



Cytogenetic divergence in two sympatric fish species of the genus *Astyanax* Baird and Girard, 1854 (Characiformes, Characidae) from northeastern Brazil

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Abstract

The fish genus *Astyanax* is widespread throughout the Neotropical region and is one of the most species-rich genera of the Characiformes. Cytogenetic studies of *Astyanax* have revealed marked intra- and interspecific diversity, with the identification of various species complexes. In this report, we describe the karyotypic structure of two sympatric species of *Astyanax* (*Astyanax* sp. and *Astyanax* aff. *fasciatus*) from the Middle Contas River basin in the northeastern Brazilian state of Bahia. Both species had $2n = 48$ but differed in their karyotypic formulae. Small heterochromatic blocks and multiple nucleolar organizer regions (NORs) were identified in both species. Terminal CMA₃/DAPI signals were observed in *Astyanax* sp. and *A. aff. fasciatus*, mostly coincident with NORs. These results show that chromosomal markers can be used to identify species in this fish complex. These markers can provide useful information for evolutionary studies and investigations on the mechanisms of chromosomal diversity in *Astyanax*.

Keywords: Ag-NOR, cytotaxonomy, fish, fluorochromes, South America.

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The Contas River basin in the northeastern Brazilian state of Bahia is characterized by a diverse ichthyofauna that includes several endemic species; this diversity reflects the independent evolutionary history of coastal rivers in eastern South America (Jacobina *et al.*, 2009). However, although a few species from this region have recently been described (Vari *et al.*, 2010), the biota of this basin is still poorly known. This is particularly true for the fish fauna of the headwaters, despite the fact that these habitats represent an excellent model for evolutionary studies because of the occurrence of isolated populations and peculiar ecological niches. Small characids, such as *Astyanax* Baird and Girard, 1854 species are usually dominant in headwaters and small tributaries throughout South America (Bertaco and Lucena, 2010).

Astyanax (Characidae, *Incertae sedis*) is one of the most cytogenetically (Kavalco *et al.*, 2011; Mendes *et al.*, 2011) and morphologically diverse fish genera within the Characiformes, with more than 100 Neotropical species (Bertaco and Lucena, 2010). Recent phylogenetic inferences based on molecular data have shown that the family Characidae is a monophyletic group, despite encompassing several polyphyletic species-rich genera such as *Astyanax*,

collectively referred to as *Incertae sedis* (Oliveira *et al.*, 2011). Because of their controversial taxonomy and extensive chromosomal variation, several species complexes have been proposed in this group, such as *Astyanax scabripinnis* (Moreira-Filho and Bertollo, 1991), *A. hastatus* (Kavalco *et al.*, 2009), *A. altiparanae* – often cited as *A. bimaculatus* (Kavalco *et al.*, 2011) – and *A. eigenmanniorum* (Mendes *et al.*, 2011). The species in the *A. fasciatus* complex are characterized by a wide distribution throughout coastal river basins in Brazil and show marked structural and numerical chromosomal variation, including the coexistence of distinct karyomorphs in a single basin (Medrado *et al.*, 2008).

In this report, we describe the karyotypic structure of two sympatric *Astyanax* species from the Middle Contas River basin in the northeastern Brazilian state of Bahia. The data described here reinforce the peculiar biogeographic history and remarkable endemism of this region.

Eighteen specimens of *Astyanax* sp. (12 females, four males and two juveniles) and 26 specimens of *Astyanax* aff. *fasciatus* (10 females, 14 males and two juveniles) were collected in sympatry and syntopy along the Preto do Crisciúma River (13°55'38" S/39°57'53" W), a tributary of the Contas River, within the Crisciúma-Guariba microbasin, northeastern Brazil (Figure 1). The specimens of *Astyanax* sp. were deposited in the Zoology Museum of the Universidade Federal da Bahia, Brazil (UFBA 5528) while

