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Molecular and karyotypic phylogeography in the Neotropical *Hoplias malabaricus* (Erythrinidae) fish in eastern Brazil

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The sedentary, predatory characin *Hoplias malabaricus* has one of the widest distributions of freshwater fishes in South America and is characterized by seven karyomorphs (A–G) that occur in sympatric and allopatric populations. Karyotypical patterns of variation in wild populations have been interpreted as evidence of multiple lineages within this nominal species, a possibility that may limit the validity of experimental data for particular karyomorphs. This study used the phylogeographic and genealogical concordance between cytogenetic ($N = 49$) and molecular (mitochondrial DNA) ($N = 73$) data on 17 samples, collected in 12 basins from south-eastern and north-eastern Brazil, to assess the systematic value of cytogenetic data. Cytogenetic patterns show a sex chromosome system in the $2n = 40$F karyomorph. Molecular and cytogenetic data indicate a long, independent evolutionary history of karyomorphs and a coastal origin of continental populations in south-eastern Brazil. The lack of fit with molecular clock expectations of divergence between groups is likely to be due to strong demographic fluctuations during the evolution of this species complex. The results indicate that karyotypical identification provides a reliable baseline for placing experimental studies on *Hoplias* spp. in a phylogenetic context.

Key words: freshwater fish; *in situ* hybridization; molecular clock; mtDNA; repetitive DNAs; South America.

INTRODUCTION

The Neotropical region harbours at least 6025 species of freshwater fishes (Reis *et al*., 2003), but perhaps as many as 8000 (Schaefer, 1998), the result of a long evolutionary history of isolation and specialization, involving mainly otophysan fishes. Freshwater fishes are highly suitable for the recovery of past biogeographic processes, due to their obligatory relationship with water (Myers, 1938). The biogeographical

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information relevant for fish diversification has been reviewed by Lundberg *et al*. (1998) and Ribeiro (2006). The former revision focused on the evolution of Andean geomorphology and the effects of this process on the South American continent, whereas the latter work dealt with the evolution of particular coastal drainages, their geomorphological interactions with continental basins and the expected levels of phylogenetic divergence among fishes derived during these complex temporal events.

Among Neotropical freshwater fishes, the trahira, *Hoplias malabaricus* (Bloch), has one of the largest distributional ranges, occurring from Panamá to the Buenos Aires Province in Argentina (Berra, 2007). *H. malabaricus* is well adapted to life in small, isolated populations, in conditions that may facilitate the stochastic fixation of chromosomal rearrangements (Sites & Moritz, 1987). This species is one of the cytogenetically most studied taxa and shows a conspicuous karyotypic diversification, with up to seven karyomorphs with diploid numbers ranging from 39 to 42. Three groups of karyomorphs are readily evident, based on distribution ranges. Two karyomorphs, $2n = 42$A and $2n = 40$C, are the most widespread and occur in sympatry in the lower Paraná–Paraguay and Amazon River basins. A second, less widely spread group is represented by three karyomorphs: (1) $2n = 40$F occurs in the São Francisco River basin, in the lower portions of the Tocantins River, in coastal drainages in Surinam and in minor basins in north-eastern Brazil; (2) $2n = 39/40$D occurs in the upper Paraná River (in sympatry with $2n = 42$A) and (3) $2n = 40/41$G occurs exclusively in the Amazonian Basin in the Aripuanã, Madeira and Trombetas Rivers.

The third group consists of karyomorphs with much restricted distributions. For example, $2n = 42$B occurs in the Doce River basin (also in sympatry with $2n = 42$A), and $2n = 42$E occurs only in the Trombetas River (Bertollo *et al*., 2000).

The lack of hybrids between sympatric karyomorphs has been interpreted as evidence for the existence of several distinct species within this nominal taxon (Bertollo *et al*., 1986, 2000). Therefore, uniparental molecular markers can be a particularly useful complement to assess gene flow in wild populations. To date, molecular studies on *H. malabaricus* have been restricted to populations in the Doce River basin (Dergam *et al*., 2002) and the Iguaçu River basin (Dergam *et al*., 1998). Both studies indicated phylogenetically related populations in the coastal and continental basins, but failed to reveal the direction of dispersal events.

Karyotypic and molecular patterns of variation may reveal vicariances and dispersals that provide insights into evolutionary processes that may involve other components of the aquatic fauna. The congruence of independent molecular and cytogenetic characters may give support to the hypothesis that at least some karyomorphs behave as valid species within *H. malabaricus*. A better understanding of *H. malabaricus* species-level systematics is needed to interpret correctly the comparative physiological, behavioural and anatomical studies in an explicit phylogenetic framework (Harvey & Pagel, 1991). Biodiversity patterns in this species complex also bear potential information to understand the palaeohydrology of the continent. In the present study, patterns of cytogenetic and molecular variation of *H. malabaricus* populations in the São Francisco, Grande and in several coastal basins along eastern Brazil were analysed using molecular and cytogenetic techniques.

MATERIALS AND METHODS

SPECIMENS AND CHROMOSOME PREPARATION

Cytogenetic analyses were carried out on 49 specimens collected in the Pandeiros River, Pará River and its headwaters in the Tombadouro Creek and in the Jacaré River (Table I and Fig. 1). Cell division was stimulated *in vivo* with two daily applications of Munolan, a commercially available antigen lysate, following Molina (2001). Mitotic chromosomes were obtained from cell suspensions of the anterior kidney, using the conventional air-drying method (Bertollo *et al*., 1978). Fish were previously anaesthetized with clove oil (Henyey *et al*., 2002). Secondary data from 10 populations with known chromosome numbers were also included in the analysis.

CHROMOSOME STAINING

In addition to the standard Giemsa method, chromosomes were analysed using silver nitrate staining (Howell & Black, 1980) to visualize the nucleolar organizing regions (Ag-NOR). C-banding was also employed to detect C-positive heterochromatin (Sumner, 1972). (4′,6′ diamidino-2-phenylindole dihydrochloride (DAPI) and chromomycin A_3 (CMA_3) fluorescence staining were used to identify the chromosome adenine–thymine (AT) and guanine-cytosine (GC) rich regions, respectively (Sola *et al*., 1992). Some populations with unknown karyotypes (or whose karyotypes are being studied by J.A.D.) were also included in this study (see below).

CHROMOSOME HYBRIDIZATION AND KARYOTYPIC ANALYSIS

Fluorescent *in situ* hybridization (FISH) was performed according to Pinkel *et al*. (1986) using three repetitive DNA sequences probes isolated from the genome of *H. malabaricus* and an 18S probe. The first probe contained a 5S rDNA repeat copy and included 120 base pairs

TABLE I. *Hoplias malabaricus* collecting localities, sample sizes, geographic coordinates and karyomorph nomenclature

Locality	Cytogenetic samples	Molecular samples	G.P.S.	Karyomorph
Pará River	8♀–7♂	14	20° 08′ 21″ S–44° 53′ 17″ W	40F
Pandeiros River	2♀–3♂	9	15° 40′ 18″ S–44° 37′ 43″ W	40F
Tombadouro Creek	4♀–3♂	5	20° 41′ 56″ S–44° 34′ 46″ W	42A
Ibimirim	—	6	8° 30′ 31″ S–37° 42′ 21″ W	Unknown
Curvelo	12	8	18° 42′ 57″ S–44° 25′ 56″ W	40F
Itapicuru River	4	1	13° 14′ 40″ S–41° 23′ 34″ W	40F
Dom Helvécio Lake	10	3	19° 46′ 34″ S–42° 35′ 19″ W	42B
São Mateus River	2	6	18° 44′ 09″ S–48° 29′ 47″ W	42A
Paraíba do Sul River	15	2	21° 46′ 26″ S–41° 30′ 01″ W	42A
Itabapoana River	8	2	21° 08′ 20″ S–41° 39′ 35″ W	42A
Macaé River	6	1	22° 17′ 43″ S–41° 52′ 48″ W	42A
São João River	8	2	22° 33′ 29″ S–42° 07′ 21″ W	42A
Ribeira River	2	1	24° 29′ 23″ S–47° 50′ 10″ W	42A
Carioca Lake	15	1	19° 45′ 32″ S–42° 37′ 15″ W	42B
Paranaguá Bay	—	1	25° 32′ 39″ S–48° 29′ 47″ W	Unknown
Macacu Waterfalls	—	1	22° 27′ 37″ S–42° 39′ 18″ W	Unknown
Jacaré River	15♀–7♂	10	20° 48′ 29″ S–44° 33′ 58″ W	42A

