



DNA barcode sheds light on systematics and evolution of neotropical freshwater trahiras

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Abstract

We assessed the presence of independent evolving lineages of the trahira, *Hoplias malabaricus*, one of the few freshwater fish species having wide distribution in the Neotropics which is the region with the highest global diversity of freshwater fish. To achieve that goal, 58 mitochondrial sequences of cytochrome c oxidase subunit I (COI; DNA barcoding) were generated from collected samples and 85 obtained from public databases, which were analyzed in comparison to chromosomal and geological data. The magnitude of genetic diversity found among different sampling sites was greater than 2%. Molecular species delimitation methods indicated the existence of a least four distinct lineages. The recognised cytotypes did not form monophyletic groups, suggesting that the karyotypic macrostructure could be a homoplastic character. The haplotype relationships suggested secondary contacts between the ecoregions of Northern and Northeastern Brazil that were shaped by coastal routes between adjacent watersheds during the Pleistocene epoch and probable exchanges of their ichthyofaunas. Our results indicated that multiple factors have driven the diversification of *H. malabaricus*, from ancient geological events linked to the reactivation of tectonic faults to more recent occurrences related to eustatic changes in ocean levels. Ultimately, the magnitude of its genetic diversity suggests the necessity of revising its taxonomic status.

Keywords *Hoplias malabaricus* · Phylogeography · Cryptic diversity · Characiformes · Species delimitation

Introduction

The Neotropical region harbours the highest amount of freshwater fish biodiversity in the world (Reis et al. 2016) and understanding the processes that underlie diversification

has been a challenge for biologists. One of the major challenges facing biogeography in the Neotropics is the reconstruction of paleohydrological patterns that are major drivers of the freshwater fish diversity (Albert and Reis 2011). Hydrological basins from this region harbour the greatest ichthyofauna diversity in the world, with more than 5000 described species (Reis et al. 2016). This elevated species richness appears to be associated with a series of intricate geomorphological, climatic and ecological processes that

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have taken place since the split of the continental blocks of Gondwana (Ribeiro et al. 2016). In that context, continental fish represent useful models for understanding diversification in hydrological systems, as they depend on the connectivity of water bodies (current or past) to maintain gene flow between distant river/lagoon populations. They can, therefore, be used to trace fluvial connections, both geographically and chronologically (Myers 1938; Montoya-Burgos 2003; Roxo et al. 2014).

Molecular studies of Neotropical freshwater fish have revealed distinct biogeographical patterns derived mainly by processes of dispersion and ancient vicariance (Roxo et al. 2014). Several studies have shown the complexity involved in reconstructing biogeographic processes, and the difficulties in identifying the patterns that can drive species diversification (Torres and Ribeiro 2009; Mai et al. 2016; Lima et al. 2016). To investigate paleohydrological histories and to explain how diversification processes affect ichthyofauna, studies should include species with wide geographic distributions as models (Albert and Reis 2011). Examples of cis-Andean Neotropical freshwater fish species with wide distributions include *Hoplias malabaricus* (Bloch, 1794), *Synbranchus marmoratus* Bloch 1795, *Gymnotus carapo* (Linnaeus 1758), *Rhamdia quelen* (Quoy and Gaimard 1824), *Callichthys callichthys* and *Astyanax bimaculatus* (Linnaeus 1758) (Reis et al. 2003).

Herein, we studied the trahira, *H. malabaricus*, as a model to better understand the spatiotemporal processes of diversification in the Neotropical hydrographic basins. *H. malabaricus* is distributed in all cis-Andean water basins. This taxon inhabit a great number of habitats, ranging from lotic and lentic environments such as rivers, as lakes and reservoirs. In all these environments, they are predators of other fish (Reis et al. 2003). It has been the focus of several chromosomal studies that have revealed notable variations in chromosome numbers and karyotypic formulas, which have resulted in the identification of seven cytotypes (Bertollo et al. 2000; Jacobina et al. 2009, 2011; Santos et al. 2009). Some of the cytotypes occurred in sympatry, possibly suggesting that *H. malabaricus* is a species complex, with each cytotype representing an independent taxon (Pazza and Júlio 2003; Born and Bertollo 2001; Rosa et al. 2009). However, few studies have undertaken integrative analyses involving molecular and chromosomal data in a geographic context (Pereira et al. 2012; Marques et al. 2013).