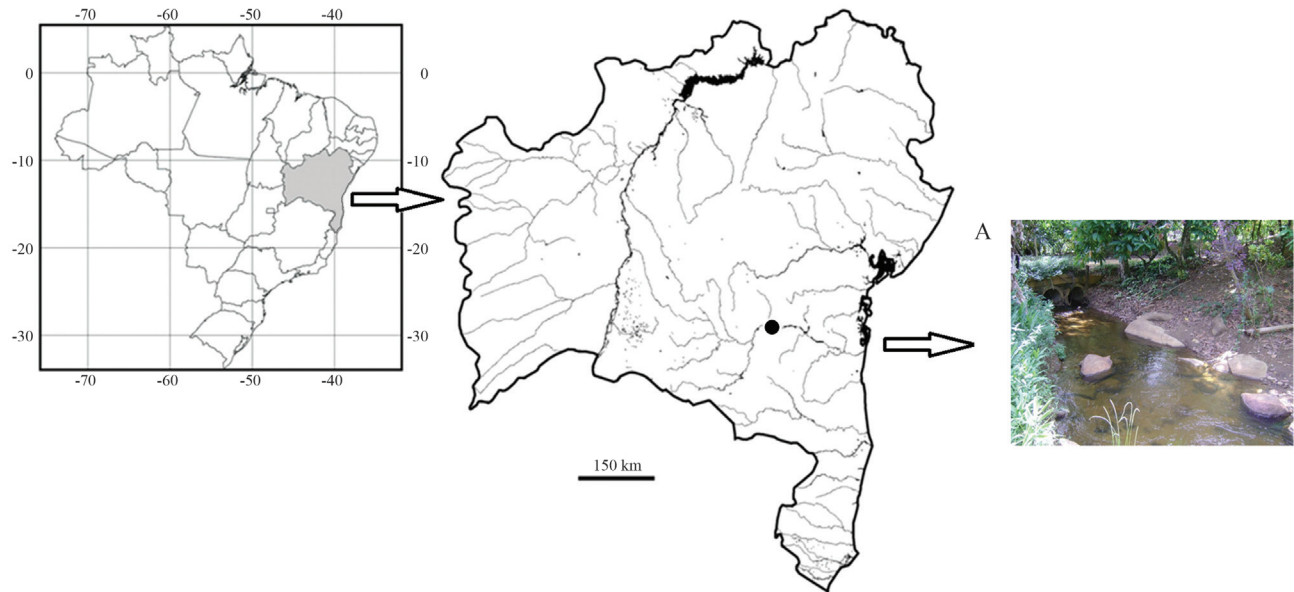


Figure 1 - Map of Bahia state, Brazil, showing Preto do Crisciúma River in the Guariba-Crisciúma microbasin of the Middle Contas River basin, with a view of the collection site (A).

Astyanax aff. fasciatus were identified and deposited in the fish collection of the Universidade Estadual do Sudoeste da Bahia, Brazil (CR 348, 349, 350, 353, 418, 482, 484, 693, 694, 728, 730, 734, 1227, 1228, 1229, 1232, 1233, 1234, 1289, 1290, 1291, 1300, 1301, 1302, 1303).

Chromosomes were obtained from anterior kidney cells after mitotic stimulation (Bertollo *et al.*, 1978). Heterochromatin was visualized by C-banding (Sumner, 1972) and nucleolar organizer regions (NORs) were located by silver nitrate staining (Howell and Black, 1980). Five specimens of each species were used in Ag-NOR studies, with at least 30 metaphase counts per individual. The GC- and AT-rich regions were detected by base-specific fluorochrome staining using chromomycin A₃ (CMA₃) and 4',6-diamidino-2-phenylindole (DAPI), respectively (Schmid, 1980). Chromosomal morphology was established based on the arm ratio and arranged in order of decreasing size (Levan *et al.*, 1964). The fundamental number (FN) was calculated by assuming that metacentric (m), submetacentric (sm) and subtelocentric (st) chromosomes are biarmed while acrocentric chromosomes (a) have a single chromosomal arm.

Astyanax sp. and *Astyanax aff. fasciatus* had $2n = 48$, although the species could be differentiated by their chromosomal morphology, *i.e.*, $10m+18sm+08st+12a$ and $FN = 84$ for *Astyanax* sp. (Figure 2A) and $08m+28sm+08st+04a$ and $FN = 92$ for *Astyanax aff. fasciatus* (Figure 2B). Furthermore, the first sm and st pairs in individuals of *A. aff. fasciatus* were remarkably larger than the others within their respective morphological classes (Figure 2B). No sex-related differences were observed in the samples analyzed.

Faintly-stained heterochromatin blocks were observed in the pericentromeric regions of all chromosomes in both species (Figure 2A,B). In *Astyanax aff. fasciatus*, some terminal heterochromatin segments were observed on long arms in one homolog from pairs 23 and 24 (Figure 2B).

Silver nitrate-stained NORs (Ag-NORs) were located in the terminal regions of up to four chromosomes, including the short arms of two st chromosomes and long arms of an st and one a chromosome in *Astyanax* sp. (Figure 2C) and the short arms of one sm pair and long arms of one st and one a chromosome in *Astyanax aff. fasciatus* (Figure 2E).

