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Molecular data reveal a diverse *Astyanax* species complex in the upper Iguaçu River

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Astyanax is among the most speciose genera in the Characidae. In this study, molecular markers were used to assess the extent to which some of the commonly recognized *Astyanax* species of the upper Iguaçu River correspond to natural groups. These results indicate that the diversity of *Astyanax* has been severely underestimated with several potential cryptic species.

Key words: *Astyanax* cryptic species; D-loop; lambari; species complex.

Species of *Astyanax* have been an important model system in genetic studies of neotropical fishes. Such a wealth of information, however, has still been insufficient to resolve the taxonomy of *Astyanax* species. This situation is particularly severe in the case of *Astyanax* spp. of the Iguaçu River basin, which has a variety of undescribed species commonly referred to in the literature as *Astyanax* sp. B, *Astyanax* sp. C, *Astyanax* sp. D, *Astyanax* sp. E and *Astyanax* sp. F (Sampaio, 1988; Agostinho *et al*., 2007). The Iguaçu River basin is considered a global biodiversity hotspot, because of the endemic and diverse small-bodied fish species, with many endangered species (Abilhoa & Duboc, 2004).

Uncovering accurate estimates of the true diversity of *Astyanax* in the Iguaçu River and their relative divergence times are crucial to understand the evolutionary diversification of the genus in this area of endemism and to foster efficient conservation measures. The main goal of the present study is to use molecular markers to assess the extent to which some of the commonly recognized *Astyanax* species of the upper Iguaçu River correspond to natural groups.

All samples were collected in the upper Iguaçu River (state of Paraná, southern Brazil): 39 individuals from Lapa (25° 47′ 40″ S, 50° 11′ 54″ W), collected

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in December 2005 and 23 individuals from São Mateus do Sul (25° 53′ 24″ S, 50° 25′ 54″ W), collected in July 2006. All individuals were identified according to the available taxonomic characters indicated by Sampaio (1988), Garavello *et al*. (1997) and Ingenito *et al*. (2004) prior to sequencing. Muscle and gill tissues were removed and preserved in EDTA-DMSO buffer and maintained at −20° C. The remaining portions were fixed in 10% formalin and preserved in 70% ethanol. DNA in muscle tissue and gills was extracted using an EZ-DNA kit (Applied Biosystems; www.appliedbiosystems.com). Approximately 800 bp of the mitochondrial control region (D-loop) was amplified using the primers H16498 (5′-CAC CTG AAG TAG GAA CCA GAT G-3′) and L15774M (5′-ACA TGA ATT GGA GGT ATA CCA GT-3′) (Prioli *et al*., 2002). Each 25 μl PCR included 3·4 mM of $MgCl_2$, 0·15 mM of each dNTP, 1× buffer, 0·15 U μl^{-1} of Taq polymerase, 3 μM of each primer, 3·2 ng μl^{-1} of DNA template and 1·6 μl of bovine serum albumin (BSA) 1% (Fluka BioChemika, Buchs, Switzerland). Thermocycling conditions included as initial denaturation at 94° C for 4 min, followed by 35 cycles at 94° C for 45 s, 60° C for 45 s and 72° C for 1·5 min and a final extension at 72° C for 10 min. The PCR product was electrophoresed on a 1·5% agarose gel, stained with ethidium bromide and visualized under UV light. The successfully amplified products were purified with Montage kit (Millipore). Sequencing reactions used the same thermocycling conditions as the PCR. Cycle sequence in 10 μl solution included the following final concentrations: 0·25× buffer, 0·16 μM of each primer, 0·5 μl of BigDye (Applied Biosystems) and 4 ng μl^{-1} of DNA template of each purified product. The final product was purified using Sephadex G50 and processed on an ABI3130 Genetic Analyzer.

Sequences were aligned using CLUSTAL W (Thompson *et al*., 1994). Maximum parsimony analyses were carried out using PAUP* 4.0b10 (Swofford, 2001), with unweighted parsimony. Branch support was obtained through 1000 replicates of non-parametric bootstrapping, each with 10 random taxon additions and a maximum of 100 trees per replicate. Maximum likelihood analyses were conducted using RAxML-VI-HPC (Stamatakis, 2006) with a GTR+G model of evolution and branch support indicated by 600 bootstrap replicates.

