DISSERTATION

PHYLOGEOGRAPHY AND CHARACTER CONGRUENCE WITHIN THE *Hoplias malabaricus* Bloch, 1794 (Erythrinidae, Characiformes, Ostariophysi) SPECIES COMPLEX

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JORGE A. DERGAM ENTITLED PHYLOGEOGRAPHY AND CHARACTER CONGRUENCE WITHIN THE *Hoplias malabaricus* Bloch, 1794 BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT

PHYLOGEOGRAPHY AND CHARACTER CONGRUENCE WITHIN THE *Hoplias malabaricus* BLOCH, 1794 (ERYTHRINIDAE, CHARACIFORMES, OSTARIOPHYSI) SPECIES COMPLEX.

I analyzed patterns of congruence of independent data sets (molecular and karyotypical) within the Neotropical fish species complex *Hoplias malabaricus* Bloch, 1794, at the local and at the macrogeographic levels. In two locales of sympatry of fish with different karyotypes (cytotypes) in Argentina and Brazil, the patterns of congruence were interpreted as evidence that the cytotypes are distinct species.

At the macrogeographical level, I analyzed the patterns of congruence of nuclear DNA, mitochondrial DNA and cytogenetic data. In the Rio Iguassu, the patterns of similarity of nuclear DNA markers (Randomly Amplified Polymorphic DNA) suggested that the population of *H. malabaricus* of this river derives from a southern-southwestern group of populations. At the continental level, nuclear DNA markers were consistent with other data sets at low levels of genetic divergence, allowing for geographic analysis of differentiation of populations and for the detection of cryptic sympatric forms. However, similarity analysis based on these markers failed to recover phylogenetic relationships as evidenced by mitochondrial DNA sequence analysis. Phylogenetic analyses of mitochondrial DNA sequences indicated

high levels of within and among population variation. In the best supported tree, a mitochondrial DNA variants (haplotype) from the middle Río Paraná derived from populations with 40 chromosomes is the sister group of all the other haplotypes of *Hoplias malabaricus*, while populations in southeastern and southern Brazil with $2n=42$ form a monophyletic clade within the complex. The pattern of geographic distribution of this clade is compatible with other, unrelated fish taxa. However, other haplotypes from populations with $2n=42$ were phylogenetically more related to haplotypes from populations with $2n=40$. Thus, grouping populations of the species complex by diploid numbers would render those groups paraphyletic (they would not include all descendants of a common ancestor). The discrepancy of patterns of variation between diploid number and mitochondrial DNA may be explained by multiple origins of diploid numbers, or by random fixation of ancestral polymorphisms following the fixation of chromosomal rearrangements in a large population.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. Introduction

In 1938, Myers stated that freshwater fishes of the primary division (intolerant to salt or brackish waters) were ideal candidates for biogeographical studies. Although in the Neotropical Region such studies have yet to be realized for most taxa, some studies have already addressed the question of the geographical distribution of taxa at the specific or populational levels (Kullander, 1983; Menezes, 1987; Weitzman and Weitzman, 1982; Weitzman and Fink, 1983; , and Weitzman et al., 1987).

Phylogenetic analysis of monophyletic taxa represents the only means of formulating scientific (i.e., subject to refutation) hypotheses of historical relations between different regions (Wiley, 1981 and references therein). Hypothetical areas of endemism may be corroborated by the distributional patterns of unrelated organisms, especially by organisms with different dispersal capabilities (for ex., birds, trees, and fish) and are more objective than traditional studies of faunal similarity based on the presence or absence of nominal species or supra specific taxa (e.g., Ringuelet, 1975 ; Gery, 1969).

When biogeographical phylogenetic analyses are applied to taxa with complex patterns of variation within and among populations, a multidisciplinary, populational approach becomes a necessity. One example of such taxa is the nominal species *Hoplias malabaricus* Bloch, 1794 a taxon

with a reported range from Río Salado (Buenos Aires Province) in Argentina (Mac Donagh, 1934) to Panamá (Berra, 1981) (Fig. 1.1a). Ringuelet (1975) extended Donagh's range farther south to the "Laguna Salada Grande", without further specifications.

In the Neotropical Region, virtually every lowland body of water harbors fish easily assignable to *H. malabaricus* (e.g, Lowe-McConnell, 1987). As a predator, *H. malabaricus* plays also a key role in those ecosystems, representing up to 47% of the biomass in roadside lagoons in Venezuela (Mago, 1970), and up to 25% of the biomass in temporary oxbow lakes in some islands of the Middle Río Paraná (Bonetto et al., 1969). Its abundance and relatively large size (up to 50 cm, usually around 35 cm) make this species a common food fish, commonly seen in markets of the cis-Andean region (Eigenmann and Eigenmann, 1889). It has an array of common names, such as "tarariras" in Uruguay and also "taruchas" in Argentina, "guavinas" in Venezuela, "perra loca" in Colombia, and "traíras" in Brazil. In the English literature they are usually cited as "trahiras" (e.g., Gery, 1977).

Together with the genera *Hoplerythrinus* Gill, 1896 and *Erythrinus* Scopoli, 1777, the genus *Hoplias* comprises the family Erythrinidae, within the order Characiformes. This family was recently hypothesized by Buckup (1991), and Vari (1995) to be a member of a clade composed by the families Lebiasinidae, the African Hepsetidae, and Ctenoluciidae. Earlier,

Fink and Fink (1981) had also suggested a close relationship of erithrynids with Ctenoluciidae and Hepsetidae.

The erythrinids comprise an array of forms that exploit different habitats, and such diversity is also reflected in their morphology and physiology. For example, *H. malabaricus* is typically abundant in sluggish and lentic waters. Within the genus *Hoplias*, this ecological difference is also reflected in the respiratory physiology of *H. malabaricus*, which shows lower values of gill ventilation and smaller tidal volumes in hypoxic conditions, when compared to *Hoplias lacerdae* Ribeiro, 1908, a member of another species complex adapted to well oxygenated waters (Rantin et al., 1992). The remaining two genera, *Hoplerythrinus* and *Erythrinus* have modified walls in the posterior chamber of the air bladder, allowing them to take up oxygen directly from atmospheric air (Stevens and Holeton, 1978).

Regarding behavior, Mago (1970) reported that *H. malabaricus* and *Hoplerythrinus* are able to move overland through wet vegetation. *H. malabaricus* is also well known for its nest-building and nest guarding habits (Ihering, 1940; Ringuelet et al., 1967).

Taxonomic revision. The current genus *Hoplias* encompasses three nominal species: *Hoplias malabaricus*, *Hoplias lacerdae*, and *Hoplias malabaricus* var. *macrophtalmus* Pellegrin, 1907. These taxa were considered as the only species within the genus *Hoplias* by Gery (1977), but each of them was considered a "species group" by Oyakawa (1990). In current taxonomic

practice, *H. malabaricus* species may be identified and differentiated from other taxa by the presence of patches of minute teeth on the tongue (Britski et al., 1984), by the tendency of the exposed internal margin of the dentary bones to converge towards the symphysis (Azevedo et al., 1965), and by the presence of four pits of the lateral line system on each side of the dentary (Oyakawa, 1990). In contrast, *H. lacerdae* and *H. macrophtalmus* lack lingual patches of teeth, the exposed margins of the dentary bones are arranged in a parallel fashion (Azevedo et al., 1965), and they have more than four sensorial pits on each dentary (Oyakawa, 1990). In this study, I have used *H. cf. lacerdae* and *Erythrinus sp.* as outgroups.

The first reference to *H. malabaricus* is the work of Marcgrave (1648), wherein he describes a riverine form, the "tareira do rio". Despite the paucity of the description, Cuvier and Valenciennes (1846) and Eigenmann and Eigenmann (1889) considered it as equivalent to the current genus *Hoplias*.

The original description of *H. malabaricus* was published by Bloch in 1794, as *Esox malabaricus*. Bloch's illustration is of an unmistakable *Hoplias*, albeit with a rather unusual coloration. Nevertheless, a more serious problem with this description was that the localities indicated for the species (Malabar and Tranquebar) are in India. This mistake was perpetuated in the specific name of the species ("*malabaricus*").

Later, Bloch (1801) would transfer this taxon to the genus *Synodus*

as *Synodus malabaricus*, a genus currently in the Order Aulopiformes. This author also considered as valid (and distinct from *S. malabaricus*), the "tareira" of Marcgrave, as *Synodus tareira*, with its locality indicated as "Brazilian rivers".

Agassiz (in Spix and Agassiz, 1829) described many species of fish that had been collected by Johann von Spix and Carl Von Martius in Brazil. Agassiz described *Erythrinus macrodon* (in Spix and Agassiz, 1829) and indicated *Erythrinus trahira* (in Spix and Agassiz, 1829) as its junior synonym.

In 1842, Muller erected the genus *Macrodon* for *Macrodon trahira*, as the senior synonym for *E. macrodon* and for *S. malabaricus*. In this study, Muller was the first to point out that the Malabar locality for *S. malabaricus* was likely mistaken. Later, Muller and Troschel (1844) added *Erythrinus trahira* as a junior synonym of *Macrodon trahira.*

Gill (1903) substituted *Hoplias* for *Macrodon*, because *Macrodon* had already been assigned by Schinz (1822) for another group of fish.

Although *E. macrodon* was presented as a senior synonym of *E. trahira*, the latter was indicated as the type species for the current genus *Hoplias* by the first revisors of the Erythrinidae, Eigenmann and Eigenmann (1889). Their consideration of *Hoplias trahira* as an available name is sufficient for its validity (art. 11e, ICZN, 1985) as the type species for the genus *Hoplias*. The holotype of *H. trahira* is deposited in the Musee

d'Histoire Naturelle de Neuchâtel (Kottelat, 1984), and its type locality is indicated as the Almada Lagoon ("*lacu* Almada"), Province of Bahia, and the Rio São Francisco (both in Brazil) (Fig. 1.1a).

Another species of this genus is *Hoplias microlepis* Gunther, 1864 and its type locality is Río Chagres (western Ecuador) (Fig. 1.1a). Its range was indicated by Eigenmann and Eigenmann (1889) to be the western slopes of South America from Guayaquil (Ecuador) to Panamá. Type material for this species is apparently deposited in the British Museum.

Gill (1858) described another species for this genus: *Hoplias ferox* from Trinidad. Oyakawa (1990) concluded that *H. microlepis* and *Hoplias ferox* also share the diagnostic characters of *H. malabaricus* such as the presence of lingual patches of teeth and margin of the dentaries converging toward the symphysis. This author also considered *Hoplias malabaricus* as a *nomen dubium*. However, two (out of the original series of three) syntypes are deposited in the Berlin Museum of Natural History, and circumstantial evidence points to Surinam (Fig. 1.1a) as their likely place of origin (pers. comm. with the curator of the fish collection of the Berlin Museum, H. J. Paepke).

Based on the availability of type material and on Oyakawa (1990) findings, I provisionally considered the following species as valid members of the *H. malabaricus* species complex:

Hoplias malabaricus Bloch, 1794

Hoplias trahira Agassiz, in Spix and Agassiz, 1829

Hoplias microlepis Gunther, 1864

Hoplias ferox Gill, 1858

Extreme patchiness of genetically differentiated populations within the species complex, and poor understanding of the patterns of morphological correlated with genetically based characters preclude the assignment of populations or set of populations to nominal taxa, and a comprehensive revision of this species complex is urgently needed.

Cytotaxonomic revision. To date, the most encompassing studies of this species complex have focused on the karyotypical patterns of variation within and among populations (e.g., Bertollo, 1978; Bertollo et al., 1979; 1983; Bertollo and Moreira, 1980; Bertollo et al.,1986; for a review see Oliveira et al., 1988).

Dergam and Bertollo (1990) suggested that the available karyotypical information of allopatric populations of *H. malabaricus* was sufficient to indicate the existence of two groups of trahiras: one with a diploid number of 42, and another with a diploid number of 40, or slight variations of 40 (Fig. 1.1b).

Although voucher specimens have not been compared with type material, preliminary cytogenetic studies of a population of *H. malabaricus* from Tigjerkreek, West Surinam revealed a diploid number of $2n=40$ (Bertollo, 1988), and the same diploid number was observed from

populations of this species from the Rio São Francisco at the southern localities of Três Marias and Lagoa da Prata (Dergam and Bertollo, 1990). The Rio São Francisco was indicated by Agassiz (in Spix and Agassiz, 1829) as one of the type localities for *H. trahira*. The diploid number of *H. microlepis* and *H. ferox* are unknown.

In the following years, researchers have detected other locales with sympatry of the two chromosome numbers: one in the Mogi-Guassu drainage (Rio Paraná Superior basin, State of São Paulo, Brazil) (Scavone et al., 1994) and another in the Uruguay basin (Río Aguapey, Province of Misiones, Argentina) (Lopez et al. unpubl.).

Objectives. Molecular tools have become commonly used for assessing all levels of organismic variation (Hillis and Moritz, 1990), and molecular studies at the infraspecific level are commonly seen in the literature (e.g., Badaracco et al., 1995; Norris et al., 1996; Ortí et al., 1994). In this study, I have explored the patterns of molecular variation within the *H. malabaricus* species complex with three techniques based on the Polynucleotide Chain Reaction (PCR) (Saiki et al., 1988). The objective was to unravel the nature of the diploid number variation at the populational level (i. e., are diploid numbers good taxonomic markers when $2n = 40$ specimens are sympatric with $2n = 42$ specimens?) and at the macrogeographical level (are populations with $2n = 42$ more related among themselves than with other

populations with $2n = 40$?). Assessment of these issues were achieved by the analysis of patterns of congruence among cytogenetic, molecular (nuclear) data sets at the populational and at the macrogeographical levels (Fig. 1.2). Because this research was not based on museum material, and I did not compare my voucher specimens with type material, further taxonomic work will be needed to make nomenclatural decisions regarding these populations. For this reason, and also because of the idiosyncrasy of mitochondrial DNA (mtDNA) data, the phylogenetic trees of this study depict gene genealogies and not species phylogenies (for a review see Avise and Ball, 1990). Large levels of mtDNA intraspecific variability and the detection of cryptic sympatric species led me to follow Avise and Ball's (1990) suggestion, itself also inspired by the patterns of variation of molecular data: exercise of taxonomic decisions must include concordance among independent genetic traits as a reflection of reproductive barriers among species. For instance, if widely divergent haplotypes within a sample are not correlated with other independent genetic markers, they are unsufficient evidence as diagnostic characters for the presence of different species within that sample or population.

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Figure 1.1

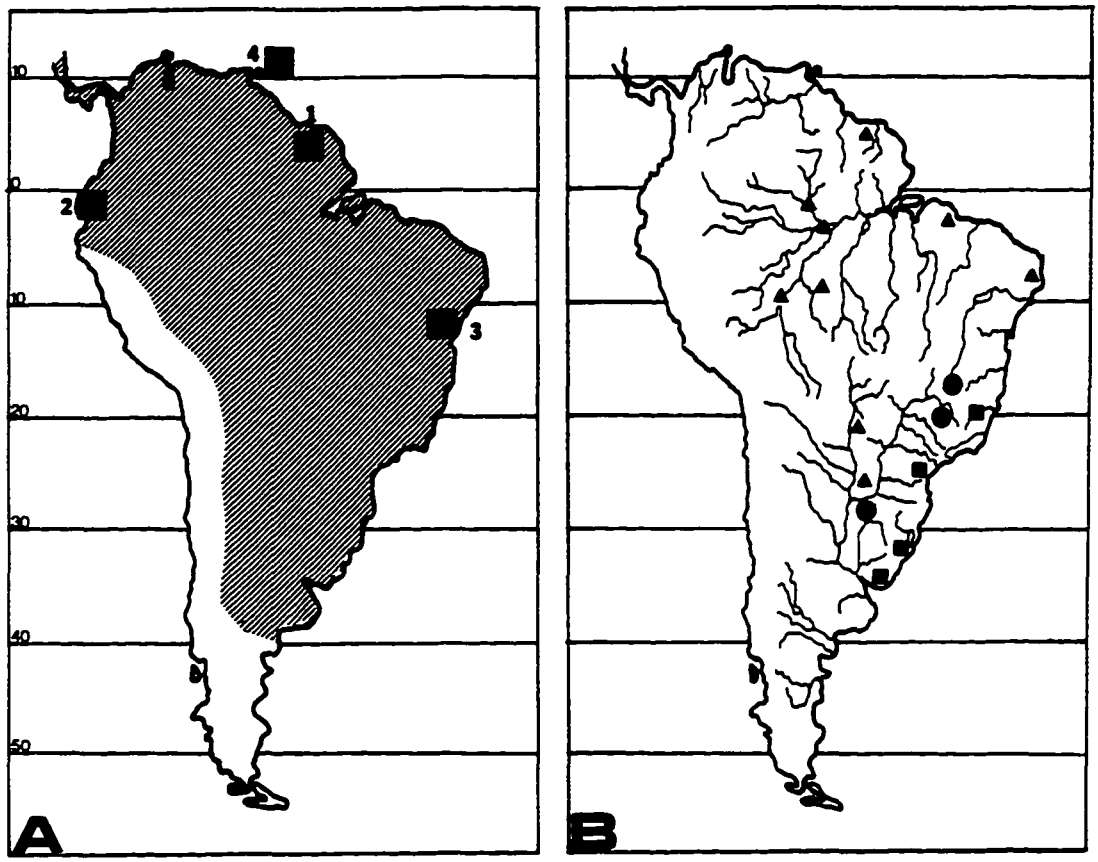
A) Map of the Neotropical Region, with range of distribution of the species *Hoplias malabaricus* Bloch, 1794. The numbers indicate type localities for taxa assumed to be members of the *Hoplias malabaricus* species complex: 1) Surinam, presumed locality of *Hoplias malabaricus*; 2) Rio Chagres, locality for *Hoplias microlepis* Gunther, 1864; 3) State of Bahia ("*Iacu* Almada") (but also São Francisco), localities for *Hoplias trahira* Spix, 1829, 4) Trinidad, type locality for *Hoplias ferox* Gill, 1858.

B) Localities with known diploid numbers for the *Hoplias malabaricus* species complex:

Squares indicate exclusive presence of populations with a diploid number of 42 chromosomes.

Triangles indicate exclusive presence of populations with diploid number of 40 chromosomes.

Circles indicate the presence of populations with specimens with both diploid numbers in syntopy.


10
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10
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3
4
A
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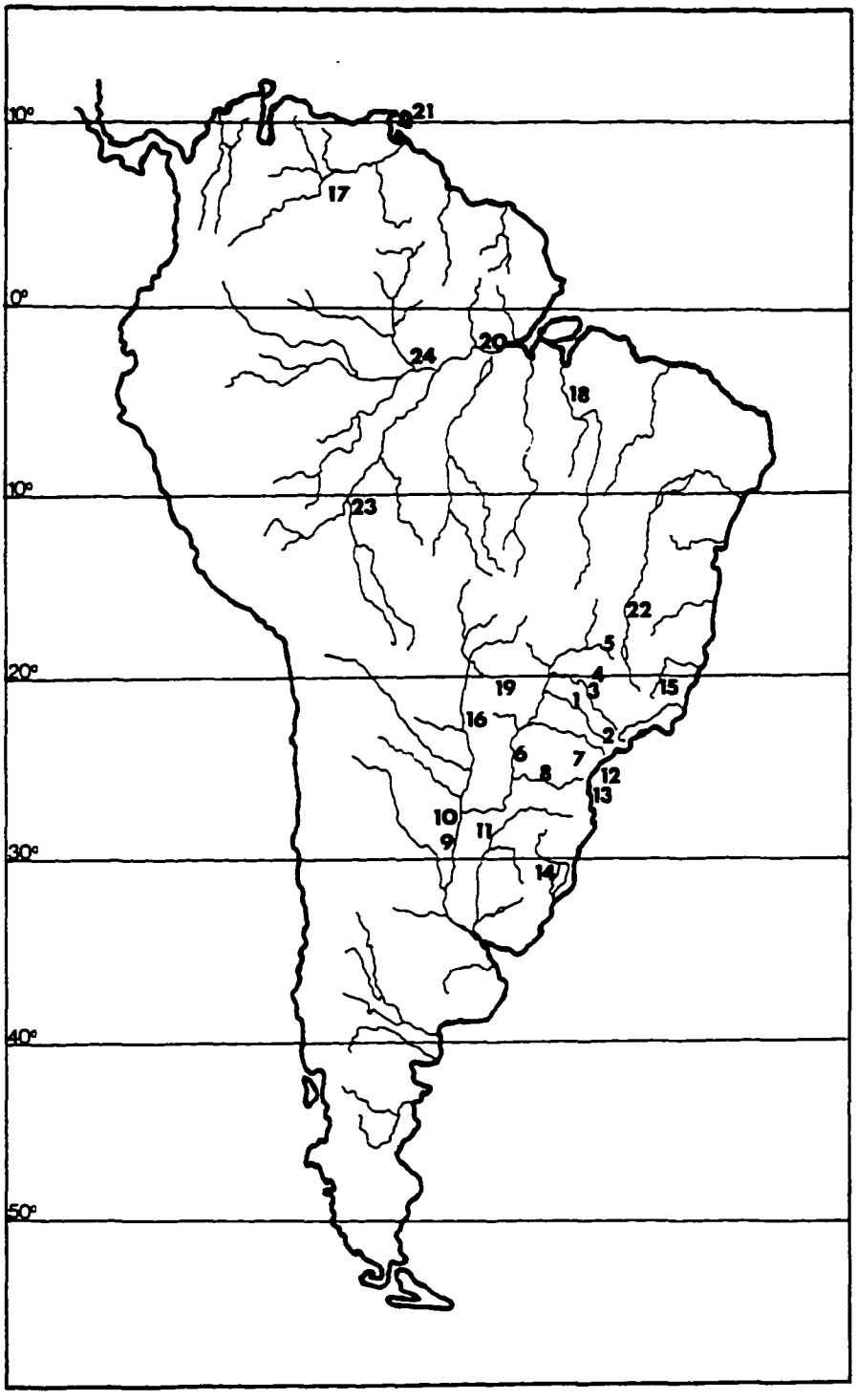
Figure 1.2. Sample localities analyzed in this study:
1) Rio Mogi-Guassu. Represa do Monjolinho, Campus of the Universidade Federal de São Carlos, São Carlos, State of São Paulo, Brazil.
2) Rio Tietê, Iracemápolis, State of São Paulo, Brazil.
3) Rio Mogi-Guassu, Represa Beija-Flor, Reserve of Jataí, State of São Paulo, Brazil.
4) Rio Grande, Conceição das Alagoas, State of Minas Gerais, Brazil.
5) Rio Paranaíba, Nova Ponte, State of Minas Gerais, Brazil.
6) Rio Paraná, Porto Rico, State of Paraná, Brazil.
7) Rio Tibagi, Ponta Grossa, State of Paraná, Brazil.
8) Rio Iguassu, Segredo Reservoir, State of Paraná, Brazil.
9) Rio Paraná, Reconquista, Province of Santa Fé, Argentina.
10) Rio Paraná, Bella Vista, Province of Corrientes, Argentina.
11) Rio Aguapey, Province of Posadas, Argentina.
12) Rio Ribeira, Registro, State of São Paulo, Brazil.
13) Rio Perequê, Paranaguá, State of Paraná, Brazil.
14) Rio Guaíba, Porto Alegre, State of Rio Grande do Sul, Brazil.
15) Rio Doce, State of Minas Gerais, Brazil.
16) Rio Paraguai, Corumbá, State of Mato Grosso, Brazil.
17) Rio Orinoco, State of Portuguesa and Apure, Venezuela.
18) Tucurui Dam, Rio Tocantins, state of Pará.
19) Barra do Garças, Rio Araguaia, State of Mato Grosso.
20) Rio Tapajós, Belém, State of Pará.
21) Caroni River, Trinidad
22) Rio São Francisco, Curvelo, State of Minas Gerais.
23) Rio Madeira, Porto Velho, State of Rondônia. and
24) Rio Mindu, Manaus, State of Amazonas.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2. Gene Flow Among Sympatric Populations of the Neotropical Fish *Hoplias malabaricus* (Erythrinidae: Teleostei) with Different Diploid Numbers.

