6212, 10 alc, rio Lageado near USITESC, Urussanga, Santa Catarina (28°31'04.92"S 49°19'10.07"W). UFRGS 9060, 8 alc, creek tributary to rio Urussanga, Urussanga, Santa Catarina (28°30'00.33"S 49°23'43.00"W). ***Epactionotus*** **d'Una**: MCP 35156, 1 tis, stream tributary to rio d'Una, Imbituba, Santa Catarina (28°11'56"S 48°47'17"W). MZUEL 07528, 51 alc, 5 tis, rio d'Una, Imarui, Santa Catarina (28°10'48.8"S 48°47'12.0"W). ***Epactionotus*** **Tubarão:** UFRGS 22941, 3 alc, 1 tis, rio Bonito, on Rio Bonito Alto, Santa Catarina (28°25'48.3"S 49°27'50.7"W). MCN 18835, 4 alc, rio Palmeiras, tributary to rio Tubarão, Lauro Müller, Santa Catarina (28°27'01"S 49°25'03"W). MCN 18844, 1 alc, rio do Rastro, tributary to rio Tubarão, Lauro Müller, Santa Catarina (28°21'50"S 49°26'43"W).

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Contributions

MLSD, TPC and RER designed the study. RER and TPC made substantial contributions to conception and acquisition of data. MLSD generated morphological and molecular data. MLSD and TPC analyzed the data. MLSD, TPC and RER interpreted results and wrote the manuscript.

Significance Statement

Distribution of *Epactionotus*, known to be endemic to Maquiné, Três Forquilhas, Mampituba and Araranguá rivers, is expanded northwards to Urussanga, Tubarão, d'Una, and Biguaçu river drainages. Species delimitation helps understanding how these populations and river drainages evolved. Data supports the endemism of each *Epactionotus* populations on isolated river drainages, a frequent hypothesis for species delimitation in freshwater fishes. Habitat specificities suggest that *Epactionotus* lineage may not have used lowland connections between former palaeodrainages.

References

Abell R., Thieme, M. L., Revenga, C., Bryer, M., Kottelat, M., Bogutskaya, N., … Petry., P. (2008). Freshwater ecoregions of the world: a new map of biogeographic units for freshwater biodiversity conservation. *BioScience*, **58**, 403–414.

Aitchison, J. (1982). The statistical analysis of compositional data. *Journal of the Royal Statistical Society: Series B (Methodological)*, **44**, 139–177.

Albert, J. S., & Carvalho, T. P. (2011). Neogene assembly of modern faunas. In J. S. Albert & R. E. Reis (Eds.), *Historical biogeography of Neotropical freshwater fishes* (pp. 119–136). Berkeley, Los Angeles, CA: University of California Press, Ltd.

Albert, J. S., Petry, P., & Reis, R. E. (2011). Major biogeographic and phylogenetic patterns. In J. S. Albert & R. E. Reis (Eds.), *Historical biogeography of Neotropical freshwater fishes* (pp. 21–57). Berkeley, Los Angeles, CA: University of California Press, Ltd.

Angrizani, R. C., & Malabarba, L. R. (2018). Morphology and molecular data reveal the presence of two new species under *Rhamdia quelen* (Quoy Gaimard, 1824)(Siluriformes: Heptapteridae) species complex. *Zootaxa*, **4388**, 41–60.

Bermingham E., McCafferty, S. S., & Martin, A. P. (1997). Fish biogeography and molecular clocks: perspectives from the Panamanian Isthmus. In T. D. Kocher & C. A. Stepien (Eds.), *Molecular systematics of fishes* (pp. 113–128). San Diego, CA: Academic Press.

Bertaco, V. A., Ferrer, J., Carvalho, F. R., & Malabarba, L. R. (2016). Inventory of the freshwater fishes from a densely collected area in South America-a case study of the current knowledge of Neotropical fish diversity. *Zootaxa*, **4138**, 401–440.

Bouckaert, R., Vaughan, T. G., Barido-Sottani, J., Duchêne, S., Fourment, M., Gavryushkina, A., ... Drummond A. J. (2019). BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *PLoS computational biology*, **15**, e1006650.

Calegari, B. B., Delapieve, M. L. S., & Souza, L. M. (2016). Tutorial para preparação de mapas de distribuição geográfica. *Boletim Sociedade Brasileira de Ictiologia*, **118**, 15–30.

Calegari, B. B., Lehmann, P. A., & Reis, R. E. (2011). A new species of *Otothyropsis* (Siluriformes: Loricariidae) from the rio Paraguay basin, Paraguay. *Neotropical Ichthyology*, **9**, 253–260.

Calegari, B. B., Silva, E. V., & Reis, R. E. (2014). *Microlepidogaster discontenta*, a new species of hypoptopomatine catfish (Teleostei: Loricariidae) from the rio São Francisco basin, Brazil. *Ichthyological Exploration of Freshwaters*, **25**, 213–221.

Carvajal-Quintero, J., Villalobos, F., Oberdorff, T., Grenouillet, G., Brosse, S., Hugueny, B., Jézéquel, C., & Tedesco, P. A. (2019). Drainage network position and historical connectivity explain global patterns in freshwater fishes' range size. *Proceedings of the National Academy of Sciences*, **116,** 13434–13439.

Carvalho, T. P. (2007). Distributional patterns of freshwater fishes in coastal Atlantic drainages of eastern Brazil: a preliminary study applying parsimony analysis of endemism. *Darwiniana*, 45(Suppl).

Chiachio, M. C., Oliveira, C., & Montoya-Burgos, J. I. (2008). Molecular systematic and historical biogeography of the armored Neotropical catfishes Hypoptopomatinae and Neoplecostominae (Siluriformes: Loricariidae). *Molecular Phylogenetics and Evolution*, **49**, 606–617.

Chuctaya, J., Bührnheim, C. M., & Malabarba, L. R. (2018). Two new species of *Odontostilbe* historically hidden under *O. microcephala* (Characiformes: Cheirodontinae). *Neotropical Ichthyology*, **16**, e170047.1–e170047.22.

Cramer, C. A., Bonatto, S. L., & Reis, R. E. (2011). Molecular phylogeny of the Neoplecostominae and Hypoptopomatinae (Siluriformes: Loricariidae) using multiple genes. *Molecular Phylogenetics and Evolution*, **59**, 43–52.

Cramer, C. A., Liedke A. M. R., Bonatto S. L., & Reis R. E. (2008). The phylogenetic relationships of the Hypoptopomatinae and Neoplecostominae (Siluriformes: Loricariidae) as inferred from mitochondrial cytochrome c oxidase I sequences. *Bulletin of Fish Biology*, **9**, 51–59.

Dagosta, F. C. P., & Pinna, M. D. (2017). Biogeography of Amazonian fishes: deconstructing river basins as biogeographic units. *Neotropical Ichthyology*, **15**, e170034.

Delapieve, M. L. S., Lehmann, P. A., & Reis, R. E. (2017). An appraisal of the phylogenetic relationships of Hypoptopomatini cascudinhos with description of two new genera and three new species (Siluriformes: Loricariidae). *Neotropical Ichthyology*, **15**, e170079.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, **32**, 1792–1797.

Ezard, T., Fujisawa, T., Barraclough, T. G. (2009). Splits: Species' Limits by Threshold Statistics. R package version 1.0-11/r29. http://www.R-Forge.R project.org/projects/splits/

Faustino-Fuster, D. R., Bockmann, F. A., & Malabarba, L. R. (2019). Two new species of *Heptapterus* (Siluriformes: Heptapteridae) from the Uruguay River basin, Brazil. *Journal of Fish Biology*, **94**, 352–373.

Ferrer, J., Donin, L. M., & Malabarba, L. R. (2015). A new species of *Ituglanis* Costa & Bockmann, 1993 (Siluriformes: Trichomycteridae) endemic to the Tramandaí-Mampituba ecoregion, southern Brazil. *Zootaxa*, **4020**, 375–389.

Fujisawa, T., & Barraclough, T. G. (2013). Delimiting species using single-locus data and the Generalized Mixed Yule Coalescent approach: a revised method and evaluation on simulated data sets. *Systematic Biology*, **62**, 707–724.

Gauger, M. F. W., & Buckup, P. A. (2005). Two new species of Hypoptopomatinae from the rio Paraíba do Sul basin, with comments on the monophyly of *Parotocinclus* and the Otothyrini (Siluriformes: Loricariidae). *Neotropical Ichthyology*, **3**, 509–518.

Guisande, C., Vari, R. P., Heine, J., García-Roselló, E., González-Dacosta, J., Perez-Schofield, G. J. B., … Pelayo-Villamil, P. (2016). VARSEDIG: an algorithm for morphometric characters selection and statistical validation in morphological taxonomy. *Zootaxa*, **4162**, 571–580.

Hammer, Ø., Harper, D. A.T., & Ryan P. D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*, **4**, 1–9.

Harvey, M. G., Seeholzer, G. F., Smith, B. T., Rabosky, D. L., Cuervo, A. M., & Brumfield, R. T. (2017). Positive association between population genetic differentiation and speciation rates in New World birds. *Proceedings of the National Academy of Sciences*, **114**, 6328–6333.

Hirschmann, A., Fagundes, N. J., & Malabarba, L. R. (2017). Ontogenetic changes in mouth morphology triggers conflicting hypotheses of relationships in characid fishes (Ostariophysi: Characiformes). *Neotropical Ichthyology*, **15**, e160073.

Hirschmann, A., Malabarba, L. R., Thomaz, A. T., & Fagundes, N. J. R. (2015). Riverine habitat specificity constrains dispersion in a Neotropical fish (Characidae) along Southern Brazilian drainages. *Zoologica Scripta*, **44**, 374–382.

IBGE. 2012. Atlas Geográfico Escolar. 6ª ed., Rio de Janeiro.

IUCN Standards and Petitions Committee. (2019). Guidelines for using the IUCN Red List Categories and Criteria. Version 14. Prepared by the Standards and Petitions Committee. Available at: http://www.iucnredlist.org/documents/ RedListGuidelines.pdf

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111–120.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, **33**, 1870–1874.

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2016) PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular biology and evolution*, **34**, 772–773.

Leal, M. E. C., & Sant'Anna, V. B. (2006). Quantitative analysis of interspecific and ontogenetic variation in *Osteoglossum* species (Teleostei: Osteoglossiformes: Osteoglossidae). *Zootaxa*, **1239**, 49–68.

Leigh, J.W., & Bryant, D. (2015). Monte Carlo strategies for selecting parameter values in simulation experiments. *Systematic Biology*, **64**, 741–751.

Lima, S. M., Berbel-Filho, W. M., Araújo, T. F., Lazzarotto, H., Tatarenkov, A., & Avise, J. C. (2017). Headwater capture evidenced by paleo-rivers reconstruction and population genetic structure of the armored catfish (*Pareiorhaphis garbei*) in the Serra do Mar mountains of southeastern Brazil. *Frontiers in genetics*, **8**, 1–8.

Lippert, B. G., Calegari, B. B., & Reis, R. E. (2014). A New Species of *Otothyropsis* (Siluriformes: Hypoptopomatinae) from Eastern Brazil. *Copeia*, **2**, 238–244.

Malabarba, L. R., & Isaia, E. A. (1992). The fresh water fish fauna of the rio Tramandaí drainage, Rio Grande do Sul, Brazil, with a discussion of its historical origin. *Comunicações do Museu de Ciências e Tecnologia da PUCRS, Série Zoologia*, **5**, 197–223.

Malabarba, L. R., Carvalho Neto, P., Bertaco, V. A., Carvalho, T. P. Ferrer, J., Artioli, L. G. S. (2013). *Guia de Identificação dos Peixes da Bacia do Rio Tramandaí*. Porto Alegre, RS: Via Sapiens.

Martins, F. O. Britski, H. A., & Langeani, F. (2014). Systematics of *Pseudotothyris* (Loricariidae: Hypoptopomatinae). *Zoological Journal of Linnean Society*, **170**, 822–874.

Melo, B. F., Benine, R. C., Mariguela, T. C., & Oliveira, C. (2011). A new species of *Tetragonopterus* Cuvier, 1816 (Characiformes: Characidae: Tetragonopterinae) from the rio Jari, Amapá, northern Brazil. *Neotropical Ichthyology*, **9**, 49–56.

Pereira, E. H. L., Vieira, F., & Reis, R. E. (2007). A new species of sexually dimorphic *Pareiorhaphis* Miranda Ribeiro, 1918 (Siluriformes: Loricariidae) from the rio Doce Basin, Brazil. *Neotropical Ichthyology*, **5**, 443–448.

QGIS Development Team. (2020). Geographic Information System (QGIS). Open Source Geospatial Foundation Project. http://www.qgis.org/.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, **67**, 901–904.

Reis, R. E., & Schaefer, S. A. (1998). New cascudinhos from southern Brazil: systematics, endemism, and relationships (Siluriformes, Loricariidae, Hypoptopomatinae). *American Museum Novitates*, **3254**, 1–25.

Ribeiro, A. C. (2006). Tectonic history and the biogeography of the freshwater fishes from the coastal drainages of eastern Brazil: an example of faunal evolution associated with a divergent continental margin. *Neotropical Ichthyology*, **4**, 225–246.

Rodriguez, M. S., Delapieve, M. L. S., & Reis, R. E. (2015). Phylogenetic relationships of the species of *Acestridium* Haseman, 1911 (Siluriformes: Loricariidae). *Neotropical Ichthyology*, **13**, 325–340.

Roxo, F. F., Albert, J. S., Silva, G. S., Zawadzki, C. H., Foresti, F., & Oliveira, C. (2014). Molecular phylogeny and biogeographic history of the armored Neotropical catfish subfamilies Hypoptopomatinae, Neoplecostominae and Otothyrinae (Siluriformes: Loricariidae). *PLoS ONE*, **9**, e105564.

Roxo, F. F., Lujan, N. K., Tagliacollo, V. A., Waltz, B. T., Silva, G. S., Oliveira, C., & Albert, J. S. (2017). Shift from slow-to fast-water habitats accelerates lineage and

phenotype evolution in a clade of Neotropical suckermouth catfishes (Loricariidae: Hypoptopomatinae). *PLoS ONE*, **12**, e0178240.

Roxo, F. F., Ochoa, L. E., Sabaj, M. H., Lujan, N. K., Covain, R., Silva, G. S., ... Alfaro, M. E. (2019). Phylogenomic reappraisal of the Neotropical catfish family Loricariidae (Teleostei: Siluriformes) using ultraconserved elements. *Molecular Phylogenetics and Evolution*, **135**, 148–165.

Sabaj M.H. 2019. Standard symbolic codes for institutional resource collections in herpetology and ichthyology: An Online Reference. Version 7.1. Washington, DC: American Society of Ichthyologists and Herpetologists, Available at www.asih.org/ (21 March 2019).

Schaefer, S. A. (1997). The Neotropical cascudinhos: systematics and biogeography of the *Otocinclus*, catfishes (Siluriformes: Loricariidae). *Proceedings of the Academy of Natural Sciences of Phyladelphia*, **148**, 1–120.

Schaefer, S. A. (1998). Conflict and resolution: impact of new taxa on phylogenetic studies of the Neotropical cascudinhos (Siluroidei: Loricariidae). In L. R. Malabarba, R. E. Reis, R. P. Vari, Z. M. Lucena & C. A. S. Lucena (Eds.), *Phylogeny and classification of Neotropical fishes* (pp. 375–400). Porto Alegre, RS: Edipucrs.

Singhal, S., Huang, H., Grundler, M. R., Marchán-Rivadeneira, M. R., Holmes, I., Title, P. O., ... Rabosky, D. L. (2018). Does population structure predict the rate of speciation? A comparative test across australia's most diverse vertebrate radiation. *The American Naturalist*, **192**, 432–447.

Taylor, W. R., & Van Dyke, G. C. (1985). Revised procedures for staining and clearing small fishes and other vertebrates for bone and cartilage study. *Cybium*, **9**, 107–19.

Thomaz, A. T., & Knowles, L. L. (2018). Flowing into the unknown: inferred paleodrainages for studying the ichthyofauna of Brazilian coastal rivers. *Neotropical Ichthyology*, **16**, e180019.

Thomaz, A. T., Malabarba, L. R., Bonatto, S. L., & Knowles, L. L. (2015). Testing the effect of palaeodrainages versus habitat stability on genetic divergence in riverine systems: study of a Neotropical fish of the Brazilian coastal Atlantic Forest. *Journal of Biogeography*, **42**, 2389–2401.

Thomaz, A. T., Malabarba, L. R., & Knowles, L. L. (2017). Genomic signatures of paleodrainages in a freshwater fish along the southeastern coast of Brazil: genetic structure reflects past riverine properties. *Heredity*, **119**, 287–294.

TABLES

Table 1 Descriptive morphometrics of species/populations of *Epactionotus* by drainage (part). Values are given as percent of standard length (SL) or head length (HL). SD = standard deviation; MQ = Maquiné; TF = Três Forquilhas; MA = Mampituba; AR = Araranguá; UR = Urussanga.

	E. bilineatus (MQ)				*E. bilineatus* (TF)				*E. itaimbezinho* (MA)				*E. gracilis* (AR)				*Epactionotus* (UR)			
	N=13				N=12				N=12				N=13				N=15			
	Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD
Standard length (mm)	32.7	36.0	34.3	1.0	32.6	35.6	33.6	1.0	33.4	38.5	36.5	1.9	30.3	37.6	34.0	2.3	28.9	36.4	32.4	2.2
Percent of SL																				
Head length	32.3	35.0	33.4	0.9	32.0	34.8	33.4	0.7	32.6	34.9	33.7	0.8	30.9	34.4	32.9	0.9	30.7	35.4	32.7	1.1
Predorsal length	44.9	48.9	46.9	1.2	46.1	48.3	47.1	0.8	46.0	48.8	47.7	0.9	45.3	49.8	47.9	1.4	44.1	49.7	47.4	1.3
Postdorsal length	41.3	47.2	43.9	1.6	41.6	43.9	43.2	0.8	42.5	45.0	43.2	0.7	39.9	45.5	43.3	2.0	41.0	46.8	45.3	1.4
Prepectoral length	24.6	26.8	25.7	0.6	24.5	27.0	25.8	0.8	24.2	26.7	25.7	0.7	24.2	27.1	25.4	0.8	24.5	27.1	25.6	0.8
Prepelvic length	40.6	43.2	42.1	0.8	40.8	43.6	42.1	1.0	40.4	43.8	42.4	1.0	40.4	43.6	41.6	0.9	39.0	44.4	41.8	1.2
Preanal length	59.8	63.4	62.2	1.0	61.3	63.4	62.2	0.7	60.6	64.4	62.5	1.0	59.8	64.3	61.8	1.5	59.4	63.3	61.3	1.1
Cleithral width	23.6	26.1	24.6	0.8	23.1	25.0	24.1	0.6	22.2	23.8	22.9	0.5	20.1	23.3	21.7	0.9	20.6	22.8	21.8	0.7
Pectoral-pelvic-fins distance	16.7	19.3	18.0	0.9	16.1	18.8	17.3	0.8	15.3	19.1	17.6	1.1	15.2	18.1	16.8	0.8	16.1	19.6	17.8	0.9
Pelvic-anal-fins distance	21.0	24.3	22.6	0.8	20.8	22.9	21.7	0.6	20.4	23.3	21.9	0.8	19.6	23.9	21.9	1.2	19.3	23.0	21.7	0.9
Dorsal-fin spine length	20.3	23.6	21.7	0.9	19.2	21.9	20.8	0.9	18.4	22.6	20.9	1.3	18.3	22.1	20.3	1.1	17.0	21.9	19.8	1.4
Dorsal-fin base length	10.6	12.8	11.9	0.7	11.1	13.0	12.0	0.7	11.3	12.6	12.0	0.4	9.9	12.8	11.2	0.8	9.1	11.1	10.2	0.5
Pectoral-fin spine length	20.2	22.7	21.5	0.8	20.5	23.1	21.9	0.8	19.1	21.6	20.7	0.8	18.0	22.4	20.9	1.5	17.4	21.5	19.8	1.2
Pectoral-fin length	22.6	26.1	24.0	0.8	22.3	24.7	24.0	0.7	20.9	23.9	22.9	0.8	20.5	24.5	22.9	1.4	19.8	23.4	21.8	1.3
First pelvic-fin unbranched ray length	16.3	19.4	17.3	0.8	16.6	17.6	17.1	0.3	15.0	16.9	16.0	0.6	14.7	18.4	16.3	1.0	13.8	16.7	15.5	0.8
First pelvic-fin unbranched ray width	6.2	8.9	7.3	0.8	6.9	9.5	7.7	0.7	6.3	8.9	7.8	0.8	6.4	8.7	7.4	0.7	5.3	9.4	6.8	1.0
First anal-fin unbranched ray length	13.0	15.7	14.5	0.9	14.0	15.7	15.1	0.5	13.1	16.9	15.0	1.0	13.1	15.9	14.7	0.9	12.0	15.5	14.0	1.0
Caudal-peduncle length	37.2	40.5	38.7	1.0	37.0	40.0	38.3	0.8	36.1	38.5	37.3	0.7	36.9	40.3	38.8	1.1	37.3	41.8	40.0	1.2
Caudal-peduncle depth	9.2	10.7	10.0	0.4	9.8	10.8	10.3	0.3	9.9	11.2	10.5	0.5	9.8	10.9	10.5	0.4	8.8	10.4	9.5	0.5
Caudal-peduncle width	5.8	7.2	6.5	0.5	4.9	5.8	5.5	0.3	4.7	5.8	5.3	0.3	4.9	5.9	5.4	0.3	5.1	8.2	6.3	0.9
Body depth at dorsal-fin origin	14.1	18.3	15.5	1.2	13.0	15.5	14.4	0.8	13.3	16.4	15.3	0.9	12.1	16.5	14.4	1.2	13.4	16.0	14.3	0.8
Body width at dorsal-fin origin	18.6	26.6	21.3	2.4	17.5	21.2	19.1	1.0	18.5	21.5	19.8	1.0	15.4	22.6	18.4	2.2	17.2	22.8	19.9	2.0
Percent of HL																				
Head depth	39.7	44.7	41.9	1.5	40.1	44.6	42.7	1.1	39.8	43.0	41.7	1.0	39.0	45.8	43.0	2.3	37.0	42.8	40.3	1.5
Head width	70.2	77.2	73.8	2.2	70.7	74.9	72.2	1.4	64.1	69.4	66.5	1.2	61.9	69.8	66.4	2.6	63.5	69.2	66.1	2.0
Snout length	51.7	56.1	53.9	1.4	51.3	55.0	53.6	1.1	51.3	54.8	53.0	1.2	50.3	55.6	53.3	1.5	49.6	52.9	51.1	1.0
Orbital diameter	13.2	15.2	14.2	0.7	14.5	15.9	15.2	0.5	12.7	14.9	13.9	0.7	13.5	15.9	14.9	0.7	12.4	16.3	14.1	1.0

Table 1 Continued.

	E. bilineatus (MQ)				*E. bilineatus* (TF)				*E. itaimbezinho* (MA)				*E. gracilis* (AR)				*Epactionotus* (UR)			
	N=13				N=12				N=12				N=13				N=15			
	Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD
Snout-opercle distance	76.2	81.7	78.4	1.7	77.2	82.9	79.9	1.7	76.2	80.7	77.8	1.5	74.9	81.3	79.0	1.5	74.2	80.8	78.1	2.0
Interorbital distance	38.4	42.3	39.6	1.1	38.4	40.9	39.5	0.8	36.6	40.5	38.9	1.3	37.0	41.2	39.4	1.1	35.7	42.6	39.2	2.6
Internareal width	11.1	14.2	13.1	0.9	11.9	15.0	13.5	0.8	11.6	14.2	12.8	0.8	10.9	14.2	12.2	1.1	11.2	14.3	12.6	1.0
Nares diameter	9.1	11.9	10.2	0.8	8.4	11.2	10.0	0.8	8.0	10.6	9.4	0.7	9.0	12.5	10.5	1.0	9.2	11.3	10.3	0.6
Prenasal length	33.1	36.6	35.0	1.0	34.6	36.7	35.7	0.6	34.4	37.3	36.0	1.0	33.0	37.9	35.1	1.6	30.9	34.9	32.9	1.0
Suborbital depth	15.1	18.0	16.7	1.0	15.2	20.3	17.8	1.2	15.7	19.1	17.6	0.9	15.0	19.4	17.4	1.4	13.3	16.5	14.9	1.0

Table 2 Descriptive morphometrics of species/populations of *Epactionotus* by drainage (part). Values are given as percent of standard length (SL) or head length (HL). Hol = Holotype; SD = standard deviation; TU = Tubarão; DU = d'Una; BI = Biguaçu.

	Epactionotus (TU)				*Epactionotus* (DU)				*Epactionotus* (BI)				
	N=6				N=12				N=23				
	Low	High	Mean	SD	Low	High	Mean	SD	Hol	Low	High	Mean	SD
Standard length (mm)	28.3	35.8	31.9	2.5	27.9	34.0	31.0	1.9	35.4	32.7	39.0	36.6	1.6
Percent of SL													
Head length	31.8	35.0	33.4	1.2	32.4	36.4	34.4	1.2	33.3	30.8	34.8	32.4	0.9
Predorsal length	46.8	49.7	48.0	1.1	48.1	52.4	49.6	1.3	48.1	46.0	49.9	47.7	0.9
Postdorsal length	44.9	46.6	45.4	0.7	42.6	47.5	44.4	1.6	44.2	41.4	47.1	44.2	1.5
Prepectoral length	25.1	28.7	26.3	1.4	25.1	28.2	26.8	1.0	25.9	23.6	26.5	24.8	0.8
Prepelvic length	41.7	44.5	42.5	1.0	39.3	42.6	40.6	0.9	43.2	38.8	43.2	40.9	1.1
Preanal length	60.4	65.5	62.1	1.8	59.3	62.1	60.8	0.9	63.1	58.8	63.1	60.8	1.2
Cleithral width	22.1	24.3	23.1	0.9	20.0	22.3	21.0	0.7	20.8	19.0	20.8	20.2	0.5
Pectoral-pelvic-fins distance	15.7	18.7	17.7	1.2	13.8	16.6	15.3	0.7	17.9	15.0	18.5	16.7	0.9
Pelvic-anal-fins distance	20.8	24.1	22.4	1.3	20.6	23.1	21.5	0.7	21.9	20.0	23.4	21.6	0.9
Dorsal-fin spine length	19.2	22.8	21.1	1.3	17.0	20.2	19.0	0.8	19.3	17.3	20.0	18.6	0.9
Dorsal-fin base length	9.6	14.1	11.4	1.7	10.2	11.6	10.8	0.5	11.8	9.6	12.3	11.1	0.8
Pectoral-fin spine length	19.3	20.7	20.2	0.5	17.9	22.3	19.2	1.4	17.2	15.5	18.8	17.0	0.9
Pectoral-fin length	22.3	23.5	23.1	0.5	18.0	22.6	20.9	1.2	20.9	19.0	22.3	20.4	0.8
First pelvic-fin unbranched ray length	15.3	16.5	15.8	0.4	15.2	17.6	16.4	0.7	16.5	13.6	16.5	15.1	0.8
First pelvic-fin unbranched ray width	6.1	9.3	7.9	1.1	4.2	7.8	6.1	0.9	6.6	6.6	9.9	8.0	1.0
First anal-fin unbranched ray length	13.5	15.7	14.6	0.8	12.8	14.9	13.8	0.7	14.2	11.2	14.6	12.9	1.1
Caudal-peduncle length	37.6	41.5	39.3	1.3	38.9	40.8	39.7	0.6	40.6	38.1	41.6	39.8	1.1
Caudal-peduncle depth	9.3	10.7	10.0	0.6	8.4	9.4	9.0	0.3	9.1	7.7	9.3	8.7	0.4
Caudal-peduncle width	5.2	6.8	6.0	0.6	4.1	6.0	4.7	0.6	5.5	4.1	5.8	4.9	0.4
Body depth at dorsal-fin origin	12.3	17.0	15.1	2.1	11.7	13.8	13.0	0.5	14.2	11.1	14.2	12.8	1.0
Body width at dorsal-fin origin	17.2	24.2	21.0	3.0	16.6	19.2	17.7	0.9	18.6	14.9	20.2	17.3	1.3
Percent of HL													
Head depth	40.5	42.6	41.4	0.8	35.6	38.8	37.5	1.1	40.2	37.0	43.2	39.2	1.6
Head width	67.1	71.6	68.9	1.8	56.6	63.6	60.4	2.3	63.6	59.5	66.6	63.0	1.6
Snout length	51.4	56.0	53.3	1.8	52.4	55.0	53.8	0.8	51.9	51.1	55.4	52.9	1.1
Orbital diameter	14.7	16.1	15.6	0.5	12.8	15.1	13.6	0.8	14.6	12.8	15.5	14.4	0.8
Snout-opercle distance	78.2	82.3	80.3	1.5	76.2	81.4	78.6	1.6	77.6	75.6	81.9	78.1	1.5
Interorbital distance	39.1	41.2	40.4	0.7	33.3	37.4	35.5	1.3	37.0	34.5	38.6	37.1	0.9
Internareal width	11.1	13.7	12.9	1.0	9.3	12.7	11.5	1.0	12.8	11.3	13.9	12.5	0.8
Nares diameter	7.9	12.3	10.5	1.5	8.9	11.8	10.1	0.8	8.9	8.4	10.6	9.6	0.6
Prenasal length	31.6	36.2	34.3	1.6	33.8	37.9	36.0	1.3	35.1	33.5	38.0	35.3	1.3
Suborbital depth	15.4	17.3	16.5	0.8	13.1	16.5	14.8	1.1	16.1	13.9	18.2	16.1	1.3

Table 3 Descriptive counts of species/populations of *Epactionotus* by drainage (part). SD = standard deviation; MQ = Maquiné; TF = Três Forquilhas; MA = Mampituba; AR = Araranguá; UR = Urussanga.

