Satellite DNA and chromosomes in Neotropical fishes: methods, applications and perspectives


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Constitutive heterochromatin represents a substantial portion of the eukaryote genome, and it is mainly composed of tandemly repeated DNA sequences, such as satellite DNAs, which are also enriched by other dispersed repeated elements, including transposons. Studies on the organization, structure, composition and in situ localization of satellite DNAs have led to consistent advances in the understanding of the genome evolution of species, with a particular focus on heterochromatic domains, the diversification of heteromorphic sex chromosomes and the origin and maintenance of B chromosomes. Satellite DNAs can be chromosome specific or species specific, or they can characterize different species from a genus, family or even representatives of a given order. In some cases, the presence of these repeated elements in members of a single clade has enabled inferences of a phylogenetic nature. Genomic DNA restriction, using specific enzymes, is the most frequently used method for isolating satellite DNAs. Recent methods such as C0–1 DNA and chromosome microdissection, however, have proven to be efficient alternatives for the study of this class of DNA. Neotropical ichthyofauna is extremely rich and diverse enabling multiple approaches with regard to the differentiation and evolution of the genome. Genome components of some species and genera have been isolated, mapped and correlated with possible functions and structures of the chromosomes. The SSHindIII-DNA satellite DNA, which is specific to Hoplias malabaricus of the Erythrinidae family, has an exclusively centromeric location. The AsS1 satellite DNA, which is closely correlated with the genome diversification of some species from the genus Astyanax, has also been used to infer relationships between species. In the Prochilodontidae family, two repetitive DNA sequences were mapped on the chromosomes, and the SATH 1 satellite DNA is associated with the origin of heterochromatic B chromosomes in Prochilodus lineatus. Among species of the genus Characidium and the Parodontidae family, amplifications of satellite DNAs have demonstrated that these sequences are related to the differentiation of heteromorphic sex chromosomes. The possible elimination of satellite DNA units could explain the genome compaction that occurs among

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INTRODUCTION

Teleostei represents more than half of the living vertebrates, with over 32,500 known species and constitutes an extremely diverse group that inhabits various aquatic environments (Nelson, 2006). A large portion of this diversity is in the Neotropical region. Chromosomal rearrangements correlated with satellite DNA are known in this group, but they remain poorly understood, which justifies a detailed discussion of this class of DNA, including (1) the significance of extensive heterochromatic domains in some species; (2) the origin and diversification of recurrent systems of sex chromosomes between families or genera; and (3) the origin, maintenance and possible functions of heterochromatic B chromosomes. More detailed studies on satellite DNA families could also contribute towards the cytotaxonomy and phylogenetic systematics of different families, genera and species that have a confusing taxonomy and poor phylogenetic relations.

In eukaryotic genomes, most segments of constitutive heterochromatin contain large amounts of repetitive DNA sequences. Satellite DNA is one of the components of heterochromatin, which is also enriched with other dispersed repeated elements, including transposons (Mazzuchelli & Martins, 2009). Tandemly repetitive DNA sequences are known as satellite DNA. Satellite DNA sequences are organized in specific sites of a chromosome pair. These repetitive units, however, most often evolve in parallel (Dover, 1986), leading to intra-species homogenization among repetitive units (Ugarkovic & Plohl, 2002). This chromosome distribution is actually a reflection of a combined evolution mode of sequences among non-homologous chromosomes (Ugarkovic & Plohl, 2002). In such cases, inter-chromosome dispersion is thought to be quicker than mutation rates or intra-chromosome homogenization. Thus, satellite DNA sequences can be used as markers for studies of genome diversification and phylogenetic relations. Considering the extremely variable evolutionary dynamics of these sequences, they can provide useful information for both microevolution studies (in the case of species-specific or chromosome-specific satellite DNA) (Ugarkovic & Plohl, 2002), as well as phylogenetic studies (in which a satellite DNA is shared by some species in a given group) (Pons et al., 2002; Pons & Gillespie, 2003; Kantek et al., 2009a). Satellite DNA families, however, may also remain conserved over time due to a slow evolution, maintained by low mutation rates with a reflection in the homogenization of the sequences (De La Herrán et al., 2001).

In Teleostei, chromosome-specific satellite DNA may be a useful cytogenetic marker (Saito et al., 2007), sometimes used to understand phylogenetic relations among different taxa (De La Herrán et al., 2001; Saito et al., 2007), clarify cytotaxonomy in species complexes, such as demonstrated in the Astyanax scabripinnis (Jenyns) species group (Mantovani et al., 2004; Vicari et al., 2008a; Kantek et al., 2009a), determine likely origins of supernumerary chromosomes (Mestriner et al., 2007), and study the genetic basis of sex chromosome diversification (Mestriner et al., 2007; Pons et al., 2002).
2000; Jesus et al., 2003; Ziegler et al., 2003; Artoni et al., 2006a) and characterize sex chromosomes (Devlin et al., 2001). Thus, the present paper reviews the current status of the knowledge of satellite DNA in various groups of Neotropical Actinopterygii, with a particular focus on B chromosomes, sex chromosomes and phylogenetic relations in order to help predict future perspectives for research in this field.

