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# Molecular taxonomy and evolutionary hypothesis concerning *Astyanax fasciatus* (Characiformes, Characidae) from Vila Velha State Park and Tibagi and Iguaçu Rivers

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**ABSTRACT.** A species complex hypothesis involving *Astyanax fasciatus* from southern Brazil was tested using 12S mtDNA sequences. Phylogenetic inferences were performed with maximum likelihood, maximum parsimony and Bayesian as phylogenetic methods and *Hemigrammus bleheri* as the outgroup. Besides 11 sequences from *A. fasciatus*, the data set was comprised of other partial 12S sequences including material from *Astyanax altiparanae* (two sequences) and *Astyanax* sp (four sequences), both from the Iguaçu River. The hypothesis of an *A. fasciatus* species complex was reinforced given the close relationship between *A. altiparanae* and *Astyanax* sp observed in the Bayesian tree. Consequently, a taxonomic revision is necessary for these species.

**Key words:** Fish; Evolution; Systematic phylogeny; Neotropical region

 

## INTRODUCTION

Molecular markers coupled with sophisticated analytical tools have contributed significantly to our understanding of the complex interplay between historical and contemporary processes that shape intraspecific diversification (Brant and Orti, 2003).

The *Astyanax* genus, typically one of the most abundant and diversified genus in the family Characidae in terms of species, presents a wide-ranging geographic distribution that is accompanied by extraordinary genetic and phenotype plasticity. Moreira-Filho and Bertollo (1991), who studied isolated samples from the same hydrographic basin, suggested that *Astyanax scabripinnis* is a species complex. Furthermore, this genus is very important for maintaining the ecology of freshwaters, and hence, it is an excellent model for studies regarding the dynamics of geological and biogeographical histories (Orti and Meyer, 1997; Calcagnotto et al., 2005). In this context, molecular markers have been currently used as tools for assessing genetic variability and endemism, as well as phylogenetic and biogeographic relationships, in *Astyanax altiparanae*, *A. schubarti*, *A. fasciatus*, *A. lacustris*, and *A. scabripinnis paranae* (Prioli et al., 2002; Moysés and Almeida-Toledo, 2002; Leuzzi et al., 2004; Kavalco et al., 2011).

The Vila Velha State Park (VVSP) is characterized by the presence of natural rocky formations assigned as sinkholes and dated from the Pleistocene. These sinkholes are in fact very deep (approximately 50 m), collapsed rocks filled with freshwater. *A. fasciatus* is abundant in inner sinkholes and on the basis of chromosome, random amplification of polymorphic DNA, and morphological studies, they might have been recently isolated from populations of the Tibagi River headwaters (Matoso et al., 2002, 2004; Gross et al., 2004).

Despite increasing efforts to understand the taxonomic, evolutionary, and/or biogeographical dynamics of *A. fasciatus* populations from the sinkholes and from Tibagi River headwaters, their phylogeographical scenario remains unknown. Thus, we aimed to yield a molecular taxonomy and phylogenetic scenario for *A. fasciatus* from VVSP and from the main course of the Tibagi River via analyses of partial mitochondrial 12S ribosomal sequences.

## MATERIAL AND METHODS

Liver samples from 11 specimens identified as *A. fasciatus* from VVSP (Sinkhole 1, Sinkhole 2, Dourada Lagoon) and from the upper Tibagi River microbasin (25° 14' 09" S, 50° 00' 17"W) (Figure 1) were used for DNA extraction by following the methodology described by Sambrook et al. (1989). The voucher specimens were identified and deposited in the ichthyological collection of the Zoology Museum of the Universidade Estadual de Londrina (Paraná, Brazil) (voucher Nos. MZUEL 1792, 1794, 1795). The 12S mitochondrial segment was amplified and sequenced from the whole samples. The polymerase chain reaction (PCR) amplifications were performed by using 50 ng DNA, 50 ng of each primer (H1478 5'-TGACTGCAGAGGGTGACGGGGCGGTGTGT-3' and L1091 5'-AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT-3') (Kocher et al., 1989), 5 mM $MgCl_2$, 2.5 U DNA *Taq* polymerase, 1X buffer solution (200 mM Tris-HCl, pH 8.4, and

500 mM KCl), and 0.4 mM of each dNTP, in a final reaction volume of 50 µL. The profile for PCR consisted of an initial denaturation temperature of 94°C for 4 min, followed by 35 cycles at 92°C for 1 min, hybridization at 60°C for 1 min, elongation at 72°C for 1 min, and a final extension at 72°C for 5 min. All reactions were performed in a thermal cycler (PTC-100 MJ Research, GMI, Ramsey, MN, USA). The images were documented using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290.