RAPD-PCR polymorphisms at 66 presumptive loci were used to determine the molecular similarity between sympatric populations of the fish *Hoplias malabaricus* that contain different chromosome numbers. Collections were from the Aguapey and Paraná rivers in Argentina, and from the Mogi-Guassu and Grande rivers in Brazil. Each of the specimens from seven locales was karyotyped and screened for nuclear (Randomly Amplified Polymorphic DNA) levels of variation. Molecular similarity was estimated by computing shared presence and absence of amplified bands, and the resulting clusters were congruent with karyological data. It was concluded that allelic differentiation as revealed by RAPD-PCR indicates lack of gene flow among these populations.

If species are independent evolutionary units, the ultimate test for the maintenance of such independence should be their sympatry with other related species (i.e., the "sympatry test", Mayr, 1963). When sympatric species lack obvious phenotypic differences, or their differences are difficult to be observed in the wild, they are referred to as cryptic species (see

Knowlton, 1993 for a recent review). This study explores the existence of cryptic species within the nominal species *H. malabaricus* Bloch, 1794, a characin widely distributed in the neotropics (Berra, 1981), and characterized by complex patterns of morphological variation within and among populations (e.g., Eigenmann and Eigenmann, 1889). Dergam and Bertollo (1990) suggested the existence of two monophyletic groups with 42 and 40 chromosomes within the species complex, the range of distribution of the group with 42 chromosomes was southern and southeastern Brazil, while the other one occurred in the remaining portions of the range (Fig. 1.1b). Subsequent studies allowed for the detection of populations with specimens bearing 40 and 42 chromosomes in three localities (Fig. 1.1b):

a) Rio Mogi-Guassu (São Paulo State) (Scavone et al., 1994), and

b) Río Aguapey, Argentina (Lopez et al., unpubl.).

c) Rio Mindu, Manaus (Porto and Feldberg, unpubl.).

The sympatry raised a question: are there chromosomal "races" (cytotypes) in *H. malabaricus* or do chromosomal differences in numbers exist as floating polymorphisms (*sensu* White, 1973; 1978)?.

In the Rio Mogi-Guassu (Rio Grande basin), lack of specimens with a putative hybrid karyotype of 41 chromosomes was interpreted by Scavone et al. (1994) as evidence for the specific status for each karyomorph, but these authors did not mention sample sizes, and $\chi 2$ goodness-of-fit

statistics for expected frequencies was not calculated. Typically, low yields of cytogenetic results relative to the number of collected specimens pose difficulties for population cytogenetic studies, and therefore analyses of samples from regions of sympatry are best approached with a multidisciplinary experimental design (including, for example, molecular techniques). The purpose of this study was to determine the congruence between Randomly Amplified Polymorphic DNA (RAPD) markers (Williams et al., 1990) and diploid numbers, and to extend the molecular analysis to specimens that did not give chromosomal data. RAPD-PCR markers allow for easy screening of mainly repetitive regions of the genome (sequences that are represent by many copies in the genome) subject to high levels of evolutionary changes (reviewed in Haymer, 1994) by means of the amplification of arbitrary regions with the polymerase chain reaction (PCR) (Saiki et al., 1988). The protocol involves the use of a 10 oligonucleotide primer with a minimum G-C content of 60% , which anneals to the template at low temperatures ($\leq$37°C) (Williams et al., 1990). By using several primers, it is possible to reveal numerous differences among individuals within a species (e.g., Apostol et al., 1993), as well as to detect molecular differences between cryptic species (e.g., Crossland et al., 1993; Khasa and Dancik, 1996; Wilkerson et al., 1993).

Because RAPD alleles are amplified with one primer, they are likely originated from repetitive regions of the genome, where reversed sequence

repeats are more abundant (Black, 1993; Haymer, 1994). These repetitive regions are represented in all chromosomes of *H. malabaricus* as blocks of constitutive heterochromatin or C-bands (Dergam and Bertollo, 1990), and therefore RAPD-PCR alleles are assumed to be independent markers. Members of a population or species are expected to share more independent alleles than with any other population or species, and thus molecular similarity may permit the detection of cryptic species in sympatry.

Taking advantage of preliminary data by Lopez et al. (unpubl.) of the existence of the two karyomorphs in syntopy (i.e., they are collected together in the same seine haul) in the Río Aguapey (Province of Corrientes, Argentina) and results of Scavone et al. (1994) for the Rio Mogi-Guassu, I collected and sampled the same locales, and some neighboring ones. Each of the collected specimens was analyzed with cytogenetic and molecular techniques. The pattern of variation of these independent data sets represents a test of the specific status of the cytotypes involved, as follows:

1) correlated bimodal patterns of karyotypical and molecular variation among the specimens will be a corroboration of the existence of two different species; while

2) lack of any correlation between these data sets will be evidence for the existence of a panmictic population (and consequently the diploid number variation would be considered as a polymorphic trait).

3) finally, partial correlation will indicate gene flow between the

cytotypes.

Materials and Methods

Field collections. Aguapey and Paraná rivers. In July 1994, four samples (N = 49) (Fig. 2.1) were seined from:

Site 1. A tributary of the Río Aguapey (Province of Corrientes) (N = 34).

Site 2. A dam 37 km south of Posadas (Province of Posadas) (N = 7), also a member of the Río Aguapey basin.

Site 3. Laguna Caré, Bella Vista, Río Paraná (Province of Corrientes). (N = 2).

Site 4. Río Paraná in Resistencia (Province of Santa Fe) (N = 6).

Rio Grande basin. During 1993 and 1994, 33 specimens were collected from the following localities:

Site 5. Monjolinho Dam, Campus of the Universidade Federal de São Carlos, Rio Mogi-Guassu, a tributary of the Rio Grande, State of São Paulo (N = 12).

Site 6. Beija-Flor lagoon, in the Ecological Reserve of Jataí, State of São Paulo (the sympatry locale indicated by Scavone et al. (1994), Rio Mogi-Guassu (N = 4). And

Site 7. Rio Grande, Conceição das Alagoas, at CEMIG hydroelectric power plant facilities, Volta Grande, State of Minas Gerais (N = 16).

Specimens were gill netted at night time or seined from dense vegetation along the margins during the day.

Laboratory analyses. Except for specimens from Site 2 (where six specimens were 37-85 mm immatures, and the only large specimen died before being processed), all others yielded enough tissue to conduct cytogenetic analyses. Protocols for cytogenetic analyses followed Dergam and Bertollo (1990).

In contrast, all specimens gave suitable material for molecular analyses. Muscle tissue was collected from freshly killed specimens and fixed in absolute ethanol or in ethanol:methanol (1v:1v). DNA extraction followed a CTAB protocol modified from Boyce et al. (1989): 0.1 g of muscle was transferred to a sterile 1.5 centrifuge tube, and the remaining ethanol in the tissue was evaporated by placing the tubes in a Speed Vac for 5 min at 60° C. The dehydrated tissue was placed in 500 μl of CTAB homogenization buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 0.05 M hexadecyltrimethylammonium bromide (pH 8.3), 0.2% β-mercaptoethanol (v:v)), and the tube contents were briefly vortexed. The tubes were incubated at 65°C for 60 min, and vortexed at 20 min intervals, then centrifuged at 14,000 RPM for 2 min. The supernatant was transferred to the second set of tubes with 500 μl of chloroform:isoamyl alcohol (24v :1v), and both layers were mixed by hand. The tubes were centrifuged for 15 min at 14,000 RPM, and the upper layer was transferred to a third set of tubes, without disturbing the interface material. After adding 250 μl of cold (-20°C) isopropanol, the material was again mixed by hand. The tubes were

placed in the refrigerator (4°C) for at least 30 min, and then they were centrifuged for 30 min at 4°C at 14,000 RPM. The supernatant was poured off, and the pellet was washed in cold (-20°C) 75% ethanol and in cold 100% ethanol. The pellet was dried out in a Speed Vac for approximately 25 min., and the DNA was resuspended overnight in the refrigerator (4°C) in a volume of 50-200 μl of TE (Tris-EDTA pH 8.0). Resuspended DNA was used immediately, or stored at -20° C.

PCR reactions followed Black and Peisman (1994): each 50 μl of reaction buffer contained 500 mM KCl, 100 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 0.1 gelatin (w/v) and 1.0% Triton X-100, 200 μM dNTPs and 1 μM of each primer in 500 or 200 μl microcentrifuge tubes with approximately 25 μl of mineral oil overlay. Before adding the template, the tubes were exposed for 12 min to ultraviolet light (620 nm) (UV Stratalinker 1800) to inactivate possible contaminant DNA. Template DNA was added afterwards, and the tubes were placed in the thermal cycler at 95°C for 5 min, and 1 unit of *Taq* polymerase (Cetus) was added at the 80°C step ("hot-start PCR", Erlich et al., 1991). Amplifications were completed in a 60-well and in a 96-well PTC-100 thermal cyclers (MJ Research, Inc., MA), and the thermal cycle was: 5 min at 95°C, 1 min at 80°C, 45 times 1 min at 92°C, 1 min at 35°C, then ramp from 37 °C, rising 1°C each 8 sec , up to 72 °C, where it remained for 2 min, 7 min at 72°C.

. RAPD alleles were obtained with three primers. After a pilot test

with 15 different primers, 3 primers (A9, A05, and C-13) (Operon Technologies, Inc.) were selected for their consistency in amplifying a large number (approximately 20 per primer) of scorable bands (intensely stained and separated from other bands). The samples were loaded in a 1X TBE 5% glycerol, 5% acrylamide, and 2% N, N' -methylenebisacrylamide nondenaturing gel in a 38x50 cm Sequi Gen II set apparatus (Bio-Rad, Richmond, California) for 16 h at 25°C, at 345 volts (13-15 mA). DNA bands were stained with silver nitrate (Hiss et al., 1994).

The gel was air- dried, and the bands were photographed, scanned and finally scored directly on the gel. Each band was identified by its position on the gel, fitted to an inverse function that relates mobility and molecular weight (Schaffer and Sederoff, 1981), with a LOTUS program MWT.WK3 (Black, unpubl.). Identity of a given band was determined by the values of the mentioned function, based on the position of the bands of the molecular ladder in subsequent gels, relative to the position of 1 kilobase molecular ladder (Bethesda Research Laboratory, Gaithesburg, MD) (Fig. 2.2 a and b).

Alleles were classified as follows:

a) diagnostic if they were present in all and only the members of a cytotype or population,

b) characteristic if they were present but not fixed in only one cytotype, and

c) shared if present in at least two members of both cytotypes.

Scoring of the bands for each individual yielded a binary matrix where 0 indicates absence and 1 indicates presence of a band (Black, 1996). The matrices were processed with the FORTRAN program RAPDPLOT of molecular similarity (Kambhampati et al., 1992; Black, 1993). RAPDPLOT calculates a matching pairwise distance matrix based on the measure 1-M, where

$$M = N_{ab}/N_T$$

N_{ab} is the total number of matches (shared presence or absence of a given band) in individuals a and b, and N_T is the total number of matches. An M value of 1 indicates that two individuals share identical banding pattern, while a value of 0 indicates no matching banding pattern (Black, 1996). An unweighted pair group method with arithmetic averaging (UPGMA) was calculated with the PHYLIP 3.5C software package (Felsenstein, 1993). Dendrograms were drawn with the DRAWGRAM option of the same package. Statistical support for the branches in the form of boostrap (Efron, 1985) was calculated by the FORTRAN program RAPDBOOT (Black, unpubl.), which is also compatible with PHYLIP 3.5C.

Results

Cytogenetic results. Paraná and Aguapey rivers. From Río Paraná, all specimens were $2n = 40$. Fifteen specimens from Río Aguapey yielded acceptable cytogenetic results (11 specimens, five males and six females)

were $2n = 42$, and four of them (two males and two females) were $2n = 40$. The morphology of the chromosome complements of both groups was similar to the one reported by Lopez and Fenocchio (1994).

Mogi-Guassu and Grande rivers. Nine specimens (three males and six females) from Monjolinho Dam were $2n = 39$ and $2n = 40$ respectively. In the Beija-Flor Dam, three female specimens were $2n = 40$, and one female was $2n = 42$. In the Rio Grande sample, four specimens (two males and two females) yielded countable metaphases of 39 and 40 chromosomes respectively, and six more specimens (two males and four females) were $2n = 42$. The morphology of the chromosome complements agreed with those reported by Dergam and Bertollo (1990), and by Scavone et al. (1994).

Molecular results. Based on band repeatability and lack of ambiguity, I selected 66 of the bands (alleles) amplified by RAPD primers and detected by silver staining (Figs. 2.2 and 2.3).

UPGMA analysis of the matching matrix permitted clustering individuals of each of the collection sites in two groups (Fig. 2.4 and 2.5). All branches were strongly supported by bootstrap values.

Río Aguapey. All specimens from Río Aguapey with $2n = 42$ clustered together with 15 more specimens for which no cytogenetic results were available, and with six specimens from Posadas (site 2). The other cluster was composed of individuals with $2n = 40$ from the Aguapey and Paraná

rivers, including four individuals from Rio Aguapey (site 1) with unknown diploid numbers, and one of the specimens from Posadas (site 2) (Fig. 2.4).

The primer C-13 was the only one to amplify diagnostic alleles for the two groups. Three alleles were diagnostic for the cluster with specimens with 42 chromosomes, while one was diagnostic for the other cluster.

In the Mogi-Guassu and Grande rivers (Fig 2.4), each of the cytotypes was diagnosed by two bands. Characteristic bands were more frequent: nine for the 39/40 cytotype, and seven for the 42 cytotype. The Monjolinho population was characterized by three bands, and two bands characterized the Monjolinho and the Mogi-Guassu populations. However, the most frequent class of bands was composed by shared alleles: 38 bands (57% of total number of bands).

Discussion

Cytogenetic results. My failure to collect specimens that could be cytogenetically identified as F_1 hybrids (that is, fishes with 41 chromosomes) confirmed reports by Fenocchio (unpubl.) and Scavone et al. (1994). Unlike RAPDs, karyotypes are codominant markers, and the lack of frequencies of combinations of putative haploid sets of chromosomes (in this case, 20 and 21) may reveal species boundaries in samples composed of morphologically similar sympatric species. Assuming Hardy-Weinberg equilibrium for the pooled collection sites in the Rio Aguapey, $2n = 42$

homokaryons appeared with a frequency (p) of 48%, while 2n=40 homokaryons occurred in a frequency (q) of 52%. The expected frequency of heterokaryons (2pq) in the combined sample size would be close to 50% (or 12 individuals), and the probability of a given individual not being heterokaryon will be 1-0.50 = 0.50 ; likewise, the probability of not finding heterozygotes in a sample size of 23 would be $(0.50)^{23}$ or $1.2x10^{-7}$. The value of this probability in the Rio Mogi-Guassu and Rio Grande is $8.6x10^{-9}$. These low probabilities are incompatible with the assumption that the samples were drawn from panmictic populations.

These results indicate that the use of many independent molecular markers such as RAPD-PCR alleles with similarity distances are also suitable for detecting species boundaries. Congruence between the molecular and karyological data sets on the discrimination of two groups of fish in the Río Aguapey and in the Mogi-Guassu and Rio Grande rivers indicates the existence in each case, of two different species with different chromosome numbers and genetic structure. Shared possession of heritable traits such as molecular and chromosomal diagnostic characters is the result of reproductive links among members of species, and such cohesion is maintained in the presence of other, similar species. Ongoing morphological analyses indicate minor morphological differences between the two species in the Rio Aguapey, but no differences between the species in the Rio Mogi-Guassu and Rio Grande.

In the Rio Aguapey, sampling data suggest the original range of each of the species: only specimens with a diploid number of 40 have been collected in the Río Paraná in Argentina, and the species with $2n=42$ is apparently absent from the Rio Paraná (Lopez and Fenocchio, 1994). Overlapping ranges of the species from the Aguapey and Paraná rivers may have been the result of two processes:

a) extensive seasonal flooding of the Río Paraná is a common phenomenon that affects extensive regions, and the wetlands between the Río Paraná and the Río Aguapey are under the influence of the flooding events.

b) since the 1930's (Fenocchio, unpubl.), drainage ditches have maintained permanent connection between the waters of the Río Paraná and the Río Aguapey.

Conceivably, flooding events have operated over a long period of time, making it difficult to estimate a minimum age for this sympatry.

Besides the recognized fact that the Río Paraná has a very complex historical geology (Beurlen, 1970), there is no current specific explanation for the origin of the sympatric situation in the Rio Mogi-Guassu and the Rio Grande.

Absence of hybrids or introgressed individuals should not be considered a necessary reflection of chromosomal differences among the species. Lack of gene flow may be the result of many factors, ranging from

pre-zygotic mechanisms (Mayr, 1963) to hybrid mortality or sterility possibly due to effects of chromosomal or genic differences (King, 1993). Ongoing analyses of mitochondrial DNA variants (haplotypes) are also consistent with lack of gene flow between the cytotypes.

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Figure 2.1. Geographic location of samples:

a) Rio Mogi-Guassu and Rio Grande in Brazil. In the Rio Mogi-Guassu two localities were sampled: Monjolinho and Beija-Flor dams. In the Rio Grande, the locality Volta Grande was sampled. The city of São Paulo is indicated.

b) Rio Aguapey and Rio Paraná in Argentina, triangles indicate two localities with specimens with 40 chromosomes. Circles indicate two localities of the Río Aguapey basin with sympatry of fish with 40 and 42 chromosomes. The city of Asunción (Paraguay) is indicated.

