Research Article

Identification of *Astyanax altiparanae* (Teleostei, Characidae) in the Iguaçu River, Brazil, based on mitochondrial DNA and RAPD markers

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Abstract

Astyanax fishes are among the most important food-web components of South America rivers. In the Iguaçu River basin, the *Astyanax* genus is represented mainly by endemic species. For millions of years, that hydrographic basin has been geographically isolated from the Paraná River basin by the Iguaçu Falls. Recently, a species from the Upper Paraná River basin identified as A*styanax bimaculatus* was revised and described as a new species named *Astyanax altiparanae* Garutti & Britski, 2000. Fauna endemism and geographic isolation triggered interest in investigations to evaluate the identification and genetic relatedness among two *A. altiparanae* populations from the Upper Paraná River basin and the population identified as *A. bimaculatus* in the Iguaçu River, upstream from the Iguaçu Falls. Mitochondrial DNA sequences and RAPD markers revealed high genetic diversity within each population, as well as low genetic distance, high gene flow, and high mitochondrial DNA similarity among all three populations. In conjunction with morphological similarities, these results demonstrated that the population presently known as *Astyanax bimaculatus* in the Iguaçu River should actually be stated as *Astyanax altiparanae*. Furthermore, it could be inferred that the *A. altiparanae* population is not endemic and most likely it was recently introduced in the Iguaçu River basin, maintaining the ancestral genetic identity.

Introduction

Species of the genus *Astyanax* Baird & Girard, 1854 are among the most important components of the food-web in South America rivers, with a significant participation in the diet of big fishes. There are about one hundred nominal species and subspecies described in this genus*,* but many aspects of their taxonomy are still unknown. In the Iguaçu River basin, Paraná State, Brazil, *Astyanax* is represented mainly by seven or eight endemic species. The Iguaçu ichthyofauna has a peculiar evolutionary history, with a high rate of endemism in a cataract-ridden fluvial environment. Approximately 80% of the species are exclusive to this hydrographic basin and non-existent elsewhere on the planet (Agostinho and Gomes, 1997). Among the many

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falls in the river's 1,060 km course, the Iguaçu Falls are the most outstanding. They are located about 22 km from the mouth of the Iguaçu River where it discharges into the Paraná River. Dated from the Oligocene, about 22 million years ago, and dropping 72 m, the Iguaçu Falls isolate the ichthyofauna of the Iguaçu River basin from that of the Paraná River basin (Haseman, 1911; Sampaio, 1988).

All *Astyanax* species existing in the Paraná River basin are absent in the hidrographic basin of Iguaçu, with exception of a fish population popularly known as *tambiú* and *lambari-de-rabo-amarelo*. In the Paraná River basin, this fish population had been identified as *Astyanax bimaculatus*, but recently it was revised and described by Garutti and Britski (2000) as a new species named *Astyanax altiparanae* Garutti & Britski, 2000. It is indicated that only the species from Surinam, apparently the holotype of this species, should a priori be called *A.*

bimaculatus. The geographical isolation of the Iguaçu River basin and the special characteristics of its ichthyofauna endemism instigated an investigation as to whether the *tambiú* population from the Iguaçu River, above the falls, referred to as *A. bimaculatus* (Garavello *et al.*, 1997) belongs to the *A. altiparanae* species found in tributaries of the Upper Paraná River basin. Current work in systematics of fishes has counted on the foundation and starting point provided by the remarkable success achieved by morphological studies. In the last decade, new suites of characters provided by molecular approaches have helped to invigorate studies of fish systematics. Interspecific and intraspecific genetic variation analyzed by mitochondrial DNA (mtDNA) sequences and Random Amplified Polymorphic DNA (RAPD) are among the molecular tools most widely used in these studies.

The mitochondrial genome co-evolves, with a certain degree of independence, at its own faster rate together with the host organism. Analyses of nucleotide variations in mtDNA sequences have been extremely helpful in polymorphism studies and phylogenetic analysis of natural fish populations (Kocher and White 1989; Stepien and Kocher 1997; Sivasundar *et al.*, 2001). The mitochondrial control region is the main noncoding region and the most variable of metazoan mtDNA, including fishes. It is characterized by the displacement loop (D-loop), a DNA segment that is complementary to the L-strand, and that displaces the H-strand. In addition to conserved segments, the control region contains interposed sequences in the 5' and 3' domains, which have high levels of polymorphism. The rate of evolution of the control region is two to five times higher than that of the mitochondrial protein coding genes. Because mutations accumulate fastest in the control region, mainly in the 5' domain, this is the main mtDNA sequence of choice for population and phylogenetic studies among closely related species, including fish species (Meyer, 1994; Faber and Stepien, 1977; Sivasundar *et al.*, 2001).

The RAPD technique consists of DNA analysis of fragments amplified by Polymerase Chain Reaction (PCR) with single primers of randomized sequences (Williams *et al.*, 1990). These primers, consisting of randomized sequences, do not discriminate between coding and noncoding regions. It may be thus admitted that the RAPD technique samples the genome in a highly randomized way (Williams *et al.*, 1990). In fishes, RAPD has been successfully used to supplement systematic and phylogenetic studies of natural populations, including species and sub-species (Stepien and Kocher 1997; Almeida *et al.* 2001; Oliveira *et al.*, 2002).

In the present work, mitochondrial DNA sequences and RAPD markers were used to study the genetic relatedness between the non-endemic *Astyanax* population from the Iguaçu River, upstream from the Iguaçu Falls, and two *A. altiparanae* populations from tributaries of the Paraná River basin, attempting to study the identification and pos-

sible origin of this *Astyanax* population existent in the Iguaçu River basin.

Materials and Methods

Fish sampling and DNA extraction

Astyanax altiparanae[1] was sampled in the Keller and Pirapó tributaries of the Upper Paraná River basin, near Maringá, Northern Paraná State, Brazil. The Pirapó belongs to the Paranapanema River sub-basin and the Keller belongs to the Ivaí River sub-basin, both in the hydrographic basin of the Upper Paraná River. In the Iguaçu River, *Astyanax*[2] specimens of interest were collected at the Salto Caxias Hydroelectric reservoir, soon after impounding, located about 200 km from Iguaçu Falls. The related species, *Astyanax* sp B[2] was chosen as an outgroup for genetic analyses. This species is endemic to the Iguaçu River basin, and it was sampled at the Salto Caxias Hydroelectric reservoir. Specimens were captured by gill nets and immediately frozen in liquid nitrogen. Species identification was undertaken on the basis of morphological characteristics, in agreement with Sampaio (1988), Garavello *et al.* (1997), and Garutti and Britski (2000). Samples of muscle tissues were taken for DNA extraction. Sampled fishes were numbered, preserved in 10% formaldehyde for a month, and then they were transferred to 70 °GL ethanol.