	E. bilineatus (MQ)				*E. bilineatus* (TF)				*E. itaimbezinho* (MA)				*E. gracilis* (AR)				*Epactionotus* (UR)			
	N=13				N=12				N=12				N=13				N=15			
Counts	Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD	Low	High	Mean	SD
Rigth premaxillary teeth	17	21	18.7	1.5	16.0	21.0	18.2	1.6	16.0	23.0	19.3	2.4	16.0	23.0	18.5	2.0	15.0	22.0	18.3	1.6
Left premaxillary teeth	17	21	19.2	1.4	16.0	21.0	18.8	1.5	17.0	22.0	19.2	1.9	16.0	23.0	18.2	1.9	16.0	21.0	18.9	1.6
Rigth dentary teeth	17	21	18.9	1.5	16.0	20.0	18.3	1.5	15.0	21.0	18.5	1.7	15.0	20.0	17.2	1.5	14.0	19.0	16.8	1.6
Left dentary teeth	17	21	18.8	1.2	15.0	21.0	18.0	1.6	16.0	21.0	18.1	1.5	15.0	20.0	16.9	1.8	13.0	19.0	16.4	1.6
Plates in median lateral series	25	27	25.8	0.6	25.0	28.0	26.4	0.9	25.0	28.0	26.7	0.9	26.0	27.0	26.7	0.5	25.0	28.0	26.5	0.7
Plates in mid-dorsal series	21	24	22.7	0.9	22.0	24.0	23.1	0.5	22.0	25.0	23.5	0.8	22.0	24.0	23.4	0.8	23.0	25.0	24.0	0.8
Plates in dorsal series	23	24	23.1	0.3	22.0	23.0	22.3	0.5	22.0	24.0	23.0	0.4	22.0	23.0	22.6	0.5	22.0	24.0	22.8	0.6
Plates in mid-ventral series	22	24	22.9	0.8	23.0	24.0	23.3	0.5	23.0	25.0	23.9	0.8	22.0	27.0	24.1	1.4	22.0	25.0	23.8	0.8
Plates in ventral series	20	24	22.7	1.3	22.0	24.0	23.1	0.8	22.0	24.0	23.4	0.7	20.0	27.0	23.1	2.0	22.0	25.0	23.7	1.0
Plates between anal-and-caudal fin series	12	13	12.3	0.5	11.0	14.0	12.3	0.8	12.0	13.0	12.7	0.5	12.0	13.0	12.8	0.4	12.0	14.0	12.9	0.5
Plates at dorsal-fin base	5	6	5.5	0.5	5.0	6.0	5.4	0.5	5.0	6.0	5.3	0.5	5.0	6.0	5.1	0.3	4.0	5.0	4.9	0.3
Plates at anal-fin base	3	4	3.1	0.3	3.0	4.0	3.3	0.5	3.0	4.0	3.2	0.4	3.0	3.0	3.0	0.0	2.0	3.0	2.9	0.3
Unpaired predorsal plates	0	1	0.5	0.5	0.0	1.0	0.6	0.5	0.0	3.0	1.6	0.8	1.0	3.0	1.5	0.7	1.0	2.0	1.3	0.5
Predorsal plates	3	4	3.8	0.4	4.0	4.0	4.0	0.0	3.0	4.0	3.9	0.3	4.0	4.0	4.0	0.0	4.0	4.0	4.0	0.0
Right abdominal plates	0	6	2.5	1.7	1.0	7.0	3.8	1.9	3.0	6.0	4.1	0.8	2.0	6.0	3.8	1.4	2.0	7.0	4.7	1.4
Left abdominal plates	1	5	2.5	1.4	1.0	7.0	4.0	1.7	3.0	7.0	4.3	1.4	2.0	5.0	3.5	1.1	3.0	7.0	4.5	1.4
Medium abdominal plates	0	1	0.4	0.5	0.0	20.0	4.8	6.0	0.0	25.0	7.8	8.2	0.0	16.0	5.6	4.8	0.0	40.0	8.5	10.3
Pectoral-fin rays	6	6	6.0	0.0	6.0	6.0	6.0	0.0	6.0	6.0	6.0	0.0	6.0	6.0	6.0	0.0	6.0	6.0	6.0	0.0
Dorsal-fin rays	7	7	7.0	0.0	7.0	7.0	7.0	0.0	7.0	7.0	7.0	0.0	7.0	7.0	7.0	0.0	7.0	7.0	7.0	0.0
Pelvic-fin rays	5	5	5.0	0.0	5.0	5.0	5.0	0.0	5.0	5.0	5.0	0.0	5.0	5.0	5.0	0.0	5.0	5.0	5.0	0.0
Anal-fin rays	5	5	5.0	0.0	5.0	5.0	5.0	0.0	5.0	5.0	5.0	0.0	5.0	5.0	5.0	0.0	5.0	5.0	5.0	0.0
Caudal-fin rays	14	14	14.0	0.0	14.0	14.0	14.0	0.0	13.0	14.0	13.9	0.3	13.0	14.0	13.8	0.4	13.0	14.0	13.9	0.4

Table 4 Descriptive counts of species/populations of *Epactionotus* by drainage (part). Hol = Holotype; SD = standard deviation; TU = Tubarão; DU = d'Una; BI = Biguaçu.

	Epactionotus (TU)				*Epactionotus* (DU)				*Epactionotus* (BI)				
	N=6				N=12				N=23				
Counts	Low	High	Mean	SD	Low	High	Mean	SD	Hol	Low	High	Mean	SD
Rigth premaxillary teeth	16.0	21.0	18.0	2.0	19.0	25.0	21.5	1.4	19.0	15.0	21.0	17.6	1.6
Left premaxillary teeth	16.0	19.0	17.5	1.2	19.0	26.0	21.0	1.8	19.0	15.0	20.0	17.7	1.2
Rigth dentary teeth	15.0	18.0	16.3	1.0	16.0	20.0	18.4	1.3	17.0	14.0	18.0	16.0	1.1
Left dentary teeth	15.0	18.0	16.3	1.0	15.0	20.0	18.0	1.6	17.0	14.0	19.0	16.3	1.3
Plates in median lateral series	26.0	27.0	26.5	0.5	25.0	27.0	25.8	0.6	28.0	26.0	29.0	27.1	0.8
Plates in mid-dorsal series	24.0	24.0	24.0	0.0	21.0	23.0	22.5	0.7	24.0	23.0	26.0	24.3	0.8
Plates in dorsal series	22.0	23.0	22.8	0.4	22.0	23.0	22.5	0.5	23.0	23.0	24.0	23.3	0.5
Plates in mid-ventral series	24.0	25.0	24.2	0.4	22.0	23.0	22.4	0.5	26.0	22.0	26.0	24.5	1.1
Plates in ventral series	24.0	25.0	24.2	0.4	21.0	24.0	22.4	0.8	26.0	23.0	26.0	25.0	0.9
Plates between anal-and-caudal fin series	13.0	13.0	13.0	0.0	12.0	14.0	12.7	0.7	14.0	12.0	14.0	13.1	0.5
Plates at dorsal-fin base	5.0	5.0	5.0	0.0	5.0	5.0	5.0	0.0	5.0	5.0	6.0	5.0	0.2
Plates at anal-fin base	3.0	3.0	3.0	0.0	3.0	3.0	3.0	0.0	3.0	2.0	3.0	3.0	0.2
Unpaired predorsal plates	0.0	2.0	1.2	0.8	0.0	2.0	1.0	0.4	3.0	1.0	3.0	2.0	0.6
Predorsal plates	4.0	5.0	4.2	0.4	4.0	4.0	4.0	0.0	4.0	4.0	5.0	4.1	0.3
Right abdominal plates	2.0	7.0	4.0	1.7	1.0	6.0	3.2	1.6	1.0	0.0	4.0	1.0	1.0
Left abdominal plates	2.0	7.0	4.2	1.6	1.0	5.0	3.6	1.2	1.0	0.0	4.0	0.9	0.9
Medium abdominal plates	0.0	12.0	5.2	4.2	0.0	13.0	1.3	3.7	0.0	0.0	0.0	0.0	0.0
Pectoral-fin rays	6.0	6.0	6.0	0.0	6.0	6.0	6.0	0.0	6.0	6.0	6.0	6.0	0.0
Dorsal-fin rays	7.0	7.0	7.0	0.0	6.0	7.0	6.9	0.3	7.0	7.0	8.0	7.0	0.2
Pelvic-fin rays	5.0	5.0	5.0	0.0	5.0	5.0	5.0	0.0	5.0	5.0	5.0	5.0	0.0
Anal-fin rays	5.0	5.0	5.0	0.0	5.0	5.0	5.0	0.0	5.0	5.0	5.0	5.0	0.0
Caudal-fin rays	14.0	14.0	14.0	0.0	12.0	14.0	13.8	0.6	13.0	13.0	14.0	13.9	0.3

Table 5 Comparison of GMYC supports for species/populations between different datasets. Bold represents groups that were found as clusters using the single threshold in GMYC. MQ = Maquiné; TF = Três Forquilhas; MA = Mampituba; AR = Araranguá; UR = Urussanga; TU = Tubarão; DU = d'Una; BI = Biguaçu.

Species (drainage)	Newly sequenced *Epactionotus*	All *Epactionotus* samples	*Epactionotus* + *Eurycheilichthys*
E. bilineatus (MQ)	**0.82**	**0.5**	0.16
E. bilineatus (TF)	**0.82**	**0.46**	0.17
E. itaimbezinho (MA)	**0.82**	**0.53**	**0.27**
E. gracilis (AR)	0.34	**0.36**	0.03
Epactionotus (UR)	singleton	singleton	singleton
Epactionotus (TU)	0.1	0.21	0.08
Epactionotus (DU)	-	singleton	singleton
Epactionotus (BI)	**0.89**	**0.62**	0.89

Table 6 Pairwise mtDNA genetic distance values (mean ± standard error) for cytochrome oxidase c subunit 1 (COI) gene between and within species/populations according to drainge using Kimura 2+G+I parameter. Diagonal bold numbers show within drainage values. Blue and red numbers show the lowest and highest genetic distance values, respectively, between and within drainages. MQ = Maquiné; TF = Três Forquilhas; MA = Mampituba; AR = Araranguá; UR = Urussanga; TU = Tubarão; DU = d'Una; BI = Biguaçu.

	Populations	1	2	3	4	5	6	7	8
1	*Epactionotus bilineatus* (MQ)	**0.36 ± 0.33**							
2	*Epactionotus bilineatus* (TF)	1.50 ± 0.48	**0.78 ± 0.46**						
3	*Epactionotus gracilis* (AR)	2.51 ± 0.29	2.78 ± 0.29	**0.45 ± 0.32**					
4	*Epactionotus* (DU)	1.90 ± 0.23	2.03 ± 0.27	1.75 ± 0.34	**- ± -**				
5	*Epactionotus* (TU)	1.80 ± 0.31	2.08 ± 0.40	1.72 ± 0.39	**1.20** ± 0.01	**0.73 ± 0.00**			
6	*Epactionotus* (UR)	3.19 ± 0.15	3.58 ± 0.10	1.57 ± 0.20	2.05 ± 0.00	2.04 ± 0.20	**- ± -**		
7	*Epactionotus itaimbezinho* (MA)	2.52 ± 0.40	2.80 ± 0.49	1.83 ± 0.28	2.15 ± 0.27	1.83 ± 0.26	2.09 ± 0.14	**0.42 ± 0.31**	
8	*Epactionotus* (BI)	2.51 ± 0.38	2.94 ± 0.56	3.30 ± 0.32	2.62 ± 0.00	2.20 ± 0.22	**4.07** ± 0.00	3.33 ± 0.38	**0.00 ± 0.00**

FIGURES

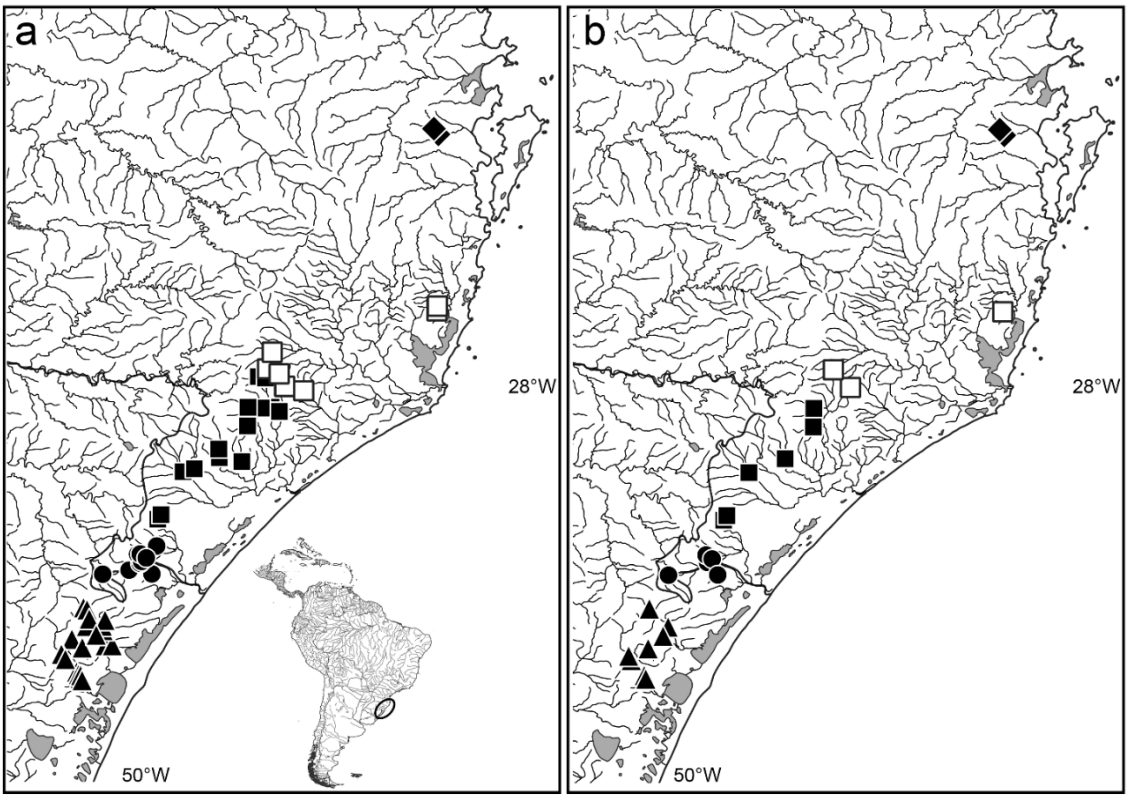

Figure 1 Geographic distribution of the species/populations of *Epactionotus* based on a) material examined and, b) sampling localities used for molecular analyzes. Triangles, *E. bilineatus* (MQ and TF); dots, *E. itaimbezinho* (MA); black squares, *E. gracilis* (AR); opened squares populations of *Epactionotus* from Urussanga (UR), Tubarão (TU) and d'Una (DU), and diamonds *Epactionotus* from Biguaçu (BI).

Figure 2 *Epactionotus bilineatus* from Maquiné (MQ), MCP 19105, female, 30.6 mm SL, Brazil, Rio Grande do Sul, Maquiné, arroio do Ouro (29°39'58"S, 50°10'59"W).

Figure 3 *Epactionotus bilineatus* from Três Forquilhas (TF), MCP 28978, male, 34.3 mm SL, Brazil, Rio Grande do Sul, Três Forquilhas, arroio Japonês, (approx. 29°32’S, 50°05’W).

Figure 4 *Epactionotus itaimbezinho* from Mampituba (MA), MCP 23683, male, 34.9 mm SL, Brazil, Santa Catarina, Morrinhos do Sul, rio Mangue (29°14’55”S, 49°55’30”W).

Figure 5 *Epactionotus gracilis* from Araranguá (AR), UFRGS 22945, male, 28.2 mm SL, Brazil, Santa Catarina, Nova Veneza (28°35’02.2”S, 49°32’31.2”W).

Figure 6 *Epactionotus* from Urussanga (UR), UFRGS 6212, female, 36.2 mm SL, Brazil, Santa Catarina, Urussanga, rio Lageado (28°31'04.92"S, 49°19'10.07"W).

Figure 7 *Epactionotus* from Tubarão (TU), UFRGS 22941, male, 31.9 mm SL, Brazil, Santa Catarina, Rio Bonito Alto (28°25'48.3"S, 49°27'50.7"W).

Figure 8 *Epactionotus* from d'Una (DU), MZUEL 7528, female, 31.2 mm SL, Brazil, Santa Catarina, Imarui, rio d'Una (28°10'48.8"S, 48°47'12.0"W).

Figure 9 *Epactionotus* from Biguaçu (BI), UFRGS 28220, holotype, female, 35.4 mm SL, Brazil, Santa Catarina, Antônio Carlos, rio Rachadel (27°29’44”S, 48°46’57”W).

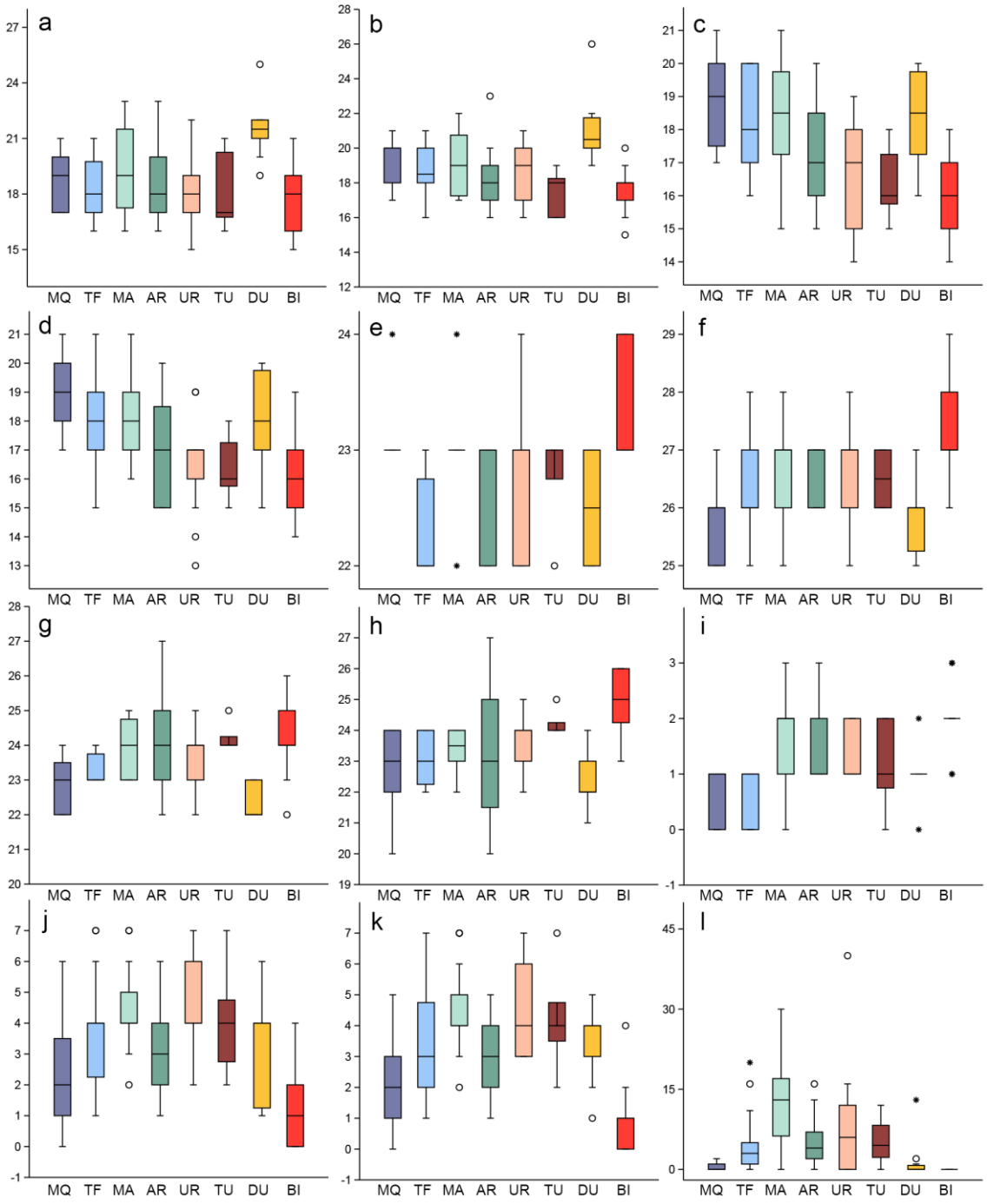

Figure 10 Box plots of the significant variable meristic data between different drainages according to Tukey's Pairwise results. Each graphic contains the number of: a) right premaxillary teeth; b) left pramaxillary teeth; c) right dentary teeth; d) left dentary teeth; e) dorsal lateral plates; f) median lateral plates; g) mid-ventral lateral plates; h); ventral lateral plates; i) unpaired predorsal plates; j) right abdominal plates; k) left abdominal plates, and l) median abdominal plates. Horizontal line inside each box indicates median values and short horizontal lines represent minimum and maximum values less than 1.5 times the box heights; circles represent outliers and black stars indicate outlier values higher than three times the box heights.

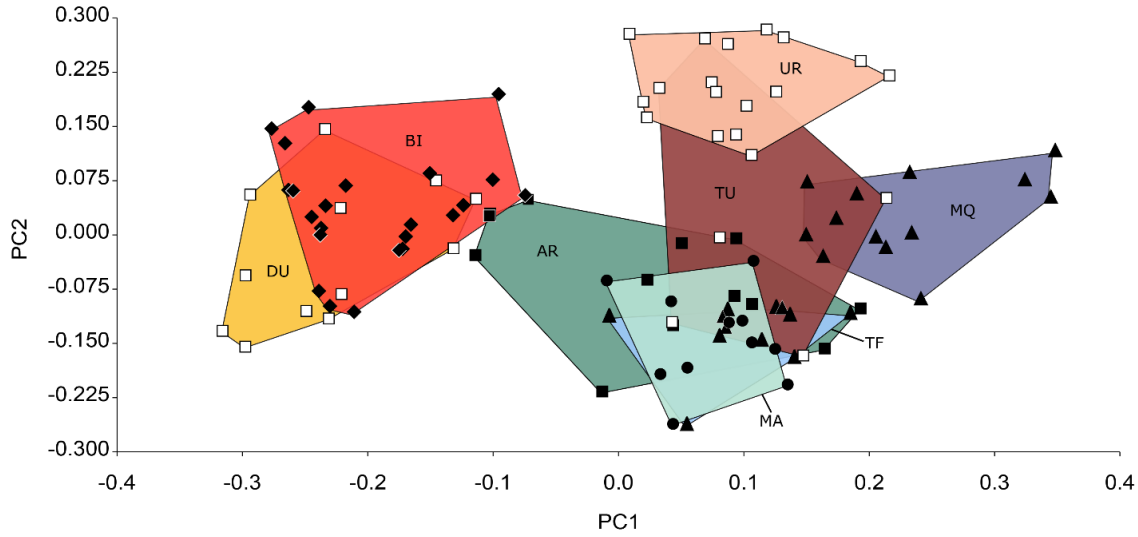

Figure 11 Plots of factor scores of principal component analysis (PCA) of the species/populations of *Epactionotus*. Triangles, *E. bilineatus* (MQ and TF); dots, *E. itaimbezinho* (MA); black squares, *E. gracilis* (AR); opened squares, populations of *Epactionotus* from Urussanga (UR), Tubarão (TU) and d'Una (DU), and diamonds *Epactionotus* from Biguaçu (BI).

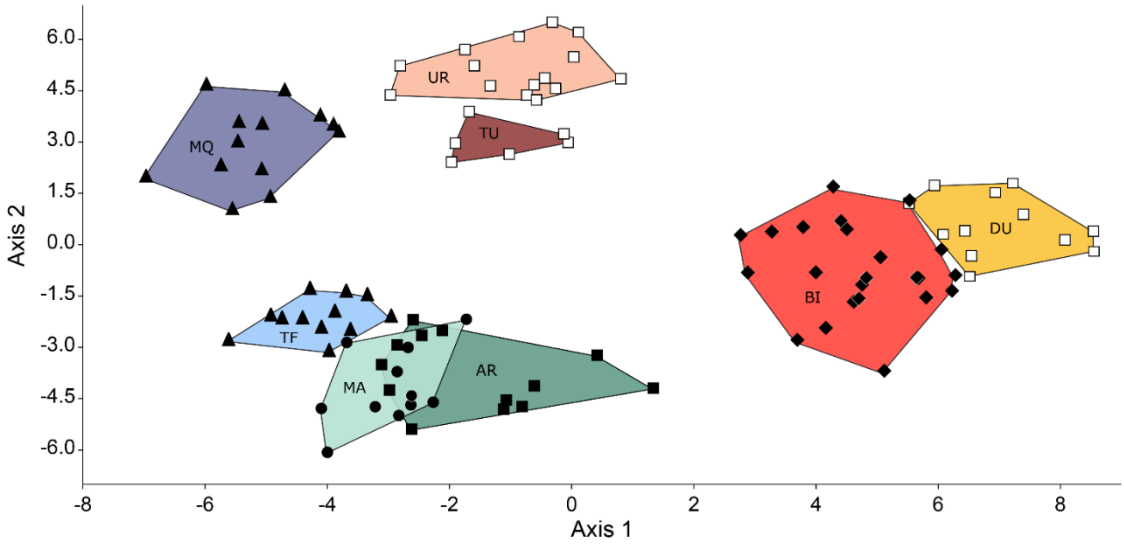

Figure 12 Plots of factor scores discriminant analysis (LDA) of the species/populations of *Epactionotus*. Triangles, *E. bilineatus* (MQ and TF); dot, *E. itaimbezinho* (MA); black squares, *E. gracilis* (AR); opened squares, populations of *Epactionotus* from Urussanga (UR), Tubarão (TU) and d'Una (DU), and diamonds *Epactionotus* from Biguaçu (BI).

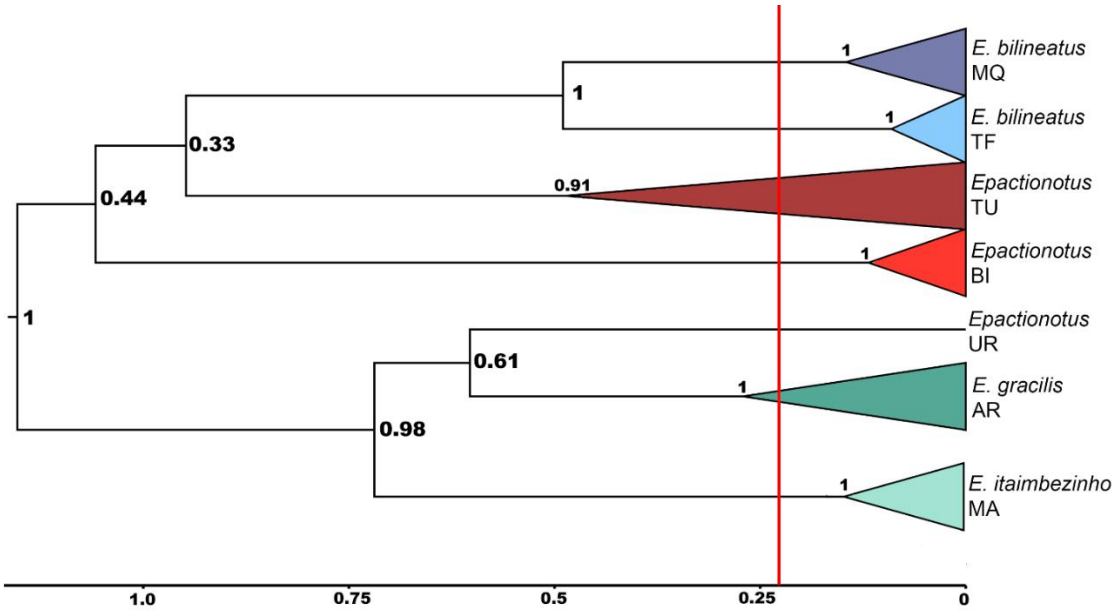

Figure 13 Bayesian phylogenetic tree of species/populations of *Epactionotus* obtained with mitochondrial (COI) loci of the newly sequenced data; the vertical red line shows the coalescent branching process of all sequences, estimated by using the single-threshold model in the general mixed Yule coalescent model (GMYC) test with birth–death speciation models. Colors correspond to each basin and node numbers correspond to BI posterior probability (PP). Bar below corresponds to divergence-time estimates in millions of years.

