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RESEARCH ARTICLE

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Abstract

The upper Paraná River system (UP) is a highly diverse biogeographic province for freshwater fishes, but little is known about processes which shaped that diversity. This study describes the phylogeographic pattern in *Hypostomus ancistroides*, a suckermouth catfish species that is widespread in the UP and also reported from the adjoining Ribeira do Iguape basin. We used complete mtDNA sequences of ATPase 6/8 of 162 specimens to infer haplotype distribution using phylogenetic and demographic analyses and a Bayesian molecular clock. Results suggest that during the Quaternary *H. ancistroides* has undergone superimposed phylogeographic histories, alternating between isolation and subsequent merging of different populations. Occurrence of an isolated population on the Ribeira de Iguape is demonstrated to be a Pleistocene headwater capture event. Widely distributed haplotypes indicate deep genetic differences and suggest that populations of *H. ancistroides* were isolated for considerable time, but did not undergo speciation because of recurrent population mixing.

Introduction

Hypostomus Lacépède is a highly diverse and widespread genus of the Neotropical catfish family Loricariidae, and is regarded as a model for the study of biogeographic patterns and speciation in Neotropical freshwaters (Montoya-Burgos, 2003). *Hypostomus ancistroides* (Ihering, 1911) is one of the most abundant and widespread species in the genus occurring along the entire upper Paraná River system (UP) and on the contiguous Ribeira de Iguape coastal basin (Oyakawa et al., 2005; Weber, 2003). The UP is traditionally considered as a distinct biogeographic province of Neotropical freshwater fishes (Géry, 1969; Hubert & Renno, 2006; Vari, 1988) and more recently has been listed as one of the freshwater ecoregions of the world (Abell et al., 2008; Albert & Carvalho, 2011). The UP drains an area of 900 000 km^2 on the central and southern Brazilian shield, and is composed of large basins which have changed little for the last nine million years (Albert & Reis, 2011; Langeani et al., 2007). The UP is known for its sizeable fish diversity, with ca. 250 species, of which 125 are endemic (Langeani et al., 2007). This represents roughly 6% of the Neotropical fish diversity (Albert et al., 2011). Despite such high endemism, fish species in the UP have variable distributional patterns. While some species are restricted to one or few river basins, such as *Microglanis garavelloi* Shibatta & Benine, 2005, *Lophiobrycon weitzmani* Castro et al., 2003, *Oligosarcus planaltinae* Menezes & Géry, 1983, *Steindachnerina corumbae* Pavanelli & Britski, 1999, *Hypostomus heraldoi* Zawadzki et al., 2008, and *H. denticulatus* Zawadzki et al., 2008, others are found in other systems contiguous to the UP as well, such as *Phalloceros spiloura* Lucinda, 2008, *Characidium xanthopterum* Silveira et al., 2008, and *Hypostomus ancistroides*. This mosaic of particular distribution patterns is suggestive of a complex paleohydrological history that, in combination with specific biological traits and habitat differentiation, resulted in a complex scenario of diversity and endemicity.

Hypostomus ancistroides is highly polymorphic in its karyotype formulae, color pattern, and body shape, and for this reason, it is often considered as a potential species complex (e.g. Endo et al., 2012). For the same reasons, the species is a promising model species to investigate microevolutionary processes, phylogeography, and their relationship to taxonomic patterns. Population genetics and phylogeographic tools have been widely used to correlate evolutionary history of organisms and their current biogeographic patterns (e.g. Hickerson et al., 2010) and have often resulted in refinements in the associated taxonomy of various species.

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Here, we describe the phylogeographic pattern of *H. ancistroides* using complete sequences of mtDNA ATP synthase (subunits 6 and 8). We sampled specimens collected along the entire distributional range of the species. Our purpose is to investigate the hypothesis that speciation processes intrinsic to the UP have led to particular distribution patterns of certain species in this area, of which *H. ancistroides* is a prime example. This is the first extensive phylogeographic research on any species of *Hypostomus* and the first analysis of evolutionary relationships among rivers of the UP using molecular tools.

