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Chromosomal Mapping of Repetitive DNA Sequences in Five Species of *Astyanax* (Characiformes, Characidae) Reveals Independent Location of U1 and U2 snRNA Sites and Association of U1 snRNA and 5S rDNA

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Fish cytogenetics · Genome evolution · Histone genes · rDNA · U snRNA

Abstract
Astyanax is a genus of Characidae fishes currently composed of 155 valid species. Previous cytogenetic studies revealed high chromosomal diversification among them, and several studies have been performed using traditional cytogenetic techniques to investigate karyotypes and chromosomal locations of 18S and 5S rDNA genes. However, only a few studies are currently available about other repetitive sequences. Here, the chromosomal location of small nuclear RNA genes, identified as U1 and U2 snRNA clusters, was established and compared to the distribution of 5S rDNA and histone clusters in 5 *Astyanax* species *(A. paranae*, *A. fasciatus*, *A. bockmanni*, *A. altiparanae,* and *A. jordani)* using FISH. The cytogenetic mapping of U1 and U2 snRNA demonstrated a conserved pattern in the number of sites per genome independent of the location in *Astyanax* species. The location of the U1 snRNA gene was frequently associated with 5S rDNA sequences, indicating a possible interaction between the distinct repetitive DNA families. Finally, comparisons involving the location of U1 and U2 snRNA clusters in the chromosomes of *Astyanax* species revealed a very diverse pattern, suggesting that many rearrangements have occurred during the diversification process of this group.

Astyanax Baird & Girard is a Neotropical fish genus within the family Characidae and is currently composed of 155 valid species [Eschmeyer, 2014] that are distributed along the southern United States to central Argentina [Ornelas-Garcia et al., 2008]. The systematics of this group is still confusing and poorly understood. Several species comprise different 'species complexes', such as the *A. fasciatus*, *A. scabripinnis,* and *A. altiparanae* complexes, which have been established based on morphological and/or cytogenetic data [Moreira-Filho and Bertollo, 1991; Fernandes and Martins-Santos, 2004; Artoni et al., 2006; Castro et al., 2015].

In general, *Astyanax* species display a wide variety of diploid chromosome numbers, ranging from 2n = 36 in *A. shubarti* [Morelli et al., 1983] to 2n = 50 in several oth-

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er species [Kavalco and Moreira-Filho, 2003; Kavalco et al., 2009a, b, 2011; Oliveira et al., 2009; Castro et al., 2015]. Moreover, the occurrence of different types of B chromosomes in their cells, the existence of distinct karyotype formulas, and the differential distribution of repetitive DNAs in closely and distantly related species all reinforce the intense and continuous dynamics of the *Astyanax* genome, which includes macro-chromosomal rearrangements (e.g. fissions, fusions, and inversions) and changes at the sub-chromosomal level of resolution [Fernandes and Martins-Santos, 2005; Mantovani et al., 2005; Pazza et al., 2006; Carvalho et al., 2008; Hashimoto et al., 2008; Vicari et al., 2008; Daniel et al., 2012; Santos et al., 2013; Silva et al., 2013].

Small nuclear RNA sequences are components of the spliceosome and may be characterized as 5 types, including U1, U2, U4, U5, and U6 [Valadkhan, 2005]. Cytogenetic mapping data of these sequences are restricted to U1 and U2 snRNA types in several organisms, including mollusks, grasshoppers, mammals, and fish [Lund and Nesbitt, 1988; Cross and Rebordinos, 2005; Cabral-de-Mello et al., 2012; Palacios-Gimenez et al., 2013]. Further investigation revealed the presence of U snRNA clusters in distinct sex and supernumerary chromosomes of some species, which allowed for inferences to be made about their origin and evolution [Bueno et al., 2013; Palacios-Gimenez et al., 2013; Utsunomia et al., 2014]. Moreover, the linkage between U snRNA genes and 5S rDNA was also reported in different organisms which may indicate a possible evolutionary trend [Barzotti et al., 2000; Pelliccia et al., 2001; Cross and Rebordinos, 2005; Manchado et al., 2006a; Vierna et al., 2011].

In the present study, the chromosomal location of U1 and U2 snRNA clusters is established and compared with the distribution of 5S rDNA and histone sequences in 5 *Astyanax* species that currently comprise different species complexes within this genus. The main objective of the study was to develop a better understanding of the genomic organization and evolutionary dynamics of repetitive sequences in the genome of Neotropical fish. This study also sought to reveal the evolutive trends involved in the differentiation process of the species using new chromosomal markers.

