Chromosomal characterization in two species of an *Astyanax bimaculatus* complex (Characidae, Characiformes) using different techniques of chromosome banding

Laura L. L. Da Silva · Angélica R. Dos Santos ·
Lucia Giuliano-Caetano · Ana L. Dias

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Abstract *Astyanax* has been the subject of extensive cytogenetic studies due to its wide karyotypic diversity. This genus comprises species complexes, namely groups of fish of difficult morphological differentiation, such as the bimaculatus complex, which includes the characids with a rounded humeral spot. Thence, the present study proposed to accomplish a cytogenetic characterization of two species of this complex: *A. asuncionensis* and *A. altiparanae*, aiming to find chromosomal markers that differentiate these species, as well as achieve a better understanding of the karyotype evolution in the genus. For this we used different techniques of chromosome banding as C-banding, impregnation by silver nitrate, fluorescence staining and FISH with 18S rDNA probe. This is the first cytogenetic study in *A. asuncionensis*, from Miranda river, which presented $2n = 50$ and $18m + 22sm + 6st + 4a$ (FN = 96) and single NORs. The populations of *A. altiparanae* also presented $2n = 50$, but with different karyotypic formulae: the population of the Quexada river presented $16m + 24sm + 4st + 6a$ (FN = 94) and the Esperança stream and Jacutinga river showed $16m + 20sm + 4st + 10a$ (FN = 90). All analyzed

populations showed an interindividual variation in the number and location of the nucleolar organizer regions (NORs). Single and multiple NORs were detected either by impregnation with silver nitrate or by FISH with 18S rDNA probe. After C-banding, the two species differed in relation to the composition and heterochromatin distribution. The meiotic cells of *A. altiparanae* male individuals were also analyzed, showing that, despite the high karyotype variability, chromosome pairing occurs normally. The data show that *A. altiparanae* and *A. asuncionensis* share some characteristics with other species of the bimaculatus complex, suggesting a close phylogenetic relationship among those species. However, some features can be used as differentiation chromosomal markers in *altiparanae/asuncionensis* morphotypes, which could result from a natural speciation process.

Keywords Chromosome markers · Meiosis · In vitro culture · Polymorphism · 18S rDNA

Introduction

According to Eschmeyer (2014), the genus *Astyanax* comprises 156 valid species distributed throughout the Neotropical watersheds and is considered a genus *Incertae Sedis*. The morphological and cytogenetic characteristics found within the genus vary widely (Kantek et al. 2007). This diversity, together with the wide geographical distribution of the genus, hinders

L. L. L. Da Silva · A. R. Dos Santos ·
L. Giuliano-Caetano · A. L. Dias (✉)
Departamento de Biologia Geral, Centro de Ciências
Biológicas (CCB), Universidade Estadual de Londrina,
P.O. Box 6001, Londrina, Paraná CEP 86051-970, Brazil
e-mail: anadias@uel.br

the determination of phylogenetic relationships. Absent shared morphological characteristics that could define *Astyanax* as a monophyletic, the genus is most usefully considered to be a species complex (Bertaco and Garutti 2007).

Garutti (1995) defined the *bimaculatus* species complex as a set of described and undescribed species and subspecies that occur in the Paraná, São Francisco and Amazon river Brazilian basins. However, Garutti and Britski (2000) asserted that the name *Astyanax bimaculatus* L. 1758 referred only to those populations of Suriname and defined a new species called *A. altiparanae* found in the Alto Paraná river basin. Moreover, according to Lima et al. (2003), the species of the Baixo Paraná, Paraguay and São Francisco river basins, previously identified as *A. bimaculatus*, came to be called *A. asuncionensis* Géry 1972 and *A. lacustris* Lütken 1875. However, the validity of the latter is controversial due to an overlap with the features of *A. altiparanae*.

Peres et al. (2012) analyzed cytogenetically specimens of the *bimaculatus* complex of the Alto Paraná and São Francisco river basins and of a channel between the two basins. These authors substantiate that *A. altiparanae* and *A. lacustris* belong to the same taxonomic unit, since they observed evidence of interbreeding between these species in the hybrid zone.