Extraction of total DNA was performed according to Whitmore *et al.* (1992), with a few modifications. Approximately 1 g of muscle tissue was macerated in the presence of liquid nitrogen. Extraction buffer (500 µL) was added, consisting of 100 mM Tris-HCl pH 8.0, 10 mM EDTA (ethylenediaminetetraacetic acid), 0.1% SDS (sodium dodecyl sulphate), 50 mM DTT (dithiothreitol), and 0.5 mg/mL proteinase K. After shaking for one hour at 60 Srpm, at 42 °C, DNA was extracted with an equal volume of phenol, and then with chloroform/isoamylic alcohol (24:1 v:v). DNA was then precipitated by addition of two volumes of cold ethanol 100%, followed by incubation at -20 °C for 12-24 h. After centrifugation at 10,000 x g for 10 min, the precipitate was washed with ethanol 80% and resuspended in diluted TE buffer (0.1 mM Tris-HCl pH 8.0, 0.01 mM EDTA), containing RNAase (20 µg/mL). The DNA suspension was stored at -21 °C. Aliquots of each sample were used for visual estimation of DNA quantity by

comparison with known quantities of phage λ DNA in agarose gel (0.8%), and stained with ethydium bromide (20 µg/100 mL).

PCR and sequencing of mitochondrial DNA

A mtDNA fragment (approx. 800 bp) was amplified by PCR from DNA samples of six to seven fishes of each *Astyanax* population. For each fish specimen, the mtDNA fragment was amplified in three independent PCR reactions and they were then sequenced and analyzed, as replicates. The primers H16498 5'-CACCTGAAGTAGGAACCA GATG-3' (Meyer *et al.*, 1990), and L15774M 5'-ACATGAATTGGAGGTATACCAGT-3', which was modified from the L15774 primer described by Irwin *et al.* (1991), were used for PCR amplifications. PCR reaction mix consisted of Tris-KCl (20 mM Tris-HCl pH 8.4 plus 50 mM KCl), 1.5 mM MgCl$_2$, 2.5 µM of each primer, 0.1 mM of each dNTP, 2.5 U *Taq*-polymerase, 15 ng total DNA and water to a total volume of 25 µL. PCR amplification was performed in a first cycle of 94 °C for 4 min, 50 °C for 30 s, and 72 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 56 °C for 30 s, 72 °C for 2 min, and a final extension step of 72 °C for 10 min. After amplification, an aliquot was taken from each PCR reaction mix, and the amplified DNA fragment was quantified in agarose gel by comparison with known quantities of phage λ DNA.

The final DNA product obtained from each PCR reaction was used directly as template for sequencing on an ABI-3100 (Perkin Elmer, Norwalk, CT, USA) automated sequencer. Sequencing was performed with BigDye™ Terminator Cycle Sequencing v. 2.0 kit (Perkin Elmer Biosystems), according to the manufacturers instructions. Approximately 50 ng of template DNA and 20 pmol of either L15774M or H16498 primer were added to each sequencing reaction. Immediately after heating the reaction mix at 94 °C for 4 min, amplifications were performed in 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min 30 s at 60 °C, followed by 5 min at 60 °C. Sequencing data, under chromatogram format, were directly collected by the Sequencing Analysis program (Perkin Elmer) and submitted to quality check, assembly and alignment on the Vector NTI Suite version 6.0 software (Informax, Inc.). The nucleotide sequence of each specimen was confirmed in at least three replicates, consisting of three independent PCR reactions.

A 569-nucleotide portion of the 5' side of the mitochondrial control region of 24 individuals was used in genetic relatedness analyses. In addition, a corresponding mtDNA segment of the control region of the closely related species *Astyanax* sp B was sequenced and included as an outgroup in the analysis. Genetic analyses were performed with MEGA version 2.1 (Kumar *et al.*, 2001). Genetic distances of Tamura and Nei (1993) with gamma distribution were calculated and clustered into a distance neigh-

bor-joining tree (Saitou and Nei, 1987). Bootstrap analyses were based on 1000 replications. The Gamma coefficient was estimated by using PAUP* version 4.0 (Swofford, 2002).

RAPD electrophoresis profile analysis

Eighteen fishes from the Iguaçu River, and eighteen from both tributaries of the Upper Paraná River basin were evaluated by RAPD markers. Initial tests of PCR primers were undertaken by using *Astyanax* DNA in amplifications with two sets of 20 decamers of Kits X and W from Operon Technologies (Alameda, CA, USA). Primers amplifying the highest number of fragments and producing accurate bands were chosen for RAPD analysis. PCR reaction mixture consisted of buffer Tris-KCl (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 2 mM MgCl$_2$, 0.46 µM primer, 0.2 mM dNTP, 1U/Reaction of *Taq*-polymerase (Gibco BRL), 10 ng DNA, and sterile deionized water to a total volume of 13 µL. The reaction mixture was heated at 92 °C for 4 min, followed by 40 cycles of 1 min at 92 °C, 1 min 30 s at 40 °C and 2 min at 72 °C. Immediately after the last amplification cycle, the reaction mixture was maintained at 72 °C for 5 min and then cooled at 4 °C for 20 min. PCR amplified DNA fragments were separated by electrophoresis in agarose gel (1.4%), stained with ethydium bromide (20 µg/100 mL), at 3 V.cm^{-1} for 5-6 h. A sample was included as a negative control in each experiment, consisting of the PCR reaction mixture without template DNA. In addition, 1 to 3 samples of PCR products, containing *Astyanax* DNA fragments previously amplified and analyzed with the same primer, were included in each agarose gel. The electrophoretic profile was visualized under UV radiation and photographed with Kodak EDAS-290. Sizes of DNA fragments were estimated by comparison with the standard Ladder 100 pb (Gibco BRL).

Unbiased genetic distances based on RAPD markers were estimated according to Nei (1978), with Lynch and Milligan's (1994) correction, using the software Rapddist (Black and Antolin, 1997). The Shannon gene diversity indexes were calculated with the software Popgen 1.31 (Yeh *et al.*, 1999). The Shannon diversity index was used to quantify levels of genetic diversity in isolated populations and for the partition of components within and between populations. Shannon's diversity index has been useful for RAPD data analysis due to its relative non-sensitivity to bias, which could be introduced in data because of the impossibility of detecting heterozygous individuals. Fixation index F_{st}, and gene flow, equivalent to the number of migrants per generation (Nm), were estimated according to Lynch and Milligan (1994), with the computer program RAPDFST (Black, 1997). The arithmetic complement of the coefficient of similarity among individuals, according to Nei and Li (1979) was estimated by using RAPDPLOT 3.0 (Black, 1997). Clustering was performed by the neigh-

bor-joining method (Saitou and Nei, 1987), with MEGA 2.1 (Kumar *et al.*, 2001).