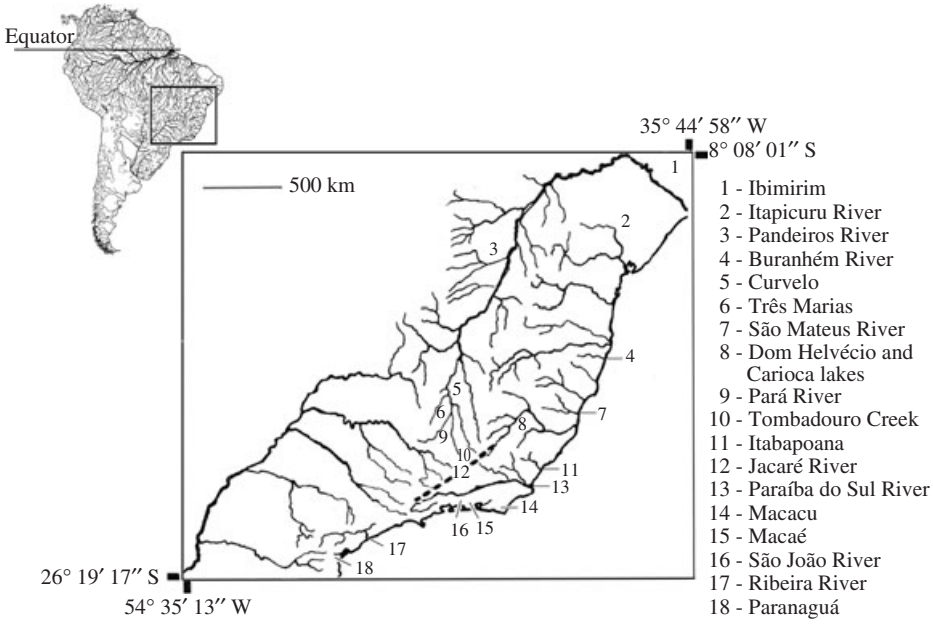

FIG. 1. *Hoplias malabaricus* collection localities. The dotted line indicates the upper Grande crustal discontinuity. The locality of Três Marias is also depicted.

(bp) of the 5S rRNA encoding gene and 200 bp of the non-transcribed spacer (NTS) (Martins *et al*., 2006). The second probe is specific to *H. malabaricus* (Ferreira *et al*., 2007) and contained a copy of the repetitive satellite 5S*Hind*III-DNA sequence with 360 bp composed of a 95 bp segment similar to the 5S rRNA gene of the first probe and a 265 bp segment similar to the NTS of the first probe (Martins *et al*., 2006). The third probe corresponded to a 1400 bp segment of the 18S rRNA gene obtained by the polymerase chain reaction (PCR) from nuclear DNA (Cioffi *et al*., 2009). The probes were labelled by nick translation with biotin-14-dATP (Bionick Labeling System, Invitrogen; www.invitrogen.com). Signal detection and its amplification were performed using conjugated avidin–fluorescein isothiocyanate (FITC) and anti-avidin–biotin (Sigma; www.sigmaaldrich.com). The chromosomes were counterstained with propidium iodide (50 $\mu g\,ml^{-1}$) and analysed with an Olympus BX50 epifluorescence microscope. The chromosomal images were captured using CoolSNAP-Pro software (Media Cybernetics; www.MediaCy.com). About 30 metaphase spreads were analysed per specimen to determine the diploid chromosome number and karyotypic structure. Chromosomes were classified as metacentrics (m) or submetacentrics (sm), according to centromeric index values proposed by Levan *et al*. (1964).

DNA EXTRACTION AND MOLECULAR ANALYSIS

DNA extraction followed Boyce *et al*. (1989). Fragments of ATPase 6 were amplified using primers L8524 (5′-AAY CCT GAR ACT GAC CAT G-3′) and H9236 (5′-GTT AGT GGT CAK GGG CTT GGR TC-3′) (Quenouille *et al*., 2004). Double-stranded DNA was synthesized in 50 µl reactions containing 10 µl dNTPs (1 mM), 5 µl reaction buffer (200 mM Tris–HCl pH 8·4, 500 mM KCl), 2 µl $MgCl_2$ (50 mM), 2 µl of each primer (10 mM), 0·5 µl (2·5 U) Taq DNA polymerase (Phoneutria), 2 µl template DNA (100 ng/µl) and 26·5 µl H_2O. PCR conditions were as follows: 94° C (2 min), five cycles of 94° C (45 s), 54° C (45 s) and 72° C (1·5 min) and 29 cycles of 94° C (45 s), 58° C (45 s) and 72° C (1·5 min). PCR products were purified using Qiaquick (Qiagen; www.quiagen.com): 5 µl of the purified PCR product were used in a 10 µl cycle sequencing reaction using a drhodamine terminator

cycle sequencing kit (PE Applied Biosystems; www.appliedbiosystems.com). Sequences were aligned using CLUSTAL X 1.83 in MEGA 4.0 (Tamura *et al*., 2007). Phylogenetic trees were constructed with neighbour joining (NJ) (Saitou & Nei, 1987), maximum parsimony (MP), maximum likelihood (ML) (Felsenstein, 1981) and Bayesian inference (MB) (Huelsenbeck & Ronquist, 2001). The model of molecular evolution that best fitted the data was chosen using Modeltest 3.7 (Posada & Crandall, 1998), and haplotype divergence was estimated within and between haplogroups using this selected model. Phylogenetic signal in NJ, MP and ML trees was assessed using bootstraps with 1000 repetitions. Bayesian inference was performed with five million Markov-Chain Monte-Carlo (MCMC) steps to produce posterior probabilities of nodes in the tree with MrModeltest 2 (Nylander, 2004). *Hoplias lacerdae* Miranda Ribeiro was used as an outgroup. Sequences were deposited in GenBank (accession numbers GQ848606–GQ848642).

RESULTS

KARYOTYPIC DATA

Karyomorph 2n = 40*F*

All specimens from the Pandeiros and Pará rivers (except those from Pará's headwaters, in Tombadouro Creek) had $2n = 40$ chromosomes for both sexes. The karyotypes were composed of 10 m + 10 sm pairs, without morphologically differentiated sex chromosomes using Giemsa conventional staining [Fig. 2(a)], and were typical $2n = 40$F karyomorphs. C-banding showed heterochromatic blocks in centromeric regions, except for chromosome pairs 9 and 10, which had less conspicuous blocks, in addition to faint telomeric marks in some chromosome pairs. Males always showed an interstitial heterochromatic block in the short arms of one homologue of the first chromosome pair [Fig. 2(c)], whereas females lacked this block [Fig. 2(e)]. This heterochromatic region was negative for DAPI staining but failed to show fluorescence with CMA_3. A proximal heterochromatic block located on the short arms of a small submetacentric pair was the only observed GC-rich segment in both populations [Fig. 3(f), (i)]. Ag-NORs were telomeric and their numbers were either fixed (four in the Pará River) or varied from 4 to 5 (Pandeiros River) [Fig. 4(a), (b)]. FISH analyses with the repetitive sequences showed no differences between these two populations. The 18S rDNA probe hybridized on the pericentromeric region of one metacentric pair and on the telomeric regions of two submetacentric pairs [Fig. 3(a)]. The 5S rDNA probe hybridized on the pericentromeric region of a medium-sized metacentric pair [Fig. 3(c)]. The repetitive 5S*Hind*III-DNA was mapped in the centromeric region of 10 chromosome pairs [Fig. 5(a)]. Although Ibimirim samples were not karyotyped, they were collected in a region with populations characterized by $2n = 40$F karyomorph and were assumed to share this karyomorph.