Material and methods

Tissue samples of muscle or fins were collected during field trips along most of the distribution range of *Hypostomus ancistroides* (Figure 1). A total of 162 samples (Supplementary Appendices A and B) were collected from the main tributaries composing the UP, which are Paranaíba, Grande, Rio do Peixe, Tietê, São José dos Dourados, Paranapanema, and Ivaí, as well as from the Ribeira do Iguape, an adjacent yet independent coastal river basin located at the eastern slope of the Serra do Mar mountain range. The aforementioned tributaries of the UP are located on the left margin of the river system and their headwaters are separated from coastal basins by the Serra do Mar, the main coastal range of

eastern neotropics. This physiographical constitution represents an ideal situation for inferring the permeability of the two main biogeographic barriers for freshwater fishes, which are the landmasses and the long distances lacking suitable habitat. Samples were preserved on ethanol 95%, stored at $-20\,°C$, and deposited at the ichthyological collection of the *Museu de Zoologia da Universidade de São Paulo* (MZUSP) and the *Laboratório de Biologia e Genética de Peixes*, *Departamento de Morfologia* (LBP-UNESP) and are listed in Supplementary Appendix A.

Genomic DNA was extracted using the Wizard DNA Extraction Kit (Promega, Madison, WI) according to the manufacturer protocol with the following changes: (i) addition of 6 μl of proteinase K 20 mg/ml to the Nuclei Lysis Solution; and (ii) all centrifugation steps were conducted for 15 min. Amplification of the subunits 6 and 8 of the mitochondrial gene ATP Synthase (ATPase) was performed by polymerase chain reaction (PCR) using PCR Master Mix (Promega, Madison, WI) in a total volume of 25 μl. Primers used were designed for the amplification of the complete subunits 6 and 8 of the ATPase mitochondrial gene at Bermingham lab (Smithsonian Tropical Research Institute: http://stri.si.edu/sites/bermingham/research/primers/index.html): 8.2 L8331 (5′ AAA GCR TYR GCC TTT TAA GC 3′) and CO3.2 H9236 (5′ GTT AGT GGT CAK GGG CTT GGR TC 3′). PCR
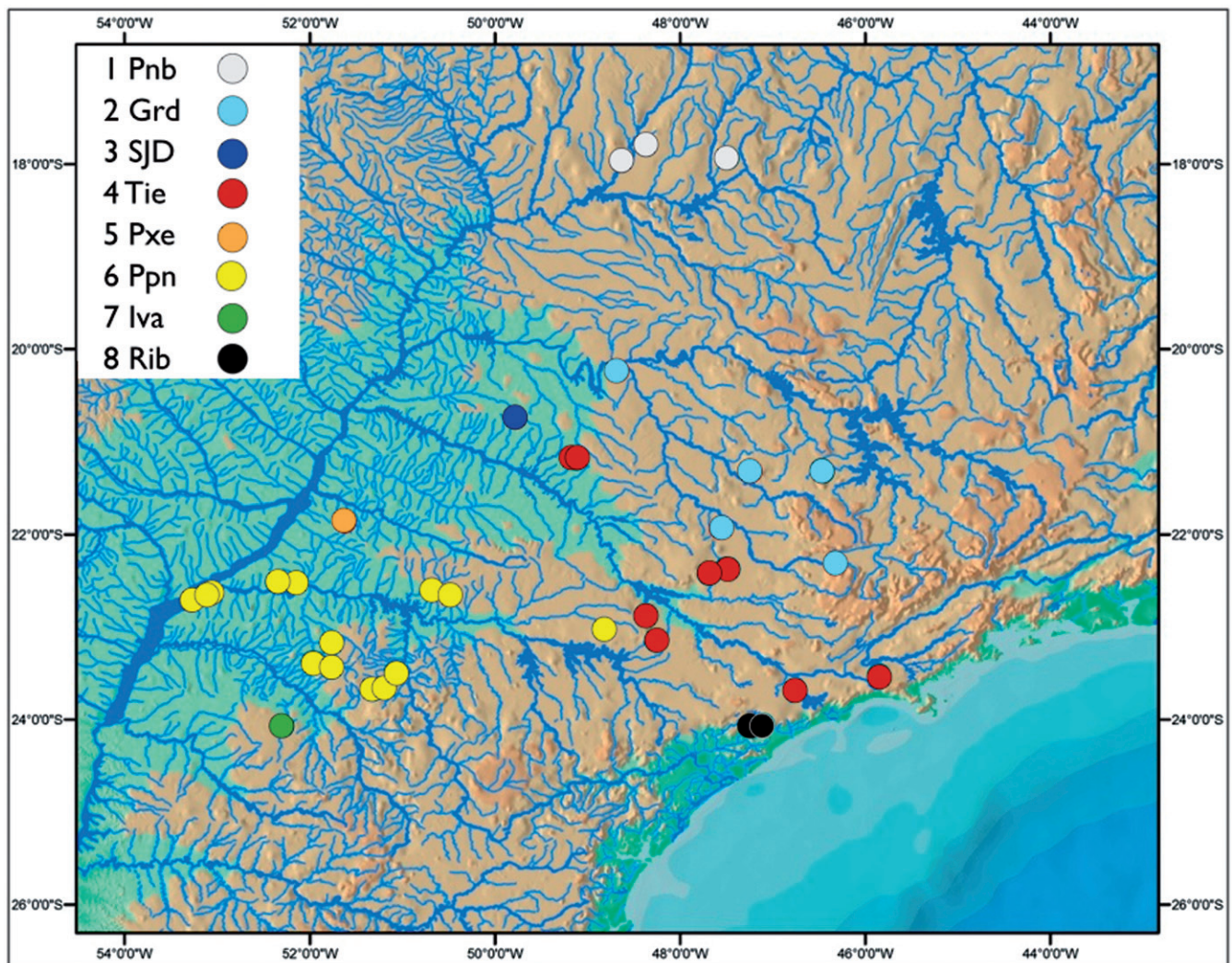