Results

Mitochondrial DNA

A mtDNA fragment of approximately 800 bp was amplified by PCR from all *Astyanax* specimens. This mtDNA fragment consisted of approximately 90 bp of the 3' end of the mitochondrial cytochrome b gene (GenBank Accession Numbers AF531481 to AF531504), immediately followed by the complete nucleotide sequences of tRNA^{Thr} and tRNA^{Pro} genes (GenBank Accession Numbers AF531874 to AF531897) and by a partial sequence of the 5' side of the control region (GenBank Accession Numbers AY125820 to AY125843). Nucleotide sequences of the control region and cytochrome b were identified by similarity to GenBank accesses via BLASTN (Altschul *et al.*, 1997) from NCBI. The tRNA genes were identified with the tRNAScan-SE program (Lowe and Eddy, 1997). Altogether, twenty mtDNA sequences, amplified from twenty specimens, were analyzed. The cytochrome b, tRNA^{Thr}, and tRNA^{Pro} nucleotide sequences were identical among individuals of all three *Astyanax* populations, thus these genes were excluded from analyses. Nucleotide polymorphism was restricted to the amplified control region sequence, from which 569 bp were analyzed in this work. As shown in Figure 1, multiple alignment of these *Astyanax* sequences revealed 27 polymorphic nucleotides, approximately 4.7%, in the Keller, Pirapó, and Iguaçu populations. Altogether, a total of eleven different control-region haplotypes were identified among twenty individuals of these populations. Changes were mostly single nucleotide substitutions. On the other hand, in comparisons of these three populations with the outgroup *Astyanax* sp B, the percentage of polymorphic sites was increased to approximately 30%. A high polymorphism level was also detected when the nucleotide sequence of the mitochondrial control region from another characid species, *Oligosarcus paranensis*, was tested as the outgroup, replacing *Astyanax* sp B (data not shown).

As shown in Table I, low genetic distances of Tamura and Nei (1993) with gamma distribution, based on control region nucleotide sequences, were estimated among the Keller, Pirapó, and Iguaçu populations, at a maximum value of 0.019. On the other hand, high genetic distances, ranging from 3.176 to 3.527, were calculated between these three populations and *Astyanax* sp B. As expected, in the neighbor-joining tree, the Keller, Pirapó, and Iguaçu specimens were combined as a single cluster, distant from the outgroup (Figure 2). Within the subtree, an intermix of individuals from the Keller, Pirapó and Iguaçu rivers clearly demonstrated the high molecular similarity among these *Astyanax* specimens. The distances among individuals of the three populations were in a similar magnitude observed

among the individuals from the outgroup population, as represented in the tree branches (Figure 2). Low bootstrap values can be attributed to the low molecular differences among individuals within the cluster. As a contrast, this subtree was clearly separated in a high distance, with 100% bootstrap value, from the related *Astyanax* sp B species used as outgroup.

RAPD markers

In each *Astyanax* population, a total of 10 selected primers were used for DNA amplifications by PCR. Amplified DNA fragments counted for 79 polymorphic bands, seven 100% monomorphic bands and one monomorphic band at 5% probability level. RAPD electrophoresis profiles are represented in Figure 3, for one of the selected primers. Sizes of polymorphic bands ranged from approximately 0.4 to 3.0 kb (Table II). The number of bands per primer varied from five to ten. Polymorphic bands were taken as RAPD markers, and they were combined in a simple matrix of binary data, with registers of presence or absence of bands. The Shannon genetic diversity index within each population was 0.58 (±0.15) for Iguaçu, 0.54 (±0.20), for Keller, and 0.50 (±0.25) for Pirapó. Estimates of Nei's unbiased genetic distances ranged from 0.050 to 0.070 (Table III). As shown in Table IV, the estimated value of fixation index F_{st}, was not significant between the Iguaçu and Keller populations, and it was significant, only at the 5% level, between the Pirapó and Keller, as well as Pirapó and Iguaçu. The migration model among the three populations was adopted to estimate gene flow (*Nm*) from F_{st} (Lynch and Milligan, 1994). Each population was considered sufficiently large to present negligible genetic drift. Allele frequency of migrants among the three populations was considered to be equal to the mean of the allele frequency in the entire population. Estimates of gene flow on all loci among populations varied from 2.4 to 4.0 migrants per generation (Table IV). The low RAPD divergence among populations, as derived from the neighbor-joining tree (Figure 4), indicated that the three studied *Astyanax* populations form a homogeneous group; therefore, a result congruent with the high genetic similarity estimated by all analyses performed for both mtDNA and RAPD markers.