Karyomorph 2n = 42*A*

In the headwaters of Tombadouro Creek, all specimens had $2n = 42$ chromosomes, with a karyotype consisting of 11 m + 10 sm pairs and without morphologically differentiated sex chromosomes [Fig. 2(b)], and were considered to be typical $2n = 42$A karyomorphs. Besides some telomeric marks, conspicuous heterochromatic bands were found in the centromeric region of all chromosomes, except for chromosome pairs 6, 19 and 20, which showed faint blocks [Fig. 2(d)]. Ag-NORs varied among and within individuals (one to two pairs), and bitelomeric NORs were evident in either one or two chromosomes [Fig. 4(c)]. In this population, the 18S rDNA probe

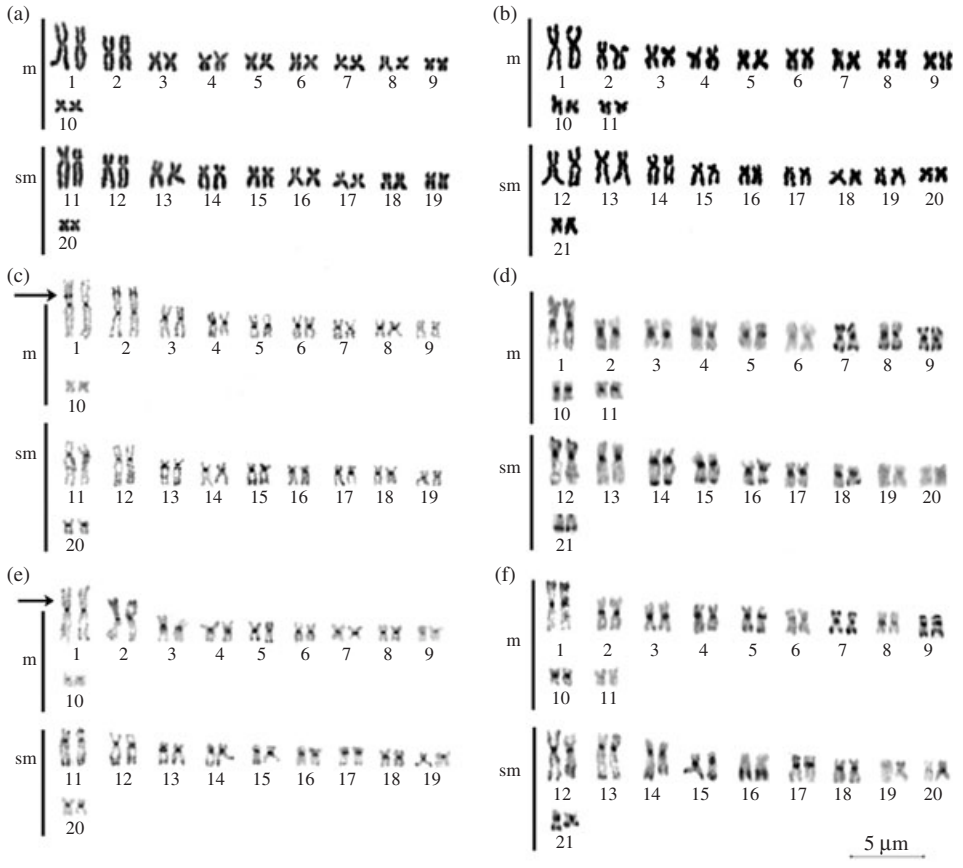

FIG. 2. Giemsa-stained and C-banded karyotypes of *Hoplias malabaricus*. (a) conventional Giemsa-stained karyotype of populations from Pandeiros and Pará rivers, (b) samples from Jacaré River and Tombadouro Creek, (c) C-banded karyotypes of males from Pará and Pandeiros Rivers, (e) C-banded karyotypes of females from Pandeiros and Pará Rivers, (d) C-banded karyotype of specimens from Tombadouro Creek and (f) C-banded karyotype from Grande River. Arrows indicate an interstitial heterochromatic block in the short arm of a homologue of the first chromosome pair. This block appeared in males of Pandeiros and Pará River populations, but not in females.

hybridized on three chromosome pairs, with interstitial, telomeric and bitelomeric sites [Fig. 3(b)]. The 5S rDNA probe hybridized on the interstitial region of a large submetacentric pair [Fig. 3(d)]. The repetitive 5S*Hind*III-DNA probe bound to the centromeric region of nine chromosome pairs [Fig. 5(b)].

Specimens from the Jacaré River in the Grande drainage had $2n = 42$ karyomorphs composed of 11 m + 10 sm pairs, without morphologically differentiated sex chromosomes [Fig. 2(b)], and represented a typical $2n = 42$A karyomorph. Heterochromatic blocks were centromeric in most chromosomes, except for pairs 6, 8, 11, 19 and 20, which showed faint bands, in addition to telomeric marks in some chromosome pairs [Fig. 2(f)]. Ag-NORs were restricted to telomeric regions of two chromosomes [Fig. 4(d)]. Fluorescent regions hybridized with 18S rDNA and 5S*Hind*III-DNA and showed a pattern similar to the Tombadouro Creek sample.

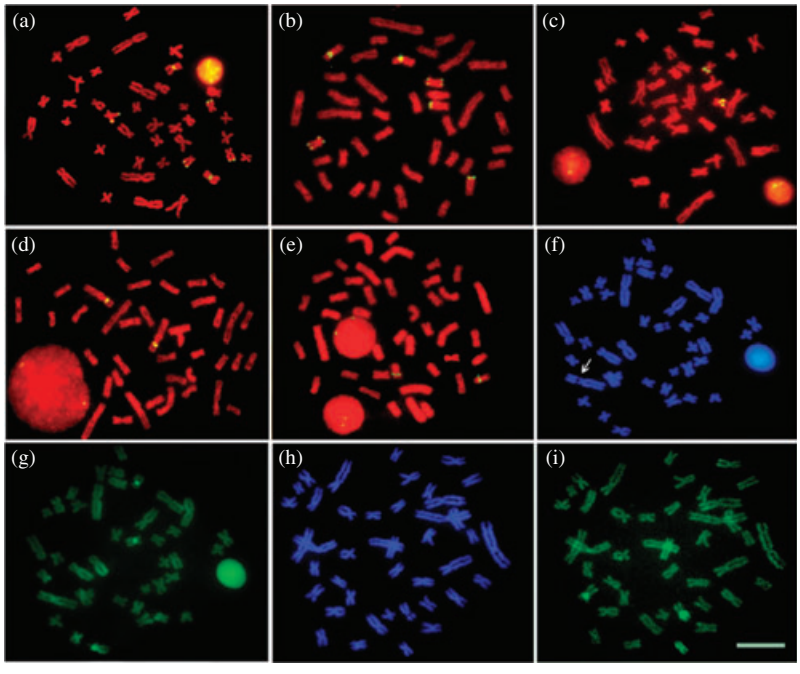

FIG. 3. Metaphase chromosome spreads of *Hoplias malabaricus* after FISH with 18S and 5S rDNA probes and DAPI–Chromomycin A3 staining. Numerical and positional variation of 18S rDNA sites in populations from (a) Pandeiros and Pará Rivers, (b) Tombadouro Creek and Jacaré River. Mapping of 5S rDNA sites for populations of (c) Pandeiros and Pará Rivers, (d) Tombadouro Creek and (e) Jacaré River. DAPI staining demonstrating absence of signals in males (f) and females (h) from Pará River. Arrow indicates the interstitial heterochromatic block negative for DAPI in one homologue of the first chromosome pair in males. Sequential CMA_3 staining showing GC-rich DNA segments located on a submetacentric chromosome pair on (g) males and (i) females. Bar = 5 μm.

Additionally, the 5S rDNA probe hybridized on the interstitial region of a small metacentric pair [Fig. 3(e)]. As was the case with the Ibimirim samples, Paranaguá and Macacu samples were collected within the range of $2n = 42$A populations (J. A. Dergam, unpubl. data) and were therefore assumed to be derived from these populations.