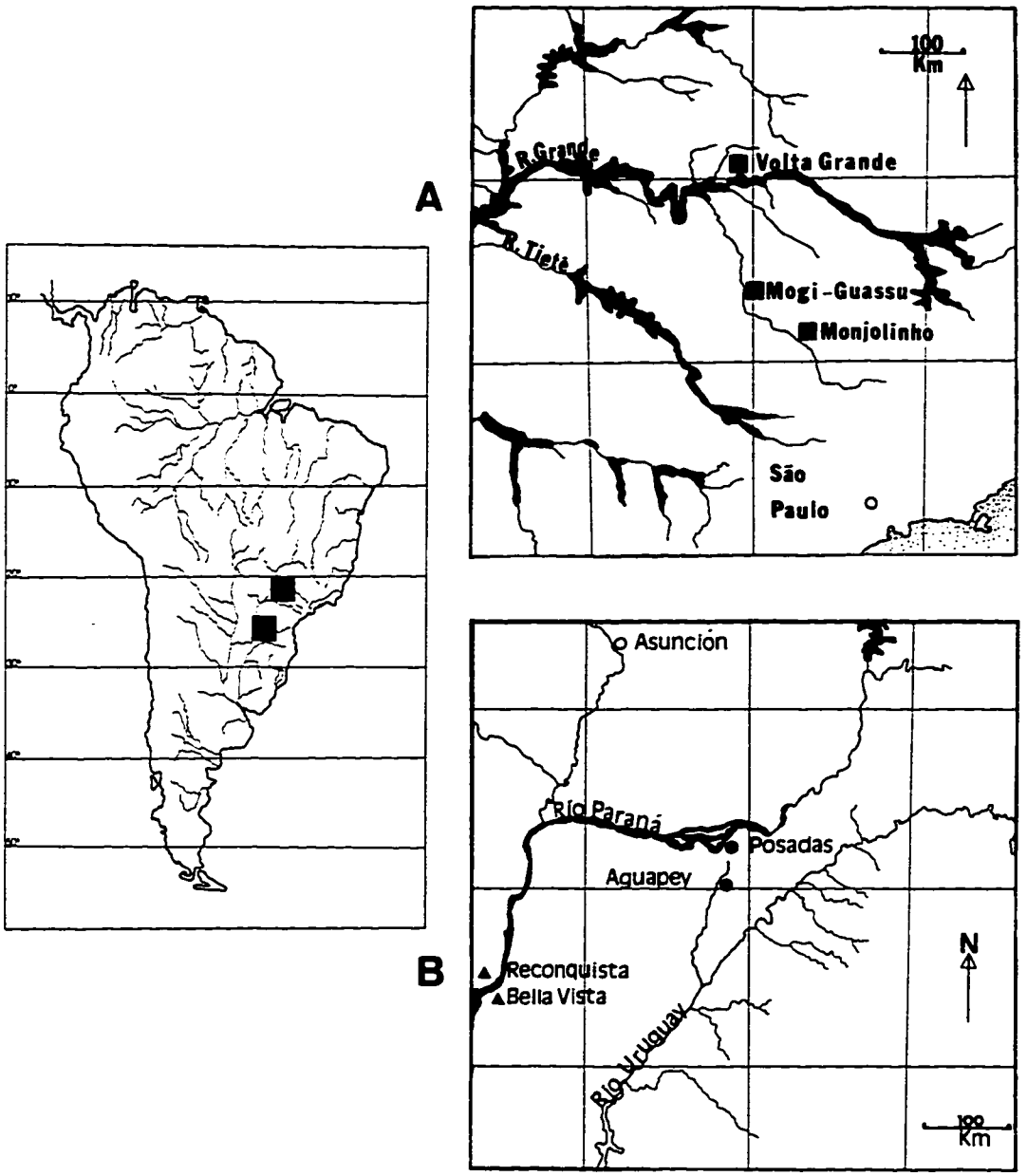

A
B
100 Km
R. Grande
Volta Grande
R. Tietê
Mogi-Guassu
Monjolinho
São Paulo
Asunción
Río Paraná
Posadas
Aquapey
Reconquista
Bella Vista
Río Uruguay
N
100 Km

Figure 2.2. RAPD bands amplified with primer C-13 of populations of the fish *H. malabaricus* from the Rio Mogi-Guassu (Monjolinho and Beija-Flor dams), and from the Rio Grande (Volta Grande). All karyotyped specimens from Monjolinho were $2n = 40/39$, and arrows indicate four specimens with $2n = 40/39$ in Beija-Flor, and four specimens with $2n = 40/39$ in Volta Grande. "L" indicates molecular markers of 1 Kb.

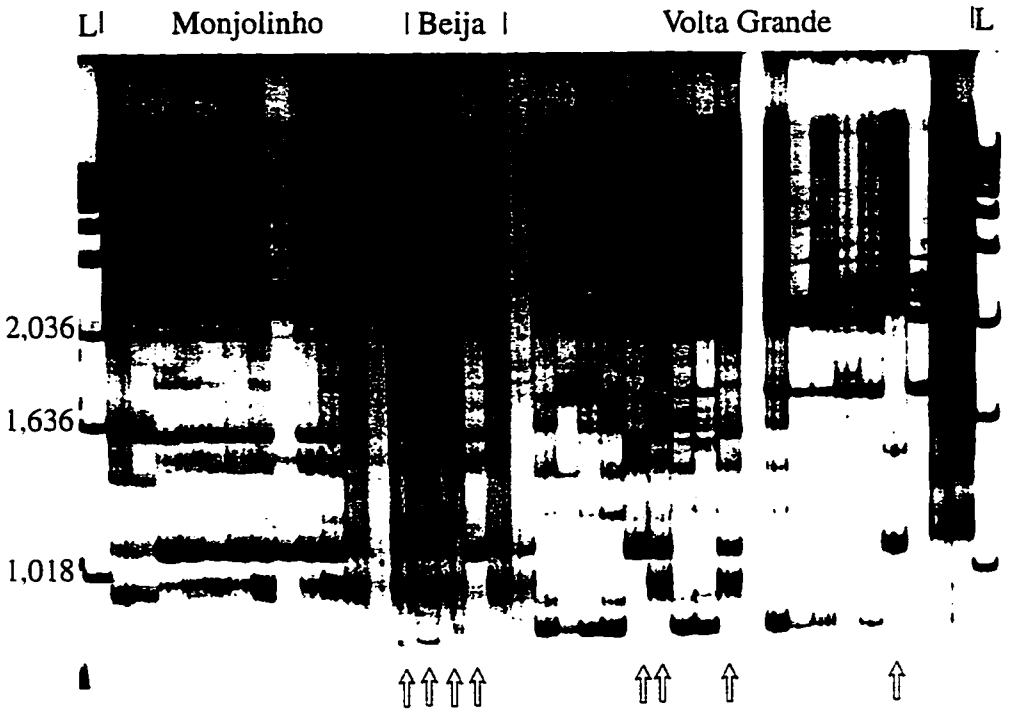

L
Monjolinho
Beija
Volta Grande
L
2,036
1,636
1,018

Figure 2.3. RAPD bands amplified with primer C-13 of populations of the fish *H. malabaricus* from the Rio Aguapey and Rio Paraná. Diploid numbers are indicated. "L" indicates molecular markers of 1 Kb.

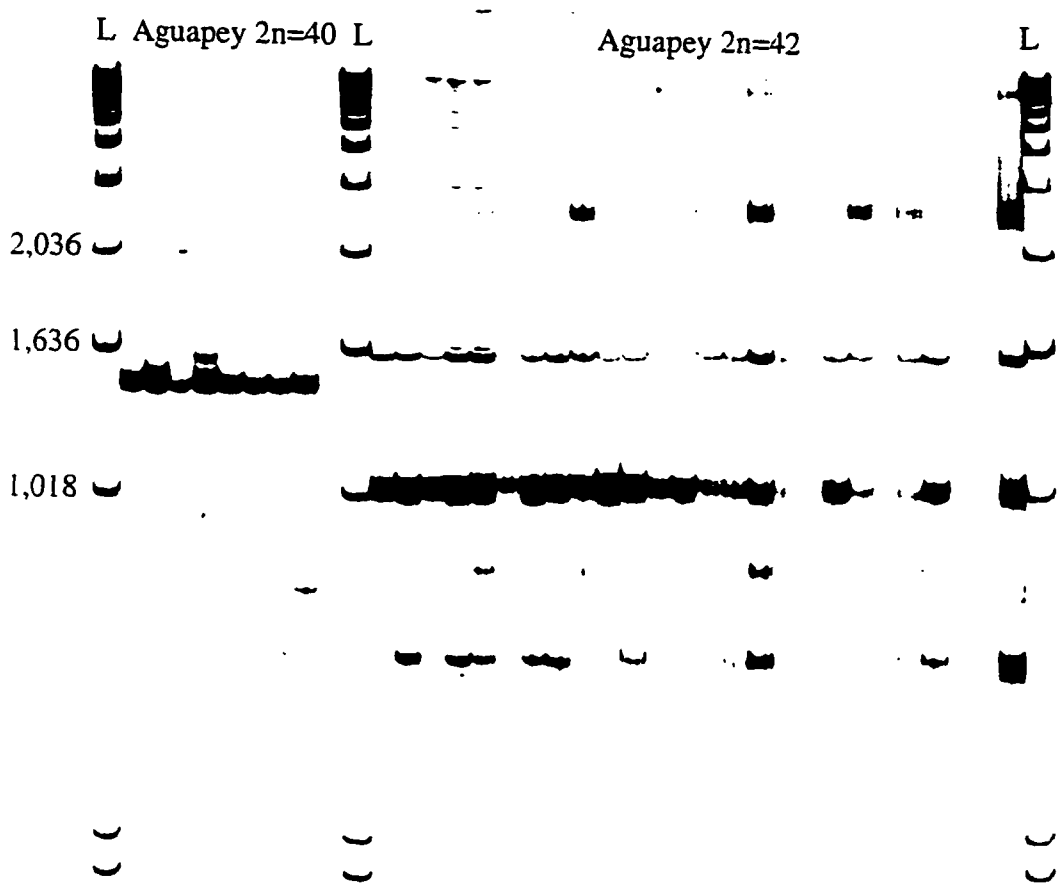

L
Aguapey 2n=40
L
Aguapey 2n=42
L
2,036
1,636
1,018

Figure 2.4. Phenogram of overall molecular similarity among specimens from sympatric populations of the fish *H. malabaricus* from the Mogi-Guassu and the Grande rivers. Terminal nodes indicate the locality name and the specimen number, and bootstrap values are indicated for some branches. Specimens with confirmed diploid numbers are indicated: specimens with $2n = 39/40$ are indicated with a circle, and specimens with $2n = 42$ are indicated with a triangle.

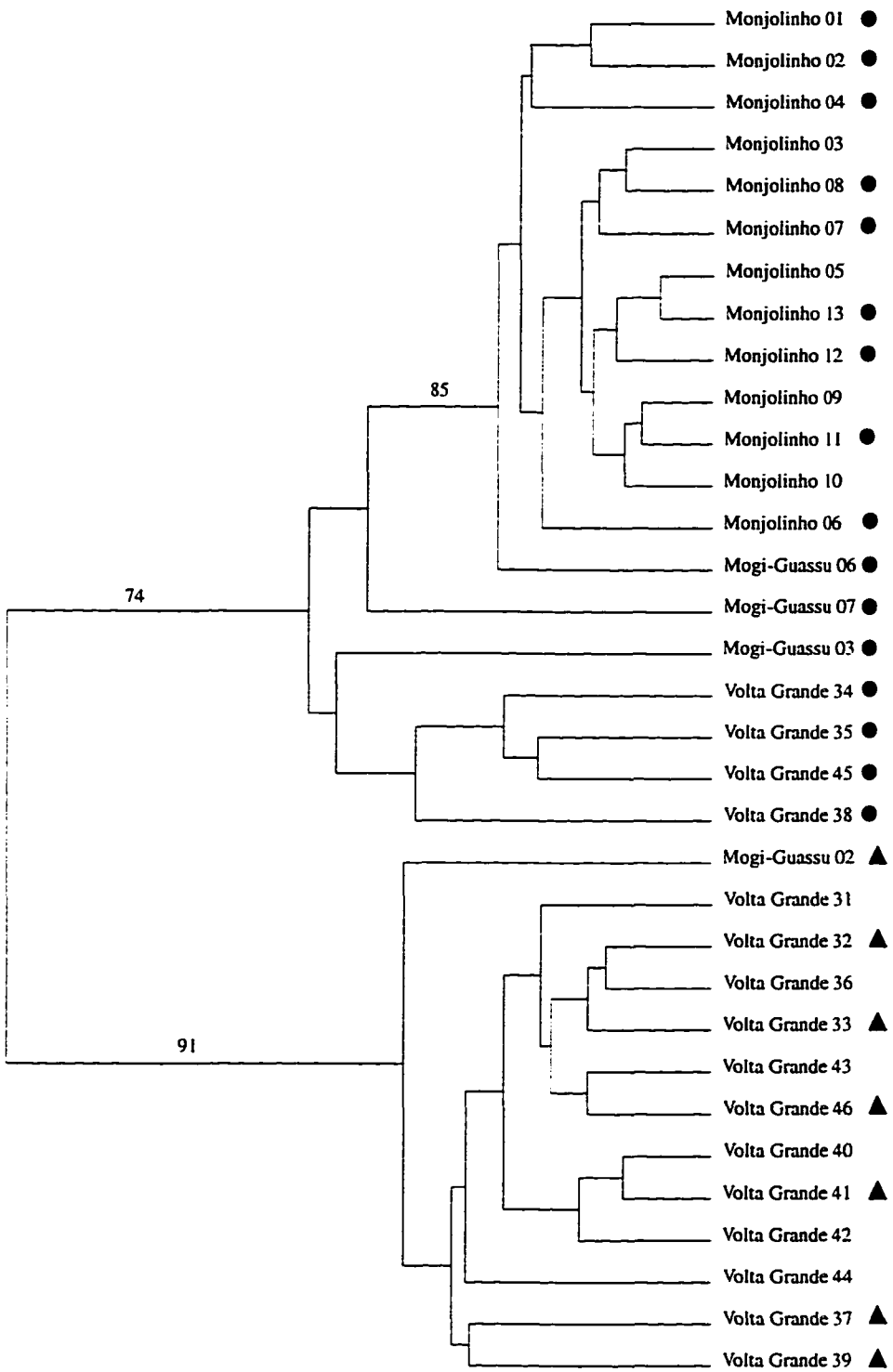
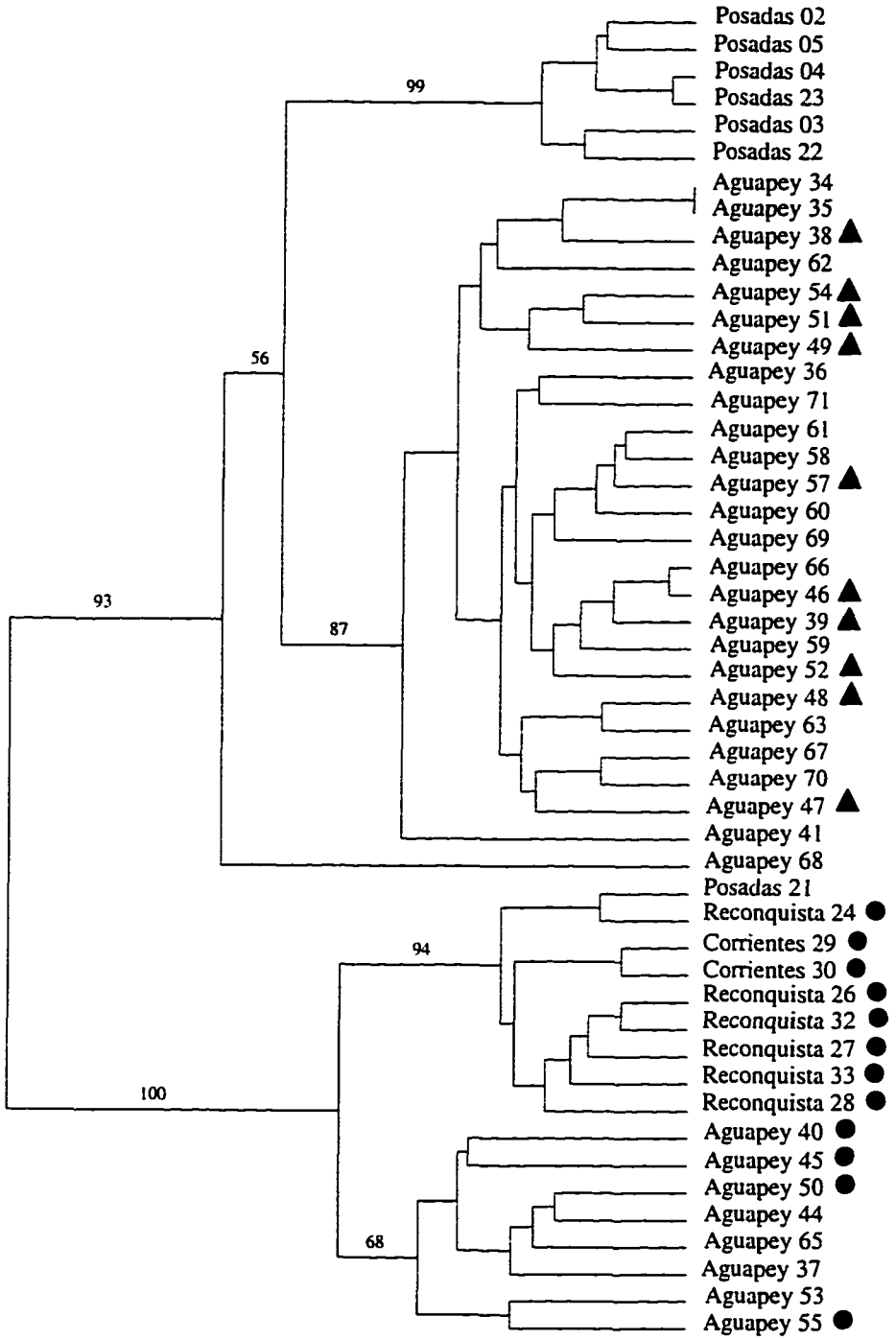

Monjolinho 01
Monjolinho 02
Monjolinho 04
Monjolinho 03
Monjolinho 08
Monjolinho 07
Monjolinho 05
Monjolinho 13
Monjolinho 12
85
Monjolinho 09
Monjolinho 11
Monjolinho 10
Monjolinho 06
Mogi-Guassu 06
74
Mogi-Guassu 07
Mogi-Guassu 03
Volta Grande 34
Volta Grande 35
Volta Grande 45
Volta Grande 38
Mogi-Guassu 02
Volta Grande 31
Volta Grande 32
Volta Grande 36
Volta Grande 33
91
Volta Grande 43
Volta Grande 46
Volta Grande 40
Volta Grande 41
Volta Grande 42
Volta Grande 44
Volta Grande 37
Volta Grande 39

Figure 2.5. Phenogram of overall molecular similarity among specimens from sympatric populations of the fish *H. malabaricus* from the Aguapey and Paraná rivers. Each terminal node indicates the locality name and the specimen number. Numbers on some branches indicate the statistical support (bootstrap values). Specimens with confirmed diploid numbers are indicated: a circle for $2n = 40$ chromosomes and a triangle for $2n = 42$.


Posadas 02
Posadas 05
Posadas 04
Posadas 23
Posadas 03
Posadas 22
Aguapey 34
Aguapey 35
Aguapey 38
Aguapey 62
Aguapey 54
Aguapey 51
Aguapey 49
Aguapey 36
Aguapey 71
Aguapey 61
Aguapey 58
Aguapey 57
Aguapey 60
Aguapey 69
Aguapey 66
Aguapey 46
Aguapey 39
Aguapey 59
Aguapey 52
Aguapey 48
Aguapey 63
Aguapey 67
Aguapey 70
Aguapey 47
Aguapey 41
Aguapey 68
Posadas 21
Reconquista 24
Corrientes 29
Corrientes 30
Reconquista 26
Reconquista 32
Reconquista 27
Reconquista 33
Reconquista 28
Aguapey 40
Aguapey 45
Aguapey 50
Aguapey 44
Aguapey 65
Aguapey 37
Aguapey 53
Aguapey 55
99
56
93
87
94
100
68

3. Molecular biogeography of the Neotropical Fish *Hoplias malabaricus* (Erythrinidae: Characiformes) in the Iguassu, Tibagi, and Paraná rivers.

Hoplias malabaricus Bloch, 1794 has extended its range into the Rio Iguassu sometime in the last decades. To determine the source of the invading population, patterns of similarity of genomic markers (Randomly Amplified Polymorphic DNA) were analyzed on samples from the Rio Paraná, the Rio Iguassu, and from the Rio Tibagi. Samples from the Paraná and Tibagi rivers showed high genetic diversity and population structure, while all the alleles of the sample from the headwaters of Rio Tibagi were present in the sample from the Rio Iguassu. This similarity suggests that the Rio Tibagi or another population with similar genetic makeup is the source for the population of the Rio Iguassu.

In the Neotropical Region, knowledge of patterns of geographical distribution of freshwater fish is still in its infancy. As discussed by Böhlke et al. (1979), researchers face difficulties such as lack of sampling, poor accessibility of type material, and high levels of diversity of the fish fauna.

In *Hoplias malabaricus* Bloch, 1794, complex patterns of morphological variation among and within populations (e.g., Eigenmann and Eigenmann, 1889) make it difficult to assess the origin of the relatively

recently established population in the Rio Iguassu (southeastern Brazil) (Fig. 3.1). The basin of this river extends from the western slope of a coastal relief, the Serra do Mar, to the Rio Paraná, as do other rivers such as the Tibagi and Ivai (Fig. 3.1).