Discussion

As inferred by the mitochondrial DNA sequences and RAPD markers, the genetic similarity among the three studied *Astyanax* populations is so high that it is not consistent with their current taxonomic identification as two different species. Genetic relatedness estimated by molecular markers indicated that the population from the Iguaçu River and the *Astyanax altiparanae* population from the Keller tributary of the Upper Paraná River basin are close to genetic homogeneity and they did not differentiate, not even by genetic drift. Pirapó was the most differentiated
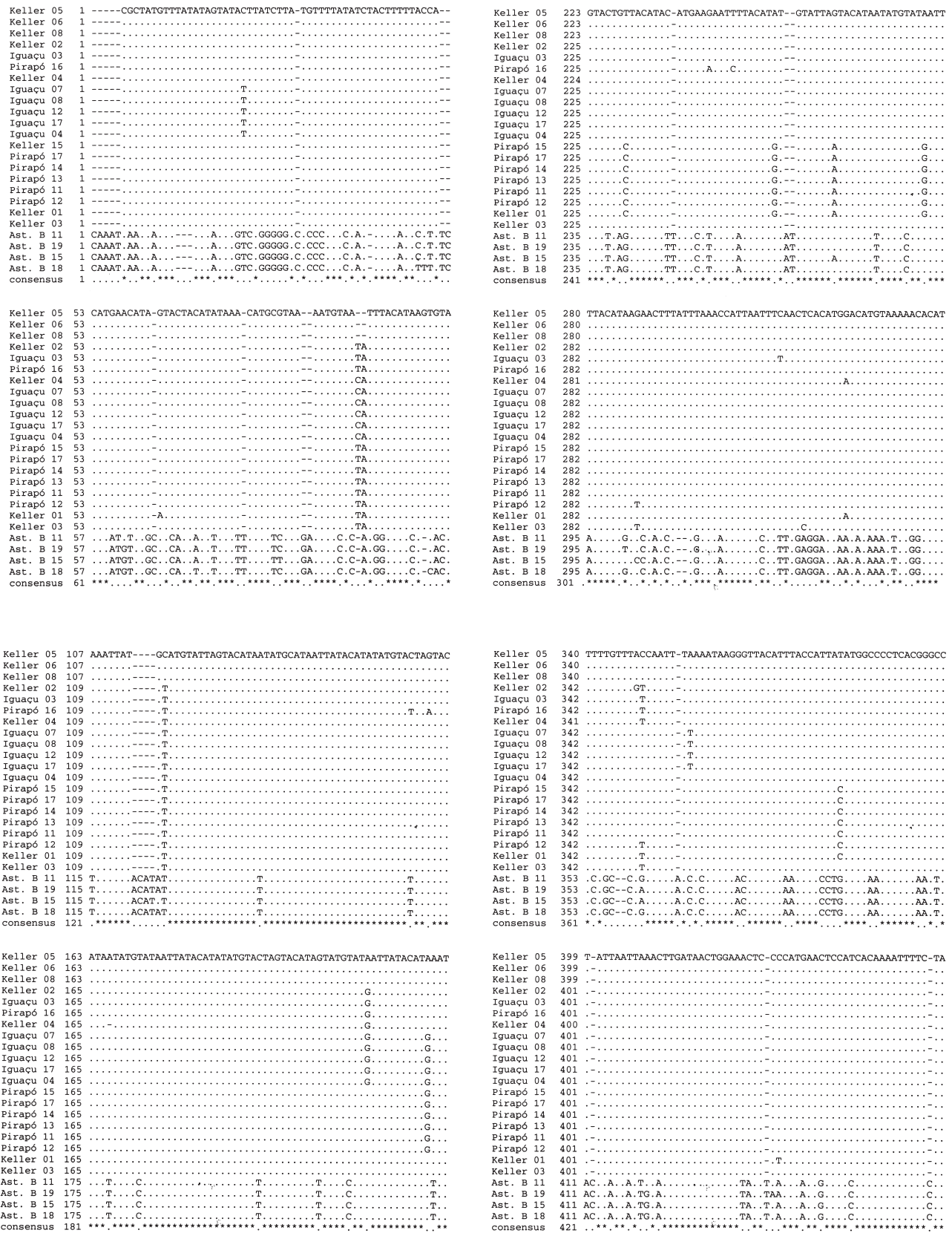
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Keller 05   1 -----CGCTATGTTTATATAGTATACTTATCTTA-TGTTTTATATCTACTTTTTACCA--
Keller 06   1 -----.................................-..................--
Keller 08   1 -----.................................-..................--
Keller 02   1 -----.................................-..................--
Iguaçu 03   1 -----.................................-..................--
Pirapó 16   1 -----.................................-..................--
Keller 04   1 -----.................................-..................--
Iguaçu 07   1 -----...................T.............-..................--
Iguaçu 08   1 -----...................T.............-..................--
Iguaçu 12   1 -----...................T.............-..................--
Iguaçu 17   1 -----...................T.............-..................--
Iguaçu 04   1 -----...................T.............-..................--
Keller 15   1 -----.................................-..................--
Pirapó 17   1 -----.................................-..................--
Pirapó 14   1 -----.................................-..................--
Pirapó 13   1 -----.................................-..................--
Pirapó 11   1 -----.................................-..................--
Pirapó 12   1 -----.................................-..................--
Keller 01   1 -----.................................-..................--
Keller 03   1 -----.................................-..................--
Ast. B 11   1 CAAAT.AA..A...--...A..GTC.GGGGG.C.CCC...C.A.-....A..C.T.TC
Ast. B 19   1 CAAAT.AA..A...--...A..GTC.GGGGG.C.CCC...C.A.-....A..C.T.TC
Ast. B 15   1 CAAAT.AA..A...--...A..GTC.GGGGG.C.CCC...C.A.-....A..C.T.TC
Ast. B 18   1 CAAAT.AA..A...--...A..GTC.GGGGG.C.CCC...C.A.-....A..TTT.TC
consensus   1 .....*..**.***...***.***...*.....*.*...***.*.*.****.**...*..

Keller 05  53 CATGAACATA-GTACTACATATAAA-CATGCGTAA--AATGTAA--TTTACATAAGTGTA
Keller 06  53 .........-..........-.........--.......--..........
Keller 08  53 .........-..........-.........--.......--..........
Keller 02  53 .........-..........-.........--...TA..............
Iguaçu 03  53 .........-..........-.........--...TA..............
Pirapó 16  53 .........-..........-.........--...TA..............
Keller 04  53 .........-..........-.........--...CA..............
Iguaçu 07  53 .........-..........-.........--...CA..............
Iguaçu 08  53 .........-..........-.........--...CA..............
Iguaçu 12  53 .........-..........-.........--...CA..............
Iguaçu 17  53 .........-..........-.........--...CA..............
Iguaçu 04  53 .........-..........-.........--...CA..............
Pirapó 15  53 .........-..........-.........--...TA..............
Pirapó 17  53 .........-..........-.........--...TA..............
Pirapó 14  53 .........-..........-.........--...TA..............
Pirapó 13  53 .........-..........-.........--...TA..............
Pirapó 11  53 .........-..........-.........--...TA..............
Pirapó 12  53 .........-..........-.........--...TA..............
Keller 01  53 ......-A...........-.........--...TA..............
Keller 03  53 .........-..........-.........--...TA..............
Ast. B 11  57 ...AT.T..GC..CA..A..T...TT....TC...GA....C.C-A.GG....C-.AC.
Ast. B 19  57 ...ATGT..GC..CA..A..T...TT....TC...GA....C.C-A.GG....C-.AC.
Ast. B 15  57 ...ATGT..GC..CA..A..T...TT....TT...GA....C.C-A.GG....C-.AC.
Ast. B 18  57 ...ATGT..GC..CA..T...TT....TC...GA....C.C-A.GG....C.-CAC.
consensus  61 ***.....**...*..**.**.***..****..***..****.*...*..****.*....*

Keller 05 107 AAATTAT----GCATGTATTAGTACATAATATGCATAATTATACATATATGTACTAGTAC
Keller 06 107 ..........----.................................
Keller 08 107 ..........----.................................
Keller 02 109 ..........----.T...............................
Iguaçu 03 109 ..........----.T...............................
Pirapó 16 109 ..........----.T...........................T..A...
Keller 04 109 ..........----.T...............................
Iguaçu 07 109 ..........----.T...............................
Iguaçu 08 109 ..........----.T...............................
Iguaçu 12 109 ..........----.T...............................
Iguaçu 17 109 ..........----.T...............................
Iguaçu 04 109 ..........----.T...............................
Pirapó 15 109 ..........----.T...............................
Pirapó 17 109 ..........----.T...............................
Pirapó 14 109 ..........----.T...............................
Pirapó 13 109 ..........----.T...............................
Pirapó 11 109 ..........----.T...............................
Pirapó 12 109 ..........----.T...............................
Keller 01 109 ..........----.T...............................
Keller 03 109 ..........----.T...............................
Ast. B 11 115 T......ACATAT...............T.............T.....
Ast. B 19 115 T......ACATAT...............T.............T.....
Ast. B 15 115 T......ACAT.T...............T.............T.....
Ast. B 18 115 T......ACATAT...............T.............T.....
consensus 121 .******.....***************.*****************.**.***