There has been considerable discussion concerning the role of chromosomal changes in the isolation of evolutionary lineages (White 1978; Marques et al. 2013). Although little is currently known concerning the relationships between karyotypic changes and geographic-evolutive isolation, two hypotheses have been formulated: (1) chromosomal changes are the moving force behind cladogenetic events, and such changes occur before the separation of lineages; and (2)

karyotypes accumulate chromosomal differences after the separation of lineages in response to distinct selective pressures. Advances in the field of phylogeography have resulted in a better understanding of the principles and processes that govern evolution at the intraspecific level (Avice 2000, 2009). As such, the integration of molecular phylogenetics with karyotypic information could help to clarify the role of chromosomal changes in the diversification of *H. malabaricus*.

The subunit I of mitochondrial gene cytochrome oxidase C (COI) has become widely used in recent years to define interspecific and intraspecific lineages, principally those linked to DNA barcode methodologies (Hebert et al. 2003; Ward et al. 2005, 2009). In addition to its usefulness in identifying species and cryptic lineages, the COI region has been widely used to examine genealogical relationships through time and space in phylogenetic and phylogeographic approaches (Lara et al. 2010; Pereira et al. 2012; Jacobina et al. 2016; Melo et al. 2016). Although, nowadays a mixture between nuclear and mitochondrial markers has been more resolute (Roxo et al. 2014).

In the present work, we investigated the historical relationships among the main ecoregions of South America using the *H. malabaricus* model. Our analyses were based on new mitochondrial COI sequences of trahira from Northeastern Brazil, a region inserted in the South American dry diagonal, located between the Amazon and the Atlantic Forest. In addition, we included georeferenced samples available from online databases that covered a substantial portion of the natural species distribution in the Neotropics. Chromosomal information obtained from the literature was also incorporated into a comparative analysis. Specifically, we addressed the following questions: (1) are there distinct genetic matrilineal lineages of *H. malabaricus* found in different hydrographic basins in Brazil? (2) What are the historical relationships among those hydrographic basins and ecoregions?; and (3) what is the relationship between mtDNA lineages and previously described karyotypes?

Materials and methods

Sampling

The fish collections were gathered under permit 32656-1/2012, issued by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio/SISBIO). Fish were collected by fishing rods and gillnets, and killed in the field using cold water and immediately transferred onto ice. Tissue samples were collected after fish euthanasia that was confirmed by a lack of operculum movement. This study did not involve an endangered or protected species. No ethics committee approval is required for this organism in Brazil.

We obtained a total of 58 samples of *H. malabaricus* from seven watersheds that belong to four ecoregions in Northeastern Brazil: São Francisco (15 individuals), (São Francisco ecoregion, SFRE); Contas (9 individuals) and Itapicuru (16 individuals) (Northeastern Atlantic Forest ecoregion, NAFE); Jaguaribe (6 individuals), Paraíba do Norte (5 individuals) and Piranhas-Açu (5 individuals) (Mid-Northeastern Caatinga ecoregion, MNCE); and Parnaíba (2 individuals) (Maranhão-Piauí ecoregion, MAPE). The collected specimens were identified based on their external morphology, using the diagnostic characteristics reported by Oyakawa and Mattox (2009). In all specimens collected, the rami of the dentary bone were convergent toward the symphysis, resulting in a “V” shape pattern. The specimens were deposited at the Museum Universidade Federal de Viçosa (UFV) and Universidade Federal do Rio Grande do Norte (UFRN), Brazil.

In addition, we used databases in the public domain (National Center for Biotechnology Information and Barcode of Life Database) to gather a dataset with 92 COI sequences belonging to *H. malabaricus* from the Lower Amazon (Amazon Estuary Ecoregion, AMEE), Paraíba do Sul (Paraíba do Sul ecoregion, PASE), the Upper Paraná basin and the Pampas Plain region in Argentina. Other complementary samples from the ecoregion Maranhão-Piauí (basin Itapecuru-Mirim) and São Francisco were also added (see supplementary material). Sequences from *Hoplias intermedius* (Gunther, 1864) were used as outgroup in the phylogenetic analyses. Allied to the molecular data, bibliographical surveys were carried out on chromosomal information of *H. malabaricus* in the studied basins (Table 1).