METHODS FOR OBTAINING AND ISOLATING SATELLITE DNA

In recent years, Neotropical Actinopterygii cytogenetics has been improving in terms of the search for more consistent answers regarding the genome diversification mechanisms of species. A large number of researchers use the heterochromatin portion of the genome to support their theories on karyotype evolution. The traditional method for isolating repetitive DNA sequences in Neotropical Actinopterygii is genomic DNA restriction. Two new strategies, however, have been successfully used: (1) re-association kinetics based on $C_{0t-1}$ DNA and (2) the microdissection of chromosomes submitted to C-banding and subsequent amplification of heterochromatic sequences using DOP-PCR (degenerate oligonucleotide primed-polymerase chain reaction).

ISOLATION OF REPETITIVE DNA THROUGH GENOMIC RESTRICTION

In this procedure, the genomic DNA is digested with specific restriction enzymes (following the manufacturer’s instructions). The digestion reaction is incubated for c. 16 h in a water-bath at 37°C and one aliquot of this reaction is submitted to electrophoresis in agarose gel (2%). DNA bands are selected and purified from the gel, cloned and submitted to nucleotide sequencing, with subsequent in situ localization of these regions in the chromosomes [Fig. 1(a)]. The major problem of this method is to test a large enough set of restriction enzymes and to find satellite DNA that has a matching sequence to the given enzyme. Among Neotropical Actinopterygii, satellite DNAs from the species Hoplias malabaricus (Bloch) (Haaf et al., 1993; Martins et al., 2006), A. scabripinnis (Mestriner et al., 2000), Parodon hilarii Reinhardt (Vicente et al., 2003) and Prochilodus lineatus (Valenciennes) (Jesus et al., 2003) have been isolated using this method.

ISOLATION OF REPETITIVE DNA THROUGH RE-ASSOCIATION KINETICS

This procedure is based on the $C_{0t-1}$ DNA method (DNA enriched with highly and moderately repetitive sequences). $C_{0t-1}$ DNA can be isolated based on the method described by Zwick et al. (1997), recently adapted by Ferreira & Martins (2008) for the isolation of repetitive DNA in Actinopterygii using the species Oreochromis niloticus (L.). Genomic DNA with a concentration between 100 and 500 ng μl$^{-1}$ in a 0.3 mol l$^{-1}$ NaCl solution is autoclaved (121°C, 1.034 × 10$^5$ Pa) for 5 min to obtain fragments ranging from 100 to 2000 base pairs (bp). Then the DNA is denatured at 95°C for 10 min, placed in ice for 10 s and subsequently
Restriction enzymes cleave DNA into smaller segments of various sizes.

**Pattern after digestion**

- 1000 bp
- 500 bp
- 100 bp
- 10 bp

**Restriction endonuclease**

- Hind III

**Overnight at 37°C**

1000 bp
500 bp
100 bp
10 bp

**Restriction enzymes cleave DNA into smaller segments of various sizes**

**Pattern after digestion**

- 1000 bp
- 500 bp
- 100 bp
- 10 bp

**Genomic DNA**

- Overnight at 37°C

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**Restriction enzymes cleave DNA into smaller segments of various sizes**

**Pattern after digestion**

- 1000 bp
- 500 bp
- 100 bp
- 10 bp

**Fig. 1.** Representative schematic of the methods for obtaining and isolating satellites DNAs: in (a) isolation of repetitive DNAs through genomic restriction; (b) isolation of repetitive DNAs using re-association kinetics based on the Cot – 1 DNA method and (c) isolation of repetitive DNAs through chromosome microdissection and DOP-PCR reactions.

placed at 65°C for re-annealing. At this step, three DNA annealing times can be tested (0, 1 and 5 min). One unit of nuclease S1 and 1× enzyme reaction buffer per 1 μg of DNA are then added and the solution incubated at 37°C for 8 min [Fig. 1(b)]. The repetitive portion of this DNA can be recovered by freezing it immediately in liquid nitrogen and performing an extraction using phenol–chloroform. The resulting DNA fragments can be cloned in blunt-ended vectors and then sequenced, or directly labelled by nick translation and used as probes for fluorescent in situ hybridization (FISH).

**ISOLATION OF REPETITIVE DNA THROUGH CHROMOSOME MICRODISSECTION AND DOP-PCR**

Metaphasic chromosomes obtained through conventional procedures and maintained in a 3:1 methanol–acetic acid solution are transferred to a 24 mm × 60 mm cover glass and submitted to the C-banding procedure (Sumner, 1972), in which the heterochromatic portion of the chromosomes remains practically intact, while the
euchromatic regions are partially or totally removed. The material is stained with 5% Giemsa (v/v) for 5 min and washed in distilled water. The procedure allows the identification of the chromosome to be microdissected through the visualization of its heterochromatic content. The chromosome preparations are then examined under an inverted microscope equipped with a mechanical microdissector. About 20 chromosomes of interest are microdissected with capillary needles with tips of c. 0.7 μm. Following microdissection, the tip of the needle containing the chromosome material is broken in the interior of a 0.2 ml microtube, in which the first DOP-PCR is performed [Fig. 1(c)].