Figure 1. Map of the upper Paraná River system (UP) and collecting sites of *Hypostomus ancistroides*. Each colour stands for one of the eight river basins sampled. Abbreviations: Paranaíba (Pnb), Grande (Grd), Sao José dos Dourados (SJD), Tietê (Tie), Peixe (Pxe), Paranapanema (Ppn), Ivaí (Iva), and Ribeira de Iguape (Rib).

cycles were 4 min at 94 °C followed by 38 cycles of 40 s at 94 °C, 50 s at 54 °C, and 70 s at 72 °C, with a final extension at 72 °C for 5 min. Sequencing was performed at the CEGH-USP (http://genoma.ib.usp.br) and at Macrogen Korea (http://dna.macrogen.com). Sequences were deposited in GenBank (accession numbers: KT276009–KT276182)

Sequences were aligned using ClustalW automated tool implemented on BioEdit 7.0.5 (Hall, 1999) and were later visually inspected and manually edited when needed. Phylogenetic and phylogeographic analyses were carried out to understand geographic partition of the genetic variation. The following species were selected as outgroups because they are considered as closely related to *H. ancistroides*: *H. affinis*, *H. boulengeri*, *H. commersoni*, *H. interruptus*, *H. piratatu*, and *H. tapijara* (e.g., Montoya-Burgos, 2003, and Hollanda Carvalho unpublished results). Maximum Likelihood (ML) analyses were performed on Mega 5.03 (Tamura et al., 2011) using substitution model TrN + G+I (Tamura & Nei, 1993) selected by Akaike Information Criteria (AIC) on jModel Test (Posada, 2008) and 1000 bootstrap replicates to estimate branch support. The ML phylogenetic hypothesis was used to formulate hypotheses about relationships between lineages from different river basins and generate hypotheses of population structure. Uncorrected p-distances between lineages and haplogroups were estimated on Mega 5.03.

Divergence time among lineages of *H. ancistroides* and between that species and outgroups was estimated through Bayesian Strict Clock implemented in Beast v.1.8 (Drummond et al., 2012). Dataset included the same outgroup used on ML analysis plus *H. plecostomus* (Linnaeus, 1758). For computational reasons, the dataset was reduced to 57 haplotypes selected by DNAsp (Librado & Rozas, 2009) covering all the haplotypes of *H. ancistroides* and those of the outgroup species. We used a constant evolutionary rate of 1.3%/Ma, reportedly consistent for ATPase 6 and 8 in fishes (Bermingham et al., 1997) and previously employed for Neotropical siluriforms (Perdices et al., 2002; Borba et al., 2013). Calibration was enforced in a normal clock rate distribution prior with $1.3E{-}8$ corresponding to the above rate as the initial and mean value, and $1.0E{-}11$ as standard deviation. Other parameters include the TN93+G + I substitution model and estimated base frequencies for the substitution model. Yule Speciation process was used as tree prior, which is the most appropriated for species-level analyses (Drummond et al., 2007). Remaining parameters used default options. The post-run analysis of 100 million MCMC chain length sampling every 1000 generation was assessed with Tracer v.1.6 to verify the effective sample size (ESS) and chain convergence. The maximum clade credibility tree was generated using TreeAnnotator v.1.8, with the first 25% discarded as burn-in. A low posterior probability of 0.5 was set as limit, in accordance with the goal of estimating the period of haplogroups formation, rather than their evolutionary relationships.