The Iguassu river is isolated from the Rio Paraná by the Iguassu Cataracts. These cataracts were formed during Oligocene times (Fulfaro, unpubl., in Sampaio, 1988) and constituted an impassable barrier for upstream dispersal of fish faunas of the Rio Paraná into the Rio Iguassu. The long isolation from other rivers certainly played a critical role on the endemism of the fish fauna of the Rio Iguassu, which was considered by Haseman (1911) as the main feature of this river. A recent survey by the limnology research group (NUPELIA) of the Fundação Universidade Estadual de Maringá, identified 52 fish species in the Rio Iguassu (FUEM/NUPELIA/COPEL, 1995), 75% of which are considered endemic, 17.3% are indigenous but not endemic, and 7.7% are introduced.

Haseman (1911) was the first to report the absence of *H. malabaricus* from the Rio Iguassu, conversely, Godoy (1979, in Sampaio, 1988) gave the first published account on the presence of this species in the Iguassu. Sampaio (1988) reports that locals assert *H. malabaricus* was likely introduced in the last decades, perhaps together with aquaculture stocking of other exotics such as carp and tilapia. These exotics are also established in the Rio Iguassu (FUEM/NUPELIA/COPEL, 1995).

To unravel the question of the origin of the Iguassu population of *H. malabaricus*, I analyzed the patterns of variation of (mainly) nuclear markers of one sample from the Rio Iguassu, and six other samples from two neighboring basins. Determining the origin of the introduced population contributes to a better understanding of the conditions of the introductions, and thus it may prove relevant for preventing the invasion of additional exotics in the Rio Iguassu.

Materials and Methods

A total of 132 specimens were collected in the following localities (Fig. 3.1):

a) Rio Tibagi at Ponta Grossa samples (N=12), by L. F. Duboc da Silva in the Lagoa Dourada, Parque do Sol, State of Paraná, in January 1994.

b) Rio Tibagi at Sertanópolis, (N=5), by O. Shibata of the Universidade Estadual de Londrina, State of Paraná, in May 1995.

c) Rio Tibagi, Ribeirão Três Bocas, (N=9) District of Londrina, by O. Shibata, between May and October 1995.

d) Rio Iguassu sample (N=55), in the Segredo Reservoir, by members of NUPELIA, in 1995.

e) Rio Paraná sample (N=43) at the Porto Rico and Rio Ivinheima localities in Brazil, by members of the NUPELIA in 1993 and 1995, and

d) Río Paraná sample (N=8) in Argentina (Reconquista and Corrientes), by

Fenocchio and collaborators.

Tissue collection and DNA extraction are described in Dergam (unpubl.). and molecular analyses were carried on at the Department of Microbiology in Colorado State University (Colorado, United States).

I used the same Randomly Amplified Polymorphic DNA (RAPD)-PCR technique described earlier (Williams et al., 1990), with primers A-9, A-05, and AC-13 (Promega). To minimize the vagaries of repeatability of RAPDs (Black, 1993), all samples were run simultaneously for each primer reaction. Recording bands and cluster analysis involved the construction of a binary matrix ("1" for presence, and "0" for the absence of bands) for each specimen, and a measure of distance was calculated for each pairwise comparison, yielding a matching matrix (where similarity was assessed by the number of shared presence or absence of bands) with the RAPDPLOT program (Kambhampati et al., 1991; Black, 1996). Molecular similarity was analyzed by unweighted pair-group mean average (UPGMA) cluster analysis computed with the NEIGHBOR option of PHYLIP 3.5C (Felsenstein, 1993). The phenogram was drawn with the DRAWGRAM program in the same package.

Results

RAPD primers amplified a total of 74 bands for all samples (Fig. 3.2). Number of scorable bands was correlated to sample size ($r=0.82$). The

population with highest number of scorable bands was Porto Rico (51 bands), and the one with the lowest number was Três Bocas (27 bands).

Although in many cases the alleles were not fixed (not present in all individuals of a given sample), some alleles were unique for some populations: in the Rio Tibagi, five alleles were unique to the Sertanópolis population, and another one was unique to the Três Bocas population. In the Rio Iguassu, only one allele was unique to the Segredo population, and in the Rio Paraná, three alleles characterized the Porto Rico population, and two alleles were typical of the Reconquista population.

Five alleles were shared at least by one member of each population. Excluding alleles shared by members of all populations, the number of shared alleles among populations was correlated with sample size ($r = 0.77$).

All alleles of Ponta Grossa (Rio Tibagi) were also present in the population of Segredo (Iguassu), although the population from Segredo showed 48 alleles, one third more than the population of Ponta Grossa (Fig. 3.2).

Molecular similarity is depicted by a phenogram in Fig. 3.3. The matching matrix grouped all specimens in three major clusters. The first one is composed by the sample from Rio Paraná at Bella Vista and Reconquista in Argentina (Dergam, unpubl.), plus three individuals from Porto Rico. The second cluster joins all the specimens from Tibagi and Segredo, except for one outlier that the third cluster formed by the specimens from Rio Paraná at

Porto Rico.

Discussion and Conclusions

RAPD patterns indicate strong genetic differentiation within and among populations in the Paraná and in the Tibagi rivers. In the Rio Paraná at Porto Rico, high levels of polymorphism detected in the RAPD patterns may be the result of a faunal mixing caused by the building of the Itaipu Dam in 1978 and the consequent drowning of Sete Quedas, another geographic barrier also dating from the Oligocene (Fulfaro, in Sampaio, 1988), which was the natural limit between the Upper and the Middle Paraná basins.

In the Rio Tibagi, the Três Bocas sample is more similar to the populations of the Rio Paraná (Figs. 3.2, 3.3, and 3.5). While the Três Bocas sample is genetically homogeneous, the sample from Sertanópolis (N=5) included one specimen with molecular profile similar to Três Bocas, and four specimens different from all other samples along the Rio Tibagi (Fig. 3.3). These four specimens were also different enough from the rest to constitute a small, third cluster on their own (Fig. 3.4).

The patterns of genomic variation obtained with RAPDs suggest a close kinship between the newly established population of *H. malabaricus* in the Rio Iguassu, and the sample from the Rio Tibagi at Ponta Grossa. Phylogenetic sequence analyses of the mitochondrial DNA of these two

populations (Dergam, unpubl.) corroborate they are more related to each other than to any of the other sampled populations from the Rio Tibagi or the Rio Paraná.

The lower genetic variation of RAPDs of the Tibagi sample when compared to the Iguassu/Segredo sample may be an artifact of the small sample size, it may be the result of the effects of random genetic drift, or still it may be a combination of both factors: the Tibagi/Ponta Grossa sample is from a lagoon close to the headwaters of the Rio Tibagi.

Although Haseman (1911) described the divide between the Rio Tibagi and the Rio Iguassu as "short and low", molecular similarity is not sufficient for supporting or rejecting the possibility that founder animals came specifically from the Rio Tibagi. RAPD analysis of two small samples (each N=3) from coastal populations of this species (from Rio Perequê in Paranaguá and from Rio Ribeira at Registro) are similar to the sample from Tibagi/Ponta Grossa (Dergam, unpubl.). A phylogenetic analysis of haplotypes from many samples of that region places the populations of the Rio Tibagi at Ponta Grossa, Iguassu, and coastal populations in a well supported clade (Dergam, unpubl.).

At any rate, molecular similarity may allow determination of the conditions of the introduction. If founder individuals were introduced from aquaculture facilities (Sampaio, 1988), the genetic composition of the populations of *H. malabaricus* at those stations should match the one from

H. malabaricus of Tibagi/Ponta Grossa.

In conclusion, genomic and mitochondrial DNA evidences suggest that the introduced population of *H. malabaricus* in the Rio Iguassu has its most likely origin from populations with a genetic composition similar to the population from the headwaters of the Rio Tibagi.

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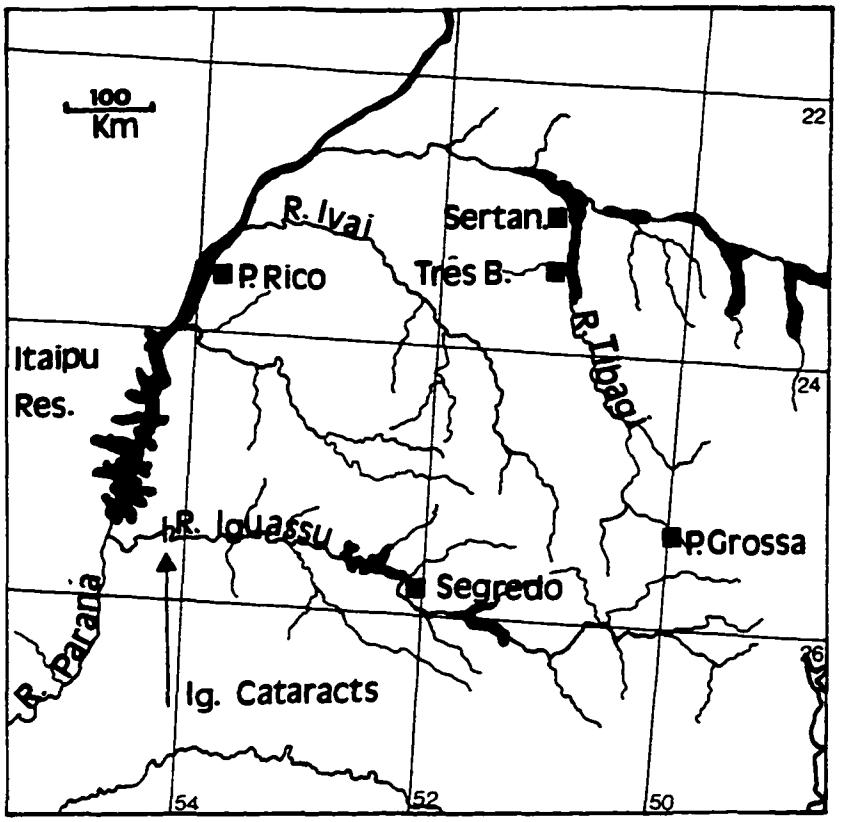
Williams, J. G. K. , A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful genetic markers. Nucleic Acids Res. 18:6531-6535.

Figure 3.1. Geographic locations of samples from the Rio Tibagi, the Rio Paraná, and the Rio Iguassu. In the Rio Tibagi, three localities were sampled: Ponta Grossa, close to the headwaters; Ribeirão Três Bocas; and Sertanópolis. In the Rio Paraná, only the Porto Rico locality is indicated, but the analysis included the samples from Reconquista and Bella Vista (Dergam, unpubl.). In the Rio Iguassu, samples were collected in the Segredo Reservoir.

The Iguassu Cataracts on the Rio Iguassu are also indicated.


100
Km
22
R. Ivai
Sertan.
P. Rico
Três B.
R. Tibagi
Itaipu
Res.
24
R. Iguassu
P. Grossa
Segredo
R. Parana
Ig. Cataracts
54
52
50

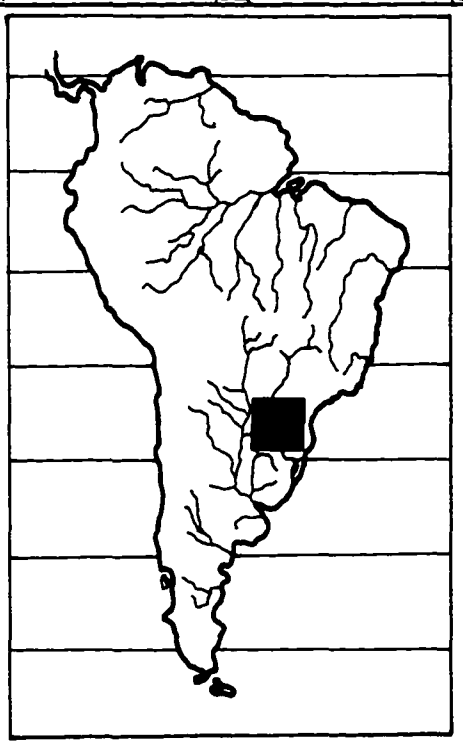


Figure 3.2. RAPD bands amplified with primer C-13 of 45 specimens (one specimen per column) from seven populations:

a) From the Rio Tibagi: Sertanópolis, Ribeirão Três Bocas, and Ponta Grossa.

b) From the Rio Iguassu: Segredo Reservoir.

c) From the Rio Paraná: Porto Rico, Bella Vista, and Reconquista.

Both Rio Tibagi and Rio Paraná are highly heterogeneous, and the two more similar populations are Ponta Grossa and Segredo. Letter "L" indicates molecular markers of 1 Kb.

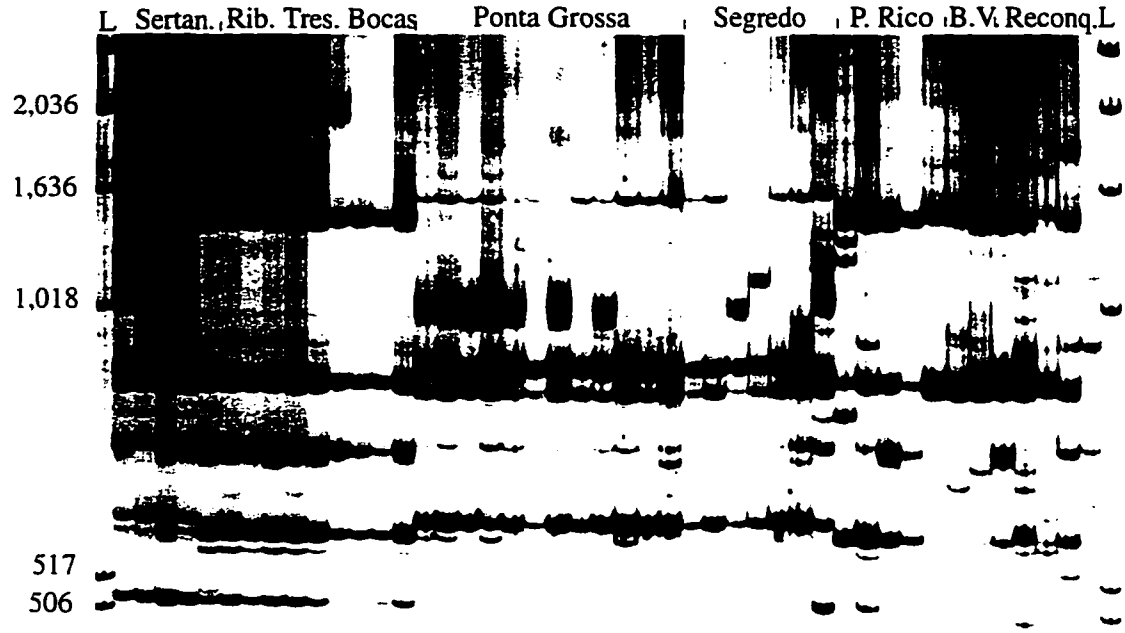

L
Sertan.
Rib. Tres. Bocas
Ponta Grossa
Segredo
P. Rico
B.V.
Reconq.
L
2,036
1,636
1,018
517
506

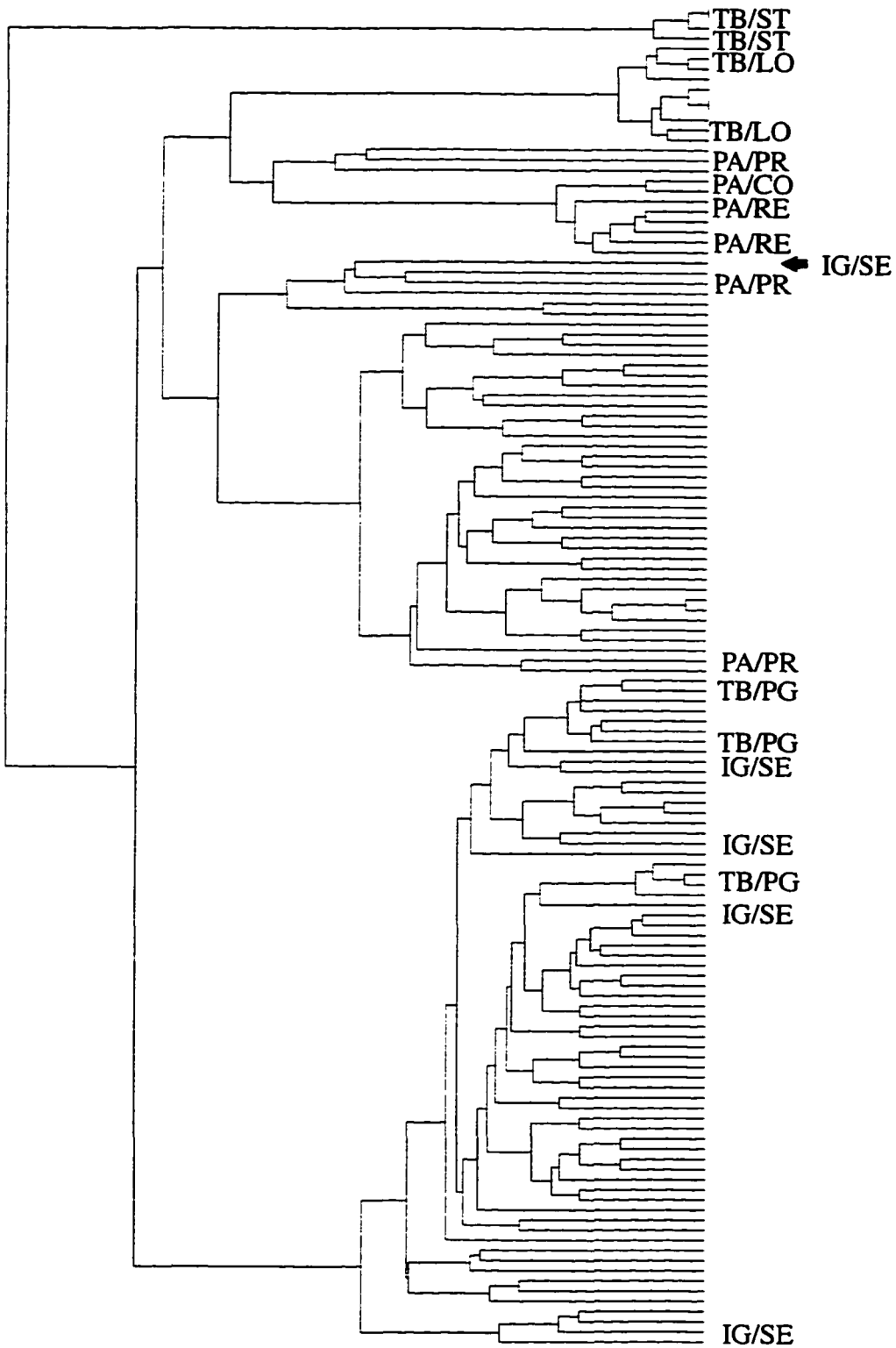
Figure 3.3. Phenogram of overall molecular similarity of RAPD bands among specimens from all localities at Rio Tibagi, Rio Paraná, and Rio Iguassu.

From Rio Tibagi, TB/ST indicates Sertanópolis, TB/LO indicates Ribeirão Três Bocas, and TB/PG indicates Ponta Grossa.

From Rio Paraná, PA/PR indicates Porto Rico, PA/CO indicates Bella Vista, and PA/RE indicates Reconquista.

From Rio Iguassu, IG/SE indicates Segredo Reservoir.

The phenogram depicts three major clusters. One of them is composed by four out of five specimens from the Tibagi/Sertanópolis sample; the fifth specimen clustered with the Tibagi/Três Bocas sample. The second cluster is composed mainly by specimens from the Rio Tibagi/ Três Bocas, and all the specimens from the Rio Paraná, plus one outlier from Rio Iguassu/Segredo (indicated by the arrow). The third cluster is composed by all the specimens from Rio Tibagi/Ponta Grossa and from the Rio Iguassu/Segredo.


TB/ST
TB/ST
TB/LO
TB/LO
PA/PR
PA/CO
PA/RE
PA/RE
IG/SE
PA/PR
PA/PR
TB/PG
TB/PG
IG/SE
IG/SE
TB/PG
IG/SE
IG/SE

4. RAPD-PCR Assessment of the Patterns of Molecular Divergence Among Populations of the Fish *Hoplias malabaricus* (Erythrinidae: Teleostei), with comments on the phylogenetic applications of RAPD-PCR data.

RAPD-PCR polymorphisms at 111 presumptive loci were used to examine the molecular divergence among 28 populations of trahiras (*Hoplias malabaricus*) in South America. Molecular similarity was assessed by means of computing unweighted pair mean average on shared absence and presence of amplified bands. Statistical support for phenogram branches was estimated by bootstrap resampling. Alleles were recorded across populations assuming identity in state among amplified and null alleles. Partial agreement was found between clustering by molecular similarity, karyotypical data, and sequences of mitochondrial DNA, and causes for disagreement are discussed.

Since the seminal paper of Williams et al. (1990) introduced the use of Randomly Amplified Polymorphic DNA obtained by the Polynucleotide Chain Reaction (RAPD-PCR) as genetic markers, data on polymorphic regions of the genome have been applied to biological problems like genetic differentiation among populations (Ballinger-Crabtree et al., 1992; Thorpe et al., 1994), fingerprinting (Apostol et al., 1993), cryptic species (Wilkerson et al., 1993; Dergam, unpubl.), and phylogenetic analyses (Demeke and

Adams, 1994; Badaracco et al., 1995). Undoubtedly, the ease of application of RAPD protocols has been the preponderant factor for their widespread use (reviewed in Black, 1993; 1996). Typically, RAPD protocols require a minimum amount of template, there is no need for a priori knowledge of specific annealing sites, and radioactive labeling is not necessary. On the other hand, RAPD-PCR markers are susceptible to amplification conditions and cycle profiles of thermocyclers (Black, 1993). This kind of markers seem to be ill suited for phylogenetic studies because homology is difficult to assess between alleles, and at least in *Xanthomonas campestris*, alleles amplified in different regions of the genome may co-migrate (Smith et al., 1994). Even if homology of presence and absence of large number of amplified alleles is assumed among members of a population, this assumption becomes increasingly untenable at higher levels of phylogenetic divergence (Black, 1996).