DNA extraction, polymerase chain reaction and sequencing

Using a DNeasy kit (QIAGEN), total DNA was extracted from samples of muscle tissue or fin clips that had been fixed in 95% ethanol. A fragment with 652 bp of the mitochondrial COI gene was amplified by polymerase chain reaction (PCR) using the universal primers BarcFishF1 and BarcFishR2 (Ward et al. 2005). PCR reactions were performed in a final volume of 25 μ l, which was comprised of 12 μ l of 2X Taq master mix (Vivantis), 2 μ l of 40 μ g/ μ l genomic DNA solution, 0.5 μ l of each primer (10 mM) and the remainder of ultrapure water. The reactions occurred for an initial two minutes at 95 °C; 35 cycles of 30 s at 94 °C, 30 s at 57 °C and 2 min at 72 °C; and a final extension of 7 min at this same temperature 72 °C for 10 min. PCR products were directly sequenced using the same amplification primers and a Big Dye terminator 3.1 cycle sequencing kit (Applied Biosystems), following the manufacturer's protocol. Sequences were analysed using the automated sequencer ABI PRISM 3130 (Applied Biosystems).

Data analysis

After editing electropherograms, the consensus sequences were aligned using ClustalW (Thompson et al. 1994), implemented in Geneious (Kearse et al. 2012), and followed by visual inspection for final adjustments. Genetic distances were estimated with the Kimura-2-parameters (K2P) substitution model, using MEGA v.6 (Tamura et al. 2013) and following the analytical routine established by the international consortium Barcode of Life (<http://www.boldsystems.org>). We also estimated a best-fit model using the PartitionFinder 2 program (Lanfear et al. 2012).

We inferred the phylogenetic relationships among haplotypes using Bayesian inference (BI) with the MrBayes 3.2

Table 1 Chromosome studies in *Hoplias malabaricus* following the nomenclature (A-F) of Bertollo et al. (2000)

Hydrographic basin	Diploid number = 2n	Karyomorph	References
Lower Amazonas	40M/SM	C	Bertollo et al. (2000)
Itapecuru-Mirim	40M/SM	F	Bertollo et al. (2000)
Itapicuru	40M/SM	F	Jacobina et al. (2009)
São Francisco	40M/SM	F	Dergam and Bertollo (1990)
Parnaíba	*_**	*_**	Not studied
Contas	40M/SM	F	Jacobina et al. (2009)
Paraná	39-40M/SM-42M/SM 40M/SM	A, C, D	Bertollo et al. (2000), Pazza and Júlio (2003)
Paraíba do Sul	42M/SM	A	Bertollo et al. (2000)
Pampa Plain	42M/SM	A	Grassi et al. (2017)
Piranhas	*_**	*_**	Not studied
Jaguaribe	*_**	*_**	Not studied
Paraíba do Norte	40M/SM	F	Bertollo et al. (2000)

program (Ronquist et al. 2012). Two independent runs were implemented, with four chains (three heated and one cold) for 20 million generations each. Parameters were sampled every 2000 generations. We visually inspected the convergence of the chains in the Tracer 1.5 program (Drummond et al. 2012) to assess convergence, making sure that all effective sample sizes (ESS) were higher than 200. We discarded the first 10% of generations as burn-in and combined the remaining trees into a 50% majority-rule Bayesian consensus tree. The MCC tree was visualised in FigTree 1.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

To infer independent evolving lineages in *H. malabaricus*, we implemented molecular species delimitation based on two coalescent tree-based methods: the Bayesian Poisson Tree Process (bPTP; Zhang et al. 2013) and the generalised mixed Yule-coalescent model (GMYC; Pons et al. 2006). The bPTP was carried out on the bPTP web server (<http://species.h-its.org/ptp/>) using the MrBayes resulting tree containing only haplotypes. We used 300,000 generations of Markov Chain Monte Carlo (MCMC) with a 10% burn-in. The GMYC was implemented using the GMYC web server (<http://species.h-its.org/gmyc/>), assuming a single threshold, and using the BEAST resulting tree containing only haplotypes. For GMYC, we used an uncalibrated MCC tree obtained from the BEAST 1.8 program (Drummond and Rambaut 2007; Drummond et al. 2012) using the Yule tree prior and uncorrelated lognormal relaxed clock. The BEAST analysis comprised two independent runs with 100 million generations for each run. The parameters were sampled every 5000 generations, and a burn-in of 10% was used. Run convergence and performance were inspected using Tracer 1.5, making sure that all ESS values were greater than 200. The resulting trees were combined in TreeAnnotator.

We calculated a distance matrix using the K2P model, with the `sppDistMatrix` function in the Spider v1.3-0 package in the R (Brown et al. 2012). From this matrix, we created an object of density using the minimum local function, which does not require prior knowledge of the species identity to indicate potential threshold, that infers possible transitions of intraspecific and interspecific variations (Brown et al. 2012). In addition, a haplotype network was inferred for the most diverse clade (Northeastern clade; see the “Results” section) using the Popart software (Leigh and Bryant 2015) with the TCS Network.