The DOP-PCRs follow the general procedure described by Telenius et al. (1992), with modifications. The PCR consists of 1× ThermoSequenase reaction buffer, 400 μM dNTPs and 2 μM DOP primer (5′ ccc act cga gnn nnn nat gtg g 3′). The microtube is heated to 95°C for 10 min, followed by the addition of 10 U of ThermoSequenase enzyme. The first amplification is carried out through RAMP-PCR: 94°C 3 min; 12 cycles of low stringency (94°C 1 min and 30 s, 32°C 2 min, increasing 0.2°C s⁻¹ until reaching 72°C and 72°C 2 min); followed by 30 cycles of high stringency (94°C 1 min and 30 s, 52°C 1 min and 30 s and 72°C 1 min and 30 s). The PCR products are checked in 1% agarose gel, in which the expected size of DNA fragments corresponds to a smear of 100 and 600 bp. Then, 2 μl of this PCR product is aliquoted and submitted to a new amplification reaction carried out at a final volume of 50 μl, containing 1× reaction buffer, 2 mM MgCl₂, 400 μM dNTPs, 2 μM DOP primer and 2 U Taq DNA polymerase under the following conditions: (1×) 94°C C 3 min; (35×) 90°C C 1 min and 30 s, 52°C C 1 min and 30 s, 72°C C 1 min and 30 s, and (1×) 72°C C 5 min. The product of this PCR can be purified from 1.5% agarose gel and cloned to an appropriate vector for subsequent sequencing analyses and in situ localization. Alternatively, this product can be submitted to a new PCR (1× reaction buffer, 2 mM MgCl₂, 400 μM dATP, dGTP and dCTP, 280 μM dTTP, 120 μM dUTP 11-digoxigenin, 2 μM DOP primer and 2 U Taq DNA polymerase), with similar conditions to the last one described, for use directly by FISH method. The overall hybridization procedure follows the protocol described by Pinkel et al. (1986), under high stringency conditions, i.e. 2.5 ng μl⁻¹ probes, 50% deionized formamide, 10% dextran sulphate and 2× SSC at 37°C overnight. In many Actinopterygii, there is difficulty in recognizing a target chromosome due to its morphological similarity with other chromosomes of the karyotype. Thus, the advantage of this procedure lies in the possibility of the initial identification of the target chromosome to be microdissected based on its heterochromatic content.

SATELLITE DNA IN NEOTROPICAL ACTINOPTERYGIID

Although relatively recent and still rarely followed considering the large diversity of ichthyofauna, the investigation of satellite DNA in Neotropical Actinopterygii has provided important advances in the analysis of the heterochromatic component of the genome and its relation with chromosome structures, the origin and diversification of B and sex chromosomes and inferences on relationships among species.
HOP SATELLITE DNA AND 5S HindIII-DNA IN HOPLIAS MALABARICUS

The first description of a satellite DNA in Neotropical Actinopterygii was carried out by Haaf et al. (1993) with *H. malabaricus* (Erythrinidae), which is endemic to South America and widely distributed throughout hydrographic basins in Brazil. Erythrinidae is related to three other families: Lebiasinidae, Ctenoluciidae and the African Hepsetidae (Buckup, 1998). Although relatively small, with only three genera, *Hoplerithrinus* Gill, *Erythrus* Scopoli and *Hoplias* Gill, the taxonomy of this group is not well resolved. In the genus *Hoplias*, the occurrence of nine species is recognized (Oyakawa, 2003), for which *Hoplias lacerdae* (Miranda Ribeiro) and *H. malabaricus* are the most representative. Each of these two species, however, may actually correspond to a species group. The chromosome data on *H. malabaricus* indeed corroborate the occurrence of a species group, with seven well-established karyomorphs, A–G (for a review, see Bertollo et al., 2000), easily characterized by the diploid number, chromosome morphology and distinct sex chromosome systems (Table I). The portion of heterochromatin in the *H. malabaricus* genome is found in the pericentromeric region of the chromosomes as well as in the terminal region of some pairs in the karyotype. In experiments involving genomic DNA digestion, mainly with the HindIII restriction enzyme, Haaf et al. (1993) isolated a repetitive portion with monomers of 333–366 bp (mean = 355 bp) and identity between 62 and 98%. This satellite DNA was denominated Hop (GenBank accession numbers L11927 and L11928), with an AT content of 67% and subdivided into two evolutionally related subfamilies (A and B). Fluorescent in situ hybridization (FISH) revealed that subfamily A was located in the pericentromeric region of several chromosome pairs of karyomorph D in *H. malabaricus*, whereas subfamily B was specific to the pericentromeric region of one chromosome pair of the same karyomorph. It was proposed that this satellite DNA sequence is evolving in concert and that its function could be related to the centromere of the chromosomes in *H. malabaricus*.