Material and Methods

Sampling of Species and Mitotic Chromosomes Preparation

We collected 7 individuals of *A. altiparanae*, 19 individuals of *A. bockmanni* and 10 individuals of *A. fasciatus* from the Capivara River (22°53′57'S/48°23′11'W), and 38 individuals of *A. paranae* from the Cascatinha River (22°53′30″S/48°28′36″W). Both rivers are components of the Tietê River Basin in Botucatu, São Paulo State, Brazil. Moreover, 6 individuals of *A. jordani* were imported from Mexico by a local aquarium store.

The samples were collected in accordance with the Brazilian environmental protection legislation (collection permission MMA/IBAMA/SISBIO-number 3245), and the procedures for collection, maintenance, and analysis of fish specimens were performed in accordance with the international protocols on animal experimentation followed by the Universidade Estadual Paulista, UNESP. After analysis and identification, all specimens were deposited at the fish collection of the Laboratório de Biologia e Genética de Peixes (LBP), UNESP, Botucatu, SP, Brazil, under the vouchers LBP13346 *(A. altiparanae)*, LBP13342 *(A. bockmanni)*, LBP13344 *(A. fasciatus)*, LBP19572 *(A. paranae)*, and LBP19575 *(A. jordani)*.

Mitotic chromosomes were obtained from tissue cell suspensions of the anterior kidney according to Foresti et al. [1981]. The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) according to Levan et al. [1964] and were arranged in descending order of size and morphology in the karyotypes.

Repetitive Probes and FISH Experiments

5S rDNA, U2 snRNA, and H1 and H3 histone gene probes were obtained using PCR amplification directly from the genome of *A. paranae* with previously described primers [Pendás et al., 1994; Colgan et al., 1998; Hashimoto et al., 2011; Bueno et al., 2013]. Due to the difficulty of amplifying U1 snRNA sequences in Characidae species using the primer described by Cabral-de-Mello et al. [2012], we designed a new set of primers for U1 snRNA using NGS sequencing data on *A. mexicanus*, which are available in GenBank, and human U1 snRNA was used as a reference. The primers were U1F (5′-GCAGTCGAGATTCCCACATT-3′) and U1R (5′-CTTACCTGGCAGGGGAGATA-3′). With these primers, we amplified sequences of U1 snRNA using DNA of *A. paranae* as a template. The reactions were performed in 1× PCR buffer, 1.5 mM of $MgCl_2$, 200 mM of each dNTP (dATP, dCTP, dGTP, dTTP), 0.5 U of Taq polymerase (Invitrogen), 0.1 mM of each primer, and 50 ng of gDNA. The basic program used to amplify these regions consisted of denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 45 s at 54°C, 45 s at 72°C, and a final extension of 5 min at 72°C. The PCR experiments were conducted in a Veriti 384-well Thermal Cycler Applied Biosystems (ABI). The probes were labeled with digoxigenin-11-dUTP or biotin-16-dUTP by PCR.

Double-FISH was performed under high stringency conditions using the method described by Pinkel et al. [1986]. Slides were incubated with RNAse (50 µg/ml) for 1 h at 37°C, and then chromosomal DNA was denatured in 70% formamide/2× SSC for 5 min at 70°C. For each slide, 30 µl of hybridization solution containing 200 ng of each labeled probe, 50% formamide, 2× SSC, and 10% dextran sulfate was denatured for 10 min at 95°C, dropped on the slides, and hybridized overnight at 37°C in a 2× SSC moist chamber. Post hybridization, slides were washed in 0.2× SSC/15% formamide for 20 min at 42°C, followed by a second wash in 0.1× SSC for 15 min at 60°C and a final wash at room temperature in 4× SSC, 0.5% Tween for 10 min. Probe detection was performed with Avidin-FITC (Sigma) or anti-digoxigenin-rhodamine (Roche), and chromosomes were counterstained with DAPI