Results

In the ecoregions analyzed here, four cytotypes have been described (Bertollo et al. 2000): the cytotype named A ($2n = 42$) has a wide distribution on from northern to southern Brazil, as well as, the cytotype C ($2n = 40$). Already the cytotype D ($2n = 40$ chromosomes in females and $2n = 39$

in males) it is found in the Upper Paraná basin, and the F ($2n = 40$ similar to C, but differentiated by a large meta-centric pair) occurs from Surinam to southeastern Brazil, although it has with a preferential distribution in the oriental part of the Brazilian continent. We obtained a COI alignment of 555 bp, of which 437 sites were conserved (78.7%) and 118 (21.3%) were variable. The intra-basin K2P distance varied from 0 to 4%, and it was the Lower Amazon site that presented the greatest genetic divergence. The smallest distance was between Contas and Itapicuru (0.3%), which belong to the same ecoregion (that is, NAFE). The largest distance was between Itapecuru–Mirim (NAFE) and Paraíba do Sul (PASE) (8.1%) (see Table 2).

The Bayesian analysis with the evolutionary model HKY + I detected four clades with statistical support greater than 0.9 (posterior probability to IB). The four main clades in *H. malabaricus* (Fig. 1) were: clade I, the Inland clade, included samples from the Upper Paraná and Lower Amazon (Fig. 1); clade II, the Pampas Plain clade, included samples from the Argentine Plains; clade III, the Paraíba do Sul clade, only included samples from the Paraíba do Sul basin; and clade IV, the Northeastern clade, included samples mainly from Northeastern Brazil. Clade IV contained the largest number of samples and the highest haplotypic diversity, and the samples were comprised of haplotypes from five ecoregions (NAFE, SFRE, MNCE and MAPE), and included samples from the AMEE (Fig. 1).

The intra-clade genetic distances we observed ranged from 0.4% in the Pampas Plain clade to 1.6% in the Northeastern clade and 1.7% in the Inland clade. We were unable to calculate the intra-clade distance for clade III because it was composed of a single individual. We also observed high inter-clade divergences: 7.0% between Inland and Pampas Plain clades, and 7.3% between Northeastern and Inland clades.

Results from GMYC and bPTP tests corroborated the presence of four independent lineages in *H. malabaricus* (Fig. 1). The threshold potential, with minimum local function in the Spider, indicated three dips with low genetic density in the analysed populations: the potential threshold limits were they: 1.2%, 4.8% and 6.7%.

Our haplotype network for clade IV included 40 haplotypes, and it revealed a star-like shape. The São Francisco and Amazon basins exhibited higher levels of diversity with very distinct haplotypes (Fig. 2). Exclusive haplotypes were detected in almost all basins: São Francisco (4), Jaguaribe (3), Parnaíba (2), Piranhas Açu (1), Paraíba do Norte (1), Itapecuru–Mirim (1) and in the Lower Amazon (13). In addition, we observed shared haplotypes among distinct basins (Fig. 2): between the Itapicuru and Contas basins (both from NAFE); between Contas (NAFE) and São Francisco; between São Francisco and Paraíba do Norte; between Paraíba do Norte and Jaguaribe (both from MNCE); and

Table 2 Mean distances within and between South American basins using the K2P model with Cytochrome Oxidase I (COI)

Distance between basins													
Basins	Distance within basins	PAR	PAM	PBS	AMZ	ITA	CON	JAG	SFR	PIR	ITE	PRN	PNB
PAR	0.009	*											
PAM	0.004	0.070	*										
PBS	–	0.079	0.077	*									
AMZ	0.040	0.052	0.073	0.080	*								
ITA	0.001	0.064	0.070	0.077	0.029	*							
CON	0.004	0.066	0.073	0.076	0.031	0.003	*						
JAG	0.006	0.061	0.071	0.076	0.031	0.006	0.008	*					
SFR	0.004	0.063	0.072	0.075	0.031	0.004	0.005	0.008	*				
PIR	0	0.062	0.073	0.079	0.031	0.006	0.008	0.005	0.008	*			
ITE	0.003	0.074	0.075	0.081	0.041	0.022	0.025	0.025	0.025	0.027	*		
PRN	0.018	0.062	0.072	0.078	0.035	0.011	0.014	0.011	0.014	0.013	0.027	*	
PNB	0.005	0.061	0.073	0.078	0.030	0.004	0.006	0.006	0.005	0.005	0.025	0.012	*