The software Arlequin 3.5 (Excoffier & Lischer, 2010) was employed to calculate population parameters for each sampling site: Φst, haplotypic (h) and nucleotide (π) diversity. Analysis of molecular variance (AMOVA) was also performed using Arlequin in order to explore hypotheses of population structure related to river basins. To that end, samples were assigned to one of eight main hydrographic systems, namely Parnaíba, Grande, São Jose dos Dourados, Paranapanema, Peixe, Tiete and Ivaí rivers (all part of the UP), plus the coastal Ribeira de Iguape basin. We generated a parsimonious haplotype network using TCS 1.21 (Clement et al., 2000) and the choice between ambiguities followed the criteria proposed by Crandall & Templeton (1993). The neutrality tests of *Fs* (Fu, 1997) and *D* (Tajima, 1989) using Arlequin were used to

test hypotheses of size fluctuation for the whole population. Contrary to the AMOVA, and following its preliminary results, neutrality tests were performed for the eight putative population merged into one, assuming panmixia. Those analyses test whether population size fluctuations could be involved in temporal segregation and subsequent diversification among lineages of the species, as suggested by our first results (see Results section).

Results

Phylogenetic results and tempo estimate

Phylogenetic results show that *Hypostomus ancistroides* is a monophyletic group composed of four main lineages widely distributed along the UP (Figure 2). The first lineage (named haplogroup 1) is the largest and is composed of fishes from effectively all the distribution range of the species, including the rivers Ivaí, Paranapanema, Tietê, Paranaíba, and Ribeira de Iguape. All specimens from the Ribeira de Iguape coastal basin belong to a single haplotype of this lineage.

The second lineage (haplogroup 2) is composed of a single haplotype from the least densely sampled area, the Ivaí river, the southernmost UP tributary. The third lineage (haplogroup 3) comprises specimens from the Paranapanema, São José dos Dourados, and Paranaíba. The fourth lineage (haplogroup 4) encompasses fishes collected at Paranapanema, Tietê, Rio do Peixe, and Rio Grande. Uncorrected p-distances between these lineages and among haplogroups are shown in Supplementary Appendices C and D. These distances were higher amid haplogroups one and two or three, with a maximum value of 2.7% between sequences. Mean distance for the species is 1.46%.

Values of uncorrected p-distance between *H. ancistroides* and its closely related species ranges from 0.7% (*H. commersoni*) to 3.2% (*H. affinis*) (Supplementary Appendix C). Bayesian molecular clock inference is highly congruent with the topology recovered by ML analysis (Figure 3). Cladogenetic events separating *H. ancistroides* from outgroup species are assigned to the Pleistocene, ca. 1.1 Ma. The four main haplogroups of *H. ancistroides* are well supported by high posterior probability (above 0.96). Emergence of haplogroups starts around 0.81 Ma with the split of two main lineages, one composed of haplogroup 1 and the other composed of haplogroups 2, 3, and 4. Subsequent cladogenetic events are estimated between 0.66 and 0.52 Ma while the most recent diversification among members of each haplogroup starts around 0.44 Ma.

Population analyses of *Hypostomus ancistroides*

The 162 complete sequences of ATPase 6 and 8 comprise 842 base pairs and 48 haplotypes. Haplotype diversity (h) is 0.8996 and nucleotide diversity (π) is 0.01165 (Supplementary Appendix B). The haplotype network (Figure 4) shows a large number of mutational steps between several of the 48 haplotypes. The most common haplotype is H2, found in 35 specimens from four localities (Pnb=3, Grd=7, Tie=5, and Rib=20), followed by H3, found in 29 specimens in four localities (Pnb=6, Grd=3, Tie=17, and Ppn=3). H8 is found in 20 specimens from the Paranapanema river and H11 in 13 specimens from Grande, Peixe and Paranapanema rivers. Remaining haplotypes are mostly restricted to a single locality and are found in one or two specimens only.