MOLECULAR DATA

Multiple sequences 511 bp in length yielded 86 phylogenetically informative sites. Translation of these sequences into the corresponding amino acid sequences resulted in eight phylogenetically informative sites. The transitions:transversions ratio was 6·4, indicating that substitution rates were not saturated. The best model fit estimated with Modeltest and MrModeltest was HKY+G, which was used for NJ. In the tree, haplotypes were separated into two large clades with variable bootstrap support (Fig. 6). The largest bootstrap values were obtained for haplogroup I, which was composed of the $2n = 42$A karyomorph from Tombadouro Creek, Ribeira, Paranaguá and Macacu rivers (Fig. 1). Haplogroup II was less supported and included all remaining *H. malabaricus* ($N = 67$), regardless of diploid number. Within this

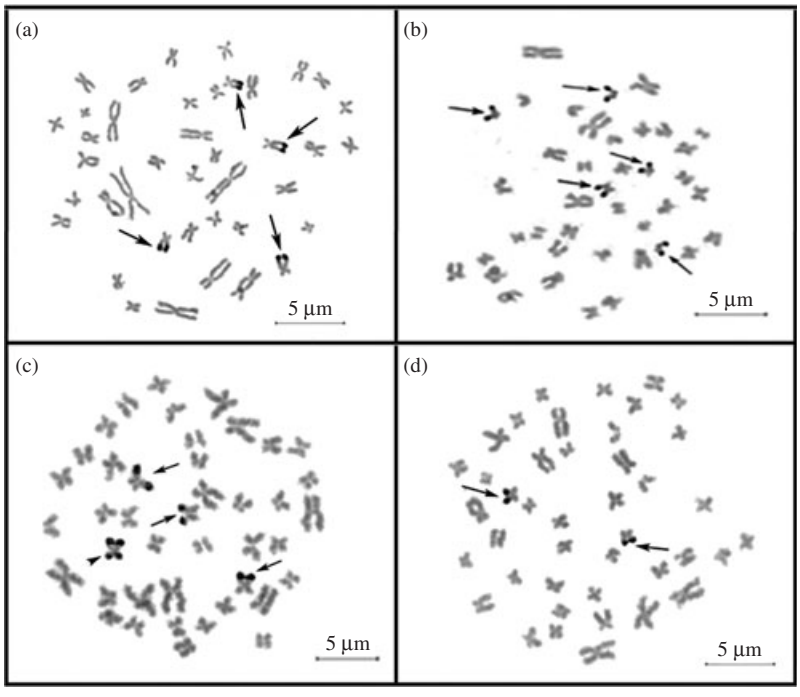

FIG. 4. Metaphase chromosome spreads of *Hoplias malabaricus* after silver staining, showing Ag-NORs in populations from (a) Pará River, (b) Pandeiros River, (c) Tombadouro Creek and (d) Jacaré River. Arrows and arrowhead indicate telomeric and bitelomeric Ag-NORs, respectively.

TABLE II. Within- and between-haplogroup molecular distances in *Hoplias malabaricus*

	Haplogroup I, $2n = 42$	Haplogroup IIA, $2n = 40$	Haplogroup IIB, $2n = 42$
Haplogroup I, $2n = 42$	0·032		
Haplogroup II A, $2n = 40$	6·82	0·0053	
Haplogroup II B, $2n = 42$	9·20	9·50	2·65

haplogroup, two subclades were highly supported: haplotypes derived from populations with the $2n = 40$F karyomorph (haplogroup IIA) and from populations with $2n = 42$A and $2n = 42$B karyomorphs (haplogroup IIB). Haplogroup IIA included all $2n = 40$ São Francisco River *H. malabaricus*, plus the Itapicuru River specimen. Haplogroup IIB included two well-defined lineages: one included haplotypes from the Doce and São Mateus Rivers with no representatives in the Grande River, and a second lineage indicated a close phylogenetic relatedness between *H. malabaricus* from the Jacaré (Grande River), the Tombadouro (São Francisco River) and the Macaé, São João, Itabapoana and Paraíba do Sul coastal basins. Within-haplogroup nucleotide molecular distances showed high levels of variation, with the smallest values in the São Francisco and Itapicuru samples and the largest in the IIB haplogroup. The range of between-haplogroup variation was much smaller (Table II).

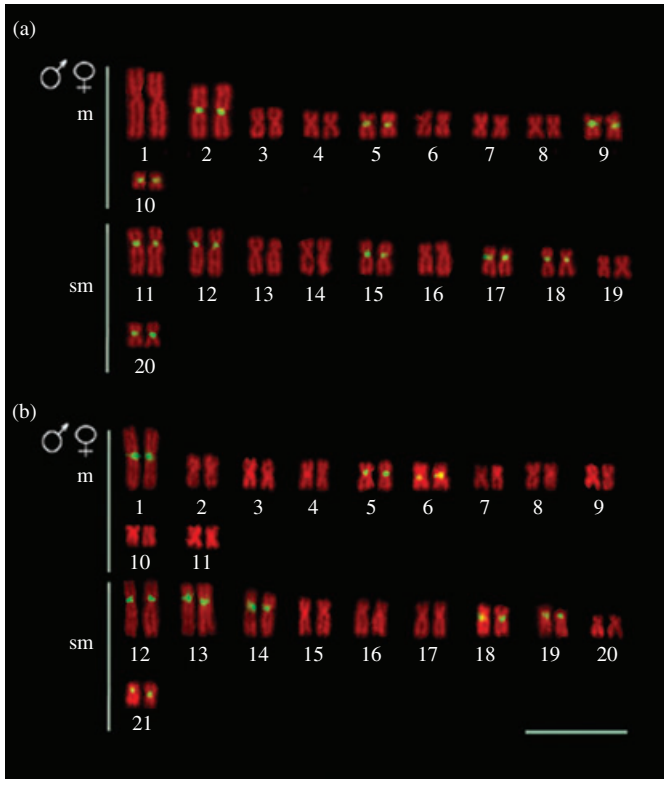

FIG. 5. Karyotypes of *Hoplias malabaricus* samples from (a) Pará and Pandeiros Rivers and (b) Tombadouro Creek and Jacaré River arranged from chromosomes probed with 5S*Hind*III-DNA counterstained with propidium iodide. Bar = 5 μm.

DISCUSSION

KARYOTYPIC DATA

Karyomorph 2n = 40*F*

In addition to specimens from Tombadouro Creek, all samples from the São Francisco drainage shared the same karyomorphic formula, which is considered to be characteristic for São Francisco basin populations (Bertollo *et al*., 2000). The distributional pattern of heterochromatin was similar to that described by Dergam & Bertollo (1990) for the Três Marias population in this basin (Fig. 1), except for the presence of a heterochromatic block that was always restricted to one homologue of the first chromosome pair in males. This pattern suggested a probable XX/XY sex chromosome system in these populations, because this variant occurred only in males and was absent in females. This sex chromosome differentiation involving only heterochromatinization is a novelty within *H. malabaricus*, because all other sex chromosome systems described for this species complex involve translocations between chromosome pairs (*e.g.* karyomorphs $2n = 39/40$D and $2n = 40/41$G) (Bertollo *et al*., 2000), or translocation and heterochromatinization, as proposed for the evolution of the $2n = 42$B karyomorph from a $2n = 42$A karyomorph ancestor