All populations of the *bimaculatus* complex analyzed so far had $2n = 50$, although a large karyotypic variability had already been reported, including the presence of a B chromosome in *A. altiparanae* of the Campo Novo river/SP (Hashimoto et al. 2008). Most populations of the complex show a pattern of multiple NORs (Almeida-Toledo et al. 2002; Fernandes and Martins-Santos 2004; 2006a; Domingues et al. 2007; Kavalco et al. 2011), however, single NORs have already been documented for some populations of *Astyanax* aff. *bimaculatus*, *A. altiparanae*, *A. jacuhiensis* Cope 1894 and *A. lacustris*. Variability also occurs in relation to the distribution of heterochromatin, which can be found in the pericentromeric, terminal and interstitial regions.

Given that the morphological characteristics traditionally used in *Astyanax* taxonomy are not sufficient to distinguish the *altiparanae/asuncionensis* morphotypes groups (Prioli et al. 2012), the main objective of this study was to identify specific chromosomal markers that could facilitate differentiation of these

morphotypes, using different techniques for obtain mitotic and meiotic chromosomes and chromosome banding using AgNOR conventional, fluorochrome staining, C-banding and fluorescence in situ hybridization (FISH) with 18S rDNA probe.

Materials and methods

We analysed six specimens of *A. asuncionensis* (three female, three unidentified sex) from Miranda river/Corumbá/MS/Brazil (19°34'34.55"S/57°01'01.23"W) and three populations of *A. altiparanae* from the Paraná river basin: six specimens (3 females, 1 male, 2 unidentified sex) from Quexada river/Borrazópolis/PR/Brazil (23°56'9.65"S/51°39'26.08"W), eleven specimens (four females, four males, three unidentified sex) from Esperança stream/Londrina/PR (23°20'8.76"S/51°12'38.42"W) and eleven (six females, three males, two unidentified sex) from Jacutinga river/Ibiporã/PR (23°13'25.3"S/50°58'47.37"W) (Fig. 1). Specimens were deposited in the Museum of Zoology of the Universidade Estadual de Londrina (MZUEL) under the numbers: 5769, 5770, 5771, 5772 and 8246. The samples were collected with the permission of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), protocol number 11399-1.

Mitotic chromosomes were obtained by direct preparation, removing the anterior kidney and short term culture using solid tissues, according to Bertollo et al. (1978) and Fenocchio et al. (1991), respectively. In the latter technique, the kidney was incubated by 6 h in culture medium, at 37 °C, followed by addition of 0.05 % colchicine in the last 30 min of incubation. This technique, while not widely used in fish cytogenetics, allowed a greater number of metaphases, as well as improved visualization of chromosomes. Meiotic chromosomes were obtained from gonadal cells by the technique developed by Kligerman and Bloom (1977). The chromosomes were organized according to Levan et al. (1964) and for determination of the fundamental number (FN), the metacentric, submetacentric, and subtelocentric chromosomes were considered biarmed, and the acrocentric, uniarmed.

Nucleolar organizer regions (NORs) and constitutive heterochromatin (C-bands) were identified using silver (Howell and Black 1980) and Giemsa staining (Sumner 1972), respectively. GC- and AT-rich

Fig. 1 Map of Brazil indicating the collection sites: Miranda river, Quexada river, Esperança stream and Jacutinga river (a). The specimens of *Astyanax asuncionensis* (b) and *A. altiparanae* (c). Scale bar 1 cm

segments were visualised using chromomycin A₃ (CMA₃) and 4-6-diamino-2-phenylindole (DAPI) fluoro-chrome staining (Schweizer 1980). In situ hybridisation was used for mapping of 18S ribosomal DNA (rDNA) sites (Pinkel et al. 1986, with slight modifications). The 18S rDNA probe of *Prochilodus argenteus* Agassiz 1829 (Hatanaka and Galetti 2004) was labelled with biotin-14-dATP by nick translation using the BioNick Labeling System (Invitrogen®, Sao Paulo, Brazil) and detected with avidin-FITC (conjugated fluorescein isothiocyanate-avidin) following the manufacturer's instructions. All images were acquired with a Leica DM 4500 B microscope equipped with a DFC 300FX camera and Leica IM50 4.0 software. Contrast and brightness were optimised with iGrafx Image software.