Significant genetic differentiation between each of the eight river basins (Table 1) ranges from $F_{ST} = 0.057$ (between Tietê and Paranaíba rivers) to 0.51 (between Paranapanema and Ribeira). Values of Φst ranging from 0.24 to 0.37 were found between the larger river basins composing the UP, i.e. Parnaíba, Grande, Tietê,

Figure 2. Maximum Likelihood tree showing four haplogroups of *Hypostomus ancistroides*. Bootstrap values above 60% for the ingroup are shown above nodes. Legend of colors for the river basins follows Figure 1. Each haplogroup is depicted in a different colour: dark green (haplogroup 1), light green (2), grey (3), and red (4). Outgroup in black.
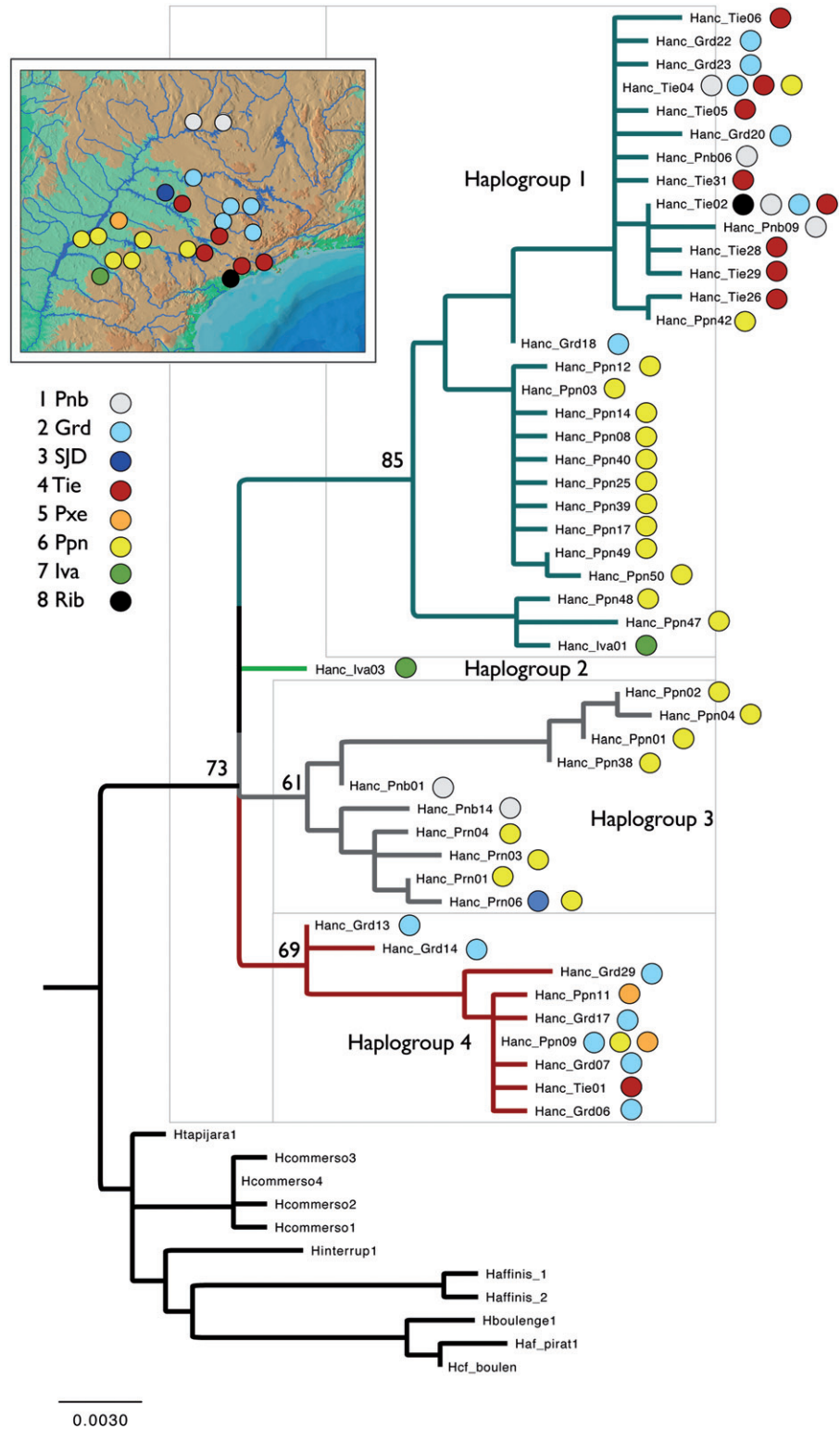
and Paranapanema. AMOVA results (Table 2) show that most of the genetic variation is found within each river basin (44%) and within each sampled locality (46%). A low proportion of the total variation (10%) is assignable to a hypothesis of basin-structured populations, but non-significantly. Neutrality test results in negative values (Tajima's D -0.70247, p value 0.27210; Fu's Fs -11.2534, p-value 0.0271), which are consistent with an event of population expansion (Table 2). However, p value is significant for Fu's Fs but not significant for Tajima's D.