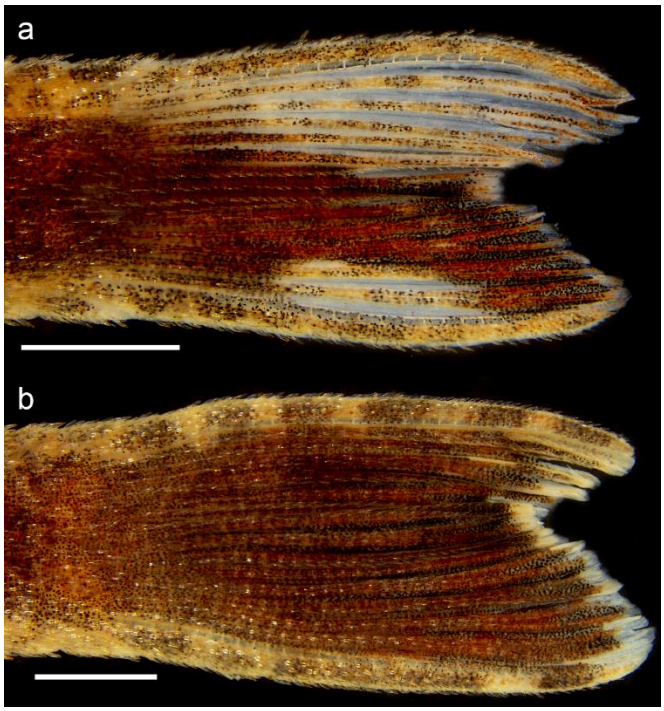

Figure 14 Caudal-fin color variation of species/populations of *Epactionotus* a) *E. gracilis* (AR), UFRGS 22945, 28.2 mm SL; b) *Epactionotus* from Biguaçu (BI), MCP uncataloged, 37.8 mm SL. Scale = 2mm.

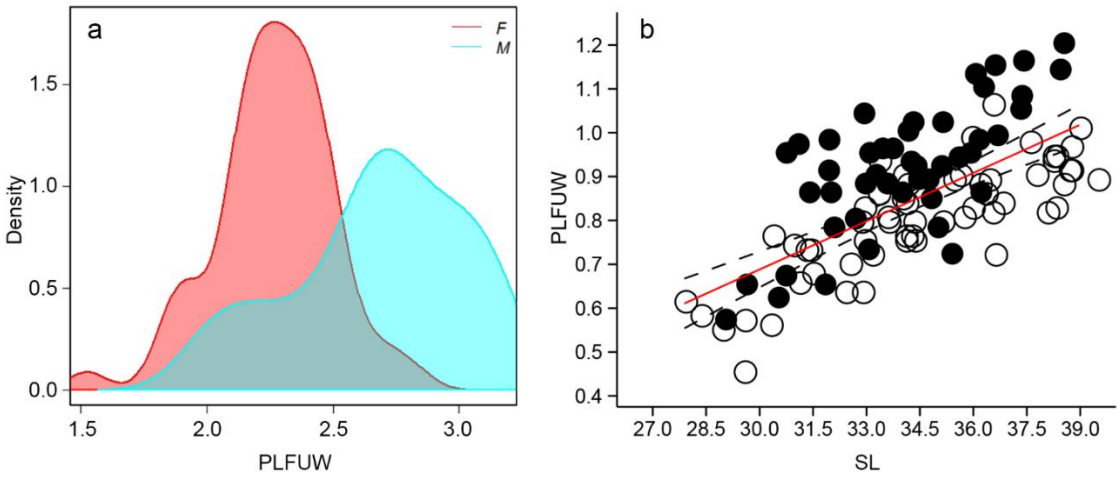

Figure 15 Sexual dimorphism in *Epactionotus* species/populations identified by VARSEDIG algorithm. a) Distribution of the first pelvic-fin unbranched ray width (PLFUW) for males (blue) and females (red), and b) bivariate plot of first pelvic-fin unbranched ray width (PLFUW) against standard length (SL) for males (dots) and females (circles).

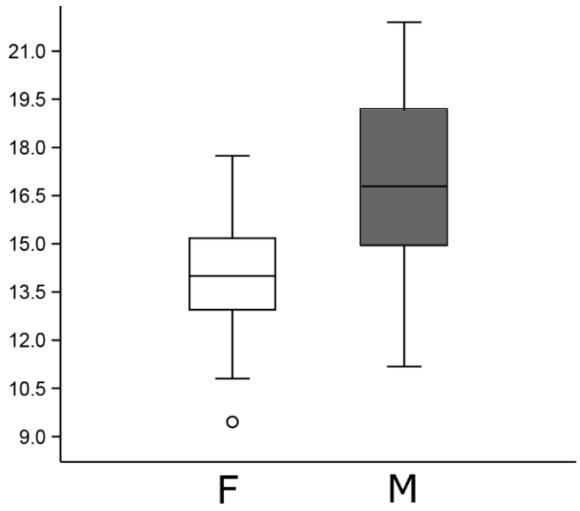

Figure 16 Box plot of first pelvic-fin unbranched ray width (PLFUW) against its length (PLFUL) for males (grey) and females (white) in species/populations of *Epactionotus*.

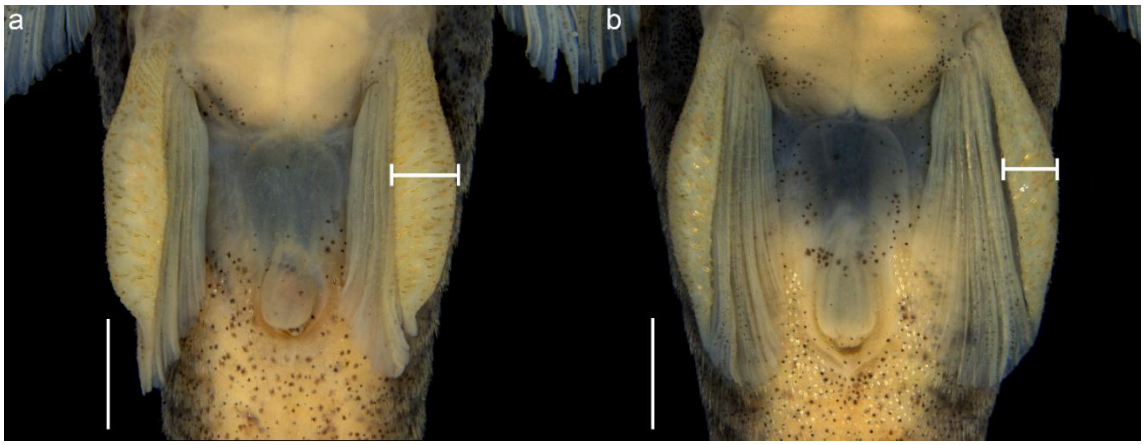

Figure 17 Pelvic region of *Epactionotus* from Biguaçu (BI). First pelvic-fin unbranched ray slightly thicker in males than females. a) Male, MCP uncataloged, 36 mm SL; b) female, UFRGS 20926, 39 mm SL. Ventral view, anterior towards top. Scale = 2mm.

Figure 18 Locality and habitat of *Epactionotus* from Biguaçu (BI), Rachadel River at Antonio Carlos, N of Rachadel, Santa Catarina State, Brazil (27°28’22.8”S 48°48’00.8”W)

SUPPLEMENTARY MATERIAL FOR MANUSCRIPT

TABLES

Table S1 Information from specimens, vouchers and sequences used in molecular analyses of *Epactionotus* species.

Species	Catalog #	Voucher specimen	Control #	Drainage	Drainage code	Location (county,state)	Geographic Coordinate	GenBank Accession #	Reference
Epactionotus bilineatus	UFRGS 17967	TEC 3640B	ML1	Maquiné	MQ	Maquiné, RS	29°38'53"S 50°13'4"W	Upon Submission	This study
	UNICTIO 1406	UNICTIO 225	ML2	Maquiné	MQ	Maquiné, RS	29°35'14.7"S 50°16'12"W	Upon Submission	This study
	UNIC 1444	UNICTIO 242	ML3	Maquiné	MQ	Maquiné, RS	29°32'28.1"S 50°12'8.9"W	Upon Submission	This study
	MCP 26964	-	-	Maquiné	MQ	Maquiné, RS	29°39'43"S 50°12'43"W	EU371009 (clone 2)	Cramer et al., 2008
	MCP 26964	-	-	Maquiné	MQ	Maquiné, RS	29°39'43"S 50°12'43"W	EU371008 (clone 1)	Cramer et al., 2008
	UFRGS 17817	TEC 3948A	ML4	Maquiné	MQ	Maquiné, RS	29°34’13.6”S 50°16’49”W	Upon Submission	This study
	UFRGS 9128	TEC 0272	ML5	Três Forquilhas	TF	São Francisco de Paula, RS	29°22'55"S 50°11'52"W	Upon Submission	This study
	UFRGS 16545	TEC 2878	ML6	Três Forquilhas	TF	Itati, RS	29°27'18"S 50° 7'22"W	Upon Submission	This study
	MCP 23679	-	-	Três Forquilhas	TF	Itati, RS	29°29'28"S 50° 8'35"W	EU371006	Cramer et al., 2008
Epactionotus itaimbezinho	UNICTIO 1908	UNICTIO 164	ML20	Mampituba	MA	Praia Grande, SC	29°14’57”S 50°07’17”W	Upon Submission	This study
	UFRGS 12719	TEC 1456B	ML21	Mampituba	MA	Praia Grande, RS	29°11’54”S 49°57’57”W	Upon Submission	This study
	UNICTIO 1993	UNICTIO 201	ML23	Mampituba	MA	Praia Grande, SC	29°10’07.2”S 49°58’17.7”W	Upon Submission	This study
	MCP 53971	151	RC92	Mampituba	MA	Rosa da Estância, Mampituba, RS	29°14’01”S 50°00’59”W	Upon Submission	This study
	MCP 23683	-	-	Mampituba	MA	Praia Grande, SC	29°14’55”S 49°55’3”W	EU371004	Cramer et al., 2008
Epactionotus gracilis	UNICTIO 1866	UNICTIO 147	ML7	Araranguá	AR	Jacinto Machado, SC	29°1'47.8"S 49°54'4.4"W	Upon Submission	This study
	UNICTIO 1882	UNICTIO 151	ML8	Araranguá	AR	Jacinto Machado, SC	29°0'42.6"S 49°53'19"W	Upon Submission	This study
	UFRGS 12544	TEC 1246	ML10	Araranguá	AR	Nova Veneza, SC	28°39'29"S 49°32'36"W	Upon Submission	This study
	UFRGS 22945	TEC 7390A	ML11	Araranguá	AR	Nova Veneza, SC	28°35’02.2”S 49°32’31.2”W	Upon Submission	This study
	MCP 23606	-	-	Araranguá	AR	Meleiro, SC	28°47’9”S 49°39’23”W	EU371005	Cramer et al., 2011
	MCP 53973	118	RC86	Araranguá	AR	Timbé do Sul, SC	28°50’25”S 49°48’02”W	Upon Submission	This study
Epactionotus DU	MCP 35156	-	-	d'Una	DU	Imbituba, SC	28°11'56"S 48°47'17"W	EU371007	Cramer et al., 2008
Epactionotus TU	UFRGS 22941	TEC 7391B	ML12	Tubarão	TU	Rio Bonito Alto, SC	28°25'48.3"S 49°27'50.7"W	Upon Submission	This study
	UFRGS 22941	TEC 7391C	ML13	Tubarão	TU	Rio Bonito Alto, SC	28°25'48.3"S 49°27'50.7"W	Upon Submission	This study
Epactionotus UR	MCP 53836	36	RC71	Urussanga	UR	Carvão Alto, Urussanga, SC	28°30’3”S 49°23’45”W	Upon Submission	This study
Epactionotus BI	UFRGS 20926	TEC 6043A	ML15	Biguaçu	BI	Antônio Carlos, SC	27°29'44”S, 48°46’57” W	Upon Submission	This study
	UFRGS 20926	TEC 6043B	ML16	Biguaçu	BI	Antônio Carlos, SC	27°29'44”S, 48°46’57” W	Upon Submission	This study
	UFRGS 22913	TEC 7392A	ML18	Biguaçu	BI	Antônio Carlos, SC	27°28’22.8”S 48°48’00.8”W	Upon Submission	This study
	UFRGS 22913	TEC 7392B	ML19	Biguaçu	BI	Antônio Carlos, SC	27°28’22.8”S 48°48’00.8”W	Upon Submission	This study
	UFRGS 22913	TEC 7392C	ML17	Biguaçu	BI	Antônio Carlos, SC	27°28’22.8”S 48°48’00.8”W	Upon Submission	This study

Table S1 Continued.

Species	Catalog #	Voucher specimen	Control #	Drainage	Drainage code	Location (county,state)	Geographic Coordinate	GenBank Accession #	Reference
Eurycheilichthys	MCP 35124	-	-	Taquari-Antas	TA	Barros Cassal, RS	29°2'53"S 52°33'19"W	EU370996	Cramer et al., 2008
apocremnus	MCP 35071	-	-	Taquari-Antas	TA	Barros Cassal, RS	29°2'51"S 52°34'6"W	EU370997	Cramer et al., 2008
E. castaneus	MCP 35049	-	-	Taquari-Antas	TA	Passo Fundo, RS	28°21'7"S 52°15'54"W	EU370999	Cramer et al., 2008
E. luisae	MCP 21207	-	-	Taquari-Antas	TA	Arvorezinha, RS	28°48'24"S 52°18'14"W	EU370995 (clone 1)	Cramer et al., 2008
	MCP 21207	-	-	Taquari	TA	Arvorezinha, RS	28°48'24"S 52°18'14"W	EU370998 (clone2)	Cramer et al., 2008
E. limulus	MCP 21270	-	-	Jacuí	JA	Tunas, RS	29°3'3"S 53°1'2"W	EU370989 (clone 1)	Cramer et al., 2008
	MCP 21270	-	-	Jacuí	JA	Tunas, RS	29°3'3"S 53°1'2"W	EU370990 (clone 2)	Cramer et al., 2008
E. pantherinus	MCP 22373	-	-	Uruguay	UY	São José dos Ausentes, RS	28°37'20"S 49°56'09"W	EU371000	Cramer et al., 2008
E. paucidens	MCP 22374	-	-	Taquari-Antas	TA	Lageado Grande, RS	29°5'34"S 50°37'30"W	EU370992	Cramer et al., 2008
	MCP 22800	-	-	Taquari-Antas	TA	Muitos Capões, RS	28°21'50"S 51°17'53.17"W	EU370994	Cramer et al., 2008
E. planus	MCP 22199	-	-	Taquari-Antas	TA	Guabiju, RS	29°38'4"S 51°36'53"W	EU370991	Cramer et al., 2008
E. vacariensis	MCP 22790	-	-	Taquari-Antas	TA	Lagoa Vermelha, RS	28°17'35"S 51°24'40"W	EU370993	Cramer et al., 2008

Table S2 Best-fit nucleotide evolution models for COI partitioned by codon position in each of the three datasets analyzed.

codon	*Epactionotus* + *Eurycheilichthys*	All *Epactionotus*	Newly sequenced *Epactionotus*
1st	K80	JC	JC
2nd	HKY+I	HKY+I	HKY+I
3rd	HKY+G+I	HKY+G+I	TRN+I

Table S3 Maximum Likelihood fit of 24 different nucleotide substitution models from a dataset containing a total of 731 positions conducted in MEGA7. BIC, AICc and lnL indicate scores according to Bayesian Information Criterion, Akaike Information Criterion, corrected, and Maximum Likelihood value, respectively. Whenever applicable, estimates of gamma shape parameter (+G) and/or the estimated fraction of invariant sites (+I), assumed or estimated values of transition/transversion bias (R) are shown for each model, followed by nucleotide frequencies (f) and rates of base substitutions (r) for each nucleotide pair. GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor. First line in bold (K2+ G+I) indicate the nucleotide model with the lowest BIC scores selected for the genetic distance analysis.

Model	# Param	BIC	AICc	lnL	Invariant	Gamma	R	Freq A	Freq T	Freq C	Freq G	A=>T	A=>C	A=>G	T=>A	T=>C	T=>G	C=>A	C=>T	C=>G	G=>A	G=>T	G=>C
K2+G+I	**56**	**3585.76**	**3145.56**	**-1516.62**	**0.79**	**0.68**	**12.61**	**0.25**	**0.25**	**0.25**	**0.25**	**0.01**	**0.01**	**0.23**	**0.01**	**0.23**	**0.01**	**0.01**	**0.23**	**0.01**	**0.23**	**0.01**	**0.01**
T92+G+I	57	3595.04	3147.00	-1516.33	0.79	0.68	12.61	0.25	0.25	0.25	0.25	0.01	0.01	0.23	0.01	0.23	0.01	0.01	0.23	0.01	0.23	0.01	0.01
K2+G	55	3597.82	3165.48	-1527.58	n/a	0.05	15.59	0.25	0.25	0.25	0.25	0.01	0.01	0.23	0.01	0.23	0.01	0.01	0.23	0.01	0.23	0.01	0.01
HKY+G+I	59	3602.35	3138.60	-1510.11	0.80	0.64	18.86	0.23	0.28	0.30	0.20	0.01	0.01	0.19	0.01	0.28	0.01	0.01	0.26	0.01	0.22	0.01	0.01
T92+G	56	3605.45	3165.26	-1526.46	n/a	0.05	15.59	0.25	0.25	0.25	0.25	0.01	0.01	0.23	0.01	0.23	0.01	0.01	0.24	0.01	0.24	0.01	0.01
TN93+G+I	60	3611.59	3139.98	-1509.80	0.80	0.64	14.13	0.23	0.28	0.30	0.20	0.01	0.01	0.17	0.01	0.29	0.01	0.01	0.27	0.01	0.2	0.01	0.01
HKY+G	58	3614.74	3158.84	-1521.24	n/a	0.05	12.93	0.23	0.28	0.30	0.20	0.01	0.01	0.18	0.01	0.27	0.01	0.01	0.25	0.01	0.21	0.01	0.01
TN93+G	59	3623.16	3159.41	-1520.52	n/a	0.05	12.80	0.23	0.28	0.30	0.20	0.01	0.01	0.19	0.01	0.26	0.01	0.01	0.25	0.01	0.22	0.01	0.01
GTR+G+I	63	3627.91	3132.74	-1503.16	0.80	0.67	11.53	0.23	0.28	0.30	0.20	0.03	0.01	0.16	0.02	0.3	0	0.01	0.28	0	0.18	0	0
GTR+G	62	3639.62	3152.31	-1513.95	n/a	0.05	9.94	0.23	0.28	0.30	0.20	0.03	0.01	0.17	0.03	0.28	0	0.01	0.26	0	0.2	0.01	0.01
K2+I	55	3639.65	3207.31	-1548.50	0.46	n/a	11.63	0.25	0.25	0.25	0.25	0.01	0.01	0.23	0.01	0.23	0.01	0.01	0.23	0.01	0.23	0.01	0.01
T92+I	56	3648.92	3208.73	-1548.20	0.46	n/a	11.63	0.25	0.25	0.25	0.25	0.01	0.01	0.23	0.01	0.23	0.01	0.01	0.23	0.01	0.23	0.01	0.01
K2	54	3662.22	3237.74	-1564.72	n/a	n/a	11.41	0.25	0.25	0.25	0.25	0.01	0.01	0.23	0.01	0.23	0.01	0.01	0.23	0.01	0.23	0.01	0.01
HKY+I	58	3664.04	3208.13	-1545.89	0.46	n/a	12.08	0.23	0.28	0.30	0.20	0.01	0.01	0.18	0.01	0.27	0.01	0.01	0.25	0.01	0.21	0.01	0.01
T92	55	3671.55	3239.21	-1564.45	n/a	n/a	11.41	0.25	0.25	0.25	0.25	0.01	0.01	0.23	0.01	0.23	0.01	0.01	0.23	0.01	0.23	0.01	0.01
TN93+I	59	3672.27	3208.52	-1545.08	0.46	n/a	11.99	0.23	0.28	0.30	0.20	0.01	0.01	0.21	0.01	0.24	0.01	0.01	0.22	0.01	0.25	0.01	0.01
HKY	57	3674.86	3226.81	-1556.23	n/a	n/a	11.49	0.23	0.28	0.30	0.20	0.01	0.01	0.18	0.01	0.27	0.01	0.01	0.25	0.01	0.21	0.01	0.01
TN93	58	3682.98	3227.08	-1555.36	n/a	n/a	11.48	0.23	0.28	0.30	0.20	0.01	0.01	0.21	0.01	0.24	0.01	0.01	0.22	0.01	0.25	0.01	0.01
GTR+I	62	3685.20	3197.88	-1536.74	0.46	n/a	8.77	0.23	0.28	0.30	0.20	0.03	0.01	0.19	0.03	0.25	0.01	0.01	0.23	0.01	0.23	0.01	0.01
GTR	61	3707.72	3228.25	-1552.93	n/a	n/a	8.50	0.23	0.28	0.30	0.20	0.03	0.01	0.2	0.03	0.24	0.01	0.01	0.22	0.01	0.23	0.01	0.01
JC+G+I	55	3710.40	3278.07	-1583.87	0.79	0.73	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC+G	54	3723.80	3299.32	-1595.51	n/a	0.05	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC+I	54	3762.90	3338.42	-1615.06	0.46	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
JC	53	3783.46	3366.83	-1630.27	n/a	n/a	0.50	0.25	0.25	0.25	0.25	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08

SUPPLEMENTARY MATERIAL FOR MANUSCRIPT

FIGURES

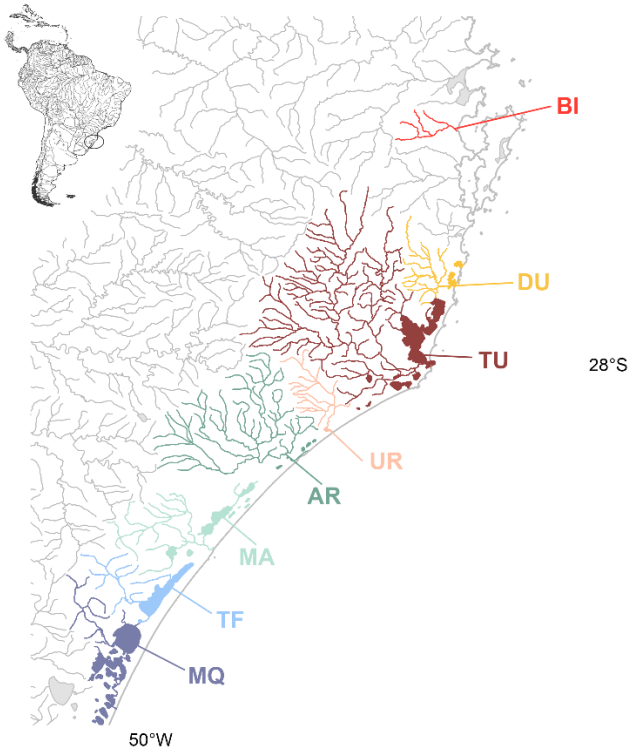

Figure S1 Updated range extension of the species/populations of *Epactionotus*, including previously known areas in Maquiné (MQ), Três Forquilhas (TF), Mampituba (MA) and Araranguá (AR), plus newly revealed areas Urussanga (UR), Tubarão (TU), d'Una (DU), and Biguaçu (BI).

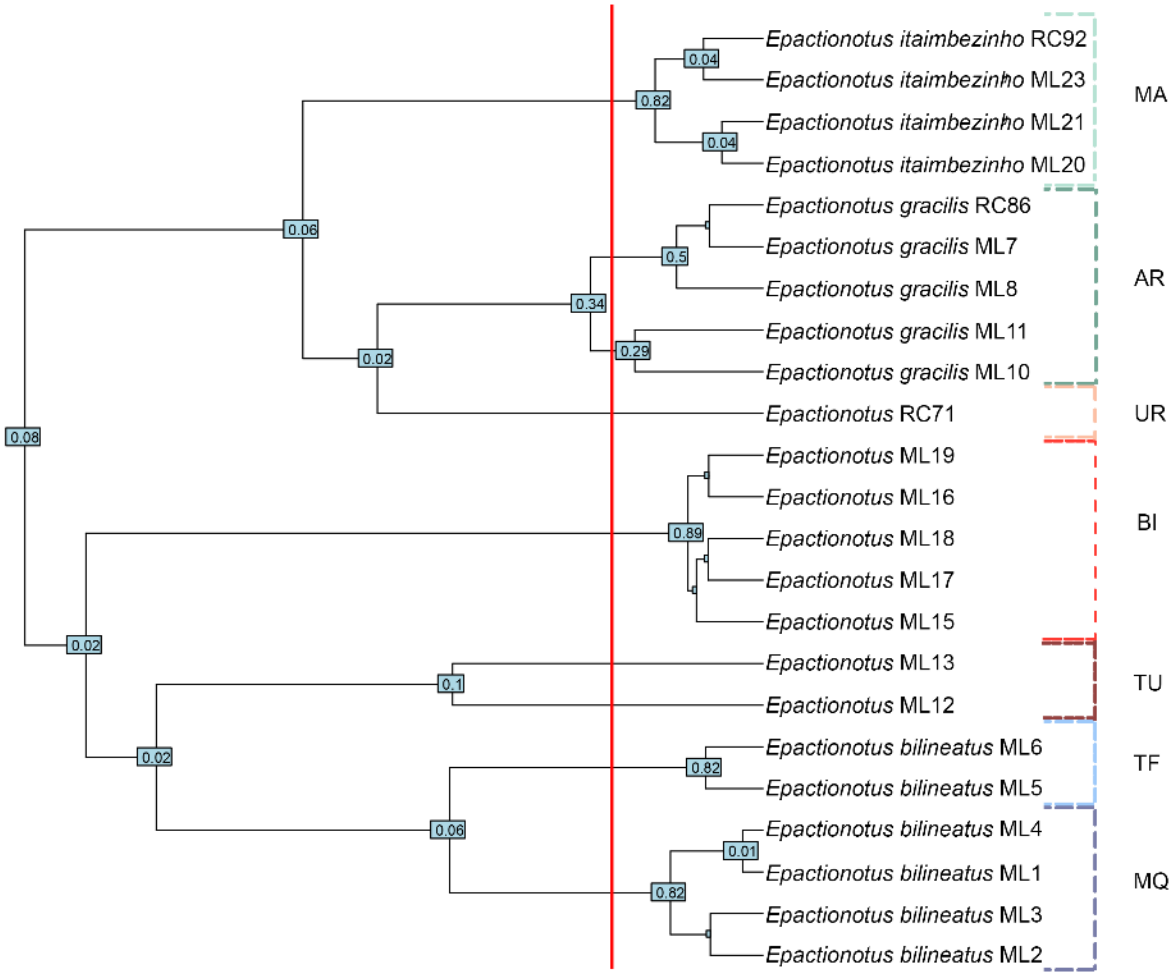

Figure S2 Bayesian phylogenetic and time-divergence coalescence analyses of newly sequenced species/populations of *Epactionotus* obtained with mitochondrial (COI) loci; the vertical red line shows the coalescent branching process of all sequences, estimated by using the single-threshold model in the general mixed Yule coalescent model (GMYC) test with birth-death speciation models. Colored dashed lines correspond to each drainage and node numbers correspond to BI posterior probability (PP).

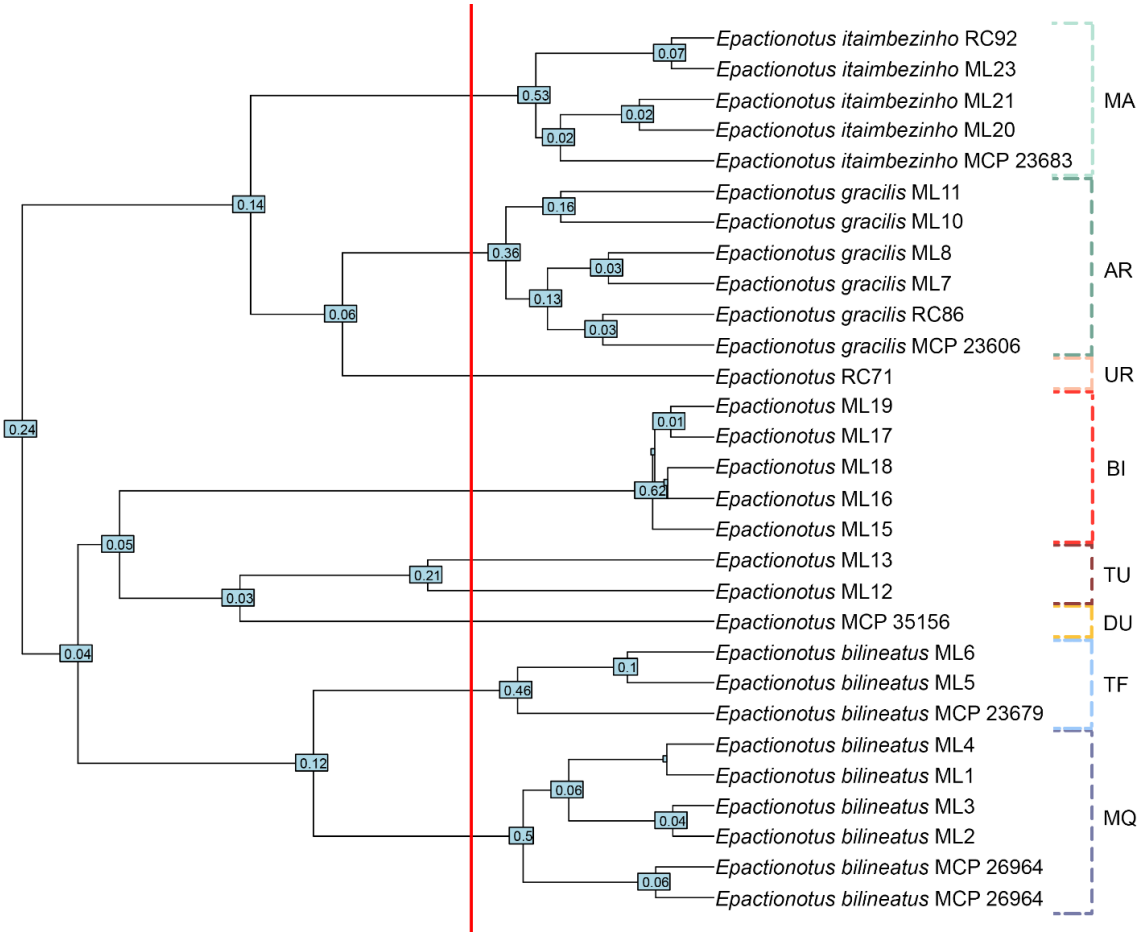

Figure S3 Bayesian phylogenetic and time-divergence coalescence analyses of all species/populations of *Epactionotus* obtained with mitochondrial (COI) loci; the vertical red line shows the coalescent branching process of all sequences, estimated by using the single-threshold model in the general mixed Yule coalescent model (GMYC) test with birth-death speciation models. Colored dashed lines correspond to each drainage and node numbers correspond to BI posterior probability (PP).