Fluorochrome staining showed differences in the distribution of GC-rich sites between both species. *Astyanax* sp. presented eight terminal CMA₃/DAPI signals on the short arms of two non-homologous m chromosomes and five st chromosomes and on the long arms of one a chromosome (Figure 2C,D). *Astyanax aff. fasciatus* showed terminal GC-rich signals on the short arms of one large m chromosome and a sm pair plus the long arms of one st pair, comprising five chromosomes (Figure 2E,F).

Numerical and structural karyotypic variation has been reported among or within populations of *Astyanax* representatives, including the occurrence of B chromosomes and polymorphism in heterochromatic bands and/or ribosomal sites. In *A. aff. fasciatus* in particular, sympatric karyomorphs with $2n = 48$ and 50 were observed in the Tibagi River basin, including a putative hybridization event (Artoni *et al.*, 2006). Similarly, karyomorphs with $2n = 45$, 46, 47 and 48 are found in this species complex from the Mogi-Guaçu River, southeastern Brazil (Pazza *et al.*, 2006).

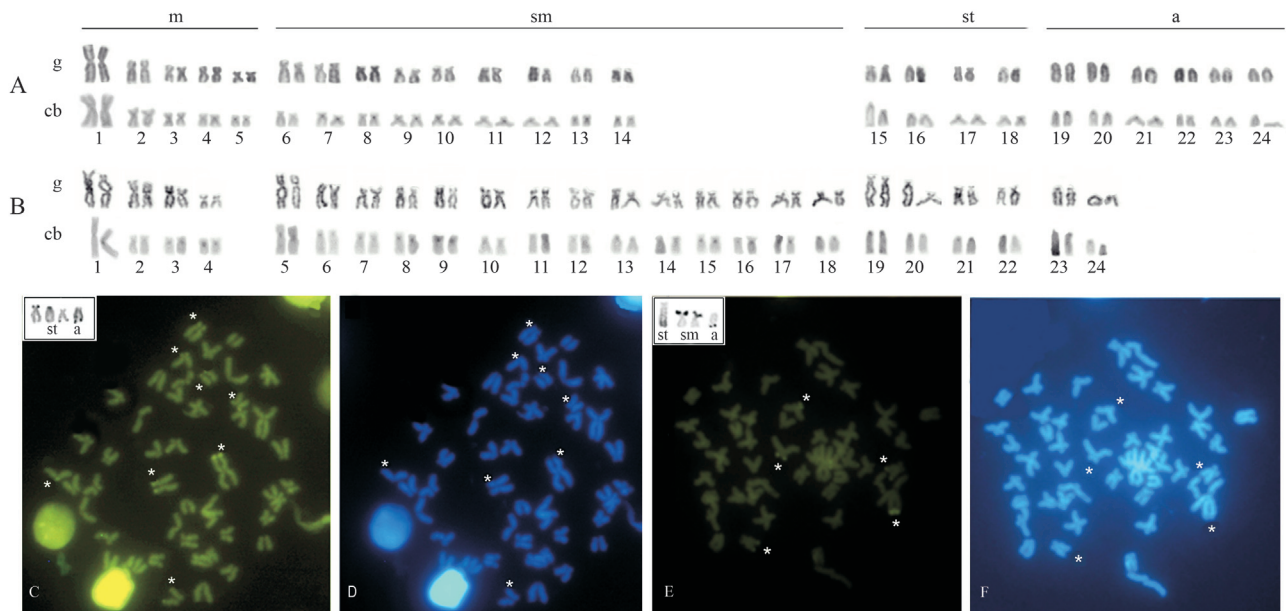


Figure 2 - Karyotypes and metaphases of *Astyanax* sp. (A,C,D) and *A. aff. fasciatus* (B,E,F) after Giemsa staining (g), C-banding (cb), CMA₃ staining (C and E) and DAPI staining (D and F). The asterisks indicate the CMA₃⁺/DAPI signals. The chromosomes bearing Ag-NORs are shown as insets in boxes (C and E).

