

Phylogeny and species delimitation based on molecular approaches on the species of the *Australoheros autrani* group (Teleostei, Cichlidae), with biogeographic comments

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Abstract

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Three distinct and independent molecular-based species delimitation analyses were performed among the species and populations included within the *Australoheros autrani* group, based on sequences of the mitochondrial gene Cytochrome b: a tree-based method proposed by Wiens and Penkrot (WP), a Character-based DNA Barcoding (CBB) and coalescent species delimitation method termed the Bayesian Implementation of the Poisson tree processes (bPTP). The congruence of WP and CBB delimited 11 independent lineages (species), while the bPTP delimited just nine lineages. We did not favour any of the methods, and we considered the possibility of two slightly variant scenarios. A time-calibrated phylogenetic analysis is proposed based on the predominant congruence of the results of these three species delimitation methods herein applied. The monophyly of the *A. autrani* species group was highly supported with maximum node support value and diagnosed by 11 nucleotide substitutions. The sister clade of the *A. autrani* species group is the clade comprising *A. sp. Timbé do Sul* and *A. minuano*. The phylogenetic analysis supports three main clades within the *A. autrani* species group, supported by maximum node support value, with the Southern Mata Atlântica clade as the most basal clade. Divergence time estimates indicate that the diversification of the *Australoheros* originated during the early Neogene, but only in the late Neogene did the processes of diversification in the southeast and north regions occur. Diversification within the *Australoheros autrani* species group occurred synchronically for the three main clades during the beginning of the Quaternary. It is demonstrated that molecular characters are valuable tools for species recognition, particularly in speciose groups with inconspicuous or difficult to record morphological characters. The resulting phylogeny of the *Australoheros autrani* group is highly compatible with the geological and biogeographic scenarios proposed for the Neogene and Quaternary shaping of the extant river basins of eastern Brazil. Despite the origin of the *A. autrani* group being dated to the late Miocene, species level diversification occurred in the Pleistocene and was probably driven by headwater capture events and sea-level fluctuations.

Introduction

Over the past two decades, research on cryptic species have exponentially increased, mainly due to the improvement of molecular methods and availability of DNA se-

quences (Bickford et al. 2006). Cryptic or hidden species are those species which are: morphologically indistinguishable, since speciation is not always accompanied by morphological change or are at least superficially morphologically difficult to be differentiated; species which

are, or have been, erroneously classified as a single nominal species on formal grounds; or any taxa comprising taxonomically confused “species”, that in fact consist of a number of valid but undiagnosed species (Bickford et al. 2006; Adams et al. 2014). Among cryptic species complexes, the number of species is likely to be greater than that estimated by traditional alpha-taxonomy, because speciation is not always accompanied by morphological differentiation (Bickford et al. 2006; Adams et al. 2014). Many species are expected cryptic, similar morphologically, usually difficult to be identified based only on preserved specimens. In particular this applies to species properly diagnosed by, for example, colouration in life, behaviour, and acoustic or electric discharge characters. In these cases, the use of additional tools and methods such as molecular and DNA data and methods are important to evaluate the diversity within taxonomically unresolved groups (Wiens and Penkrot 2002; Bickford et al. 2006; De Queiroz 2007; Goldstein and Desalle 2010; Costa et al. 2012, 2014). However, diagnosing species using only molecular characters is possible, but is not yet a widespread practice (Cook et al. 2010; Pante et al. 2015), especially in animals, although recent papers have increasingly included molecular data in species descriptions (Goldstein and Desalle 2010; Pante et al. 2015).

The ongoing destruction and disturbance of natural ecosystems and the resultant increase in extinction rates, makes it urgent to catalogue and describe biodiversity, as well as to develop approaches directed to the study of species complexes (Brook et al. 2006; Costa et al. 2012). Molecular data proved to be very useful in revealing cryptic species (Bickford et al. 2006; Adams et al. 2014), which have been subsequently supported by morphological, ethological, and/or ecological data, or in evidencing a hypothesis of species identity when these are morphologically quite similar or undistinguishable. Thus, molecular methods should be incorporated into alpha taxonomy, thereby improving the accuracy of biodiversity estimates. Underestimation of species diversity and misidentification of cryptic species could have severe consequences, mainly in the field of conservation biology. Among the main consequences related to species misidentification is the possibility of endangered species being hidden in cryptic species complexes, and consequently not being taken into account by conservation policies and protective efforts. Other consequences were widely discussed by Bickford et al. (2006).

The Neotropical region, known for its rich species diversity, has inspired classical studies on evolutionary biology. In this region the most diverse fauna of freshwater fishes in the world is found, exhibiting intriguing and unique specializations (e.g. Reis et al. 2003, 2016). Cichlids are among the main components of the Neotropical ichthyofauna; they are a teleost family occurring in tropical and subtropical regions of the Americas, Africa, and Asia (Kullander 1998, 2003; Nelson et al. 2016). Cichlidae is one of the most species-rich vertebrate families (McMahan et al. 2013; Near et al. 2013; Nelson et al.

2016), comprising more than 1700 valid species (Fricke et al. 2018). Despite the predominantly marine habits of closely related families in the Ovalentaria clade (Betancur et al. 2013; Near et al. 2013, Nelson et al. 2016), cichlids inhabit freshwater environments, with species occasionally tolerating brackish water (Kullander 1998; Nelson et al. 2016).

Australoheros Říčan & Kullander, 2006 is a South American cichlid genus of the tribe Heroini, which was described to include species previously placed in *Cichlasoma* Swainson, 1839. Before 1995, all the 29 nominal species presently contained in *Australoheros* were considered to belong to a single species, *Cichlasoma facetum*, which was thought to be geographically widespread between southeastern Brazil and northeastern Argentina (Casciotta et al. 1995; Říčan and Kullander 2003; Ottoni and Costa 2008). In recent years, taxonomic studies have revealed high species diversity, mostly concentrated in the lower La Plata river basin and adjacent coastal river basins (Casciotta et al. 1995, 2006; Říčan and Kullander 2003, 2006, 2008; Ottoni and Cheffe 2009; Říčan et al. 2011) as well as in coastal basins of eastern Brazil and adjacent headwaters of the upper Paraná and upper São Francisco river basins (Ottoni and Costa 2008; Ottoni 2010, 2012, 2013a, b; Ottoni et al. 2011).

Říčan and Kullander (2008) delimited four *Australoheros* species groups for taxa endemic to the La Plata river basin which occur in the rivers system of the Paraná–Paraguay–Uruguay based on both morphological and molecular data [cytochrome b (CYTB)]: the *A. scitulus* species group, *A. forquilha* species group, *A. facetus* species group, and *A. kaaygua* species group. A fifth species group was later proposed by Ottoni (2010, 2012), and named the *A. autrani* group based on morphological characters, to include species from eastern Brazilian basins.

Presently, the *A. autrani* group includes 16 nominal species (Ottoni and Costa 2008; Ottoni 2010, 2012; Ottoni et al. 2011), including *A. sanguineus*, not previously placed in any species group (Ottoni 2013). However, Říčan et al. (2011) suggested that the number of valid species in this group was overestimated, with all nominal species belonging to a single species (probably *A. facetus*), in spite of the several morphological characters previously used to diagnose and distinguish them (see Ottoni et al. 2008, 2011; Ottoni and Costa 2008; Ottoni 2010, 2011). The assumption that all species of the *A. autrani* group belong to a single lineage (*A. facetus*) relied on morphology-based phylogenetic analyses, including only 38 characters. Of these, only 10 characters were applicable for species of the *A. autrani* group (Ottoni 2012), resulting in a low resolution and a poorly supported tree. In addition, no nominal species of the *A. autrani* species group have been examined for the analyses (the information was obtained only from original descriptions), therefore weakening the original basis of the statement. Subsequently, Ottoni (2012, 2013a) contested this hypothesis and presented a set of morpho-

logical character states useful to distinguish species of the *A. autrani* group from *A. facetus*, and some papers providing evidence of the morphological discontinuity between species and populations within the *A. autrani* group were published (e.g. Ottoni 2012, 2013a, b; Ottoni et al. 2014; Ottoni and Schindler 2014). The present study is the first to approach the diversity of the *A. autrani* group through molecular data and different species delimitation methods, providing a clearer panorama on the *A. autrani* group species and lineage diversification. In view of the two confronting perspectives on diversity in the *A. autrani* group, testing species limits within this group is the main goal of the present study. It is especially warranted when considering future conservation policies and efforts. In addition, this study provides the first phylogenetic analysis of the *A. autrani* group, testing its monophyly and establishing its intrarelationships. Another aim of the present research is to approach the *A. autrani* group diversification in a temporal perspective, through a time calibrated analysis, looking for major paleogeographic and paleoclimatic events that may have contributed to species diversification and distribution.

