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# Detection of natural hybridization and delimitation of two closely related operational taxonomic units of the *Astyanax fasciatus* (Teleostei: Characidae) complex through integrative approaches

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*Astyanax* is a species-rich, non-monophyletic genus composed of several supraspecific taxa that are poorly delimited. The *Astyanax fasciatus* complex is one of these taxa and shows high taxonomic complexity. To elucidate the evolutionary history of the *A. fasciatus* complex from southern South America, we conducted cytogenetic, molecular and morphological analyses in specimens from the Uruguay River basin. Cytogenetic characters demonstrated two closely related operational taxonomic units: *Astyanax* sp. 1 (8*m*+22*sm*+10*st*+6*a*), *Astyanax* sp. 2 (8*m*+24*sm*+10*st*+4*a*) and natural hybrids (8*m*+23*sm*+8*st*+5*a*). 5S ribosomal DNA sites were found in two pairs of *m* chromosomes and one pair of *a* chromosomes in *Astyanax* sp. 1, two pairs of *a* chromosomes and one pair of *m* chromosomes in *Astyanax* sp. 2, and three *m* chromosomes and three *a* chromosomes in hybrids. *As*51 sites were found in three chromosomes in *Astyanax* sp. 1 and in five chromosomes in *Astyanax* sp. 2 and hybrids. Mitochondrial sequence analyses did not separate the two units and hybrids. Morphological analyses revealed differences between *Astyanax* sp. 2 and hybrids. This secondary contact with gene flow between lineages that diverged long ago might slow or reverse the differentiation/speciation process. These results help us to understand the evolutionary history of this highly complex clade of *Astyanax* in southern South America.

ADDITIONAL KEYWORDS: *COI* – *Cytb* – cytogenetics – grey zone – karyotype – mitochondrial DNA – secondary contact zone – sibling species.

## INTRODUCTION

Considered one of the hotspots for new fish species, the Neotropical region contains the largest known freshwater ichthyofauna, with estimates of between 5600 and 9000 species (Reis *et al.*, 2016; Birindelli & Sidlauskas, 2018). One of the most species-rich groups is the characiform *Astyanax* Baird & Girard, 1854, with ~160 valid species (Eschmeyer & Fong, 2019) and several taxonomic uncertainties (Rossini *et al.*, 2016; Mirande, 2018; Pazza *et al.*, 2018). It is a non-monophyletic group with morphologically

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similar species and indefinite taxonomic delimitations, which makes species identifications and phylogenies imprecise in many subgroups (Oliveira *et al.*, 2011; Mirande, 2018). Furthermore, several species have been described based on a few individuals or have been identified erroneously, causing problems such as synonymies or under/overestimation of species diversity within the genus (Mirande & Koerber, 2015).

The number of new fish species described has been increasing in recent years (Reis *et al.*, 2016). However, the presence of cryptic species and high morphological similarity, mainly within species complexes, i.e. groups of species that share very similar morphological characteristics and high chromosomal variability (Moreira-Filho & Bertollo, 1991), has made identification difficult, and it is possible that the number of species is even higher. Thus, integrative approaches have been gaining strength in recent years, combining different tools and new concepts and methods for the delimitation and classification of species boundaries (Dayrat, 2005; Valdecasas *et al.*, 2008; Padial *et al.*, 2010), including those of Neotropical freshwater fishes (e.g. Guimarães *et al.*, 2019; Serrano *et al.*, 2019).

The question of species delimitation has long been confused with that of species conceptualization, and several different concepts of species have emerged, each using different properties or characteristics to delimit it. However, the process of speciation is not uniform; it does not lead to changes in all aspects of the organism (morphological, ecological, genetic, etc.) at the same time, generating a grey zone (De Queiroz, 2007). According to De Queiroz (2007), the grey zone is the moment in the speciation process when, depending on the characters evaluated, it is possible to reach different conclusions regarding the existence of one or more species.

In order to avoid the conflicts generated using alternative concepts of species, many researchers define species as lineages of populations or metapopulations evolving independently (De Queiroz, 2007; Padial *et al.*, 2010). In addition, the use of different characters proposed by integrative taxonomy can provide greater reliability and minimize underestimation/overestimation of biodiversity (Padial *et al.*, 2010; Venkatraman *et al.*, 2019), especially in complex groups, such as *Astyanax*.

Cytogenetic (Centofante *et al.*, 2003; Pazza *et al.*, 2008a, b) and morphological (Melo & Buckup, 2006) approaches suggest that *Astyanax fasciatus* (Cuvier, 1819) corresponds to a species complex composed of several species, in which only those specimens from the São Francisco River (type locality of the species) would be the *A. fasciatus s.s.*, whereas specimens from other regions, widespread in almost all South American river basins, would be named *Astyanax* aff. *fasciatus*. Those specimens reported from the Amazon River basin and other basins (in Ecuador, Bolivia, Colombia, Uruguay and Venezuela) could either belong to new species or represent misidentifications (Eschmeyer & Fong, 2019); for instance, Mirande & Koerber (2015) identified specimens from Argentina and Uruguay as *A.* aff. *fasciatus* that might be *A. aramburui* Protogino, Miquelarena & López, 2006 or *A. rutilus* (Jenyns, 1842), thus requiring a detailed taxonomic revision of the group.

In this study, we aimed to expand the cytogenetic and molecular data for specimens currently identified as belong to the *A. fasciatus* complex from the Uruguay River basin, southern South America. We intended to identify possible efficient markers: (1) to recognize operational taxonomic units (OTUs); and (2) and to verify whether the chromosomal evolution is compatible with the molecular and morphological evolution. We also described the cytogenetics for the *A. fasciatus* complex, compared the mitochondrial DNA with that available from the data of Rossini *et al.* (2016) and investigated morphological traits, in an integrative approach.

## MATERIAL AND METHODS

Voucher specimens were deposited in the fish collection of the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura, Universidade Estadual de Maringá, Maringá, Brazil (NUP). All specimens were identified, morphologically, as *Astyanax* aff. *fasciatus* (NUP 15740, NUP 17346 and NUP 17784) from the Ijuí River, Ijuí, Rio Grande do Sul, Brazil (28°18′06.30″S; 53°53′33.60″W), in the upper-middle Uruguay River basin (Fig. 1). Samples were collected in three periods (November 2013, April 2014 and May 2015) and were split into three subgroups based on a preliminary cytogenetic analyses: *Astyanax* sp. 1 (one male and one female); *Astyanax* sp. 2 (eight males and six females); and natural hybrids (six males and two females). This study was carried out in strict accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals*, approved by the Committee on the Ethics of Animal Experiments of the Universidade Estadual do Oeste do Paraná (licence number: protocol 13/09—CEEAAP/Unioeste). All individuals were anaesthetized and killed by an overdose of clove oil, following Griffiths (2000).

### Cytogenetic analyses

For all cytogenetic analyses, we used all specimens sampled from the Ijuí River. Chromosome preparations were obtained from cells of the anterior region of the kidney, following Bertollo *et al.* (1978).

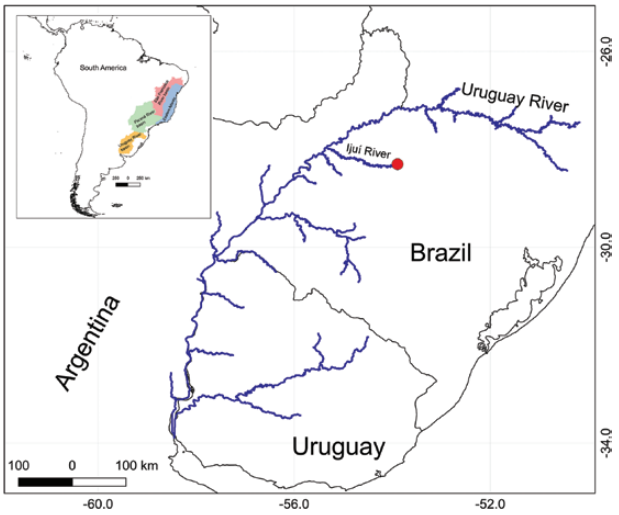


**Figure 1.** Map of South America, showing the distribution of *Astyanax fasciatus s.s.* in the São Francisco basin and other *Astyanax* aff. *fasciatus*, usually identified in the Paraná River basin, Uruguay River basin and Eastern Atlantic hydrographical region. The red dot represents the material analysed in this study from the Ijuí River, upper-middle Uruguay River basin.