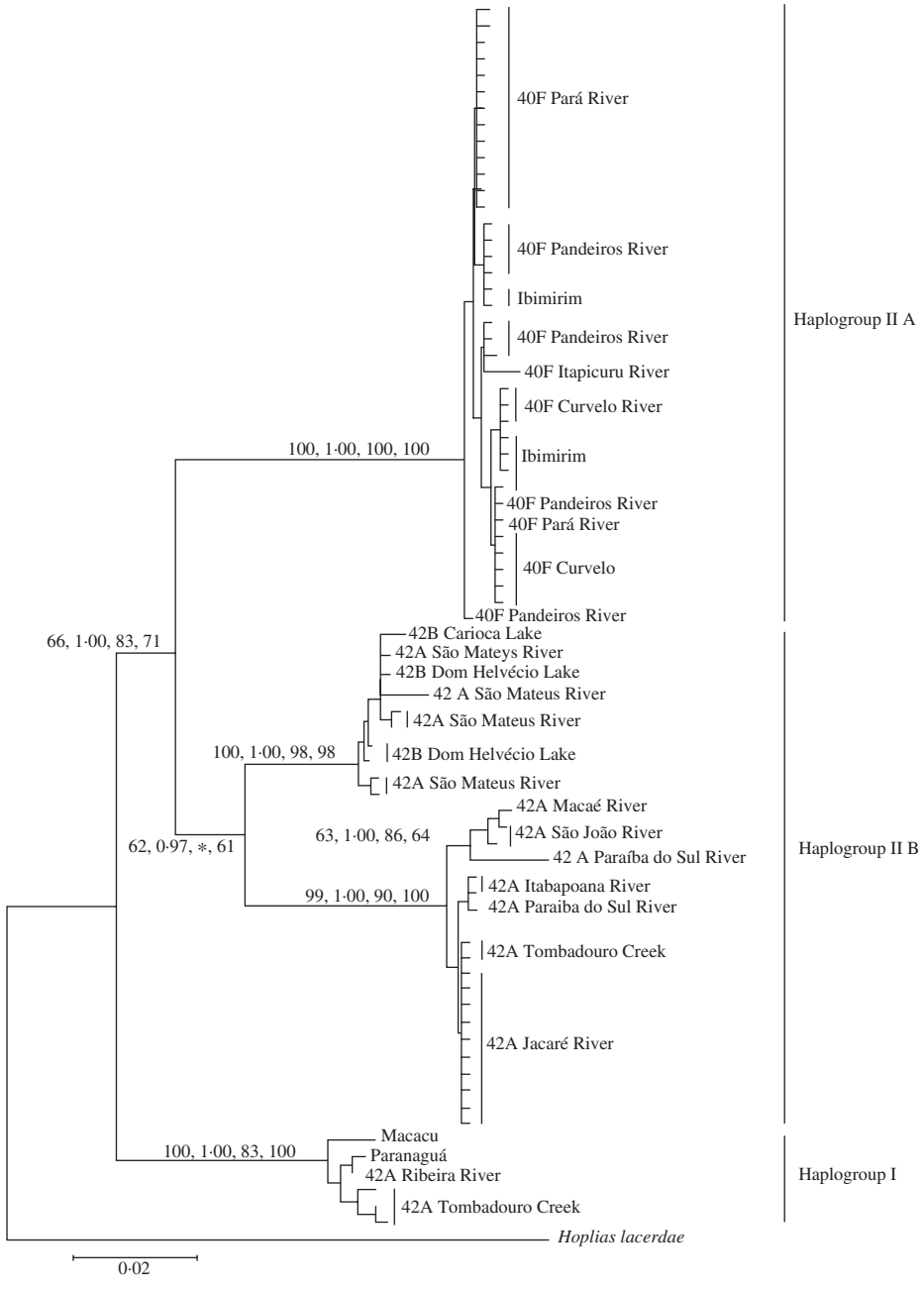

FIG. 6. Phylogenetic relationships of ATPase 6 haplotypes derived from *Hoplias malabaricus* karyomorphs. The topology was obtained with neighbour joining analysis. Bootstrap values are expressed as neighbour joining–Bayesian–maximum likelihood–maximum parsimony. The asterisk indicates a polytomy with maximum likelihood analysis. Bar = molecular distance.

(Born & Bertollo, 2000). The $2n = 40/41$G karyomorph also has a large first pair of metacentrics and is therefore morphologically closest to the $2n = 40$F karyomorph. Females with both karyomorphs have identical karyotypes and may be sister karyomorphs or species. Ongoing studies including these karyomorphs, using additional chromosomal markers will allow for a more thorough hypothesis of the evolutionary origins of this sex chromosome system.

C-positive heterochromatic bands were always located in the centromeric/pericentromeric region of all chromosomes and in the telomeric region of some pairs, in addition to multiple Ag-NORs sites. These heterochromatic and NOR characteristics were similar to those reported for other populations or karyomorphs of *H. malabaricus* (Dergam & Bertollo, 1990; Haaf *et al*., 1993; Bertollo, 1996, 1997; Born & Bertollo, 2000; Vicari *et al*., 2003, 2005). In the Pandeiros River sample, Ag-NORs were restricted to telomeres of some chromosomes and appeared in larger numbers than 18S rDNA sites, but the latter also hybridized to the pericentromeric region of one chromosome pair [Fig. 3(a)]. There is currently no explanation for the apparently non-specific nature of some telomeric Ag-NORs in the Pandeiros sample, which is as large as seven in the Três Marias region (Dergam & Bertollo, 1990). On the other hand, pericentromeric 18S rDNA sites were not visualized by the silver nitrate method (these data and Dergam & Bertollo, 1990), which may be due to preferential telomeric NOR activation, as already pointed out for other *Hoplias* spp. karyomorphs (Vicari *et al*., 2005). Hybridization sites of the 5S rDNA probe were interstitially located in a small metacentric pair in both populations, a pattern also reported for *H. malabaricus* samples from Três Marias (Ferreira *et al*., 2007). The patterns obtained with repetitive DNA class 5S*Hind*III-DNA were similar to those obtained for the Três Marias population (Ferreira *et al*., 2007).

Karyomorph $2n = 42A$

The karyotypes of specimens from Tombadouro Creek and Jacaré River were characterized as karyomorph $2n = 42$A, a widespread karyotypic form in the Neotropics (Bertollo *et al*., 2000). C-banding slowed conspicuous centromeric blocks in almost all chromosomes, an overall pattern also reported by Born & Bertollo (2001) for other Grande River $2n = 42$A populations. High levels of Ag-NOR variation observed in the Tombadouro Creek population have also been reported elsewhere in the Grande River basin (Born & Bertollo, 2001). Jacaré River *H. malabaricus* showed Ag-NORs restricted to one chromosome pair, characterizing the lowest number of activated NORs reported so far as a fixed character state for any *H. malabaricus* population. FISH with an 18S rDNA probe, however, showed a larger number of NORs cistrons in both $2n = 42$A karyomorph populations.

On the other hand, FISH mapping with a 5S rDNA probe revealed differences between the Tombadouro Creek and the Jacaré River populations, where they hybridized on a large submetacentric and a small metacentric pair, respectively. This difference contrasted with previous reports of other *H. malabaricus* populations from the upper Paraná Basin, the Araguá River, where both pairs showed fluorescence with this probe (Martins *et al*., 2006). Considering that at least some specimens from both basins shared identical haplotypes (see below), these population differences highlighted the great potential of this probe for population studies.

Patterns of variation of 18S rDNA were substantially different from the ones reported by Cioffi *et al*. (2009). These authors indicated the presence of five sites in

the $2n = 42$B karyomorph and four fluorescent sites in a $2n = 42$A karyomorph from the upper Paraná River basin, whereas the results of this study showed that the same karyomorph in the Jacaré and Tombadouro populations had three fluorescent sites. This reduction in numbers was also evident in the $2n = 40$F karyomorph, when compared with the $2n = 40$C and $2n = 39/40$D karyomorphs, which showed five fluorescent sites. Thus, this probe appears to be especially informative for studying among-karyomorph variation.