Keller 05 163 ATAATATGTATAATTATACATATATGTACTAGTACATAGTATGTATAATTATACATAAAT
Keller 06 163 ..............................................
Keller 08 163 ..............................................
Keller 02 165 ..............................G.............
Iguaçu 03 165 ..............................G.............
Pirapó 16 165 ..............................G.............
Keller 04 165 ...-..........................G.............
Iguaçu 07 165 ..............................G.......G...
Iguaçu 08 165 ..............................G.......G...
Iguaçu 12 165 ..............................G.......G...
Iguaçu 17 165 ..............................G.......G...
Iguaçu 04 165 ..............................G.......G...
Pirapó 15 165 ..............................G.............
Pirapó 17 165 ..............................G.............
Pirapó 14 165 ..............................G.............
Pirapó 13 165 ..............................G.............
Pirapó 11 165 ..............................G.............
Pirapó 12 165 ..............................G.............
Keller 01 165 ..............................................
Keller 03 165 ..............................................
Ast. B 11 175 ...T....C................T.........T....C........T..
Ast. B 19 175 ...T....C................T.........T....C........T..
Ast. B 15 175 ...T....C................T.........T....C........T..
Ast. B 18 175 ...T....C................T.........T....C........T..
consensus 181 ***.****.******************.*********.****.**.*********..**
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Keller 05 223 GTACTGTTACATAC-ATGAAGAATTTTACATAT--GTATTAGTACATAATATGTATAATT
Keller 06 223 ..............-.................--..................
Keller 08 223 ..............-.................--..................
Keller 02 225 ..............-.................--..................
Iguaçu 03 225 ..............-.................--..................
Pirapó 16 225 ..............A....C.............--..................
Keller 04 224 ..............-.................--..................
Iguaçu 07 225 ..............-.................--..................
Iguaçu 08 225 ..............-.................--..................
Iguaçu 12 225 ..............-.................--..................
Iguaçu 17 225 ..............-.................--..................
Iguaçu 04 225 ..............-.................--..................
Pirapó 15 225 ......C.......-........G.--.....A..........G...
Pirapó 17 225 ......C.......-........G.--.....A..........G...
Pirapó 14 225 ......C.......-........G.--.....A..........G...
Pirapó 13 225 ......C.......-........G.--.....A..........G...
Pirapó 11 225 ......C.......-........G.--.....A..........G...
Pirapó 12 225 ......C.......-........G.--.....A..........G...
Keller 01 225 ......C.......-........G.--.....A..........G...
Keller 03 225 ......C.......-........G.--.....A..........G...
Ast. B 11 235 ...T.AG......TT...C.T....A......AT.............T....C.....
Ast. B 19 235 ...T.AG......TT...C.T....A......AT.............T....C.....
Ast. B 15 235 ...T.AG......TT...C.T....A......AT.......A......T....C.....
Ast. B 18 235 ...T.AG......TT...C.T....A......AT.............T....C.....
consensus 241 ***.*..*******..***.*.***..*****.*..******.******.****.**.***

Keller 05 280 TTACATAAGAACTTTATTTAAACCATTAATTTCAACTCACATGGACATGTAAAAACACAT
Keller 06 280 ..............................................
Keller 08 280 ..............................................
Keller 02 282 ..............................................
Iguaçu 03 282 ......................T...................
Pirapó 16 282 ..............................................
Keller 04 281 .........................................A...
Iguaçu 07 282 ..............................................
Iguaçu 08 282 ..............................................
Iguaçu 12 282 ..............................................
Iguaçu 17 282 ..............................................
Iguaçu 04 282 ..............................................
Pirapó 15 282 ..............................................
Pirapó 17 282 ..............................................
Pirapó 14 282 ..............................................
Pirapó 13 282 ..............................................
Pirapó 11 282 ..............................................
Pirapó 12 282 ......T...............................
Keller 01 282 .........................................A...
Keller 03 282 ......T...............................C...
Ast. B 11 295 A.....G..C.A.C.--.G...A......C..TT.GAGGA..AA.A.AAA.T..GG....
Ast. B 19 295 A.....T..C.A.C.--.G..A......C..TT.GAGGA..AA.A.AAA.T..GG....
Ast. B 15 295 A.......CC.A.C.--.G..A......C..TT.GAGGA..AA.A.AAA.T..GG....
Ast. B 18 295 A.....G..C.A.C.--.G..A......C..TT.GAGGA..AA.A.AAA.T..GG....
consensus 301 .*****.*..*.*.*..*.***.******.**..*.....**..*.*...*.**..****

Keller 05 340 TTTTGTTTACCAATT-TAAAATAAGGGTTACATTTACCATTATATGGCCCCTCACGGGCC
Keller 06 340 ...............-.................................
Keller 08 340 ...............-.................................
Keller 02 342 ........GT....-.................................
Iguaçu 03 342 .........T....-.................................
Pirapó 16 342 .........T....-.................................
Keller 04 341 .........T....-.................................
Iguaçu 07 342 ..............-.T.................................
Iguaçu 08 342 ..............-.T.................................
Iguaçu 12 342 ..............-.T.................................
Iguaçu 17 342 ..............-.T.................................
Iguaçu 04 342 ..............-..................................
Pirapó 15 342 ..............-...........................C.....
Pirapó 17 342 ..............-...........................C.....
Pirapó 14 342 ..............-...........................C.....
Pirapó 13 342 ..............-...........................C.....
Pirapó 11 342 ..............-...........................C.....
Pirapó 12 342 .........T....-...........................C.....
Keller 01 342 .........T....-...........................C.....
Keller 03 342 .........T....-.................................
Ast. B 11 353 .C.GC--.C.G.....A.C.C....AC......AA....CCTG....AA......AA.T.
Ast. B 19 353 .C.GC--.C.A.....A.C.C....AC......AA....CCTG....AA......AA.T.
Ast. B 15 353 .C.GC--.C.A.....A.C.C....AC......AA....CCTG....AA......AA.T.
Ast. B 18 353 .C.GC--.C.G.....A.C.C....AC......AA....CCTG....AA......AA.T.
consensus 361 *.*.......*****.*.*.*****..******..****....****..******..*.*