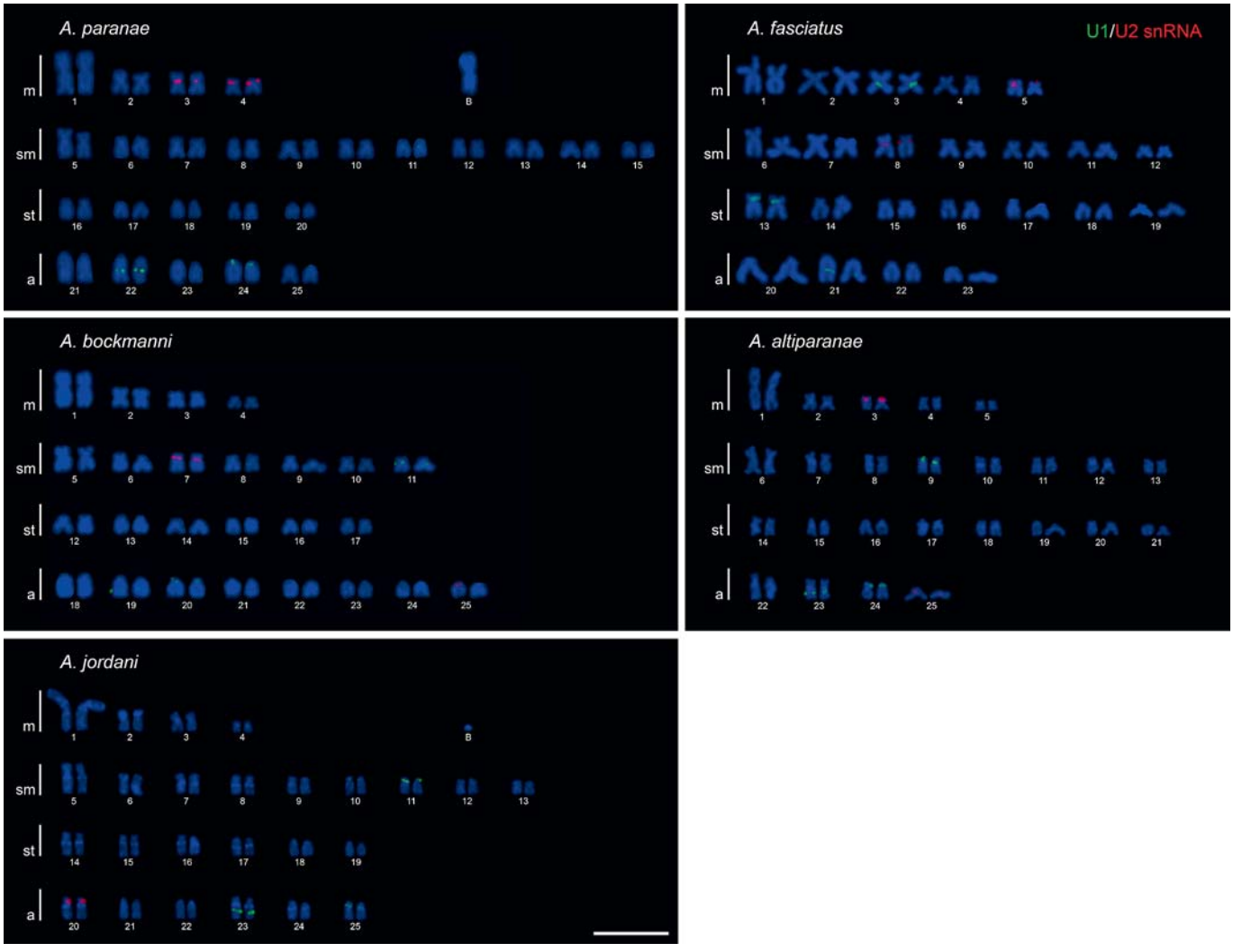

Fig. 1. Karyotypes of the *Astyanax* species after FISH with U1 (green) and U2 snRNA (red) probes and counterstaining with DAPI. Bar = 10 μm.

(4′,6-diamidino-2-phenylindole, Vector Laboratories). For each species, a minimum of 20 cells was analyzed to confirm FISH results.

Molecular Analysis

U1 snRNA sequences of *A. paranae* were obtained to confirm the nucleotide composition of the probe as well as the quality of the primers described. PCR bands obtained for this gene were ligated to a TOPO TA cloning vector for transformation of One Shot TOP10 Competent Cells. Eight clones were chosen for DNA sequencing. The plasmid DNA was then isolated with the Perfectprep Plasmid Mini kit (Eppendorff), and PCR was performed with M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) primers. The PCR products were purified using the ExoSAP-IT kit (USB Corporation) and sequenced in both directions with the Big Dye TM Terminator v 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's instructions. The sequences were initially analyzed with BioEdit 5.0.9 software [Hall, 1999] and used as queries for BLAST searches [Altschul et al., 1990] against the NCBI nucleotide collection (http://www.ncbi.nlm.nih.gov/blast). Next, the U1 snRNA sequence for *A. paranae* was deposited in the NCBI database under the access number KP411800.