According to the haplotype-based trees, 5S*Hind*III-DNA patterns reflected a small increase in site numbers (from 18 to 20); Cioffi *et al*. (2009) also found variation in site numbers for other $2n = 42$ and $2n = 40$ populations. Also, the fluorescent centromeric region in the first or second chromosome pairs appears to be particularly informative. The presence of the fluorescent region in the first chromosome pair was observed in the $2n = 42$A karyomorph, as is the case for $2n = 42$B, $2n = 40$C and $2n = 39/40$D karyomorphs (Cioffi *et al*., 2009), representing a plesiomorphic state. On the other hand, the $2n = 40$F karyomorph showed an apparently apomorphic condition, where the fluorescent region occurred in the second chromosome pair. Absence of the fluorescent site in the first chromosome pair might be attributed to a more recent origin of the first chromosome pair and its possible origin from chromosomes lacking this site. This hypothesis, however, involves a particularly complex evolutionary process, because the persistence of diploid number would also involve alterations of other chromosome pairs. Alternatively, the $2n = 40$F karyomorph might have evolved directly from a $2n = 42$A ancestor, causing a reduction in diploid number and producing an independent $2n = 40$ lineage. This hypothesis is consistent with the hypothesis of Bertollo *et al*. (2000), based on gross chromosome morphology, that the $2n = 40$F, $2n = 40/41$G and $2n = 42$E karyomorphs represent a monophyletic group within *H. malabaricus*.

In the submetacentric chromosome group, the pattern of fluorescent centromeric sites in three major chromosome pairs also sets apart the $2n = 40$F karyomorph from other karyomorphs. The $2n = 42$A karyomorph and the three karyomorphs reported by Cioffi *et al*. (2009) have conspicuous fluorescent centromeric regions in these pairs, while this number was reduced to two pairs in the $2n = 40$F karyomorph. Homeologies among other chromosome pairs seem less reliable and must await further data. These FISH results and the presence of a unique sex chromosome system clearly indicate that the $2n = 40$F karyomorph is a taxon with the largest suit of derived karyotypic characters in this species complex.

MOLECULAR DATA

Molecular data are congruent with the cytogenetic results and suggest a long reproductive isolation between the karyomorphs. Haplogroup I included some of the $2n = 42$A karyomorph haplotypes from the Tombadouro Creek and haplotypes derived from three coastal basins located south of the Paraíba do Sul River. This haplogroup showed low levels of within variation, suggesting that these haplotypes were closely related. Some haplogroup II haplotypes were sympatric with haplogroup I haplotypes at the Tombadouro Creek. Within haplogroup IIB, all haplotypes from the Grande River were closely related to haplotypes from four coastal basins. These haplotypes showed a sister group relationship with haplotypes from the São Mateus and Doce coastal basins that were not represented in samples from the São Francisco

and Grande Rivers. Some $2n = 42$ trahiras from the Tombadouro Creek and Jacaré River shared identical haplotypes, suggesting a recent range expansion from the Grande to the São Francisco basins.

These results provide insights into the evolution of *H. malabaricus* karyomorphs and the history of contact between coastal and continental hydrological basins. The most inclusive study of ND2 and ATPase 6 divergence between freshwater fishes is Bermingham *et al.* (1997), who estimated a rate of divergence for this gene segment of 1·3% per million years. Overall sequence (p-distance) divergence of the divergent haplogroup I haplotypes versus haplogroup II haplotypes would suggest a lineage split at 4·2 million years ago, an age that is younger than the inferred Amazon–Paranean vicariance, which has been dated at 11·8–10 million BP (reviewed in Lundberg *et al.*, 1998). This isolation left $2n = 42$ and $2n = 40$ karyomorphs in both basins (Bertollo *et al.*, 2000), indicating that karyotypic evolution was well underway during the Late Miocene Epoch. Miocene fossils are also considered to be similar to present-day taxa (Lundberg, 1998). The information content of molecular variation of this species complex may be more elusive: a preliminary analysis of ATPase 6 sequences in *H. malabaricus* populations from the Amazon–Paranean boundary basins reveals two clades that show large differences in p-distance divergence within each clade (0·10 and 0·34% per million years, respectively) (J. A. Dergam, unpubl. data), suggesting that molecular evolution within the *H. malabaricus* complex may be highly variable and influenced by other causes. Factors such as efficiency in DNA repair mechanism (Britten, 1986), generation time (Wu & Li, 1985), metabolic rate (Martin & Palumbi, 1993) and demographic processes (Ohta, 2002) may affect substitution rates among taxa. Hence, it is plausible to hypothesize a strong effect of demographic processes on the rates of molecular substitution in these populations, considering the close phylogenetic relationships among *H. malabaricus* karyomorphs and the adaptation of *H. malabaricus* to life in small populations, which is also reflected in their high levels of karyotypical variation. Slightly disadvantageous substitutions can drift to high frequencies in small populations (Ohta, 2002), thereby altering substitution rates in the DNA. Therefore, *H. malabaricus* and other species with similar ecology may be particularly poor candidates for using the molecular clock hypothesis to estimate divergence times. In the highly migratory species, *Prochilodus* spp., substitution rates of ATPase 6,8 range from 0·8 to 2·5% (Sivasundar *et al.*, 2001), suggesting haplotypes in *H. malabaricus* are among the most divergent in the Neotropical region. The present study is the first to indicate such a deep molecular divergence within the same karyomorph, although deep cytochrome *b* divergences were also observed among populations of the catfish *Pimelodus albicans* (Valenciennes) in the River Plate basin (Vergara *et al.*, 2008).

The location where lineage splitting occurred is limited to the distribution of current karyomorphs. The Paraná River basin harbours at least three karyomorphs, whereas the $2n = 40$F karyomorph appears to be widespread in the São Francisco River basin and the $2n = 42$A karyomorph is apparently restricted to the Pará River headwaters. North-eastern coastal populations harbour the $2n = 40$F karyomorph as far as the Buranhém River to the south (J. A. Dergam, unpubl. data) (Fig. 1), and these haplotypes had the lowest degree of divergence. This macro-geographical pattern suggests that lineage splitting occurred elsewhere and not in the relatively isolated São Francisco River basin. In haplogroup IIB, the close relationship between the $2n = 42$A and $2n = 42$B karyomorphs indicated that karyotypical differentiation

involved few alterations that resulted in a differentiated XX/XY chromosome system (Bertollo *et al*., 2000).

STREAM PIRACY

On a local scale, the apparently restricted range of the Tombadouro Creek $2n = 42$A karyomorph may have resulted from stream piracy from a former Jacaré River tributary. The Tombadouro Creek is 12 km from the Jacaré across the Galga watershed. Three hypotheses might explain this dispersal. First, boundary crossing may have resulted from either human mediated activities. Second, dispersal may have occurred through a high-altitude wetland that used to drain into both basins. For example, the connection may have been destroyed by the construction of BR 494 Highway, which is 8 km away at its closest point from the Tombadouro Creek. Third, headwater capture in the region may have occurred by relatively recent reactivation of ancient faults (Saadi *et al*., 2002). Altitudinal characteristics are also consistent with the direction of stream capture. Tombadouro is at 860 m altitude, whereas the Jacaré River is at 1038 m. This area is within the range of a continental fault known as the Upper Grande River Crustal Discontinuity (Saadi *et al*., 2002). This fault represents the southern limit of the São Francisco craton and the movable belts of southern Minas Gerais State (Fig. 1) and has been active in the last 15 000 years.

The phylogenetic origins of two highly divergent haplotypes in the Tombadouro Creek became clear only after haplotypes from coastal basins were included in the analysis. Most critically, haplotypes found in sympatry in the São Francisco River are related to haplotypes in different coastal basins. Under the allopatric model of speciation, this asymmetry suggests that ancestral fish dispersed from the coastal to the continental drainages, and the present study is the first to indicate unambiguously the direction of this dispersal. The timing of the dispersal between coastal and inland drainages, however, could not be estimated, because molecular clock expectations are not suitable for *H. malabaricus*. Therefore, the phylogeographic history of this group does not fit any of the three phylogenetic patterns proposed by Ribeiro (2006). On the other hand, low genetic divergence between the São Francisco and Itapicuru River drainages may be part of a recent faunal exchange in the arid region of north-eastern Brazil that is apparent in many other species of fish (Rosa *et al*., 2004).