Discussion

Results show that *Hypostomus ancistroides* has a complex and multi-layered evolutionary history as reflected in phylogenetic results, tempo estimate, genetic distances between extant lineages and geographic distribution of haplotypes. In view of the vast extent and age of the UP, more clearly structured populations among different river basins were expected. The UP is a well-established hydrographic entity and conserves its main
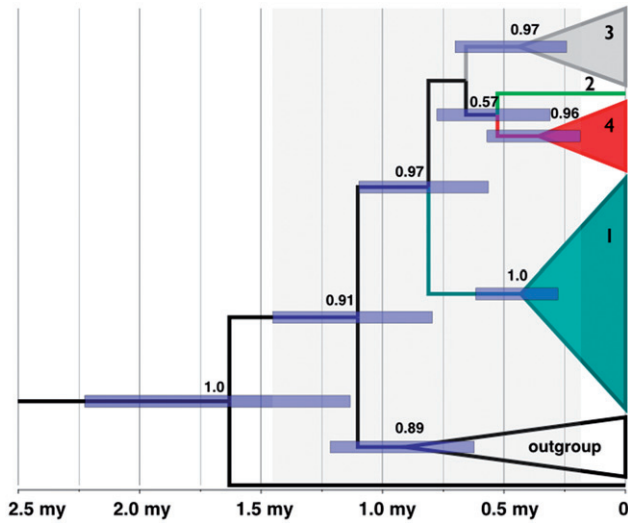
Figure 3. Tempo (years) estimate for the divergence of *Hypostomus ancistroides* and diversification of its four haplogroups. Branches within each haplogroup are collapsed. Haplogroups are numbered and colored as in Figure 2. Posterior probability values higher than 0.5 are shown above nodes. Blue bar indicates 95% credibility intervals.