Results

Basic karyotype information revealed differences among the analyzed species. While specimens of *A. fasciatus* presented a karyotype of 2n = 46 chromosomes composed of 10m + 14sm + 14st + 8a, the other 4 species showed the same 2n = 50 diploid chromosome number

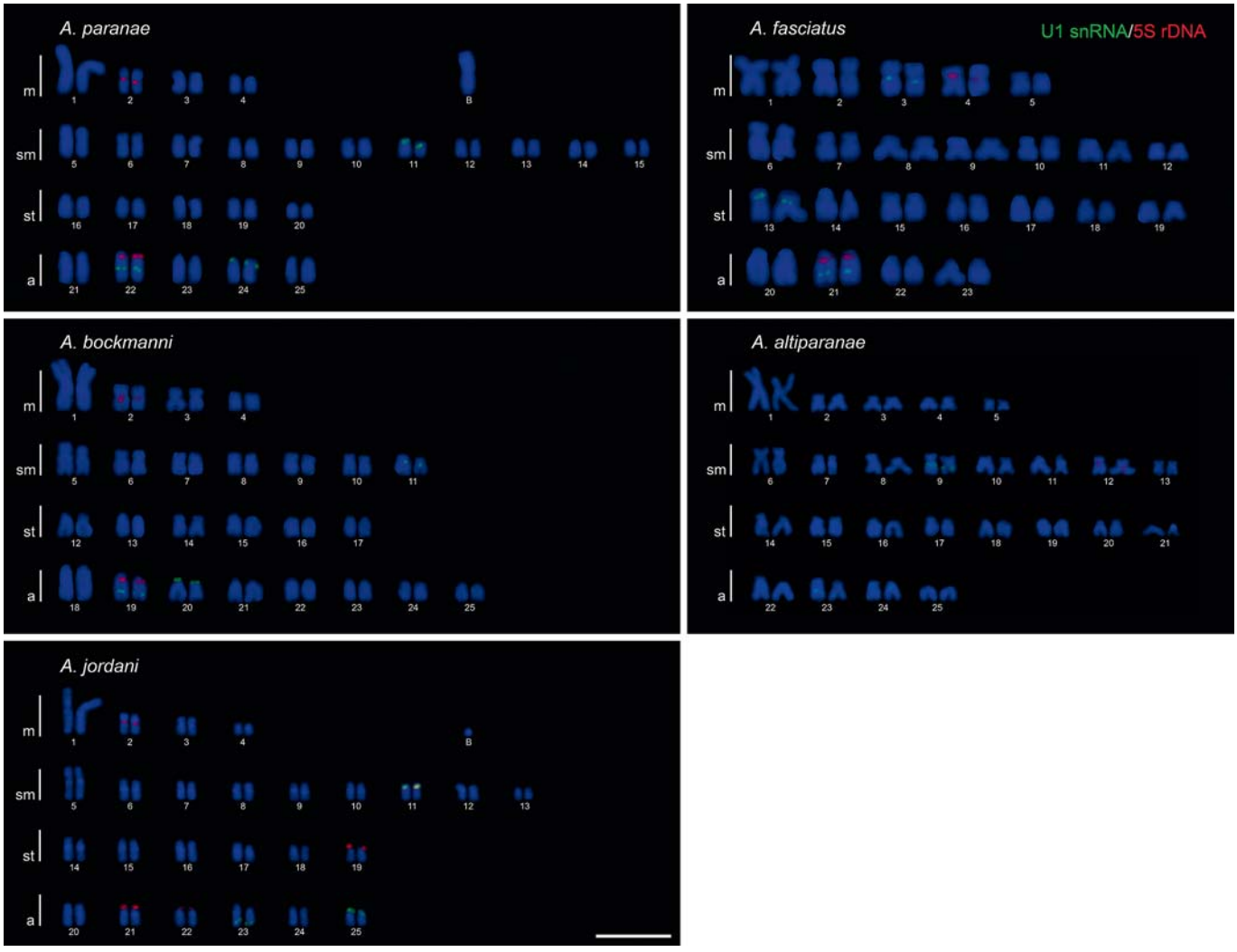

Fig. 2. Karyotypes of the *Astyanax* species after FISH with U1 snRNA (green) and 5S rDNA (red) probes and counterstaining with DAPI. Note the chromosomes bearing both markers. In *A. jordani* the sequences are co-localized on chromosomes of pair 11, on pair 22 of *A. paranae*, on pair 21 of *A. fasciatus,* and on pair 19 of *A. bockmanni*. Bar = 10 μm.

(fig. 1). However, the following differences in the karyotype formula for these species were detected: (1) *A. paranae,* 8m + 22sm + 10st + 10a; (2) *A. bockmanni,* 8m + 14sm + 12st + 16a; (3) *A. altiparanae,* 10m + 16sm + 16st + 8a; (4) *A. jordani,* 8m + 18sm + 12st + 12a (fig. 1). Moreover, 23 out of 38 specimens (60.5%) of *A. paranae* had 1 (20 individuals) or 2 (3 individuals) B chromosomes in 100% of the cells. Likewise, a variation from 0 to 2 B chromosomes was also observed in the cells of 1 out of 6 individuals of *A. jordani*.