Ribeiro (2006) indicated that the Atlantic drainages (Doce, Paraíba do Sul and Ribeira) have existed since the break-up of Gondwana. The results of the present study suggested that populations in these basins represent three biogeographic units, for which only two have close representatives in the boundaries of the Jacaré and Pará drainages. Dergam *et al*. (2002) previously reported a close phylogenetic relatedness between two haplotypes in the Doce and lower Grande River basins. Fish dispersal from coastal to continental drainages may have been restricted to only a few taxa adapted to headwater conditions, because coastal drainages are characterized by high levels of endemism (Ribeiro, 2006) and the Proterozoic Mantiqueira Range is considered to be an efficient barrier between coastal and continental drainages (Ingenito & Buckup, 2007). To the south, stream piracy has been particularly intensive in the Paraíba do Sul, Ribeira and Upper Paraná basins (Ribeiro, 2006).

The present study provided important information on the population distribution of *H. malabaricus* in São Francisco River basin and on historical relationships with populations in the Grande River and coastal basins. The $2n = 40$F karyomorph and

its associated haplotypes were widely distributed throughout the São Francisco basin, maintaining an apparently stable karyotypic structure of the chromosomal markers analysed here. This karyomorph was characterized by a XX/XY sex chromosome system. In contrast, the $2n = 42$A karyomorph showed a more restricted distribution in São Francisco basin and was closely associated with populations in a Grande River basin tributary, where the $2n = 42$A karyomorph predominated. The $2n = 42$A karyomorph populations isolated in drainages, however, showed striking differences for some markers, notably the Ag-NORs and 5S rDNA sites. The patterns of variation in repetitive DNA sequences as revealed by 5S rDNA probes indicated that these markers appeared to be useful population markers, showing significant differences among localities and karyomorphs. Patterns of 5S*Hind*III-DNA variation yielded the largest phylogenetic signal and placed the $2n = 40$F karyomorph in a high position in the phylogeny of karyomorphs that occur in eastern Brazil.

Given the current status of phylogenetic data, an understanding of distribution patterns for *H. malabaricus* will be particularly challenging. Studies of the distribution patterns of freshwater fishes have considered endemism (Vari, 1988), faunal composition including endemic and widespread species (Hubert & Renno, 2006), molecular data (Sivasundar *et al*., 2001; Montoya-Burgos, 2003) or a combination of morphological and molecular data (Menezes *et al*., 2008). When integrated into a wider geographic context, molecular and cytogenetic patterns can be informative of faunal dispersals between drainages. The results of the present study suggested that recent fish dispersal may occur even within apparently stable geological regions and that headwater stream piracy between coastal and continental (upper Paraná tributaries) basins may have played a role in producing the high levels of freshwater fish diversity in the Neotropics.

Finally, the topology of mtDNA-based tree suggests that the proposal of Dergam & Bertollo (1990) to separate species by diploid numbers would result in a paraphyletic arrangement, in which some *Hoplias* spp. haplotypes associated with $2n = 42$ karyomorphs would be more closely related to trahiras with $2n = 40$ karyomorphs than to other fish with $2n = 42$ karyomorphs. Although haplogroup I appeared as the sister group of the remaining trahiras, haplogroup II is less supported and the possible ancestry of karyomorphs must wait for more data. A time frame for dispersal between coastal and continental drainages is elusive for the *H. malabaricus* species complex. Nevertheless, geographical isolation during coastal diversification has enhanced speciation processes and genetic isolation between karyomorphs, a process that has resulted in molecular and cytogenetic differentiation between populations. These results are consistent with the existence of multiple independent lineages that can be easily detected with standard cytogenetic techniques.

Although *Hoplias* spp. are common members of lowland freshwater communities (Bonetto *et al*., 1969; Winemiller, 1991), at least part of their widespread distribution in the Neotropics is due to their ability to colonise high-altitude headwaters, such as in the Jacaré and Tombadouro localities. The question of how this non-migratory sedentary species has adapted to sluggish waters and to waters at high altitudes is not explained by current geological data. Nevertheless, this broad ecological diversity indicates a long history of *Hoplias* spp. in south-eastern Brazil.

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References

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

Berra, T. M. (2007). *Freshwater Fish Distribution*. Chicago, IL: University of Chicago Press.

Bertollo, L. A. C. (1996). The nucleolar organizer regions of Erythrinidae fish. An uncommon situation in the genus *Hoplias*. *Cytologia* **61,** 75–81.

Bertollo, L. A. C., Born, G. G., Dergam, J. A., Fenocchio, A. S. & Moreira-Filho, O. (2000). A biodiversity approach in the neotropical Erythrinidae fish, *Hoplias malabaricus*. Karyotypic survey, geographic distribution of cytotypes and cytotaxonomic considerations. *Chromosome Research* **8,** 603–613.

Bertollo, L. A. C., Moreira-Filho, O. & Fontes, M. S. (1997). Karyotypic diversity and distribution in *Hoplias malabaricus* (Pisces, Erythrinidae): cytotypes with $2n = 40$ chromosomes. *Genetics and Molecular Biology* **20,** 237–242.

Bertollo, L. A. C., Moreira-Filho, O. & Galetti, P. M. Jr. (1986). Cytogenetics and taxonomy considerations based on chromosome studies of freshwater fish. *Journal of Fish Biology* **28,** 153–159.

Bertollo, L. A. C., Takahashi, C. & Moreira-Filho, O. (1978). Cytotaxonomic considerations on *Hoplias lacerdae* (Pisces, Erythrinidae). *Brazilian Journal of Genetics* **1,** 103–120.

Britten, R. J. (1986). Rates of DNA sequence evolution differ between taxonomic groups. *Science* **231,** 1393–1398.

Bonetto, A., Dioni, W. & Pignalberi, C. (1969). Limnological investigations on biotic communities in the Middle Paraná River Valley. *Verhandlungen Internationalen Vereinigung für Theoretische und Angewandte Limnologie* **17,** 1035–1050.

Born, G. & Bertollo, L. A. C. (2000). An XX/XY sex chromosome system in a fish species, *Hoplias malabaricus*, with a polymorphic NOR-bearing X chromosome. *Chromosome Research* **8,** 111–118.

Born, G. & Bertollo, L. A. C. (2001). Comparative cytogenetics among allopatric populations of the fish, *Hoplias malabaricus*. Cytotypes with $2n = 42$ chromosomes. *Genetics and Molecular Biology* **110,** 1–9.

Boyce, T. M., Zwick, M. E. & Aquadro, C. F. (1989). Mitochondrial DNA in the bark weevils: size, structure and heteroplasmy. *Genetics* **123,** 825–836.

Cioffi, M. B., Martins, C. & Bertollo, L. A. C. (2009). Comparative chromosome mapping of repetitive sequences. Implications for genomic evolution in the fish, *Hoplias malabaricus*. *BMC Genetics* **10,** 34. doi: 10.1186/1471-2156-10-34

Dergam, J. A. & Bertollo, L. A. C. (1990). Karyotypic diversification in *Hoplias malabaricus* (Osteichthyes, Erythrinidae) of the São Francisco and Alto Paraná basins, Brazil. *Genetics and Molecular Biology* **13,** 755–756.

Dergam, J. A., Suzuki, H. I., Shibatta, O. A., Duboc, L. F., Júlio, H. F., Caetano-Giuliano, L. & Black, W. C. I. V. (1998). Molecular biogeography of the Neotropical fish *Hoplias malabaricus* (Erythrinidae: Characiformes) in the Iguaçu, Tibagi, and Paraná rivers. *Genetics and Molecular Biology* **21,** 493–496.

Dergam, J. A., Paiva, S. R., Schaeffer, C. E., Godinho, A. L. & Vieira, F. (2002). Phylogeography and RAPD-PCR variation in *Hoplias malabaricus* (Bloch,1794) (Pisces, Teleostei) in southeastern Brazil. *Genetics and Molecular Biology* **25,** 379–387.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* **17,** 368–376.

Ferreira, I. A., Bertollo, L. A. C. & Martins, C. (2007). Comparative chromosome mapping of 5SrDNA and 5S *Hin* dIII repetitive sequences in Erythrinidae fishes (Characiformes)

with emphasis on the *Hoplias malabaricus* 'species complex'. *Cytogenetic and Genome Research* **118,** 78–83.