AMZ Amazonas, (AMEE ecoregion), PNB Parnaíba, ITE Itapecuru-Mirim (MAPE ecoregion), PRN Paraíba do Norte, JAG Jaguaribe, PIR Piranhas-Açu (MNCE ecoregion), SFR São Francisco (SFRE ecoregion), ITA Itapicuru, CON Contas (NAFE ecoregion), PBS Paraíba do Sul (ecoregion, PASE), PAM Pampa Plain, PAR Upper Paraná

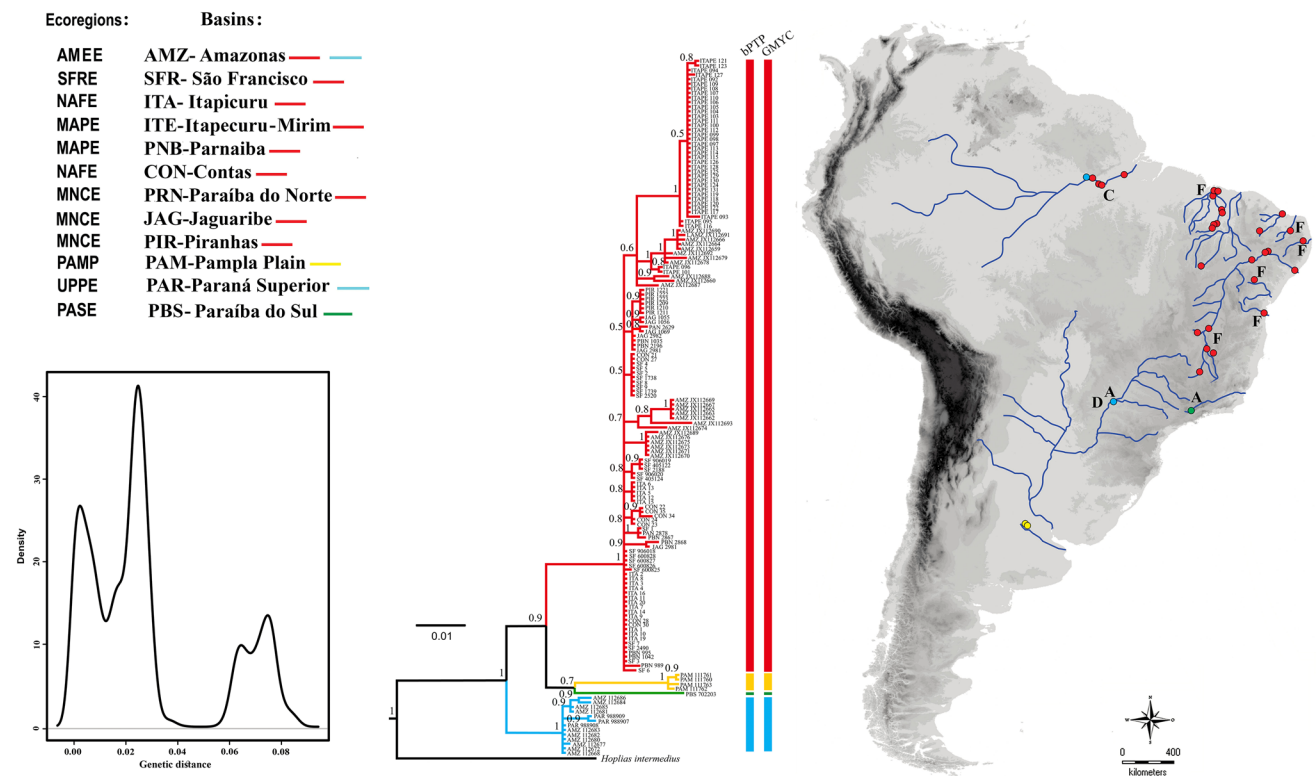
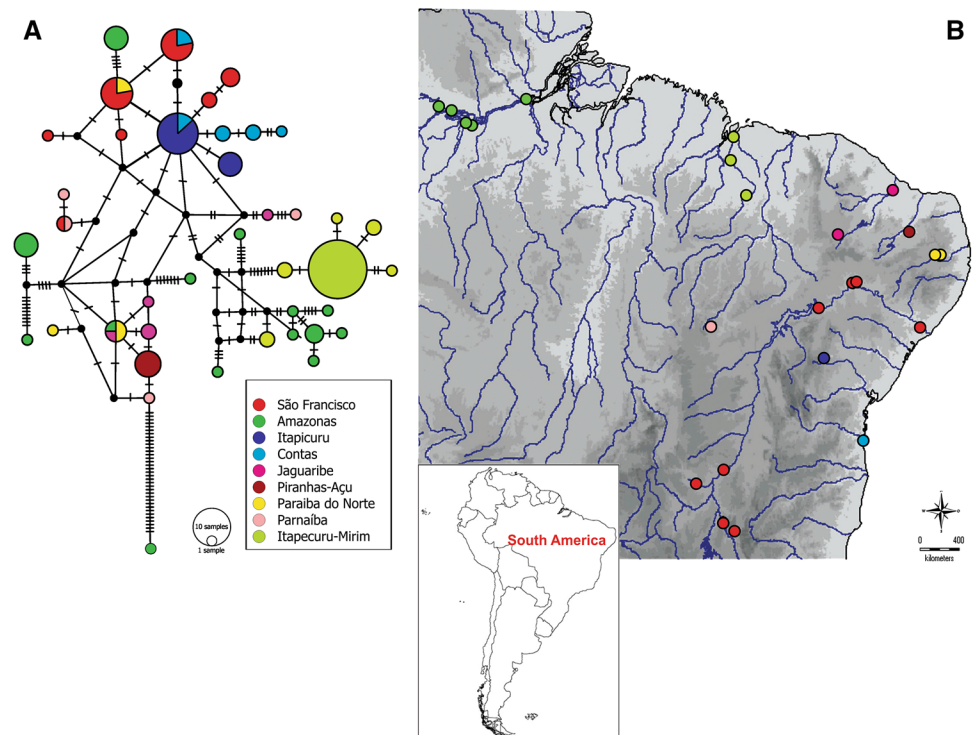