FISH mapping with the U2 snRNA probe revealed signals in the proximal region in one chromosomal pair of *A. jordani* and in 2 chromosomal pairs of the other analyzed species (fig. 1). However, differences in the morphology of the U2-bearing chromosomes for these species were also found. In *A. paranae*, 2 metacentric chromosome pairs (No. 3 and 4) had U2 snRNA clusters (fig. 1), while in *A. fasciatus* these sequences were identified in 1 metacentric (No. 5) and 1 submetacentric pair (No. 8) (fig. 1). Additionally, U2 snRNA clusters were also identified in 1 submetacentric and 1 acrocentric chromosome pair of *A. bockmanni*, in 1 metacentric and 1 acrocentric chromosome pair of *A. altiparanae*, and in only 1 acrocentric pair of *A. jordani*. The U2 snRNA clusters were not associated with 5S rDNA and/or H1 histone clusters in the chromosomes of the 5 species analyzed.

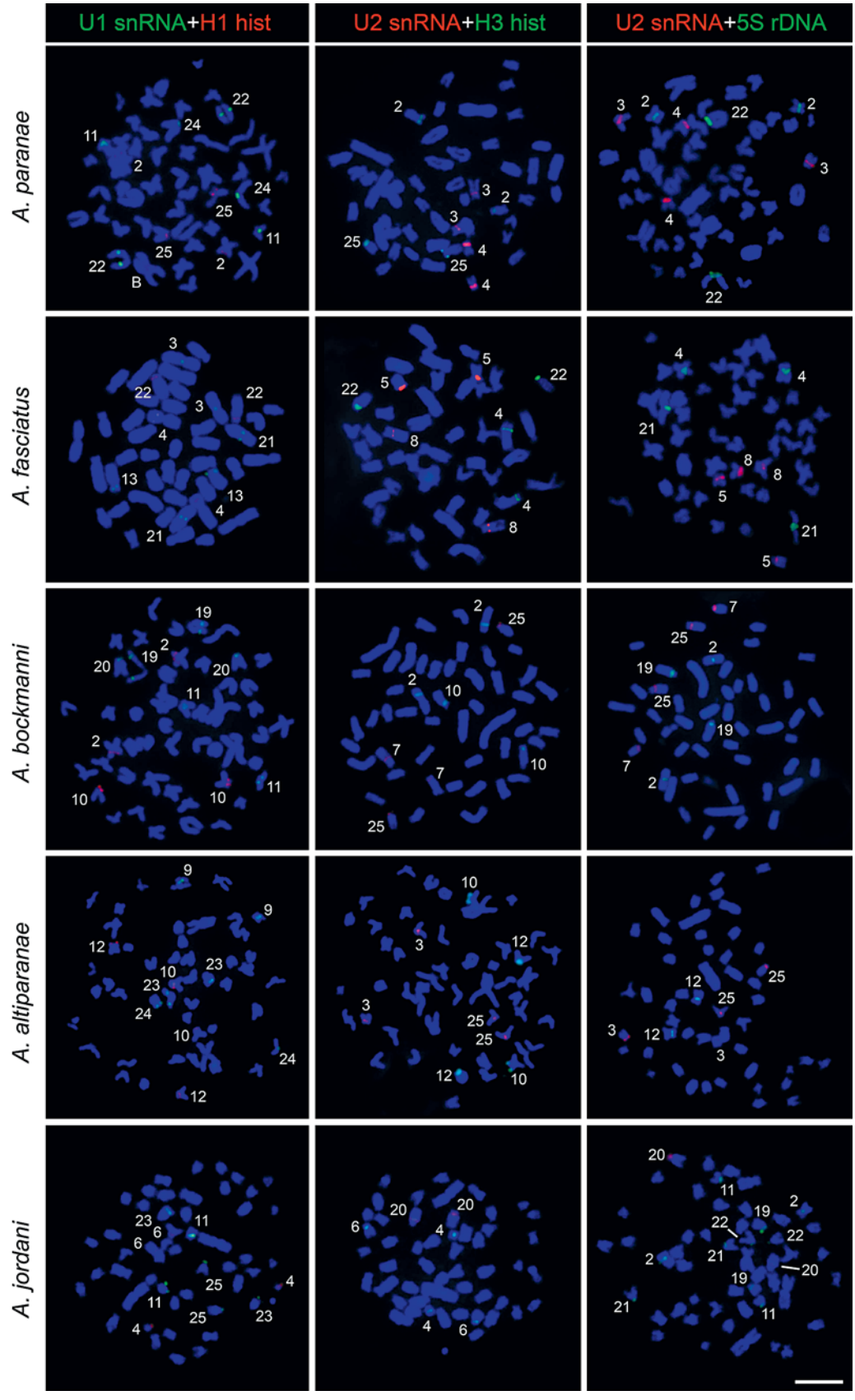

Fig. 3. Metaphases of *Astyanax* species after FISH with repetitive DNA probes for U1 and U2 snRNA, H1 and H3 histone genes, and 5S rDNA. Numbers indicate the chromosomes that carry marked gene sequences in the metaphase plates. Bar = 10 μm.

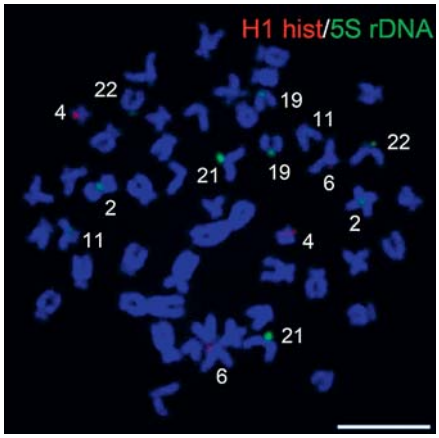

Fig. 4. Metaphase of *A. jordani* after FISH with repetitive DNA probes for H1 histone gene and 5S rDNA and counterstaining with DAPI. Note the independent location of 5S rDNA and histone gene sites. Numbers indicate the chromosomes that carry marked gene sequences. Bar = 10 μm.

U1 snRNA clusters were detected in 6 chromosome pairs in the 5 species analyzed (figs. 2, 3). In *A. paranae*, *A. bockmanni*, *A. altiparanae,* and *A. jordani*, the U1 snRNA clusters were identified in the proximal region of 1 submetacentric and 1 acrocentric chromosome pair and in the interstitial region of 1 acrocentric pair. These chromosomes correspond to pairs 11, 22, and 24 in *A. paranae*; 11, 19, and 20 in *A. bockmanni*; 9, 23, and 24 in *A. altiparanae;* and 11, 23, and 25 in *A. jordani* (figs. 1–3). Furthermore, in *A. fasciatus*, the U1 snRNA clusters were localized to the proximal region of 1 metacentric chromosome pair (No. 3) and 1 subtelocentric (No. 13) and to the interstitial region of 1 acrocentric pair (No. 21) (figs. 1–3).

5S rDNA sequences were identified in various sites in the *Astyanax* species. In *A. altiparanae*, 1 submetacentric chromosome pair (No. 12) showed 5S rDNA sequences in the proximal region, while *A. paranae, A. bockmanni,* and *A. fasciatus* had these sequences in the proximal region of 1 metacentric chromosome pair (No. 2, 2, and 4, respectively) and in the distal region of one acrocentric chromosome pair (No. 22, 19, and 21, respectively). Notably, the 5S rDNA and U1 snRNA sites were syntenic for a single pair of chromosomes in *A. paranae, A. bockmanni,* and *A. fasciatus* (fig. 2). In *A. jordani*, 5S rDNA clusters were observed in 5 chromosomal pairs (No. 2, 11, 19, 21, and 22), and the 5S rDNA site is co-located with U1 snRNA in the submetacentric chromosome pair 11 (fig. 2).

The histone clusters were localized in accordance with previous studies performed by Hashimoto et al. [2010] and Silva et al. [2013, 2014], showing conserved positions in 4 chromosome pairs in all the analyzed species. Notably, *A. jordani* was the only species that did not have synteny between histone genes and minor ribosomal sites (fig. 4). Among the repetitive DNA probes for U1 and U2 snRNA, 5S rDNA and histone genes were mapped, and only H1 histone sequences were located on the B chromosome of *A. paranae*, confirming previous results [Silva et al., 2014].

The nucleotide sequence obtained for U1 snRNA from *A. paranae* (119 bp) was similar to the sequences obtained from other organisms that were available in the NCBI database. This sequence showed 94.1% similarity with the U1 snRNA gene sequence obtained from *A. mexicanus*.