Material and methods

Material

Specimens of the *A. autrani* group were fixed in absolute ethanol immediately after collection and later preserved in the same solution (see Suppl.material 1: Table S1 for list of specimens, taxonomic names, author and year of publication of analysed taxa, respective geographic location, and GenBank accession numbers; for localities see also Fig. 1). The voucher specimens are deposited in the ichthyological collection of the Institute of Biology, Federal University of Rio de Janeiro (UFRJ). For each focal species, specimens were collected at type localities or nearby, but some additional populations were also sampled. Sequences of congeners belonging to other species groups were obtained from GenBank, following identifications proposed by Říčan et al. (2011), as well as sequences from other *Heroini* genera (Suppl. material 1: Table S1).

The collected specimens were euthanized in a buffered solution of tricaine methane sulphonate (MS-222) at a concentration of 250 mg/L, for a period of 10 min, following the guidelines of the Journal of the American Veterinary Medical Association (AVMA Guidelines) (Leary et al. 2013) and European Commission DGXI consensus for fish euthanasia (Close et al. 1996, 1997).

DNA extraction, amplification, sequencing and alignment

The genomic material was extracted from muscle tissue of the caudal peduncle region through the commercial DNeasy Blood and Tissue Kit (Qiagen). We used the primers CytB-F (Palumbi et al. 1991) and TrucCytB-R (Martin and Bermingham 1998) to amplify a fragment of 1039 bp of the mitochondrial encoded gene Cytochrome

b (CYTB). Polymerase chain reaction (PCR) was performed in 50 µl reaction mixtures containing 5× Green GoTaq (Promega), 3.2 mM MgCl₂, 1 µM of each primer, 75 ng of total genomic DNA, 0.2 mM of each dNTP and 1U of Taq polymerase. The thermocycling profile was: (1) 1 cycle of 1 minutes at 94 °C, (2) 40 cycles of 1 min at 92 °C, 1 min at 45–60 °C and 1 min at 72 °C, and (3) 1 cycle of 4 min at 72 °C. Negative controls were used to check DNA contamination in all PCR reactions. Amplified PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The sequencing reactions were purified, and the samples were run on an ABI 3130 Genetic Analyzer. Sequences were edited using MEGA 7.0 (Kumar et al. 2016), aligned using ClustalW (Chenna et al. 2003), and subsequently the aligned fragments were translated into amino acid residues to verify the presence of premature stop codons or indels.

Species delimitation

Three distinct operational criteria (species delimitation methods), based on molecular data, were performed: a tree-based method as proposed by Wiens and Penkrot (2002) [hereafter WP, following Sites and Marshall (2003)], a coalescent species delimitation method termed the Bayesian implementation of the Poisson tree processes [hereafter bPTP, following Zhang et al. (2013)], and a character-based DNA barcoding as proposed by Desalle et al. (2005) (hereafter CBB). All species delimitation methods here adopted were performed including only Cytochrome b (Cytb) sequences, as it is a mitochondrial gene with fast evolution rate, indicated for species delimitation approaches (Avice 2000).

The WP is based on the direct inspection of haplotype trees generated from the phylogenetic analyses having as terminals at least two individuals (haplotypes) of each focal species. In this method, the term “exclusive” is used instead of monophyletic, since the term monophyly is considered inapplicable below the species level (Wiens and Penkrot 2002). Clustered haplotypes with concordant geographic distribution forming mutual and well supported clades (exclusive lineages) are considered strong evidence for species discrimination (absence of gene flow with other lineages). The failure of haplotypes from the same population to cluster together instead, is considered potential evidence for gene flow within populations, therefore suggesting the lack of divergence between lineages (species) (Wiens and Penkrot 2002). Statistical support for clades is assessed by the posterior probability value, considered as significant at values about 0.95 or higher (Alfaro and Holder 2006). When only one haplotype (specimen) from one putative population was available, the species delimitation was based on the exclusivity of the sister clade of this single haplotype, supported by significant values, allowing us to perform the test in populations with only one haplotype (Wiens and Penkrot 2002). In addition, the method allows recognition of nonexclusive lineages as species since their sister clades are exclusive and supported by significant values (Wiens