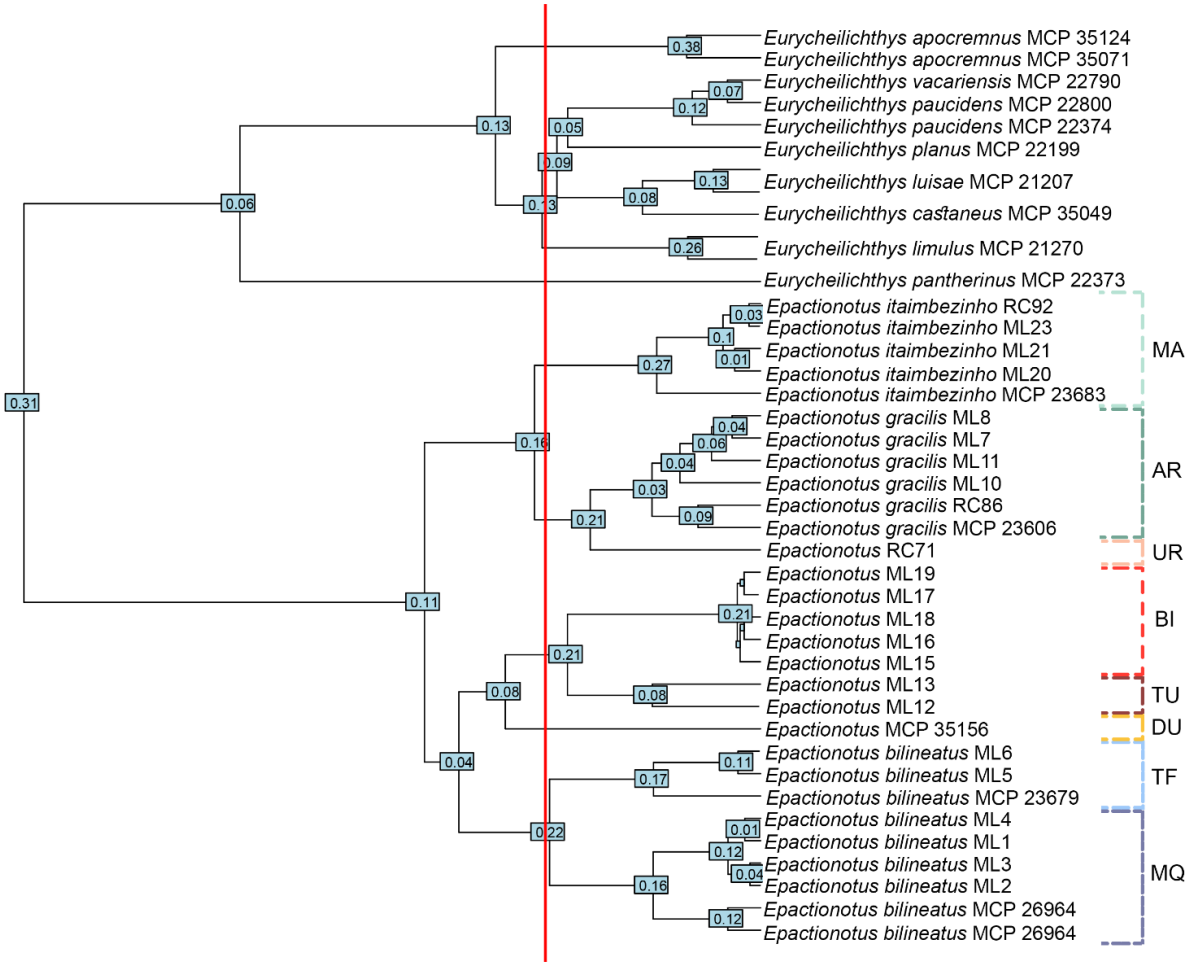

Figure S4 Bayesian phylogenetic and time-divergence coalescence analyses of all species/populations of *Epactionotus* plus sequences of *Eurycheilichthys* obtained with mitochondrial (COI) loci; the vertical red line shows the coalescent branching process of all sequences, estimated by using the single-threshold model in the general mixed Yule coalescent model (GMYC) test with birth-death speciation models. Colored dashed lines correspond to each drainage and node numbers correspond to BI posterior probability (PP).

Capítulo II

Title: Phylogenomics of the narrowly endemic genus *Eurycheilichthys* (Siluriformes: Loricariidae): a history of recent and rapid radiation in Southern Neotropical Freshwaters.

Running title: Phylogenomics of *Eurycheilichthys*

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Data Accessibility Statement: archival location upon submission

Title

Phylogenomics of the narrowly endemic genus *Eurycheilichthys* (Siluriformes: Loricariidae): a history of recent and rapid radiation in Southern Neotropical Freshwaters.

Abstract

Eurycheilichthys is a Neotropical freshwater genus comprising nine species of small catfishes endemic and restrictedly distributed through two river basins in Southern Brazil. The genus is better known by *E. pantherinus*, from the upper Uruguay River basin and *E. limulus*, from the upper reaches of the Jacuí River basin. The seven additional, and recently described, species of *Eurycheilichthys*, however, are all distributed through higher altitudes of the Taquari-Antas River basin, a tributary to the lower Jacuí River. Its diversity and endemism make *Eurycheilichthys* an important focal group for studying and understanding evolutionary biology. In this study, the phylogenetic relationships and time divergence of the species are presented and interspecific genetic structure comparing rare and common polymorphisms were estimated based on new genomic data created for 65 individuals of the nine species using ddRADseq protocol. Analyses support *Eurycheilichthys* as a monophyletic genus comprised by two species-inclusive clades, with absolute support and suggesting two and very recently diverged lineages on the Taquari-Antas species. Except for *Eurycheilichthys luisae*, all remaining species were recovered as monophyletic. Discussion on the likely processes responsible for these results and the distribution of the species is presented.

Keywords: Cascudinhos, ddRADseq, Endemism, Hypoptopomatinae, Phylogeny

Introduction

Species with restricted geographic patterns of distribution (*i.e*. endemic species) have always drawn the attention of scientists. They are unique and irreplaceable results of evolution (Feng et al. 2019) being of high interest to conservation, evolution, and biogeography as they can help define biodiversity hotspots (Myers et al. 2000) and infer evolutionary processes of speciation (Alonso et al. 2012; Welch et al. 2016).

In a worldwide scenario, endemic fish groups, especially cichlids, have been helpful to understand evolution and infer hypothesis in African lakes (Wagner et al. 2013; Ford et al. 2015; Gante et al. 2016). As for the Neotropical Region, endemic fishes have been the focus of significant evolutionary and ecological studies in Guatemala (Rosen 1979), Nicaragua (Kautt et al. 2016), and the Guiana Shield highlands (Rull 2005, 2007; Vari and Ferraris 2009).

Neotropical freshwater fishes comprise the most species-rich vertebrate fauna on the planet with more than 6,000 known species (Albert et al. 2011; Reis et al. 2016; Carvalho et al. 2018). This diverse pattern can be linked to the geomorphological history of the river basins in which they live (Smith 1981; Mayden 1988; Lundberg et al. 1998; Albert and Carvalho 2011) as freshwater fishes dispersal is dependent on drainage connections (Lundberg 1993; Carvajal-Quintero et al. 2019).

Eurycheilichthys is a Neotropical freshwater genus and comprises nine species of small size armored catfishes among the 247 valid hypoptopomatines (van der Laan and Fricke 2019). In spite of being a small number of species within the subfamily, the genus is endemic to Southern Brazil and Misiones (Argentina), showing a high species diversity restrictedly distributed in two river basins chiefly in the southern portion of the Araucaria forest plateau of Southern Brazil (Reis 2017).

The genus is better known by *E*. *pantherinus*, the most widespread species, from the upper Uruguay River basin and *E*. *limulus*, from the upper reaches of the Jacuí River

basin, which is part of the Laguna dos Patos drainage (Figs. 1A and S1). The seven additional and recently described species of *Eurycheilichthys* (Figs. 1A and S1), however, are all distributed through higher altitudes of the Taquari-Antas River basin, a tributary to the lower Jacuí River. Hence, eight out of nine species of the genus occur in the headwaters of the Laguna dos Patos basin (Reis 2017).

When considering distribution of only the Taquari-Antas species (Fig. 1A), *Eurycheilichthys apocremnus* and *E. castaneus* are geographically limited to only few streams located in Western portions of the basin, while *E. coryphaenus*, *E. planus*, and *E. vacariensis* are somewhat confined to Eastern creeks. The remaining species *E. luisae* and *E. paucidens* are more widely distributed through a middle range having populations relatively distant from each other and being sympatric to other species.

Most localities of *Eurycheilichthys* in Uruguay and Taquari-Antas basins are small rivers and creeks with fast flowing, clear water and the substrate comprised of rocks and stones. In the upper Jacuí River basin, *E. limulus* is very common and found in nearly every creek with medium to fast-flowing water (Reis, 2017). The rivers in the upper Taquari-Antas basin are characterized by being high-energy streams, with high average declivity and deeply excavated valleys (Liedke 2007). Regardless of the river basin, species of *Eurycheilichthys* are typically associated with the river bottom, dwelling among the stones and rocks. The rivers inhabited by the species of *Eurycheilichthys* run on the ancient crystalline rocks of the Serra Geral formation in Grande do Sul and Santa Catarina States of Brazil and Misiones in Argentina (Reis and Schaefer 1998; Liedke 2007; Carvalho and Reis 2011; Reis 2017).

The genus has been studied before based on both morphology and molecular aspects. The morphological diversity of the genus was explored and described by Reis (2017), and even though some species are more similar to each other (Fig. S1) they can be easily diagnose and identified based on several morphological features such as color

variation, abdominal plate morphology, number of dermal plates, number of teeth, and body shape and proportions.

As for molecular work, based on mitochondrial genes COI and ND2, Liedke (2007, unpublished thesis) conducted the first phylogenetic and phylogeographic study of *Eurycheilichthys*, including the species not yet described at the time, and found both phylogenetic trees and network structured by basin. Additionally, the molecular clock estimation made by Liedke (2007) suggested two main and very recent divergence events within the genus. The first one dated to late Pliocene (2.4–1.2 Ma) between *E. pantherinus* (upper Uruguay) and all remaining species from the Laguna dos Patos basin. The second, dated to middle Pleistocene (1.0–0.5 Ma) between *E. limulus* (upper Jacuí) and all Taquari-Antas species (Fig. S2). However, only three (*E. apocremnus*, *E. castaneus*, and *E. planus*) out of the seven species from Taquari-Antas were monophyletic, the other species showed several paraphyletic clades with restrict geographical distribution.

The amount of generated data and the possibility of having no previous known genome have made Next-Generation Sequencing approaches very popular (Leaché et al. 2015a). Additionally, even though methods like Sequence Capture have been more commonly used to infer phylogenetic relationships, RADseq methods, for instance, have been proved to provide highly supported phylogenomic resolutions (Rubin et al. 2012; Eaton & Ree 2013; Wagner et al. 2013; Leaché et al. 2015a), especially in the case of closely related and, which seem to be the case of *Eurycheilichthys*, recent species. Yet, bioinformatics developments and assembling methods must be properly applied aiming for a more accurate classification of homology and the detection of paralogs (Philippe et al. 2011; Treangen et al. 2014; Leaché et al. 2015b).

The high species diversity to such a restricted distribution of *Eurycheilichthys* make them an important focal group for studying evolutionary biology. However, the

relationships between the nine species suggested by Liedke (2007) have not been tested yet and understanding evolution requires a well-established phylogeny (Wielstra et al. 2019). In this study, new molecular data was generated for several populations of each species using double digest RADseq protocol to examine interspecific genetic structure based on rare versus common polymorphisms and to estimate phylogenetic relationships and time divergence between the species of *Eurycheilichthys*.

Material and Methods

Sampling and specimen collection

Aiming to generate genomic data, approximately 10 individuals were sampled from different populations of each species of *Eurycheilichthys*, except for those species with very restrictive distribution pattern (Fig. 1) which had only 3-5 sampled individuals. The total sampling was comprised of 65 individuals of *Eurycheilichthys*, collected from upper and middle Uruguay, upper Jacuí, and Taquari-Antas River basins. Mostly muscle but also fin tissues were collected in the field from January to June 2016, preserved in 99.8% ethanol and stored at -20°C at the Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul (MCP). Additional tissue samples were also loaned from the ichthyological collections of the Universidade Federal do Rio Grande do Sul (UFRGS) and Universidade do Vale do Rio dos Sinos - UNISINOS (UNICTIO). The complete list of tissue samples is in Tab. S1.

RADSeq library preparation and sequencing

DNA was extracted using DNeasy Blood and Tissue extraction kits (Qiagen, Valencia, CA) following the manufacturer's protocol for animal tissue, and extractions were stored at -20ºC. After measuring genomic DNA concentrations following DNA quality verification and quantification with NannoDrop (Thermo Fisher Scientific, Waltham,

MA) and, most importantly, Qubit fluorometer (Invitrogen, Carlsbad, CA), library construction was conducted using only samples with concentrations equal to 10ng/µl.

A RADSeq library was prepared following Peterson et al. (2012) double digest protocol with few modifications for 65 individuals of *Eurycheilichthys*, plus seven outgroups (outgroup list in Tab. S1). Succinctly, DNA was double-digested with two restriction enzymes (SphI and MluCl), followed by a ligation step and amplification by PCR, where unique barcodes (5 bp) and Illumina adapters were added to the digested DNA so that individuals could be pooled together. PCR products were cleaned and pooled DNA was size-selected using BluePippin (Sage Science, Beverly, MA) with selected fragment size between 376-450 bp (aimed size: 400bp). The unique Illumina indices were incorporated into the P2-adaptor end of DNA fragments using a real-time library amplification kit (Kapa Biosystems, Wilmington, MA). The concentration of each pool was standardized and combined for Illumina sequencing and quantified using a High Sensitivity DNA Kit on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Libraries were sequenced at the qb3 Berkeley facility on an Illumina HiSeq 2500, with 100 base pair single-end reads. Sequencing generated 331 million total reads which passed initial quality control at the sequencing facility. All STACKS modules and IPYRAD steps mentioned below were run under parallel execution in LAD – Laboratório de Alto Desempenho at PUCRS.

Processing Illumina reads to assess interspecific relationships

The raw reads were processed using IPYRAD version 0.7.30 (Eaton and Ree 2013; Eaton 2014) which allows for indel variation within and between samples, and is able to recover more shared homologous loci across disparate taxa. More specifically, raw reads were de-multiplexed (step 1), by trimming the portion of the Illumina adapters plus the reverse complement of the second cut site and the barcodes. Trimming was done by using a code

from the software *cutadapt*, which allows for errors within the adapter (max_barcodes_mismatch 2). Individuals were identified allowing no mismatch in the barcode sequence (filter_adapters), since several barcodes used were only one mismatch away from each other. De-multiplexed raw reads were then filtered (step 2), allowing an upper limit of five 'Ns' in a read (*i.e.* low quality bases) (max_low_qual_bases) and bases were trimmed from the 3' end of reads using default offset for quality scores of 33 (Phred_Qscore_offset). The reads of each sample were then clustered into putative loci *de novo* (step 3 - Assembly_method) using a 90% similarity threshold (clust_threshold), a minimum depth for making a statistical base call at each site (steps 4 and 5) of five (mindepth_majrule & mindepth_statistical) and a maximum number of indels per consensus sequence (max_Ns_consens) of three. The same similarity threshold and number of indels allowed were used for clustering loci across individuals (step 6). Finally, loci with heterozygous alleles shared across more than eight individuals were discarded (step 7) in order to remove potential paralogs. All the 29,801 remaining loci shared by at least two samples were exported for further processing. The aligned loci had 6 bp of their 3' ends trimmed off and were retained. From IPYRAD, two different filtering approaches were used in order to create a more inclusive and a less inclusive datasets.

To obtain a more inclusive dataset a series of customized R scripts (available at https://github.com/airbugs/Dynastes_introgression) provided by Huang (2016) were applied. First, using the cout_n_varsitesfrom_locifile.r script, filtering and clustering results from IPYRAD (specifically, the .loci output file) were visualized. A second R script (chop_loci_file.r) was used to exclude downstream sites for all aligned clusters, since dataset contained an increase in sequence variation towards the 3' ends of the alignments after site 75. With the third script (EDchopped_locifile.r), the pairwise sequence divergence based on uncorrected p-distances between samples were calculated

for each locus. Loci with a maximum pairwise sequence divergence of 16% were then excluded to remove suspicious clusters of paralogous loci with too many variable sites (threshold based on the maximum pairwise sequence divergence between one outgroup and *Eurycheilichthys* species in mitochondrial COI available in GenBank). Additionally, all invariable loci and loci that contained samples from less than four species in the dataset were removed. The comparison between before and after data filtering can be found in Fig. S3. Finally, a last R script (concate_SNPs.r) was used to select only putatively unlinked biallelic single-nucleotide polymorphism (SNP) from each locus (specifically, the one closest to the 5' end to reduce possibility of sequencing error), assuming that all loci in the final dataset were unlinked and randomly distributed in the genome. The use of only one SNP per locus was preferred since other variable sites may contain singletons and be, therefore, less informative for phylogenetic reconstruction. The parsimony-informative chosen SNPs were then used to produce a dataset/alignment and converted to a file in PHYLIP format, and afterwards manually edited and saved in NEXUS format.

As for the less inclusive dataset, the program PLINK version 1.9 (Purcell et al. 2007), was used to apply filters and test different levels of missing data. More specifically, the Variant Call Format file (.vcf) containing all SNPs per loci from IPYRAD was filtered calling only the SNP closest to the 5' end (--bp-space) of each loci. The species with smaller proportion of shared loci (*Pareiorhaphis hystrix*, *Pseudotocinclus juquiae*, *Schizolecis guntheri*, and *Hisonotus armatus*) were removed, and each of the remaining species was represented by two-four individuals. The dataset with unlinked SNPs was further filtered keeping only SNPs with a 100% genotyping rate (--geno 0). The filtered SNPs were exported from PLINK in a .vcf format, and a Python script (vcf2phylip.py) was used to convert the dataset to PHYLIP.

Species-tree and divergence-time estimation

The NEXUS file containing the more inclusive SNPs dataset was then used to infer the phylogeny of the group and evolutionary relationships among species and populations. More specifically, a species-tree analysis was performed using SVDQUARTETS, a coalescent-based method (Chifman and Kubatko 2014) implemented in PAUP* version 4.0a166 (Swofford 2002). This approach accounts for the differences in the genealogical history of individual loci expected to arise under a multispecies coalescent model and has been developed specifically for SNP data (Papadopoulou and Knowles 2017). The species tree was constructed with an exhaustive search of all possible quartets, which were assembles with Quartet FM (Fiduccia and Mattheyses 1982) amalgamation algorithm (Reaz et al. 2014) and each SNP was treated as an independent locus. The reliability of the branches of the species tree was evaluated using the same search options, with 1000 bootstrapping replicates in PAUP*.

An additional and exploratory phylogenetic analysis was conducted using Maximum Parsimony (MP). The above-mentioned NEXUS matrix was exported and submitted to heuristic analyses in TNT version 1.5 (Tree Analysis using New Technology - Goloboff et al. 2008) using New Technologies and extensive branch swapping with the algorithm Tree Bissection Reconnection (TBR). To estimate branch support, Bremer decay index was calculated also in TNT.

The PHYLIP format with the less inclusive dataset was used to prepare a .xml input file for running SNAPP version 1.5.0 (Bryant et al. 2012) implemented in BEAST2 version 2.6.0 The .xml file was created with a Ruby script (snapp_prep.rb) provided by Stange et al. (2018) (available at https://github.com/mmatschiner/snapp_prep). The model settings of the script assumes that population sizes of all species are linked and the substitution rate, which is assumed to be according to a strict clock, was calibrated with a lognormal distribution age constraint centered on the node between *Epactionotus* and

Eurycheilichthys. The divergence between the two genera was estimated at 3.3781 Ma with a 95% HPD interval from 2.6751 to 4.085 Ma (Delapieve et al. *in prep*) with a standard deviation, of 0.06 in real space. An independent theta (θ=4μN) was estimated for each branch under default gamma (γ) prior distribution values (α=11.75, β=109.73). A run with chain length of one million MCMC generations was performed sampling every 1000 generations. Performance of MCMC was assessed considering trace plots and effective sample sizes (ESS) with values >200 using program TRACER version 1.6 (Rambaut et al. 2014) excluding 10% of runs as burn-in. Resulting trees from SNAPP were visualized using program DENSITREE (Bouckaert 2010) and the posterior probabilities of clades were quantified in a Maximum-Clade-Credibility Tree using TreeAnnotator version 2.6.

Processing Illumina reads to assess interspecific structure

The raw sequenced reads were also de-multiplexed and filtered, using the *process_radtags.pl* script in STACKS version 1.42 (Catchen et al. 2011; 2013). Using default settings, reads with Phred scores below 33 were discarded.

From de-multiplexed reads, the assembling process to assess structure information was also conducted using STACKS. Based on preliminary phylogenetic results, three datasets were separately processed. 1) The nine species of *Eurycheilichthys* (hereafter referred to as full dataset, which included *E. pantherinus*, *E. limulus*, and the species from Taquari-Antas); 2) Eastern Taquari-Antas clade species (*E. coryphaenus*, *E. luisae*, *E. planus*, and *E. vacariensis*), and 3) Western Taquari-Antas clade species (*E. apocremnus*, *E. castaneus*, and *E. paucidens*). Aiming to align resulting reads into exactly matching stacks (i.e. putative alleles) and to form a set of putative loci so that SNPs could be detected at each locus, the short-reads for each individual were assembled *de novo* with USTACKS (mean coverage of each dataset in Tab. S2). The program was run to the

three datasets with a minimum depth coverage of three (-m 3) to create a stack, allowing a maximum nucleotide distance of two (-M 2, default) between stacks. The Removal algorithm (-r) and the Deleveraging algorithm (-d), were also included to remove overrepresented stacks and resolve overmerged loci, respectively, with model type equal bounded (--model_type), and an error bound for ε of 0.1 (--bound_high). These parameters increase the likelihood of a locus with a number of alternative reads to be called a homozygous site with excessive error and decreases the chance of calling a homozygote when the true genotype is heterozygous (Catchen et al. 2013). From the samples processes by USTACKS a catalog of genomic sequences (i.e. consensus homologous loci) was built in CSTACKS, creating a set of consensus loci by merging the alleles together. Finally, loci for each individual 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 for two mismatches between individuals (-n 2).

Interspecific structure and differentiation

From SSTACKS the POPULATIONS program was run for the three datasets under loose parameters (-r 0 -p 2 -m 5 --min_maf 0 --max_obs_het 0.5), treating each species as a different population and creating a .vcf output with all SNPs per locus. The presence of artificially overestimated SNPs on the 3' end of each locus as well as loci with exceedingly high genetic diversity (represented in the upper 95% quantile of the distribution of the genetic diversity) can be highlighted as possible consequences of both sequencing and data assembly errors (Thomaz et al. 2017). Aiming to eliminate both plausible error, the SNP output .vcf file from SSTACKS was processed using an R script. More specifically, loci that were within the upper 95% of the estimated theta values (Fig. S4) were removed. Additionally, the number of SNPs per sequence position was plotted

and the five positions at the 3' end with an increased number of SNPs (when compared to other sites) were manually trimmed (Fig. S4). A whitelists with the ID of the selected SNPs was created and the program POPULATIONS was run again using the whitelists as input and exporting filtered new .vcf file.

The program PLINK 1.9 was then used differently to each dataset in order to filter and test different levels of missing data. The full dataset was checked for missingness per SNP (--geno) and individual (--mind), totaling 16 tested datasets (Tab. S3). Once datasets generated include ddRADseq data from individuals of nine species, they were expected to present high mutational rate and, therefore, a large number of missing data. Additionally, when excluding too much missing data important types of loci can also be excluded; i.e. loci with a high mutational rate which are useful for historical inferences (Huang and Knowles 2016). Thus, the parameters used to filter missing data with PLINK were not too strict.

Among the 16 tested levels of missing data, all further analyses were conducted with a dataset containing a maximum of 30% of missing data per unlinked SNP and 80% per individual (i.e., 62 individuals and 43,712 SNPs, with a genotyping rate 0.46). These parameters were chosen based on the low number of individuals excluded, the high number of SNPS kept and by the correspondence between population genomic structure and the putative morphological species.

As for the Eastern and Western Taquari-Antas datasets, individuals with more than 60% of missing data (--mind 0.6) were removed and two different filters were applied to each of them aiming to obtain two different sub-datasets. One containing only rare variants (calling SNPs with minor allele frequency, maf ≤ 0.05; --max-maf 0.05), and other containing common variants (maf > 0.05; flag --maf 0.05).

From each dataset (full, Eastern and Western – Tab. S2) the most likely number of genetic clusters (i.e. K-value) was searched with ADMIXTURE (Alexander et al. 2009)

which clusters samples into populations and estimates ancestry in a model-based manner from large autosomal SNP genotype datasets. Ranging K from one to 15 (number of species +6 when analyzing the full dataset) and from one to the number of species +1 in the case of Eastern and Western datasets cross-validation procedure (--cv) was used, where the best value of K exhibits the lowest cross-validation error (Fig. S5). Based on the best K value, ADMIXTURE's and CV's graphics were created using R scripts (version 3.5.1). Separated PCAs were run with common and rare variants of both Eastern and Western datasets using ADEGENET R package (Jombart 2008; Jombart and Ahmed 2011) to visualize the major axes of genetic variation.

Results

Processing Illumina reads

From the 331 million raw reads here sequenced for 72 individuals, 249 million reads passed initial filters and were retained from IPYRAD and 226 million from STACKS assembling processes (average of 3.460,822 ± 1.464,635 reads per individual and 3.144,637 ± 1.359,274 sequences per individual, respectively – Tab. S1; Fig. S6).

From IPYRAD two datasets were created, one for SVDQUARTETS coalescent species-tree and MP analyses including all individuals and 29,350 loci. The other dataset was used for SNAPP, a more sensitive analysis, having outgroups and individuals removed according to levels of missing data, which resulted in 1,355 loci and 33 individuals. From STACKS assembling, three basic dataset were created. The full dataset included 62 *Eurycheilichthys* individuals and 43,712 SNPs, with a genotyping rate 0.46. The Eastern Taquari-Antas dataset included 30 individuals and 114,815 loci (genotyping rate = 0.6), and Western included 21 individuals and 58,216 loci (genotyping rate = 0.7). See following section (Interspecific structure and differentiation) and Tab. S2 for further filtering information.

Species-tree and divergence-time estimation

The exhaustive search analysis performed with SVDQUARTETS of all 607,161 possible quartets from 29,350 unlinked SNPs resulted in a species-tree topology with most clades supported by a bootstrap ≥ 98.4 (except for two outgroup clades weakly supported – Fig. 2A). The SNAPP analysis of the less inclusive dataset with 1,355 SNPs (Fig. 2B) also produced a strongly supported topology with all PP>0.94. Analyses recovered *Eurycheilichthys* as a monophyletic group comprised of two species-inclusive clades, with absolute support and suggesting two different lineages on the Taquari-Antas species. One of the subclades comprises *E. limulus* (species from upper Jacuí River basin) as sister to Western species of the Taquari-Antas basin, *E. apocremnus, E. castaneus,* and *E. paucidens*. The other clade is formed by *E. pantherinus* (species from Uruguay River basin) as sister to the Eastern species of the Taquari-Antas basin, *E. coryphaenus, E. luisae, E. planus,* and *E. vacariensis*. In this Taquari-Antas lineage, however, *E. luisae* was not recovered as monophyletic. The samples of *E. luisae* (from Camisas River, *i.e.* upper Antas) were found as sister to *E. coryphaenus* (upper Antas) and another sample of *E. luisae* (from Guaporé River) was found as sister to the clade *E. vacariensis* plus remaining *E. luisae*. Both SVDQUARTETS and SNAPP analyses obtained the same species-tree topology where, except for *E. luisae*, all remaining species of *Eurycheilichthys* were found as monophyletic. MP exploratory phylogenetic analysis (Fig. S7), which resulted in one single most parsimonious tree with a length of 36,525 steps, consistency index of 0.75, and retention index of 0.93, recovered a very similar species-tree topology, but with both *E. luisae* and *E. vacariensis* paraphyletic.