Nucleolar organizing regions (NORs) were revealed by silver impregnation according to Howell & Black (1980), and C-banding followed Sumner (1972), with modifications suggested by Lui *et al.* (2012). Physical mapping of 5S ribosomal DNA (rDNA), 18S rDNA and *As*51 satellite DNA was carried out by fluorescence *in situ* hybridization (FISH) according to Pinkel *et al.* (1986) and modifications suggested by Margarido & Moreira-Filho (2008), using DNA probes obtained from *Megaleporinus obtusidens* (cited as *Leporinus elongatus*; Martins & Galetti-Jr, 1999), *Prochilodus argenteus* (Hatanaka & Galetti-Jr., 2004) and *Astyanax scabripinnis* (Mestriner *et al.*, 2000), respectively. Probes were labelled by the nick translation method with digoxigenin-11-dUTP (5S rDNA and *As*51) and biotin-16-dUTP (18S rDNA) (Roche). Detection of signals was performed with antidigoxigenin–rhodamine (Roche) to probe the 5S rDNA and *As*51, and amplified avidin–fluorescein isothiocyanate with biotinylated anti-avidin (Sigma-Aldrich) to probe the 18S rDNA, with the chromosomes counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 50 µg/mL). Metaphases were photographed using a BX 61 epifluorescence microscope, coupled with an Olympus DP 71 digital camera (Olympus America, Inc.) with the Olympus DP Controller software v.3.2.1.276. Chromosomes were classified and organized in accordance with Levan *et al.* (1964) as metacentric (*m*), submetacentric (*sm*), subtelocentric (*st*) and acrocentric (*a*). The fundamental number (FN) was calculated considering *m*, *sm* and *st* chromosomes as having two arms and *a* chromosomes as having only one chromosome arm.

### Molecular analyses

For molecular analyses of mitochondrial data, we used 11 specimens of *Astyanax* aff. *fasciatus* from the Ijuí River. Extraction of total DNA from hepatic tissue preserved in 100% ethanol was performed with the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) following the manufacturer's recommendations. The genomic DNA was quantified on a nanospectrophotometer NanoK (Kasvi) and then diluted to a concentration of 10 ng/µL.

For amplification of mitochondrial genes cytochrome *c* oxidase subunit I (*COI*) and cytochrome B (*Cytb*), we used the primers FISH F1 and FISH R1 (Ward *et al.*, 2005), GluDGF (Palumbi, 1996) and H16460R (Perdices *et al.*, 2002), respectively. The final polymerase chain reaction (PCR) products were purified using the Wizard Kit SV Gel and PCR Clean-up System (Promega), according to the manufacturer's guidelines. Samples were sequenced at the Centro de Pesquisa do Genoma Humano, Universidade de São Paulo, Brazil.

Sequences were then edited with BioEdit (Hall, 1999) and aligned with the MUSCLE algorithm (Edgar, 2004) implemented in Geneious v.11.1.2 (Kearse *et al.*, 2012). Two matrices were assembled: the first included a total of 1688 terminals, mostly retrieved from Rossini *et al.* (2016), in order to determine the phylogenetic position of our focal taxa; and the second included 88 terminals selected after examination of major subgroups from the first matrix. Saturation signal was checked by DAMBE v.7.0.28 (Xia, 2018), and the best-fitting model was chosen using the Akaike information criterion and jModelTest2 (Darriba *et al.*, 2012).

Sequences of the second matrix were binned into species groups to calculate the overall mean distance (distance among all specimens), the intraspecific distances (among specimens of each species) and interspecific distances (among species groups), with 1000 bootstrap replicates. Groups (total = 10) were ordered based on preliminary topologies [neighbour-joining (NJ) and maximum likelihood (ML) analyses]. The NJ tree with 1000 bootstrap replicates was generated in Geneious v.11.1.2. The best ML tree was obtained in RAxML PTHREADS-SSE3 implemented in RAxML v.8.019 (Stamatakis, 2014) using the GTRGAMMA model (Stamatakis *et al.*, 2008) and 1000 bootstrap replicates. Maximum likelihood analyses were performed in both matrices of *COI* and also for *Cytb*.

### Morphological analysis

Morphological analyses usual for taxonomic studies of *Astyanax* were also performed for comparative purposes. The measurements were taken from the left side of the specimens using digital callipers and followed Fink & Weitzman (1974) and Menezes & Weitzman (1990). For a better interpretation of the morphometric data, the values were square root transformed, and summarized with a principal coordinates analysis (PCoA; Legendre & Legendre, 2012), in a Euclidean similarity matrix with 9999 randomizations. Principal coordinates analysis is a generalization of principal components analysis, in which the eigenvalues are extracted from a similarity or distance matrix (Borcard *et al.*, 2011; Legendre & Legendre, 2012). The main advantage of this method is that it can be applied when the relationships between the variables are not linear. Axes with positive eigenvalues were retained for interpretation (Borcard *et al.*, 2011). To test for significant differences in morphometric characters square root transformed between *Astyanax* sp. 2 and natural hybrids (because only two individuals *Astyanax* sp. 1 were recorded, it was excluded from this analysis), we used a permutational multivariate analysis of variance (Permanova; Anderson, 2001). This test is sensitive to differences in dispersion among the groups (Anderson, 2004). Differences among the groups (the two fish species) detected using Permanova might be attributable either to differences in morphometric characters between fish species or to the inter-individual variability in the characters within each fish species. Thus, if the Permanova result showed a significant difference, a permutation analysis of multivariate dispersions (Permdisp; Anderson, 2004) was used. The Permdisp was used to measure the distance between each individual fish specimen and their group median (centroid) and to evaluate the difference in the centroid distances among the groups (Anderson, 2004). This analysis allowed the verification of which fish species showed a more uniform morphometric character matrix (smaller inter-individual variation) and whether this variability varied significantly between fish species. The resulting $F$ statistic of this analysis was tested using the Monte Carlo method with 9999 randomizations. The permutational ANOVA was used to determine whether the morphometric characters differed in relationship to the dispersion of a fish species to another. If Permanova identified a difference in morphometric characters between fish species, a similarity percentage analysis (Simper) using the Bray–Curtis dissimilarity measure (Clarke, 1993) was performed to identify which morphometric character metric contributed most to the dissimilarity between the fish species. All statistical analyses were conducted in the R programming environment (R Core Team, 2019) using the vegan package (Oksanen *et al.*, 2015; The R Project for Statistical Computing, http://www.r-project.org/). The level of statistical significance for all analyses was $P < 0.05$.

## RESULTS

### Chromosomal markers

#### *Astyanax sp. 1 (OTU 1)*

The diploid number was 46 chromosomes (8*m*+22*sm*+10*st*+6*a*, NF = 86) for the male and the female (Fig. 2A). A single pair of NORs was located in a terminal position on the short arm of chromosome *st* pair 19 and confirmed by 18S rDNA FISH [Fig. 2A (box), C]. C-Banding showed centromeric heterochromatin blocks, coincident with NORs (Fig. 2B). FISH revealed multiple 5S rDNA sites in a centromeric position in the *m* pair 3 and in the *a* pairs 22 and 23 (Fig. 2C). *As*51 satellite DNA was located in an interstitial position on the long arm in one of the *sm* chromosomes of pair 11 and coincident with NORs [Fig. 2C (box)].

#### *Astyanax sp. 2 (OTU 2)*

The diploid number was 46 chromosomes (8*m*+24*sm*+10*st*+4*a*, NF = 88) for males and females (Fig. 2D). A single pair of NORs was located in a terminal position on the short arm of chromosome *st* pair 20 and confirmed by 18S rDNA FISH [Fig. 2D (box), F]. C-Banding showed centromeric heterochromatin blocks, coincident with NORs (Fig. 2E). FISH revealed multiple 5S rDNA sites in a centromeric position in the *m* pairs 2 and 3 and in the *a* pair 23 (Fig. 2F). *As*51 satellite DNA was located in an interstitial position in the long arm of pair 11, in a terminal position in the long arm in one of the *a* chromosomes of the pair 23 and coincident with NORs [Fig. 2F (box)].