Results

All specimens of *Astyanax asuncionensis* presented $2n = 50$ chromosomes and a karyotype formula of $18m + 22sm + 6st + 4a$ (FN = 96) (Fig. 2a). The three populations of *Astyanax altiparanae* all exhibited $2n = 50$ chromosomes but had different karyotypic formulae: the population of the Quexada river had $16m + 24sm + 4st + 6a$ (FN = 94) (Fig. 2b), while the populations of the Esperança stream and Jacutinga river had $16m + 20sm + 4st + 10a$ (FN = 90) (Fig. 2c).

NORs impregnated with silver nitrate (AgNORs) were observed in *Astyanax asuncionensis* on the short arm of the subtelocentric chromosome pair 21, a region that was also stained by CMA₃⁺/DAPI⁺; FISH

Fig. 2 Karyotypes of: *Astyanax asuncionensis* from Miranda river (**a**), *A. altiparanae* from Quexada river (**b**), Esperança stream and Jacutinga river (**c**). Scale bar 5 μ m

performed with an 18S rDNA probe confirmed the single NOR pattern (Fig. 3).

In contrast, the three populations of *A. altiparanae* showed intra- and inter-individual variations in the number and location of AgNORs: the *A. altiparanae* population of the Quexada river showed interindividual variation at 2–3 AgNORs sites in the terminal region of the short arm of the acrocentric and subtelocentric chromosomes. One individual exhibited extreme variation, with up to 14 AgNORs sites distributed on the short arms of metacentric, subtelocentric and acrocentric chromosomes; on the long arms of small and large metacentric and submetacentric chromosomes; and on medium-sized submetacentric chromosomes with bitelomeric markings. Most sites were also coincident with CMA₃⁺/DAPI[−] signals (Fig. 3).

The population of the Esperança stream showed interindividual variations at 2–5 AgNORs sites, totalling eight observations: three on long arms (a pair of submetacentric chromosomes and one large acrocentric chromosome) and five on short arms (one metacentric chromosome, a pair of a large subtelocentric chromosomes and a pair of acrocentric chromosomes). All markings were terminal and some are coincident with CMA₃ signals (Fig. 3).

The population of the Jacutinga river showed interindividual variations at 2–6 AgNORs sites, totalling 10 observations: on the short arms of metacentric and subtelocentric chromosomes as well as medium and small acrocentric chromosomes, and on the long arms of a submetacentric pair of

chromosomes and an acrocentric chromosome. All markings were terminal and a few are CMA₃⁺ and DAPI[−] (Fig. 3).

FISH also revealed interindividual variability at 18S rDNA sites in all three populations of *A. altiparanae*, being all the markings located in the terminal region of the chromosomes. In Quexada and Jacutinga rivers populations ten chromosomes showed the sites and in Esperança stream population only six chromosomes were observed. However, 18S rDNA was not found at every NOR site identified by silver staining, probably this signals are related with heterochromatin regions. And two chromosomes in the Jacutinga river population had 18S rDNA signals but no apparent silver impregnation (Fig. 3).

After C-banding, it was possible to observe that heterochromatin is weakly distributed in the pericentromeric regions of most chromosomes of *A. asuncionensis* (Fig. 4a). After sequential staining with base-specific fluorochromes, terminal CMA₃⁺ sites related to NORs were observed, as were DAPI⁺ sites in the remaining heterochromatic regions. Pair 21 presented a DAPI⁺ pericentromeric heterochromatic block adjacent to the CMA₃⁺ terminal block that corresponds to the NOR (Fig. 4b, c). The three populations of *A. altiparanae* showed little heterochromatin distributed in the pericentromeric and interstitial regions that revealed terminal CMA₃⁺ sites (most of which were proximate to the NORs) and DAPI⁺ sites in the remaining heterochromatic regions (Fig. 5).