Even though most outgroups and some individuals of *Eurycheilichthys* were removed from SNAPP analyses (Fig. 2B), the sister relationship between *Eurycheilichthys* and *Epactionotus* species was recovered. The first divergent event within the genus was estimated to the Pleistocene (1.37 Ma, 95% confidence intervals

1.62-1.16 Ma; Fig. 2B). Also, when considering total phylogenetic branch length, divergence between Jacuí species and Western Taquari-Antas clade showed relatively deeper interspecific divergences when compared to Uruguay and Eastern Taquari-Antas. More specifically, split between *E. pantherinus* (Uruguay basin) and Eastern Taquari-Antas were estimated between 1.06-0.73 Ma and between *E. limulus* (upper Jacuí basin) and Western Taquari-Antas clade, 0.95-0.66 Ma. *Eurycheilichthys luisae* and *E. vacariensis* were recovered as the most recent paired taxa divergence within *Eurycheilichthys* (0.15 Ma, 95% confidence intervals 0.19-0.11 Ma).

Interspecific structure and differentiation

Results from the ADMIXTURE analysis using the full dataset (62 individuals and 43,712 SNPs) indicate a K=9, showing strong clustering among samples of each species, corresponding to the morphological delimitation (Fig. 3A). However, it shows one sample of *Eurycheilichthys luisae* (from Camisas River) with strong patterns of admixture with *E. coryphaenus* and *E. vacariensis*, (>70-80% and ~15%, respectively). Additionally, samples of *E. vacariensis* (from Ituim River) were recovered as having ancestry pattern of ~50% admixture between *E. luisae* and *E. planus*.

ADMIXTURE and PCA analyses of common and rare variants of the Eastern and Western datasets showed, in general, a strong clustering among individuals of the corresponding morphological species, yet, Western dataset showed a higher degree of separation between species when compared to the Eastern dataset (Figs. 3B-E and 4A-D). ADMIXTURE analyses of the Eastern dataset cross-validation procedure suggest different numbers of clusters when comparing common and rare variants (Fig. 3D-E). Common variants (71,670 SNPs) analysis indicates a K=4 with patterns of ancestry similar to the pattern showed in the full dataset (for these four species). Yet, having more admixture between species, one individual of *E. luisae* from Guaporé River basin, was

recovered with high patterns of admixture between all four species with higher percentages of *E. planus* and *E. vacariensis* (~20% and ~60% respectively) and the individuals of *E. vacariensis* from Ituim River were recovered as having ancestry pattern of 100% *E. luisae* (Fig. 3D). Rare variants (43,843 SNPs) analysis suggest a K=3 recognizing *E. coryphaenus*, *E. planus*, and *E. vacariensis* as three independent clusters with a lack of clustering among individuals of *E. luisae* (Fig. 3E). When analyzing PCA of both common and rare variants of the Eastern dataset (Fig. 4C and D, respectively), cluster individuals of *E. luisae* and *E. vacariensis* are highly overlapped with each other. Exceptions to that are the sample of *E. luisae* from Camisas River that based on both common and rare variants is plotted outside the two 95% overlapping ellipses and the sample of *E. luisae* from Guaporé River basin that is plotted outside its 95% ellipses, but only when considering rare variants.

Discussion

A single freshwater fish species has a geographical median area of 77,322 km^2 (Carvajal-Quintero et al. 2019) which is almost the same range covered by all nine species of *Eurycheilichthys*, since together they occupy an area of approximately 80,000 km^2 in the headwaters of two river basins in the southern South America (Reis 2017). Endemism in *Eurycheilichthys* species has often been explained by the combination of its species being ecologically associated to fast-flowing watercourses (Reis, 2017) with the fact that these basins are located in a mountain relief with high declivity and presence of waterfalls, which isolate and limit their distribution (Liedke 2007; Carvalho and Reis 2011; Ferrer and Malabarba 2013; Reis 2017).

A new population of *Eurycheilichthys luisae* was sampled during species inventory in a very low portion of Taquari-Antas basin nearby Travesseiro County at an altitude of 105-125 meters above sea level. Before this occurrence, all specimens of

Eurycheilichthys sampled were restricted to an altitudinal range from approximately 400 to 1,400 meters above sea level never reaching lower portions of the rivers (Reis and Schaefer 1998; Reis 2017). Yet, even though the new population was found in a low altitude, *Eurycheilichthys luisae* is the most widespread species in the Taquari-Antas, being known from several localities in both upper and lower courses of the Forqueta, Carreiro, Turvo, and Antas rivers (Reis 2017). Additionally, that watercourse is 50 km distant from the type locality and is a very small creek with the same physionomic and ecological characteristics from the headwater habitats in which the species is commonly found.

From the three phylogenetic approaches herein applied the same congruent species-tree topology was recovered by two of them (SVDQUARTETS and SNAPP). Even though, MP topology was not exactly the same, it shared most of the patterns recovered by SVDQUARTETS and SNAPP. All analyses suggest a monophyletic and well supported *Eurycheilichthys* divided into two subclades estimated (by SNAPP) to the Pleistocene between 1.62-1.16 Ma. These results differ from Liedke (2007) which indicated three major mitochondrial clades and *Eurycheilichthys* hierarchically structured by basin. Two of these three clades encountered by Liedke (2007) were represented by *E. pantherinus* and *E. limulus* (divergence estimated between 2.4-2.2 Ma), this last one being sister to the third clade containing all seven species of Taquari-Antas basin, with an estimation for the divergence between the two Laguna dos Patos river basins dated to 1.0-0.5 Ma. Internal clades of Taquari-Antas showed a recent radiation around 0.167-0.08 Ma.

Alternatively, one of the subclade division herein recovered is represented by *Eurycheilichthys limulus* (from upper Jacuí River basin) as sister to Western species of the Taquari-Antas basin *E. apocremnus* and *E. castaneus* plus the more widely distributed *E. paucidens*. These findings are congruent with the more similar

morphological features shared by *E. apocremnus* and *E. castaneus* with *E. limulus*. Also, this geographic pattern coincides with the distribution described for *Cambeva poikilos*, which occurs through upper tributaries of the Jacuí basin and Western portions of Taquari-Antas tributaries (Ferrer and Malabarba, 2013).

The other sub-clade of *Eurycheilichthys* comprises *E. pantherinus* (from Uruguay River basin) as sister to the Eastern species of the Taquari-Antas basin, *E. coryphaenus*, *E. planus*, *E. vacariensis*, plus the more widely distributed species *E. luisae* which is also congruent to morphological similarities between these species. Geographically speaking, this composition is consistent with the distribution pattern found for *Pareiorhaphis hystrix*, a widely distributed loricariid species in upper and middle portions of Uruguay River and Taquari-Antas basins. Even though a recent integrative taxonomy study (Fagundes et al. *in prep*) suggests *P. hystrix* as being one single biological species, the populations sampled from the Pelotas River (upper Uruguay basin) clustered with populations from Eastern Taquari-Antas areas based on morphologic data, genetic distance, and haplotype analyses.

Another interesting point to highlight is the lower levels of gene flow across the Western clade added to their relatively deeper interspecific divergence, which can be explained by the fact that two of its species are isolated and restrictedly distributed resulting in a higher population persistence through time. Yet, all those lineages, including the species from Uruguay and upper Jacuí basins, have very similar life-history and ecological traits and speciation time is almost the same between the two sub-clades herein encountered. So, what explains the higher diversity present in the Taquari-Antas basin when compared to the conserved patterns of Uruguay and upper Jacuí basins?

It is acknowledged that dispersal is favored in spatially and temporally variable habitats (Papadopoulou and Knowles 2017), so one potential explanation is that the Taquari-Antas basin has had more temporal and spatial habitat instability. Thereafter, a

more dynamic landscape could sustain more variable population densities through space, selecting higher dispersal rates through time in comparison to more static landscapes of the other two river basins where geological space is more stable.

The formation of the river basins involved precedes the time herein estimated for the speciation events (Ribeiro 2006). Additionally, a good amount of their shallow headwaters are very close to each other (Liedke 2007; Ferrer and Malabarba 2013; Reis 2017) and the species of *Eurycheilichthys* are mainly distributed through those upper stream portions. The combination of the aforementioned factors suggest that basins formation do not explain the observed differences in diversity across this system. In other words, it is very likely that headwater capture events among geographically close small tributaries, especially in the Taquari-Antas, are responsible for the phylogenetic, diversity, and geographic patterns observed in *Eurycheilichthys* (Liedke 2007; Carvalho and Reis 2011; Ferrer and Malabarba 2013; Reis 2017). This falls into the statement that the average of the stream orders where a species occurs and the historical connectivity (*i.e.* a measure of past connections among drainage basins) can be considered two of the most important drivers of geographical range size variation in freshwater fish species in the Neotropical region (Carvajal-Quintero et al. 2019).

SVDQUARTETS and SNAPP analyses recovered all species as monophyletic, except for *Eurycheilichthys luisae*, while MP analysis found both *E. luisae* and *E. vacariensis* as paraphyletic. This is also inconsistent with Liedke's (2007) findings, where only *E. pantherinus*, *E. limulus* and the three very restrictedly distributed species from Taquari-Antas, *i.e. E. apocremnus, E. castaneus*, and *E. planus* were monophyletic; the remaining four species being paraphyletic.

The high mutational rate of animal mitochondrial genes, the fact that they are exclusively transmitted through the maternal lineage, and that they do not (generally) undergo recombination (Avise 2000; Brito and Edwards 2009) were considered ideal for

using them to infer recent history of populations and species and processes of speciation as the study presented by Liedke (2007). However, molecular phylogenies based on single or few genes have often lead to conflicting results and incongruent topologies and are more susceptible to stochastic and sampling errors (Jeffroy et al. 2006; Philippe et al. 2005; Philippe et al. 2011) since they reflect how genetic lineages evolve across species (Degnan and Rosenberg 2009, Knowles 2009).

When considering the non-monophyly of *Eurycheilichthys luisae*, all phylogenetic analyzes recovered the population of *E. luisae* from the Camisas River as sister to *E. coryphaenus*, a species from the nearby Tainhas River. This result is consistently supported by PCA of both rare and common variants and ADMIXTURE analyzes of full dataset and common variants. Both Camisas and Tainhas rivers are located at extreme Eastern portion of the upper Antas, and some of their headwaters are very close to each other. Additionally, even though they were collected near the type locality of *E. coryphaenus*, all individuals from the Camisas River were positively identified as *E. luisae* and lack the diagnostic features provided by Reis (2017) for *E. coryphaenus* of having the parieto-supraoccipital conspicuously elevated. Therefore, this signature of interspecific gene flow may be a possible case of introgression.

Yet, incomplete lineage sorting (ILS), the sharing of ancestral polymorphisms across speciation events (Philippe et al 2011), is also a plausible explanation for this pattern. Since it results in the failure of two or more lineages to coalesce in a species-tree (Jeffoy et al. 2006; Degnan and Rosenberg 2009) and especially because recent processes of speciation derived from large reproductive populations, which is probably the case in the Eastern clade of *Eurycheilichthys*, increase the likelihood of ILS (Liedke 2007; Philippe et al. 2011).

One sample of *Eurycheilichthys luisae* from Guaporé River basin was recovered as sister to *E. vacariensis* (upper Turvo) plus remaining samples of *E. luisae* in all

phylogenetic analyses and having patterns of admixture between *E. luisae*, *E. planus* and *E. vacariensis* (based on common variants). This monophyletic group formed by *Eurycheilichthys luisae* and *E. vacariensis* is known from several tributaries of the Taquari-Antas basin, but populations of *E. vacariensis* are restrictedly distributed through more northern headwaters, while *E. luisae* is more widely distributed. The pattern of gene flow and the paraphyly of this individual from Guaporé River basin may be indicative of a hybrid population, especially considering that these species are sympatric in this area. However, it has been advocated that gene flow between diverging or diverged species can also facilitate the formation of genomic islands, increasing genomic incompatibility between species, helping to finalize speciation (Wu 2001; Seehausen 2013; Huang 2016; Payseur and Rieseberg 2016). Hence, a possible case of introgression can be considered, but it should be further investigated.

All analyses recovered *Eurycheilichthys luisae* as paraphyletic and *E. vacariensis* as monophyletic, but MP suggests both being paraphyletic. Morphologically, the two species can be easily diagnosed from each other based on body and head color patterns and length of lower lip, which is longer in *E. luisae* extending past the anterior margin of the pectoral girdle. Considering the results herein obtained, those morphological differences found between *E. luisae* and *E. vacariensis* may as well suggest ecological differences regarding their different latitudinal distributions along the basin. Another possibility is a between-species morphological differentiation smaller than the within-species variation (Wiens and Reeder 1997; Wiens and Penkrot 2002). On the other hand, there might be more morphological character not yet explored to diagnose *E. luisae* and *E. vacariensis*. Considering all those possibilities, further species delimitation study including morphology should be performed. Species delimitation studies can help identify the moment when population-level processes begin to produce phylogenetic patterns in order to understand evolutionary processes (Carstens et al. 2013). It was,

therefore, preferable to take a more conservative course of action and maintain both species pending on further studies that target the patterns found among Eastern clade using micro evolutionary approaches and integrative species delimitation.

Conclusions

This study presents the first phylogenomic study of *Eurycheilichthys*, an endemic genus of southern Neotropical catfishes, including all and densely-sampled species. Analyses support the monophyly of the genus and suggest two species-inclusive clades with absolute support and very recently diverged species. One clade containing *Eurycheilichthys limulus* (from upper Jacuí River basin) as sister to Western species of the Taquari-Antas basin plus *E. paucidens*, and other with *E. pantherinus* (from Uruguay River basin) as sister to the Eastern species of the Taquari-Antas basin plus *E. luisae*. These findings corroborate previous geographic patterns described for *Cambeva poikilos* and *Pareiorhaphis hystrix*, respectively. The more diverse lineages on the Taquari-Antas when compared to Uruguay and upper Jacuí River basins suggest a more dynamic landscape with several headwater capture events. Except for *Eurycheilichthys luisae*, all remaining species of the genus were recovered as monophyletic, but the choice for a more conservative course of action was taken pending future studies that should aim to understand the patterns among Eastern clade using micro evolutionary approaches and integrative species delimitation.

References

Albert, J. S., P. Petry, and R. E. Reis. 2011. Major biogeographic and phylogenetic patterns. Pp. 21–57 *in* J. S. Albert and R. E. Reis, eds. Historical biogeography of Neotropical freshwater fishes. University of California Press, Ltd Berkeley, Los Angeles, CA.

Albert, J. S., and T. P. Carvalho. 2011. Neogene assembly of modern faunas. Pp. 119–136 *in* J. S. Albert and R. E. Reis, eds. Historical biogeography of Neotropical freshwater fishes. University of California Press, Ltd Berkeley, Los Angeles, CA.

Alexander, D. H., J. Novembre, and K. Lange. 2009. Fast model-based estimation of ancestry in unrelated individuals. Genome Res. 19:1655–1664.

Alonso, R., A. J. Crawford, and E. Bermingham. 2012. Molecular phylogeny of an endemic radiation of Cuban toads (Bufonidae: Peltophryne) based on mitochondrial and nuclear genes. 2012. J. Biogeogr. 39:434–451.

Avise, J. C., 2000. Phylogeography: the history and formation of species. Harvard University Press, Cambridge, MA.

Bouckaert, R. R. 2010. DensiTree: making sense of sets of phylogenetic trees. Bioinformatics 26:1372–1373.

Brito, P. H., and S. V. Edwards. 2009. Multilocus phylogeography and phylogenetics using sequence-based markers. Genetica 135:439–455

Bryant, D., R. Bouckaert, J. Felsenstein, N. A. Rosenberg, and A. RoyChoudhury. 2012. Inferring species trees directly from biallelic genetic markers: bypassing gene trees in a full coalescent analysis. Mol. Biol. Evol. 29:1917–1932.

Carstens, B. C., T. A. Pelletier, N. M. Reid, and J. D. Satler. 2013. How to fail at species delimitation. Mol. Ecol. 22:4369–4383.

Carvajal-Quintero, J., F. Villalobos, T. Oberdorff, G. Grenouillet, S. Brosse, B. Hugueny, C. Jézéquel, and P. A. Tedesco. 2019. Drainage network position and historical connectivity explain global patterns in freshwater fishes' range size. Proc. Natl. Acad. Sci. 116:13434–13439.

Carvalho, T. P., and R. R. Reis. 2011. Taxonomic review of *Hisonotus* Eigenmann & Eigenmann (Siluriformes: Loricariidae: Hypoptopomatinae) from the laguna dos Patos system, southern Brazil. Neotrop. Ichthyol. 9:1–48

Carvalho, T. P., M. Arce H., R. E. Reis, and M. H. Sabaj. 2018. Molecular phylogeny of Banjo catfishes (Ostaryophisi: Siluriformes: Aspredinidae): A continental radiation in South American freshwaters. Mol. Phylogenet. Evol. 127:459–467.

Catchen, J. M., A. Amores, P. Hohenlohe, W. Cresko, and J. H. Postlethwait. 2011. Stacks: building and genotyping loci de novo from short-read sequences. G3 1:171–182.

Catchen, J., P. A. Hohenlohe, S. Bassham, A. Amores, and W. A. Cresko. 2013. Stacks: an analysis tool set for population genomics. Mol. Ecol. 22:3124–3140.

Chifman, J., and L. Kubatko. 2014. Quartet inference from SNP data under the coalescent model. Bioinformatics 30:3317–3324.

Degnan, J. H., and N. A. Rosenberg. 2009. Gene tree discordance, phylogenetic inference and the multispecies coalescent. Trends Ecol. Evol. 24:332–340.

Eaton, D. A. R. 2014. PyRAD: assembly of de novo RADseq loci for phylogenetic analyses. Bioinformatics 30:1844–1849.

Eaton, D. A. R., and R. H. Ree. 2013. Inferring phylogeny and introgression using RADseq data: an example from flowering plants (*Pedicularis*: Orobanchaceae). Syst. Biol. 62:689–706.

Feng, G., Z. Ma, B. Sandel, L. Mao, S. Normand, A. Ordonez, and J. C. Svenning. 2019. Species and phylogenetic endemism in angiosperm trees across the Northern Hemisphere are jointly shaped by modern climate and glacial–interglacial climate change. Global Ecol. Biogeogr. 28:1393–1402.

Ferrer, J., and L. R. Malabarba. 2013. Taxonomic review of the genus *Trichomycterus* Valenciennes (Siluriformes: Trichomycteridae) from the laguna dos Patos system, Southern Brazil. Neotrop. Ichthyol. 11:217–246.

Fiduccia, C. M., and R. M. Mattheyses. 1982. A linear time heuristic for network partitions. Pp. 175–181 *in* Proc. 19th IEEE Design Automation Conf.

Ford, A. G. P., K. K. Dasmahapatra, L. Ruber, K. Gharbi, T. Cezard, and J. J. Day. 2015. High levels of interspecific gene flow in an endemic cichlid fish adaptive radiation from an extreme lake environment. Mol. Ecol. 24:3421–3440.

Gante, H. F., M. Matschiner, M. Malmstrø, K. S. Jakobsen, S. Jentoft, and W. Salzburger. Genomics of speciation and introgression in Princess cichlid fishes from Lake Tanganyika. Mol. Ecol. 25:6143–6161.

Goloboff, P. A., J. S. Farris, and K. C. Nixon. 2008. TNT: a free program for phylogenetic analysis. Cladistics 24:774–786.

Huang, H., and L. L. Knowles. 2016. Unforeseen consequences of excluding missing data from next-generation sequences: simulation study of RAD sequences. Syst. Biol. 65:357–365.

Huang, J. P. 2016. Parapatric genetic introgression and phenotypic assimilation: testing conditions for introgression between Hercules beetles (*Dynastes*, Dynastinae). Mol. Ecol. 25:5513–5526.

Jeffroy, O., H. Brinkmann, F. Delsuc, and H. Philippe. Phylogenomics: the beginning of incongruence? Trends Genet. 22:225–231.

Jombart, T., and I. Ahmed. 2011. adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. Bioinformatics 27:3070–3071.

Jombart, T., S. Devillard, and F. Balloux. 2008. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet. 11:94.

Kautt, A. F., G. Machado-Schiaffino, and A. Meyer. 2016. Multispecies outcomes of sympatric speciation after admixture with the source population in two radiations of Nicaraguan crater lake cichlids. PLoS Genet. 12:e1006157

Knowles, L. L. 2009. Estimating species trees: methods of phylogenetic analysis when there is incongruence across genes. Syst. Biol. 58:463–467.

Leaché, A. D., A. S. Chavez, L. N. Jones, J. A. Grummer, A. D. Gottscho, and C. W. Linkem. 2015a. Phylogenomics of Phrynosomatid Lizards: conflicting signals from sequence capture versus restriction site associated DNA sequencing. Genome Biol. Evol. 7:706–719.

Leaché, A. D., B. L. Banbury, J. Felsenstein, A. N. M. Oca, and A. Stamatakis. 2015b Short tree, long tree, right tree, wrong tree: new acquisition bias corrections for inferring SNP phylogenies. Syst. Biol. 64:1032–1047.

Liedke, A. M. R. 2007. Filogenia e Filogeografia do gênero *Eurycheilichthys* (Siluriformes: Loricariidae) (Masters thesis). Available from PUCRS Library http://tede2.pucrs.br/tede2/handle/tede/8.

Lundberg, J. G. 1993. African–South American freshwater fish clades and continental drift: problems with a paradigm. Pp. 156–199 *in* P. Goldblatt, ed. Biological Relationships between Africa and South America, Yale University Press, New Haven, CT.

Lundberg, J. G., O. J. Linares, M. E. Antonio, and P. Nass. 1988. *Phractocephalus hemiliopterus* (Pimelodidae, Siluriformes) from the Upper Miocene Urumaco Formation,

Venezuela: a further case of evolutionary stasis and local extinction among South American fishes. J. Vertebr. Paleontol. 8:131–138.

Mayden, R. L. 1988. Vicariance biogeography, parsimony, and evolution in North American freshwater fishes. Syst. Zool. 37:329–355.

Myers, N., R. A. Mittermeier, C. G. Mittermeier, G. A. B. Fonseca, and J. Kent. 2000. Biodiversity hotspots for conservation priorities. Nature 403:853–858.

Papadopoulou, A., and L. L. Knowles. 2017. Linking micro- and macroevolutionary perspectives to evaluate the role of Quaternary sea-level oscillations in island diversification. Evolution 71: 2901–2917.

Payseur B. A., and L. H. Rieseberg. 2016. A genomic perspective on hybridization and speciation. Mol. Ecol. 25:2337–2360.

Peterson, B. K., J. N. Weber, E. H. Kay, H. S. Fisher, and H. E. Hoekstra. Double Digest RADseq: an inexpensive method for *de novo* SNP discovery and genotyping in model and non-model species. PLoS ONE 7:e37135.

Philippe, H., F. Delsuc, H. Brinkmann, and N. Lartillot. 2005. Phylogenomics. Annu. Rev. Ecol. Evol. Syst. 36:541–62.

Philippe, H., H. Brinkmann, D. V. Lavrov, D. T. J. Littlewood, M. Manuel, G. Wörheide, and D. Baurain. 2011. Resolving difficult phylogenetic questions: why more sequences are not enough. PLoS Biol. 9:e1000602.

Purcell, S., B. Neale, K. Todd-Brown, L. Thomas, M. Ferreira, D. Bender, J. Maller, P. Sklar, P. de Bakker, M. Daly, et al. 2007. PLINK: a toolset for whole-genome association and population-based linkage analysis. Am. J. Hum. Genet. 81:559–575.

Rambaut, A., M. A. Suchard, D. Xie, and A. J. Drummond. 2014. Tracer version 1.6, Available at http://beast.bio.ed.ac.uk/Tracer. Accessed December, 2019.

Reaz, R., M. S. Bayzid, and M. S. Rahman. 2014. Accurate phylogenetic tree reconstruction from quartets: a heuristic approach. PLoS ONE 9:e104008

Reis, R. E. 2017. Unexpectedly high diversity in a small basin: A taxonomic revision of *Eurycheilichthys* (Siluriformes: Loricariidae), with descriptions of seven new species. Neotrop. Ichthyol. 15:e160068

Reis, R. E., J. S. Albert, F. Di Dario, M. M. Mincarone, P. Petry, and L. A. Rocha. 2016. Fish biodiversity and conservation in South America. J. Fish Biol. 89:12–47.

Reis, R. E., and S. A. Schaefer. 1998. New Cascudinhos from southern Brazil: systematics, endemism, and relationships (Siluriformes, Loricariidae, Hypoptopomatinae). Am. Mus. Novit. 3254:1–25.

Ribeiro, A. C. 2006. Tectonic history and the biogeography of the freshwater fishes from the coastal drainages of eastern Brazil: an example of faunal evolution associated with a divergent continental margin. Neotrop. Ichthyol. 4:225-246.

Rosen, D. E. 1979. Fishes from the uplands and intermontane basins of Guatemala: Revisionary studies and comparative geography. Bull. Am. Mus. Nat. 162:1–176.

Rubin, B. E. R., R. H. Ree, and C. S. Moreau. 2012. Inferring Phylogenies from RAD sequence data. PLoS ONE 7:e33394.

Rull, V. 2005. Biotic diversification in the Guayana highlands: a proposal. J. Biogeogr. 32:921–927.

Rull, V. 2007. The Guayana highlands: a promised (but threatened) land for ecological and evolutionary science. Biotropica 39:31–34.

Seehausen, O. 2013. Conditions when hybridization might predispose populations for adaptive radiation. J. Evol. Biol. 26:279–281.

Smith, G. R. 1981. Late Cenozoic freshwater fishes of North America. Annu. Rev. Ecol. Evol. Syst. 12:163–193.

Stange, M., M. R. Sánchez-Villagra, W. Salzburger, and M. Matschiner. 2018. Bayesian divergence-time estimation with genome-wide single-nucleotide polymorphism data of sea catfishes (Ariidae) supports Miocene closure of the Panamanian Isthmus. Syst. Biol. 67:681–699.

Swofford, D. L. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Ver. 4.0a166. Sinauer, Sunderland, MA.

Thomaz, A. T., L. R Malabarba, and L. L. Knowles. 2017. Genomic signatures of paleodrainages in a freshwater fish along the southeastern coast of Brazil: genetic structure reflects past riverine properties. Heredity 119:287–294.

Treangen, T. J., B. D Ondov, S. Koren, and A. M. Phillippy. 2014. The Harvest suite for rapid core-genome alignment and visualization of thousands of intraspecific microbial genomes. Genome Biol. 15:524.

van der Laan, R., and R. Fricke. 2019. Family-Group names. (http://www.calacademy.org/scientists/catalog-of-fishes-family-group-names/). Electronic version accessed December, 2019.

Vari, R. P., and C. J. Ferraris. 2009. Fishes of the Guiana Shield. Proc. Biol. Soc. Wash. 17:9–18.

Wagner, C. E., I. Keller, S. Wittwer, O. M. Selz, S. Mwaiko, L. Greuter, A. Sivasundar, and O. Seehausen. 2013. Genome-wide RAD sequence data provide unprecedented

resolution of species boundaries and relationships in the Lake Victoria cichlid adaptive radiation. Mol. Ecol. 22:787–798.

Welch, A. J., K. Collins, A. Ratan, D. I. Drautz-Moses, S. C. Schuster, and C. Lindqvist. 2016. The quest to resolve recent radiations: Plastid phylogenomics of extinct and endangered Hawaiian endemic mints (Lamiaceae). Mol. Phylogenet. Evol. 99:16–33.

Wielstra, B., E. McCartney-Melstada, J. W. Arntzen, R. K. Butlin, and H. B. Shaffer. 2019. Phylogenomics of the adaptive radiation of *Triturus* newts supports gradual ecological niche expansion towards an incrementally aquatic lifestyle. Mol. Phylogenet. Evol. 133:120–127.

Wiens, J. J., and T. A. Penkrot. 2002. Delimiting species using DNA and morphological variation and discordant species limits in spiny lizards (Sceloporus). Syst. Biol. 51:69–91.

Wiens, J. J., and T. W. Reeder. 1997. Phylogeny of the spiny lizards (Sceloporus) based on molecular and morphological evidence. Herpetol. Monogr. 11:1–101.

Wu, C.I. 2001. The genic view of the process of speciation. J. Evol. Biol. 14:851–865.