#### *Natural hybrid*

The diploid number was 46 chromosomes (8*m*+23*sm*+10*st*+5*a*, NF = 87) for males and females (Fig. 2G). A single pair of NORs was located in a terminal position on the short arm of chromosome *st* pair 19 and confirmed by 18S rDNA FISH [Fig. 2G (box), I]. C-Banding showed centromeric heterochromatin blocks, coincident with NORs (Fig. 2H). FISH revealed multiple 5S rDNA sites in a centromeric position in the *m* pair 3 and one of the *m* chromosomes of pair 2, and in the *a* pair 23 and one of the *a* chromosomes of the pair 24 (Fig. 2I). *As*51 satellite DNA was located in an interstitial position

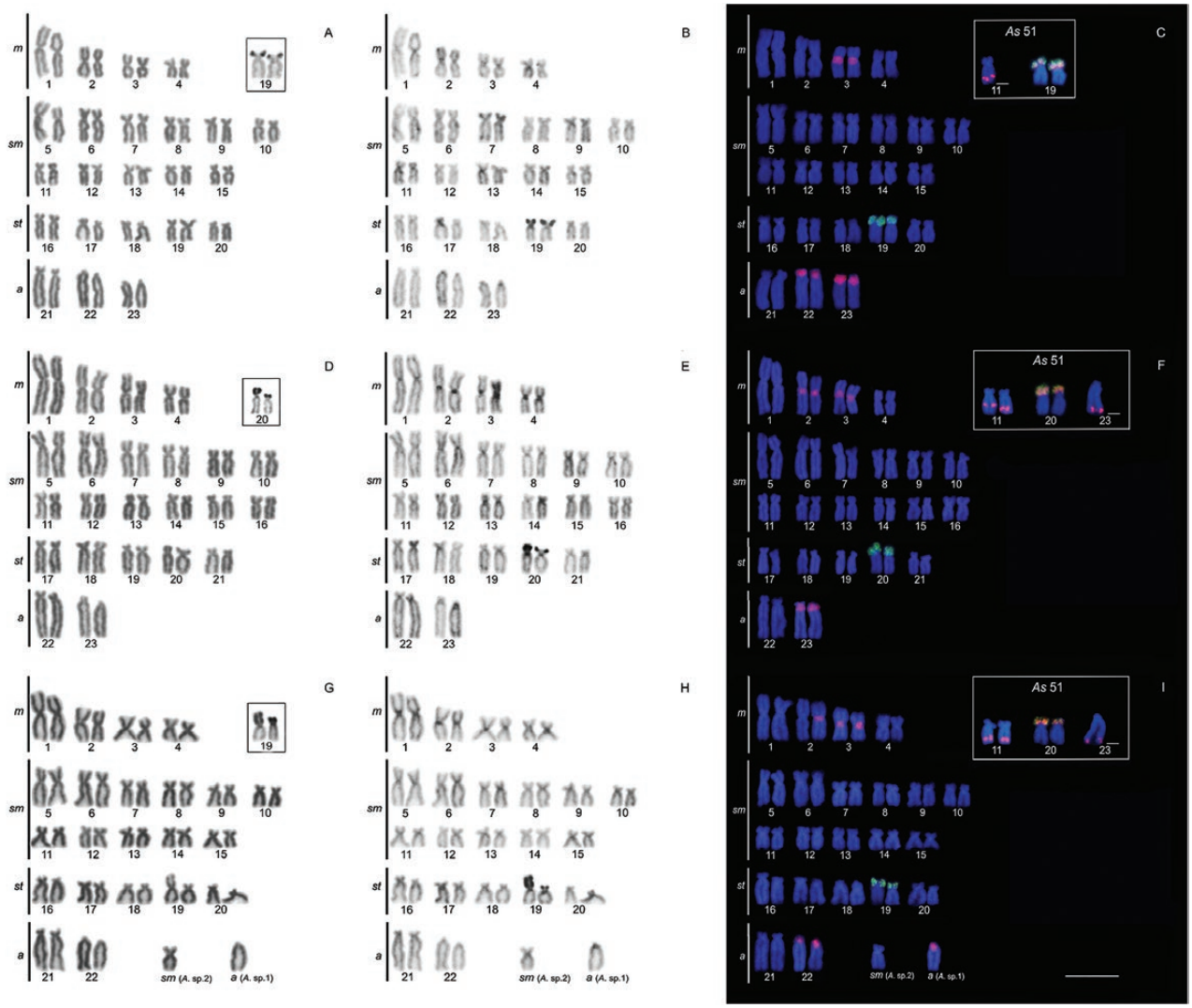


**Figure 2.** Karyotypes stained with Giemsa (NORs in box), C-banded and fluorescence *in situ* hybridization (FISH) with 5S rDNA (red) and 18S rDNA (green) probes (*As*51 satellite in box): A–C, *Astyanax* sp. 1; D–F, *Astyanax* sp. 2; and G–I, hybrids. Scale bar=10 μm.

in the long arm of pair 11, in a terminal position in the long arm in one of the *a* chromosomes of pair 23, coincident with NORs [Fig. 2I (box)].

### Phylogenetic analyses of mitochondrial data

Two mitochondrial genes were used for phylogenetic analyses: *COI* (two matrices) and *Cytb* (one matrix) (Supporting Information, Files S1–S3). Sequences are available in GenBank with the following accession numbers: *COI*, MN820987–MN820997 and *Cytb*, MN802998–MN821007. The first *COI* matrix contained 1688 terminals and 633 bp, with 351 variable sites (55%). The results showed clearly that the analysed specimens of the *A. fasciatus* complex from the Ijuí River were embedded within group 1, containing the *A. fasciatus* species complex (Supporting Information, Fig. S1). We then included representatives of the closer lineages based on the ML tree to construct a reduced matrix containing 88 terminals, 633 bp and 62 variable sites (9%). We report the distance analysis only for the reduced matrix (genetic lineages of closely related species of *Astyanax* group 1). The overall mean distance was 0.013 ± 0.003. The results showed very low distance values between lineages of *Astyanax*, with 28 out of 45 pairwise estimates (62%) < 0.02. Pairwise genetic distances ranged from 0.004 between *Astyanax bockmanni*, *Astyanax pampa* and *Astyanax* sp., to 0.048 ± 0.010 between *Astyanax xavante* and *Astyanax goyanensis* (Table 1). The *Cytb* matrix consisted of 1038 bp and 154 variable sites (14%). The low genetic variation among individuals of *A.* aff. *fasciatus* was

**Table 1.** Pairwise Kimura two-parameter genetic distance among genetic lineages of closely related species of *Astyanax* group 1 (below diagonal) and values of standard error (above diagonal)

| | Species | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *Astyanax xavante* | – | 0.010 | 0.009 | 0.009 | 0.009 | 0.009 | 0.009 | 0.009 | 0.009 | 0.009 |
| 2 | *Astyanax goyanensis* | 0.048 | **0.001** | 0.009 | 0.009 | 0.009 | 0.008 | 0.008 | 0.008 | 0.008 | 0.009 |
| 3 | *Astyanax scabripinnis* | 0.041 | 0.037 | **0.000** | 0.006 | 0.005 | 0.005 | 0.005 | 0.006 | 0.006 | 0.006 |
| 4 | *Astyanax parahybae* | 0.044 | 0.042 | 0.019 | **0.001** | 0.006 | 0.005 | 0.006 | 0.005 | 0.006 | 0.006 |
| 5 | *Astyanax bifasciatus* | 0.046 | 0.038 | 0.016 | 0.016 | **0.001** | 0.004 | 0.004 | 0.004 | 0.004 | 0.005 |
| 6 | *Astyanax bockmanni* | 0.045 | 0.033 | 0.015 | 0.015 | 0.008 | **0.001** | 0.003 | 0.003 | 0.003 | 0.003 |
| 7 | *Astyanax* sp. (present study) | 0.045 | 0.033 | 0.017 | 0.017 | 0.010 | 0.004 | **0.000** | 0.003 | 0.003 | 0.003 |
| 8 | *Astyanax biotae* | 0.043 | 0.035 | 0.017 | 0.013 | 0.010 | 0.004 | 0.004 | **0.000** | 0.003 | 0.003 |
| 9 | *Astyanax pampa* | 0.043 | 0.035 | 0.017 | 0.017 | 0.010 | 0.004 | 0.004 | 0.004 | **0.001** | 0.003 |
| 10 | *Astyanax fasciatus* | 0.045 | 0.037 | 0.019 | 0.019 | 0.012 | 0.006 | 0.006 | 0.006 | 0.006 | **0.000** |

Numbers in bold represent the intraspecific genetic distance. Groups were ordered based on the maximum likelihood analysis.

also observed in the analysis of *Cytb* (Supporting Information, Fig. S2).

Phylogenetic analysis based on the ML tree of the *COI* gene showed that specimens from the Ijuí River analysed here were closer to specimens of *A. fasciatus*, *Astyanax eigenmanniorum* and *Astyanax* sp. from the Uruguay River and the lower Paraná River (Fig. 3). This species appeared closer to *A. pampa* from coastal drainages of the Brazilian state of Rio Grande do Sul and *Astyanax biotae* from the Paranapanema River, upper Paraná River basin.