Haaf, T., Schmid, M., Steinlein, C., Galetti, P. M. Jr & Willard, H. F. (1993). Organization and molecular cytogenetics of satellite DNA family from *Hoplias malabaricus* (Pisces, Erythrinidae). *Chromosome Research* **1,** 77–86.

Harvey, P. H. & Pagel, M. D. (1991). *The comparative method in evolutionary biology*. *Oxford Series in Ecology and Evolution*. p. 263. New York, NY: Oxford University Press.

Henyey, E., Kynard, B. & Zhuang, P. (2002). Use of eletronarcosis to immobilize juvenile lake and shortnose sturgeons for handling and the effects on their behavior. *Journal of Applied Ichthyology* **18,** 502–504.

Howell, W. M. & Black, D. A. (1980). Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* **36,** 1014–1015.

Hubert, N. & Renno, J. F. (2006). Historical biogeography of South American freshwater fishes. *Journal of Biogeography* **33,** 1414–1436.

Huelsenbeck, J. P. & Ronquist, F. (2001). MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17,** 754–755.

Ingenito, L. F. S. & Buckup, P. A. (2007). The Serra da Mantiqueira, south-eastern Brazil, as a biogeographical barrier for fishes. *Journal of Biogeography* **34,** 1173–1182.

Levan, A., Fredga, K. & Sandberg, A. A. (1964). Nomenclature for centromeric position on chromosome. *Hereditas* **1,** 201–220.

Lundberg, J. G., Marshall, L. G., Guerrero, J., Horton, B., Malabarba, M. C. S. L. & Wesselingh, F. (1998). The stage for Neotropical fish diversification: a History of tropical South American rivers. In *Phylogeny and Classification of Neotropical Fishes* (Malabarba, L. R., Reis, R. E., Vari, R. P., Lucena, C. A. S. & Lucena, Z. M. S., eds), pp. 13–48. Porto Alegre, Brazil: Edipucrs.

Lundberg, J. G. (1998). The temporal context for the diversification of Neotropical fishes. In *Phylogeny and Classification of Neotropical Fishes* (Malabarba, L. R., Reis, R. E., Vari, R. P., Lucena, C. A. S. & Lucena, Z. M. S., eds), pp. 49–68. Porto Alegre, Brazil: Edipucrs.

Martin, A. P. & Palumbi, S. R. (1993). Body size, metabolic rate, generation time, and the molecular clock. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 4087–4091.

Martins, C., Ferreira, I. A., Oliveira, C., Foresti, F. & Galetti, P. M. Jr. (2006). A tandemly repetitive centromeric DNA sequence of the fish *Hoplias malabaricus* (Characiformes: Erythrinidae) is derived from 5S rDNA. *Genetica* **127,** 133–141.

Menezes, N. A., Ribeiro, A. C., Weitzman, S. & Torres, R. A. (2008). Biogeography of Glandulocaudinae (Teleostei: Characiformes: Characidae) revisited: phylogenetic patterns, historical geology and genetic connectivity. *Zootaxa* **1726,** 33–48.

Molina, W. F. (2001). An alternative method of mitotic stimulation in fish cytogenetics. *Chromosome Science* **5,** 149–152.

Montoya-Burgos, J. I. (2003). Historical biogeography of the catfish genus *Hypostomus* (Siluriformes: Loricariidae), with implications on the diversification of Neotropical ichthyofauna. *Molecular Ecology* **12,** 1855–1867.

Myers, G. S. (1938). Fresh-water fishes and the West Indian zoogeography. *Annual Report of the Smithsonian Institution* **1937,** 339–364.

Nylander, J. A. A. (2004). MrModeltest v2. Program distributed by the author. Uppsala, Sweden: Evolutionary Biology Centre, Uppsala University.

Ohta, T. (2002). Near neutrality in evolution of genes and gene regulation. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 16134–16137.

Pinkel, D., Straume, T. & Gray, J. (1986). Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. *Proceedings of the National Academy of Sciences of the United States of America* **83,** 2934–2938.

Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics* **14,** 817–818.

Quenouille, B., Bermingham, E. & Planes, S. (2004). Molecular systematics of the damselfishes (Teleostei: Pomacentridae): Bayesian phylogenetic analyses of mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution* **31,** 66–88.

Reis, R. E., Kullander, S. O., & Ferraris, C. J. Jr. (2003). *Check List of the Freshwater Fishes of South and Central America*. Porto Alegre, Brazil: Edipucrs.

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

Rosa, R. S., Menezes, N. A., Britski, H. A., Costa, W. J. E. M. & Groth, F. (2004). Diversidade, padrões de distribuição e conservação dos peixes da caatinga. In *Ecologia e conservação da Caatinga* (Tabarelli, I. R. M. & da Silva, J. M. C., eds), pp. 135–180. Recife: Edufpe.

Saadi, A., Dart, R. L. & Machette, M. N. (2002). Map of quaternary faults and lineaments of Brazil. Project of international lithosphere program task group II-2, major active faults of the world, University of Minas Gerais and U.S. Geological Survey.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4,** 406–425.

Schaefer, S. A. (1998). Conflict and resolution: Impact of new taxa on phylogenetic studies of the neotropical cascudinhos (Siluriformes: Loricariidae). In *Phylogeny and Classification of Neotropical Fishes* (Malabarba, L. R., Reis, R. E., Vari, R. P., Lucena, C. A. S. & Lucena, Z. M. S., eds), pp. 375–400. Porto Alegre, Brazil: Edipucrs.

Sites, J. W. Jr & Moritz, C. (1987). Chromosomal evolution and speciation revisited. *Systematic Zoology* **36,** 153–174.

Sivasundar, A., Bermingham, E. & Ortí, G. (2001). Population structure and biogeography of migratory freshwater fishes (*Prochilodus*:Characiformes) in major South American rivers. *Molecular Ecology* **10,** 407–417.

Sola, L., Rossi, A. R., Laselli, V., Rasch, E. M. & Monaco, P. J. (1992). Cytogenetics of bisexual/unisexual species of *Poecilia* II. Analysis of heterochromatin and nucleolar organizer regions in *Poecilia mexicana mexicana* by C-banding and DAPI, quinacrine, chromomycin A3, and silver staining. *Cytogenetics and Cell Genetics* **60,** 229–235.

Sumner, A. T. (1972). A simple technique for demonstrating centromeric heterocromatin. *Experimental Cell Research* **75,** 304–306.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24,** 1596–1599.

Vari, R. P. (1988). The Curimatidae, a lowland Neotropical fish family (Pisces: Characiformes); distribution, endemism, and phylogenetic history. In *Proceedings of a Workshop on Neotropical Distribution Patterns*. (Heyer, W. R. & Vanzolini, P. E., eds), pp. 343–377. Rio de Janeiro: Academia Brasileira de Ciências.

Vergara, J., Azpelicueta, M. M. & Garcia, G. (2008). Phylogeography of the Neotropical catfish *Pimelodus albicans* (Siluriformes: Pimelodidae) from Río de la Plata Basin, South America, and conservation remarks. *Neotropical Ichthyology* **6,** 75–85.

Vicari, M. R., Artoni, R. F. & Bertollo, L. A. C. (2003). Heterochromatin polymorphism associated with 18S rDNA. A differential pathway among the fish *Hoplias malabaricus* from Southern Brazil. *Cytogenetic and Genome Research* **101,** 24–28.

Vicari, M. R., Artoni, R. F. & Bertollo, L. A. C. (2005). Comparative cytogenetics of *Hoplias malabaricus* (Pisces, Erythrinidae): a population analysis in adjacent hydrographic basins. *Genetics and Molecular Biology* **28,** 103–110.

Winemiller, K. O. (1991). Ecomorphological diversification in lowland freshwater fish assemblages from five biotic regions. *Ecological Monographs* **61,** 343–365.

Wu, C. I. & Li, W. H. (1985). Evidence for higher rates of nucleotide substitution in rodents than in man. *Proceedings of the National Academy of Sciences of the United States of America* **82,** 1741–1745.