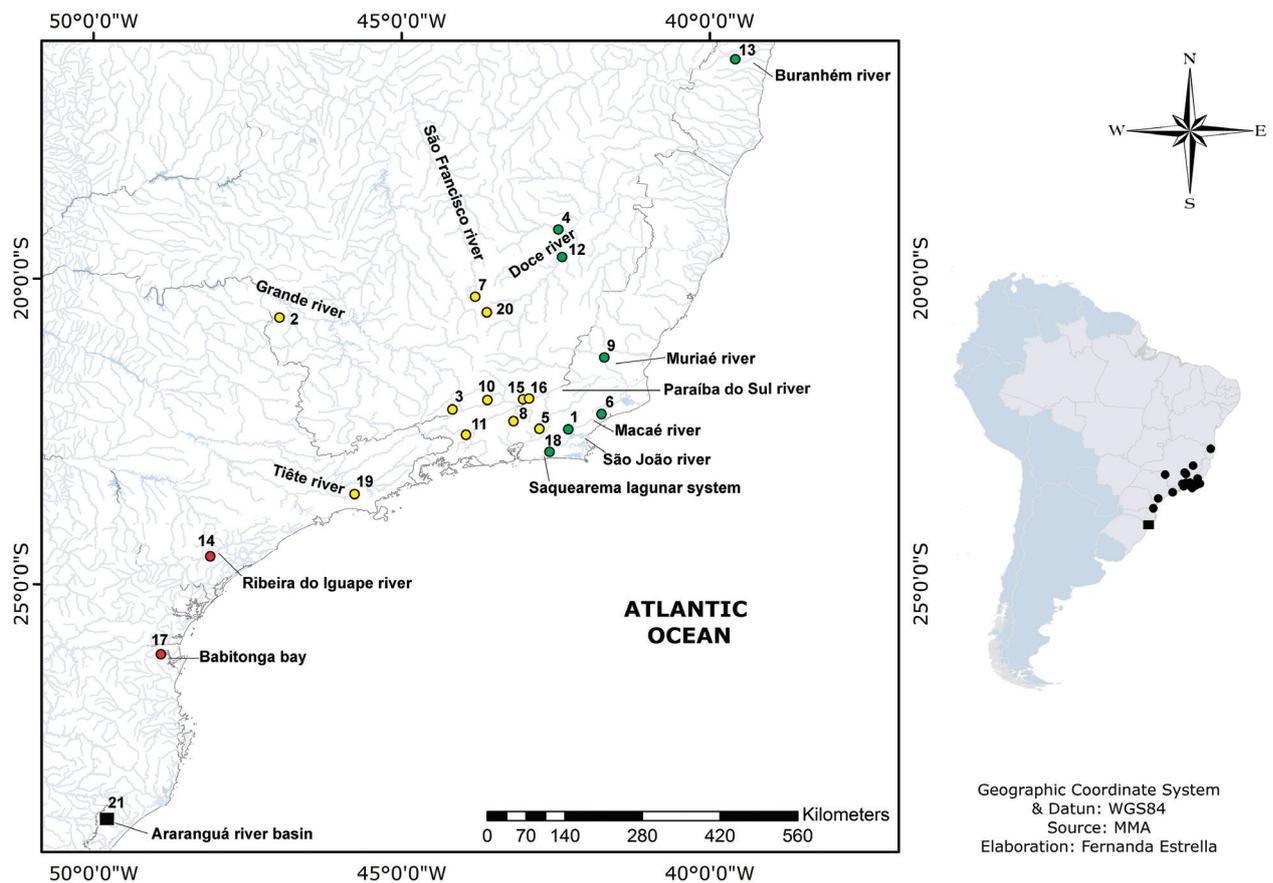


Figure 1. Map of the samples obtained for the present work. Circles = *Australoheros autrani* species group; Red circles = Southern Mata Atlântica clade; Yellow circles = Upper/middle Paraíba do Sul river basin and adjacent drainages clade; Green circles – Northern Mata Atlântica clade; and Square = *A. sp.* Timbé do Sul. Localities: *A. autrani* = 1 and 18, *A. barbosa* = 2, 3, 10, 11 and 19, *A. ipatinguensis* = 4, *A. macacuensis* = 5, *A. macaensis* = 6, *A. muriae* = 9, *A. perdi* = 12, *A. ribeirae* = 14, *A. robustus* = 7, 8, 15, 16 and 20, *A. sanguineus* = 17, *A. cf. capixaba* = 13, and *A. sp.* Timbé do Sul = 21.

and Penkrot 2002). Terminal in-group taxa were the a priori morphologically defined species and populations of the *A. autrani* group. Terminal out-group taxa included several lineages representing all the other *Australoheros* species groups proposed by Řičan and Kullander (2008), an additional population from the south of Santa Catarina state, and more distantly *Trichromis salvini* (Suppl. material 1: Table S1). When performing the WP species delimitation method, the protein-coding CYTB sequences were partitioned by codon position. Jmodeltest 2.1.7 program (Darriba et al. 2012) was used to define the most appropriate evolutionary model for each partition, and the choice for the best model was based on the Akaike information criterion. First codon position HKY+I, 2nd codon position F81 and 3rd codon position GTR+I.

The topology for the WP approach was generated by Bayesian inference analysis performed in the MrBayes 3.2.5 program (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with the following settings: two Markov chain Monte Carlo (MCMC) runs of four chains each for 10 million generations, and sampling frequency of 100. All parameters between partitions except

topology and branch lengths were unlinked. The convergence of the MCMC chains were graphically assessed by evaluating the stationary phase of the chains using Tracer v. 1.6 (Rambaut et al. 2014). Consensus topology and posterior probabilities were obtained after applying a burn-in of the first 25% of the generated trees. The resulting haplotype tree topology is presented in Figure 2.

The CBB is similar to the population aggregation analysis proposed by Davis and Nixon (1992), but directed to nucleotides as an alternative method for diagnosing taxa through DNA barcodes. This is because the original method is based on subjective cut-off distance measures to make inferences about species designation (e.g. Hebert et al. 2003a, b, 2004a, b), which have been questioned by several authors both as theoretical and practical aspects (e.g. Desalle et al. 2005; Brower 2006; Meier et al. 2006). This method delimits species based on a unique combination of nucleotides within a site shared by individuals of the same population or group of populations. In addition, species were molecularly diagnosed by nucleotide substitutions as proposed by Costa et al. (2014). Optimization of nucleotide substitutions among

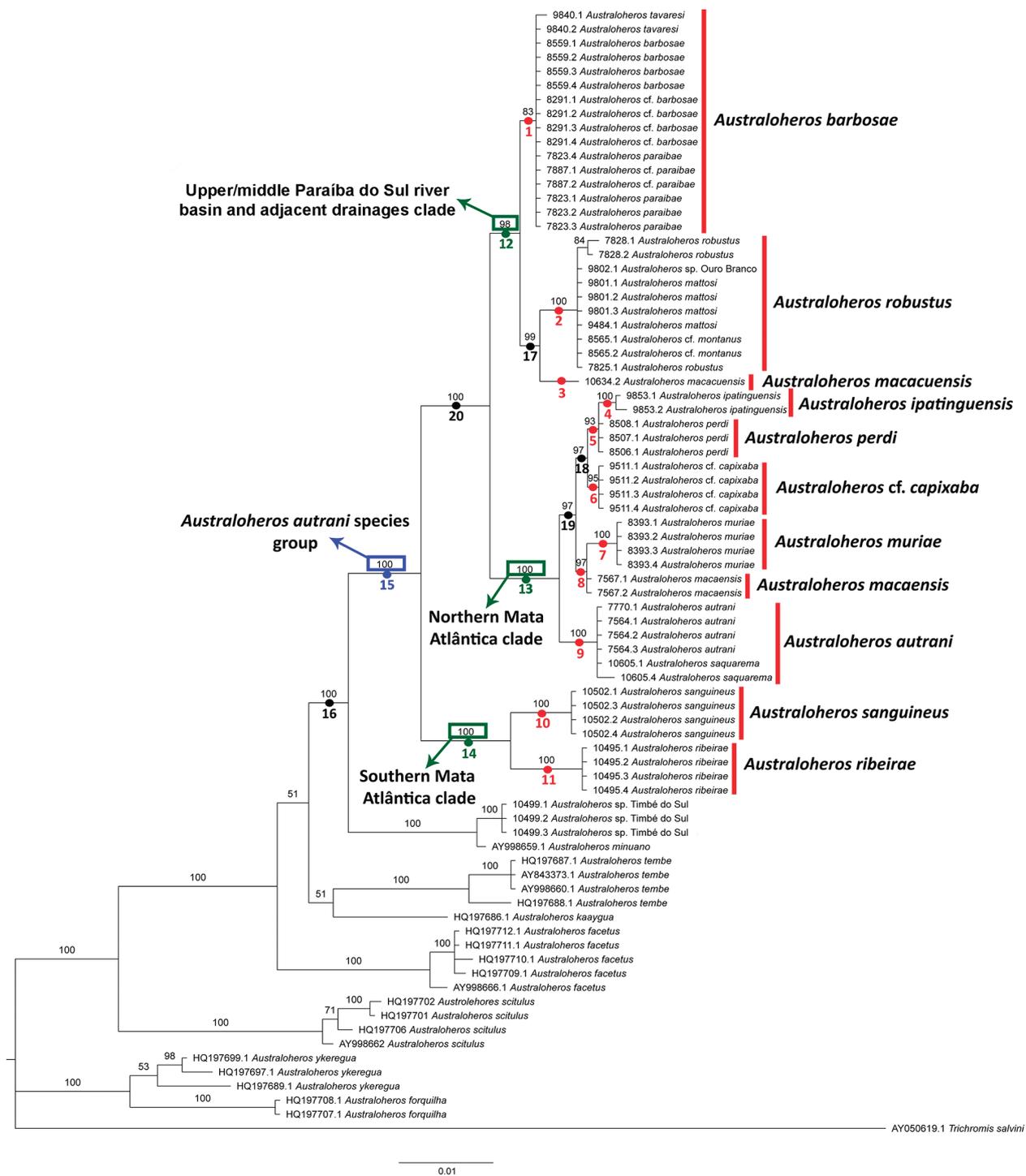


Figure 2. Phylogenetic haplotype tree based on Bayesian Inference (BI). Numbers above branches are posterior probability values, and below branches are numbered nodes which represent the combination of nucleotide substitution which define the species (in CBB) or clades. The nucleotide substitutions (CBB) can be checked in box1. Posterior probability value supporting the *Australoheros autrani* group is indicated in blue, as well as, the three clades herein proposed within this species group are indicated in green. Species of the in-group delimited through the tree based method (WP) are indicated with red bars, as well as, the species of the in-group delimited by nucleotide substitution method (CBB) have their nodes marked in red.

lineages of *Australoheros* were obtained from the Bayesian topology, using PAUP4 (Ronquist et al. 2002). Each nucleotide substitution is represented by its relative numeric position determined through sequence alignment

with the complete mitochondrial genome of *Astronotus ocellatus* (Mabuchi et al. 2007), followed by the specific nucleotide substitution in parentheses. The results are presented in Figure 2 and Box 1.