Fig. 1 Bayesian topology and species delimitation GMYP and bPTP of the trahira *Hoplias malabaricus*. Below, the left side of the graph shows the density dive related to the genetic distances of *H. malabaricus* populations studied. On the right side, the map shows the

occurrence of haplotypes with colors related to species delimitations. The letters indicate the places where the cariomorphs were described in the basins

Fig. 2 Map with geographic distribution (A) and haplotype network (B) of the cytochrome oxidase subunit 1 (COI) gene of samples from the red clade (Fig. 1) of *Hoplias malabaricus*. In haplotype network each color represents basins according to the map, and the size of the circle is proportional to the number of individuals. Marks in lines connecting the haplotypes refer to mutational steps



between Paraíba do Norte (MNCE), Parnaíba (MAPE) and Lower Amazon. The Lower Amazon shared haplotypes with two distinct basins in the Northeastern region (Fig. 2). Apart from two of the more distant locations coming out together in the same clade, in general, we observed a concordance between genetic variation and geography, with low levels of haplotype sharing between more distant ecoregions.

Discussion

The groupings in our phylogenetic and phylogeography analysis, together with chromosomal information, provided sufficient evidence to answer the main questions we asked.

Are there distinct genetic lineages of *H. malabaricus* in different hydrographic regions?

Our data indicated the presence of genetically distinct lineages of *H. malabaricus* from different Neotropical ecoregions. These distinct lineages were observed in geographically distant drainage basins, as well as in adjacent ecoregions and even in the same watershed (Fig. 1; Table 1). The use of the COI gene (known popularly as one of the main DNA barcode gene) has improved taxonomic diagnosis, and it has been shown to be efficient in identifying species and detecting cryptic taxa (Ward et al. 2005, 2009; Hubert et al. 2008; Lara et al. 2010). The molecular identification system that uses COI opens the door to various

methods of delimiting lineages based on genetic distances (Hebert et al. 2003; Brown et al. 2012; Fujisawa and Barracough 2013). While some studies used a 1% cut-off for defining divergence (Ratnasingham and Hebert 2007), a 2% threshold is more common in studies with Neotropical fish and is within the limits of intraspecific variation (Hubert et al. 2008; Lara et al. 2010; Pereira et al. 2012).

The magnitude of genetic divergence found among some *H. malabaricus* lineages was four times larger (8.1%) than the established cut-off for species delimitations (2% rule). Geographically close ecoregions (such as those in Northeastern Brazil) demonstrated variations greater than 2%, as observed in the MAPE and MNCE (2.7%). The distances detected herein demonstrated that using 2% sequence divergence as the threshold value significantly increased the number of lineages (see Table 2), mainly within clade IV (Northeastern), which was the region with the greatest number of drainage basins. It is worth mentioning that the Lower Amazon (AMEE) basin exhibited the greatest intra-basin genetic distances (4.0%) within sympatric lineages, as previously reported (Marques et al. 2013).