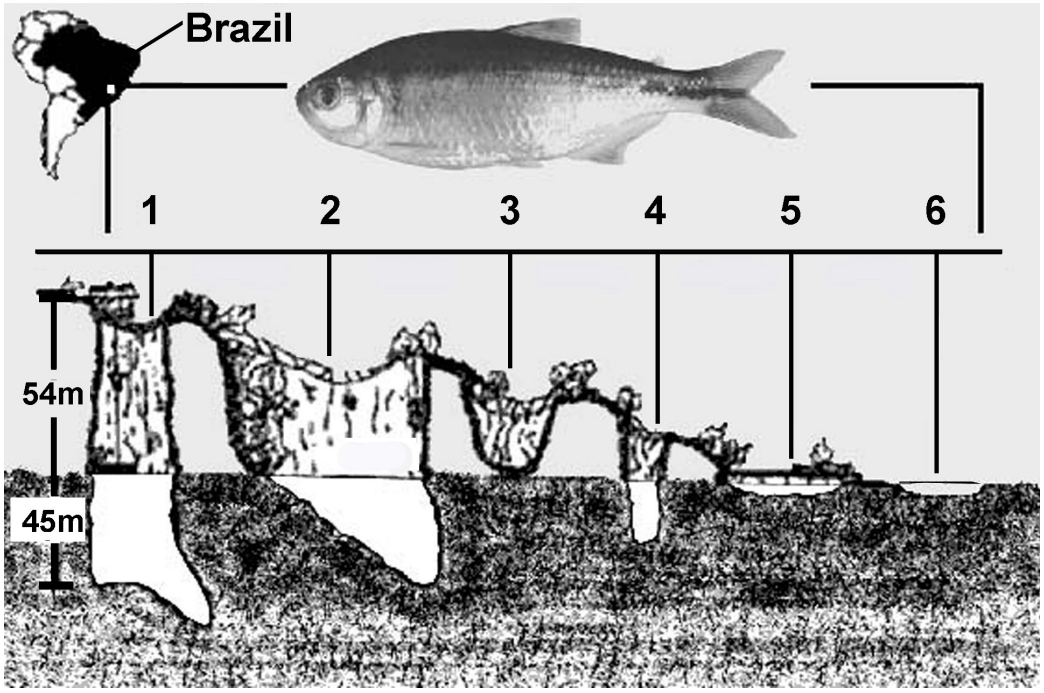


**Figure 1.** Collection sites in the South of Brazil, Ponta Grossa, Paraná. Profile from Vila Velha State Park (Soares, 1989), showing the connection among collection sites. Sinkholes (1, 2, 3, 4) and Dourada Lagoon (5) and Tibagi River (6).

The samples were purified using the Wizard PCR Preps DNA Purification System (Promega) and sequenced using DYEnamic ET Terminator Cycle Sequencing (Amersham-Biosciences), according to manufacturer instructions. All templates were sequenced in both directions by using an ABI 377 DNA sequencer (Applied Biosystems Inc.). These sequences were deposited in GenBank under the accession Nos. AY371869-AY371879. An additional 12S data set was assembled from GenBank by using other *Astyanax* species (Table 1).

The 12S sequences were aligned and edited using CLUSTAL W (Thompson et al., 1994) and improved visually by using BioEdit v.5.0.6 (Hall, 1999). Because of asymmetry as well as bad fluorescent signals in the sequencing reactions, several (initial and final) sites were omitted from the analysis. Pairwise comparisons, statistical information, and phylogenetic analysis were conducted with Paup v. 4.0b10 (Swofford, 2002) by using the maximum

likelihood, maximum parsimony and general time-reversible + gamma distribution models for gene evolution, in accordance with the optimal model of nucleotide evolution for the data set obtained by Modeltest 3.0 (Posada and Crandall, 1998). The posterior probability values obtained using the Bayesian method are shown above the nodes (Figure 2).