In a similar study, Martins et al. (2006) used the genomic DNA restriction method with HindIII on karyomorph A in *H. malabaricus* (*2n* = 42 chromosomes, with

<table>
<thead>
<tr>
<th>Karyomorph</th>
<th>2n</th>
<th>Sex chromosome system</th>
<th>Localities</th>
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<tr>
<td>A</td>
<td>42</td>
<td>Not differentiated</td>
<td>From northern to southern Brazil, Uruguay and northern Argentina</td>
</tr>
<tr>
<td>B</td>
<td>42</td>
<td>♀:XX</td>
<td>Vale do Rio Doce (São Francisco basin) and upper Iguaçu river</td>
</tr>
<tr>
<td>C</td>
<td>40</td>
<td>Not differentiated</td>
<td>From northern Brazil to north-eastern Argentina</td>
</tr>
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| D          | 39/40 | ♀: X₁X₁X₂X₂  
♂: X₁X₂Y | Upper Paraná hydrographic basin |
| E          | 42  | ♀: unknown           | Trombetas River (PA State), Brazil |
| F          | 40  | Not differentiated    | From Surinam to south-eastern Brazil |
| G          | 40/41 | ♀:XX  
♂:XY₁Y₂            | Amazonian rivers |
no sex chromosome heteromorphism) and isolated a satellite DNA composed of 350 bp in length, with the presence of base insertions, deletions and substitutions between the clones. Analyses of this sequence revealed considerable identity with the 5S rDNA of vertebrates, including Teleostei species, and with the Hop satellite DNA isolated by Haaf et al. (1993). Subsequently, the 5S rDNA (gene + NTS fraction) in *H. malabaricus* was isolated using specific PCR primers, which confirmed that the satellite sequence denominated 5SHindIII-DNA had considerable similarity with the genes and spacers of the 5S rRNA, differing only in the presence of an imperfect expansion of a TAAA microsatellite sequence and two short deletions (GenBank accession numbers AY624052 and AY624061) (Martins et al., 2006). The hybridization of this sequence on the chromosomes of karyomorph A of *H. malabaricus* revealed labelling in the centromeric region of nine chromosome pairs, and the 5S rDNA probe only in the proximities of the centromeres of pairs three and 14, thereby demonstrating that these two sites are true 5S rDNA sites. Therefore, duplicate copies of 5S rDNA probably originated the 5SHindIII-DNA, and unequal exchanges, transposition, RNA-mediated transposition and gene conversion could be the mechanisms acting on the diversification of 5SHindIII-DNA (Martins et al., 2006). A-rich motifs, which are characteristic of centromeric satellite DNA in vertebrates (Vinãs et al., 2004) and are also present in this satellite sequence support the notion that 5SHindIII-DNA plays some structural or functional role in the chromosomes of *H. malabaricus*.

Haaf et al. (1993) had previously considered the potential of satellite DNA to discriminate probable cryptic species of Hoplias. A comparative analysis among karyomorphs A, D and F in *H. malabaricus*, *H. lacerdae*, *Hoplerythrinus unitaeniatus* (Spix & Agassiz) and *Erythrinus erythrinus* (Bloch & Schneider) revealed differences in the number and position of 5S rDNA and 5SHindIII-DNA sites between these species (Ferreira et al., 2007). Although 18 5SHindIII-DNA sites constitute the most frequently found situation for karyomorph A, an analysis of different allopatric populations of this karyomorph revealed 22 sites in one of them. Thus, the distribution of 5S rDNA sites exhibited inter-population differences in terms of number and location (Fig. 2). This makes 5SHindIII-DNA and 5S rDNA sites good or even specific population markers in *H. malabaricus*, demonstrating evolutionary divergences even between populations of a single karyomorph (Cioffi et al., 2009). Other minor chromosomal divergences among populations pertaining of Karyomorph A corroborate this hypothesis (Vicari et al., 2005).

Significantly, the 5SHindIII-DNA sequences were exclusive to *H. malabaricus*, as they were not found in any of the chromosomes of the other species, even the congeneric species *H. lacerdae* (Ferreira et al., 2007; Blanco et al., 2010). Thus, 5SHindIII-DNA must have emerged during the divergence of *H. malabaricus* from the other groups of Erythrinidae and before the diversification of *H. malabaricus* karyomorphs, and has accompanied the differentiation of these karyotype forms (Ferreira et al., 2007; Blanco et al., 2010). One important question is whether *H. malabaricus* corresponds to a basal or derived species among the Erythrinidae. Although this remains an open question, some cytogenetic characteristics allow us to infer that the group *malabaricus* may be apomorphic among the Erythrinidae, as all the karyomorphs in *H. malabaricus* are dominated by meta–submetacentric chromosomes and a lower diploid number in relation to the other species of the same family.
Fig. 2. Representative idiograms of three populations [(a)–(c)] belonging to karyomorph A of *Hoplias malabaricus* showing differentiations concerning the distribution of *5S*HindIII-DNA (black) and *5S* rDNA (red) sites on the chromosomes. (d) Metaphase plate of a population showing eighteen *5S*HindIII-DNA sites. Bar = 10 μm.

(Bertollo *et al.*, 2000). Blanco *et al.* (2010) also propose that *H. malabaricus* (karyomorph A) has a set of likely apomorphic cytogenetic characteristics in relation to *H. lacerdae*, considering the FISH analysis using chromosome markers. It is also more prudent to explain the emergence of *5S*HindIII-DNA in *H. malabaricus* than its loss in a series of other species.