Our BI phylogeny indicated the presence of four clades in *H. malabaricus*, which was corroborated by GMYC and bPTP methods for delimiting lineages. These results provided evidence of at least four distinct evolving lineages within species. The threshold value of the minimum distance, which evaluates possible interspecific to intraspecific genetic transitions (Brown et al. 2012), indicated low-density genetic declines in the populations analysed. The first

was only partial (1.2%), and appears to have been related to intraspecific variations, although a low and accentuated genetic density of 4.8% was detected. Considering this value, the threshold of *H. malabaricus* would be much greater than that inferred by delimitations among Neotropical fish species (2%) (Pereira et al. 2012). A new species of *Hoplias* (*H. missioneira*) was recently described in the Uruguay, Paraguay and Paraná basins, and it showed a 5.61% genetic divergence from *H. malabaricus* (Rosso et al. 2016). As such, results from our study are in accordance with values to delimitate species within *H. malabaricus*.

What are the historical relationships among hydrographic basins and ecoregions?

Our data showed that most of the ecoregions did not include exclusive lineages, which indicated possible dispersal events between basins, mainly in the Inland and Northeastern clades. The Lower Amazon region included haplotypes that clustered into two distinct clades—clade I (Inland) and clade IV (Northeastern). The Northeastern clade was comprised of the São Francisco, Northeastern Atlantic Forest, Mid-Northeastern Caatinga and Maranhão-Piauí ecoregions. The Pampa Plain and Paraíba do Sul ecoregions were found to contain monophyletic lineages.

Although the haplotypes of the Inland clade are currently separated by large geographic distances, some populations still share genetic signatures among their haplotypes. A hydrological connection was established during the Miocene period (10 million year ago), when the ocean invaded the continent and linked the Amazon and Paraná basins (Sempere et al. 1990; Lundberg et al. 1998; Albert and Reis 2011).

The close relationship between clades II and III (Pampas Plain and Paraíba do Sul) may reflect ancient geomorphological events. Currently, little is understood about the complex palaeohydrology of the Paraíba do Sul River basin, with inversions along its course during both the Neogene period and the Miocene epoch (Ab'Saber 1957). Possible captures of upper courses between adjacent basins may have extended the geographic distribution of trahiras, and could be supported by evidence of close relationships between ecoregions (Ab'Saber 1957; Malabarba 2003). The high degree of sharing in clade IV between the ecoregions of Northeastern Brazil (MAPE, MNCE, SFRE and NAFE) and the Amazon Estuary (AMEE) reveal the dynamics of recent connections between basins and along the coast, as previously reported (Hubert and Renno 2006; Ribeiro 2006). The geomorphological studies of Ab'Saber (1998) demonstrated that the São Francisco River basin has a hybrid origin, with a paleohydrological history closely related to the coastal basins of the MNCE and MAPE ecoregions, due to complex tectonic activities beginning in the Tertiary period and

associated with fault systems that would have allowed the exchange of ichthyofaunas between neighbouring regions. An alteration in the course of the São Francisco River was detected, which demonstrates that it temporally drained to the north 400,000 years ago, and may have been connected to the Parnaíba basin in MAPE (Potter 1997; Werneck et al. 2015). The São Francisco River later changed its course to the east, allowing possible connections with coastal basins such as those of Paraíba do Norte, Contas and Itapicuru that share haplotypes with the São Francisco basin (Potter 1997; Brito-Neves et al. 2004; Costa 2010; Werneck et al. 2015). Those processes may also have been shaped by the dynamics of the Pleistocene epoch; historical changes in the courses of those rivers allowed multiple connections, with possible links between their basins to enable gene flow between populations (Pereira et al. 2012). Other studies have also found evidence of the sharing of species between coastal Northeastern areas (in hydrographic basins that are currently isolated) and the São Francisco River via headwater capture events that permitted ichthyofauna exchanges (Weitzman et al. 1988; Beheregaray et al. 2002; Rosa et al. 2004).