Scoring and processing of RAPD-PCR data only address overall molecular similarity, a phenetic method unrelated to phylogeny reconstruction (i.e., the search for the genealogical relationships between ancestral and descendant taxa) (see Wiley, 1981 for a review). Moreover, phylogenetic systematics require that only shared derived or advanced characters be used in phylogeny reconstruction. Usually this is determined by designating an outgroup (a taxon outside the taxon subject of analysis). In this case, RAPD alleles shared with the outgroup would be considered

primitive and thus uninformative. Owing to their high mutation rates, however, RAPD-PCR markers may evolve too fast in putative outgroups.

Nevertheless, RAPDs are very useful as taxonomic markers (Black, 1996) for detecting cryptic species in sympatry or genetic relationships between conspecific or subspecific populations. In the chromosomally polytypic fish *Hoplias malabaricus* Bloch, 1794, RAPD-PCR markers and mitochondrial DNA (mtDNA) sequencing data provided evidence for lack of gene flow between sympatric populations with different diploid numbers (Dergam, unpubl.). Within the family Erythrinidae, *H. malabaricus* and *Hoplerythrinus unitaeniatus* Agassiz, (in Spix and Agassiz, 1829) are characterized by populations with different diploid numbers (Bertollo et al., 1986), a pattern probably associated to the limited dispersal capabilities of these species (White, 1978; for a revision, see Sites and Moritz, 1987). The purpose of this study is to examine the consistency of clustering analysis obtained with RAPD-PCR markers with patterns of variation of cytochrome b mtDNA (expressed as maximum percent sequence divergence (MPSD)) within and among populations of *Hoplias malabaricus* in South America, and with karyotypical data (when available).

Materials and Methods

During 1993-1995, 298 specimens of *H. malabaricus* were collected by seining or gill netting in 28 localities indicated in Table 4.1 and in Fig.

4.1. Muscle samples were taken in the field and fixed in absolute ethanol or in 1v:1v ethanol:methanol. In the laboratory, DNA was extracted following Dergam (unpubl.), and PCR reactions were performed in 50-μl volume mixtures under conditions described in Black and Peisman (1994). Three primers: A09, A05, and C-13 (Promega) were chosen for their ability to amplify a large number of alleles (circa 20 per primer) and for the good quality of the bands (strongly stained and separated from other bands). PCR products were run onto a nondenaturing polyacrylamide 38 by 50 cm gel (Sequigen, BioRad, Richmond, CA) at 345 V with 1XTBE buffer for approximately 16 h at room temperature. DNA bands (alleles) were silver stained (Hiss et al., 1994), and the gels were dried, photographed, and scanned. Alleles were identified by their mobility on the gel relative to DNA fragments of a 1 kilobase molecular ladder (Bethesda Research Laboratory, Gaithesburg, MD) (Fig. 2.3) fitted to a function that relates molecular weight with fragment mobility (Schaffer and Sederoff, 1981), using the LOTUS program MWT.WK3 (Black, unpubl.), and named after the primer's name and their position on the gel. Presence and absence of alleles were scored in a binary matrix, and shared presence and absence were computed into a pairwise distance matching matrix with the FORTRAN program RAPDPLOT (Black, 1996). This matrix was used to produce a dendrogram using the unweighted pair-group method with arithmetic averaging (UPGMA) option of the NEIGHBOR program in PHYLIP 3.5C (Felsenstein, 1993), and the treefile

was plotted with DRAWGRAM of PHYLIP 3.5C. Bootstrap (Efron, 1985) values higher than 50% calculated with the FORTRAN program RAPDBOOT (Black, unpubl.) were added to the branches of the phenogram.

Specimens were deposited in the Museum of Zoology of the Universidade Federal de Viçosa, Viçosa, Minas Gerais State, Brazil, except for the following samples: a) Centro de Pesquisas do Pantanal, Corumbá, State of Mato Grosso, Brazil, b) systematic collection of the NUPELIA research group, Fundação Universidade Estadual de Maringá, Maringá, State of Paraná, Brazil, c) systematic collection of the Universidade Estadual de Londrina, Londrina, State of Paraná, Brazil, and d) systematic collection of the Departamento de Citogenética, Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Posadas, Provincia de Misiones, Argentina.

Results

The resulting dendrogram is depicted in Figure 4.2 ; diploid numbers for each population are also indicated. Levels of congruence with diploid numbers and geographically close populations are high:

a) clusters of populations from the Rio Doce, where all of them have 42 chromosomes, and a MPSD of 5.7.

b) cluster of the Rio Doce populations with populations from Mogi-Guassu and Rio Grande with same diploid number is also consistent with the

phylogeny derived from mtDNA analysis. The MPSD between these two clusters is 6.3.

c) cluster of samples from Rio Tocantins at Tucurui and at Araguaia, with a MPSD of 0.3.

d) cluster of samples (Mogi-Guassu + Tietê/Monjolinho + Rio Grande). These populations share haplotypes and chromosome numbers.

e) cluster of samples from (Tibagi/Ponta Grossa + Perequê + Rio Ribeira), with a MPSD of 0.3.

f) cluster of samples from (Paraná/Reconquista + Paraná/Corrientes + Posadas), which share chromosome numbers. Their MPSD is 9.7.

Sympatric species with different diploid numbers and lack of gene flow (Dergam, unpubl.) are correctly placed as distinct units (two populations from Aguapey and two from Rio Grande/Mogi-Guassu).

On the other hand:

g) the Rio Iguassu/Segredo population clusters together with the population from Paraná/Porto Rico instead of clustering with the Tibagi/Pereque/Rio Ribeira populations, contradicting results based on larger sample sizes, suggesting a close similarity between Iguassu and Tibagi/Ponta Grossa (Dergam, unpubl.). The MPSD between the two clusters is 8.3.

Other inconsistencies with phylogenetic reconstructions based on mtDNA are also evident: the widely divergent clusters of Rio Doce,

Tibagi/Ponta Grossa + Pereque + Rio Ribeira and Segredo constitute a well supported monophyletic group (clade) of haplotypes. Also, haplotypes from populations with 40 chromosomes from Paraná + Posadas + Aguapey together with haplotypes from Mogi-Guassu + Tietê/Monjolinho + Rio Grande represent the most basal clade in the phylogeny, yet they do not cluster together.

Discussion

My results indicate that although RAPDs are efficient for discriminating cryptic species and for clustering closely related populations, they perform poorly at higher levels of genetic divergence, and this lack of fit is not related to MPSD in a linear fashion. Real differences of RAPD patterns may be confounded with lack of band repeatability, experimental error due to unbalanced sample sizes (N = 3-56), and possible lack of homology (Black, 1993). Assuming that these potentially distorting factors are minimized, the incongruence between mtDNA sequences and the RAPD markers may result from many sources: the pattern of inheritance of mtDNA is uniparental and cytoplasmic, while RAPD markers are biparental and (mainly) nuclear. More importantly, sequences coding for RAPD markers from repetitive regions (Haymer, 1994) are traditionally seen as devoid of encoding functions, and therefore selective constraints are expected to be relaxed. Consequently, mutation rates are extremely high, an assumption

supported by the vast amount of polymorphism revealed by the RAPD-PCR protocol. Thse mutation rates might explain the efficiency of RAPD-PCR markers as molecular taxonomy tools for detecting genomic differences among species or populations, and also why these markers are misleading for evaluating phylogenetic relationships among more distantly related taxa.

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Table 4.1. Locales, sample sizes, and diploid number of samples analyzed in this study. Histological sex identification is indicated whenever it is available.

Locales	Sample size	Diploid number
Upper Rio Paraná		
Rio Tietê, Monjolinho Dam, State of São Paulo, Brazil	12 (4♀ / 2♂)	M 39 / F 40
Rio Mogi-Guassu, Jataí Natural Reserve, State of São Paulo, Brazil	3 (2♀)	M 39 / F 40
Rio Grande, Volta Grande, State of Minas Gerais, Brazil	4(2♀ /1♂)	M 39 / F 40
Rio Grande, Volta Grande, State of Minas Gerais, Brazil	12 (3♀ /9♂)	42
Rio Tibagi, Ponta Grossa, State of Paraná, Brazil	12	?
Middle Rio Paraná		
Rio Iguassu Segredo, State of Paraná, Brazil	7	?
Rio Paraná, Reconquista, Province of Santa Fe, Argentina	6	40
Rio Paraná, Bella Vista Province of Corrientes, Argentina	3	40

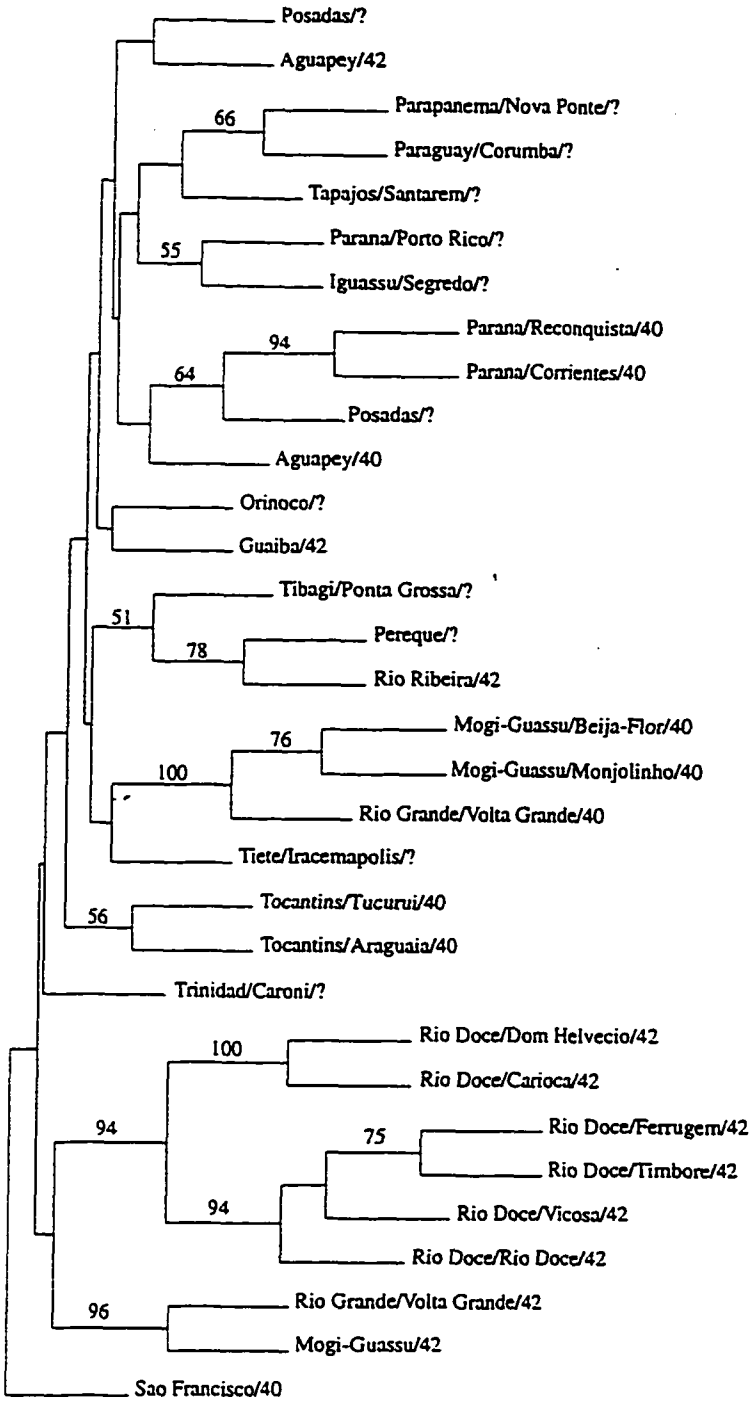
Table 4.1 (cont.)

Locales	Sample size	Diploid number
Rio Uruguay		
Rio Aguapey, Province of Corrientes, Argentina	8 (2♀ /3♂)	40
Rio Aguapey, Province of Corrientes, Argentina	27(12♀ /10♂)	42
Coastal Basins		
Rio Ribeira do Iguape, Registro, State of São Paulo, Brazil	3(1♀ /2♂)	42
Rio Perequê, Paranaguá, State of Paraná, Brazil	3	?
Rio Guaíba, Porto Alegre State of Rio Grande do Sul, Brazil	4	42
Rio São Francisco		
Rio das Velhas, Curvelo, State of Minas Gerais, Brazil	56(8♀ /5♂)	40
Rio Doce		
Rio Doce, Oriente, State of Minas Gerais, Brazil	4	42
Rio Turvo, Viçosa, State of Minas Gerais, Brazil	9(6♀)	42
Lagoa Timboré, Dionísio, State of Minas Gerais, Brazil	3	42
Lagoa Ferrugem, Dionísio, State of Minas Gerais, Brazil	3	42
Lagoa Dom Helvécio, Dionísio, State of Minas Gerais, Brazil	33	42
Lagoa Carioca, Dionísio, State of Minas Gerais, Brazil	9	42

Table 4.1 (cont.)

Locales	Sample size	Diploid number
Rio Amazonas Rio Tocantins, Marabá, Tucurui Dam, State of Pará, Brazil	4	40
Rio Araguaia (tributary of the Rio Tocantins), Barra do Garças, State of Mato Grosso, Brazil	8	40
Rio Madeira, Porto Velho, State of Rondônia, Brazil	4	40

Figure 4.1. Phenogram derived from 111 RAPD alleles amplified for 298 specimens from populations indicated in Table 4.1. Bootstrap values higher than 50% are indicated.


Posadas/?
Aguapey/42
66
Parapanema/Nova Ponte/?
Paraguay/Corumba/?
Tapajos/Santarem/?
55
Parana/Porto Rico/?
Iguassu/Segredo/?
94
Parana/Reconquista/40
64
Parana/Corrientes/40
Posadas/?
Aguapey/40
Orinoco/?
Guaiba/42
Tibagi/Ponta Grossa/?
51
78
Pereque/?
Rio Ribeira/42
76
Mogi-Guassu/Beija-Flor/40
100
Mogi-Guassu/Monjolinho/40
Rio Grande/Volta Grande/40
Tiete/Iracemapolis/?
Tocantins/Tucurui/40
56
Tocantins/Araguaia/40
Trinidad/Caroni/?
100
Rio Doce/Dom Helvecio/42
Rio Doce/Carioca/42
94
75
Rio Doce/Ferrugem/42
Rio Doce/Timbore/42
94
Rio Doce/Vicosa/42
Rio Doce/Rio Doce/42
96
Rio Grande/Volta Grande/42
Mogi-Guassu/42
Sao Francisco/40

5. Mitochondrial DNA Sequence Variation in the Neotropical Fish *Hoplias malabaricus* (Erythrinidae: Teleostei): Implications for its Phylogeographic and Karyotypic Evolution.

The trahiras (*Hoplias malabaricus*) are widely distributed in the Neotropical Region. Cytogenetic data indicate that this species complex is formed by sympatric and allopatric populations with diploid numbers of 40 and 42 chromosomes. In order to determine phylogenetic congruence with diploid and geographic patterns, I carried on phylogenetic analyses of PCR-amplified sequences of mitochondrial DNA (553 bases of 16 S ribosomal RNA and 383 bases of cytochrome b). Combined sequences of 20 haplotypes indicate the existence of a basal clade represented by one haplotype from a $2n=40$ populations from the Middle Rio Paraná , a second clade composed by haplotypes from $2n=42$ populations from southeastern and southern Brazil, and a third clade from populations with $2n=40$ and $2n=42$ from elsewhere. Monophyly of the second clade is congruent with distribution patterns of other fish taxa, and inconsistency between mitochondrial DNA and diploid number patterns in the third clade suggests multiple origins of diploid numbers and/or gene flow during or following fixation of chromosomal rearrangements.

The nominal taxon *H. malabaricus* Bloch, 1794 is characterized by a complex pattern of morphological variation within and among populations. In spite of the lack of morphological studies or taxonomic revisions of this taxon, cytogenetic information starting with the studies of Bertollo (1978) has allowed to detect macrogeographic patterns of variation in diploid numbers (Fig. 1.1b), and also populations with multiple sex chromosome mechanisms (Dergam and Bertollo, 1990; for a review, see Bertollo et al., 1986). When these chromosome races (cytotypes) are found in sympatry (e.g., Scavone et al., 1994; Lopez et al., unpubl.), RAPD markers corroborate lack of gene flow among the cytotypes (unpubl.). Lack of G-banding data has hampered the understanding of the phylogeny of karyotypical rearrangements, and thus polarity (primitive vs. derived) and monophyly (sharing of a common ancestor) of the two diploid numbers may be provisionally addressed with molecular data sets. Given the cytoplasmic and maternal mode of inheritance of mitochondrial DNA (mtDNA), the patterns of variation of mtDNA variants (haplotypes) are useful for detecting numbers of maternal clones in population bottlenecks or in founding populations (Avise et al., 1987). In taxa with chromosomal polytypy, mtDNA diversity should reflect the population conditions involved in the fixation of chromosomal rearrangements (for a review, see Sites and Moritz, 1987; King, 1993), i.e., if negatively heterotic chromosomal rearrangements arose once and in small demes, this event should be reflected on the

monophyly of haplotypes of the new rearrangement.

Another aspect of this study involved the analysis of geographic patterns of mtDNA variation. Molecular evidences for patterns of historical biogeography in species of fish have been reported by many authors (e.g., Bermingham and Avise, 1986; Bernatchez and Dodson, 1990; Ortí et al., 1994). Avise et al. (1987) coined the term "phylogeography" for the study of geographic patterns of genealogical lineages including supra- and infraspecific levels of variation (for a review, see Avise, 1994), and nowadays phylogeographic studies have become a common approach (e.g., Bowen et al., 1994; Paetkau and Strobeck, 1996). In the more general context of vicariance biogeography (Rosen, 1978; Nelson and Platnick, 1981; see Wiley, 1988 for a review), disjunct patterns of distribution of sister groups of organisms (i.e., sharing an ancestor) are seen as the result of fragmentation of the continuous range of an ancestral taxon, and areas of endemism proposed for a given monophyletic group may be corroborated by the distribution patterns of other unrelated monophyletic groups. An important contribution of phylogeographic analysis to vicariance biogeography was demonstrated by Bermingham and Avise (1986), who found that genetic markers of widespread, "redundant" taxa may be consistent with patterns of distribution of ranges of different species.

Materials and Methods

From 1993 to 1995, 298 specimens of *H. malabaricus* were collected by seining or gill netting in the localities indicated in Table 5.1, and in Fig. 1.2. All specimens were sampled for molecular studies, but in three samples, DNA was successfully extracted from leftovers of former cytogenetic studies. DNA was extracted following a CTAB protocol modified from Boyce et al. (1989)(unpubl.). Sequencing efforts involved a pilot study on mtDNA (a 553 base long fragment of the 16 S ribosomal RNA molecule) with primers L2510 5'-CGCCTGTTTATCAAAAACAT-3' and H3080 5'-CCGGTCTGAACTCAGATCACGT-3' (Palumbi et al., 1991).

Later on, cytochrome b (cyt b) was chosen as potentially more useful for lower levels of phylogenetic divergence, as reported by Buckup and Chernoff (1993) for characins of genus *Bryconops*. For cytochrome b amplification the following primers were used (Meyer et al., 1990; Kocher et al., 1989), L14724: 5'-CGAACGTTGATATGAAAAACCATCGTTG-3' and H15149: 5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3' .

Amplifications were performed from total genomic DNA by the polymerase chain reaction (PCR) (Saiki et al., 1988) performed in 50-μl reactions, in 60-wells and 96-wells PTC-100 thermal cyclers (MJ Research, Inc., MA) with the following step-cycles: a) for 16 S: 5 min at 95°C, 2 min at 80°C, 10 cycles of 1 min at 92°C, 1 min at 49°C, 2 min at 72°C, 32 cycles of 1 min at 92°C, 35 s at 54°C, 2 min at 72°C, and a final extension

of 7 min at 72°C.

b) for cyt b: 5 min at 95°C, 2 min at 80°C, 10 cycles of 3 min at 92°C, 1 min at 50°C, 1:30 min at 72°C, 32 cycles of 1 min at 92°C, 35 s at 54°C, 1:30 min at 72°C, and a final extension of 7 min at 72°C.

Amplified products were sequenced directly with cycle sequencing (Black and Piesman, 1994), and sequences were read manually from autoradiographs with the SEQAID II 3.6 software (Rhoads and Roufa, 1989), and multiple sequences were aligned with CLUSTAL V (Higgins and Sharp, 1989).