The evidence described above demonstrates the importance of repetitive DNA sequences in the analysis of the karyotypic and evolutionary differentiation in *H. malabaricus*. The use of other satellite DNA families, as well as robust morphological and molecular phylogenies, however, is needed to establish consistent phylogenetic relations for this group.

**As51 SATELLITE DNA IN ASTYANAX SCABRIPINNIS**

The genus *Astyanax* has wide distribution throughout central and South America and, until recently, belonged to the subfamily Tetragonopterinae (Géry, 1977). Lima *et al.* (2003) carried out a review of morphological characteristics and detected a lack of consistent evidence for monophyletism, classifying this group as *incertae sedis* among the Characidae. *Astyanax* encompasses 121 valid species (Froese & Pauli, 2009), many with a confusing taxonomy due to the extreme inter-population phenotypic plasticity and the lack of reliable morphological characteristics for identification.

This genus exhibits considerable karyotype diversity, with the diploid number ranging from $2n = 36$ in *Astyanax schubarti* Britski to $2n = 50$ in *Astyanax altiparanae* Garutti & Britski, *A. scabripinnis*, *Astyanax giton* Eigenmann and others. Based on morphological and chromosome characteristics, Moreira-Filho & Bertollo (1991) proposed that *A. scabripinnis* is a species complex. There are currently 15 species listed for this group (Bertaco & Lucena, 2006), although cytogenetic evidence indicates a greater number of species (Vicari *et al.*, 2008b).

Mestriner *et al.* (2000) identified a satellite DNA that is 59% AT-rich in the *A. scabripinnis* genome, with monomeric units of 51 bp, denominated As51. FISH
analyses revealed that this DNA is located mainly in the distal heterochromatin, in some nucleolus organiser regions and in chromosome B in the population analysed (Mestriner et al., 2000). This sequence has 58.8% similarity with a portion of the retrotransposon RT2 in Anopheles gambiae and lesser homology with the transposase gene of the transposon TN4430 in Bacillus thuringiensis, suggesting that it originated from a transposable element of DNA. Subsequent studies identified this satellite DNA family in other populations of A. scabripinnis and in Astyanax fasciatus (Cuvier), Astyanax janeiroensis Eigenmann, Astyanax sp. D and Astyanax paranae Eigenmann (Mantovani et al., 2004; Abel et al., 2006; Vicari et al., 2008a; Kantek et al., 2009a), thereby demonstrating its occurrence not only in the group scabripinnis but also among different species of the genus. The distribution pattern of constitutive heterochromatin is a good chromosome marker for discriminating local populations of A. scabripinnis (Moreira-Filho & Bertollo, 1991) and A. fasciatus (Artoni et al., 2006b), indicating that the heterochromatin–As51 differentiation processes may be directly involved in the chromosome evolution of this group.

Mantovani et al. (2004) found that distal blocks of heterochromatin with homology to the As51 sequence (present in two different populations of A. scabripinnis) exhibited different responses to base-specific fluorochromes that could not be attributed to the composition of the As51 satellite DNA bases. Studying the heterochromatin composition in A. janeiroensis, Vicari et al. (2008a) found that the 14 large heterochromatin blocks in the subtelocentric and acrocentric chromosomes of this species were composed of two repetitive DNA families that are co-localized, the satellite As51 and 18S rDNA (Fig. 3). In teleost fishes, the spacer portion of the major rDNA is generally GC-rich (Schmid & Guttenback, 1988), whereas As51 is 59% AT. Thus, it is possible that the co-localization of GC sites of the major rDNA and AT of As51 may lead to a competition effect between base-specific fluorochromes, Chromomycin A3 and 4′, 6 diamidino-2-phenylindole dihydrochloride (DAPI), with consequent attenuated or discordant responses in these chromosome domains (Vicari et al., 2008a). Transposable DNA elements are known to be potent inducers of changes in the host genome (Kidwell, 2002). As51 probably has its origin from a transposable DNA element (Mestriner et al., 2000). Significantly, the rDNA 18S sequences in the large As51 heterochromatic domains of A. janeiroensis have proven to be inactive, revealing the silencing of rRNA genes when co-localized with As51 (Vicari et al., 2008a).

Recently, Kantek et al. (2009a) carried out a comparative mapping project among some Astyanax species (A. scabripinnis, A. paranae, A. janeiroensis, Astyanax sp. D, A. altiparanae and A. fasciatus) using the FISH method with As51 probes. Astyanax altiparanae was the only species that did not exhibit satellite DNA sites in any of the populations analysed. The other species have extensive variations in the number of chromosomes labelled (one to 16 As51 sites). Astyanax paranae, A. janeiroensis, Astyanax sp. D and A. altiparanae have a diploid number of 50 chromosomes (Artoni et al., 2006b; Domingues et al., 2007; Vicari et al., 2008a, b; Ferreira Neto et al., 2009; Kantek et al., 2009b), whereas different populations of A. scabripinnis and A. fasciatus can have variations in their diploid number ranging from 2n = 46 to 2n = 50 chromosomes (Moreira-Filho & Bertollo, 1991). Although satellite DNA sites are likely targets for chromosome breaks and fusions, As51 has not been associated with chromosome rearrangements that modify the diploid number in A. scabripinnis or A. fasciatus (Kantek et al., 2009a).
**Fig. 3.** Male karyotypes of *Astyanax janeiroensis*: (a) C-banded chromosomes; (b) FISH with As51 satellite DNA probe showing 14 distinct sites (red); (c) mapping of the 18S rDNA by FISH showing 22 sites on the chromosomes (green); (d) double FISH with 18S rDNA and As51 probes showing a co-localization of the 14 As51+/18S+ rDNA heterochromatic domains on the chromosome pairs 11, 12, 14, 18, 19, 22 and, 23; and eight sites bearing just 18S rDNA (green). Bar = 10 μm.