Discussion

The basic karyotype information obtained from *A. paranae, A. altiparanae,* and *A. fasciatus* was consistent with previous reports on the diploid chromosome number and general karyotype structure. Several studies have found that *A. fasciatus* samples may have different diploid numbers in sympatric morphotypes, a phenomenon that seems to be common for this species in several Brazilian river basins [Artoni et al., 2006; Pazza et al., 2006; Pansonato-Alves et al., 2013]. In the present study, only individuals showing 2n = 46 chromosomes were collected at the sites sampled, and the karyotype found in individuals of this population is very similar to that analyzed by Hashimoto et al. [2011], although differences regarding their karyotypic formulas were observed. Notably, this phenomenon also occurred in the *A. altiparanae* sample analyzed here, indicating the occurrence of continuous karyotypic changes (e.g. chromosomal rearrangements) among *Astyanax* species and populations [Vicari et al., 2008; Pansonato-Alves et al., 2013].

The results from cytogenetic mapping of histone and 5S rDNA genes were congruent with similar data obtained in these same species but sampled at other collection sites [Hashimoto et al., 2011; Pansonato-Alves et al., 2013; Silva et al., 2013], reinforcing the conserved location of these clusters in *Astyanax* (fig. 5). Remarkably, *A. jordani* was the only species that did not show syntenic 5S rDNA and histone sites, which is probably due to the dynamics of the minor ribosomal sites in this species. In a previous study, Kavalco et al. [2007] reported the occurrence of six 5S rDNA clusters in the genome of *A. mexicanus*, an ancestor of *A. jordani* [Popper, 1970]. Here, 10 clusters of 5S rDNA were observed for *A. jordani*, pointing to a striking dispersion process for 5S rDNA sequences during the evolutionary history of this species.