Although phylogenetic analyses have confirmed that *Astyanax* and other small characins are polyphyletic groups (Oliveira *et al.*, 2011), it has been suggested that $2n = 50$ would be a plesiomorphic feature for most characids (Pazza and Kavalco, 2007; Kavalco *et al.*, 2011). Consequently, the populations of *A. aff. fasciatus* and *Astyanax* sp. may represent a derived group in which chromosomal fusions reduced the diploid number from $2n = 50$ to $2n = 48$. Besides sharing the same chromosomal number and ecological traits, both species are also characterized by an asymmetric karyotype. Another cytogenetic similarity in these samples was the presence of an initial large m pair in relation to other chromosomes. In contrast to $2n = 48$, this pattern is frequent in small characins and is considered another symplesiomorphic feature for *Astyanax* (Kavalco *et al.*, 2011; Mendes *et al.*, 2011).

The karyotype formulae differed between the two species studied and reinforced the role of pericentric inversions in the chromosomal evolution of *Astyanax* (Medrado *et al.*, 2008). It should also be noted that *A. aff. fasciatus* showed accentuated size differences in the first sm and st pairs in relation to other pairs, in agreement with a trend reported in ' $2n = 48$ ' karyomorphs of *A. fasciatus* from southeastern Brazil (Pazza *et al.*, 2006). Furthermore, no intermediate or variant karyotypic forms were detected, *i.e.*, there was no evidence of hybridization among specimens bearing polymorphic chromosomes (Pazza *et al.*, 2006; Kavalco *et al.*, 2009).

Apart from macrostructural traits, Ag-NORs were also useful as cytotaxonomic markers. The multiple Ag-NOR system observed here, which involves the terminal regions of up to four chromosomes, was similar to the pattern

observed in other populations of *A. fasciatus* (Medrado *et al.*, 2008) and *A. aff. bimaculatus* (Pamponet *et al.*, 2008) from the Contas River basin. However, the species we examined could be differentiated by the chromosomal location of the NORs (Figure 2). This location was possibly influenced by the translocation or transposition of rDNA to distinct chromosomes and revealed the dynamic evolution of ribosomal cistrons in this genus (Mantovani *et al.*, 2000).

Despite the importance of C-banding for karyoevolutionary inferences, little is known about the distribution of heterochromatin in *Astyanax* from coastal basins in northeastern Brazil. As with other *Astyanax* species, such as *A. giton* and *A. intermedius* (Kavalco *et al.*, 2007) and *A. jacuhiensis* (Pacheco *et al.*, 2010), the heterochromatin in the specimens studied here was restricted mainly to pericentromeric regions; such a distribution is regarded as a basal C-banding pattern in fish. Some species or populations of *Astyanax* from coastal basins tend to have a reduced amount of heterochromatin, usually close to centromeres and NORs (Rosa *et al.*, 2009; Kavalco *et al.*, 2011). Our findings corroborated this hypothesis and extended this trend to northeastern coastal rivers in South America.

The fluorochrome staining described here is the first for populations of *Astyanax* from northeastern Brazilian coastal rivers. In both species studied, the CMA₃⁺/DAPI signals were usually coincident with NORs. Although a correlation between 45S rDNA clusters and GC-rich blocks is common in fish (Verma *et al.*, 2011) this technique is more appropriate for characterizing repetitive DNA segments, as proposed for *Astyanax scabripinnis* (Mantovani *et al.*, 2004). Another peculiar feature observed in both spe-

cies was the presence of size differences and heterozygous fluorescent signals, which suggested quantitative differences in the GC content between homologs. In addition, the location of GC-rich sites revealed striking species-specific distribution patterns that confirmed their evolutionary isolation.

The high karyotypic diversity of *Astyanax* species when compared to other Neotropical fish groups is thought to be related to the biological traits of these small characins. Their common geographic isolation in headwaters would favor the fixation of interpopulation differences, resulting in the formation of species complexes (Bertaco and Lucena, 2010). Also, the occurrence of such isolates in small streams could maximize the effects of genetic drift and cause intraspecific polymorphism. The present data for *A. aff. fasciatus* and *Astyanax* sp. support both inferences since chromosomal differences were observed in each species close to headwaters in a small stream from the Middle Contas River basin.

The origin of these sympatric species remains unclear since they share a similar habitat and most likely have overlapping ecological niches. Pre-zygotic isolation related to species-specific reproductive behavior or ancient geographic barriers followed by headwater capture could account for the speciation and chromosomal differentiation of both forms. This possibility requires further investigation.

In conclusion, the chromosomal analysis described here was effective in detecting single evolutionary units in *Astyanax* from the Contas River basin, one of several isolated coastal basins in northeastern Brazil for which there is insufficient knowledge and increased habitat degradation. We suggest that cytogenetic analyses, together with traditional taxonomic methods, would provide a low-cost, highly informative approach for characterizing the biodiversity of these tropical fish.

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