Is the As51 DNA implicated in speciation events in the genus *Astyanax*? For now there is no clear answer to this question. This DNA family, however, is basal for *A. scabripinnis, A. paranae, A. janeiroensis, Astyanax* sp. D and *A. fasciatus*, thereby constituting a more related species group with regard to this characteristic than other species of the genus that do not have the As51 satellite DNA, such as *A. altiparanae* (Kantek *et al.*, 2009a). Thus, the considerable variety in the number and position of As51 sites among different populations of the same species must be due to geographic isolation. Assuming that the As51 satellite DNA is derived from a transposable element, each population or species could follow independent evolutionary paths, whether by the accumulation or invasion of other chromosome sites, or the elimination of sequences in the genome, thereby accentuating the karyotype divergences between populations or species. In this sense, we cannot discard the potential role of As51 satellite DNA in speciation processes in the genus *Astyanax*.

**pPH2004 SATELLITE DNA IN PARODON HILARII**

The Parodontidae family is another group of Neotropical Actinopterygii with a confusing taxonomy. This family is formed by a relatively small group, sustained by few morphological diagnostic characteristics and represented by three genera, *Parodon* Valenciennes, *Apareiodon* Eigenmann and *Saccodon* Kner. In this family, 13 species of *Apareiodon*, 18 species of *Parodon* and three species of *Saccodon* are recognized. After a detailed revision of this family, however, this number was
reduced to 21 valid species (Pavanelli & Britski, 2003). Two other species, *Parodon moreirai* Ingenito & Buckup (see Ingenito & Buckup, 2005) and *Apareiodon vladii* Pavanelli (Pavanelli, 2006), were later described. Parallel to these recent descriptions, new questions emerge regarding the systematics of this family, considering the lack of strong diagnostic characteristics for a correct phylogenetic grouping of its representatives. Ingenito (2008) carried out a phylogenetic analysis of this group using the parsimony cladistic method for osteological and morphological characters and found no phylogenetic evidence to sustain the maintenance of the genus *Apareiodon*, now considered a junior synonym of *Parodon*. Thus, the genus *Parodon*, in its new delimitation, is sustained by at least nine exclusive synapomorphies within Parodontidae.

The cytogenetically studied species from this family have a conserved diploid number of $2n = 54$ chromosomes. Despite this apparent conservation, there are a large number of inter-species divergences in relation to the karyotype (Moreira-Filho et al., 1980; Moreira-Filho et al., 1984; Jesus & Moreira-Filho, 2000a) as well as intra-species differences associated with an accentuated structural chromosome polymorphism (Jorge & Moreira-Filho, 2000, 2004). Although a large portion of the species studied in this family has no heteromorphic sex chromosomes, two distinct sex chromosome systems have been described: (1) *Apareiodon affinis* (Steindachner), with a multiple ZZ/ZW$_1$W$_2$ sex chromosomes system (Moreira-Filho et al., 1980; Jesus et al., 1999; Jorge & Moreira-Filho, 2000) and (2) *P. hilarii*, *P. moreirai* (cited as *Parodon* sp.), *A. vladii* (cited as *Apareiodon* sp.), *Apareiodon ibitiensis* Amaral Campos and *Apareiodon* sp., with a single ZZ/ZW sex chromosome system (Moreira-Filho et al., 1993; Jesus & Moreira-Filho, 2000a; Centofante et al., 2002; Vicente et al., 2003; Rosa et al., 2006; Vicari et al., 2006a; Bellafronte et al., 2009).

Until recently, the ZZ/ZW system was restricted to the genus *Parodon* (Moreira-Filho et al., 1993; Jesus & Moreira-Filho, 2000b; Centofante et al., 2002; Vicente et al., 2003). The description of two new cases of this system in *Apareiodon* (Rosa et al., 2006; Vicari et al., 2006a; Bellafronte et al., 2009), however, raised new considerations regarding the karyotype evolution in this family. It is likely that the ZZ/ZW is not homoplasy for *Parodon* and *Apareiodon*, as species with and without differentiated sex chromosomes occur in both genera. Moreover, when present, the sex chromosomes have a similar morphology and a likely identical differentiation mechanism of the W chromosome, thereby supporting the notion that *Parodon* and *Apareiodon* may not be distinct genera. Species that have the ZZ/ZW sex chromosome system are thought to form a monophyletic group, exhibiting this characteristic with exclusivity among other Parodontidae. This hypothesis gains strength, as the identification of species of *Parodon* and *Apareiodon* is sustained by only a few, subtle diagnostic characteristics (Pavanelli & Britski, 2003; Pavanelli, 2003) and is based mainly on the presence or absence of a few small teeth in the lower jaw. In light of such facts, DNA and chromosome markers, together with morphological characteristics, are extremely efficient for the identification of species of Parodontidae and the phylogenetic relationship among them.