### Morphological analyses

The PCoA summarized the morphometric characters and distinguished *Astyanax* sp. 2 and natural hybrids (Fig. 4). The first two axes explained 94% of the overall variance and were retained for interpretation. The position of each fish specimen along axis 1 (Fig. 4) revealed that the morphometric characters separated *Astyanax* sp. 2 and natural hybrids. The permutational multivariate ANOVA revealed significant differences in the morphometric characters between *Astyanax* sp. 2 and natural hybrids (Permanova; pseudo-$F$ = 5.28; $P$ = 0.02). In addition, these differences were not promoted by the inter-individual variability in the characters in each fish species, because the permutation analysis of multivariate dispersions revealed no differences in dispersion within *Astyanax* sp. 2 and within natural hybrids (Permdisp; $F$ = 3.12, $P$ = 0.09; Fig. 4). The Simper analysis showed that the individual contribution of each variable to species separation was low. The contribution of 11 added variables accounted for 50% of the dissimilarity between *Astyanax* sp. 2 and natural hybrids. The highest mean values for all morphometric variables were recorded for natural hybrids. Of all the measurements used in the analysis, standard length was the one that contributed the most to separate *Astyanax* sp. 2 and natural hybrids (Table 2).

## DISCUSSION

The cytogenetic data of the present study showed the existence of two closely related OTUs and the presence of natural hybrid individuals, through cytogenetic markers 5S rDNA, *As*51 and the karyotype formula. The 5S rDNA sites presented distinct patterns for each species and an intermediate pattern for the hybrid individuals. Studies using this marker in the *A. fasciatus* complex indicate a conserved condition of this character on a pair of *m* chromosomes and on a pair of *a* chromosomes (Pazza *et al.*, 2008b; Hashimoto *et al.*, 2011; Ferreira-Neto *et al.*, 2012). Exceptions

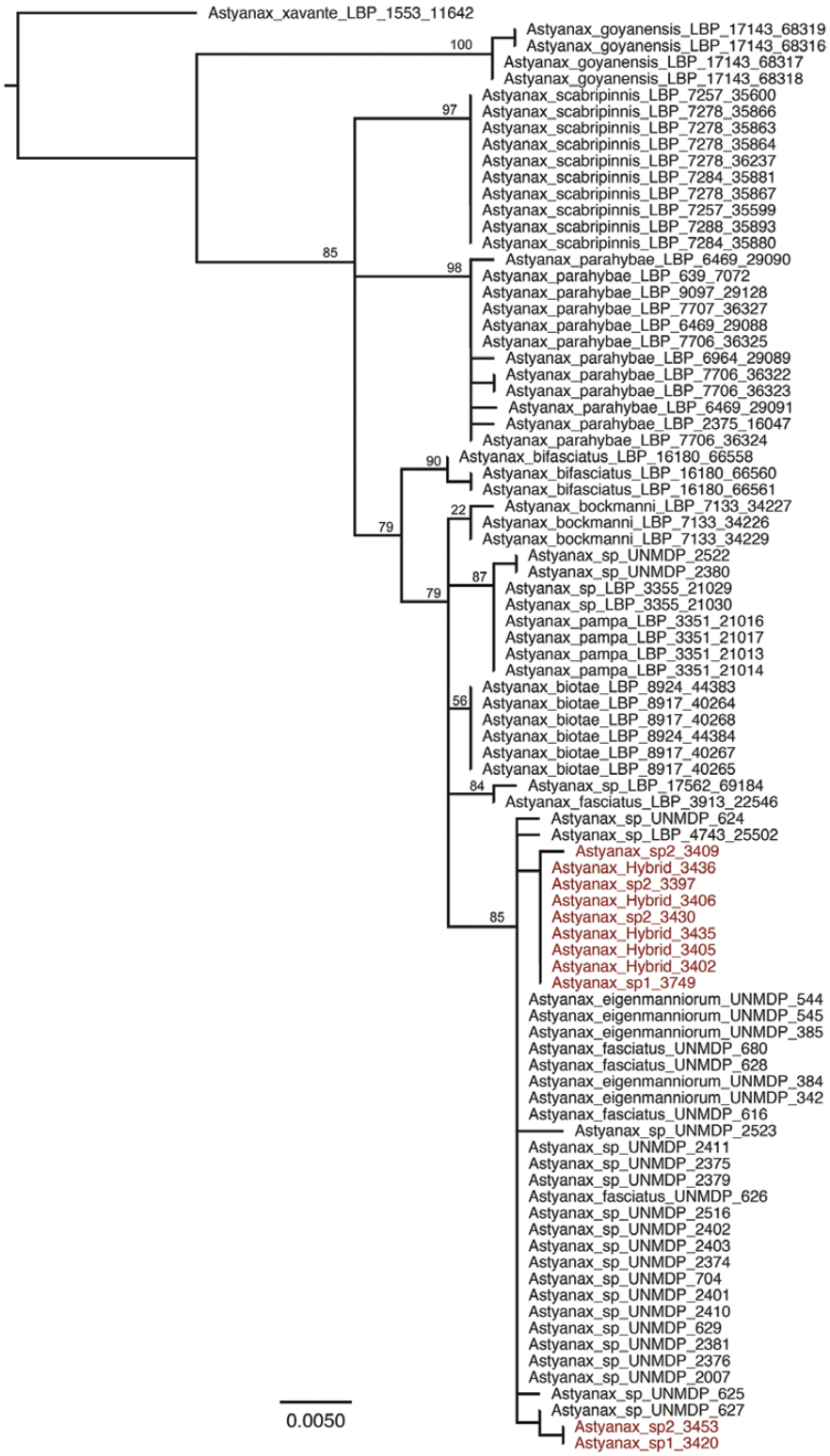


**Figure 3.** Best maximum likelihood tree based on the cytochrome *c* oxidase subunit I gene, showing the position of analysed *Astyanax fasciatus* complex (*Astyanax* sp. 1, *Astyanax* sp. 2 and *Astyanax* hybrids) from the Ijuí River.

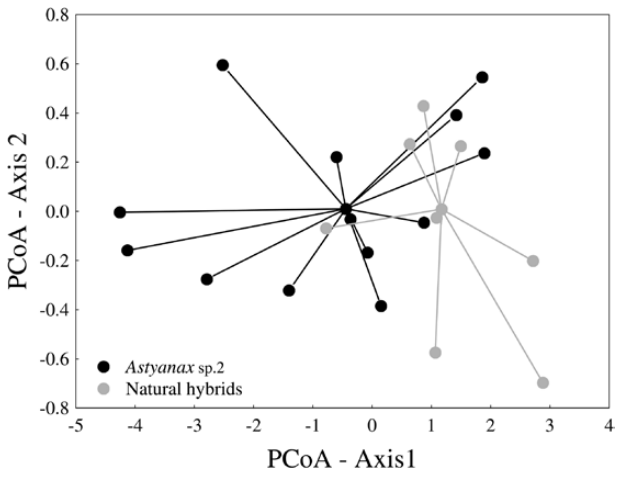


**Figure 4.** Principal coordinates analysis (PCoA) of the morphometric characters for *Astyanax* sp. 2 and natural hybrids caught in the Ijuí River, in November 2013, April 2014 and May 2015.

are found in populations of the São Francisco River basin (Kantek *et al.*, 2009; Peres *et al.*, 2009), Ribeira de Iguape River (Kavalco *et al.*, 2016), in the Eastern Atlantic hydrographical region (Medrado *et al.*, 2015) and in the Uruguay River basin (present study), indicating that these genes might have dispersion mechanisms similar to those described for other repeating sequences.

The localization of 5S rDNA genes in the genus is considered to be conserved in the pericentromeric region of chromosomes (Almeida-Toledo *et al.*, 2002; Vicari *et al.*, 2008), hindering the occurrence of unequal exchange events, which act mainly in the dispersion of sequences located in terminal positions on chromosomes (Martins & Wasko, 2004). However, the dispersion of the 5S rDNA genes found in the present study and in other characiform species, such as *A. scabripinnis* (Castro *et al.*, 2015), *Characidium* (Pucci *et al.*, 2014) and *Hoplias* (Blanco *et al.*, 2010), seems to indicate that, although protected, these genes do not present a conserved evolution and represent an efficient marker for the identification and diagnosis of the species (Almeida-Toledo *et al.*, 2002; Hashimoto *et al.*, 2011; Kavalco *et al.*, 2016; Gavazzoni *et al.*, 2018). Furthermore, 5S rDNA proved effective for the identification of natural hybrids, as also observed in some cyprinids (He *et al.*, 2012, 2013; Zhang *et al.*, 2015).