Box 1. List of nucleotide substitutions from each lineage (species) and some crucial points of the cladogram of the Fig. 2. **1–11** the delimited species of the *Australoheros autrani* species group according to the CBB, **12–14** the three clades herein proposed within *A. autrani* group, **15** the *A. autrani* group, **16–20** other points of the cladogram. When a nucleotide substitution is exclusive from that lineage or clade, not occurring in any other point of the cladogram it is marked with “*”, when a nucleotide substitution is exclusive within the *A. autrani* group it is marked with “***”.

CBB:

1 (exclusive combination of nucleotide substitution of *A. barbosa*) - Cytb258 (C→T*), Cytb474 (T→C), **2** (exclusive combination of nucleotide substitution of *A. robustus*) - Cytb348 (G→A**), Cytb612 (G→A), Cytb 684 (C→T**), Cytb 978 (G→A), Cytb 1.032 (A→G*), **3** (exclusive combination of nucleotide substitution of *A. macacuensis*) - Cytb120 (T→C), Cytb 127 (G→A**), Cytb 561 (T→C), Cytb 822 (T→C**), Cytb 939 (A→G*), **4** (exclusive combination of nucleotide substitution of *A. ipatinguensis*) - Cytb 78 (T→C), Cytb 1.070 (T→C*), **5** (exclusive combination of nucleotide substitution of *A. ipatinguensis* + *A. perdi*. *Australoheros perdi* differs from *A. ipatinguensis* by the absence of the nucleotide substitutions listed in number 4) - Cytb 700 (A→G*), **6** (exclusive combination of nucleotide substitution of *A. cf. capixaba*) - Cytb 837 (C→T**), **7** (exclusive combination of nucleotide substitution of *A. muriae*) - Cytb 435 (C→T**), Cytb 519 (T→C), Cytb 653 (A→G*), Cytb 960 (A→G**), **8** (exclusive combination of nucleotide substitution of *A. macaensis* + *A. muriae*. *Australoheros macaensis* differs from *A. muriae* by the absence of the nucleotide substitutions listed in number 7) - Cytb 1.041 (T→C**), **9** (exclusive combination of nucleotide substitution of *A. autrani*) - Cytb 270 (C→T*), Cytb 357 (T→C**), Cytb 519 (T→C), Cytb 564 (C→T**), Cytb 864 (A→G*), **10** (exclusive combination of nucleotide substitution of *A. sanguineus*) - Cytb 63 (C→A*), Cytb 120 (T→C), Cytb 204 (C→T*), Cytb 219 (A→G*), Cytb 405 (A→G**), Cytb 474 (T→C), Cytb 967 (T→C*), Cytb 1.044 (T→C**), **11** (exclusive combination of nucleotide substitution of *A. ribeirae*) - Cytb 114 (A→C*), Cytb 364 (C→T), Cytb 390 (C→T*), Cytb 408 (A→G), Cytb 528 (A→C*), Cytb 741 (T→C**), Cytb 786 (G→A**), Cytb 813 (A→G*), Cytb 870 (G→A**), Cytb 897 (T→C**), Cytb 1.003 (T→C*).

Other relevant nucleotide substitutions:

12- Cytb 465 (C→T**), Cytb 846 (A→G*), Cytb 917 (T→C**), **13** - Cytb 364 (C→T), Cytb 408 (A→G), Cytb 540 (C→T*), Cytb 552 (C→T*), Cytb 784 (T→C**), Cytb 825 (A→G*), Cytb 867 (A→G**), Cytb 954 (G→A**), Cytb 978 (G→A), Cytb 993 (T→C**), **14-** Cytb 352 (G→A**), Cytb 354 (T→C*), Cytb 438 (T→C*), Cytb 630 (T→C*), Cytb 688 (T→C**), Cytb 690 (A→G*), Cytb 726 (T→C*), Cytb 906 (A→G*), Cytb 916 (G→A**), Cytb 945 (T→C**), Cytb 1.020 (A→G**), Cytb 1.047 (T→C**), Cytb 1.074 (T→C), **15** - Cytb 345 (T→C), Cytb 474 (C→T), Cytb 589 (C→T*), Cytb 723 (C→T), Cytb 741 (C→T), Cytb 795 (G→A), Cytb 807 (A→G*), Cytb 852 (T→C), Cytb 897 (C→T), Cytb 978 (A→G), Cytb 1.038 (A→C), **16** - Cytb 303 (G→A), Cytb 768 (C→T*), Cytb 954 (A→G), Cytb 960 (G→A*), Cytb 1.026 (C→A*), **17** - Cytb 585 (T→C**), Cytb 721 (G→A**), **18-** Cytb 561 (T→C), **19** - Cytb 114 (A→G**), Cytb 612 (G→A), **20** - Cytb 78 (C→T**), Cytb 141 (A→G*), Cytb 174 (T→C*), Cytb 351 (T→C*), Cytb 600 (T→C**), Cytb 750 (G→A**), Cytb 891 (C→T*), Cytb 924 (C→T**).

The bPTP is a coalescent phylogeny-based species delimitation method intended to delimit species based on single locus molecular data (Zhang et al. 2013). An advantage of bPTP is that it does not need an ultrametric calibration like other coalescent approaches, avoiding errors and computer intensive processes (Zhang et al. 2013). The method relies on the number of substitutions between haplotypes and assumes that more molecular variability is expected between species than within a species (Zhang et al. 2013). In the present bPTP analysis, the dataset was reduced to include only unique haplotypes from species of the *A. autrani* group. Outgroups were restricted to *A. facetus* (AY998666.1), *A. kaaygua*, *A. minuano* and *Australoheros* sp. Timbé do Sul. The evolution model HKY+G for the reduced dataset was calculated in Jmodeltest 2.1.7 program (Darriba et al. 2012). The input phylogenetic tree was performed in Beast v.1.8 (Drummond et al. 2012) with a lognormal uncorrelated relaxed clock model and coalescent constant size tree priors. Bayesian inference was performed with the following settings: 50 million MCMC generations, and a sampling frequency of 100. The value of parameters of the analyses, sample size and the stationary phase of the chains were evaluated using Tracer v. 1.6 (Rambaut et al. 2014). A birth-death speciation process was applied as the tree prior (Gernhard 2008).

The bPTP analysis was performed in the Exelixis Lab's web server <http://species.h-its.org/ptp/>, following the default parameters except for a 20% burn-in and the tree was rooted on *A. facetus*. The results are presented in Figure 3.

Phylogenetic analysis

After performing the different species delimitation methods, only one haplotype from each species recovered by the congruence of all species delimitation methods was included (Suppl. material 1: Table S1) to avoid confusion, and a phylogenetic analysis was conducted. The terminal out-group taxa comprised several lineages representing all *Australoheros* species groups proposed by Řičan and Kullander (2008), an additional population from southern Santa Catarina state, and more distantly *Trichromis salvini*, *Heros appendiculatus*, and more distantly *Hoplarchus psittacus* (Suppl. material 1: Table S1).