Population variation of mtDNA variants (haplotypes) was assessed with the Single Strand Conformation Polymorphism (SSCP) protocol (Orita et al., 1989 a and b): under suitable conditions, fragments of DNA bearing different sequences show different band patterns on a nondenaturing gel. The SSCP protocol was carried on a 400 base pair long fragment of the 16 S rRNA mtDNA with the nested primer: 5'-GGATCTTTTGGTCAGAAG-3' together primer L14724, with a mixed site of thymine substituting guanine at the 3' end. This primer was designed by choosing stable segments of sequences of trahiras, amplified with the 16 S primers mentioned above.

Each SSCP profile (haplotype) was identified with arbitrary numbers, based on the mobility and configuration of the bands on the gels (e.g., Fig. 5.1). Because the ability of the technique to detect point mutations is inversely related to the fragment length (Hayashi, 1991), the sensitivity was

determined by sequencing two pairs of haplotypes with identical SSCP profiles. SSCP profiles derived from 16 S rRNA were more easily interpretable than the ones derived from a similar size fragment of cyt b (Dergam, unpubl.), and they were thus used for haplotype screening for all specimens.

Phylogenetic reconstruction was performed using PHYLIP 3.5C (Felsenstein, 1993). The sequences were bootstrapped 100 times, a maximum parsimony analysis was run for each of the bootstrapped samples, and a majority consensus was then obtained for all the trees. A genetic distance analysis was calculated from a Kimura 2-parameter distance matrix (Kimura, 1980), set at observed transition/transversion (TS/TV) levels (see results), and a Neighbor Joining (NJ) (Saitou and Nei, 1987) analysis was conducted on the distance matrix. Again, a majority consensus tree was calculated from each of the obtained data set. Bootstrap values of the consensus tree from parsimony and distance analyses were added to the tree obtained with maximum likelihood option. For phylogenetic analyses, outgroup taxa were represented by two erythrinids: *Hoplias cf. lacerdae*, collected in the Beija-Flor Dam, Jatai Natural Reserve, Rio Mogi-Guassu, State of São Paulo, Brazil, and *Erythrinus sp.*, collected in the Rio Paraná in Resistencia, Argentina. All voucher specimens are deposited in the Museu de Zoologia of the Universidade Federal de Viçosa, Minas Gerais State, Brazil, except for the following samples:

a) Rio Paraguai/Corumbá, Centro de Pesquisa Agropecuária do Pantanal, Embrapa, Corumbá, State of Mato Grosso, Brazil, and

b) Rio Paraná/Porto Rico, in the systematic collection of the limnological research group NUPELIA of the Fundação Universidade Estadual de Maringá, Maringá, State of Paraná, Brazil.

Results

SSCP analysis. Sequencing of haplotypes with identical SSCP profiles (haplotypes "AL45"/"AL50", and "AL38"/"AL39" in Fig. 5.1) allowed to determine 100% sensitivity of the technique (i.e., identical SSCP profiles correspond to identical sequences) (Table 5.1). Haplotype screening revealed high levels of within and among population variation (Table 5.2), and sympatric forms with different diploid numbers (cytotypes) (Scavone et al., 1994; Dergam, unpubl.) revealed unique haplotypes for each cytotype, corroborating lack of gene flow between the cytotypes.

DNA sequencing. 1)16 S rRNA. A phylogenetic analysis of a 552 nucleotide fragment of the 16 S rRNA sequence for 18 specimens (Table 5.1), yielded 41 phylogenetically informative sites. Within the ingroup the ratio between transitions (substitutions between purines or between pyrimidines) (TS) and transversions (substitution of a purine for a pyrimidine or vice versa) (TV) was 6.0; within TS, 15 involved A-G and 15 involved C-T changes; monophyly of *H. malabaricus* was supported by six TS and two

TV.

A consensus tree of parsimony analysis yielded low bootstrap values overall, with only five lower nodes supported above the 70% level (Fig. 5.2). A NJ distance analysis showed a slight raise of bootstrap values, but the basic topology of the tree was maintained. Low resolution was interpreted as the result of paucity of phylogenetic information of the 16 S fragment at this level of evolutionary divergence.

2) Cyt b. Multiple alignment of a 383 nucleotide long fragment of cyt b for 22 haplotypes yielded 76 phylogenetically informative sites (Table 5.2). The most parsimonious tree is shown in Fig. 5.3. Statistical support for some branches was increased in relation to the tree based on 16 S rRNA sequences, and the tree topology was altered by placing three haplotypes from the Aguapey and Paraná rivers as the basal clade for the other haplotypes. Eight TS supported the monophyly of *H. malabaricus*, and within the species complex, 66 TS were observed (56 involving cytosine-thymine (C-T) and 10 adenine-guanine (A-G) changes), and three TV. Of the three codon positions, the first and the third positions were involved in 15% and 85% of the changes, respectively.

Uncorrected percent sequence divergence of haplotypes (Table 5.3) was high within the Rio Aguapey population, where one of the haplotypes ("AL53") showed a difference between 9.4 and 9.7 from three other haplotypes derived from the same panmictic population as revealed by

cytogenetic and genomic markers (unpubl.). This difference was even higher than the one observed between AL53 and the haplotype from a sympatric species (unpubl.) of the complex ("AL39") (3.9%). Among clades, percent sequence divergence was also high: it varied from 0 to 7 within the southern-southeastern clade, and from 0.8 to 5.5 within the less supported clade. Uncorrected percent sequence divergence was on average higher between the ingroup and *Erythrinus sp.* (AL74 haplotype) ($P > 0.01$) than between the ingroup and *H. cf. lacerdae* (haplotype MG01).

At the amino acid level, most substitutions were silent and restricted to the third position in codons. Non-silent substitutions occurred at positions 16, 43, and 118 and they involved the first position in the codon. The two outgroups differ at the position 16, while *H. cf. lacerdae* has an asparagine residue, *Erythrinus* has a histidine residue. All members of the southeastern, well-supported clade share the asparagine residue with *H. cf. lacerdae*, and the remaining haplotypes have an aspartic acid residue.

At the position 43, the outgroups have different residues, which are shared with subsets of the trahiras haplotypes: *H. cf. lacerdae* has a valine residue, that is shared with all of the haplotypes from $2n = 40$ populations (R66, AL26, AI21, SA29, TU1, 176, AL53, and AL50), some haplotypes from specimens with unknown diploid numbers (PT5, Y1, and K04), and haplotypes from the Rio Amazonas from $2n = 42$ specimens (JP10, and JP42). On the other hand, *Erythrinus sp.* has an isoleucine residue, which is

shared with all the southern-southeastern clade, plus haplotypes AL39 from the Rio Aguapey, and W1, a haplotype from the Rio Orinoco, with unknown diploid number.

Finally, at the position 118, both outgroups show a valine residue, which is shared with scattered some haplotypes of all clades: R66, AL26 and AL21, AL50, PF33, and K04.

A noteworthy finding was that the two haplotypes (JP10 and JP42) from Amazon specimens with $2n=42$ clustered together, although in a distant position relative to the other haplotype from the Rio Aguapey (AL39) from a specimen with $2n=42$, and from the southern-southeastern clade, composed by haplotypes from populations with $2n=42$ populations.

3) Sequence combination. The best supported tree was obtained by combining the sequences of haplotypes for which both sequences were available (Fig. 5.4). The cladogram conserved the same topology obtained with cyt b sequences alone.

Except for the cladogram obtained from 16 S sequences, the tree topology suggests the existence of three clades of haplotypes: the most basal one is derived from populations from the Paraná and Aguapey rivers. However, in the Rio Aguapey, these basal haplotypes are polymorphic with a derived haplotype (AL53) which is closely related to a haplotype from the São Francisco river (SA29). A second well supported clade is composed by haplotypes from populations distributed along the coastal region of

southeastern and southern Brazil and from some other rivers such as the Rio Doce, and tributaries of the Rio Paraná on the western slope of the Serra do Mar, such as the Rio Tibagi, Mogi-Guassu, and Rio Grande. In this clade, all the populations that have been studied cytogenetically have $2n = 42$.

The third clade, with low bootstrap support, gathers an array of haplotypes from many disjunct populations (Fig. 5.3). Here, the basal haplotype is represented by AL39, a haplotype from the Rio Aguapey from a population with $2n = 42$. The other moderately well supported branches ($\geq 70\%$) are two haplotypes from the Rio Tocantins basin (176 and TU1) from specimens with $2n = 40$, two haplotypes from the Rio São Francisco and from the Rio Aguapey (AL53 and SA29) from populations with $2n = 40$ specimens, and two haplotypes from the Rio Mindu from specimens with $2n = 42$.

Discussion

The observed patterns of variation of the two mtDNA genes were consistent with what is already known of the dynamics of base substitutions in the mtDNA of fishes (Meyer, 1993 a and b) and in other vertebrates (Brown and Simpson, 1982; Brown et al., 1982; Meyer, 1993c). As expected for closely related species, TS outnumbered TV. In cyt b sequences, third positions were more frequently involved in base substitutions, followed by first positions, while no substitutions were

observed at second positions, as reported for *Gasterosteus aculeatus* (Orti et al., 1994). However, and constrasting with *G. aculeatus*, pyrimidine transitions exceeded purine transitions, as reported for the blue marlin (Finnerty and Block, 1992). While in *G. aculeatus* (Orti et al., 1994) the most divergent haplotypes differed by 3.08%, *H. malabaricus* showed much higher levels of haplotype differentiation within and among populations, suggesting older evolutionary divergences than the ones observed in sticklebacks.

Sequence data and phylogeography. Although the most parsimonious tree derived from the 16 S fragment yielded low bootstrap values for higher nodes, well supported low nodes were also maintained with cyt b data.

The haplotypes of the second clade conform a well defined geographic unit that has been indicated as an endemic region for four species of characins of the genus *Oligosarcus* (Menezes, 1987). The coastal region has also been considered endemic for seven species of the genus *Mimagoniates* (Weitzman et al., 1987) . My sequence data not only corroborate the existence of this region, but the phylogenetic relationships of the haplotypes are also partially consistent with the phylogenetic relatedness of the species of *Mimagoniates* (Weitzman et al., 1987). The most basal haplotype of the southern-southeastern clade was collected from a population in the Rio Guaíba. This region is inhabited by *Mimagoniates inequalis*, one of the most basal members of the genus.

Though the coastal endemic region is nowadays represented by coastal rivers isolated by the sea, at least part of these rivers may have been continuous during the Wisconsin Glaciation (60,000-16,000 years B.P.), a time when the sea level was apparently 100 meters below the the present level (Schwarzbold and Schafer, 1984). Another less inclusive sister group is represented by a haplotype from the Rio Grande (VG32), a member of the Upper Paraná basin, where two other species of *Oligosarcus* occur. The haplotype from the Rio Doce (PF33) is the sister group of a strongly supported clade of derived haplotypes from the Rio Ribeira, Rio Perequê (Paranaguá), Rio Tibagi, and Rio Iguassu; this pattern of geographic distribution parallels the range of advanced species of *Mimagoniates*, such as *Mimagoniates lateralis*, *Mimagoniates* sp. B, and *Mimagoniates microlepis* (Weitzman et al., 1987). While the relatedness of the haplotypes from the Rio Ribeira and the Rio Perequê is consistent with the existence of a Central Coastal Region, their close relationships with haplotypes on the other slope of the Serra do Mar indicate that recent speciation scenarios may have not been restricted to the coastal slope of the Serra do Mar. In an attempt to explain the range of distribution of *Mimagoniates microlepis*, which has populations on the coastal rivers and in the headwaters of the Rio Iguassu, Weitzman et al. (1987) suggested alternative explanations: the population in the headwaters of the Rio Iguassu might have been established as a result of human activities, stream capture from coastal rivers, or it might have

reached the Rio Iguassu via the Rio Ribeira system (the same basin where haplotype IG1 was collected). Although the presence of *H. malabaricus* in the Rio Iguassu is recent (Sampaio, 1988), and it is thus uninformative in relation to the population of *M. microlepis* in this river, the occurrence of a haplotype closely related to the Rio Ribeira and Rio Perequê (a coastal stream) in a population of *H. malabaricus* in the headwaters of the Rio Tibagi is additional evidence for natural events of dispersion (e.g., stream capture).

Another result with phylogeographic implications is the coexistence in the Rio Aguapey (Argentina) (locality 11 of Fig. 1.2) of widely diverse haplotypes (AL50 in figs. 5.2-5.4, and AL 26, and AL 21 in 5.3) constituting the most basal clade within the species complex, with a derived haplotype (AL53 in Fig. 5.3) closely related to the haplotype sampled in the Rio São Francisco (locality SA in Fig. 5.4). Coexistence of widely different haplotypes within a population was explained in the case of Hawaiian *Drosophila* (De Salle et al., 1986) by migration, population subdivision, and/or survival of more than one ancestral lineage after a recent speciation event. In present times, the Rio São Francisco and the Middle Paraná are isolated from each other, but both of them have been connected in the past: the first connection happened in the early Tertiary when the Upper Paraná drained north into the Rio São Francisco and the Rio Tocantins, and a late connection when the Rio São Francisco drained to the Paranaíba until the Pleistocene/Pliocene (Beurlen, 1970). The Paranaíba is today a northern

member of the Upper Paraná basin. Given the fact that haplotype AL53 shares an advanced position in the cladogram with haplotype SA29 from the Rio São Francisco, I am prone to consider the presence of the former in the Middle Paraná as a result of migration from a population that underwent recent separation from the trahiras of the Rio São Francisco. Conceivably, haplotypes sharing a recent ancestor with the trahiras of the Rio São Francisco occur somewhere in the Upper Paraná, probably in the Rio Paranaíba. The relatedness between fish faunas of these rivers was also indicated by Kullander (1983), who reported a sister-group relationship for two species of cichlid fishes: *Cichlasoma sanctifranciscense* occurring in the Rio São Francisco, and *Cichlasoma paranaense* of the Upper Paraná.

Congruence among haploid and diploid data sets. As already mentioned, mtDNA is inherited maternally, and it does not recombine. Its pattern of transmission parallels that of female surnames (Avise, 1994). In contrast, the nuclear material is inherited by both parents and undergoes recombination. This independent pattern of inheritance allows for independent tests on the tempo and mode of evolution of nuclear markers, such as diploid numbers. The already well established fact that related species frequently differ in chromosome numbers or chromosome morphology (White, 1973, 1978) has prompted a large amount of papers (reviewed in Sites and Moritz, 1987, and King, 1993) on the dynamics of the establishment of the chromosomal novelties. The central issue of

whether these chromosomal differences are the cause or the effect of the speciation process remains uncertain, particularly because, as indicated by many authors (e.g., White, 1978; Templeton, 1981; Sites and Moritz, 1987), theoretical models lack testability. The only one that involves precise conditions for the fixation of chromosomal rearrangements is the one proposing low heterozygosity in the ancestral population (e.g., Lande, 1979). To complicate the situation further, G-banding techniques, which allow to identify chromosome arm homologies across species by means of transversal banding of the chromosomes arms, have been historically unsuccessful on fish. Consequently, researchers have been unable to conduct refined analyses of events involved in karyotype changes in fish, as it has been possible in other groups, particularly the mammals (e.g., Borowik, 1995; Baker et al., 1987). Only relatively recently (Gold and Li, 1991) trypsin G-banding has been successfully obtained in fish, and this also holds for *H. malabaricus* (Bertollo, unpubl.).

A multidisciplinary approach involving chromosome differences and mtDNA data (e.g., Arévalo et al., 1994; Taberlet et al., 1994) is a direct test for this model: owing to its maternal pattern of inheritance and extensive homoplasmy (only one copy per genome), haplotypes are ideal markers of bottlenecks or founding events in the past (Avise et al., 1987). In *H. malabaricus*, lack of phylogenetic relatedness among haplotypes with the same diploid number is evident in all cladograms: despite the paucity of

informative sites in the 16 S sequence, two haplotypes from $2n=42$ specimens (AL39 and AL38) appeared as more related to haplotypes derived from populations with $2n=40$ specimens, and this pattern was supported by cyt b data and by the addition of two additional unrelated haplotypes from $2n=42$ populations from Rio Mindu. The same scattered pattern is evident for $2n=40$ populations, whose haplotypes are variably related to $2n=42$ populations, and display a range from the basal position to the advanced position of haplotypes from the Amazon Basin.

Lack of concordance between mtDNA and diploid number data sets determines that grouping populations within the species complex by either of the data sets would determine paraphyletic groups (groups that do not include all the taxa derived from an ancestor). The inconsistency may be the result of many factors such as the transference of haplotypes between closely related species (e.g., Ferris et al., 1983) and/or the fixation of ancestral polymorphisms (for a review, see Avise, 1994; Avise and Ball, 1990). In relation to the first factor, data from sympatric populations with different diploid numbers (unpubl.) indicate that those populations behave as good biological species (there is no gene flow among them). In relation to the second factor, the fixation of random polymorphisms is compatible with independent origins of diploid numbers, or with single origin of diploid numbers in conditions not involving small demes or population bottlenecks (Chesser and Baker, 1986; for reviews, see Sites and Moritz, 1987, and

King, 1993). Poor fit of mtDNA data with the pattern of cytogenetic variation in chromosomally polytypic species complexes has been reported by Arévalo et al. (1994) for *Sceloporus grammicus*, and by Taberlet et al. (1994) for *Sorex araneus*.

Unfortunately, data are unavailable for time estimates of the molecular divergence within this species complex, but percent sequence divergence may aid in estimating the relative timing of lineage-splitting of 40's and 42's along the evolutionary history of the species complex. Within the southern-southeastern clade, the closest related haplotypes ("OH3" from Rio Perequê and "IG1" from Rio Ribeira), have a 0.3% sequence divergence, as a result of probable isolation during the Quaternary. On the other hand, maximum percent sequence divergence within this clade is 7.0 between "5753" and "OH3" haplotypes (Table 5.2). This value, however, is close to average of the percent sequence divergence between this clade and the other "2n = 42" haplotypes ("AL39", "JP10", and "JP42"), with values ranging from 6.3 - 6.5 (for "5753" the most basal haplotype in the clade), to 7.8-8.1% (for "TB1", "OH3", and "PF33" haplotypes). Assuming one cladogenetic event as the origin of the $2n = 42$ and the $2n = 40$, the southern-southeastern clade may represent merely one more of the $2n = 42$ - gene lineages. This clade seems to have differentiated from a relatively homogeneous population, and it may be relatively oversampled in relation to other neotropical "$2n = 42$" haplotypes.

As a conclusion, mtDNA reveals high levels of clonal divergence within and among populations. The most extensively sampled river (Rio Paraná) is highly heterogeneous, probably as a result of its complex geological history (Beurlen, 1970). This contrast with the relative homogeneity of the coastal region of southeastern and southern Brazil, subjected to more recent geological events. An overlap of these two regions is represented by the sympatry of unrelated haplotypes in some of the tributaries of the Upper Paraná (e.g., Rio Grande). Besides the well supported clade of southern and southeastern Brazil, there is a poor fit between mtDNA data and diploid number, which suggests multiple origins of diploid numbers or a single origin involving dynamics of fixation chromosomal rearrangements not requiring extreme reductions of the effective number of females in the ancestral population (e.g., models 6 and 7 in Sites and Moritz, 1987).

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Figure 5.1. Partial view of a gel with haplotypes selected for sequencing:
a) From Rio Aguapey, haplotypes from specimens 45 and 50, with 2n = 40.
b) From Rio Aguapey, haplotypes from specimens 38 and 39, with 2n = 42.
c) MO From Rio Mogi-Guassu, haplotype from specimen 01, 2n = 40.
d) MG From the Rio Mogi-Guassu, haplotype from specimen 02, 2n = 42.
e) PF From the Rio Doce, haplotype from specimen number 33, 2n = 42.
f) 17 From the Rio Araguaia, haplotype from specimen number 176, 2n = 40.
g) TU From the Rio Tocantins, haplotype from specimen 01, 2n = 42.
h) R6 From Rio Madeira, haplotype from specimen number 4966, 2n = 40.
i) IG From Rio Ribeira, haplotype from specimen number 01, 2n = 42.
j)TB From Rio Tibagi/Ponta Grossa, haplotype from specimen 01, 2n unknown.
k) OH From Rio Perequê, haplotype from specimen number 03, 2n unknown.