FIGURES

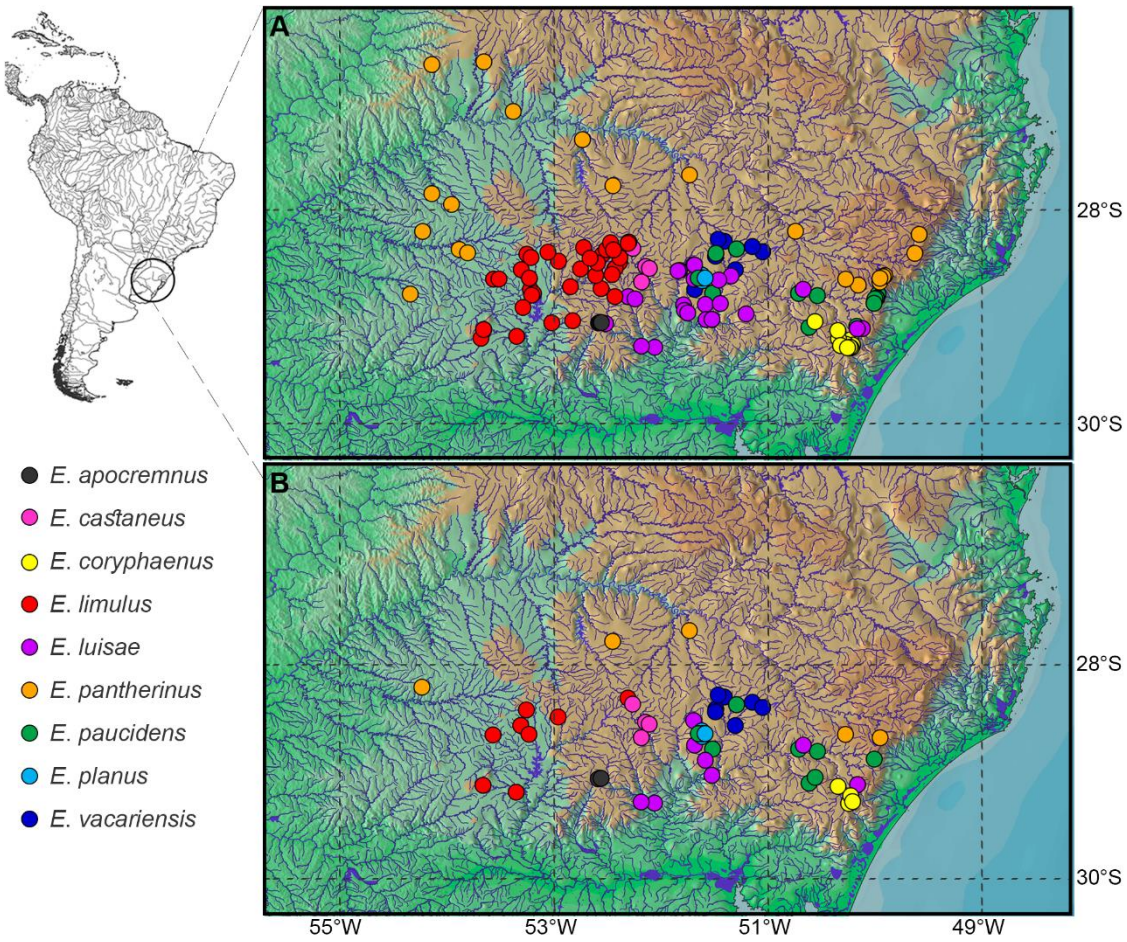

Figure 1. Distribution of the nine species of *Eurycheilichthys* across Southern Neotropical Region based on A) material listed by Reis (2017) and, B) sampling localities of ddRADseq libraries.

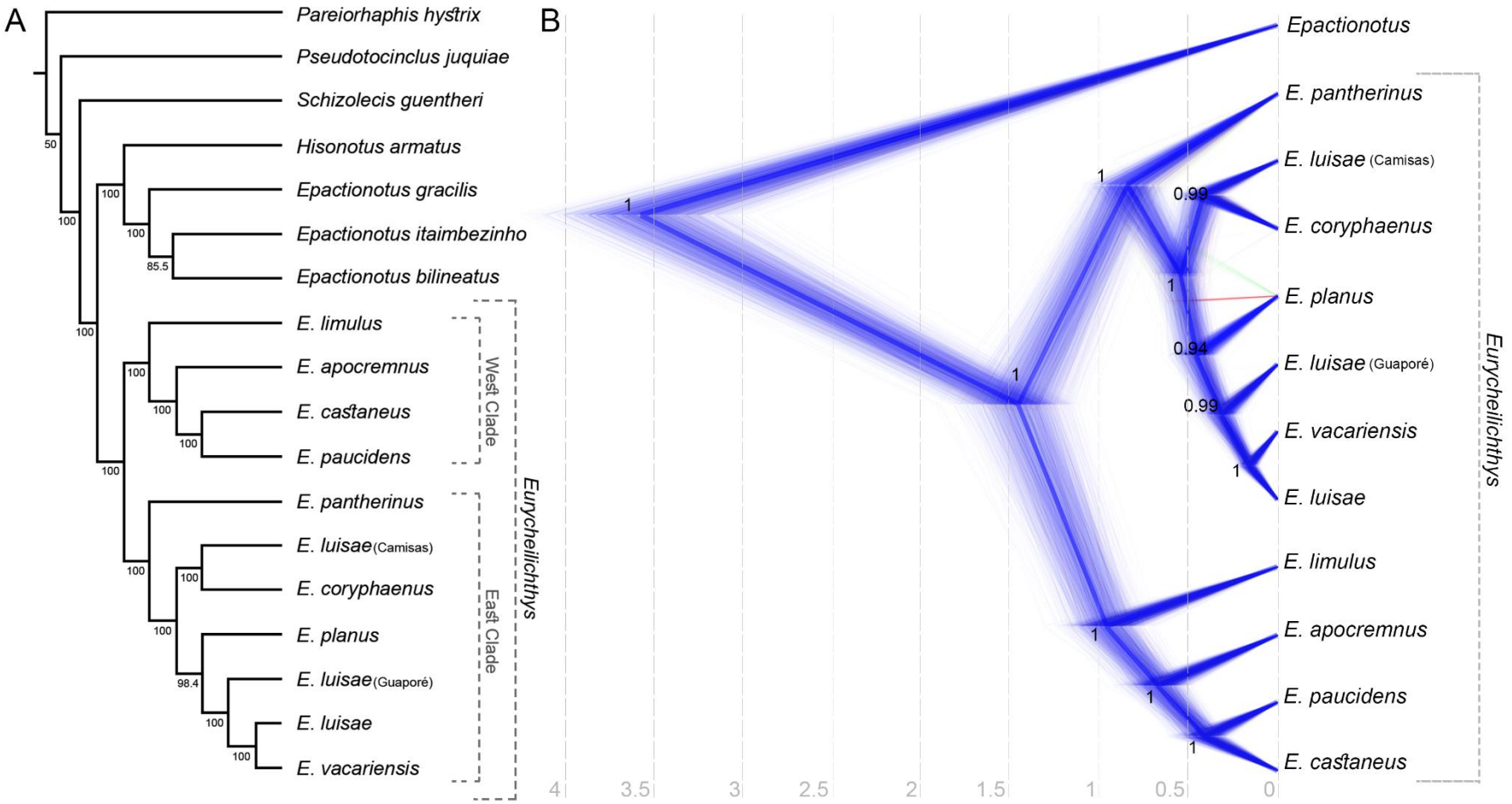

Figure 2. Species-tree topologies of all nine *Eurycheilichthys* species. A) SVDQUARTETS analysis of 29,350 unlinked SNPs. Values on the branches indicate clade support, based on 1,000 bootstrap pseudoreplicates B) SNAPP analysis based on 1,355 unlinked SNPs, after removing outgroups and individuals with higher proportion of missing data. Trees were visualized using DENSITREE. Values on the branches indicate posterior probability. Grey numbers and dashed lines indicate time lines (Ma).

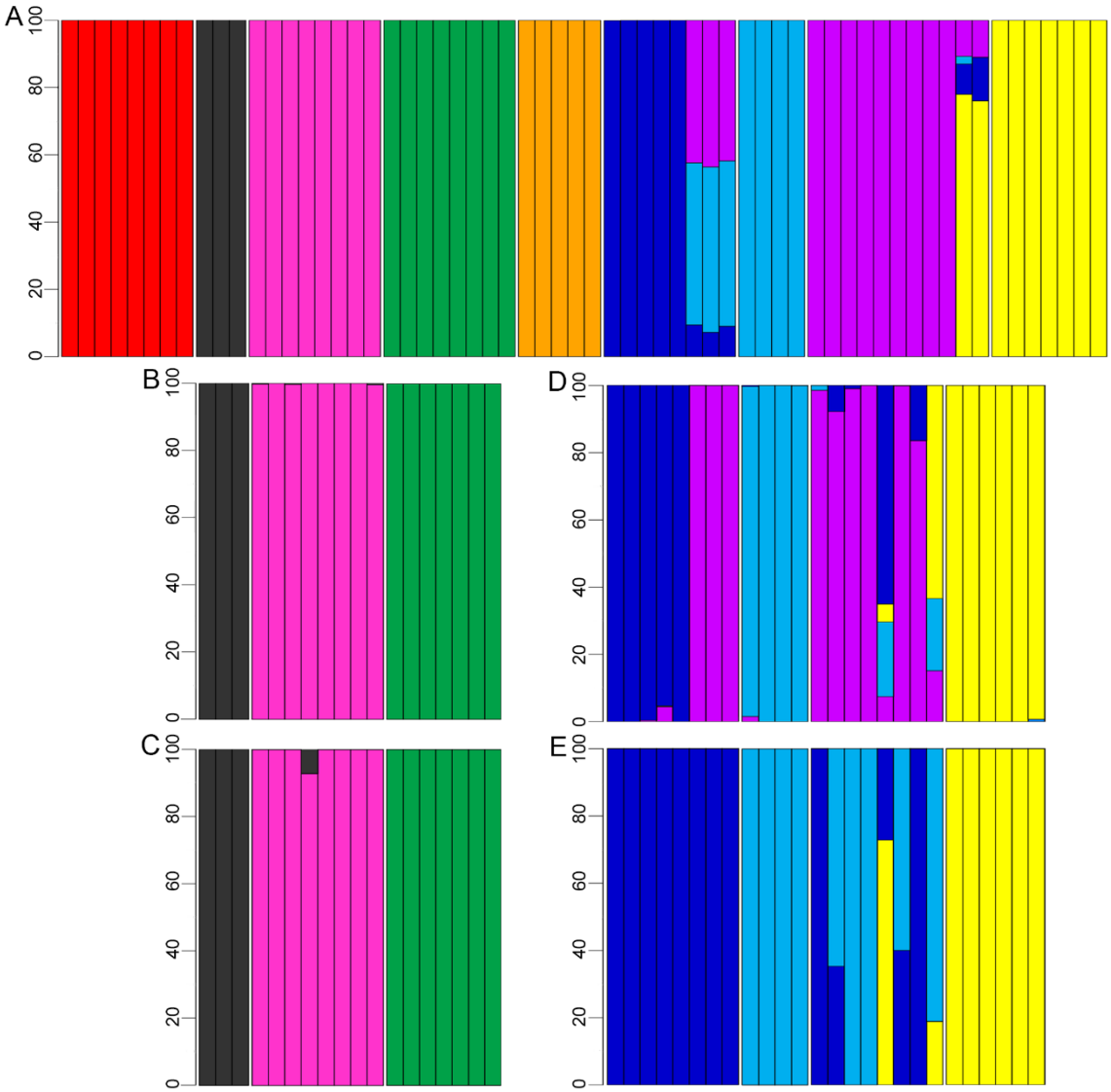

Figure 3. Results from best K by ADMIXTURE analyses for A) full dataset, B) common variants of Western dataset, C) rare variants of Western dataset, D) common variants of Eastern dataset, E) rare variants of Eastern dataset. Vertical black lines indicate individuals; color pattern corresponds to same species code from Fig. 1.

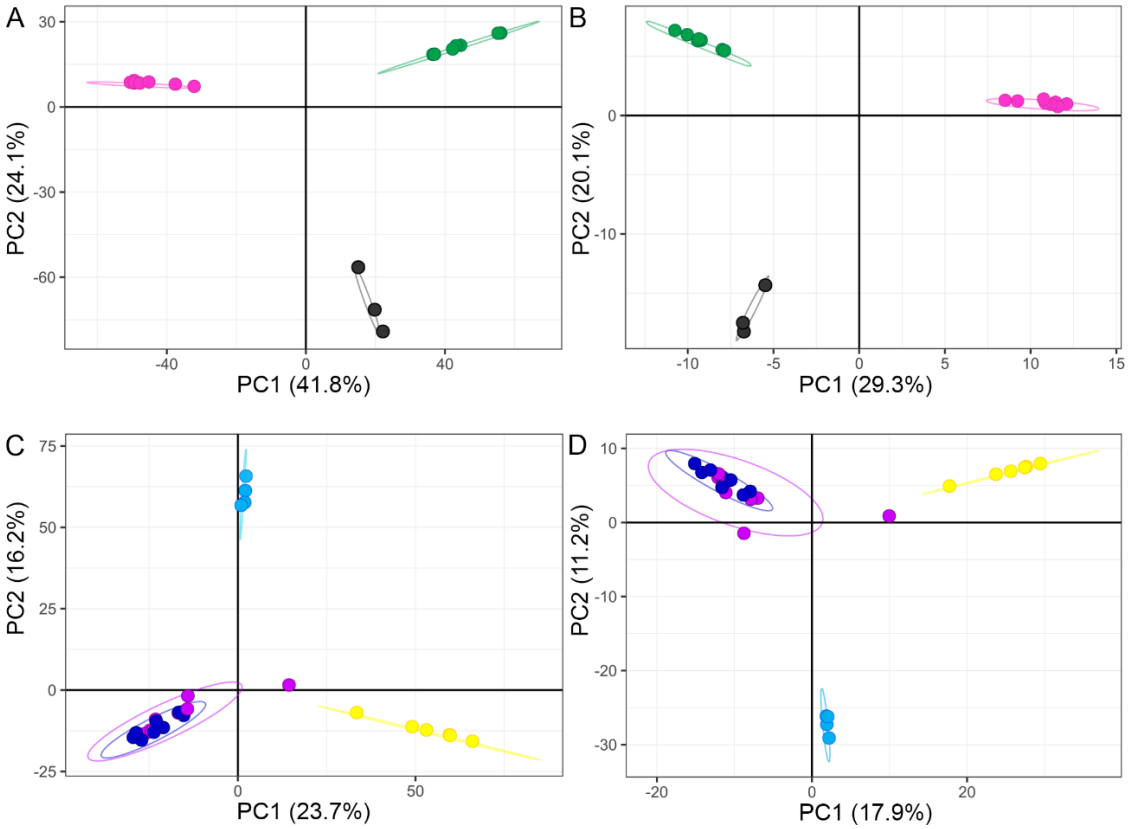

Figure 4. Results of population clustering and structure based on the distribution of individuals along principal component 1 and 2 (PC1 and PC2) for A) common and B) rare variants of Western dataset, and C) common and D) rare variants of Eastern dataset; color pattern corresponds to same species code from Fig. 1.

SUPPLEMENTARY MATERIAL FOR MANUSCRIPT

TABLES

Table S1. Sampling and genomic information of individuals used in ddradseq libraby, containig pre- and post-processing numbers of reads from STACKS and IPYRAD.

Species	Catalog #	Voucher	Code	Location (county, state)	Drainage	Latidude	Longitude	Raw read counts STACKS	Utilized Reads STACKS	Raw read counts IPYRAD	Utilized Reads IPYRAD
E. apocremnus	MCP 50110	ML94	ML94	Barros Cassal, RS	Taquari-Antas	29° 3'11.00"S	52°34'54.00"W	2148959	1880705	1914179	1913187
E. apocremnus	MCP 50214	ML97	ML97	Barros Cassal, RS	Taquari-Antas	29° 2'51.17"S	52°34'5.40"W	2199301	1940248	2713583	2712270
E. apocremnus	MCP 50114	ML99	ML99	Barros Cassal, RS	Taquari-Antas	29° 2'53.12"S	52°33'20.61"W	2221206	1963770	2357922	2356827
E. castaneus	MCP 50199	ML110	ML110	Nova Alvorada, RS	Taquari-Antas	28°40'15.00"S	52°10'55.00"W	3181046	2805476	3147430	3145850
E. castaneus	MCP 50199	ML112	ML112	Nova Alvorada, RS	Taquari-Antas	28°40'15.00"S	52°10'55.00"W	3234044	2853356	5703035	5700484
E. castaneus	MCP 50184	ML129	ML129	Vila Maria, RS	Taquari-Antas	28°31'36.00"S	52° 8'37.00"W	3486570	3085670	4659498	4656483
E. castaneus	MCP 50184	ML131	ML131	Vila Maria, RS	Taquari-Antas	28°31'36.00"S	52° 8'37.00"W	3604969	3151043	5131610	5129082
E. castaneus	MCP 50217	ML138	ML138	Vila Maria, RS	Taquari-Antas	28°32'42.00"S	52° 6'32.15"W	3584046	3171194	4411974	4409998
E. castaneus	MCP 50217	ML139	ML139	Vila Maria, RS	Taquari-Antas	28°21'32.00"S	52°15'48.00"W	3545754	3172543	3922225	3916865
E. castaneus	MCP 50175	ML146	ML146	Marau, RS	Taquari-Antas	28°21'32.00"S	52°15'48.00"W	3537478	3172994	1180539	1179928
E. castaneus	MCP 50175	ML147	ML147	Marau, RS	Taquari-Antas	28°21'32.00"S	52°15'48.00"W	3709739	3308688	2130373	2129319
E. castaneus	MCP 50175	ML148	ML148	Marau, RS	Taquari-Antas	28°21'32.00"S	52°15'48.00"W	3722421	3309274	3971420	3969588
E. coryphaenus	MCP 48715	A	ML76	Tainhas, RS	Taquari-Antas	29°16'53.36"S	50°15'28.06"W	5028340	4137843	3633085	3631091
E. coryphaenus	MCP 48715	B	ML77	Tainhas, RS	Taquari-Antas	29°16'53.36"S	50°15'28.06"W	4983474	4408680	6204815	6201761
E. coryphaenus	MCP 48715	C	ML78	Tainhas, RS	Taquari-Antas	29°16'53.36"S	50°15'28.06"W	5119050	4532321	2085355	2084305
E. coryphaenus	MCP 50091	ML224	ML224	Tainhas, RS	Taquari-Antas	29°12'36.43"S	50°14'18.80"W	5167765	4643600	3474882	3473484
E. coryphaenus	MCP 50091	ML225	ML225	Tainhas, RS	Taquari-Antas	29°12'36.43"S	50°14'18.80"W	5336594	4657612	2080416	2079491
E. coryphaenus	MCP 50162	ML228	ML228	Tainhas, RS	Taquari-Antas	29°15'52.96"S	50°13'16.09"W	5238162	4712228	3493890	3492398
E. coryphaenus	UNICTIO 125	UNICTIO 125	ML258	Tainhas, RS	Taquari-Antas	29° 7'23.72"S	50°21'22.82"W	5493922	4941932	4067499	4065841
E. limulus	UFRGS 15012	TEC 1793	ML45	Júlio de Castilhos, RS	Jacuí	29° 6'50.00"S	53°39'4.00"W	1229307	1057748	4109917	4107699
E. limulus	UFRGS 15016	TEC 1797 A	ML46	Cruz Alta, RS	Jacuí	28°38'42.00"S	53°33'32.00"W	1323361	1139232	3077579	3072996
E. limulus	MCP 49456	Caixa XXXV-E3	ML79	Pinhal Grande, RS	Jacuí	29°10'37.00"S	53°20'36.00"W	1333371	1142668	1403493	1402604
E. limulus	MCP 50168	ML161	ML161	Passo Fundo, RS	Jacuí	28°18'9.00"S	52°18'23.00"W	1509624	1243510	2691340	2689971
E. limulus	MCP 50168	ML163	ML163	Passo Fundo, RS	Jacuí	28°18'9.00"S	52°18'23.00"W	1603321	1351215	3638008	3636120
E. limulus	MCP 50095	ML165	ML165	Colorado, RS	Jacuí	28°28'41.00"S	52°57'15.00"W	1605952	1355066	5005191	5002490

Table S1. Continued.

Species	Catalog #	Voucher	Code	Location (county, state)	Drainage	Latidude	Longitude	Raw read counts STACKS	Utilized Reads STACKS	Raw read counts IPYRAD	Utilized Reads IPYRAD
E. limulus	MCP 50135	ML175	ML175	Ibirubá, RS	Jacuí	28°38'22.00"S	53°13'28.00"W	1579755	1380295	5073716	5071339
E. limulus	MCP 50088	ML177	ML177	Ibirubá, RS	Jacuí	28°33'26.00"S	53°18'4.00"W	1881163	1661917	7693884	7689970
E. limulus	MCP 50130	ML180	ML180	Sta. Bárbara do Sul, RS	Jacuí	28°24'46.00"S	53°14'55.00"W	1921089	1680915	4371410	4369318
E. luisae	MCP 49457	ML01	ML01	Veranopolis, RS	Taquari-Antas	29° 1'11.10"S	51°31'38.80"W	2295574	2036555	4572545	4570178
E. luisae	MCP 25566	17 (XVIII-60)	ML65	Veranopolis, RS	Taquari-Antas	29° 1'11.10"S	51°31'38.80"W	2398268	2122181	2463140	2461489
E. luisae	MCP 49458	ML06	ML06	Flores, RS	Taquari-Antas	28°52'48.80"S	51°35'19.20"W	2441687	2122587	5172542	5170001
E. luisae	MCP 49460	ML15	ML15	Guabiju/São Jorge, RS	Taquari-Antas	28°30'41.70"S	51°41'47.90"W	2486820	2188482	2554213	2552878
E. luisae	UFRGS 17196	TEC 3201	ML49	Cambará do Sul, RS	Taquari-Antas	29° 6'22.00"S	50°10'30.00"W	2531397	2215533	1539226	1526353
E. luisae	UNICTIO 133	UNICTIO 133	ML260	Cambará do Sul, RS	Taquari-Antas	29° 6'23.00"S	50°10'31.90"W	2594166	2239872	2333690	2332577
E. luisae	MCP 50350	ML255	ML255	Nova Bassano, RS	Taquari-Antas	28°44'24.70"S	51°41'16.70"W	4705984	4119378	3708651	3707019
E. luisae	MCP 50177	ML85	ML85	Travesseiro, RS	Taquari-Antas	29°16'42.00"S	52° 3'33.00"W	2619932	2292844	1969002	1967955
E. luisae	MCP 50220	ML90	ML90	Marques de Souza, RS	Taquari-Antas	29°16'5.00"S	52°10'60.00"W	2720036	2368379	1533496	1532721
E. luisae	MCP 50198	ML119	ML119	Nova Alvorada, RS	Taquari-Antas	28°40'15.00"S	52°10'55.00"W	2804644	2452862	4118147	4116259
E. luisae	MCP 48712	B	ML75	Bom Jesus, RS	Taquari-Antas	28°44'24.00"S	50°40'43.00"W	2768524	2457661	4302413	4299946
E. pantherinus	MCP 41475	TEC A	ML63	Paim Filho, RS	Uruguay	27°40'34.00"S	51°44'10.00"W	1988646	1714714	3911840	3909393
E. pantherinus	MCP 46775	Caixa XXVI-D3	ML69	Erebango, RS	Uruguay	27°46'34.00"S	52°26'54.00"W	2016097	1733854	4855895	4853134
E. pantherinus	MCP 50166	ML219	ML219	Bom Jesus, RS	Uruguay	28°38'25.05"S	50°17'14.41"W	2060589	1778109	2615241	2614008
E. pantherinus	UFRGS 21811	TEC 6748	ML238	Santo Ângelo, RS	Uruguay	28°12'6.20"S	54°13'6.90"W	2095861	1864568	1825331	1824524
E. pantherinus	UFRGS 21818	TEC 6761	ML239	São José dos Ausentes, RS	Uruguay	28°40'16.80"S	49°57'56.20"W	2170533	1864568	1864153	1862187
E. paucidens	MCP 49459	ML11	ML11	Nova Prata, RS	Taquari-Antas	28°46'32.33"S	51°31'14.72"W	3813877	3350315	2532056	2530487
E. paucidens	UFRGS 16488	TEC 2811	ML47	Monte Alegre dos Campos, RS	Taquari-Antas	28°46'39.00"S	50°43'17.00"W	3780190	3391448	1529114	1527919
E. paucidens	UFRGS 16495	TEC 2822	ML48	Bom Jesus, RS	Taquari-Antas	28°47'53.00"S	50°32'49.00"W	3808594	3408489	3088171	3086283
E. paucidens	MCP 22800	JAC 029	ML58	Muitos Capões, RS	Taquari-Antas	28°21'50.00"S	51°17'53.17"W	3835872	3431885	2881946	2880387
E. paucidens	MCP 50108	ML214	ML214	Lageado Grande, RS	Taquari-Antas	29° 5'35.95"S	50°37'31.75"W	3971471	3516027	6581603	6578635
E. paucidens	MCP 50158	ML216	ML216	Lageado Grande, RS	Taquari-Antas	29° 2'22.39"S	50°34'9.14"W	4220789	3538606	1267822	1267204
E. paucidens	MCP 50157	ML222	ML222	Cambará do Sul, RS	Taquari-Antas	28°52'11.47"S	50° 1'14.50"W	4017169	3550679	2409041	2408031

Table S1. Continued.

Species	Catalog #	Voucher	Code	Location (county, state)	Drainage	Latidude	Longitude	Raw read counts STACKS	Utilized Reads STACKS	Raw read counts IPYRAD	Utilized Reads IPYRAD
E. paucidens	MCP 50355	ML243	ML243	André da Rocha, RS	Taquari-Antas	28°39'34.90"S	51°37'4.80"W	4009681	3578132	2983293	2982025
E. paucidens	MCP 50355	ML244	ML244	André da Rocha, RS	Taquari-Antas	28°39'34.90"S	51°37'4.80"W	4047311	3629265	5401145	5398894
E. planus	MCP 50346	ML248	ML248	André da Rocha, RS	Taquari-Antas	28°38'5.50"S	51°36'52.00"W	3019810	2608682	5985898	5983463
E. planus	MCP 50346	ML251	ML251	André da Rocha, RS	Taquari-Antas	28°38'5.50"S	51°36'52.00"W	2958979	2614796	3764988	3763477
E. planus	MCP 50348	ML253	ML253	André da Rocha, RS	Taquari-Antas	28°36'26.50"S	51°37'16.40"W	3455526	2656993	4506989	4505050
E. planus	MCP 50354	ML240	ML240	André da Rocha, RS	Taquari-Antas	28°39'34.90"S	51°37'4.80"W	3056353	2731480	3736699	3735209
E. vacariensis	MCP 49461	ML19	ML19	Turvo, RS	Taquari-Antas	28°25'52.60"S	51°29'39.90"W	4144447	3722890	2919257	2917661
E. vacariensis	MCP 49462	ML24	ML24	Lagoa Vermelha, RS	Taquari-Antas	28°24'19.00"S	51°29'25.80"W	4221572	3724664	3492393	3490447
E. vacariensis	MCP 49463	ML31	ML31	Lagoa Vermelha, RS	Taquari-Antas	28°16'26.90"S	51°28'7.90"W	4207054	3762807	3864047	3862003
E. vacariensis	MCP 49464	ML35	ML35	Muitos Capões, RS	Taquari-Antas	28°20'29.80"S	51° 9'7.40"W	4392937	3919944	2503761	2502351
E. vacariensis	MCP 49465	ML41	ML41	Muitos Capões, RS	Taquari-Antas	28°21'53.50"S	51°17'53.20"W	4457280	4004758	5870322	5867416
E. vacariensis	UFRGS 17884	TEC 3557C	ML54	Muitos Capões, RS	Taquari-Antas	28°23'25.00"S	51° 3'21.00"W	4507164	4051608	3727831	3725912
E. vacariensis	MCP 22790	JAC 033	ML57	Lagoa Vermelha, RS	Taquari-Antas	28°17'35.00"S	51°24'40.00"W	4600428	4105092	1933592	1932342
E. vacariensis	MCP 48384	Caixa XXIX-E6	ML72	Muitos Capões, RS	Taquari-Antas	28°33'30.00"S	51°18'35.00"W	2654770	2258719	7118701	7115045
Epactionotus bilineatus	UFRGS 17817	TEC 3498C	ML230	Maquiné, RS	Maquiné	29°34’13.6”S	50°16’49”W	5807001	5224269	3394403	3392993
Epactionotus gracilis	UFRGS 12544	TEC 1246	ML231	Nova Veneza, RS	Araranguá	28° 39'29"S	49° 32'36"W	5993628	5353918	1269755	1269107
Epactionotus itaimbezinho	UFRGS 12719	TEC 1456C	ML232	Praia Grande, RS	Mampituba	29° 11'54"S	49° 57'57"W	6088170	5500566	3471963	3470430
Pseudotocinclus juquiae	MCP 45129	Caixa XXII-51	ML234	Juquitiba, SP	Paraíba	23° 59'49"S	46° 55'59"W	6338152	5668703	2228958	2227904
Hisonotus armatus	MCP 50179	TEC01	ML235	Travesseiro, RS	Taquari-Antas	29°16'42.00"S	52° 3'33.00"W	6699971	6043020	3973219	3970218
Pareiorhaphis hystrix	MCP 50089	TEC22	ML236	Itapuca, RS	Taquari-Antas	28°45'06.0"S	52°11'13.0"W	7244872	6529181	2033928	2033064
Schizolecis guntheri	MCP 31722	Caixa XVIII-71	ML256	Guaraquecaba, PR	Sudeste	25° 10'23"S	48° 25'12"W	7810150	7089992	2165029	2163872

Table S2. Summary of the datasets processed with STACKS. The number of individuals (# inds) and unlinked SNPs (# loci) used for each analysis are indicated.

Dataset	Mean Coverage after USTACKS	# Inds	# Inds after filtering with Plink	# loci (CV/RV)	Analysis	Program
Full Dataset	15.7	65	62	43,745	Admixture	Admixture
Taquari-Antas Eastern Dataset	16.3	30	26	71,670/ 43,843	Admixture (common/rare)	Admixture
					PCA (common/rare)	adgenet
Taquari-Antas Western Dataset	15.6	21	18	44,952/ 10,461	Admixture (common/rare)	Admixture
					PCA (common/rare)	adgenet

Table S3. Summary of the16 tested datasets from the full dataset. The number of SNPs after whitelist (SNPs), different filters of missing data per individual (--mind) and SNP (--geno), remaining number of individuals (# Inds), genotyping rate and final number of SNPS (# SNPS) are indicated. Bold numbers indicate selected dataset.