The protein-coding sequences were partitioned by codon position. The Jmodeltest 2.1.7 program (Darriba et al. 2012) was used to define the most appropriate evolutionary model for each partition, and the choice for the best model was based on the Akaike information criterion. The first codon position was SYM+I+G, the 2nd codon position HKY+I and the 3rd codon position GTR+G. The topology was generated by Bayesian inference (BI)

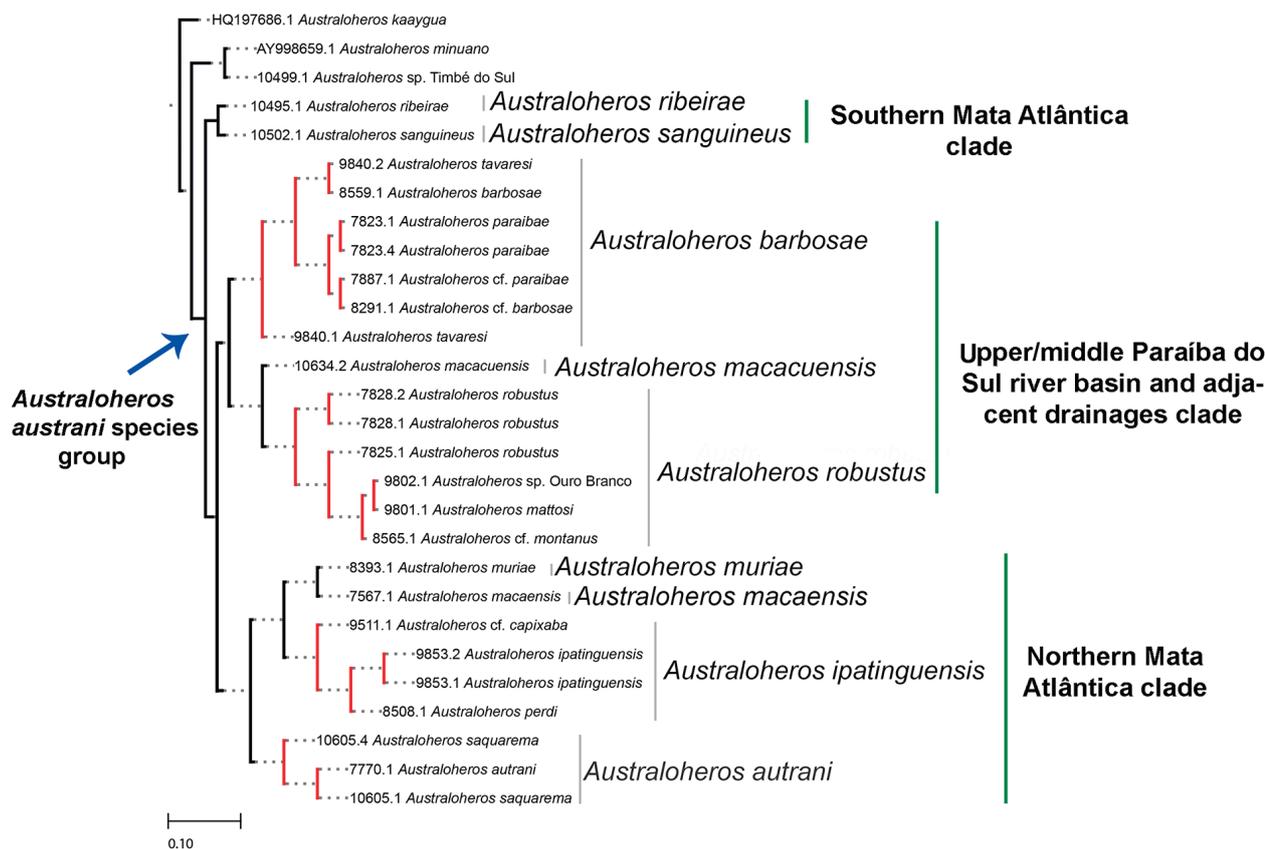


Figure 3. Species delimitation tree generated by the Bayesian Poisson Tree Processes (bPTP) model, using a fragment of the mitochondrial gene CYTB. Black lines indicate branching processes among species, red lines indicate branching processes within species. Species of the *A. austrani* species group delimited through bPTP are indicated with grey bars.

using MrBayes 3.2.5 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). It was performed with the following settings: two Markov chain Monte Carlo (MCMC) runs of four chains each for 20 million generations, sampling frequency of 1000. All parameters between partitions except topology and branch lengths were unlinked. The convergence of the MCMC chains were graphically assessed by evaluating the stationary phase of the chains using Tracer v. 1.6 (Rambaut et al. 2014). Consensus topology and posterior probabilities were obtained after applying a burn-in of the first 25% of the generated trees.

The divergence time analysis was performed in Beast v.1.8 (Drummond et al. 2012), using the same dataset, partitions and evolution models as described above, and a lognormal uncorrelated relaxed clock model. Bayesian inference was performed with 50 million generations of MCMC runs and a sampling frequency of 1000. A Yule speciation process was applied as the tree prior (Gernhard 2008). Due to the absence of closely related fossils, we used indirect calibration points, comprising two node date estimates by Matschiner et al. (2017). The oldest node was the divergence between the clade *Hoplarchus* Kaup, 1860 plus *Hypselecara* Kullander, 1986 and the clade containing the *Heros* Heckel, 1840 clade plus the

Australoheros clade (prior setting: normal distribution, mean = 53 and standard deviation = 2.0). The second node was the divergence between the clade comprising the genera *Heros*, *Symphysodon* Heckel, 1840, *Mesonauta* Günther, 1862, *Uaru* Heckel, 1840, and the *Australoheros* clade (prior setting: normal distribution, mean = 47 and standard deviation = 2.0).

Results

Species delimitation

WP and CBB

These species delimitation analyses produced identical results, delimiting 11 lineages (species) within the *A. austrani* group (Fig. 2, Box 1). Among them, seven species (*A. ipatinguensis*, *A. macaensis*, *A. macacuensis*, *A. muriae*, *A. perdi*, *A. sanguineus*, and *A. ribeirae*) previously delimited on the basis of morphological characters are corroborated. One species tentatively identified as *A. capixaba* is herein corroborated. Seven nominal species (*A. austrani*, *A. barbosae*, *A. mattosi*, *A. paraibae*, *A. robustus*, *A. saquarema*, and *A. tavaresi*) and one species tentatively identified as *A. montanus* were clustered into