Keller 05 399 T-ATTAATTAAACTTGATAACTGGAAACTC-CCCATGAACTCCATCACAAAATTTTC-TA
Keller 06 399 .-.............................-..................-.-.
Keller 08 399 .-.............................-..................-.-.
Keller 02 401 .-.............................-..................-.-.
Iguaçu 03 401 .-.............................-..................-.-.
Pirapó 16 401 .-.............................-..................-.-.
Keller 04 400 .-.............................-..................-.-.
Iguaçu 07 401 .-.............................-..................-.-.
Iguaçu 08 401 .-.............................-..................-.-.
Iguaçu 12 401 .-.............................-..................-.-.
Iguaçu 17 401 .-.............................-..................-.-.
Iguaçu 04 401 .-.............................-..................-.-.
Pirapó 15 401 .-.............................-..................-.-.
Pirapó 17 401 .-.............................-..................-.-.
Pirapó 14 401 .-.............................-..................-.-.
Pirapó 13 401 .-.............................-..................-.-.
Pirapó 11 401 .-.............................-..................-.-.
Pirapó 12 401 .-.............................-..................-.-.
Keller 01 401 .-...........................-..T...............-.-.
Keller 03 401 .-.............................-..................-.-.
Ast. B 11 411 AC..A..A.T..A.............TA..T.A...A..G....C............C..
Ast. B 19 411 AC..A..A.TG.A.............TA..TAA...A..G....C............C..
Ast. B 15 411 AC..A..A.TG.A.............TA..T.A...A..G....C............C..
Ast. B 18 411 AC..A..A.TG.A.............TA..T.A...A..G....C............C..
consensus 421 ..**.**.*..*..*************..**...***.**.****.***********..*.**
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Figure 1 - Multialignment on Clustal W of nucleotide sequences from the mitochondrial control region of *Astyanax altiparanae* specimens from the Iguaçu River and from the Keller and Pirapó tributaries of the Upper Paraná River basin, and specimens of the closely related species *Astyanax* sp B from Iguaçu River.

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Keller 05   456 TGCACGGCCCCAACTAAAATTGCACCTCAGAATCTTAATGTAGTAAGAGACCACCAACCA
Keller 06   456 ............................................................
Keller 08   456 ............................................................
Keller 02   458 ............................................................
Iguaçu 03   458 ............................................................
Pirapó 16   458 ............................................................
Keller 04   457 ............................................................
Iguaçu 07   458 ............................................................
Iguaçu 08   458 ............................................................
Iguaçu 12   458 ............................................................
Iguaçu 17   458 ............................................................
Iguaçu 04   458 ............................................................
Pirapó 15   458 ............................................................
Pirapó 17   458 ............................................................
Pirapó 14   458 ............................................................
Pirapó 13   458 ............................................................
Pirapó 11   458 ............................................................
Pirapó 12   458 ............................................................
Keller 01   458 .......................T....................................
Keller 03   458 ............................................................
Ast. B 11   471 ....A.AG........G.....GTA....AT..T...........................
Ast. B 19   471 ....A.AG........G.....GTA....AC..T...........................
Ast. B 15   471 ....A..G........G.....GTA....AT..T...........................
Ast. B 18   471 ....A.AG........G.....GTA....AC..T...........................
consensus   481 ****.*..********.*****....***..**.***************************


Keller 05   516 GTTTATATAAAAGTCCACGGTCCTTGATAGGTCAGGGACGAATTATTGTGGG
Keller 06   516 ...................................................
Keller 08   516 ...................................................
Keller 02   518 ...................................................
Iguaçu 03   518 ...................................................
Pirapó 16   518 ...........C.......................................
Keller 04   517 ...................................................
Iguaçu 07   518 ...................................................
Iguaçu 08   518 ...................................................
Iguaçu 12   518 ...................................................
Iguaçu 17   518 ...................................................
Iguaçu 04   518 ...................................................
Pirapó 15   518 ...................................................
Pirapó 17   518 ...................................................
Pirapó 14   518 ...................................................
Pirapó 13   518 ...................................................
Pirapó 11   518 ...................................................
Pirapó 12   518 ...................................................
Keller 01   518 ...................................................
Keller 03   518 ...................................................
Ast. B 11   531 ..A..GT......CTA...-..TA.....G........G.-------------
Ast. B 19   531 ..A..GT......CTA.....TA.....G........G.-------------
Ast. B 15   531 ..A..GT......CTA.....TA.....G........G.-------------
Ast. B 18   531 ..A..AT......CTA.....TA.....G........G.-------------
consensus   541 **.**..******...*****.*****.********.*..............
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Figure 1 - (Cont.)

Table I - Genetic distances of Tamura and Nei (1993) based on mitocondrial control region sequences, with gamma distribution, among *Astyanax altiparanae* individuals from Iguaçu River and from the Keller and Pirapó tributaries of the Upper Paraná River basin, and *Astyanax* sp B individuals from the Iguaçu River.

	Keller	Pirapó	Iguaçu	*A.* sp B
Keller	0.000			
Pirapó	0.013	0.000		
Iguaçu	0.017	0.019	0.000	
A. sp B	3.259	3.176	3.527	0.000

population, as compared either to Keller or to Iguaçu. The molecular genetic evidence, in conjunction with morphological similarities (Graça and Pavanelli, 2002), demonstrated that the species status of *Astyanax bimaculatus* from the Iguaçu River should actually be stated as *Astyanax altiparanae* Garutti & Britski, 2000. Furthermore, high genetic diversity within and high similarities between the populations, as indicated by of RAPD markers, suggested that this species was most probably introduced recently in the Iguaçu River basin, and that this introduction involved a large number of individuals.