SNPs After Whitelist	Filter/ individual		# Inds	Genotyping rate	Filter/SNP		# SNPs
180276	- - mind	0.6	41	0.528689	- - geno	0.1	30900
					- - geno	0.2	50979
					- - geno	0.3	64577
					- - geno	0.4	74823
	- - mind	0.7	55	0.485951	- - geno	0.1	16542
					- - geno	0.2	36617
					- - geno	0.3	52477
					- - geno	0.4	68171
	- - mind	**0.8**	**62**	**0.459784**	- - geno	0.1	12054
					- - geno	0.2	26489
					- - geno	**0.3**	**43745**
					- - geno	0.4	60339
	- - mind	0.9	65	0.446437	- - geno	0.1	9748
					- - geno	0.2	23443
					- - geno	0.3	39549
					- - geno	0.4	58748

SUPPLEMENTARY MATERIAL FOR MANUSCRIPT

FIGURES

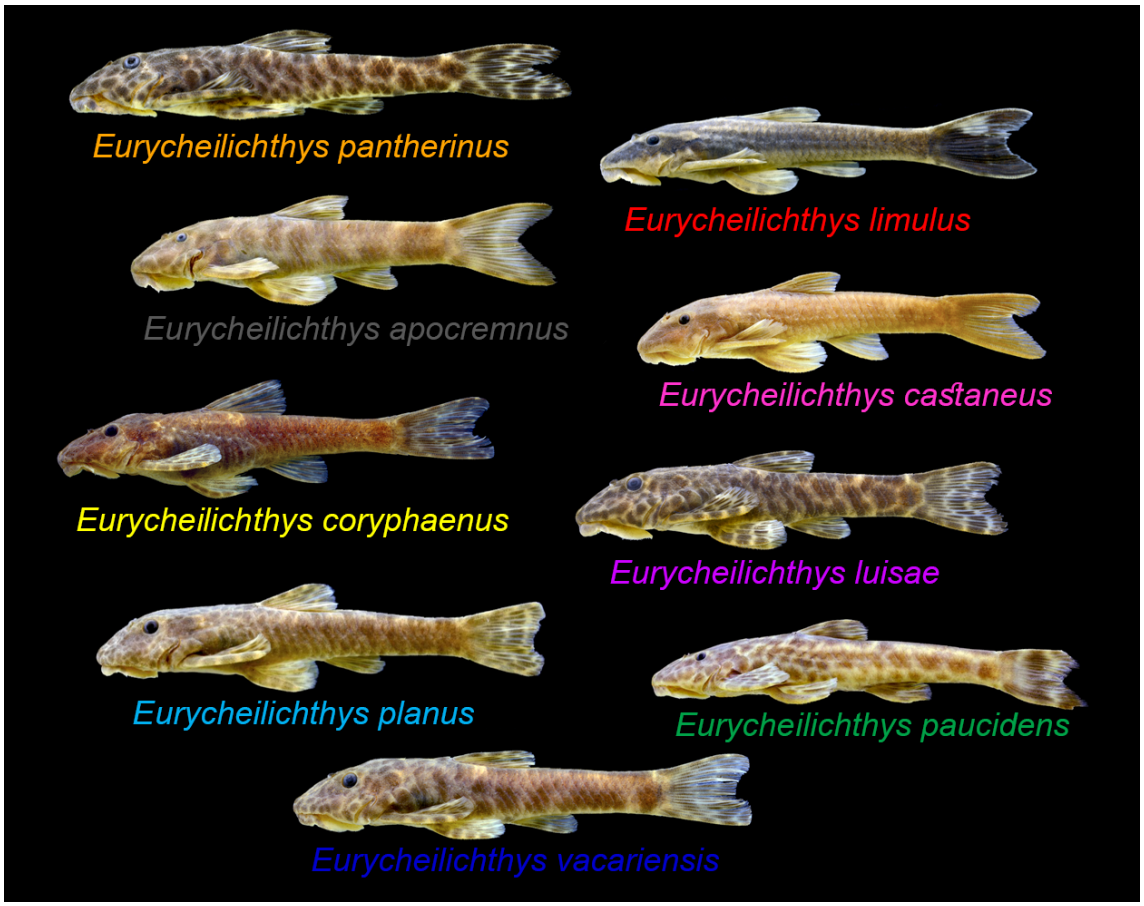

Figure S1. Diversity of *Eurycheilichthys*. Color pattern corresponds to same species code from Fig. 1.

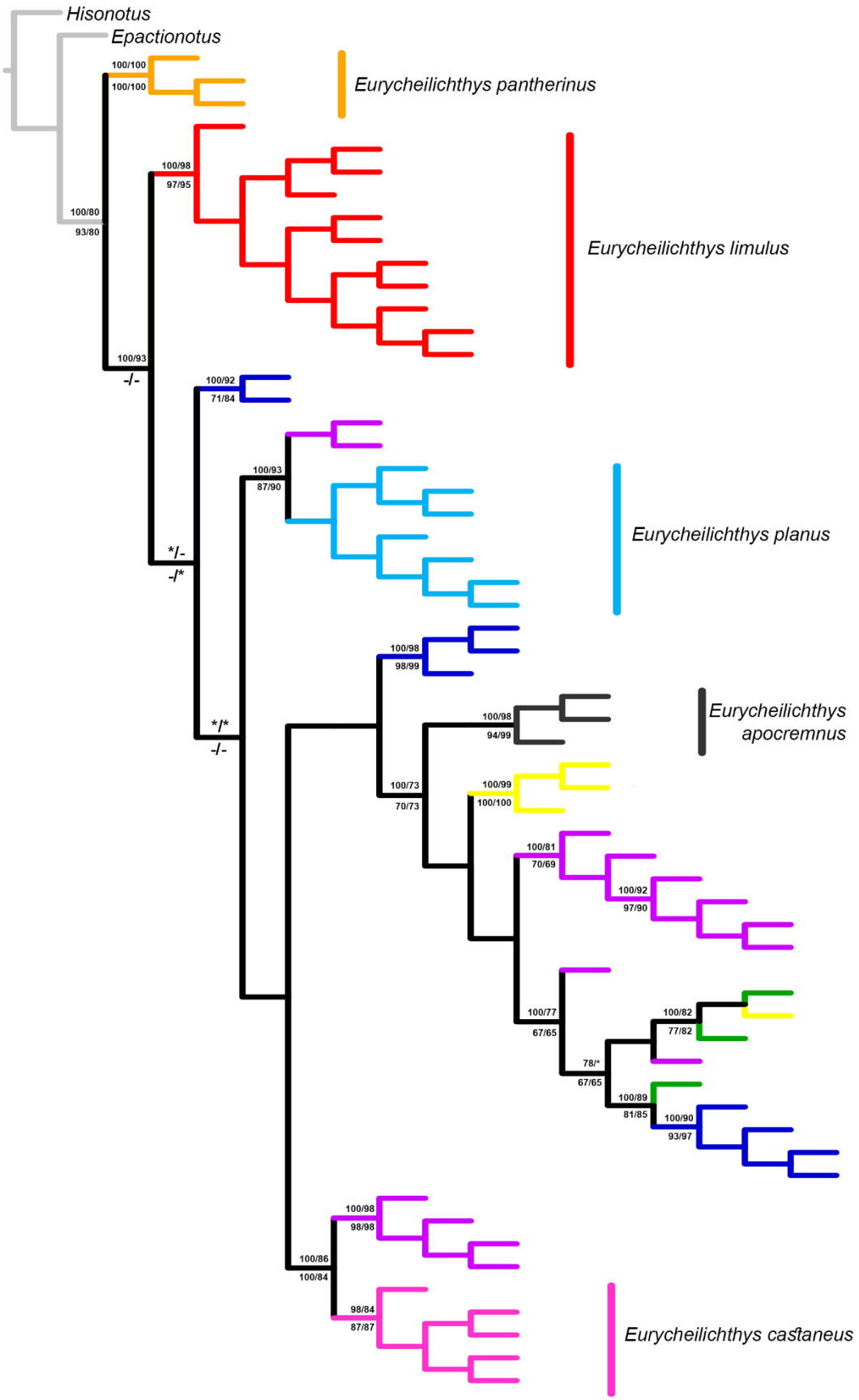

Figure S2. Bayesian phylogenetic tree presented by Liedke (2007, unpublished thesis) for the species of *Eurycheilichthys* based on mitochondrial genes COI and ND2. Support values are on the nodes. The * is for nodes with support <50 and - for nodes not found in the respective tree.

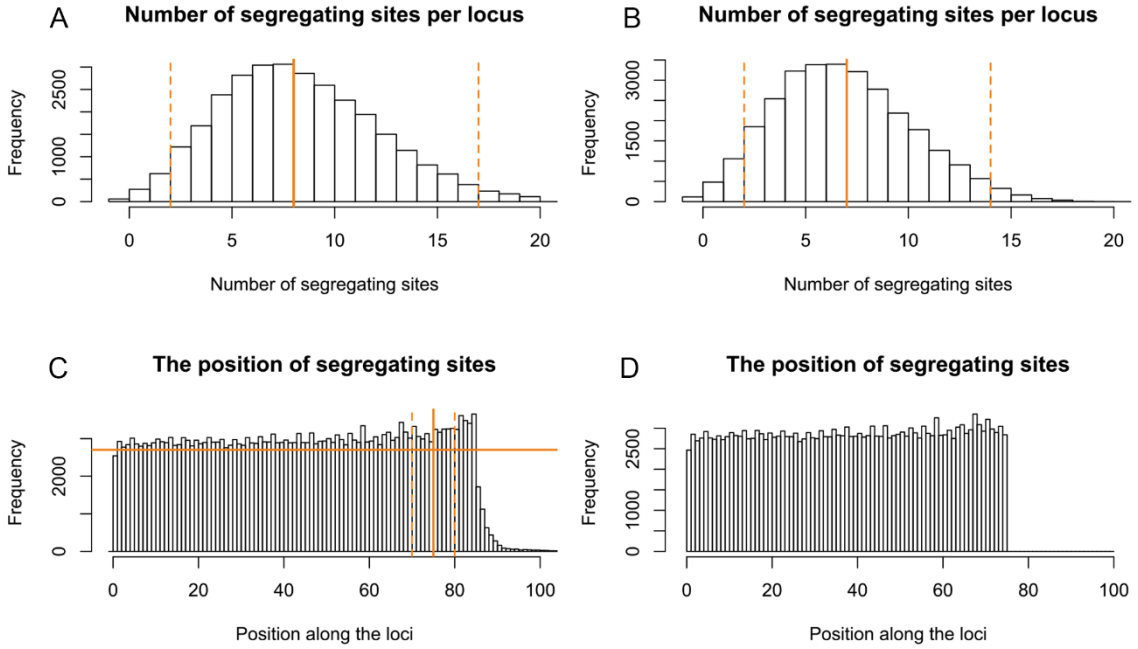

Figure S3. Summaries of filtering the dataset processed with IPYRAD (output file .loci). A) Minimum, Maximum and median (orange solid line) number of SNPs per loci before, and B) after data filtering. C) The increase in the frequency of variable site (SNP) close to the 3' end among the aligned sequences (orange solid line), and D) Sites after position 75 excluded in the final dataset in all loci.

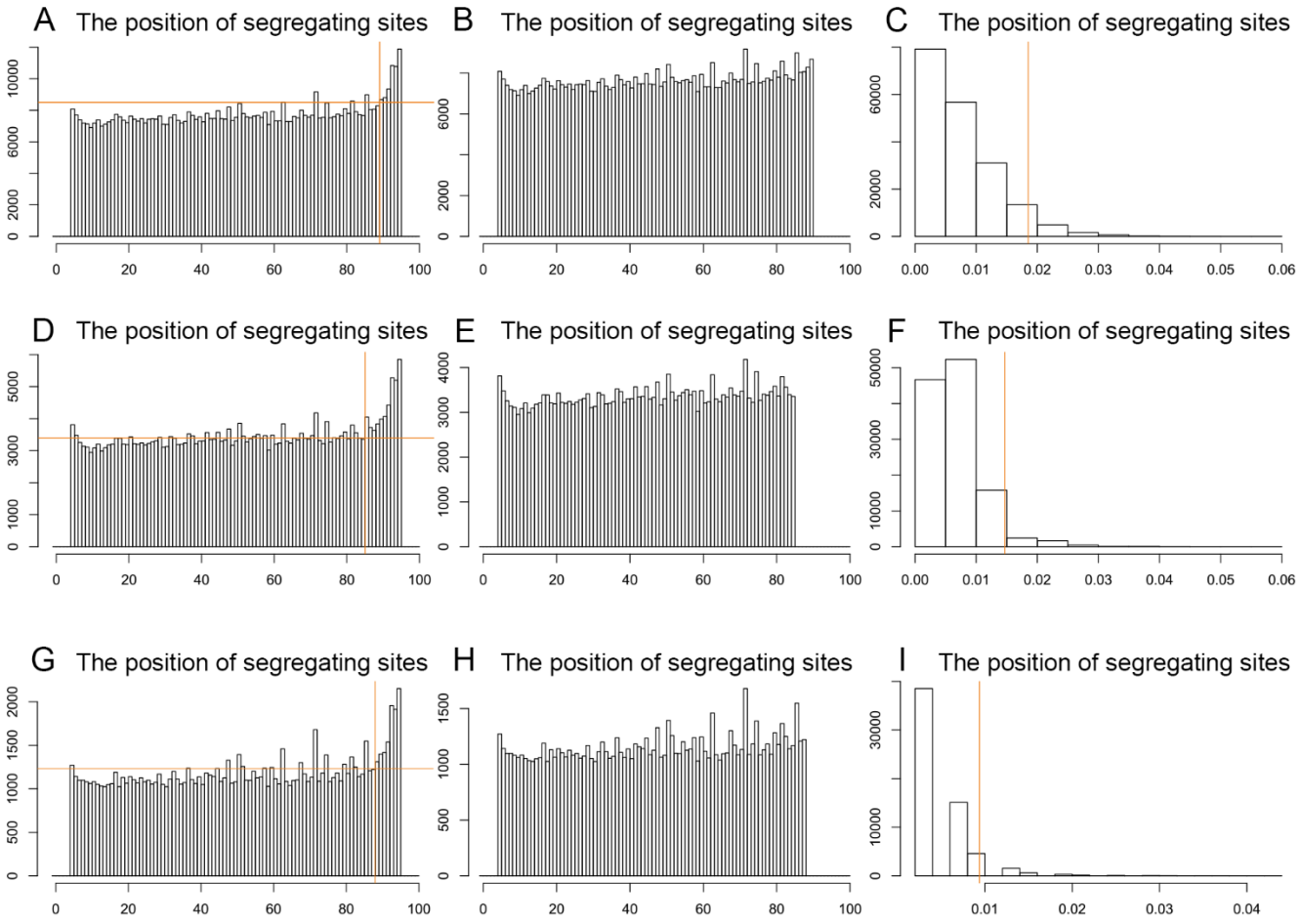

Figure S4. Summaries of filtering full (A, B and C), Eastern (D, E and F) and Western (G, H and I) datasets processed with STACKS (output file .vcf). Graphics show in A, D, G) the increase in the frequency of variable site (SNP) close to the 3' end among the aligned sequences (orange solid line), and in B, E, H) sites after position 84 excluded in the final datasets. C, F, I) The distribution of theta, θ, per loci, orange line marking the θ-values in the 95 percentile that were excluded from analyses to avoid including variation likely reflective of sequencing and/or assembly errors.

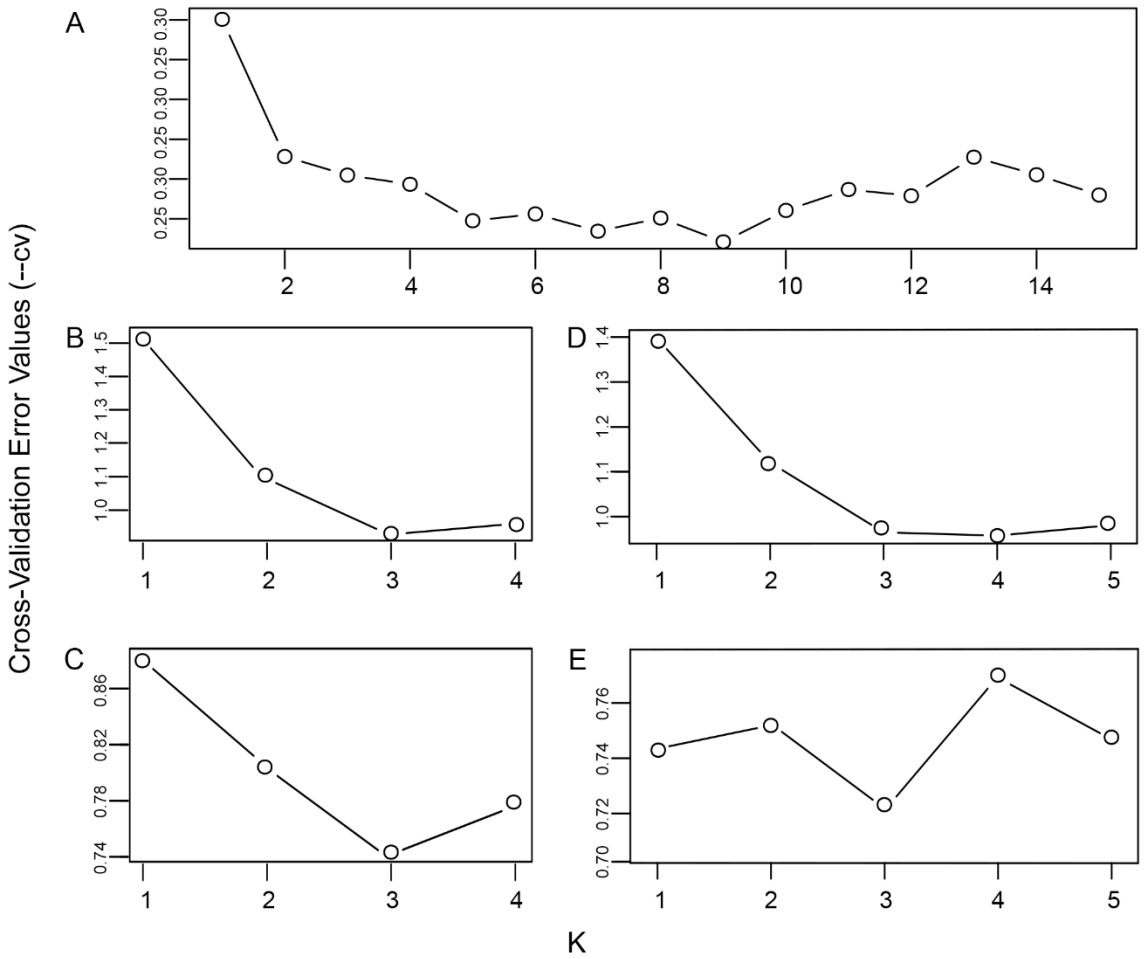

Figure S5. Cross-validation procedure (--cv) for A) Full dataset, B) common variants of Western dataset, C) rare variants of Western dataset, D) common variants of Eastern dataset, E) rare variants of Eastern dataset. The lowest cross-validation error exhibits the best value of K (clusters).

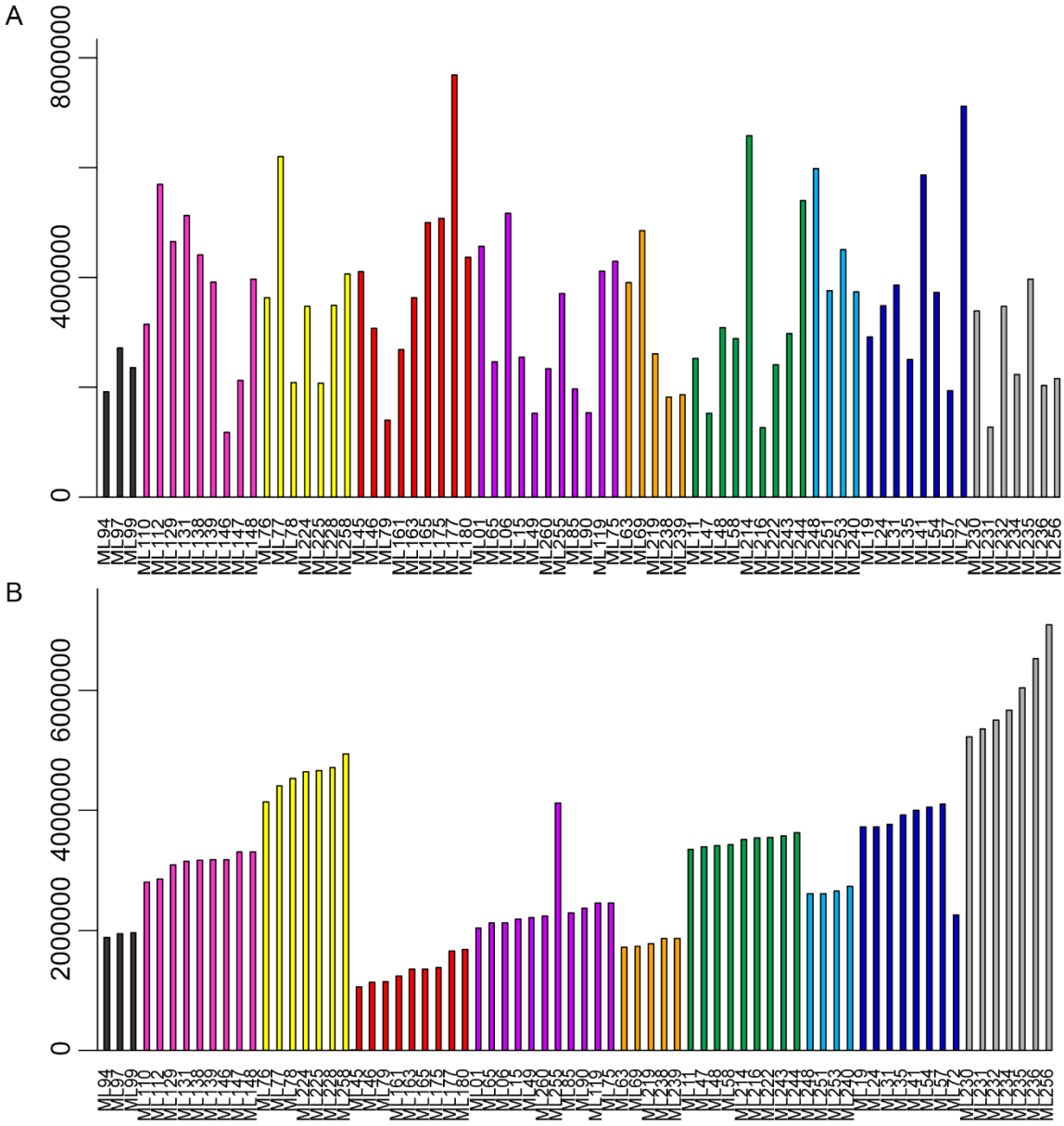

Figure S6. Summaries of the distribution and number of filtered (good) Illumina reads processed by A) IPYRAD and B) STACKS for each individual. Color of each species follows code of Fig. 1, plus light grey for outgroups; sample codes follow the ones described in Tab. S1.

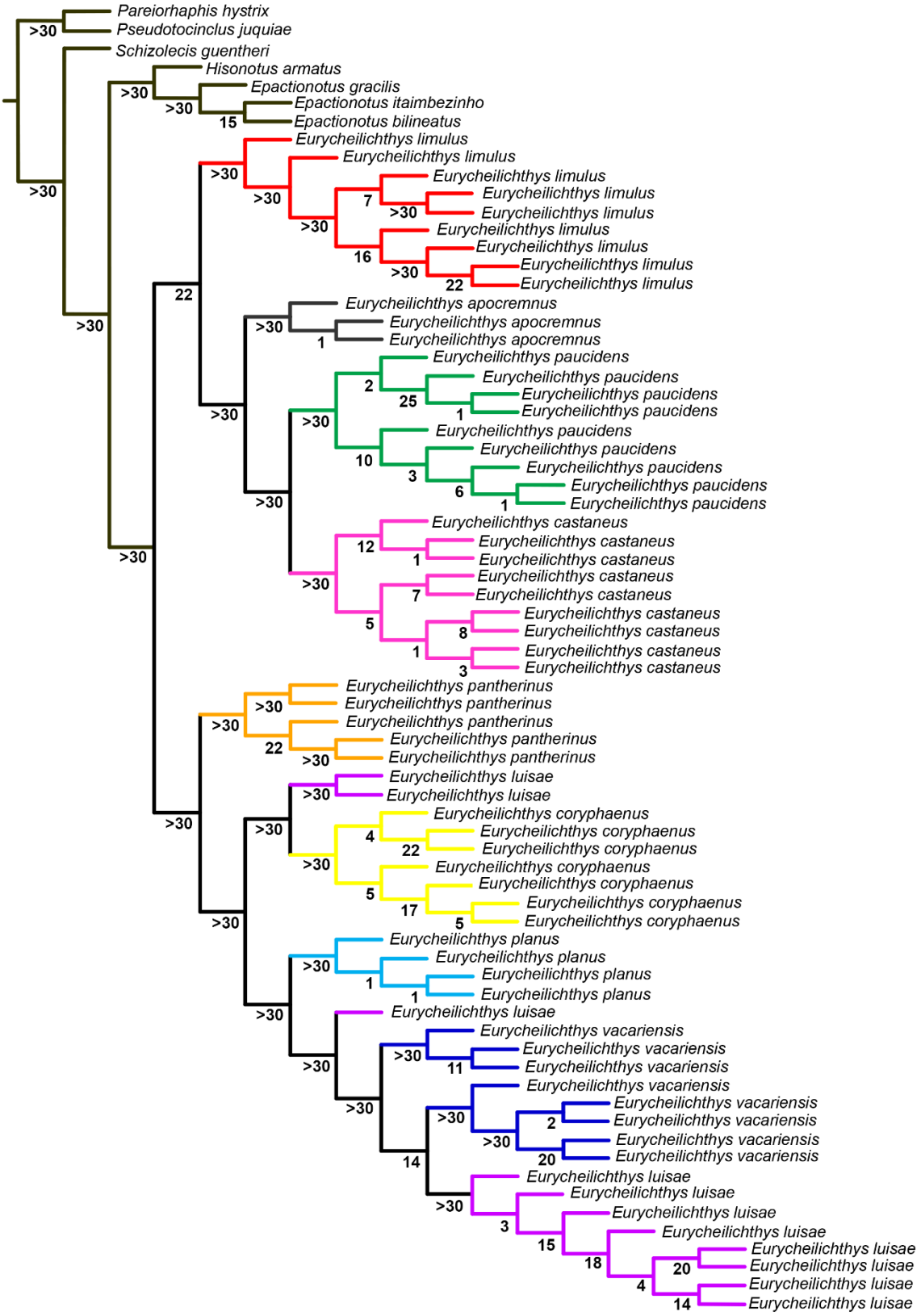

Figure S7. Most parsimonious tree (MP analysis) of all nine *Eurycheilichthys* species plus outgroups based on 29,350 unlinked SNPs. Length of 36,525 steps, consistency index of 0.75, and retention index of 0.93. Numbers below bellow branch are Goodman-Bremer support values. Color pattern corresponds to same species code of Fig. 1.

Conclusões Gerais

A distribuição de *Epactionotus*, previamente restrita aos rios Maquiné, Três Forquilhas, Mampituba e Araranguá, é expandida ao norte para as drenagens dos rios Urussanga, Tubarão, d'Una e Biguaçu. Análises de delimitação de espécies permitiram compreender e inferir processos envolvidos na evolução dessas populações e drenagens. Os dados suportam o endemismo de cada população de *Epactionotus* nas drenagens isoladas, o que é usualmente utilizado como hipótese para delimitação de espécies em peixes de água doce. A especificidade de habitat sugere que as espécies de *Epactionotus* não devem ter utilizado as conexões de planície formadas entre as paleodrenagens.

O monofiletismo e a divergência recente de *Eurycheilichthys* são confirmados com base em análises filogenômicas. Os resultados sugerem a presença de dois clados fortemente suportados: um contendo *Eurycheilichthys limulus* (espécie do alto rio Jacuí) como irmã das espécies localizadas em porções à oeste do Taquari-Antas e *E. paucidens*; e, o outro clado formado por *E. pantherinus* (espécie do alto rio Uruguai), como irmã das espécies distribuídas à leste do Taquari-Antas e *E. luisae*. Esses resultados suportam padrões geográficos descritos previamente para as espécies *Cambeva poikilos* e *Pareiorhaphis hystrix*, respectivamente. A maior diversidade encontrada no Taquari-Antas, e os menores padrões de estruturação do clado leste, sugerem um cenário bastante dinâmico, com a possibilidade de diversos eventos de captura de cabeceira. Exceto por *E. luisae*, todas as demais espécies foram recuperadas como sendo monofiléticas, porém uma medida mais conservativa foi tomada, visando estudos futuros que visem compreender os padrões encontrados no clado leste, a partir de abordagens micro evolutivas e delimitação integrativa de espécies.

NORMAS DE SUBMISSÃO

JOURNAL OF FISH BIOLOGY

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Contents

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A cover letter is not mandatory.

An **Original Research Article** will have the following 12 essential parts.