physiography since around 9.0 Ma (Albert & Reis, 2011). However, an opposite scenario is suggested by the extant haplogroups with broad distribution range and the considerable number of haplotypes found across different basins. The haplotype network shows that each river basin is inhabited by individuals belonging to distinct evolutionary lineages depicted in the phylogenetic analysis. The existence of deep lineages originated during the Pleistocene indicates periods of isolation, which have allowed the origin of such ancient haplotypic groups across the distribution range. In fact, the UP was subjected to alternating cycles of humidity and dryness along the Quaternary (e.g. Albert & Reis, 2011; Sallun & Suguio, 2010; Santos & Stevaux, 2000; Stevaux, 1994; Stevaux & Souza, 2004) revealed by palynology, sedimentology, and geomorphology data from floodplains of the UP. The level of contact between populations of freshwater organisms is strongly dependent on the availability of water, which directly determines the degree of contact among streams and rivers (Jones & Johnson, 2009). Changes in local meso- and microhabitat conditions or contact between watercourses are examples of such hydrological changes that may interfere on population structure of fishes. Consequently, the occurrence of such hydric oscillations for considerable periods of time can explain successive periods of isolation and merging of different populations by alternatively enabling and restraining
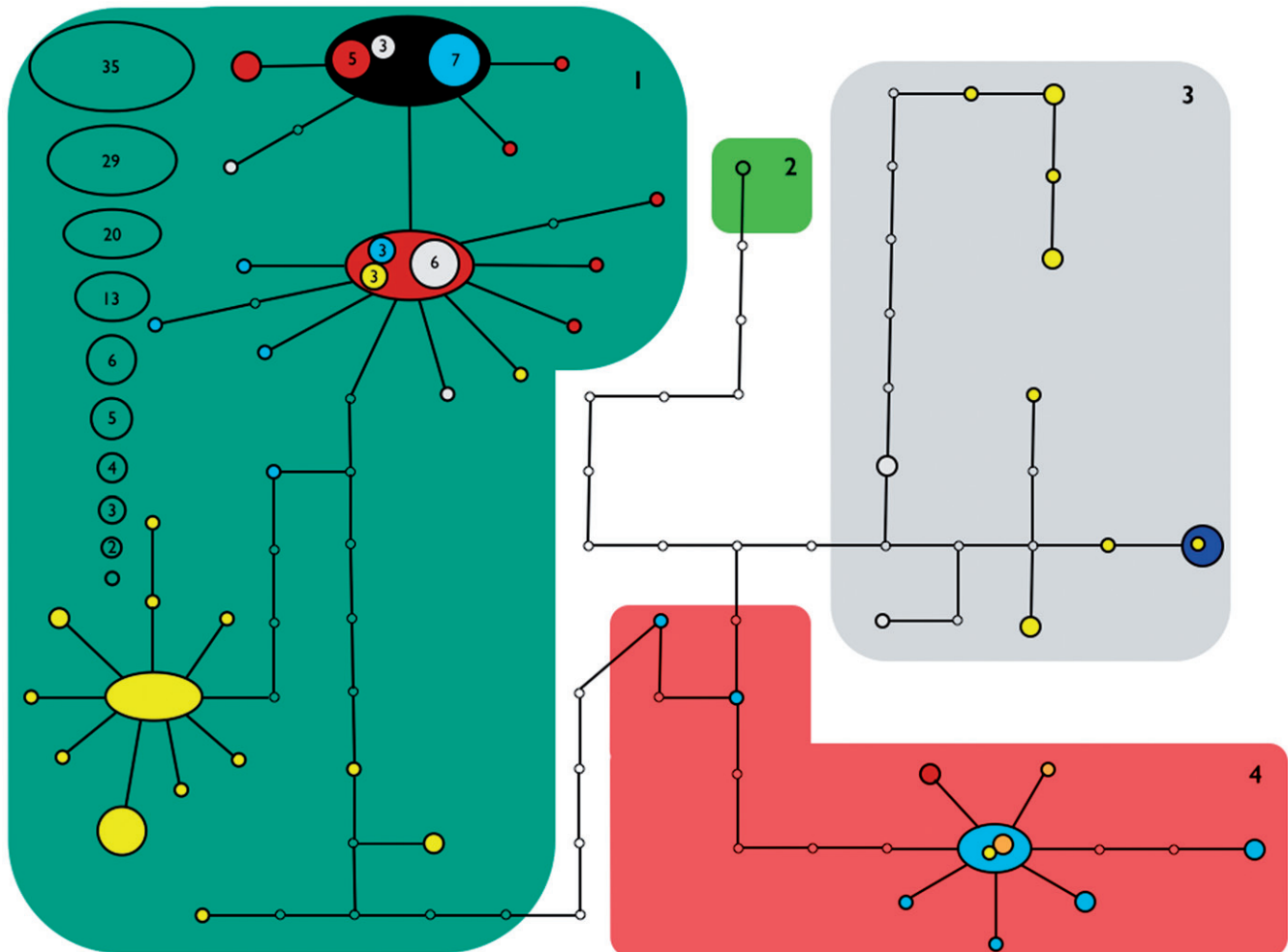
Figure 4. Haplotype network of 162 specimens of *Hypostomus ancistroides*. Sizes of symbols are proportional to the number of specimens bearing the respective haplotype, as shown in legend on left. Circles comprised by larger symbols bear the number of specimens found at the basin it represents. The number of mutational steps is represented by empty circles. Different haplogroups are indicated by numbers on the upper right of the shapes. Colors as in Figure 2. Locality abbreviations as in Figure 1.

Table 1. Pairwise Φst values (below diagonal) and number of samples (first row) for each river basin.

	Grd	Pnb	SJD	Tie	Iva	Pxe	Ppn	Rib
N (=162)	32	14	4	33	3	3	53	20
Grd	0							
Pnb	0.245	0						
SJD	0.479*	0.694*	0					
Tie	0.377	0.057	0.838*	0				
Iva	0.252*	0.382*	0.649*	0.636*	0			
Pxe	0.180*	0.709*	0.980*	0.834*	0.606*	0		
Ppn	0.296	0.285	0.518*	0.427	0.205*	0.565*	0	
Rib	0.474	0.269	1*	0.180	0.884*	0.997*	0.509	0

Pnb, Paranaíba; Grd, Grande; SJD, São José dos Dourados river; Tie, Tietê; Pxe, Peixe; Ppn, Paranapanema; Rib, Ribeira de Iguape.
*Unsupported values ($p > 0.05$).