Molecular tools associated to morphological analyses have been effectively applied in defining relationships of fishes in different levels, including among genus and species populations. The mitochondrial control region has been used extensively as a molecular marker because of its
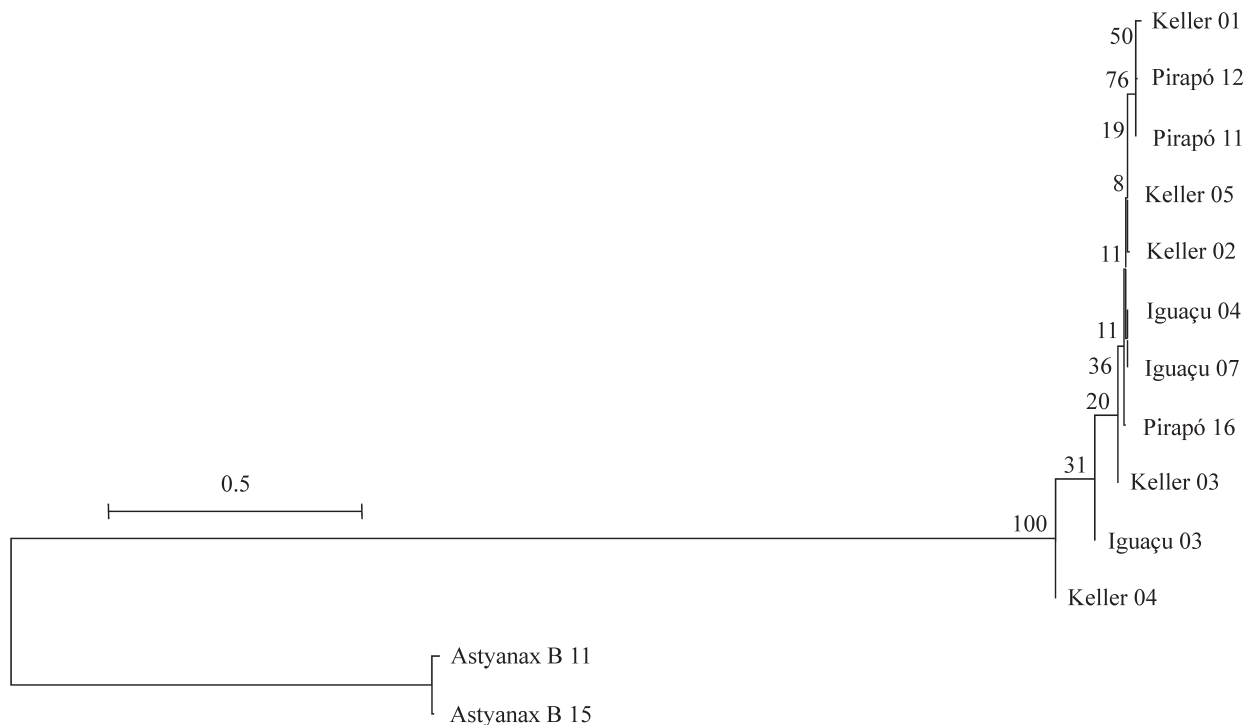
Figure 2 - Neighbor-joining tree for mitochondrial control region sequences of *Astyanax altiparanae* specimens from the Iguaçu River and from the Keller and Pirapó tributaries of the Upper Paraná River basin. Genetic distance was computed by using the Tamura and Nei (1993) method. The tree is rooted to the closely related species *Astyanax* sp B. Number on each node indicates bootstrap probability based on 1000 replications.
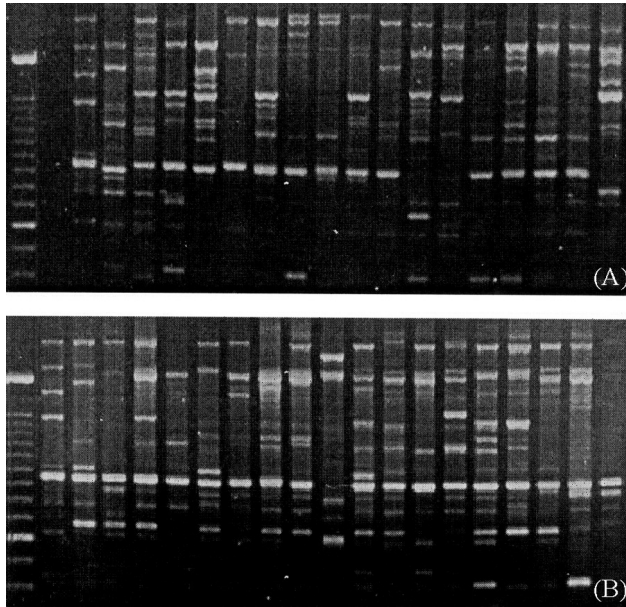
Figure 3 - RAPD profiles for *Astyanax altiparanae* using primer OPW-09. (**A**) Individuals from the Keller River (lanes 3-11) and from the Pirapó River (Lanes 12-20), both from the Upper Paraná basin. Lane 2 is a negative control, without DNA. (**B**) Individuals from the Iguaçu River (Lanes 3-20). Lane 2 is a PCR replicate from Lane 3 of A. Lane 1 in A and B contains DNA molecular weight markers (Ladder 100 bp, Gibco BRL).

the population from the Iguaçu River. These results are consistent with findings in other vertebrate species, showing that although the mitochondrial protein encoding gene and the tRNAs do not evolve as rapidly as the control region, their nucleotide sequences are highly conserved at the intraspecific level, while they have been found polymorphic at the interspecific level (Meyer, 1994; Saccone *et al.*, 2002). Furthermore, the genetic relatedness of all three *Astyanax* populations was obvious upon the contrast of their mtDNA sequences with those of the related species *Astyanax* sp B. As shown in Figure 2, individuals from the Iguaçu and Upper Paraná rivers were not discriminated by the neighbor-joining method, but they were clearly separated, with 100% bootstrap value, from *Astyanax* sp B. The genetic distances among specimens of all three studied *Astyanax* populations were in similar magnitudes to those observed among the individuals of the outgroup *Astyanax* sp B (Figure 1). The molecular genetic relatedness inferred from mtDNA sequences indicated that the studied *Astyanax* population sampled in the Iguaçu River should be joined together with the Keller and Pirapó populations, within the *Astyanax altiparanae* species.

RAPD markers corroborated the inference of high genetic similarity among the three *Astyanax* populations, as estimated from mtDNA sequences, and they indicated high nuclear genetic diversity within each population. The Shannon indexes based on RAPD markers revealed high genetic diversity within the Iguaçu population, at very similar levels to those of the Keller and Pirapó populations. This result indicated that the genetic variability was not lost during the establishment of these three populations. The highest Nei's unbiased genetic distance, estimated as 0.070 from RAPD markers (Table III), is considered low and consistent with intraspecific distances. Nei's genetic distances between species from the same genus have been found no lower than 0.162 and over 0.3 in most species, as estimated from enzyme studies (Thorpe and Solé-Cava, 1994). Analyses of

rapid rate of evolution compared to nuclear DNA and the significant literature available on the theory and application of mtDNA sequence analysis. In the present study, low genetic distances, according to Tamura and Nei (1993), were found among the Iguaçu, Keller, and Pirapó populations, as inferred from control region sequences (Table I; Figure 2). The high genetic similarity data estimated on the basis of the control region were reinforced by the cytochrome b partial sequence and the complete sequences of tRNA^{Pro} and tRNA^{Thr}, which were identical among all *Astyanax* individuals, from both populations of the Upper Paraná basin and

Table II - Approximate size, in number of base pairs (bp), of DNA fragments of polymorphic loci of *Astyanax altiparanae* from the Keller and Pirapó tributaries of the Upper Paraná River basin, and from the Iguaçu River, amplified by PCR with ten RAPD primers.

Primers	Bands (~pb)									
	a	b	c	d	e	f	g	h	i	j
OPX05	1700	1600	1200	900	700	650	500			
OPX06	2400	2200	1600	1350	1200	1150	950	900	750	400
OPX07	1800	1600	1350	1300	1000	850	800	450		
OPX08	2000	1900	1600	1500	1300	1150	1000	700		
OPX19	1700	1500	1250	1000	900	750				
OPW03	2200	2000	1500	1300	950					
OPW04	2800	2600	1900	1700	1500	1400	500			
OPW09	3000	2200	2000	1500	1200	950	650	400		
OPW17	2300	2100	1700	1450	1200	1100	900	700	650	600
OPW19	1600	1500	1300	1000	900	850	750	700	650	600

Table III - Unbiased Nei's distances among three *Astyanax altiparanae* populations from the Keller and Pirapó tributaries of the Upper Paraná River basin and from the Iguaçu River, based on RAPD markers.