Meiotic cells in all males of the three populations of *A. altiparanae* were also analyzed. The following

Fig. 3 Chromosomes of *A. asuncionensis* and *A. altiparanae* submitted to treatment with silver nitrate, in situ hybridization with rDNA 18S probe and fluorochrome chromomycin A₃. All populations have a pair of subtelocentric marked terminally on the short arm. The population from Quexada river showed ten NORs sites marked by *FISH* and up to 14 marked by *silver*

(AgNOR). The population from Esperança stream has 6 NORs sites marked by *FISH* and 8 marked by *silver*. And the population from Jacutinga river show 10 NORs sites marked by *FISH* and 10 marked by *silver*. *m* metacentrics, *sm* submetacentrics, *st* subtelocentrics, *a* acrocentrics

Fig. 4 Somatic metaphases of *Astyanax asuncionensis* submitted to C-banding with sequential staining with Giemsa (a), CMA₃ (b) and DAPI (c). In (a) heterochromatin associated with NORs, pair 21 (arrows) and in pericentromeric and interstitial regions; in (b) heterochromatin CMA₃⁺ associated with NORs (arrowheads);

while in (c) the other regions are DAPI⁺ and the pair 21 presented a DAPI⁺ pericentromeric heterochromatic block adjacent to the CMA₃⁺ terminal block. In the box the pair 21 with CMA₃⁺ and DAPI⁺ staining. Scale bar = 5 μm

stages were detected: spermatogonial with 50 chromosomes, pachytene and metaphase I with 25 bivalents, and metaphase II, with 25 chromosomes (Fig. 6).

Discussion

Little is known about the cytogenetics of *A. asuncionensis*. The only available literature consists of a recent work on the Iguazu river population realized by Paiz et al. (2015). In contrast, *A. altiparanae* has one of

the most extensively studied karyotypes in the genus. All studies of the bimaculatus species complex, including the present study, have found $2n = 50$ chromosome number, although there is substantial variability in the karyotypic macrostructure. Neither the karyotypic formula of *A. asuncionensis* nor those of the three populations of *A. altiparanae* have been previously reported (Ferreira Neto et al. 2009; Pacheco et al. 2011; Paiz et al. 2015).

Both populations of *Astyanax asuncionensis* analyzed so far, the Miranda river of this study and the

Fig. 5 Metaphases of *A. altiparanae* submitted to C-banding with sequential staining with Giemsa (a, b, c), CMA₃ (d, e, f) and DAPI (g, h, i). All populations showed little heterochromatin distributed in the pericentromeric and interstitial regions (a, b, c); with terminal CMA₃⁺ sites associated with NORs

(arrowheads) in (d, e, f). The remaining heterochromatic regions showed DAPI⁺ sites in all populations. The DAPI⁺ heterochromatin is more conspicuous in populations from Quexada river (g) and Esperança stream (h) and more discreet in populations from Jacutinga river (i). Scale bar = 5 μm

Fig. 6 Meiotic cells of *Astyanax altiparanae* from Quexada river, Esperança stream and Jacutinga river. Spermatogonial (a), Pachytene initial (b), Pachytene median (c), Pachytene end (d), Metaphase I (e) and Metaphase II (f)

Iguaçu river analyzed by Paiz et al. (2015), showed the NOR on the short arm, in a pair st-a group, that may be considered a species-specific chromosomal marker.

Individuals with single NORs and individuals with multiple NORs were found in each of the three *A. altiparanae* populations. The presence of more than one pair of NOR-bearing chromosomes is common in the genus *Astyanax* and considered a derived characteristic in fish. However, in *A. altiparanae*, the presence of a single pair of NOR-bearing chromosomes has already been documented for populations of the Iguaçu river (Domingues et al. 2007), Monjolinho river (Peres et al. 2008), Pântano stream (Ferreira Neto et al. 2009) and Igapó lake (Pacheco et al. 2011). Thus, the presence of single NORs in *A. asuncionensis* and in some individuals of *A. altiparanae* may indicate a close phylogenetic relationship with other members of the bimaculatus species complex that also exhibit this pattern, such as *A. lacustris* (Peres et al. 2008, 2012), *Astyanax* aff. *bimaculatus* (Kavalco et al. 2011) and *A. jacuhiensis* (Pacheco et al. 2010; Silva et al. 2012).