Sequences were deposited in GenBank with accession numbers GQ387315–GQ387357. The phylogenetic relationships among the samples indicated a poor correspondence between the present taxonomies of *Astyanax* spp. from the upper Iguaçu River, as defined by morphological characteristics, and their putative monophyly. In particular, several well-supported clades were inconsistent with previously recognized species (Fig. 1). In addition, the only described species, *Astyanax altiparanae* Garutti & Britski, consisted of two divergent lineages, as indicated by a distinctive 32 base pair (bp) deletion (which was absent in all other species and therefore is unlikely to be an insertion). The substantial genetic variability observed within putative morphospecies of *Astyanax* revealed in the present study indicates that the actual species diversity in the genus might have been severely underestimated in the Iguaçu River basin. There seems to be clear discordance between the commonly used morphological traits to discriminate among morphospecies and the genetic data.

Two explanations could account for this pattern: either the morphological characters used in these taxonomies might not provide sufficient resolution to efficiently discriminate these species, and therefore might overlook the existence of cryptic species, or the time span among speciation events has been insufficient for the sorting of mitochondrial lineages, or both. More extensive analyses, particularly including

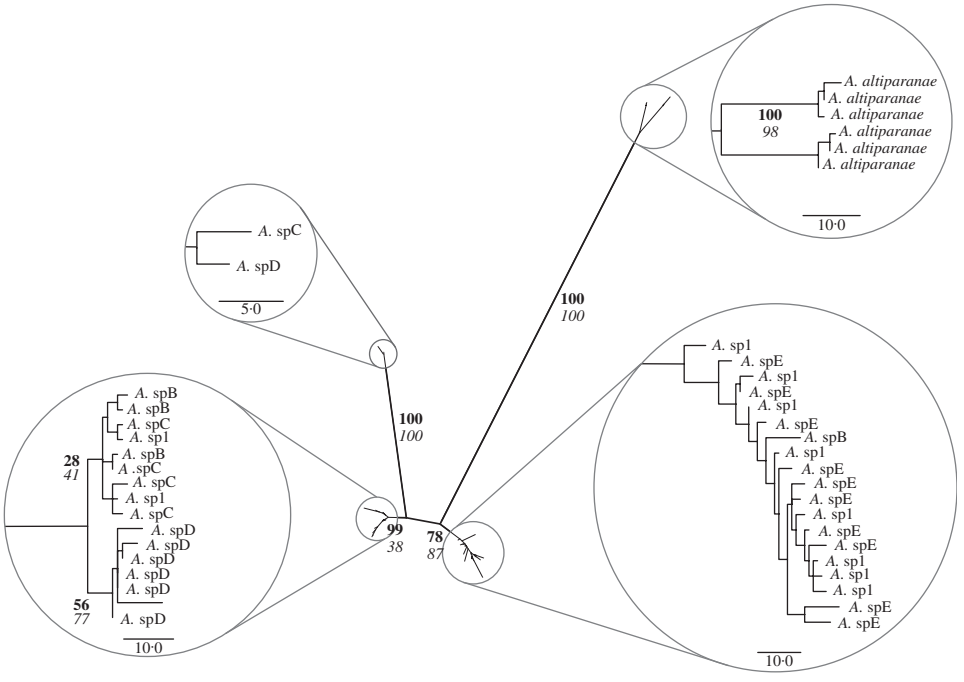

FIG. 1. Unrooted phylogeny of mitochondrial DNA control region haplotypes of *Astyanax* spp. from the upper Iguaçu region. Values near branches indicate bootstrap support values, with bold, and italicized values represent maximum parsimony and maximum likelihood analyses, respectively.

unlinked loci, are essential to discriminate these hypotheses. The repeated pattern of discordance in several putative species, however, suggests that incomplete lineage sorting may have occurred multiple times in several taxa, which seems unlikely at face value. Moreover, cytogenetic variability has been observed among populations of *Astyanax* sp. B, as well as in the nucleolus organizing regions (NOR) of *Astyanax* sp. A, *Astyanax* sp. B, *Astyanax* sp. C and *A. altiparanae* (Kantek *et al*., 2007).

Even though intermediate morphological variation could be associated with hybridization, the diagnostic features used to identify *Astyanax* species, in general, are based on a combination of characters, sometimes with high intraspecific variation. Some morphometric and meristic characters overlap widely, making species identifications difficult. In addition, several authors refer to some species of *Astyanax* as a 'complex of similar species' [*e.g*. *Astyanax* aff. *fasciatus* (Cuvier), *Astyanax* aff. *scabripinnis* (Jeyns)], using chromosomal evidence (Artoni *et al*., 2006) and morphological analyses (Garutti & Britski, 2000). These results indicate that the morphological characters proposed by Sampaio (1988) are still insufficiently informative to distinguish sympatric morphospecies of *Astyanax* in the Iguaçu River basin.