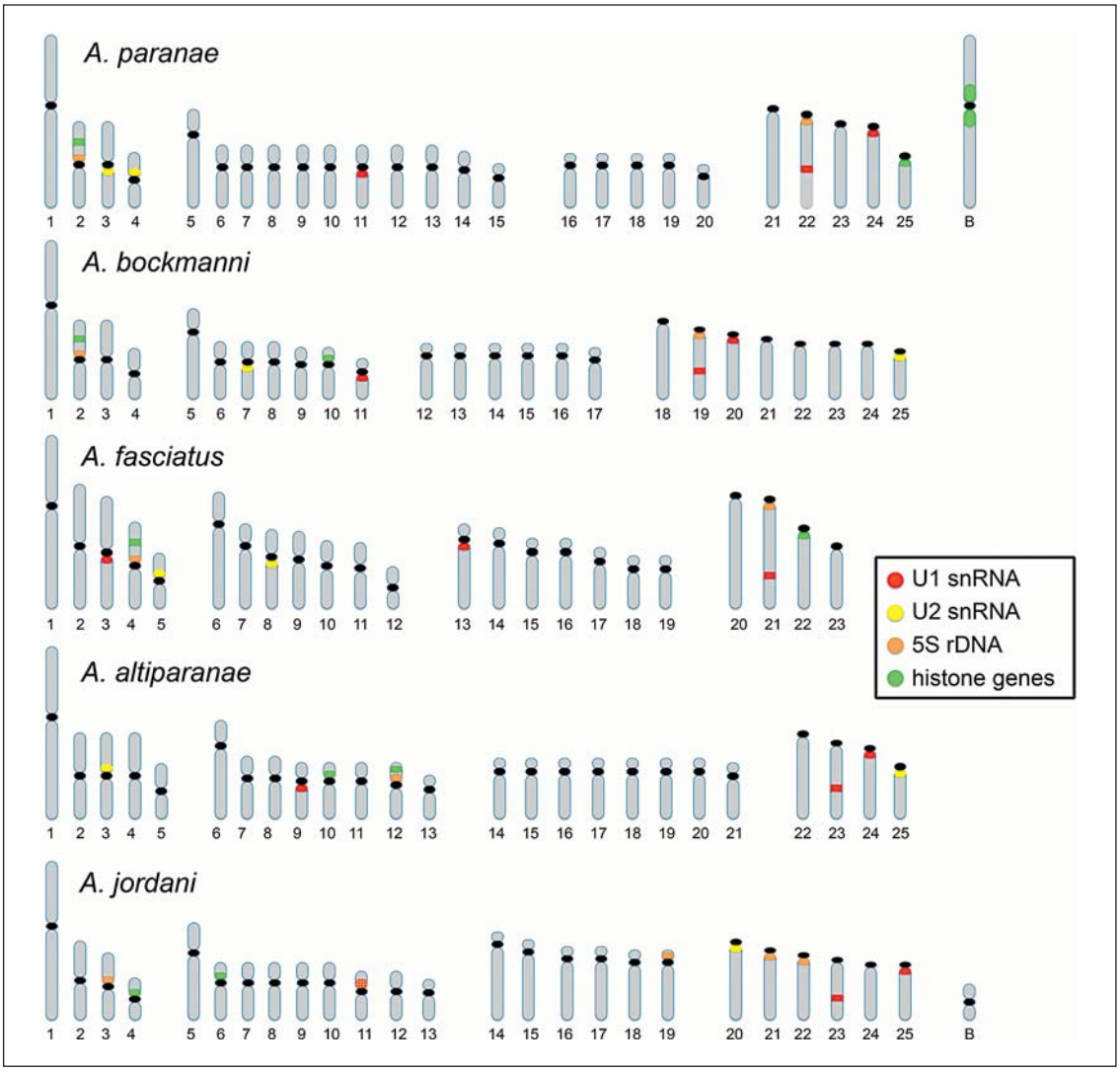

Fig. 5. Ideogram representing the karyotypes of *Astyanax* species with the location of the repetitive DNA sequences marked.

U1 and U2 snRNA genes showed a strong conservation in the number of sites per genome. Such a conserved feature seems to be common in different groups of fishes, with only 1 or few chromosomes per genome bearing these genes [Merlo et al., 2010; Cabral-de-Mello et al., 2012; Merlo et al., 2012 a, b; Supiwong et al., 2013; Utsunomia et al., 2014]. In several cichlid species, the U1 snRNA is clustered in a single pair, in the same manner as can be found in *Gymnotus* species for the U2 snRNA. However, several examples of diversification of U2 snRNA genes have already been described, including their differential dispersion and organization in closely related species [Úbeda-Manzanaro et al., 2010; Utsunomia et al., 2014]. Additionally, the apparent absence of a linkage between these 2 small nuclear RNAs seems to be normal in fish, with few exceptions reported in the literature [Manchado et al., 2006a, b]. Moreover, the separate location of U1 and U2 snRNA clusters found in the genome of *Astyanax* species (fig. 1) was demonstrated in grasshoppers species as well [Bueno et al., 2013; Palacios-Gimenez et al., 2013].

Synteny between repetitive sequences can provide interesting insights into genome evolution and organization [Andrews et al., 1987; Hashimoto et al., 2012]. The proximity of histone and 5S rDNA cluster sequences in one chromosome pair of several Characidae species led some authors to suggest a possible selective advantage provided by

such association [Hashimoto et al., 2011; Silva et al., 2013, 2014]. Remarkably, other co-localization occurrences involving different repetitive sequences have been reported in *Astyanax*, which can be useful for understanding the organization of the genome in these species, e.g. the possible role of As51 satellite DNA in silencing major ribosomal genes [Vicari et al., 2008] or the association and potential spreading of 18S rDNA genes by the *Rex3* transposable element [Silva et al., 2013]. Here, we describe a novel syntenic event between U1 snRNA and 5S rDNA sequences in 4 species of *Astyanax*. While in *A. jordani* these sequences are co-located in 1 submetacentric chromosome, in *A. paranae*, *A. bockmanni,* and *A. fasciatus* the U1 snRNA and 5S rDNA are clustered at different regions in the same chromosome. In *A. jordani* this association occurs in a submetacentric chromosome, while in the other 3 species the associated sequences are present in an acrocentric chromosome, which suggests a difference in the origin of the association process in these 4 species. The molecular association between U1 snRNA and 5S rDNA sequences has already been reported in different organisms, including several razor shell species [Vierna et al., 2011] and the fish *Solea senegalensis* [Pelliccia et al., 2001; Manchado et al., 2006a]. However, whether it provides any selective advantage remains unclear.

U1-bearing chromosomes with similar morphology in distinct *Astyanax* species suggest the homology of this chromosome pair in the different species. This possibility includes those species that harbor 5S rDNA sequences, but not the chromosome pair 3 of *A. fasciatus*, which is probably the result of a fusion with another chromosome, a hypothesis already proposed by Pansonato-Alves et al. [2013]. Conversely, the genomic location of U2 snRNA does not allow the precise identification of chromosome homology. Considering the proximal position of these sites, it can be hypothesized that multiple pericentric inversions may have occurred in these specific chromosome pairs to generate the present configuration.

Our results provided novel information about the distribution of repetitive sequences in the genome of Neotropical fish. The new set of primers provided here for the amplification of U1 snRNA works precisely in fish samples and was useful for the construction of FISH probes. The cytogenetic mapping of U1 and U2 snRNA in 5 *Astyanax* species revealed a conserved number of sites per genome, independent of the location, even in distantly related species. Moreover, chromosome mapping of U1 snRNA genes in *Astyanax* provided reliable results that may be a useful cytogenetic approach for research in other fish species.

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