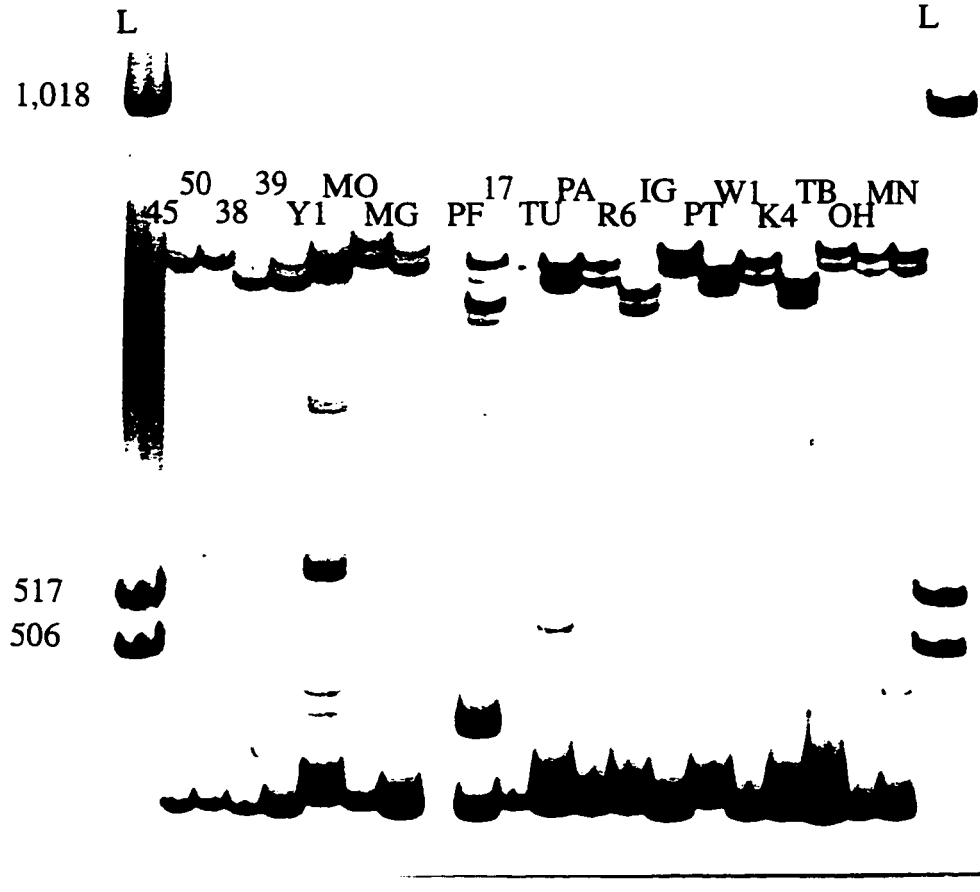
L
L
1,018
50
39
MO
17
PA
IG
W1
TB
MN
45
38
Y1
MG
PF
TU
R6
PT
K4
OH
517
506

Figure 5.2. Maximum likelihood tree with of a 553 nucleotide long fragment of the 16 S ribosomal mitochondrial DNA. Each terminal node represent an individual haplotype; the specimen diploid number is also indicated. Terminology follows Fig. 5.1, with the addition of:
a) SA29, haplotype from the Rio São Francisco, from specimen number 29.
b) AL53, haplotype from the Rio Aguapey, from specimen number 53.
c) VG32, haplotype from the Rio Grande, from specimen number 32.
d)5753, haplotype from the Rio Guaíba, from specimen with same number.
e)AL74, haplotype from Erythrinus sp., from the Rio Aguapey.
f) MG01, haplotype from Hoplias lacerdae, from the Rio Grande.

Statistical support (bootstrap values) above 50% are indicated for each branch: parsimony analysis above the branch, and below for distance analysis.

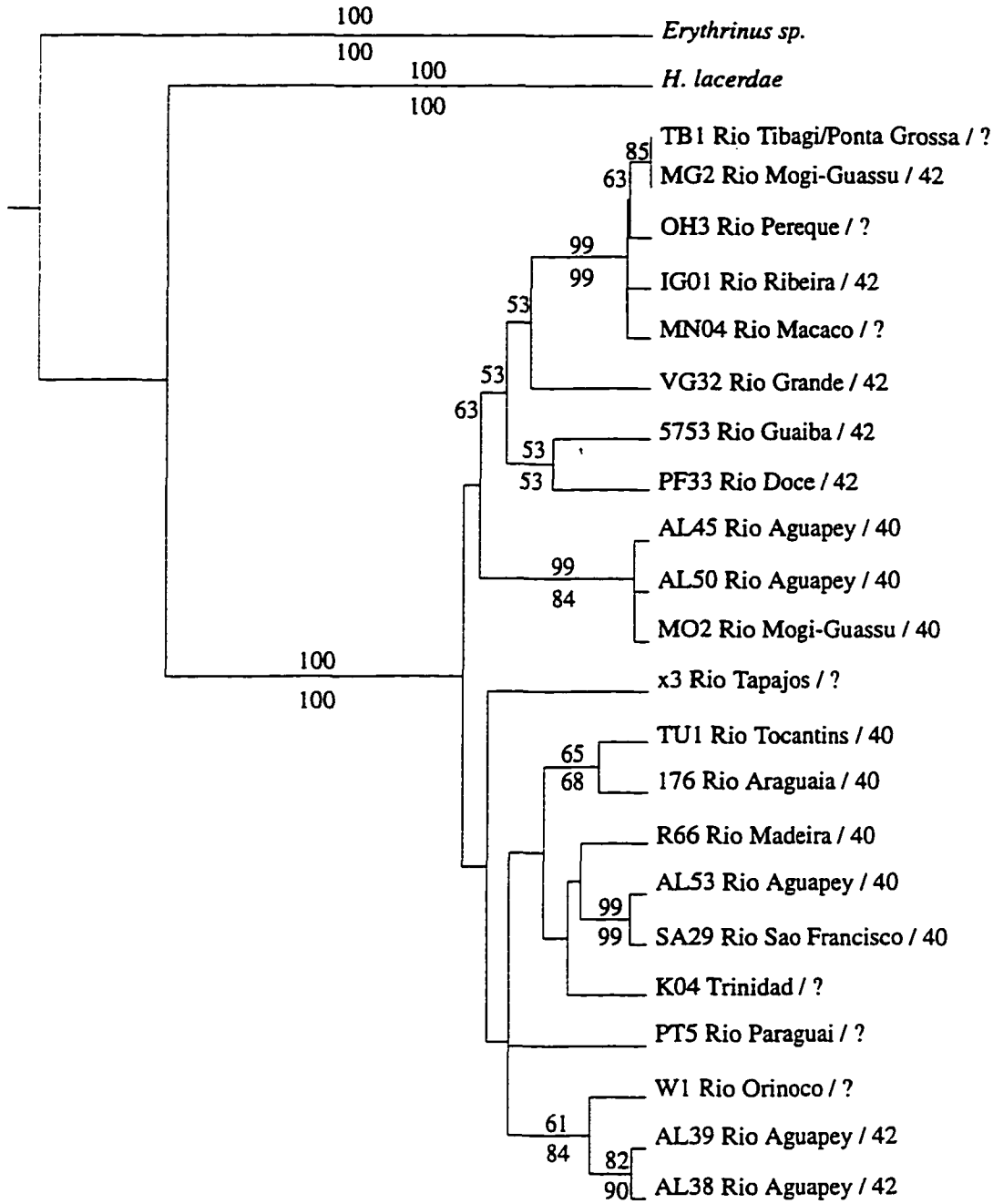
100
100
Erythrinus sp.
100
100
H. lacerdae
85
63
TB1 Rio Tibagi/Ponta Grossa / ?
MG2 Rio Mogi-Guassu / 42
99
99
OH3 Rio Pereque / ?
IG01 Rio Ribeira / 42
53
MN04 Rio Macaco / ?
53
63
VG32 Rio Grande / 42
53
53
5753 Rio Guaiba / 42
PF33 Rio Doce / 42
AL45 Rio Aguapey / 40
99
84
AL50 Rio Aguapey / 40
MO2 Rio Mogi-Guassu / 40
100
100
x3 Rio Tapajos / ?
65
68
TU1 Rio Tocantins / 40
176 Rio Araguaia / 40
R66 Rio Madeira / 40
AL53 Rio Aguapey / 40
99
99
SA29 Rio Sao Francisco / 40
K04 Trinidad / ?
PT5 Rio Paraguai / ?
W1 Rio Orinoco / ?
61
84
AL39 Rio Aguapey / 42
82
90
AL38 Rio Aguapey / 42

Figure 5.3. Maximum likelihood tree of a 383 nucleotide long fragment of the mitochondrial cytochrome b for 18 haplotypes from Figure 6.4, with the addition of the following six haplotypes:
a) Haplotype SE01 from the Rio Iguassu/Segredo Reservoir, 2n unknown.
b) Haplotype JP42 from the Rio Amazonas at Manaus, 2n = 42.
c) Haplotype JP10 from the Rio Amazonas at Manaus, 2n = 40.
d) Haplotype Y01 from the Rio Paraná at Porto Rico, 2n unknown.
e) Haplotype AL26 from the Rio Paraná at Bella Vista, 2n = 40.
f) Haplotype AL21 from the Rio Aguapey at Posadas, 2n probably 40.

Boostrap values higher than 50% are indicated: above the branches from majority consensus tree of parsimony analysis, and below the branches from the consensus tree of distance analysis.

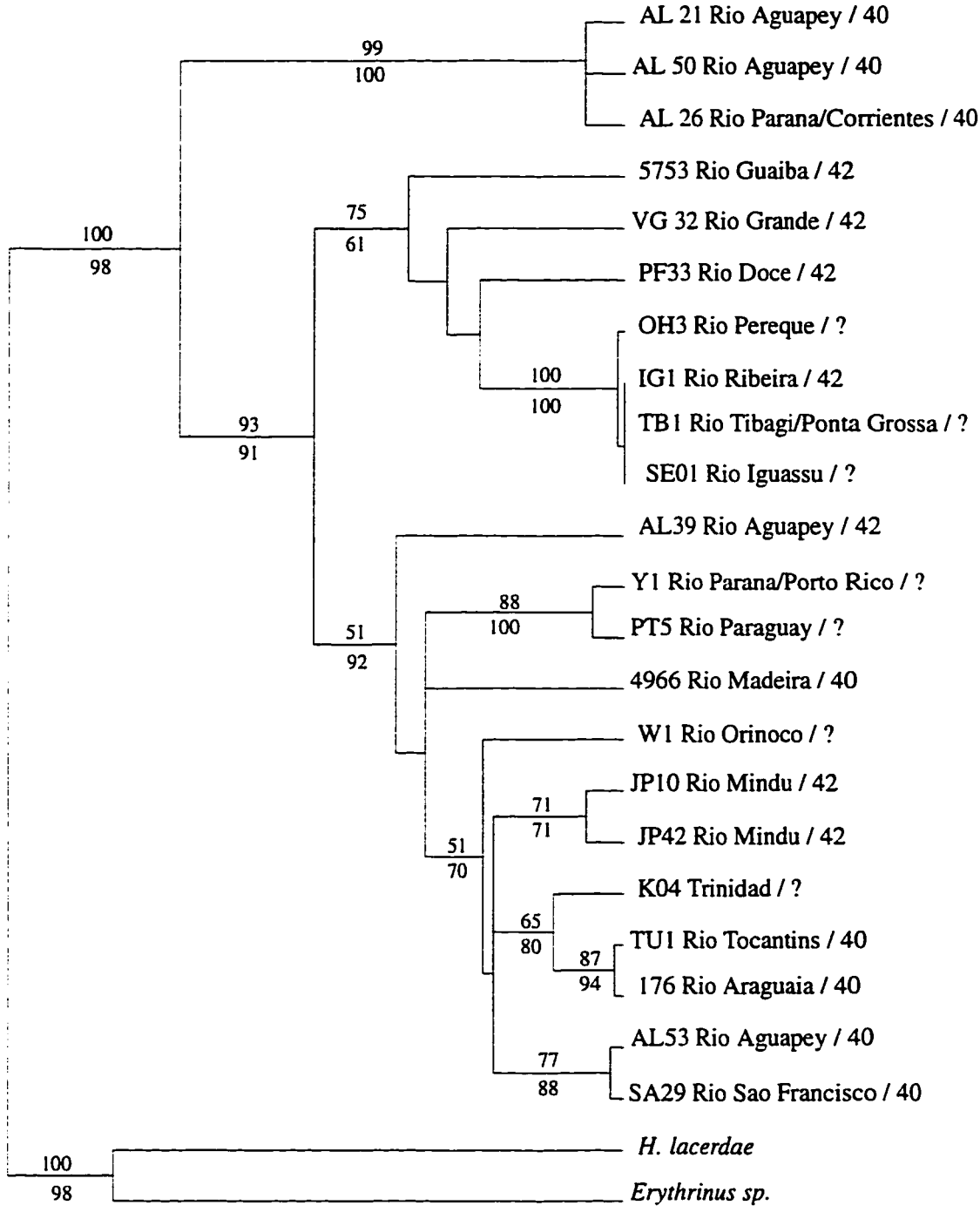
AL 21 Rio Aguapey / 40
AL 50 Rio Aguapey / 40
AL 26 Rio Parana/Corrientes / 40
5753 Rio Guaiba / 42
VG 32 Rio Grande / 42
PF33 Rio Doce / 42
OH3 Rio Pereque / ?
IG1 Rio Ribeira / 42
TB1 Rio Tibagi/Ponta Grossa / ?
SE01 Rio Iguassu / ?
AL39 Rio Aguapey / 42
Y1 Rio Parana/Porto Rico / ?
PT5 Rio Paraguay / ?
4966 Rio Madeira / 40
W1 Rio Orinoco / ?
JP10 Rio Mindu / 42
JP42 Rio Mindu / 42
K04 Trinidad / ?
TU1 Rio Tocantins / 40
176 Rio Araguaia / 40
AL53 Rio Aguapey / 40
SA29 Rio Sao Francisco / 40
H. lacerdae
Erythrinus sp.
99
100
100
98
75
61
100
100
93
91
88
100
51
92
71
71
51
70
65
80
87
94
77
88
100
98

Figure 5.4. Maximum likelihood tree of a 936 bp combined fragment of 16s and cyt b mitochondrial DNA. Haplotypes are indicated by population, specimen number, river, and diploid number. Bootstrap values are indicated as in Fig. 5.3.


TU
176
SA
PT
AL
5753

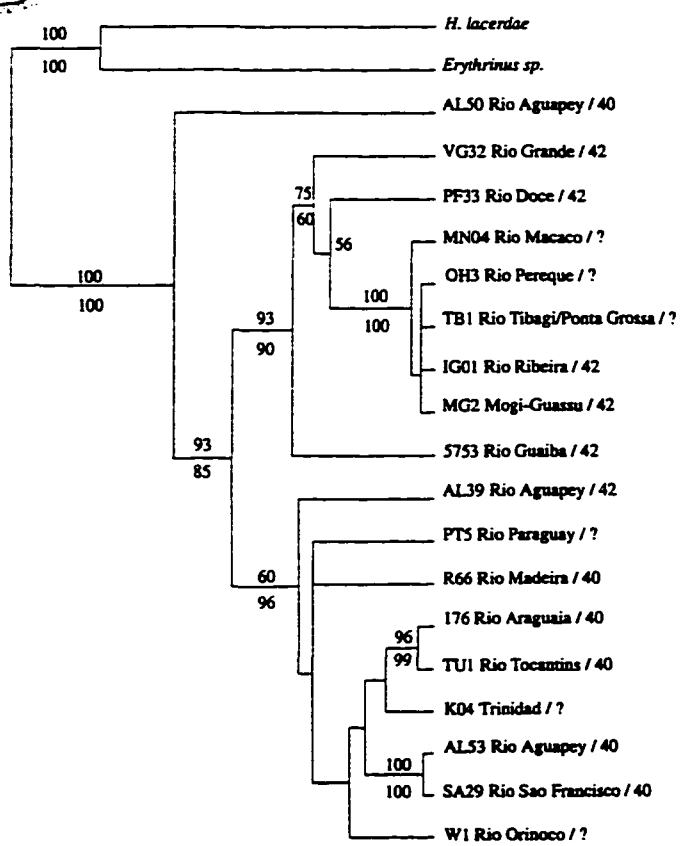
H. lacerdae
Erythrinus sp.
AL50 Rio Aguapey / 40
VG32 Rio Grande / 42
PF33 Rio Doce / 42
MN04 Rio Macaco / ?
OH3 Rio Pereque / ?
TB1 Rio Tibagi/Ponta Grossa / ?
IG01 Rio Ribeira / 42
MG2 Mogi-Guassu / 42
5753 Rio Guaiba / 42
AL39 Rio Aguapey / 42
PT5 Rio Paraguay / ?
R66 Rio Madeira / 40
176 Rio Araguaia / 40
TU1 Rio Tocantins / 40
K04 Trinidad / ?
AL53 Rio Aguapey / 40
SA29 Rio Sao Francisco / 40
W1 Rio Orinoco / ?
100
100
75
60
56
100
100
100
100
93
90
93
85
60
96
96
99
100
100

Table 5.1. The mtDNA sequences for all taxa examined in this study for 16 S ribosomal RNA.

```
                          11111112 2222222222 2222223333 333333
                         1933334771 2223444455 5556771344 444555
                         1045783298 1489035801 3453360501 258249
AL45 AGUAPEY/40          GATCCAAATT TTTTCAGAGG CTAAAACTTA CCTAAT
AL50 AGUAPEY/40          .......... .......... .......... ......
AL50 AGUAPEY/40          .......GC. .C..T.A..A T.GG..T... ....G.
AL38 AGUAPEY/42          ........C. ......A... T.G..T.... ..C.G.
al39 AGUAPEY/42          ........C. ......A... T.G..T.... ..C.G.
MO2  MOGI-GUASSU/40      .......... .......... .......... ......
VG32 RIO GRANDE/42       .G..TGG.C. CC....A... .C..G..... ..C.G.
MG2  MOGI-GUASSU/42      AG...G..C. ...C.TATA. .C........ ....G.
TB1 TIBAGI/?             AG...G..C. ...C.TATA. .C........ ....G.
5753 GUAIBA/42           ........CC ......A... .C.....C.. ..C.G.
SA29 SAO FRANCISCO/40.....GC. .C..T.A..A T.GG..T... ....G.
OH3 PEREQUE/?            AG...G..C. ...C.TATA. .C........ ....G.
MN04 MACACO/?            AG...G..C. ...C.TATA. .C........ ....G.
PF33 DOCE/42             ....T.G.C. ....T.A... .C........ ..C.G.
PT5 PARAGUAY/?           A......... ...C..A... T.GG...... ....G.
IG1 RIBEIRA/42           AG...G..C. ...C.TATA. .C........ ....G.
X3 TAPAJOS/?             ........C. C.....A... TC........ ....G.
176 ARAGUAIA/40          .G........ ....T.A..A T.G....... ....G.
TU1 TOCANTINS/40         .G........ ....T.A... T.G....... ....G.
W1 ORINOCO/?             ........C. ......A... T.G....... ..C.G.
R66 MADEIRA/40           .......GC. .C..T.A... T.G....C.. ....G.
K04 TRINIDAD/?           .......GC. ....T.A... T.G.G..... ....G.
Erythrinus sp./50        ..CTT....A .ACCT.T..A A..T.C..CT AAAGGC
H. lacerdae/50           ..CTT..GAA .ACCT.T... TC......CT AAAGGC
```

Table 5.1 (cont.)

	3333 444445
	6799 000055
	2467 123933
AL45 AGUAPEY/40	CACA CAGAAT
AL50 AGUAPEY/40	....
AL50 AGUAPEY/40	TC.T .G....
AL38 AGUAPEY/42	TC.T C
al39 AGUAPEY/42	TC.T ......
MO2 MOGI-GUASSU/40	....G..
VG32 RIO GRANDE/42	TC.. A.A...
MG2 MOGI-GUASSU/42	TC.. A.....
TB1 TIBAGI/?	TC.. A.....
5753 GUAIBA/42	TC.. AG....
SA29 SAO FRANCISCO/40	TC.T .G....
OH3 PEREQUE/?	TC.. A.....
MN04 MACACO/?	TC.. A....C
PF33 DOCE/42	TC.. AG....
PT5 PARAGUAY/?	TC.T ......
IG1 RIBEIRA/42	TC.. A.A...
X3 TAPAJOS/?	TC.T ......
176 ARAGUAIA/40	TC.T ......
TU1 TOCANTINS/40	.C.T ......
W1 ORINOCO/?	TCTT ..A...
R66 MADEIRA/40	TC.T ....G.
K04 TRINIDAD/?	TC.T ......
Erythrinus sp./50	TC.. ..ACG.
H. lacerdae/50	TCT. T.TC.C

Table 5.2. The mtDNA sequences for all taxa examined in this study for cyt b.

				1	111	111	111	111	111	111
	334	455	666	670	011	122	223	445	555	566
	192	647	036	982	824	701	676	450	136	925
AL50 AGUAPEY/40	TCC	GCT	CTC	TTC	TCG	CCT	TGT	CCT	TAC	GTT
AL21 AGUAPEY/40	...	...	...	...	...	...	...	...	...	...
AL26 CORRIENTES/40	...	...	...	...	...	...	...	...	...	...
AL39 AGUAPEY/42	CT.	...	.C.	C..	...	...	.A.	..C	CGT	A..
AL53 AGUAPEY/40	.T.	...	TC.	CC.	C.A	.T.	...	T.C	CGT	A..
SA29 SAO FRANCISCO/40	.T.	...	TC.	CC.	..A	.T.	...	T.C	CGT	A..
SE01 IGUASSU/?	.T.	A.C	...	C.T	.TA	..C	.AC	..C	CGT	A..
W1 ORINOCO/?	CT.	...	.C.	CC.	C.A	...	.A.	TTC	CGT	A..
K04 TRINIDAD/?	.T.	...	TC.	CC.	C.A	...	...	T.C	CG.	A..
Y1 PARANA/P. RICO/?	...	...	.C.	CC.	...	...	...	T.C	CGT	A..
PT5 PARAGUAY/?	...	...	.C.	CC.	...	...	...	T.C	CGT	A..
5753 GUAIBA/42	.T.	A..	.C.	CCT	.TA	...	.AC	..C	C.T	AC.
JP10 MINDU/42	.T.	...	TC.	CC.	C.A	T..	...	T.C	CGT	A..
176 ARAGUAIA/40	.T.	...	TC.	CC.	C.A	...	...	..C	CGT	A..
JP42 MINDU/42	.T.	...	.C.	CC.	C.A	T..	...	..C	CGT	A..
VG32 RIO GRANDE/42	.T.	A..	.C.	C.T	..A	...	CAC	..C	C.T	A..
TU1 TOCANTINS/40	.T.	...	TC.	CC.	C.A	...	...	..C	CGT	A..
PF33 DOCE/42	.T.	A.C	.C.	C..	CTA	...	AAC	..C	CGT	A..
TB1 TIBAGI/?	.T.	A.C	...	C.T	.TA	..C	.AC	..C	CGT	A..
IG1 RIBEIRA/42	.T.	A.C	...	C.T	.TA	..C	.AC	..C	CGT	A..
OH3 PEREQUE/?	.T.	A.C	...	C.T	.TA	..C	.AC	..C	CGT	A..
R66 MADEIRA/40	.T.	..C	.C.	CC.	..A	...	...	T.C	CGT	A..
Erythrinus sp./50	CTT	CT.	.CT	C..	C.A	..C	C.C	...	...	.CC
H. lacerdae/50	CTT	AT.	.CT	CC.	...	..C	.A.	.T.	...	.CC

Table 5.2 (cont.)