The case of *A. altiparanae*, one of the most common fishes in the Paraná floodplains (Agostinho *et al*., 2007), is of particular interest. Fernandes & Martins-Santos (2004) studied the cytogenetics of specimens from two locations (Índios River, in the Ivaí River basin, and Paraná River, in the region of Porto Rico) and found differences both in karyotype formulae and the number of NOR-bearing chromosomes. Although,

these differences were interpreted as indicating the existence of an *altiparanae* complex in the Paraná basin, distances among collection sites could potentially lead to the sampling of geographical differences among locally isolated populations. Geographical differentiation has indeed been uncovered in *A. altiparanae* using restriction fragment-length polymorphisms (RFLP) of mtDNA, random amplification of polymorphic DNA (RAPD) and allozymes (Moysés & de Almeida-Toledo, 2002; Leuzzi, *et al*., 2004). The present results, however, indicate substantial differentiation among sympatric haplotypes and not among haplotypes from geographically differentiated populations.

The conclusions of the present study are inconsistent with those presented by Prioli *et al*. (2002), in which *A. altiparanae* from the Paraná and Iguaçu Rivers were examined for sequences amplified by the same PCR primers as in the present study and in which all specimens were interpreted to belong to the same species. To resolve this issue, GenBank sequences (accession numbers AY125820-AY125839 and AY395494-AY395513; Prioli *et al*., 2002) were examined. Both groups of haplotypes were present in fish from the Iguaçu and Paraná Rivers based on the annotations of the deposited sequences. The existence of two divergent lineages was independently confirmed by pair-wise differences between haplotypes, either including or excluding the above-mentioned 32 bp deletion (Fig. 2).

Several authors have indicated considerable ecological diversity in *A. altiparanae*, both in diet (Luz & Okada, 1999) and behaviour (Suzuki & Orsi, 2008). The deep divergence observed among sympatric haplotypes, however, indicates that two biological species might be currently recognized by the same name, which could partially explain its ecological versatility. Similar cases have been uncovered in other contexts. For instance, a butterfly species thought to express generalist feeding

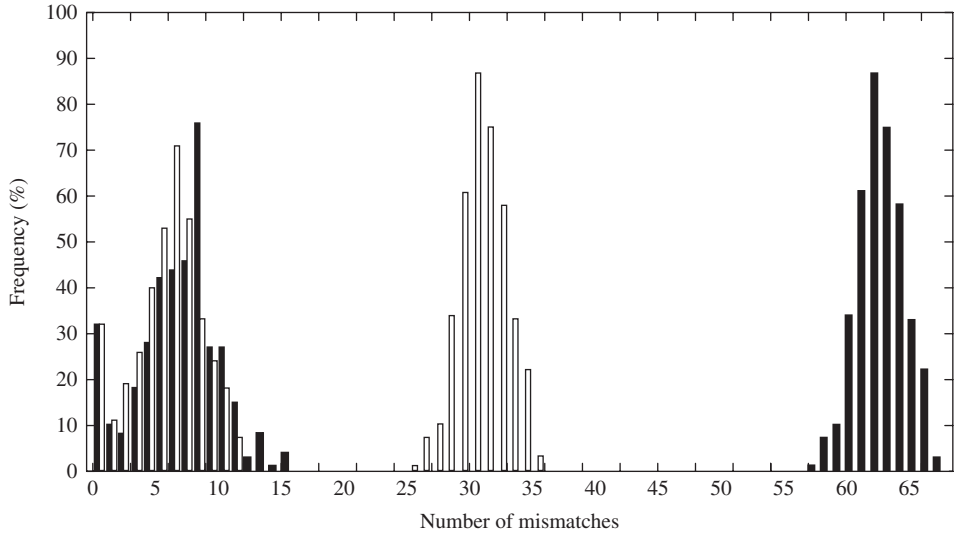

FIG. 2. Distribution of pair-wise differences between mitochondrial DNA control region haplotypes of *Astyanax altiparanae*, available in GenBank. The clearly bimodal distribution is observed either including (■) or excluding (□) the 32 bp deletion from the computations.

behaviour as caterpillars were shown using molecular methods to represent a species complex with several specialist species (Hebert *et al*., 2004).

The existence of widespread species complexes with several cryptic species that are difficult to distinguish based on morphology alone seems to be a recurrent pattern within *Astyanax*, as recently suggested by Artoni *et al*. (2006) for *A*. aff. *fasciatus* and by Moreira-Filho & Bertollo (1991) for *A. scabripinnis*. The results of the present study strongly suggest that molecular data are essential to advance the knowledge of species boundaries and the actual species diversity in *Astyanax*.

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