Despite maintenance of the diploid number of 46 chromosomes, the OTUs analysed here presented differences regarding the karyotype formula with respect to the number of *sm* and *a* chromosomes and the NF, with hybrid individuals presenting an intermediate pattern (Fig. 1). These differences might be attributable to chromosomal rearrangements, such as pericentric inversion. Studies in the *A. fasciatus* complex indicate a variation in the diploid number ($2n$ = 46 to $2n$ = 50, with or without the presence of a B chromosome) and karyotype formula (Pazza *et al.*, 2008a, b; Ferreira-Neto *et al.*, 2012; Kavalco *et al.*, 2016). In the present study, the karyotype formula contributed to the identification and differentiation of the two OTUs in sympatry, in addition to the existence of the natural hybrids. The karyotype formula was also important for differentiation of cryptic or morphologically similar species in other studies of *Astyanax* (e.g. Paiz *et al.*, 2015; Kavalco *et al.*, 2016; Gavazzoni *et al.*, 2018).

The *As*51 satellite DNA was also efficient for distinguishing the OTUs analysed here [Fig. 2 (boxes)]. In addition to *As*51 sites associated with NORs, *Astyanax* sp. 1 presented an *sm* chromosome with an interstitial site different from *Astyanax* sp. 2 and from the hybrids, which presented three carrier chromosomes. This group of satellite DNA can contribute to the evolution of the genome, promoting chromosomal rearrangements and presenting rapid differentiation owing to intragenomic mobility (Mestriner *et al.*, 2000; Vicari *et al.*, 2008).

Studies on the *A. fasciatus* complex have found two distinct patterns of *As*51 DNA distribution: conspicuous blocks, preferably terminal, on various *st* and *a* chromosomes (Abel *et al.*, 2006; Pazza *et al.*, 2008a; Kavalco *et al.*, 2013); and small blocks on few complementary chromosomes (Kantek *et al.*, 2009; Peres *et al.*, 2009; Kavalco *et al.*, 2013; present study). According to Kavalco *et al.* (2013), the populations and species of *A. fasciatus* that possess a small amount of *As*51 DNA are distributed along the coastal basins and the middle São Francisco River, whereas populations of the upper Paraná River basin and nearby drainages of the Grande, Tietê and Paranapanema rivers have a larger quantity of this DNA, more widely dispersed in the chromosomes. The data obtained here are the first for the upper-middle Uruguay River basin and show a distribution pattern of the *As*51 DNA satellite, similar to the populations of the coastal basins and middle São Francisco River.

The 18S rDNA sites were found to be variable in the *A. fasciatus* complex: simple sites in the populations/species of the São Francisco River basin (Peres *et al.*, 2009) and Uruguay River basin (present study); and multiple sites in the Paraná River basin and nearby drainage (Almeida-Toledo *et al.*, 2002; Pazza *et al.*, 2008b; Peres *et al.*, 2009; Ferreira-Neto *et al.*, 2012). In relationship to the distribution of heterochromatin, two main patterns are found: heterochromatin in distal regions in most *sm* and *a* chromosomes in populations of the Paraná River basin (Centofante *et al.*, 2003; Pazza *et al.*, 2008a, b; Kavalco *et al.*, 2013); and preferentially

**Table 2.** Results of the SIMPER analyses for the dissimilarity of the proportion of the morphometric characters for *Astyanax* sp. 2 and natural hybrids caught in the Ijuí River basin, Brazil, in November 2013, April 2014 and May 2015

| Mean ± SE values in each fish species | | Morphometric character (mm) | Average dissimilarity | Contribution (%) | Cumulative contribution (%) |
|---|---|---|---|---|---|
| *Astyanax* sp. 2 | Natural hybrids | | | | |
| 95.24 ± 4.24 | 109.71 ± 3.23 | Standard length | 0.213 | 6.56 | 6.56 |
| 59.95 ± 2.49 | 69.46 ± 2.20 | Preanal distance | 0.167 | 5.16 | 11.72 |
| 37.08 ± 1.83 | 43.52 ± 1.64 | Distance from eye to dorsal fin | 0.153 | 4.73 | 16.45 |
| 48.25 ± 2.15 | 54.95 ± 1.75 | Predorsal distance | 0.148 | 4.58 | 21.03 |
| 42.77 ± 1.99 | 49.69 ± 1.49 | Prepelvic distance | 0.147 | 4.54 | 25.57 |
| 36.38 ± 1.83 | 41.85 ± 1.54 | Dorsal-fin origin to adipose-fin origin | 0.146 | 4.44 | 30.01 |
| 32.70 ± 1.88 | 37.87 ± 1.01 | Anal-fin length | 0.145 | 4.54 | 34.55 |
| 32.12 ± 1.48 | 38.33 ± 1.70 | Body depth | 0.145 | 4.48 | 39.03 |
| 28.82 ± 1.62 | 33.69 ± 0.98 | Length of anal-fin base | 0.141 | 4.34 | 43.37 |
| 21.74 ± 0.90 | 26.95 ± 1.69 | Dorsal-fin length | 0.136 | 4.20 | 47.57 |
| 22.85 ± 1.09 | 26.82 ± 0.97 | Head depth | 0.117 | 3.61 | 51.18 |
| 26.02 ± 1.30 | 29.41 ± 1.15 | Supraoccipital to dorsal-fin origin | 0.116 | 3.57 | 54.75 |
| 21.42 ± 0.85 | 24.80 ± 0.67 | Head length | 0.098 | 3.02 | 57.77 |
| 23.40 ± 0.93 | 26.79 ± 0.74 | Prepectoral distance | 0.097 | 3.01 | 60.78 |
| 19.77 ± 0.89 | 22.68 ± 0.60 | Pectoral-fin length | 0.097 | 2.99 | 63.77 |
| 11.45 ± 0.71 | 13.72 ± 0.22 | Caudal peduncle length | 0.096 | 2.95 | 66.72 |
| 16.10 ± 0.81 | 18.24 ± 0.39 | Pelvic-fin length | 0.090 | 2.78 | 69.50 |
| 12.24 ± 0.60 | 14.30 ± 0.45 | Length of dorsal-fin base | 0.086 | 2.66 | 72.16 |
| 10.17 ± 0.50 | 12.17 ± 0.37 | Caudal peduncle depth | 0.082 | 2.53 | 74.69 |
| 8.74 ± 0.44 | 10.11 ± 0.36 | Interorbital distance | 0.070 | 2.16 | 76.85 |
| 9.56 ± 0.39 | 11.06 ± 0.21 | Length of upper maxilla | 0.063 | 1.95 | 80.77 |
| 6.01 ± 0.20 | 7.16 ± 0.25 | Snout length | 0.058 | 1.80 | 82.57 |
| 36.64 ± 0.52 | 34.47 ± 0.49 | Orbital diameter | 0.049 | 1.53 | 84.10 |
| Percentage of head length | | | | | |
| 28.23 ± 0.51 | 28.88 ± 0.67 | Snout length | 0.046 | 1.43 | 85.53 |
| 44.71 ± 0.67 | 44.70 ± 0.80 | Upper maxilla | 0.043 | 1.34 | 86.87 |
| 106.32 ± 1.48 | 108.00 ± 1.66 | Head depth | 0.064 | 1.97 | 78.82 |
| 40.64 ± 0.72 | 40.71 ± 0.48 | Interorbital distance | 0.041 | 1.28 | 88.15 |
| Percentage of standard length | | | | | |
| 30.11 ± 0.57 | 30.73 ± 0.35 | Length of anal-fin base | 0.041 | 1.26 | 89.41 |
| 33.73 ± 0.41 | 34.87 ± 0.71 | Body depth | 0.040 | 1.23 | 90.64 |
| 7.80 ± 0.23 | 8.54 ± 0.18 | Orbital diameter | 0.039 | 1.22 | 91.86 |
| 11.94 ± 0.33 | 12.55 ± 0.23 | Caudal peduncle length | 0.039 | 1.21 | 93.07 |
| 20.81 ± 0.40 | 20.71 ± 0.38 | Pectoral-fin length | 0.035 | 1.07 | 94.14 |

**Table 2.** Continued

| Mean ± SE values in each fish species | | Morphometric character (mm) | Average dissimilarity | Contribution (%) | Cumulative contribution (%) |
|---|---|---|---|---|---|
| *Astyanax* sp. 2 | Natural hybrids | | | | |
| 50.70 ± 0.51 | 50.09 ± 0.49 | Predorsal distance | 0.030 | 0.93 | 95.07 |
| 16.90 ± 0.29 | 16.67 ± 0.24 | Pelvic-fin length | 0.026 | 0.82 | 95.89 |
| 24.67 ± 0.32 | 24.45 ± 0.32 | Prepectoral distance | 0.026 | 0.79 | 96.68 |
| 22.57 ± 0.24 | 22.64 ± 0.33 | Head length | 0.024 | 0.76 | 97.44 |
| 63.06 ± 0.34 | 63.31 ± 0.53 | Preanal distance | 0.022 | 0.68 | 98.12 |
| 12.85 ± 0.20 | 13.04 ± 0.16 | Length of dorsal-fin base | 0.021 | 0.65 | 98.77 |
| 10.67 ± 0.15 | 11.10 ± 0.13 | Caudal peduncle depth | 0.021 | 0.63 | 99.40 |
| 44.86 ± 0.28 | 45.30 ± 0.33 | Prepelvic distance | 0.019 | 0.60 | 100.00 |

centromeric/pericentromeric heterochromatin in several complementary chromosomes, in populations of the São Francisco River basin and Eastern Atlantic hydrographical region (Kavalco *et al.*, 2013; Medrado *et al.*, 2015) and Uruguay River basin (present study). The 18S rDNA cistrons and the distribution pattern of heterochromatin, together with the distribution pattern of *As*51, contribute to our understanding of the biogeography of the group, because they indicate greater chromosomal similarity between the populations/species of the *A. fasciatus* complex of the Uruguay River basin and the São Francisco River basin, when compared with populations/species of the Paraná River basin.