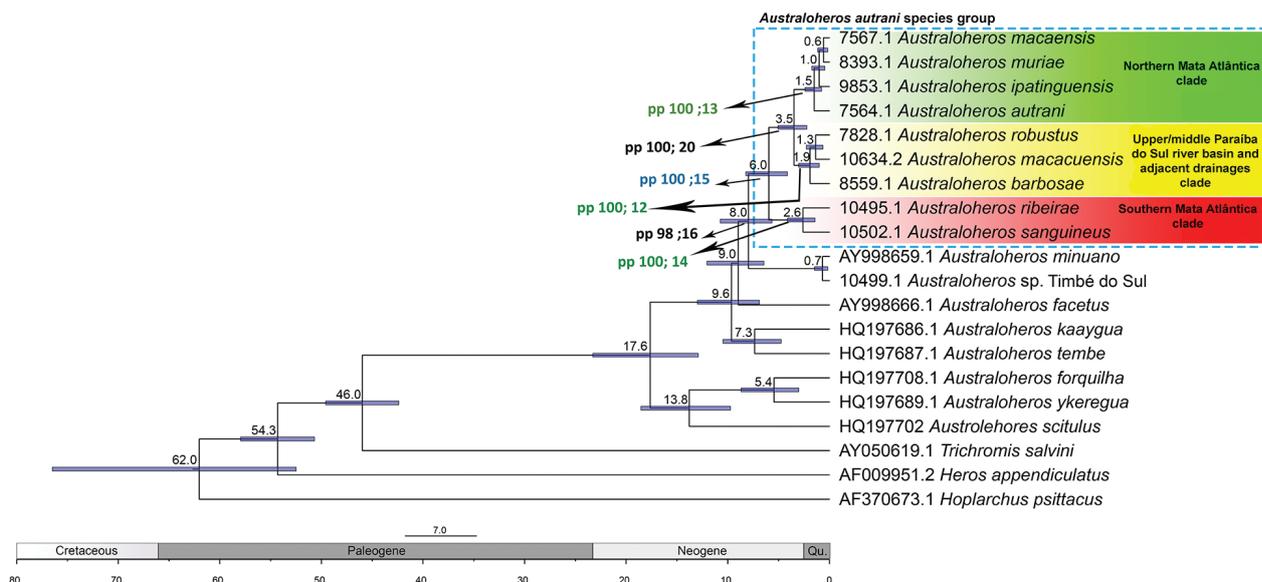


Figure 4. Time-scaled phylogeny obtained from the Bayesian analysis in BEAST. Values above nodes are mean average ages of the nodes, followed below blue bars representing the 95% highest posterior densities intervals for estimated ages; numbers indicated by arrows are the posterior probability obtained from the Bayesian analysis in MrBayes followed by the node number, corresponding to the node numbers of the Figure 2 and Box 1. PP means posterior probability.

three species, which following chronological priority for zoological species names are hereafter called *A. austrani*, *A. barbosa*, and *A. robustus* (Fig. 2, Box 1).

bPTP

This species delimitation analysis indicates nine lineages (species) within the *A. austrani* group (Fig. 3). Among them, five species (*A. macacuensis*, *A. macaensis*, *A. muriae*, *A. ribeirae*, and *A. sanguineus*) previously delimited on the basis of morphological characters are corroborated. Nine nominal species (*A. austrani*, *A. barbosa*, *A. ipatinguensis*, *A. mattsosi*, *A. paraibae*, *A. perdi*, *A. robustus*, *A. saquarema*, and *A. tavaresi*) and two species tentatively identified as *A. capixaba* and *A. montanus* were clustered into four species, which following chronological priority for zoological species names are hereafter called *A. austrani*, *A. barbosa*, *A. ipatinguensis*, and *A. robustus* (Fig. 3).

Phylogeny

The monophyly of the *A. austrani* species group was highly supported with maximum node support value and diagnosed by 11 nucleotide substitutions (Fig. 4, Box 1). The sister clade of the *A. austrani* species group was the clade comprising *Australoheros* sp. Timbé do Sul and *A. minuano*, and this sister group relationship was supported by 98% of posterior probability and by five synapomorphic nucleotide substitutions (Fig. 4, Box 1).

The phylogenetic analyses support three main clades within the *A. austrani* species group. First an Upper/middle Paraíba do Sul river basin and adjacent drainages clade: including *A. barbosa*, *A. macacuensis*, and *A. ro-*

bustus, sister group of the Northern Mata Atlântica clade, supported by maximum value, and three synapomorphic nucleotide substitutions (Fig. 4, Box 1). This clade occurs along the upper/middle Rio Paraíba do Sul basin, Rio Macacu basin, upper Rio Tietê, and Rio Grande drainages, tributaries of the upper Rio Paraná river basin, and upper Rio Paraopebas, and Rio das Velhas drainages, tributaries of the upper Rio São Francisco basin, as well as, in a headwaters border area between the Rio Doce and São Francisco river basins, eastern Brazil (Fig. 1).

Second, a Northern Mata Atlântica clade: including *A. austrani*, *A. ipatinguensis*, *A. macaensis*, and *A. muriae*; sister group to the aforementioned clade, corroborated by maximum node support value and by 10 synapomorphic nucleotide substitutions (Fig. 4, Box 1). This clade occurs along the coastal river basins from the Saquarema lagoon system, in south-eastern Brazil, to the Buranhém river basin, in northeastern Brazil, including tributaries of the lower Rio Paraíba do Sul and from the Rio Doce basin (Fig. 1).

Third, a Southern Mata Atlântica clade: including *A. ribeirae* and *A. sanguineus*, the most basal clade within the *A. austrani* species group, corroborated by maximum node support value and by 13 synapomorphic nucleotide substitutions (Fig. 4, Box 1). This clade occurs in the Rio Ribeira do Iguape basin, and in the Rio Cubatão basin of Baía de Babitonga system, eastern Brazil (Fig. 1).

Divergence-time estimation

Divergence time estimates (Fig. 4) indicate that the origin of the *Australoheros* and its diversification occurred around 46 million years ago (hereafter MYA), during the middle Eocene, and around 17.6 MYA (ranging from 12.2 to 22.7 MYA following 95% highest posterior density in-

terval [HPD]), during the middle Miocene, respectively. The origin of the *A. austrani* group occurred during the late Miocene (8 MYA; 95% HPD: 5.5–10.4 MYA), and its diversification started at about 6 MYA. The diversification within the three main clades of the *Australoheros austrani* species group occurred almost synchronically, between 2.6 and 1.5 MYA during the Pleistocene (Fig. 4).

Discussion

Species delimitation and species diversity

Many species of *Australoheros* can be definitely considered cryptic species for three reasons. This is especially true of the species which are distributed in eastern Brazil. (1) In previous studies, these were identified and classified as *Australoheros facetus*, a species whose type locality is in the north of Uruguay. Even now, in some cases, species and populations of the *A. austrani* group have been erroneously classified and considered as populations of *A. facetus*. (2) According to Řičan et al. (2011), many of the species from the *A. austrani* group are very similar morphologically and difficult to diagnose. Some of those are distinguished only based on characters of colouration in life, that cannot be observed in preserved specimens deposited in collections and museums, or based on characters of internal anatomy (e.g. Ottoni et al. 2008; Ottoni 2010, 2011, 2012, 2013a, b; Ottoni and Schindler 2014; Ottoni et al. 2014). And, for practical reasons, are not used in the vast majority of ichthyological inventories. (3) The genus *Australoheros* has a taxonomically “confused” and controversial history, including different lists of nominal species.

The coastal river basins of eastern Brazil are characterized by a low diversity of fish groups when compared to other areas, such as the Amazon basin. However, in spite of this relatively low diversity, the groups that occur in this region present a high degree of endemism along the river drainages, and speciation events along eastern Brazil are probably related to a complex palaeogeographical history (Ribeiro 2006; Buckup 2011; Thomaz and Knowles 2018). The high degree of endemism of freshwater fish in this region has been already suggested by Bizerril (1994) and Ribeiro (2006). This has provided the basis for the delimitation of this region as constituting a particular biogeographical area named the “south-eastern Brazil province” and different versions of the “eastern Brazil province” (Eigenmann 1909; Géry 1969; Ringuet 1975; Lévêque et al. 2008). More recently, these provinces were subdivided into a series of ecoregions or subprovinces, based on specific ecological and faunistic characteristics (e.g. Carvalho 2007; Abell et al. 2008). It is interesting to note that there is general correspondence between Pleistocene paleodrainages and these proposed biogeographic regions (Thomaz and Knowles 2018). In addition, the present geomorphologically isolated eastern coastal river basins were probably isolated in the past as suggested by the limits of proposed Pleistocene paleodrainages (Thomaz and Knowles 2018). Thus,

it is clear that the river drainages of eastern Brazil do not correspond to a single uniform biogeographic area of endemism (Menezes 1988; Bizerril 1994; Carvalho 2007; Abell et al. 2008; Buckup 2011; Thomaz and Knowles 2018). The existence of mountainous areas and elevated regions, often more than 1000 m in altitude in south-eastern Brazil, also contributed to the isolation of river drainages and populations, and probably promoted speciation events (Buckup 2011).