	Keller	Pirapó	Iguaçu
Keller	0.000		
Pirapó	0.070	0.000	
Iguaçu	0.050	0.059	0.000

Table IV - Matrix of fixation indexes F_{st} (lower-left) and of number of migrants per generation Nm (upper-right) among three populations of *Astyanax altiparanae* from the Keller, Pirapó and Iguaçu rivers, based on RAPD markers.

	Keller	Pirapó	Iguaçu
Keller	0.0	2.4	4.0
Pirapó	0.093*	0.0	2.9
Iguaçu	0.059[n.s.]	0.078*	0.0

*Significant at the 5% probability level by the χ^2 test.
n.s. Non-significant at the 5% level of probability by the χ^2 test.

RAPD markers of fish species from the floodplain of the Upper Paraná River basin revealed relatively high Nei's unbiased genetic distances, as 0.504 between *Pseudoplatystoma corruscans* ('pintado') and *Pseudoplatystoma fasciatum* ('cachara'), and 0.729 between *Steindachnerina brevipinna* and *Steindachnerina insculpta* (Sekine *et al.*, 2000; Oliveira *et al.,* 2002). Even though the studied species within each of these two genus are so closely related that they crossbreed generating hybrids, their genetic distances were obviously much higher than those among the three *Astyanax* populations. Therefore, the low genetic distances among the two *Astyanax altiparanae* populations from the Upper Paraná river basin and the studied *Astyanax* population from Iguaçu river are coherent with expected intraspecific distances. Nei's unbiased genetic distances are considered suitable for long evolutionary processes, with populational divergences due to genetic drift and mutational events (Weir, 1990), and its value is proportional to the time of divergence and the rate of gene substitution per locus and per generation. Nei's unbiased estimates of low genetic distances based on RAPD did not discriminate the *Astyanax* populations from the Iguaçu and Upper Paraná river basins, indicating that they maintain the genetic identity of a single recent common ancestor. Intraspecific differentiation was significant only at 5% level for the Pirapó population as compared to both the Iguaçu and Keller populations, by the fixation index F_{ST}. This result seems to indicate an expected level of intraspecific variation. RAPD estimates of gene flow on all loci among all populations was high, ranging from 2.4 to 4.0 migrants per generation (Table IV), indicating that the all three populations are very similar, and did not differentiate, not even by genetic drift.
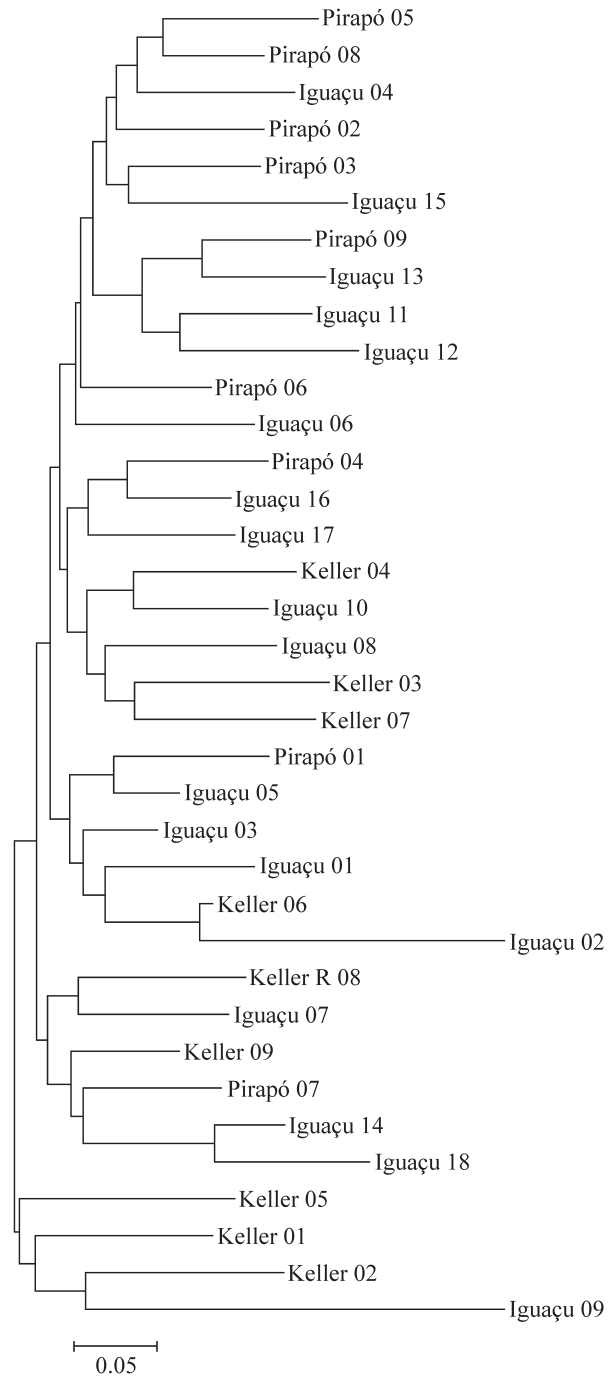
Figure 4 - Neighbor-joining tree, based on the matrix of the arithmetic complement of the similarity coefficient of Nei and Li (1979), obtained from RAPD markers of *Astyanax altiparanae* individuals from the Iguaçu River, and from the Keller and Pirapó tributaries of the Upper Paraná River basin.

The Iguaçu Falls have been considered the principal factor for fish isolation and speciation in the Iguaçu River basin, as initially suggested by Haseman (1911). In fact, most fish species from the Iguaçu River basin have been characterized as endemic, including species of the genus *Astyanax* (Sampaio, 1988; Severi and Cordeiro, 1994; Ga-

ravello *et al*., 1997). Nevertheless, introduced species, admittedly not very abundant, have also been reported in this hydrographic basin (Agostinho and Gomes, 1997). The high genetic similarity and high gene flow estimated among *A. altiparanae* populations from tributaries of the Upper Paraná River basin and from the Iguaçu population, as well as the high genetic diversity within each population, at very similar levels in the two hydrographic basins, demonstrated that the *Astyanax altiparanae* population from the Iguaçu River is not endemic. Such high genetic similarity agrees with the hypothesis of introduction of this species in the Iguaçu River from the Upper Paraná River basin (Garavello *et al*., 1997). Furthermore, the molecular data in the present work suggest a recent separation of the Iguaçu and Upper Paraná populations, mantaning the intrapopulational genetic diversity representative of the ancestral population. In addition, the time or number of generations would not be yet sufficient for mutation accumulation of mutations, as detected by the methodology used. Another possible hypothesis to explain these results, apparently less likely, would be an old introduction of *A. altiparanae* from the Upper Paraná River basin into the Iguaçu River, followed by recurrent migration or transposition of a high number of individuals, to such a degree that the genetic differentiation would be mitigated or eliminated.

Acknowledgments

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