Data obtained from satellite DNA in Parodontidae demonstrated the usefulness of this marker in evolutionary studies in this group. Vicente et al. (2003) isolated and identified a satellite DNA family using genomic DNA restriction on *P. hilarii*. This DNA fragment, denominated pPh2004, is a monomeric sequence of 200 bp and is 60% AT-rich. FISH analysis using the pPh2004 probe revealed 14 sites in
Fig. 4. Localization of the pPh2004 satellite DNA by FISH in the karyotypes of Parodon hilarii and Parodon moreirai. (a) Female of P. hilarii showing 15 autosomal pPh2004 sites plus one site terminal of long arm of the Z chromosome and one site terminal of short arm of the W chromosome; and (b) female of P. moreirai showing the ninth chromosome pair with pPh2004 sites plus one site terminal of long arm of the Z chromosome and one site terminal of short arm of the W chromosome. Bar = 5 μm.

autosomes, one site in the terminal region of the long arm of the Z chromosome and one site in the terminal region of the short arm of the W chromosome in P. hilarii (Vicente et al., 2003). The pPh2004 probe was also located in the chromosomes of P. moreirai (Centofante et al., 2002), in the same sites in the Z and W chromosomes as in P. hilarii, and two small additional sites in the autosomes (Fig. 4).

As mentioned above, all Parodontidae species with a ZW system apparently have the same W chromosome differentiation mechanism. A heterochromatic block in the short arms of the Z chromosome appears to have undergone an amplification in size from its ancestral homologue (primitive W), giving rise to the long arms of the current W chromosome, which is much larger than the Z chromosome (Centofante et al., 2002; Vicente et al., 2003; Rosa et al., 2006; Vicari et al., 2006a). Thus, this process could explain the location of the pPh2004 satellite in the short arms of the Z chromosome and in the long arms of the W chromosome in the species P. hilarii and P. moreirai (Fig. 4). Apareiodon vladii, A. ibitiensis and Apareiodon sp., however, do not have the pPh2004 satellite DNA (Silva, 2009). Because the Parodontidae species that have differentiated ZW sex chromosomes form a derived group from species without differentiated heteromorphic sex chromosomes, the following can be inferred: (1) the pPh2004 satellite DNA is not the only genome component involved in the differentiation of the W chromosome in these species; and (2) this satellite DNA probably has a later origin than the differentiation of the ZW system in Parodontidae. Although these data are preliminary, it is clear that a detailed study on the origin and dispersion of pPh2004 and other satellite DNA sequences among species would enable more consistent conclusions regarding the evolution of the ZW system and the relationships among Parodontidae species.

SATH1 AND SATH2 SATELLITE DNA IN PROCHILODUS LINEATUS

The Prochilodontidae family has a vast geographic distribution throughout South America, with 21 valid species grouped in three genera: Ichthyoelephas Posada, Prochilodus Agassiz and Semaprochilodus Fowler (Froese & Pauli, 2009). The Prochilodus genus has a conserved karyotype, with a diploid number of 2n = 54 biarmed chromosomes (Pauls & Bertollo, 1990). Some species and populations of
this genus, such as Prochilodus lineatus (Valenciennes), have demonstrated intra-population and inter-population variation stemming from the presence of supernumerary microchromosomes (Pauls & Bertollo, 1990; Oliveira et al., 1997; Cavallaro et al., 2000; Maistro et al., 2000; Jesus et al., 2003; Artoni et al., 2006a; Vicari et al., 2006b). Prochilodus is an interesting model for the origin and evolution of B chromosomes. Despite their apparent adaptive inertia, the B chromosomes in P. lineatus have proven diverse with regard to chromosome type and the presence of satellite DNA (Jesus et al., 2003; Artoni et al., 2006a).

Two satellite DNA families, denominated SATH1 and SATH2 (Jesus et al., 2003), were isolated through genomic DNA restriction in P. lineatus with the HindIII enzyme. The nucleotide sequencing of the SATH1 family revealed monomers ranging from 897 to 901 bp, 57% AT-rich and an identity of 91–94%, with few mutation sites. Southern blot analyses revealed identity in two congeneric species tested, Prochilodus affinis Lütken and Prochilodus marggravii (Walbaum). FISH analysis revealed 22 pericentromeric sites of the SATH1 family on the chromosome of the A complement in P. lineatus and on all B chromosomes, although on sites of different sizes and locations (Jesus et al., 2003). The SATH2 family consists of monomers of 441 bp, 57% AT-rich and 98% identity. Southern blot analysis revealed a high identity of the SATH2 sequence in P. affinis. In P. marggravii, however, this satellite DNA was more divergent and identified only under stringency conditions (Jesus et al., 2003). Sixteen pericentromeric SATH2 sites were mapped on the chromosomes of the A complement in P. lineatus and on some additional small sites. None of these were present on the B chromosomes. Simultaneous hybridization with SATH1 and SATH2 probes (Fig. 5) revealed co-localized sites in the chromosome domains (Jesus et al., 2003).