Table 2. AMOVA results for the hypothesis of river-basin structured populations including the seven main rivers composing the UP plus the Ribeira de Iguape.

Source of variation	Variance components	% of variation	p-Value
Among river basins	0.51760	9.73	0.0 ± 0.0
Within river basins	2.35480	44.29	0.0 ± 0.0
Within sampled localities	2.44489	45.98	0.20 ± 0.1

in-waterconnections between UP basins. Recent haplotypes might have originated in specific localities and then underwent population growth and/or range expansion, as shown by the star-like shape of the network and Fu's neutrality test. Accordingly, a reduced value of nucleotide diversity associated with the large haplotype diversity is suggestive of bottleneck events followed by the rise of new haplotypes during population growth (Grant & Bowen, 1998).

The situation of the Ribeira de Iguape coastal basin is particularly interesting. All specimens from that drainage bear the same haplotype, which is also widely found throughout the northern ranges of UP basins. Those ranges include the Tietê river, located in the adjacent slope of the Serra do Mar mountains. Significantly, *H. ancistroides* is restricted to the northeastern headwaters of the Rio Ribeira de Iguape, close to the watershed of the Upper Paraná (Oyakawa et al., 2005), suggesting a recent colonization event. Fishes inhabiting the Ribeira de Iguape drainage have not yet fixed new haplotypes and have not expanded their geographic range along its southwestern stretches. Probably, colonisation occurred by capture of headwaters originally draining into the UP, most probably from the Tietê basin itself, which today surrounds the Ribeira de Iguape basin. Similar patterns have been reported to the south-eastern coastal portion of South America during the Paleogene and Quaternary (Ribeiro, 2006) and account for the shared fauna between coastal basins and the UP (Ribeiro, 2006; Ribeiro et al., 2006). Our estimate of the rise of the haplotype found at Ribeira de Iguape and other basins is around 30 000 years ago (not shown in Figure 3). However, relationship between that haplotype and closely related ones is supported by a low PP and it is impossible to determine that putative range expansion with certainty. On the contrary, a larger clade including that haplotype is supported by 0.76 PP and is confidently estimated to have arisen before 77 000 years before present. In view of those auxiliary values, it is possible to hypothesize that the Ribeira de Iguape basin was colonised by *H. ancistroides* during the late Pleistocene.

The evidence discussed above suggests that the population dynamics of *H. ancistroides* alternates between periods of prolonged isolation and secondary contact. Such alternating pattern is likely strongly associated with climatic oscillations but also, on occasion, with geomorphological processes. The colonisation of the Ribeira de Iguape basin, for example, was likely caused by erosive retreat of the coastal slope of the Serra do Mar basin. Further data on geomorphological processes at work on the Brazilian crystalline shield are awaited to further test this idea.

Finally, the pronounced genetic divergence among lineages of *H. ancistroides* from UP is striking. Our results show genetic distance values similar to those found between different species of *Hypostomus*. Such divergence suggests relevant historical periods of population segregation that may be regarded as genuine vicariant events. However, secondary contact among populations has prevented full speciation in *H. ancistroides*. We found no associative evidence for the existence of more than one species in the taxon currently considered as *H. ancistroides*. The specimens assignable to the species form a monophyletic group and its different lineages are found in sympatry. Past events in the UP are obviously profound enough to have resulted in speciation in other lineages of fishes, as evidenced by the contemporary species richness and endemics inhabiting that basin. However, such events have not affected all fish taxa uniformly, as shown by the case of *H. ancistroides*.

In conclusion, we point out that Neotropical freshwater fish species with widespread distributions spanning more than one major drainage have often been singled out as good candidates for more in-depth taxonomic work. Current interpretation is that such species are in fact complexes, composed of cryptic species hardly diagnosable phenotypically and each endemic to smaller portions of drainages. Our results with *H. ancistroides* show that this need not necessarily be the case. The complexity of drainage history may account for instances of uniformity in otherwise widely separated populations of widespread species of freshwater fishes.

Acknowledgements

Declaration of interest

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