The speciation events within the *A. austrani* species group occurred about 2.6–0.6 MYA during the Pleistocene (Fig. 4). The species delimitation methods conducted herein demonstrate a high degree of endemism within this group, as expected according to the biogeographic history of the region. The WP and CBB were more sensitive than the other method (bPTP), delimiting 11 lineages within the *Australoheros austrani* species group: *A. austrani*, *A. barbosa*, *A. ipatinguensis*, *A. macaensis*, *A. macacuensis*, *A. muriae*, *A. perdi*, *A. ribeirae*, *A. robustus*, *A. sanguineus*, and *A. cf. capixaba* (Fig. 2, Box 1). The bPTP method was more conservative, delimiting just nine lineages: *A. austrani*, *A. barbosa*, *A. ipatinguensis*, *A. macaensis*, *A. macacuensis*, *A. muriae*, *A. ribeirae*, *A. robustus*, and *A. sanguineus* (Fig. 3). Despite the distinct number of delimited lineages among these methods, the results are fully congruent if considering two of the three clades of the *A. austrani* species group, except for the Northern Mata Atlântica clade. In this specific clade, the WP and CBB delimit the same lineages and more species than the bPTP (six against four) (Figs 2, 3, Box 1). According to De Queiroz (2007) and Goldstein and Desalle (2010), species delimited by multiple pieces of evidence and different species delimitation methods produce stronger hypotheses. However, as argued by De Queiroz (2005, 2007), any criteria (species delimitation methods) may separately provide evidence about the species limits independently from other criteria, and any property that provides evidence of lineage separation is relevant to infer boundaries and number of species. In addition, the same author also argued that distinct operational criteria could delimit different lineages, as they could have different degrees of sensitivity in relation to the lineages divergence (speciation) and different cut-offs for considering a separately evolving lineage (De Queiroz 2007: fig. 1). It is important to emphasize that every species is a hypothesis, which potentially is refuted or corroborated by subsequent studies (De Queiroz 2005, 2007). Therefore, we do not favour any of the methods, and we consider the possibility of two slightly variant scenarios: one based on the congruent results between WP and CBB, considering 11 lineages (species) along the river drainages of eastern Brazil (Fig. 2, Box 1) and the other based on the result of bPTP, considering only nine lineages (species) (Fig. 3). However, for the phylogenetic analysis we decided to follow the congruence of these species delimitation methods herein applied to avoid confusion. Thus, we considered just nine species for the time calibrated phylogenetic analysis.

Similar to the *A. autrani* group, in that several species occur along the latitudinal zonation of eastern Brazil, a congruent distribution pattern and endemism is also shared by other freshwater fish groups in this region, such as: Delturinae (family Loricariidae) (Reis et al. 2006; Buckup 2011), Neoplecostominae (family Loricariidae) (Abell et al. 2008; Buckup 2011; Roxo et al. 2014), the genera *Mimagoniates* Regan, 1907 and *Oligosarcus* Günther, 1864 (family Characidae) (Menezes et al. 2007, 2008; Buckup 2011; Ribeiro and Menezes 2015), the genus *Brycon* Müller & Troschel, 1844 (Bryconidae) (Menezes et al. 2007; Abe et al. 2014; Travenzoli et al. 2015), the genus *Characidium* Reinhardt, 1867 (Crenuchidae) (Menezes et al. 2007; Poveda-Martínez et al. 2016), the genera *Listrura* de Pinna, 1988, *Microcambeva* Costa & Bockmann, 1994, and *Trichomycterus* Valenciennes, 1832 (family Trichomycteridae) (Barbosa and Costa 2010; Abell et al. 2008; Buckup 2011; Mattos and Lima 2012; Villa-Verde et al. 2013; Katz and Barbosa 2014), the genus *Microglanis* Eigenmann, 1912 (family Pseudopimelodidae) (Sarmiento-Soares et al. 2006; Ruiz and Shibatta 2010; Mattos et al. 2013), several killifish genera (family Aplocheiliidae) (Abell et al. 2008; Buckup 2011; Costa 2014, Costa and Amorim 2014; Costa et al. 2014), the genus *Phalloceros* Eigenmann, 1907 (family Poeciliidae) (Lucinda 2008), and the “*Geophagus brasiliensis*” species group and the genus *Crenicichla* Heckel, 1840 (family Cichlidae) (Kullander and Lucena 2006; Mattos 2014; Mattos et al. 2015; Mattos and Costa 2018).

In addition to the complex paleogeographical history of eastern Brazil, which probably promoted speciation in *Australoheros*, we also cannot ignore the behavioural characteristics of cichlids. Species from this family are usually territorial, not carrying out extensive migratory movements, forming pairs during the reproductive periods, and with different strategies of parental care, from protection of eggs to juveniles in their early stages of life (Kullander 2003). Such ethological characteristics, together with the history of the drainages, probably reflect species dispersal. It usually favours restricted geographic distribution to a single river, or even in one or a few streams and tributaries (Kullander 2003). This is taken as supporting evidence, reinforcing the present result recognizing several species of *Australoheros* along eastern Brazil and making the idea of the existence of just one species of *Australoheros* along the river systems of eastern Brazil more unlikely.

Monophyly, phylogeny and age of the *Australoheros autrani* species group, and internal clades

The origin of the genus *Australoheros* lineage is dated herein from about 46 MYA, during the middle Eocene (Fig. 4), and its diversification from about 17.6 MYA age, during middle Miocene. The sister group of the *A. autrani* species group is a clade comprising *Australoheros* sp. Timbé do Sul, known from a coastal river drainage in southern Brazil and *A. minuano*, a species distributed in tributaries of the middle and lower Uruguay river in

Brazil (Rio Grande do Sul state) and Uruguay (Řičan and Kullander 2008). This relationship is herein suggested for the first time and is highly supported by 98% of the posterior probability value and by five synapomorphic nucleotide substitutions (Fig. 4, Box 1). The *A. autrani* species group is corroborated as a monophyletic clade by maximum node support value and by 11 nucleotide substitutions (Fig. 4, Box 1). The origin of the *A. autrani* species group is dated from about 8 MYA, during the late Miocene, when this lineage diverged from its sister group (including *Australoheros* sp. Timbé do Sul and *A. minuano*). However, its diversification occurred only about 6 MYA. The *A. autrani* species group is divided into three clades. The Southern Mata Atlântica clade is the most basal clade within the *A. autrani* species group, and its origin is dated from about 6 MYA, during the late Miocene, when this clade diverged from the lineage comprising the two other clades within the *A. autrani* species group (Fig. 4). The Southern Mata Atlântica clade includes the species that occur in the Ribeira do Iguape river basin, and in the Cubatão river basin of the Babitonga bay system, eastern Brazil (Fig. 1). The Northern Mata Atlântica clade includes the species that occur along the coastal river basins from the Saquarema lagoon system, in south-eastern Brazil, to the Buranhém river basin, in north-eastern Brazil, including the Doce river tributaries and the lower Paraíba do Sul river basin (Fig. 1). This clade has its origin about 3.5 MYA, during the middle Pliocene, when it diverged from the upper/middle Paraíba do Sul river basin and adjacent drainages clade with the same age of origin (Fig. 4). This latter clade includes the species which occur along the upper/middle Paraíba do Sul river basin, Macacu river basin, upper Tietê and Grande river drainages, of the upper Paraná river basin, and upper Paraopebas and das Velhas river drainages, of the upper São Francisco river basin, eastern Brazil (Fig. 1).

Biogeographic history of the river systems of eastern Brazil and its impact on *Australoheros* diversification pattern

Geological evidence suggests that the continental margin of south-eastern Brazil passed through instability events during three main phases (90–75 MYA; 50–40 MYA; and 25–0 MYA), separated by quieter intervals, coinciding with phases of Andean orogeny (Ribeiro 2006). In the more recent geological instability phase of the eastern region of the Brazilian shield, several cases of changing boundaries, headwater captures and connections of drainage systems between coastal and inland rivers occurred (Ribeiro 2006; Buckup 2011). These headwater captures promoted faunistic exchange between river basins, but likewise changing headwater catchments and boundaries of drainage systems certainly would have contributed towards isolation of populations too, which in some cases resulted in speciation events. This is considered responsible for shaping the extant freshwater fauna to large extent.