The haplotype shared between three distinct basins in the Northeastern clade may be associated with an retention of ancient polymorphism, on the basis of its large geographic distribution through the Amazon, Jaguaribe and Paraíba do Norte basins. The relationships that link the ichthyofauna of the Northeastern and Amazon regions have been documented in other studies (Vari 1988; Rosa et al. 2004; Hubert and Renno 2006). Phylogenetic analyses of the genus *Loricariidae* and an annual fish of the family Cynolebiidae also suggested that the Pleistocene epoch had an important role in promoting the dispersal of species along coastal regions (Montoya-Burgos 2003; Hubert and Renno 2006; Costa 2010, 2014). Such a scenario may have occurred with *H. malabaricus*, which is a sedentary species that demonstrates high physiological tolerance and that may have occupied flooded plains with low oxygen concentrations, such as those encountered in lowland or coastal plains (Araújo-Lima et al. 2001; Basílio et al. 2009). This biological flexibility may have allowed its populations to take advantage of dispersal routes along the coast from Northern to Northeastern regions, or vice versa. Hubert et al. (2007) also supported the hypothesis of species dispersal along the coast through swampy coastal areas (as some species of Characiformes are shared among coastal basins in Northern and Northeastern Brazil).

In general, our results reflect two patterns: (1) phylogenetic evidence of older vicariant events from Inland rivers to the Atlantic coast, in which rivers of crystalline Inland lands became connected with adjacent coastal basins (Ribeiro 2006); and (2) recent connections involving dispersal events, as evidenced by haplotype networks.

What is the relationship between mtDNA divergence and the karyotypes?

Most genetic studies with *H. malabaricus* have been undertaken with a chromosomal focus, which included the examination of chromosome numbers, karyotype formulas and differences, and the presence of simple and multiple sexual chromosomes (Bertollo et al. 2000; Jacobina et al. 2009, 2011; Santos et al. 2009). Marques et al. (2013) analysed populations in the Lower Amazon that exhibited cytotype C ($2n=40$ and without a sexual pair; sensu Bertollo et al. 2000) and observed high intra-population genetic diversity above 2%. *H. malabaricus* with cytotype C ($2n=40$) in the haplogroups of the hydrological basins in Northeastern Brazil have been characterised as another cytotype, designated F (which is like cytotype C ($2n=40$), but without differentiation between males and females). Its distinctive feature is the presence of a large metacentric pair, the number 1 in the karyotype, which constitutes also the largest chromosome known for *H. malabaricus* (Dergam and Bertollo 1990; Jacobina et al. 2009). Indeed, this suggests that the macrostructural karyotypic classification of that taxon is not adequate for the recognition of monophyletic groups, but rather represents a homoplastic character. Cytotype C has been found from Northern Brazil to Northeastern Argentina, and it has been encountered, for example, in the Paraná basin sympatrically with cytotypes A ($2n=42$ meta- and sub-metacentric chromosomes in both sexes, without an apparent sex chromosome system) and D ($2n=40$ chromosomes in females, with a reduction to $2n=39$ in males, all of them meta- and sub-metacentrics) (Bertollo et al. 2000). The species *H. ambigua* (Azpeticueta et al. 2015) was recently described in the Paraná basin, based on morphometric methods, suggesting that it may have been previously characterised as cytotype C of *H. malabaricus* (Grassi et al. 2017).

Cytotype A has also been described for populations of *H. malabaricus* in the Misiones Province, Argentina. This province also harbours a new species, *H. misioneira* (Rosso et al. 2016), which has a wide distribution in the principal basins of Southern Brazil and Northern Argentina and may have been characterised as cytotype A (in the same way as reported earlier for *H. ambigua*). Cytotype A occurs in the Paraíba do Sul and upper Paraná basins, and was recently described near the Pampa Plain region in Argentina (Bertollo et al. 2000; Grassi et al. 2017). Cytotype A has been found in three clades (Inland, Pampa Plain, and Paraíba do Sul), suggesting that the large accumulation of molecular genetic differences detected in the present study apparently did not result in macrostructural karyotypic alterations in *H. malabaricus* lineages. All these results indicating that a single cytotype could harbour more than one cryptic lineage.

Conclusions

Our data suggest that at least four MOTUS are found in the ecoregions studied. In this context, many processes could have shaped the distribution and diversification of *H. malabaricus* in *cis*-Andean neotropical watersheds. Tertiary events related to the possible reactivation of tectonic faults and headland catches, as well as more recent processes related to sea level fluctuations during the Pleistocene era, may have contributed and shaped the observed diversity. All these processes, together with the molecular and chromosomal divergence detected in this taxon, reinforce the need to revise their taxonomic status, including the combined use of nuclear and mitochondrial markers, as well as the search for more diagnostic morphological characters that can be used for a delimitation more precise within this taxon, that despite showing a slow morphological evolution, its genetic divergence has been quite significant.

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Compliance with ethical standards

Conflict of interest All authors declare that have no conflict of interest.

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