The molecular analyses show low genetic differentiation between the OTUs analysed here and great proximity to other species of Uruguay River basin, and these data still indicate the formation of a cluster composed by *Astyanax* sp. 1, *Astyanax* sp. 2 and hybrid individuals. Although DNA barcoding studies can be very efficient for species identification, with many efficient results in Neotropical fishes (Pereira *et al.*, 2013), this tool has not been completely conclusive for recent clades with low genetic variation, mainly within species complexes or taxonomically problematic groups, such as *Astyanax* (Kavalco *et al.*, 2016; Pazza *et al.*, 2018), *Rineloricaria* (Costa-Silva *et al.*, 2015), *Prochilodus* (Melo *et al.*, 2018) and, in the present study, in the *A. fasciatus* complex, as observed by their closer relationship to *A. pampa* from coastal drainages of the Brazilian state of Rio Grande do Sul and to *A. biotae* from upper Paraná River basin.

Morphological analyses did not separate the OTUs for the *A. fasciatus* complex from the Ijuí River. In contrast, *Astyanax* sp. 2 had morphological traits distinct from natural hybrids. For all measurements used in the analyses, standard length was the major contributor to separation of *Astyanax* sp. 2 and natural hybrids. In addition, another ten morphometric traits also contributed to distinguish them. Nevertheless, the visual diagnosis between the two groups is not simple. Pinheiro *et al.* (2019) also found morphological differences between hybrids and the parental species (*A. fasciatus* × *Astyanax paranae*) from the Rio Grande basin, corroborated by the molecular analyses, in that case.

Although not effective for differentiation of the OTUs, the diploid number, heterochromatin and 18S rDNA, combined with morphological and molecular evidence, indicate a pattern of sibling species occurring in the grey zone, reinforced by the occurrence of natural hybrid individuals with an intermediate chromosomal pattern between both units. According to Knowlton (1986, 1993), the concept of sibling species goes beyond cryptic species (species with high morphological similarity and consequent difficulty

in identification), because these species have a more recent common ancestry, implying a relationship between the sister species. Moreover, recent changes in the environment, owing to the construction of a small hydroelectric power station (SHPS) in the Ijuí River (PCH RS-155), inaugurated in 2012 (Ceriluz, 2019), might have favoured the emergence of a hybrid zone between OTUs.

It is obvious that a hydroelectric power plant can drastically alter the aquatic environment, causing abrupt fragmentation of river systems and habitat loss, constituting one of the greatest threats to aquatic biodiversity (Agostinho *et al.*, 2016). It can lead to homogenization of the environment, mixing or separation of fauna and changes in the characteristics of microhabitats, among other changes, which consequently affects the biological composition. The new environment established after formation of the reservoir presents very different characteristics from the original environment, and the communities are significantly different from those that occurred in the original or remnant lotic sections (Agostinho *et al.*, 2016; Souza *et al.*, 2019). Thus, the construction of the SHPS in the Ijuí River might have favoured the contact between species with recent diversification. The hybrid individuals verified by the present study reinforce this hypothesis. Crossing between sympatric species of *Astyanax* (*A. fasciatus* × *A. paranae*), with hybrids in the natural environment (Grande River, Paraná River basin), have already been recorded (Pinheiro *et al.*, 2019), thus indicating that hybridization between different *Astyanax* species is not an isolated event.

The presence of many natural hybrid individuals (eight individuals) might indicate better performance than the parental species, called hybrid vigour or heterosis (Hashimoto *et al.*, 2015). However, hybrid individuals could be the result of a unique sporadic event, given that they were collected only in the second sampling (April 2014), and it is possible that the hybrids could persist in a suboptimal adaptive condition owing low fitness (Arnold *et al.*, 2012). Moreover, if the reproductive isolating mechanisms were weak and insufficient to prevent introgression, this secondary contact, with gene flow between these lineages that diverged long ago, might slow or reverse the differentiation/speciation process. The low occurrence of *Astyanax* sp. 1 in relationship to *Astyanax* sp. 2, even with sampling at different time points (2013: *Astyanax* sp. 2, $N = 1$; 2014: *Astyanax* sp. 1, $N = 1$ and *Astyanax* sp. 2, $N = 9$; 2015: *Astyanax* sp. 1, $N = 1$ and *Astyanax* sp. 2, $N = 4$), might indicate greater susceptibility and difficulty in adapting to the environmental changes caused by the construction of the SHPS, or could still be related to artefacts of detectability and/or sampling efforts.

## Conclusion

Based on the analysed characters, it is possible to verify that the morphological and mitochondrial DNA differentiation did not follow the chromosome differentiation. Although efficient, molecular analyses are not always conclusive to diagnose species, revealing different results from morphological or cytogenetic data. The cytogenetic characters used here were effective to differentiate sibling species and natural hybrids (5S rDNA, *As*51 and karyotype) and contributed to our comprehension of the complex biogeography of *A. fasciatus* (18S rDNA, heterochromatin and *As*51). These results reinforce the importance of integrative taxonomy, combining different tools to verify the real diversity of species and to understand their evolutionary relationships better.

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## REFERENCES

**Abel LDS**, **Mantovani M**, **Moreira-Filho, O. 2006.** Chromosomal distribution of the *As*-51 satellite DNA in two species complexes of the genus *Astyanax* (Pisces, Characidae). *Genetics and Molecular Biology* **29:** 448–452.

**Agostinho AA**, **Gomes LC**, **Santos NCL**, **Ortega JCG**, **Pelicice FM. 2016.** Fish assemblages in Neotropical reservoirs: colonization patterns, impacts and management. *Fisheries Research* **173:** 26–36.

**Almeida-Toledo LF**, **Ozouf-Costaz C**, **Foresti F**, **Bonillo C**, **Porto-Foresti F**, **Daniel-Silva MFZ. 2002.** Conservation of the 5S bearing chromosome pair and co-localization with

major rDNA clusters in five species of *Astyanax* (Pisces, Characidae). *Cytogenetic and Genome Research* **97:** 229–233.

**Anderson MJ. 2001.** A new method for non-parametric multivariate analysis of variance. *Austral Ecology* **26:** 32–46.

**Anderson MJ. 2004.** *PERMDISP: a FORTRAN computer program for permutational analysis of multivariate dispersions (for any two-factor ANOVA design) using permutation tests*. Auckland: Department of Statistics, University of Auckland.

**Arnold ML**, **Balleroni ES**, **Brothers AN. 2012.** Hybrid fitness, adaptation and evolutionary diversification: lessons learned from Louisiana Irises. *Heredity* **108:** 159–166.

**Bertollo LAC**, **Takahashi CS**, **Moreira-Filho O. 1978.** Cytotaxonomic considerations on *Hoplias lacerdae* (Pisces, Erythrinidae). *Brazilian Journal of Genetics* **1:** 103–120.

**Birindelli JLO**, **Sidlauskas BL. 2018.** Preface: how far has Neotropical Ichthyology progressed in twenty years? *Neotropical Ichthyology* **16:** e180128.

**Blanco DR**, **Lui RL**, **Bertollo LAC**, **Diniz D**, **Moreira-Filho O. 2010.** Characterization of invasive fish species in a river transposition region: evolutionary chromosome studies in the genus *Hoplias* (Characiformes, Erythrinidae). *Reviews in Fish Biology and Fisheries* **20:** 1–8.

**Borcard D. Gillet F**, **Legendre P. 2011.** *Numerical ecology with R*. New York: Springer.

**Castro JP**, **Moura MO**, **Moreira-Filho O**, **Shibatta OA**, **Santos MH**, **Nogaroto V**, **Vicari MR**, **Almeida MC**, **Artoni RF. 2015.** Diversity of the *Astyanax scabripinnis* species complex (Teleostei: Characidae) in the Atlantic Forest, Brazil: species limits and evolutionary inferences. *Reviews in Fish Biology and Fisheries* **25:** 231–244.