	111	111	122	222	222	222	222	222	222	233
	677	889	900	111	122	444	555	677	888	900
	847	692	817	036	928	369	258	103	258	446
AL50 AGUAPEY/40	TCC	CCC	GCT	CTC	ACG	CCT	CCT	TCT	TTT	TCA
AL21 AGUAPEY/40	...	...	...	...	..A	...	...	...	...	...
AL26 CORRIENTES/40	...	...	...	...	..A	...	...	...	...	...
AL39 AGUAPEY/42	..T	...	A.C	T..	...	T..	..C	C.C	.C.	CT.
AL53 AGUAPEY/40	..T	T..	ATC	T..	...	...	..C	C.C	...	CT.
SA29 SAO FRANCISCO/40	..T	T..	ATC	T..	...	...	..C	C.C	...	CT.
SE01 IGUASSU/?	CTT	T..	ATC	...	...	.T.	..C	..C	...	CTG
W1 ORINOCO/?	..T	T..	A.C	T..	...	...	..C	C.C	C..	C..
K04 TRINIDAD/?	..T	T..	A.C	T..	...	.T.	T.C	C.C	...	CT.
Y1 PARANA/P. RICO/?	..T	...	A.C	.C.	..A	...	..C	C..	...	CT.
PT5 PARAGUAY/?	..T	.T.	..C	.C.	..A	...	..C	C..	...	CT.
5753 GUAIBA/42	...	T..	A.C	...	..A	...	..C	CTC	.C.	C..
JP10 MINDU/42	..T	T..	A.C	T..	G..	...	..C	C.C	...	CT.
176 ARAGUAIA/40	..T	T..	A.C	T..	..A	.T.	T.C	C.C	.C.	CT.
JP42 MINDU/42	..T	T..	A.C	T..	G..	...	..C	C.C	...	CT.
VG32 RIO GRANDE/42	C..	T..	..C	...	..A	...	..C	C..	.CC	CT.
TU1 TOCANTINS/40	..T	T..	A.C	T..	..A	.T.	T.C	C.C	.C.	CT.
PF33 DOCE/42	CT.	T.T	A.C	.C.	..A	...	..C	C.C	.C.	CT.
TB1 TIBAGI/?	CTT	T..	ATC	...	...	.T.	..C	..C	...	CTG
IG1 RIBEIRA/42	CTT	T..	ATC	...	...	.T.	..C	..C	...	CTG
OH3 PEREQUE/?	CTT	T..	ATC	...	...	.T.	..C	..C	...	CTG
R66 MADEIRA/40	C.T	...	A.C	T..	..A	...	.TC	C.C	..C	CT.
Erythrinus sp./50	...	.TT	A..	..T	.TA	TTC	.TG	.TC	CCC	...
H. lacerdae/50	CT.	T..	T.C	..T	TTC	.TC	..C	...	..C	...

Table 5.2 (cont.)

	333	333	333	333	333
	011	234	445	556	666
	928	462	581	240	349
AL50 AGUAPEY/40	TTC	CCC	CAC	GCC	TTT
AL21 AGUAPEY/40	...	...	...	...	...
AL26 CORRIENTES/40	...	...	...	...	...
AL39 AGUAPEY/42	.C.	TTT	T.A	ATT	..C
AL53 AGUAPEY/40	...	TTT	T.A	ATT	.CC
SA29 SAO FRANCISCO/40	...	TTT	T.A	ATT	.CC
SE01 IGUASSU/?	..T	...	.T.	A.T	C.C
W1 ORINOCO/?	...	TTT	T.T	ATT	.CC
K04 TRINIDAD/?	...	.TT	..A	.TT	.CC
Y1 PARANA/P. RICO/?	...	TTT	T.A	ATT	...
PT5 PARAGUAY/?	...	TTT	T.A	ATT	...
5753 GUAIBA/42	C..	T.T	T..	ATT	C..
JP10 MINDU/42	...	TTT	T.A	ATT	.CC
176 ARAGUAIA/40	...	.TT	T.A	ATT	.CC
JP42 MINDU/42	...	TTT	T.A	ATT	.CC
VG32 RIO GRANDE/42	..T	T..	TT.	ATT	C.C
TU1 TOCANTINS/40	...	..T	T.A	ATT	.CC
PF33 DOCE/42	C.T	...	TT.	.TT	..C
TB1 TIBAGI/?	..T	...	.T.	A.T	C.C
IG1 RIBEIRA/42	..T	...	.T.	A.T	C.C
OH3 PEREQUE/?	..T	...	.T.	A.T	C.C
R66 MADEIRA/40	...	.TT	T.A	.T.	..C
Erythrinus sp./50	CCT	..T	T.A	.AA	C.A
H. lacerdae/50	.C.	..T	T.A	..T	.CA

Table 5.3. Locales, sample sizes, and diploid number for populations studied. Histological sex identification is indicated whenever it is available for specimens of each sample.

Locales	Sample size	Diploid number
Upper Rio Paraná		
Rio Tietê, Monjolinho Dam, State of São Paulo, Brazil	12 (4♀ / 2♂)	M 39 / F 40
Rio Mogi-Guassu, Jataí Natural Reserve, State of São Paulo, Brazil	3 (2♀)	M 39 / F 40
Rio Grande, Volta Grande, State of Minas Gerais, Brazil	4(2♀ /1♂)	M 39 / F 40
Rio Grande, Volta Grande, State of Minas Gerais, Brazil	12 (3♀ /9♂)	42
Rio Tibagi, Ponta Grossa, State of Paraná, Brazil	12	?
Middle Rio Paraná		
Rio Iguassu Segredo, State of Paraná, Brazil	7	?
Rio Paraná, Reconquista, Province of Santa Fe, Argentina	6	40
Rio Paraná, Bella Vista Province of Corrientes, Argentina	3	40
Rio Uruguay		
Rio Aguapey, Province of Corrientes, Argentina	8 (2♀ /3♂)	40
Rio Aguapey, Province of Corrientes, Argentina	27(12♀ /10♂)	42

Table 5.3 (cont.)

Locales	Sample size	Diploid number
Coastal Basins		
Rio Ribeira do Iguape, Registro, State of São Paulo, Brazil	3(1♀ /2♂)	42
Rio Perequê, Paranaguá, State of Paraná, Brazil	3	?
Rio Guaíba, Porto Alegre State of Rio Grande do Sul, Brazil	4	42
Rio São Francisco Rio das Velhas, Curvelo, State of Minas Gerais, Brazil	56(8♀ /5♂)	40
Rio Doce Rio Doce, Oriente, State of Minas Gerais, Brazil	4	42
Rio Turvo, Viçosa, State of Minas Gerais, Brazil	9(6♀)	42
Lagoa Timboré, Dionísio, State of Minas Gerais, Brazil	3	42
Lagoa Ferrugem, Dionísio, State of Minas Gerais, Brazil	3	42
Lagoa Dom Helvécio, Dionísio, State of Minas Gerais, Brazil	33	42
Lagoa Carioca, Dionísio, State of Minas Gerais, Brazil	9	42
Rio Amazonas		
Rio Tocantins, Marabá, Tucurui Dam, State of Pará, Brazil	4	40

Table 5.3 (cont.)

Locales	Sample size	Diploid number
Rio Araguaia (tributary of the Rio Tocantins), Barra do Garças, State of Mato Grosso, Brazil	8	40
Rio Madeira, Porto Velho, State of Rondônia, Brazil	4	40

Table 5.4. Percent sequence divergence and number of transitions versus transversions among haplotypes of the *H. malabaricus* species complex. In the first section, the three values are indicated in the same cell. In the second and third sections, sequence divergence is indicated below the diagonal, and transitions versus transversions are indicated above the diagonal. Section 1.

	176	5753	AL 21	AL 26	AL 39	AL 50	AL 53	AL 74	IG 01	JP 10	JP 42	K 04
MG 01	11.8 40 / 5	11.8 39 / 6	10.2 33 / 6	10.4 34 / 6	11.8 40 / 5	10.4 34 / 6	12.5 43 / 5	13.0 37 /13	12.3 40 / 7	12.3 42 / 5	10.4 36 / 4	8.1 29 / 2
OH 3	7.8 29 / 1	7.0 26 / 1	7.0 35 / 1	9.7 36 / 1	8.1 29 / 2	9.4 35 / 1	7.8 28 / 2	17.5 56 /11	0.3 1 / 0	8.3 30 / 2	8.1 28 / 3	8.1 29 / 2
PF 33	7.0 24 / 3	6.3 26 / 1	10.2 37 / 2	10.4 38 / 2	7.8 28 / 2	10.7 39 / 2	8.1 28 / 3	15.7 48 /12	5.7 21 / 1	8.1 28 / 3	7.8 26 / 4	7.8 28 / 2
PT 05	4.7 18 / 0	6.3 24 / 1	6.8 25 / 1	7.0 26 / 1	4.4 17 / 0	7.3 27 / 1	4.2 16 / 0	17.0 55 / 10	9.1 33 / 2	4.2 16 / 0	4.4 16 / 1	5.5 21 / 0
R 66	3.6 12 / 4	7.0 25 / 4	7.3 28 / 2	8.1 29 / 2	4.4 16 / 1	8.3 30 / 2	3.6 14 / 0	15.7 50 /10	7.8 27 / 3	3.6 13 / 1	3.9 13 / 2	3.9 14 / 1
SA 29	2.3 9 / 0	6.3 23 / 1	9.1 34 / 1	9.4 35 / 1	3.6 14 / 0	9.1 34 / 1	0.3 1 / 0	17.7 60 / 8	7.3 26 / 2	1.3 5 / 0	2.1 7 / 1	2.6 10 / 0
SE 01	7.6 27 / 2	6.8 25 / 1	9.1 34 / 1	9.4 35 / 1	7.8 28 / 2	9.1 34 / 1	7.6 27 / 2	17.2 55 / 11	0	7.8 28 / 2	7.8 27 / 3	7.8 28 / 2
TB 01	7.6 27 / 2	6.8 25 / 1	9.1 34 / 1	9.4 35 / 1	7.8 28 / 2	9.1 34 / 1	7.6 27 / 2	17.2 55 /11	0	7.8 28 / 2	7.8 27 / 3	7.8 28 / 2
TU 01	0.3 1 / 0	5.7 21 / 1	8.6 32 / 1	8.9 33 / 1	4.2 16 / 0	9.1 34 / 1	2.3 9 / 0	15.7 51 / 9	7.3 26 / 2	2.3 9 / 0	2.6 9 / 1	2.1 8 / 0
VG 32	7.6 28 / 1	5.2 19 / 1	9.1 34 / 1	9.4 35 / 1	7.0 25 / 2	9.7 36 / 1	8.1 29 / 2	16.4 52 /11	6.3 24 / 0	8.1 30 / 1	7.8 26 / 4	9.4 34 / 2
W 01	3.6 13 / 1	6.3 24 / 0	9.7 37 / 0	9.9 38 / 0	3.9 14 / 1	9.7 37 / 0	2.6 9 / 1	17.0 55 /10	8.3 31 / 1	2.6 9 / 1	3.6 13 / 1	4.0 14 / 1
Y 01	4.0 15 / 0	5.7 21 / 1	6.5 24 / 1	6.8 25 / 1	3.6 14 / 0	7.0 26 / 1	3.4 13 / 0	17.0 53 /12	8.3 30 / 2	3.4 13 / 0	3.6 13 / 1	4.7 18 / 0

Table 5.4. Section 2.

	MG1	OH3	PF33	PT05	R66	SA 29	SE01	TB1	TU 1	VG 32	W01	Y01
MG 01	-	41 / 6	45 / 7	42 / 5	39 / 6	43 / 4	40 / 6	40 / 6	39 / 5	43 / 7	36 / 8	40 / 5
OH 3	12.3	-	23 / 0	34 / 2	27 / 4	27 / 2	1 / 0	1 / 0	27 / 2	25 / 0	32 / 1	31 / 2
PF 33	13.6	6.0	-	30 / 2	24 / 4	29 / 3	20 / 2	20 / 2	23 / 3	21 / 2	30 / 2	27 / 3
PT 05	12.3	9.4	8.3	-	15 / 1	15 / 0	33 / 2	33 / 2	19 / 0	27 / 2	19 / 1	3 / 0
R 66	11.7	8.1	7.3	4.2	-	12 / 1	28 / 3	28 / 3	14 / 1	26 / 3	16 / 2	12 / 1
SA 29	12.3	7.6	8.3	3.9	3.4	-	26 / 2	26 / 2	10 / 0	29 / 1	10 / 1	12 / 0
SE 01	12.0	0.3	5.7	9.1	8.1	7.3	-	0 / 0	26 / 2	24 / 0	30 / 1	30 / 2
TB 01	12.0	0.3	5.7	9.1	8.1	7.3	0	-	26 / 2	24 / 0	30 / 1	30 / 2
TU 01	11.5	7.6	6.8	5.0	4.0	2.6	7.3	7.3	-	26 / 2	14 / 1	16 / 0
VG 32	13.0	6.5	6.0	7.6	7.6	7.8	6.3	6.3	7.3	-	31 / 1	26 / 2
W 01	11.5	8.6	8.3	5.2	4.7	2..9	8.1	8.1	4.0	8.3	-	16 / 1
Y 01	11.7	8.6	7.8	0.8	3.4	3.1	8.3	8.3	0.3	7.3	4.4	-

Table 5.4. Section 3.

	176	5753	AL 21	AL 26	AL 39	AL 50	AL 53	AL 74	IG01	JP10	JP 42	K04
176	-	22 / 1	33 / 1	34 / 1	15 / 0	35 / 1	8 / 0	53 / 7	27 / 2	8 / 0	8 / 1	7 / 0
575 3	6.0	-	33 / 0	34 / 0	23 / 1	35 / 0	24 / 1	48 / 8	25 / 1	24 / 1	8 / 1	7 / 0
AL 21	8.9	8.6	-	1 / 0	32 / 1	2 / 0	35 / 1	46 / 9	32 / 3	35 / 1	22 / 2	29 / 1
AL 26	9.1	8.9	0.3	-	33 / 1	3 / 0	36 / 1	46 / 10	35 / 1	36 / 1	33 / 2	32 / 1
AL 39	4.0	6.3	8.6	8.9	-	32 / 1	15 / 0	51 / 9	28 / 2	15 / 0	13 / 1	20 / 0
AL 50	9.4	9.1	0.5	0.8	8.6	-	35 / 1	46 / 11	34 / 1	35 / 1	33 / 2	32 / 1
AL 53	2.1	6.5	9.4	9.7	3.9	9.4	-	58 / 9	27 / 2	4 / 0	6 / 1	9 / 0
AL 74	16.0	14.6	4.4	14.6	15.7	14.9	17.5	-	56 /10	58 / 9	56 /10	55 / 9
IG 01	7.6	6.8	9.1	9.4	7.8	9.1	7.6	17.2	-	29 / 2	27 / 3	28 / 2
JP 10	2.1	6.5	9.4	9.7	3.9	9.4	1.0	17.5	8.1	-	2 / 1	9 / 0
JP 42	2.3	6.3	9.1	9.4	3.6	9.1	1.8	17.2	7.8	0.8	-	11 / 1
K 04	1.8	7.8	8.6	8.9	5.2	8.6	2.3	16.7	7.8	2.3	3.1	-

Table 5.5. Mitochondrial DNA variants (haplotypes) within samples, as detected by the Single Strand Conformation Polymorphism (SSCP) protocol. Types and (proportions).

Locales	Haplotype	Diploid number
Upper Rio Paraná		
Rio Tietê, Monjolinho Dam, State of São Paulo, Brazil	21(100)	M 39 / F 40
Rio Mogi-Guassu, Jataí Natural Reserve, State of São Paulo, Brazil	21(100)	M 39 / F 40
Rio Mogi-Guassu, Jataí Natural Reserve, State of São Paulo, Brazil	22(100)	42
Rio Grande, Volta Grande, State of Minas Gerais, Brazil	21(100)	M 39 / F 40
Rio Grande, Volta Grande, State of Minas Gerais, Brazil	22(75) 23(25)	42
Rio Tibagi, Ponta Grossa, State of Paraná, Brazil	16(100)	?
Middle Rio Paraná		
Rio Iguassu, Segredo, State of Paraná, Brazil	16(65) 17(7) 18(5) 19(20) 20(3)	?

Table 5.5 (cont.)

Locales	Haplotype	Diploid number
Rio Paraná, Reconquista, Province of Santa Fe, Argentina	8(50)	
	6(50)	40
Rio Paraná, Bella Vista Province of Corrientes, Argentina	8(80)	
	6(20)	40
Rio Uruguay		
Rio Aguapey, Province of Corrientes, Argentina	1(90)	
	2(10)	40
Rio Aguapey, Province of Corrientes, Argentina	3(30)	
	4(69)	
	5(1)	42
Coastal Basins		
Rio Ribeira do Iguape, Registro, State of São Paulo, Brazil	60(100)	42
Rio Perequê, Paranaguá, State of Paraná, Brazil	65(100)	?
Rio Guaíba, Porto Alegre State of Rio Grande do Sul, Brazil	70(100)	42

Table 5.5 (cont.)

Locales	Haplotype	Diploid number
Rio São Francisco		
Rio das Velhas, Curvelo, State of Minas Gerais, Brazil	24(92)	
	24B(5)	
	25(3)	40
Rio Doce		
Rio Turvo, Viçosa, State of Minas Gerais, Brazil	27(78)	
	28(22)	42
Lagoa Timboré, Dionísio, State of Minas Gerais, Brazil	27(67)	
	28(33)	42
Lagoa Ferrugem, Dionísio, State of Minas Gerais, Brazil	27(33)	
	28(67)	42
Lagoa Dom Helvécio, Dionísio, State of Minas Gerais, Brazil	25(3)	
	26(16)	
	27(62)	
	28(16)	
	30(3)	42
Lagoa Carioca, Dionísio, State of Minas Gerais, Brazil	27(89)	
	28(11)	42
Amazone Basin		
Rio Tocantins, Marabá, Tucurui Dam, State of Pará, Brazil	35(100)	40
Rio Araguaia (tributary of the Rio Tocantins), Barra do Garças, State of Mato Grosso, Brazil	36(100)	40

Table 6.3 (cont.)

Locales	Haplotype	Diploid number
Rio Madeira, Porto Velho, State of Rondônia, Brazil	37(100)	40
Rio Amazonas, Manaus, State of Amazonas, Brazil	40(100)	42

6. Conclusions

The combined analysis of independent data sets on populations of the species complex *Hoplias malabaricus* revealed the following patterns of variation:

At the local population level, sympatry of specimens with different diploid numbers was interpreted in each case as a range overlap of two different species, with no gene flow between them. It was not possible to assess the nature of this isolation (i. e., whether pre-zygotic or pos-zygotic factors are responsible for this isolation). Although RAPD-PCR markers were useful for detecting genomic differences at the local level, they performed poorly at the macrogeographical level.

Indirect evidence derived from mtDNA data does not support monophyly of the diploid number groups. Except for one geographically restricted clade with populations with 42 chromosomes, other haplotypes from populations with the same diploid number were more related to haplotypes derived from populations with 40 chromosomes. This pattern may be explained by independent origins of diploid numbers, or by a single origin for 40's or 42's. The scattered occurrence of diploid numbers may be explained by a speciation process that allowed for extensive retention of ancestral mtDNA polymorphism, or that involved gene flow during initial phases of the lineage-splitting process.

The phylogeographic patterns mtDNA variation confirmed the

existence of an endemic region on southern and southeastern Brazil, with a range however that is not restricted to the coast, but that also encompasses other rivers like the Rio Doce, the Rio Grande, Rio Mogi-Guassu, and the Rio Tibagi (all of them are members of the Upper Paraná system). The population recently established in the Rio Iguassu also shares its mtDNA with this clade. The high diversity of haplotypes in the Middle and Upper Paraná reflects the complex historical geology of this river.