**Table 1.** Species names, geographical sites of each samples and GenBank accession number for 12S sequences.

| Species name | Geographical sites of samples | GenBank accession No. |
|---|---|---|
| | | 12S |
| *Astyanax fasciatus* | VVSP - Sinkhole 1 | AY371869 |
| *Astyanax fasciatus* | VVSP - Dourada Lagoon | AY371870 |
| *Astyanax fasciatus* | Tibagi River | AY371871 |
| *Astyanax fasciatus* | Tibagi River | AY371872 |
| *Astyanax fasciatus* | Tibagi River | AY371873 |
| *Astyanax fasciatus* | VVSP - Sinkhole 2 | AY371874 |
| *Astyanax fasciatus* | VVSP - Dourada Lagoon | AY371875 |
| *Astyanax fasciatus* | VVSP - Sinkhole 2 | AY371876 |
| *Astyanax fasciatus* | VVSP - Sinkhole 1 | AY371877 |
| *Astyanax fasciatus* | Tibagi River | AY371878 |
| *Astyanax fasciatus* | Tibagi River | AY371879 |
| *Astyanax altiparanae* | Iguaçu River | AF317206 |
| *Astyanax altiparanae* | Iguaçu River | AF317207 |
| *Astyanax* sp | Iguaçu River b2 | AF319509 |
| *Astyanax* sp | Iguaçu River c19 | AF319511 |
| *Astyanax* sp | Iguaçu River f15 | AF319512 |
| *Astyanax* sp | Iguaçu River f13 | AF319513 |
| *Hemigrammus bleheri* | - | AY130855 |

VVSP = Vila Velha State Park.

The analysis was performed with *Hemigrammus bleheri* as the outgroup. Heuristic searches were performed by stepwise taxon addition (100 replicates), combined with tree-bisection-reconnection as the branch swapping algorithm. Bootstrap analyses were performed to assess the support of the resulting topology and were based on 1000 replicates of the heuristic search described above.

## RESULTS

Aligned sequences totaled 325 bp in length, 238 sites of which were constant and 52 of which were parsimony informative. The parameter in the gamma distribution was $\alpha = 0.2319$, and the estimated base frequencies were A = 0.31150, C = 0.24140, G = 0.21950, and T = 0.22760. A single well-supported topology was found in this study with the 3 methods used. The most parsimonious tree with 151 evolutionary steps is shown in Figure 2. The consistency index was 0.7219, the homoplasy index was 0.2781, the retention index was 0.8909, and the rescaled consistency index was 0.6431. The posterior probability values are given above the nodes as Bayesian inference. According to this set data, *A. fasciatus* from VVSP and the Tibagi River is a paraphyletic taxon because it has been grouped with *A. altiparanae* and *Astyanax* sp, both of which are from the Iguaçu River.