This geological instability of the region resulting in headwater stream capture events and connections of

drainage systems was first observed by Ihering (1898), who proposed an ancient connection between the Paraíba do Sul river basin and the upper Tietê river drainage (tributary of the Paraná river basin). Several other authors have also suggested this ancient connection between these two river basins based on ichthyofaunistic, geological, or paleontological similarities (e.g. Ab'saber 1957; Menezes 1970; Langeani 1989; Riccomini 1990; Lundberg et al. 1998; Malabarba 1998; Ribeiro 2006). Thereby part of the Paraíba do Sul river basin (its upper portion) was subject to a faunistic exchange with the upper Tietê river basin (Menezes 1970; Langeani 1989; Buckup 2011).

The results of the present study corroborate the idea of this complex biogeographic history. The Paraíba do Sul is the river basin of south-eastern Brazil revealing the highest diversity for the genus *Australoheros*. The present paper demonstrates three lineages (species) of *Australoheros* occurring along that river basin: *A. barbosa*, occurring in its upper/middle portion, *A. robustus*, occurring in the middle portion, and *A. muriae*, occurring in the lower portion. This fact is not common along the river basins of south-eastern Brazil, most of which only include one *Australoheros* species (Fig. 1).

Australoheros barbosa, besides occurring in the upper/middle Paraíba do Sul river basin, also occurs in the upper Tietê river drainage, corroborating the suggested hypothesis of the existence of an ancient connection between these river basins. The species also occurs in the upper Grande river drainage (another river drainage of the Paraná river basin), which also suggests a possible ancient connection between these river drainages. *Australoheros robustus*, besides occurring in the middle portions of the Paraíba do Sul river basin, also occurs in the river drainages of the Paraopeba and Rio das Velhas (tributaries of the upper São Francisco river basin) (Fig. 1). This indicates that faunistic exchange also occurred between the Paraíba do Sul river basin and other hydrographic basins of south-eastern Brazil, caused by headwater captures during phases of instability in the Brazilian Shield (Buckup 2011). In addition, a similar case occurs with *A. macacuensis* which occurs in the Macacu river basin, a coastal river discharging at the Guanabara Bay, despite being member of the Upper/middle Paraíba do Sul river basin and adjacent drainages clade. This suggests a former close relationship between these river systems, and possibly another stream capture event (Figs 1–3).

However, the geologically documented separation of the Tietê river basin from the Upper Paraíba do Sul river basin, which in ancient times were connected discharging directly into the Atlantic ocean, occurred between 20–11.8 MYR (Lundberg et al. 1998). This dating is well before the molecular dating of the origin and diversification of the *A. autrani* species group (about 8.0 and 6.0 MYA, respectively) and even more so before the origin and diversification of the Upper/middle Paraíba do Sul river basin and adjacent drainages clade (about 3.5 and 1.9 MYA, respectively) (Fig. 4). Therefore, the occurrence of *A. barbosa* both in the Paraíba do Sul and in

the Upper Tietê river basins must be the result of more recent headwater captures and river drainages connections between these river systems, allowing species dispersal, although not followed by a speciation event. Another kind of event that may contribute to the distribution pattern and diversity of the freshwater fish species of the region were sea-level fluctuations (Weitzman et al. 1988; Lundberg et al. 1998). These continuously isolated and reconnected river and basins estuaries, allowing both dispersals of species (when rivers or basins were connected) or isolation of populations, which in some cases was reflected in speciation events. Sea-level fluctuation occurred all through the history of the South American continent (Lundberg et al. 1998), convincingly demonstrated from the Late Miocene to today (8 MYA to the present). Mainly during the Pleistocene, this phenomenon was cyclical and may have resulted directly in the connection and isolation of river systems. However, as pointed out by Thomaz and Knowles (2018), the Brazilian Pleistocene coastal paleodrainages were characterized by pronounced isolation and past connections and disconnections forced by sea-level changes have not been broadly considered as a factor greatly interfering with speciation processes within freshwater fish groups.

However, in the *A. autrani* group some species could have been affected by the sea-level fluctuation. One example is *A. autrani*, which nowadays forms isolated populations in small isolated coastal river systems (São João river basin and Saquarema lagoon system). These isolated populations had some degree of contact during the Pleistocene regression of the sea level. Similarly, we cannot discard the role of sea-level fluctuations in the Pleistocene diversification patterns among the Northern Mata Atlântica clade, opposing to the idea presented by Thomaz and Knowles (2018). In contrast, the biogeographical pattern corroborates the isolation of *A. ribeirae* and *A. sanguineus*, from the Southern Mata Atlântica clade, as suggested by Thomaz and Knowles, arguing that Pleistocene paleodrainages are much more isolated than previously suggested, promoting a high degree of endemism.

Conclusions and future perspectives

This study confirms the importance of integrating different methods for species delimitation, as suggested by several proponents of “Integrative Taxonomy” (e.g. Wiens and Penkrot 2002; De Queiroz 2007; Goldstein and Desalle 2010; Padial et al. 2010, Costa et al. 2012, 2014). The application of different approaches and reconciliation of different lines of evidence makes the delimitation of species more reliable and accurate (Goldstein and Desalle 2010). The recognition and confirmation of morphologically hidden or intricate lineages (species) is especially promising by using molecular methods (Bickford et al. 2006; Adams et al. 2014). This type of approach is highly recommended for groups including cryptic species or/and with a confusing and controversial taxonomy.

Accurate estimates of biodiversity are of utmost importance due to the rapid loss, degradation, intense destruction and modification of natural environments by anthropic actions, especially in tropical areas (Wilson 1985; Brook et al. 2006). Thus, cataloguing and recognizing the biological diversity of different taxonomic groups with high accuracy is crucial for the field of species conservation and should be treated as a priority (Wilson 1985; Brooks et al. 2002; Brook et al. 2006; Wheeler 2008; Costa et al. 2012).

The present study recognizes nine lineages (species) or 11 within the *A. autrani* species group, depending on the species delimitation method, grouped into three distinct and highly supported clades: the Southern Mata Atlântica clade, the Upper/middle Paraíba do Sul river basin and adjacent drainages clade, and the Northern Mata Atlântica clade. Four to five formerly described species are not resolved with the genetic species delimitation methods used in this study, but the majority of the species of the *A. autrani* group are well supported. However, it does not mean that knowledge of the number of lineages (species) occurring along the coastal river basins of eastern Brazil is complete. If more haplotypes of other populations are included in these species delimitation tests more lineages (species) may be recovered, whereas some lineages could be coalesced. Therefore, analyses including more populations and more genes are encouraged, in particular for the Northern Mata Atlântica clade, which is the clade within the *A. autrani* species group that presents incongruences related to the number of delimited species between methods here employed.

According to the present study, is clear that the *A. autrani* species group represents a valid and monophyletic group. It also contrasts the hypothesis that all species represent a widely distributed *A. facetus*. In fact, *A. facetus* was not even recovered as sister to the *A. autrani* group. This study thus represents a major contribution towards the knowledge and conservation of *Australoheros*. Finally, the time calibrated analysis provides dates of origin and diversification of *Australoheros* for the first time. Despite the origin of the *A. autrani* group in the late Miocene, species level diversification occurred in the Pleistocene and was probably driven by headwater capture events and sea-level fluctuations.

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Supplementary material 1

Taxon sampling, voucher catalogue number, GenBank accession number, gene sequenced country, drainage and new sequence in the present study

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