**Centofante L**, **Bertollo LAC**, **Justi AJ**, **Moreira-Filho O. 2003.** Correlation of chromosomal and morphologic characters in two *Astyanax* species. *Ichthyological Exploration of Freshwaters* **14:** 361–368.

**Ceriluz**. **2019.** *Usina RS-155*. Available at: http://ceriluz.com.br/index.php/geracao-de-energia/usina-rs-155

**Clarke KR. 1993.** Non-parametric multivariate analysis of changes in community structure. *Austral Ecology* **18:** 117–143.

**Costa-Silva GJ**, **Rodriguez MS**, **Roxo FF**, **Foresti F**, **Oliveira C. 2015.** Using different methods to access the difficult task of delimiting species in a complex Neotropical hyperdiverse group. *PLoS ONE* **10:** e0135075.

**Darriba D**, **Taboada GL**, **Doallo R**, **Posada D. 2012.** JModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9:** 772.

**Dayrat B. 2005.** Toward integrative taxonomy. *Biological Journal of the Linnean Society* **85:** 407–415.

**De Queiroz K. 2007.** Species concepts and species delimitation. *Systematic Biology* **56:** 879–886.

**Edgar RC. 2004.** MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32:** 1792–1797.

**Eschmeyer WN**, **Fong JD. 2019.** *Species by family / subfamily*. Available at: http://researcharchive.calacademy.org/research/ichthyology/catalog/SpeciesByFamily.asp#Characidae/

**Ferreira-Neto M**, **Artoni RF**, **Vicari MR**, **Moreira-Filho O**, **Camacho JPM**, **Bakkali M**, **Oliveira C**, **Foresti F. 2012.** Three sympatric karyomorphs in the fish *Astyanax fasciatus* (Teleostei, Characidae) do not seem to hybridize in natural populations. *Comparative Cytogenetics* **6:** 29–40.

**Fink WL**, **Weitzman SH. 1974.** The so-called cheirodontin fishes of Central America with descriptions of two new species (Pisces: Characidae). Smithsonian Institution. *Smithsonian Contributions to Zoology* **172:** 1–46.

**Gavazzoni M**, **Paiz LM**, **Oliveira CAM**, **Pavanelli CS**, **Graça WJ**, **Margarido VP. 2018.** Morphological cryptic species of the *Astyanax bimaculatus* "caudal peduncle spot" subgroup diagnosed through cytogenetic characters. *Zebrafish* **15:** 382–388.

**Griffiths SP. 2000.** The use of clove oil as an anaesthetic and method for sampling intertidal rockpool fishes. *Journal of Fish Biology* **57:** 1453–1464.

**Guimarães EC**, **de Brito PS**, **Feitosa LM**, **Costa LFC**, **Ottoni FP. 2019.** A new cryptic species of *Hyphessobrycon* Durbin, 1908 (Characiformes, Characidae) from the Eastern Amazon, revealed by integrative taxonomy. *Zoosystematics and Evolution* **95:** 345–360.

**Hall TA. 1999.** BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41:** 95–98.

**Hashimoto DT**, **Ferguson-Smith MA**, **Rens W**, **Foresti F**, **Porto-Foresti F. 2011.** Chromosome mapping of H1 histone and 5S rRNA gene clusters in three species of *Astyanax* (Teleostei, Characiformes). *Cytogenetic and Genome Research* **134:** 64–71.

**Hashimoto DT**, **Prado FD**, **Senhorini JA**, **Foreti F**, **Porto-Foresti F. 2015.** Aquaculture of neotropical catfish hybrids: genetic strategies for conservation and management. In: Regan B, ed. *Carp and catfish: biology, behavior, and conservation strategies*. New York: Nova Science Publishers, 1–30.

**Hatanaka T**, **Galetti-Jr PM. 2004.** Mapping 18S and 5S ribosomal RNA genes in the fish *Prochilodus argenteus* Agassiz, 1929 (Characiformes, Prochilodontidae). *Genetica* **122:** 239–244.

**He W**, **Qin Q**, **Liu S**, **Li T**, **Wang J**, **Xiao J**, **Xie L**, **Zhang C**, **Liu Y. 2012.** Organization and variation analysis of 5S rDNA in different ploidy-level hybrids of red crucian carp × topmouth culter. *PLoS ONE* **7:** e38976.

**He WG**, **Xie LH**, **Li TL**, **Liu SJ**, **Xiao J**, **Hu J**, **Wang J**, **Qin Q**, **Liu Y. 2013.** The formation of diploid and triploid hybrids of female grass carp × male blunt snout bream and their 5S rDNA analysis. *BMC Genetics* **14:** 110.

**Howell WM**, **Black DA. 1980.** Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* **36:** 1014–1015.

**Kantek DLZ**, **Vicari MR**, **Peres WAM**, **Cestari MM**, **Artoni RF**, **Bertollo LAC**, **Moreira-Filho O. 2009.** Chromosomal location and distribution of As51 satellite DNA in five species of the genus *Astyanax* (Teleostei, Characidae, *Incertae sedis*). *Journal of Fish Biology* **75:** 408–421.

**Kavalco KF**, **Pazza R**, **Brandão KO**, **Almeida-Toledo LF. 2013.** Biogeographic patterns in the chromosomal

distribution of a satellite DNA in the banded tetra *Astyanax fasciatus* (Teleostei: Characiformes). *Organisms Diversity and Evolution* **13:** 67–76.

**Kavalco KF**, **Pazza R**, **Brandão KO**, **Garcia C**, **Bertollo LAC**, **Almeida-Toledo LF. 2016.** Chromosomal diversification higher than molecular variation in *Astyanax* aff. *fasciatus* (Teleostei, Characidae). *Zebrafish* **13:** 345–353.

**Kearse M**, **Moir R**, **Wilson A**, **Stones-Havas S**, **Cheung M**, **Sturrock S**, **Thierer T. 2012.** Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28:** 1647–1649.

**Knowlton N. 1986.** Cryptic and sibling species among the decapod Crustacea. *Journal of Crustacean Biology* **6:** 356–363.

**Knowlton N. 1993.** Sibling species in the sea. *Annual Review of Ecology and Systematics* **24:** 189–216.

**Legendre P**, **Legendre L. 2012.** *Numerical ecology*, **Vol. 24**. *3rd edn*. Amsterdam: Elsevier.

**Levan A**, **Fredga K**, **Sandberg AA. 1964.** Nomenclature for centromeric position on chromosomes. *Hereditas* **52:** 201–220.

**Lui RL**, **Blanco DR**, **Moreira-Filho O**, **Margarido VP. 2012.** Propidium iodide for making heterochromatin more evident in the C-banding technique. *Biotechnic & Histochemistry* **87:** 433–438.

**Margarido VP**, **Moreira-Filho O. 2008.** Karyotypic differentiation through chromosome fusion and number reduction in *Imparfinis hollandi* (Ostariophysi, Heptapteridae). *Genetics and Molecular Biology* **31:** 235–238.

**Martins C**, **Galetti-Jr PM. 1999.** Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). *Chromosome Research* **7:** 363–367.

**Martins C**, **Wasko AP. 2004.** Organization and evolution of 5S ribosomal DNA in the fish genome. In Williams CR, ed. *Focus on genome research*. New York: Nova Science Publishers, 335–363.

**Medrado A**, **De Mello Affonso PRA**, **Carneiro PLS**, **Vicari MR**, **Artoni RF**, **Costa MA. 2015.** Allopatric divergence in *Astyanax* aff. *fasciatus* Cuvier, 1819 (Characidae, *Icertae sedis*) inferred from DNA mapping and chromosomes. *Zoologischer Anzeiger* **257:** 119–129.

**Melo BF**, **Dorini BF**, **Foresti F**, **Oliveira C. 2018.** Little divergence among mitochondrial lineages of *Prochilodus* (Teleostei, Characiformes). *Frontiers in Genetics* **9:** 107.

**Melo FAG**, **Buckup PA. 2006.** *Astyanax heseli*, a new name for *Tetragonopterus aeneus* Hensel, 1870 from Southern Brazil (Teleostei: Characiformes). *Neotropical Ichthyology* **4:** 45–52.

**Menezes, NA**, **Weitzman, SH. 1990.** Two new species of *Mimagoniates* (Teleostei: Characidae: Glandulocaudinae), their phylogeny and biogeography and a key to the glandulocaudin fishes of Brazil and Paraguay. *Proceedings of the Biological Society of Washington* **103:** 380–426.

**Mestriner CA**, **Galetti-Jr PM**, **Valentini SR**, **Ruiz IRG**, **Abel LDS**, **Moreira-Filho O**, **Camacho JPM. 2000.** Structural and functional evidence that a B chromosome in the characid fish *Astyanax scabripinnis* is an isochromosome. *Heredity* **85:** 1–9.