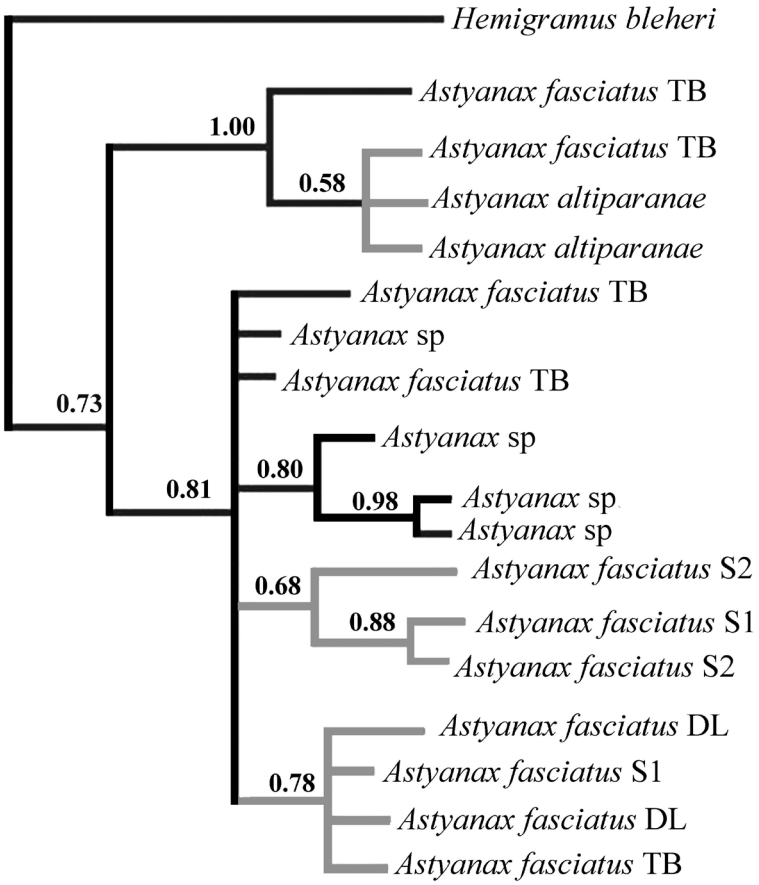


**Figure 2.** Topology obtained for three phylogenetic methods: maximum likelihood, maximum parsinomy and Bayesian. Values above the branches indicate the posterior probability values. The gray clades show the phylogenetic discountinuity among the samples of *Astyanax fasciatus* from Tibagi River (TB), Sinkoles 1 and 2 (S1 and S2), and Dourada Lagoon (DL).

## DISCUSSION

Systematic studies based on mtDNA have been increasingly used to understand diversification processes at several levels of organization, from populations and species to higher taxonomic levels (Sivasundar et al., 2001). Thus, according to our results, there is a remarkable overlapping of morphological autapomorphic features of *A. fasciatus*, given that the resulting topology shows that this species is closely related to *A. altiparanae* as well as *Astyanax* sp. Indeed, the trichotomy involving *A. fasciatus* from the Tibagi River and the two *A. altiparanae* from the Iguaçu River might be the result of the lack of a clear morphological identity among them. However, the admixture between morphologically close species and mtDNA introgression cannot be rejected.

Apart from the present approach that used nucleotide sequence data, several genetic tools have been used to translate the relationship and define the taxonomic status of *A. fasciatus*. In this context, Matoso et al. (2004) and Artoni et al. (2006) have previously suggested the occurrence of a cryptic species complex in the upper Tibagi River. The clustering between *Astyanax* sp (Iguaçu River) and *A. fasciatus* (Tibagi River) obtained in this study provides additional support for this hypothesis. According to Funk and Omland (2003), cryptic and taxonomically controversial species might interfere in the resolution of evolutionary trees; however, a polyphyletic condition might represent the real tree for the selected mitochondrial gene, and it might not be a simple artifact, depending on the branch-supporting values found in the tree (Kavalco et al., 2011).

Furthermore, Domingues et al. (2007) have questioned the processes responsible for the biogeographic relationships between Tibagi and Iguaçu headwaters, despite empirical observations that suggest the opposite - a vicariant model could explain the present distribution of *Astyanax*, given that both the hydrographic basins share natural fish fauna since the uplifts of Brazilian crystalline shields in the late Cretaceous period (Ribeiro, 2006). Therefore, the close relationships between both fauna headwaters could be the remnant of a recent common ancestry, a hypothesis reinforced by weak bootstrap values and high genetic homogeneity found for populations of *A. fasciatus* in the Iguaçu River (Prioli et al., 2002), in addition to the occurrence of *Mimagoniates microlepis* throughout both river headwaters (Ingenito et al., 2004; Santanna et al., 2006; Torres et al., 2008).

The results of this study suggest three possible explanations for the data: i) for the trichotomy observed, the occurrence of a single species, *A. fasciatus* or *A. altiparanae* or another species; ii) an mtDNA introgression between *A. altiparanae* and *A. fasciatus* and consequent sharing of mtDNA haplotypes, and iii) a misidentification of the species. Kavalco et al. (2011) found monophyly in the *A. altiparanae* group, in agreement with the nominal redefinition proposed by Garutti and Britski (2000) for this species. However, when these authors analyzed the ND2 region of mtDNA, they found a possible paraphyly in the species that was attributable to sharing of haplotype groups between the Tietê and Paranapanema Basins.

In conclusion, all the evidence reinforces *A. fasciatus* as an obscure taxon and emphasizes the need for a systematic revision to distinguish and delimit the species complex. The data emphasize the need for a broad revision in the taxa comprising the *Astyanax* genus, specifically *A. fasciatus* species from VVSP and Tibagi River headwaters. Therefore, studies about the geomorphologic evolution of the region and dispersal events need to be performed to develop the evolutionary scenario of the natural species and populations in these localities.

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