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The title page must contain the following information:

3.1.1.1. **Title of the paper**, which should be short, informative and avoid any geographical or regional references, unless they are fundamental to the scientific thrust of the paper. If a species name is used in the title, we require a common name (if available) followed by the full scientific name. Avoid the use of abbreviations unless they include the name of a group that is best known by its acronym (e.g., CONSORT statement). See Wiley's tips for search engine optimization: **https://authorservices.wiley.com/author-resources/Journal-Authors/Prepare/writing-for-seo.html**;
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3.1.1.3. The **address in full of each author's primary affiliation** (research institute, university, city, state/province, country) as a numbered list below the Author list;
3.1.1.4. The **corresponding author's name, full postal address** and **email address**.
3.1.1.5 An author's **current address** can be listed here if different from that at the head of

the page.
3.1.1.6 **Funding Information** is listed here.
3.1.1.7 **Joint first and/or senior authorship** can be indicated by stating in a footnote that 'X and Y should be considered joint first author' or '… made an equal contribution to this work'.

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The **Abstract** must be a concise and accurate summary of the **significant findings** of the paper without any introductory or contextual information. Abstracts should not be structured with headings. Methods can be identified only as part of a result (e.g., Respirometry revealed that exercise increased…; GWAS identified a significant number of SNPs…). A species name in the Abstract appears as in the title, a common name (if available) followed by the full scientific name.

Provide a list of up to 6 descriptive **Key Words** (maximum 100 characters) in alphabetical order. Specific geographical (e.g., Baffin Island, Amazon Basin) or regional references (e.g., south-east Asia) can be included here. Keywords are listed underneath the abstract and separated by commas.

3.1.3 Introduction

The **Introduction** alerts readers to literature relevant to the research discovery so that the originality of the research cannot be easily assigned. Also, the Introduction must state the intent of the research in the form of a research question or hypothesis so that no confusion arises as to what advance in fish biology is being sought. Footnotes to the text are not allowed.

3.1.3.1 **Text citations of references** use the style "author, date" and multiple references are list in alphabetical order.

For example: '…as demonstrated by McKenzie (2001) and by McKenzie and Farrell (2010)'; '…as suggested previously in some works (Sloman, 2010), but not others (McKenzie and Farrell, 2010)'; '…consistent with earlier studies (Blaber, 1975, 1988; Lujan, 2011a,b; Prodöhl, 1988)'. Three or more authors are cited with the name of the first author followed by et al. (in italics): e.g., (Sloman et al., 2002) or Sloman et al. (2002). Authors sharing the same surname and year of publication are distinguished by their initials: e.g., (Young, L., 2012; Young, T., 2012).

3.1.4 Materials and Methods

The **Materials and Methods** may contain up to two levels of sub-headings and must provide sufficient detail so that the work can be replicated by others. Established methods can be simply referenced, preferably acknowledging the original work (rather than a recent user of that method), even if minor methodological changes were made (which should be described). Materials and Methods must also include information on how observations were analysed to derive the quantitative results. Statistics should be based on independent biological samples. Technical replicates should be averaged before statistical treatment and not used to calculate deviation parameters. In the case of multiple comparisons (e.g., microarray data), the probability of false positives should be considered in the analysis. Citations to tables, figures, and equations are capitalized and not contracted (e.g., Table 1, Figure 3, Equation 5). Parts of figure should be in lowercase (a), (b), etc., in legend as well as in the figure. For example: Figure 1; Figure 2a; Figure 1a–c; Figures 2a–d and 5.

3.1.5 Results

The **Results** section presents a concise and accurate description of the results of the research. It may contain up to two levels of sub-headings. Figures and Tables, which are numbered consecutively in order of their mention in the text, increase the clarity and conciseness of the result presentation; excessive duplication of material in text, figures and tables is not permitted. All statements concerning quantitative differences between experimental conditions require quantitative data and adequate statistical treatment. The deviation parameter, the number of biological samples and the statistical procedures should be provided for each dataset either in the main text or as part of a Figure or Table.

3.1.6 Discussion

The **Discussion**, which may contain up to two levels of sub-headings, places the results of the study into a broader context so that the significance, quality and novelty of the work can be established with respect to existing literature. The Discussion should directly address the original research question or hypothesis, as stated in the Introduction. Excessive repetition of results is not permitted. The potential for future work or a brief perspective on the findings can be included.

3.1.7 Acknowledgements

Contributions from anyone who does not meet the criteria for authorship should be listed here without titles or honorifics, e.g., A. P. Farrell, but not Prof. Tony Farrell. Thanks to editors and anonymous reviewers are not appropriate. Authors are responsible for the accuracy of their funder designation. If in doubt, please check the Open Funder Registry for the correct nomenclature: **https://www.crossref.org/services/funder-registry/**

3.1.8 Contributions

The contributions of each author, including ideas, data generation, data analysis, manuscript preparation and funding, must be listed here using their initials only, e.g., A. P. F..

3.1.9 Significance Statement

The **Significance Statement** (no more than 75 words) will ultimately appear directly below the online title within the online table of contents (it is not in the published paper). It will be available for reviewers as part of the peer review process and should concisely and accurately explain the significance and relevance of the findings of the study to a broad readership. Suggested content includes: an introductory sentence and/or why a problem/unanswered question was important to address; what has been shown/what does the manuscript do to fill a gap in our knowledge; what it means to the field as a whole. A Significance Statement may undergo editorial revision.

3.1.10 References

All published citations mentioned in the text, tables or figures must be listed in the reference list, which includes all key elements of each reference, including the names of journals in full (not abbreviated). Authors are responsible for checking the accuracy of their references. Manuscript submissions are not required to use JFB reference formatting until the article is provisionally accepted. Corrections may be made during the publication process. A manuscript title must appear exactly as in the original publication. ***JFB* uses APA style referencing with some minor style changes**. Examples of *JFB* reference content requirements are shown below.

Journal Article:

Flowers, K. I., Henderson, A. C., Lupton, J. L., & Chapman, D. D. (2017). Site affinity of whitespotted eagle rays *Aetobatus narinari* assessed using photographic identification. *Journal of Fish Biology*, **91**, 1337– 1349 Gill, A. B. (2003).

Gill, A. B. (2003). The dynamics of prey choice in fish: The importance for prey size and satiation. *Journal of Fish Biology*, **63**, 105– 116

Online Article Not Yet Published in an Issue:

Mussen, T. D., & Cech Jr, J. J. (2018). Assessing the use of vibrations and strobe lights at fish screens as enhanced deterrents for two estuarine fishes. Advance online publication. https://doi.org/10.1111/jfb.13776

An online article is cited by its Digital Object Identifier (DOI), which remains valid and allows article tracking even after its allocation to an issue. It has no volume, issue or page numbers.

Book:

Halver, J. E., & Hardy, R. W. (2002). *Fish nutrition*. San Diego, CA: Academic Press

Chapter in a Book:

Mench, J. A., & Mason, G. J. (1997). Behaviour. In M. C. Appleby & B. O. Hughes (Eds.), *Animal Welfare* (pp. 127– 142). New York, NY: CAB International

Docotral Thesis: These must have a permanent record of where they are held (e.g., thesis has been lodged at the individual's University or Institution Library as a permanent addition to the collection there), e.g., Al-Badran, A. A. (1987). *Factors influencing river bank stability in the Tigris and Shatt Al‐Arab water ways, Iraq* (Doctoral thesis, University of Dundee, UK). Retrieved from **https://ethos.bl.uk/Home.do;jsessionid=DA702A005B56B131D4E776CD6A605544**

Master's Thesis: These must be readily available electronically and the URL provided, e.g., Cox, G. K. (2010). Anoxic survival and cardiovascular responses of the Pacific hagfish, Eptatretus stoutii (Masters thesis). Available from UBC Library **https://open.library.ubc.ca/** .

Electronic References: These include references not subject to peer review and formal publication and can be set out as shown given below. ICES (2016). Report of the Baltic salmon and trout assessment working group (WGBAST). ICES CM 2016/ACOM:09. Available at: http://ices.dk/sites/pub/Publication%20Reports/Expert%20Group%20Report/acom/2016/WGBAST/wgbast_2016.pdf

Marshall, A., Bennett, M. B., Kodja, G., Hinojosa-Alvarez, S., Galvan-Magana, F., Harding, M., Stevens, G. & Kashiwagi, T. (2011). *Manta birostris*. In *IUCN Red List of Threatened Species* Version 2013.2. Available at http://www.iucnredlist.org/details/198921/0 (last accessed 9 December 2013).

3.1.11 Tables

Tables complement but do not duplicate information contained in the text. If required, they are submitted as a separate text files (not pasted as images). Tables contain **no** vertical lines and are numbered consecutively in order of appearance in the text. The table caption is concise and descriptive, and understandable without reference to the main text. It includes the full scientific name(s) of the species to which the table relates. Statistical measures, such as SD or SE, should be identified in the caption. Dimensions for the units should appear in parentheses in the column headings and not in the legend or body of the table. All abbreviations must be defined in footnotes. Footnote symbols: †, ‡, §, ¶, should be used (in that order) and *, **, *** should be reserved for P-values.

3.1.12 Figures

Figures complement information contained in the text, but without unnecessary duplication. Figures that contain data are intended to accurately, clearly and concisely represent the research results, while other figures may better orientate the reader, e.g., maps.

3.1.12.1 **Preparing Figures**

Figures are submitted in digital format and as separate files. **Native file formats are not accepted**. Figures are numbered consecutively in order of appearance in the text. A wide variety of formats, sizes and resolutions of high quality figures are accepted for initial peer review. More information is found at: https://authorservices.wiley.com/asset/photos/electronic_artwork_guidelines.pdf

Line artwork (vector graphics) are prepared in black and white with shades of grey, unless colour is essential for clarity. Error bars must be included and the method used to derive them explained in the caption. Line artwork must be saved as Encapsulated PostScript (EPS) file.

Photographs should illustrate something that cannot adequately be displayed in any other manner. Electron and light microscope photographs must embed a magnification as a **scale bar**. Staining techniques should be described in the caption. Photographs must be saved as bitmap files (half-tones or photographic images) as Tagged Image Format (TIFF) file. **Maps and charts** should be contained within a frame and show either a latitude and longitude or a single co-ordinate (N, S, E or W). *JFB* use The Times Concise Atlas of the World. London: Times Books as its standard for geographical names, countries, seas, rivers, etc.

3.1.12.2 **Figure captions**

A Figure caption is a concise and self-contained description of the figure that can be understood without reference to the main text. Figure captions are submitted as a separate text file along with the Figures. They begin with a short title for the figure, which **include the**

full scientific name(s) of the species to which the illustration relates. Any lines fitted through data points in the figure must be statistically significant and be supported by the mathematical equation and statistical information (P-values and R2 or R values). Keys to the symbols, formulae and regression values can be included in the figure itself or the caption, but not both. The minimum reduction for a figure may be indicated. If material has previously been published, authors must obtain permission from the copyright owner (usually the publisher) to use such material and cite the author in the caption (or text), e.g., 'Reproduced with permission from Blaber (1975).'. (This requirement also applies to the reproduction of a previously published Table or an extended quotation from material.)

3.1.13 Supporting Information

When appropriate, submissions may include **Supporting Information** specifically files containing videos and animations, and long datasets, tables and figures. Supporting Information contains information that is not essential to the article but is a valuable addition by providing greater depth and background. Supporting Information will be reviewed, will appear without typesetting and be hosted only online. The availability of Supporting Information is indicated in the main text after the Acknowledgements, headed "Supporting Information". Short captions list the titles of all supporting material. Supporting Information should be supplied as separate files, and not incorporated into the main manuscript text file. Wiley's FAQs on Supporting Information is found at: **https://authorservices.wiley.com/author-resources/Journal-Authors/Prepare/manuscript-preparation-guidelines.html/supporting-information.html**

3.2 Preparing a Brief Communication

A **Brief Communication** is confined to a **single point or issue of progress** such as an unusual occurrence, an interesting observation, a particularly topical and timely finding or an important technical advance. The point or issue must have relevance beyond the species or locality under consideration. First records should adhere to best practices proposed by Bello et al. (2014. A proposed best practice approach to overcome unverified and unverifiable "first records" in ichthyology. *Cybium* 38, 9-14) and should strive to aggregate and report regional historical records for the same species. *JFB* no longer considers short technical notes describing molecular markers (e.g., microsatellites). A **Brief Communication** is limited in length (**no more than 5 printed pages**; c. 2500 words of text) and normally includes no more than **one (multi-panel) figure and one table**. It follows the same format as Research Articles with respect to the Title, Authors and Affiliations, Abstract, Key Words, Statement of Significance, Acknowledgements and References (see Section 3.1), but the main text is written in freeform **without any headings**. The Abstract is **no more than 90 words.**

3.3 Preparing a Review Article

Prospective authors will submit a synopsis (two pages maximum) of their article to an Associate Editor or the Editor-in-Chief. The synopsis should outline why the review is topical, its main points and objectives, and how it will stimulate debate and research. When the proposal has been accepted, the authors will submit a manuscript within a mutually agreed upon time and page limit.

3.4 Preparing an Opinion Piece

An **Opinion Piece**presents a brief, personal view on a topical or emerging issue in Fish Biology that has broad readership appeal. It may be offered to or commissioned by the Editor-in-Chief. The submission includes a Title page, Main Text and References. It contains no Abstract or Key Words but can contain Tables or Figures. It will be peer reviewed.

3.5 Preparing a Comment to the Editor

Comments are no more than c. 750 words of text and deal with single significant finding or point for discussion concerning a recent published paper in *JFB* and needs rapid publication. The submission includes a Title page, Main Text and References (maximum four). It contains no Abstract, Key Words, Tables or Figures. After satisfactory peer review, it will be sent to the original corresponding author for a Reply. The reply will take the same form and will be peer reviewed. Publication will end the debate.

4. ETHICAL CONSIDERATIONS

4.1 Ethical considerations for the use of animals

JFB takes its responsibility towards animal welfare very seriously, whether it concerns fish collection, predator-prey interactions or invasive surgical procedures. At the same time *JFB* recognizes that permitting requirements for animal collections and animal welfare have regional differences and therefore may not be exactly the same as those stipulated in the United Kingdom, which is the home of *JFB*.

Therefore, when a research paper that involves animal experimentation or harm is submitted to *JFB*, authors are accepting and acknowledging that appropriate permits for animal collections and animal welfare issues were sought and approved by the local committee(s) responsible for such permits. If a submission is received from a country where no such permitting is required, then any decision with regards to ethics rests solely with the Editor-in-Chief, who will seek advice from the Editorial Team, referees and other qualified scientists as needed.

Furthermore, as specific evidence of the permitting, a clear ethical statement must be provided in the Materials and Methods under a subheading Ethical Statement for any submissions to the *JFB*. This statement may take a form similar to the following:

The care and use of experimental animals complied with [Insert the local or national body] animal welfare laws, guidelines and policies as approved by [Insert the local or national permitting authority and the permit reference number].

Independent of any such permits, the *JFB* still reserves the right to reject papers on an ethical basis should valid concerns emerge from the contents of the research paper. Therefore, it is essential that within their ethical statement authors clearly identify any welfare implications arising from their experimental design including steps taken to minimise impact on fish welfare. Studies which may require additional information in the ethical statement include (but are not limited to) those where: fishes were collected as part of faunal surveys; experimental conditions caused severe distress or lasting harm to sentient fishes (e.g. predation studies, toxicity testing, disease trials); surgical procedures were used; sentient un-anaesthetised animals were subjected to chemical agents that induce neuromuscular blockade, such as muscle relaxants. In addition, ethical statements should say whether fishes were killed at the end of the experiment (e.g. for tissue sampling).

Ahead of submission, authors will benefit greatly from reading our Editorials on animal welfare: **http://onlinelibrary.wiley.com/doi/10.1111/j.0022-1112.2006.01035.x/full** (2006) and **http://onlinelibrary.wiley.com/doi/10.1111/j.1095-8649.2010.02900.x/full** (2011).

If the research did not involve animal experimentation or harm, and required no permits then no ethical statement is required.

4.2 Publication Ethics

The Fisheries Society of the British Isles (FSBI) considers that scientists should avoid research threatening the conservation status of any species of fish that is already regarded as threatened according to the IUCN Red List of Threatened Species and the associated current Red List Categories and Criteria (**http://www.iucnredlist.org/technical-documents/categories-and-criteria**) or which is listed as such in a Red Data Book appropriate to the country or geographical area concerned. In accordance with this view, papers based on such research will not be accepted, unless the work had clear conservation objectives.

4.3 Authorship

The list of authors should accurately illustrate who contributed to the work. Any person listed as an author, by definition, will have contributed substantially to the article's conception and design, or acquisition of data, or analysis and interpretation of data. All listed authors will be contacted by email after a manuscript is submitted to confirm their contribution. Listed authors should meet the following criteria:

- Have made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; given final approval of the version to be

published and have participated sufficiently in the work to take public responsibility for appropriate portions of the content;
- Been involved in drafting the manuscript or revising it critically for important intellectual content; and
- Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Contributions from anyone who does not meet the criteria for authorship should be listed, with permission from the contributor, in an Acknowledgments section (for example, to recognize contributions from people who provided technical help, collation of data, writing assistance, acquisition of funding, or a department chairperson who provided general support). Prior to submitting the article all authors should agree on the order in which their names will be listed in the manuscript. (https://www.crossref.org/services/funder-registry/).

How individual authors specifically contributed to the work is listed in the **Contributions** statement (see Section 3.8).

5. EDITORIAL POLICIES AND JOURNAL STYLE

As *JFB* serves an international community of fish biologists, some conventions are required that deviate from the APA style. For a full explanation of style requirements for *JFB*, with examples, please click **here.**

5.1 Abbreviations and acronyms:

All abbreviations and acronyms in the text and in all figure and table captions must be given in the fully expanded form on first mention and abbreviated thereafter, except for the small number of abbreviations and acronyms that are scientifically accepted, e.g., DNA. Useful resources are:

BSI (1967). *Recommendations for Letter Symbols, Signs and Abbreviations:* BS 1991, Part I. London: British Standards Institute.
Baron, D. N. (Ed.) (1977) *Units, Symbols and Abbreviations. A Guide for Biological and Medical Editors and Authors*, 3rd edn. London: The Royal Society of Medicine.

5.2 Units: Physical measurements only use metric units in accordance with the Systeme International d'Unites (SI), e.g., m, mm3, s (h and day are acceptable), g, m s−1, g l−1, mg l−1 (not ppm), J (not calories).
The 24-h clock is used for time of day, e.g., 1435 hours, not 2.35 p.m. Calendar dates use day month year, e.g., 15 June 1998. Salinity has no units; do not use psu, ‰ or similar. Ship's speed is given in km h−1; knots (nautical miles h−1) can follow in parentheses. Latitude and longitude can be given either as degrees minute seconds, or decimal degrees, at a level of precision proportionate to the accuracy of the fix. (0.1 second of latitude is equivalent to 185 m, but this decreases for longitude by the cosine latitude).

5.3 Statistics, equations & mathematical expressions: A useful resource for equations and mathematical expressions:
Journal of Fish Biology **8**2, 1771–1772 DOI: 10.1111/jfb.12146 (2013); A useful resource for reporting statistics: *Journal of Fish Biology* **78**, 697–699 DOI: 10.1111/j.1095-8649.2011.02914.x (2011)

Where decimal values are given, the number of decimal places must reflect the accuracy of the work. Thus, means and error (S.D., S.E., 95% C.L., etc.), should have the same number of decimal places, e.g., 15.1 + 0.2 and not 15.1 + 0.19. In mathematical expressions, italicized single letters are used for dimensions, qualified by subscripts (roman) as required, e.g., mass (not weight) M, wet mass (M_W), length L, fork length L_F (not FL), standard length L_S, index I, gonadosomatic index I_G, hepatosomatic index I_H, etc.

Statistics are presented as follows: name of test, test statistic with associated degrees of freedom (d.f.; *N.B*. an *F* distribution has two d.f. values) and probability level (*P*). Although ANOVA and regression are robust, the real *P*-values are likely to be different from the precise values provided by the statistics program, because of violations of the assumptions. If the manuscript clearly states that data conform fully to all the assumptions of the statistical method used, then precise *P*-values can be cited with three decimal places. Otherwise, *P*-

values are normally limited to: > 0.05, 0.05, 0.01 and 0.001. Confidence intervals (95% C.I.) can be provided for parameters estimated by ANOVA and regression analysis. Where numerical resampling (e.g. bootstrapping) is used to assess the statistical significance of a given parameter (e.g. F_{ST}), in addition to resulting confidence intervals, the number of replicates should be also provided (e.g. 1000 bootstrap replicates).

5.3 Species nomenclature, authority and nomenclature: The plural of more than one individual of a single species is 'fish', but it is 'fishes' if there is more than one species. After its first mention, a fish species is **only** referred to by its scientific name. There should then be no further reference to the common name, describing author or date. The genus name can be abbreviated to a single letter (e.g., *C. carpio* and *O. mykiss*), except either at the start of a sentence, or where confusion arises from multiple genera with the same first letter, when either the genus is given in full, or the first three letters of the genus is used to provide a clear distinction.

First use of a fish species name in the Title and Abstract must include common (if available) and scientific name without describing the authority and date of authorship. First mention of a fish species in the main text must include the common name (if available), the binomial scientific name (in italics) **and the describing authority and date of authorship**, e.g., rainbow trout *Oncorhynchus mykiss* (Walbaum 1792), not (Walbaum, 1792). Naming authorities must appear in full except Linnaeus, 1758, e.g., Atlantic salmon *Salmo salar* L. No commas are necessary to separate either the common name from the species, or the authority from the date. **The use or absence of parentheses around the naming authority's name and date is covered by strict scientific rules.** If the current accepted genus and species name is the same as that given by the original naming author, the name appears without parentheses, e.g., *Pleuronectes platessa* L. 1758, but if the current accepted scientific name differs from that given by the original naming author, the original author's name appears within parentheses, e.g., *Platichthys flesus* (L. 1758).

For **correct scientific names and formatting of naming author** please use the following: Eschmeyer, W. N. (Ed.) *Catalog of Fishes* electronic version (15 November 2013). http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp

For **accepted common names of fishes**:
Wheeler, A. (1992). A list of the common and scientific names of fishes of the British Isles. *Journal of Fish Biology* **41**(Suppl. A), 17–26. doi: 10.1111/j.1095-8649.1992.tb05644.
Wheeler, A. C., Merrett, N. R. & Quigley, D. T. G. (2004). Additional records and notes for Wheeler's (1992) List of the Common and Scientific Names of Fishes of the British Isles. *Journal of Fish Biology* **65**(Suppl. B), 1–40. doi: 10.1111/j.0022-1112.2004.00583.x
Nelson, J. S., Crossman, E. J., Espinosa-Perez, H., Findley, L. T., Gilbert, C. R., Lea, R. N. & Williams, J. D. (2004). Common and scientific names of fishes from the United States, Canada, and Mexico, 6th edn. Special Publication 29. Bethesda, MD: American Fisheries Society.
Froese, R. & Pauly, D. (Eds) (2013). *FishBase*. World Wide Web electronic publication. Available at http://www.fishbase.org/Search.php
FAO (2013). A*SFIS List of Species for Fishery Statistics Purposes*. Rome: Fisheries & Aquaculture Department, FAO. Available at http://www.fao.org/fishery/collection/asfis/en

5.4 Synonyms for a species

Synonyms require the following style: *Eptatretus cirrhatus* (Forster 1801) *Homea banksii* Fleming 1822: 375 (original description; type locality: South Seas; holotype: unknown); *Bdellostoma heptatrema* Muller 1836: 79 (original description; type locality: South seas; holotype: unknown); *Bdellostoma forsteri* Muller 1836: 80 (original description; type locality: Queen Charlotte Sound, New Zealand; holotype: unknown). Conel, 1931: 76 *Bdellostoma forsteri* var. *heptatrema*. Muller, 1838: 174 (new combination); *Bdellostoma cirrhatum*. G¨unther, 1870: 511 (in part). Hutton, 1872: 87 (in part). Putnam, 1874: 160 (in part); Gunther, 1880: 27. (Note that species names that are modifications of an existing binomial, rather than an original description, are separated from the author name by a full stop, *Bdellostoma cirrhatum*. Gunther, 1870: 511 (in part). [based in part on: Mincarone, M. M. & Fernholm, B. (2010). Review of the Australian hagfishes with description of two new

species of *Eptatretus* (Myxinidae), *Journal of Fish Biology* **77**, 779–801. doi: 10.1111/j.1095-8649.2010. 02661.x]

5.5 New species: The International Code of Zoological Nomenclature (Article 8.5, amendment) requires that a work bearing a new taxonomic name, issued and distributed electronically must be registered in the Official Register of Zoological Nomenclature (ZooBank) and contain evidence in the work itself of such registration. Any manuscript dealing with the description of new species, genera, or family submitted to *JFB* must be registered in ZooBank and the name of each new taxonomic name (e.g., new family, genus or species) should be added to ZooBank. Read http:**//zoobank.org/** and associated video tutorials (**http://zoobank.org/VideoGuide**) and the Editorial on this subject in ***JFB* 90, 1167–1169.**

5.6 Curation of taxonomic specimens

Name-bearing type specimens of taxa that are described in *JFB* as new to science must be deposited in recognized national or international institutions that can meet ICZN (2012) criteria for Recommendations 72F.1-5 into the foreseeable future: ICZN (2012). *The International Code of Zoological Nomenclature*, 4th edn. London: The International Trust for Zoological Nomenclature 1999.

Other specimens used for taxonomic analyses should, wherever possible, be deposited in appropriate scientific collections (e.g., museums and university collections, or private collections when there is good evidence that these are adequately maintained), with identifying catalogue numbers, so that they are accessible to the scientific community for subsequent examination and taxonomic revision **http://iczn.org/iczn/index.jsp**. Distribution of paratype series among more than one recognized national or international institution is at the discretion of the authors, but is encouraged for paratype series whenever the paratype series can be split into two or more representative samples for deposit at different institutions. Institutions and their official abbreviations are listed in Eschmeyer's *Catalog of Fishes* *Online* : **https://www.calacademy.org/scientists/projects/catalog-of-fishes** and in Poss, S. G. & Collette, B. B. (1995). Second survey of fish collections in the United States and Canada. *Copeia* 1995, 48–70. **http://www.jstor.org/stable/1446799**

5.7 Genetic nomenclature: Authors are responsible for ensuring correct style for naming genes, etc. to avoid delay publication at the final proofreading stage. To differentiate genes, proteins etc., by fish origin, *JFB* uses the zebrafish system: **https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines** . On first mention, the name of a gene, etc. should be given in full (roman) with its abbreviated form immediately after in parentheses. Thereafter, an abbreviated format should be used, as shown below.

Full name	Abbreviated form
Heat-specific protein-I gene	*hspI* (all lower case italic)
Heat-specific protein-I	Hsp1 (all roman, capital first letter)
Xxx-x microsatellite locus	Gsp-1 (all roman, capital first letter of genus, followed by: two first initials of species name and the clone number; *e.g.* Gmo-1 for *Gadus morhua*)
zzz exon	*zzz-ex1* (all lower case italic followed by *-ex* also italics and a number for the exon number)
aaa intron	*aaa-in1* (*cf.* exon)
Enhanced fluorescent green protein-N3 plasmid	pEGFP-N3 (all roman capitals preceded by roman lowercase p)
Bbb-x primer	*Gsp-1* (all italic, capital first letter of genus, followed by: two first initials of species name and the clone number; *e.g. Gmo-1* for *Gadus morhua*)
Plasmids	All roman
Mammalian heat-specific protein-I gene	*HSPI* (all capital italic)
Mammalian heat-specific protein-I	HSPI (all capital roman)

5.8 Sequence data: Descriptions of novel amino-acid sequences of proteins or novel nucleotide sequences (e.g., primer sequences) are only be accepted if they carry a statement that all the data have been deposited with an appropriate data bank, e.g., the European Molecular Biology Laboratory (EMBL) or GenBank Data Libraries, and the database accession number must be given in the Materials and Methods. Data deposited in genetic data banks should include: specimen catalogue numbers (for specimens preserved in collections); a note identifying sequences that are derived from type specimens; and the collection locality data. For taxonomic papers that refer to sequences derived from specimens preserved in collections, authors should include a Table that clearly links each sequence accession number with the specimen from which it was derived. Sequences from type specimens should be clearly identified by bold text in this table and the significance of the bold text explained as a table footnote. For appropriate nomenclature for genetic sequences of type specimens please see: Chakrabarty, P. (2010). Genetypes: a concept to help integrate molecular phylogenetics and taxonomy. *Zootaxa* **2632**, 67–68. **http://www.mapress.com/zootaxa/2010/f/zt02632p068.pdf**. Sequences from holotypes are identified as hologenetypes, those from topotypes are topogenetypes, and the genetic marker(s) used are incorporated into the nomenclature (e.g., paragenetype ND2). Lengthy nucleotide sequences will only be published in the text if, in the judgement of the Editorial Team, these results are of general interest and importance. Where sequences are already published, reference to the original source will suffice.

RAPD –randomly amplified polymorphic DNA: Papers submitted to *JFB* must not include data generated by RAPD technology because conclusions derived from them may be unreliable.

6. PUBLICATION PROCESS AFTER ACCEPTANCE

6.1 Author Licensing

If a paper is accepted for publication, the author identified as the formal corresponding author will receive an email prompting them to log in to Author Services, where via the Wiley Author Licensing Service (WALS) they will be required to complete a copyright license agreement on behalf of all authors of the paper. Authors may choose to publish under the terms of the journal's standard copyright agreement, or **OnlineOpen** under the terms of a Creative Commons License. General information regarding licensing and copyright is available **here**. To review the Creative Commons License options offered under OnlineOpen, please **click here**. (Note that certain funders mandate a particular type of CC license be used; to check this please **click here**)

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