**Mirande JM. 2018.** Morphology, molecules and the phylogeny of Characidae (Teleostei, Characiformes). *Cladistics* **34:** 1–19.

**Mirande JM**, **Koerber S. 2015.** On some species of *Astyanax* reported erroneously from Argentina. *Ichthyological Contributions of Peces Criollos* **35:** 1–8.

**Moreira-Filho O**, **Bertollo LAC. 1991.** *Astyanax scabripinnis* (Pisces; Characidae): a "species complex". *Brazilian Journal of Genetics* **14:** 331–357.

**Oksanen J**, **Blanchet FG**, **Kindt R**, **Legendre P**, **Minchin PR**, **O'Hara RB**, **Simpson GL**, **Solymos P**, **Stevens MHH**, **Wagner H. 2015.** *Vegan: community ecology package. R package vegan, v.2.2-1*. Available at: http://www.cran.r-project.org/package=vegan

**Oliveira C**, **Avelino GS**, **Abe KT**, **Mariguela TC**, **Benine RC**, **Ortí G**, **Vari RP**, **Corrêa-e-Castro RM. 2011.** Phylogenetic relationships within the speciose family Characidae (Teleostei: Ostariophysi: Characiformes) based on multilocus analysis and extensive in group sampling. *BMC Evolutionary Biology* **11:** 275.

**Padial JM**, **Miralles A**, **De-la-Riva I**, **Vences M. 2010.** The integrative future of taxonomy. *Frontiers in Zoology* **7:** 16.

**Paiz LM**, **Baumgartner L**, **Graça WJ**, **Margarido VP. 2015.** Basic cytogenetics and physical mapping of ribosomal genes in four *Astyanax* species (Characiformes, Characidae) collected in Middle Paraná River, Iguassu National Park: considerations on taxonomy and systematics of the genus. *Comparative Cytogenetics* **9:** 54–65.

**Palumbi SR. 1996.** Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Moritz C, Mable BK, eds. *Molecular systematics*. Sunderland: Sinauer Associates, 205–247.

**Pazza R**, **Dergam JA**, **Kavalco KF. 2018.** Trends in karyotype evolution in *Astyanax* (Teleostei, Characiformes, Characidae): insights from molecular data. *Frontiers in Genetics* **9:** 131.

**Pazza R**, **Kavalco KF**, **Bertollo LAC. 2008a**. Chromosome polymorphism in *Astyanax fasciatus* (Teleostei, Characidae). 2 – chromosomal location of a satellite DNA. *Cytogenetic and Genome Research* **122:** 61–66.

**Pazza R**, **Kavalco SAF**, **Penteado PR**, **Kavalco KF**, **Almeida-Toledo LF. 2008b.** The species complex *Astyanax fasciatus* Cuvier, 1819 (Teleostei, Characiformes) a multidisciplinary approach. *Journal of Fish Biology* **72:** 2002–2010.

**Perdices A**, **Bermingham E**, **Montilla A**, **Doadrio I. 2002.** Evolutionary history of the genus *Rhamdia* (Teleostei: Pimelodidae) in Central America. *Molecular Phylogenetics and Evolution* **25:** 172–189.

**Pereira LHG**, **Hanner R**, **Foresti F**, **Oliveira C. 2013.** Can DNA barcoding accurately discriminate megadiverse Neotropical freshwater fish fauna? *BMC Genetics* **14:** 20.

**Peres WAM**, **Buckup PA**, **Kantek DLZ**, **Bertollo LAC**, **Moreira-Filho O. 2009.** Chromosomal evidence of downstream dispersal of *Astyanax fasciatus* (Characiformes, Characidae) associated with river shed interconnection. *Genetica* **137:** 305–311.

**Pinheiro APB**, **Melo RMC**, **Teixeira DF**, **Birindelli JLO**, **Carvalho DC**, **Rizzo E. 2019.** Integrative approach detects natural hybridization of sympatric lambaris species and emergence of infertile hybrids. *Scientific Reports* **9:** 4333.

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

**Pucci MB**, **Barbosa P**, **Nogaroto V**, **Almeida MC**, **Artoni RF**, **Pansanoto-Alves JC**, **Foresti F**, **Moreira-Filho O**, **Vicari MR. 2014.** Population differentiation and speciation in the genus *Characidium* (Characiformes: Crenuchidae): effects of reproductive and chromosomal barriers. *Biological Journal of the Linnean Society* **111:** 541–553.

**R Development Core Team. 2019.** *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing. Available at: http://www.R-project.org/

**Reis RE**, **Albert JS**, **Didario F**, **Mincarone MM**, **Petry P**, **Rocha LA. 2016.** Fish biodiversity and conservation in South America. *Journal of Fish Biology* **89:** 12–47.

**Rossini BC**, **Oliveira CAM**, **Melo FAG**, **Bertaco VA**, **Astarloa JMD**, **Rosso JJ**, **Foresti F**, **Oliveira C. 2016.** Highlighting *Astyanax* species diversity through DNA barcoding. *PLoS ONE* **11:** e0167203.

**Serrano ÉA**, **Melo BF**, **Freitas-Souza D**, **Oliveira ML**, **Utsunomia R**, **Oliveira C**, **Foresti F. 2019.** Species delimitation in Neotropical fishes of the genus *Characidium* (Teleostei, Characiformes). *Zoologica Scripta* **48:** 69–80.

**de Souza CA**, **Vieira LCG**, **Legendre P**, **de Carvalho P**, **Velho LFM**, **Beisner BE. 2019.** Damming interacts with the flood pulse to alter zooplankton communities in an Amazonian river. *Freshwater Biology* **64:** 1040–1053.

**Stamatakis A. 2014.** RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30:** 1312–1313.

**Stamatakis A**, **Hoover P**, **Rougemont J. 2008.** A rapid bootstrap algorithm for the RAxML Web servers. *Systematic Biology* **57:** 758–771.

**Sumner AT. 1972.** A simple technique for demonstrating centromeric heterochromatin. *Experimental Cell Research* **75:** 304–306.

**Valdecasas AG**, **Williams D**, **Wheeler QD. 2008.** 'Integrative taxonomy' then and now: a response to Dayrat (2005). *Biological Journal of the Linnean Society* **93:** 211–216.

**Venkatraman MX**, **Deraad DA**, **Tsai WLE**, **Zarza E**, **Zellmer AJ**, **Maley JM**, **Mccormack JE. 2019.** Cloudy with a chance of speciation: integrative taxonomy reveals extraordinary divergence within a Mesoamerican cloud forest bird. *Biological Journal of the Linnean Society* **126:** 1–15.

**Vicari MR**, **Artoni RF**, **Moreira-Filho O**, **Bertollo LAC. 2008.** Colocalization of repetitive DNAs and silencing of major rRNA genes. A case report of the fish *Astyanax janeiroensis*. *Cytogenetic and Genome Research* **122:** 67–72.

**Ward RD**, **Zemlak TS**, **Innes BH**, **Last PR**, **Hebert PD. 2005.** DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B: Biological Sciences* **360:** 1847–1857.

**Xia X. 2018.** DAMBE7: New and improved tools for data analysis in molecular biology and evolution. *Molecular Biology and Evolution* **35:** 1550–1552.

**Zhang C**, **Ye L**, **Chen Y**, **Xiao J**, **Wu Y**, **Tao M**, **Xiao Y**, **Liu S. 2015.** The chromosomal constitution of fish hybrid lineage revealed by 5S rDNA FISH. *BMC Genetics* **16:** 140.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

**File S1.** *COI* matrix with 1688 terminals, based on Rossini *et al.* (2016) and the *A. fasciatus* complex (*Astyanax* sp. 1, *Astyanax* sp. 2 and *Astyanax* hybrids) from the Ijuí River.
**File S2.** *COI* matrix with 88 terminals containing representatives of the closer lineages of the *A. fasciatus* complex.
**File S3.** *Cytb* matrix with 11 terminals of the *A. fasciatus* complex (*Astyanax* sp. 1, *Astyanax* sp. 2 and *Astyanax* hybrids) from the Ijuí River.
**Figure S1.** Best maximum likelihood tree based on the cytochrome *c* oxidase subunit I gene, showing the position of the analysed *A. fasciatus* complex (*Astyanax* sp. 1, *Astyanax* sp. 2 and *Astyanax* hybrids) from the Ijuí River within group 1, containing the *A. fasciatus* species complex, based on Rossini *et al.* (2016).
**Figure S2.** Best maximum likelihood tree based on the B gene in the *A. fasciatus* complex (*Astyanax* sp. 1, *Astyanax* sp. 2 and *Astyanax* hybrids) from the Ijuí River.