Variations in the number and/or position of 18S rDNA sites have been previously observed in *A. altiparanae* of the Keçaba stream (Fernandes and Martins-Santos 2006a), as well as in other *Astyanax* species, such as *A. eigemanni* Cope 1894 (Mendes et al. 2011) *A. intermedius* Eigenmann 1908, *A. giton* Eigenmann 1908, *A. paraguayae* Eigenmann 1908 (Kavalco and Moreira-Filho 2003) and in some populations of *A. scabripinnis* Jenyns 1942 (Mantovani et al. 2005; Fernandes and Martins-Santos 2006b). The interindividual variation of 18S rDNA sites can be explained by the Rabl model proposed by Schweizer and Loidl (1987). According to this model, the proximity of telomeric regions in the interphase nucleolus could facilitate the transfer of genetic material, and therefore, the terminal NOR sites would more easily suffer dispersion, causing variability in number and position within the same population. The ribosomal gene polymorphisms described in the three populations of *A. altiparanae* may be the result of this dispersal mechanism.

The NORs in *A. asuncionensis* and most of those in *A. altiparanae* were CMA_3^+ , indicating that GC-rich regions are interspersed with ribosomal sites. This relationship has already been reported for the bimaculatus species complex (Domingues et al. 2007; Pacheco et al. 2010; Silva et al. 2012). However, CMA_3^+ sites cannot be considered synonymous with NORs because the three populations of the *A. altiparanae* analysed had CMA_3^+ sites that were not identified by 18S rDNA FISH. These results demonstrate that the NORs may or may not be related to the GC-rich regions. Moreover, there are AgNORs without 18S rDNA FISH that are probably heterochromatic sites with acid proteins that have affinity to silver. These data show the importance of combining multiple differential staining techniques in chromosome studies, to achieve a greater karyotype characterization and to obtain a better estimate of the genetic diversity of the species.

The distribution of heterochromatin in the pericentromeric and interstitial regions is considered a conserved characteristic in *A. altiparanae* (Fernandes and Martins-Santos 2004; Domingues et al. 2007). However, terminal heterochromatin has previously been observed (Ferreira Neto et al. 2009).

The three populations of *A. altiparanae* exhibited slight differences in DAPI^+ heterochromatin. Those from the Quexada river and Esperança stream had more conspicuous DAPI^+ , while the population of the Jacutinga river had fewer, more discrete sites. These differences are most likely due to natural differentiation of these populations because *A. altiparanae* is not considered a migratory species.

Therefore, the presence of multiple NORs in *A. altiparanae*, together with the occurrence of single pair in *A. asuncionensis* and the heterochromatin distribution pattern, may help differentiate these species because the morphological characteristics usually are not sufficient.

Given the high karyotypic diversity in the three populations of *A. altiparanae*, the study of meiotic cells becomes interesting to investigate whether such variation affects chromosome pairing. Through the meiotic analysis, it was possible to confirm that chromosome segregation occurs without alterations, since bivalents and univalents were observed during regular meiosis as expected. Therefore, the formation of viable gametes allows the maintenance of this diversity in the populations analyzed. Silva et al.

(2012) observed the same results in analyzing meiotic cells of *A. jacuhiensis*.

Two characteristics shared by *A. asuncionensis* and some *A. altiparanae* individuals the diploid number and the single NOR pattern is also shared with other members of the species complex, indicating that they still maintain a close phylogenetic relationship attributable to a recent divergence. We cannot discount the possibility that the low variability observed in *A. asuncionensis* is due to the small number of individuals analysed. Karyotypic differences and morphological variation may be due to the process of speciation or may only be interpopulational variations of the same biological unit, as suggested by Peres et al. (2012) for *A. altiparanae* of the Grande river and *A. lacustris* of the São Francisco river.

Indeed, Garutti and Britski (2000) observed that populations of *A. altiparanae* are found in a variety of microenvironments and are not morphologically homogenous. Future assignments of new species to the bimaculatus complex should therefore be undertaken with caution. Consideration must be given not only to the traditional morphological characteristics but also to the cytogenetic, reproductive and ecological characteristics. Only by applying this approach can we build a group with valid species and consistent phylogenetic relationships.

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