Many hypotheses have been suggested to explain the origin of B chromosomes, such as those of an intra-species nature through meiotic non-disjunction, the formation of isochromosomes, the accumulation of disperse repetitive elements and those of an inter-species nature through hybridizations (Camacho et al., 1997; Maistro et al., 2000; Néo et al., 2000; Jesus et al., 2003; Ziegler et al., 2003; Artoni et al., 2006a). The investigation of the SATH1 satellite DNA allowed inference of an intra-species origin for B chromosomes in P. lineatus, which may have originated from chromosomes of the A complement with this DNA family (Jesus et al., 2003). Regarding the intraspecific origin, both a non-disjunction event and the formation of isochromosomes could explain the differentiation of the B microchromosomes in P. lineatus, provided that subsequent structural changes had occurred [Fig. 5(b)], including deletions and modifications in the number of copies of this satellite DNA (Jesus et al., 2003).

In a similar study, Artoni et al. (2006a) used SATH1 and SATH2 probes for mapping of these sites in a different population of P. lineatus. The data corroborated with those described by Jesus et al. (2003), as the B chromosomes also exhibited compositional homology with the chromosomes of the A complement of this species. FISH/SATH1 labelling in the B chromosomes, however, was different from that found in the population analysed by Jesus et al. (2003). Essentially, one metacentric B chromosome exhibited SATH1 in both terminal regions, which gives strong evidence for an isochromosomal origin (Artoni et al., 2006a). For this model, B chromosomes with and without SATH1 DNA in their composition could be originated [Fig. 5(b)]. These results also support the notion of a non-recent origin.
for B chromosomes in *P. lineatus*, as previously proposed by Maistro *et al.* (2000) using chromosome banding through restriction enzymes. The occurrence of a set of differentiated B chromosomes was also found between the two *P. lineatus* populations studied, suggesting a population structure for this migrating species, which has differences in the number of B chromosomes as well as the form and composition of satellite DNA.

**ADVANCES AND PERSPECTIVES ASSOCIATED WITH SATELLITE DNA IN NEOTROPICAL FISHES**

Recent studies have been carried out by the authors of the present paper that seek a better understanding of the following: the origin and composition of the B chromosome in *A. scabripinnis*; the diversification of the ZZ/ZW sex chromosome system in the Chrenuchidae family; phylogenetic grouping associated with the diversification of the Z and W sex chromosomes in the Parodontidae family; and genome evolution in Neotropical representatives of Tetraodontiformes.

**Astyanax scabripinnis**

The *A. scabripinnis* population from the region of Campos de Jordão, SP, Brazil, has a high intra-individual and interindividual frequency of a completely heterochromatic B macrochromosome. The populations with a high frequency of this chromosome are those isolated by waterfalls at heights generally greater than 1920 m
above sea level (Néo et al., 2000). Although located in apparently inhospitable environments that are not inhabited by any other fish species, these endemic populations exhibit a normal reproductive biology. A central question associated with these populations refers to the strategy that must be used to maintain high levels of variability and escape endogamic problems. Does the As51 satellite DNA in the genome of this species have any relation to genetic variability of the population?

Metaphasic cells in the A. scabripinnis population of Campos de Jordão were submitted to C-banding and subsequent microdissection of the B macrochromosome. The microdissected chromosomes were amplified using DOP-PCR and labelled with biotin dATP in a new DOP-PCR for use as probes in FISH procedures. The data revealed that this chromosome is highly differentiated in relation to the chromosomes of the A complement, exhibiting only small terminal sites of the B probe in the autosomes [Fig. 6(a)]. Double FISH with the B probe and As51 probe revealed that the As51 satellite DNA is found in the B chromosome at an equidistant position in both arms (Fig. 6), which is in agreement with the results and isochromosome origin model proposed by Mestriner et al. (2000). Following up this issue, nucleotide sequencing and a meiotic investigation associated with these probes will be carried out in an attempt to map possible recombination events. The following are among the questions to be investigated: (1) with the accumulation of mutations differentiating its sequences in relation to the repetitive DNA sequences of the A complement, is the B macrochromosome composed of transposable elements?; (2) Does this heterochromatic macrochromosome cause a meiotic delay and possible greater recombination rate?

Chrenuchidae family

In the Chrenuchidae family, the occurrence of heteromorphic ZZ/ZW sex chromosome systems in some representatives of the genus Characidium provides a good model for studies on the diversification of sex chromosomes. In this genus, the Z and W chromosomes may vary in shape and size (Vicari et al., 2008c). Among the
previous *Characidium* species studied, *Characidium gomesi* Travassos, *Characidium alipioi* Travassos and *Characidium lanei* Travassos exhibit differentiated sex chromosome systems (Centofante *et al*., 2001, 2003; Maistro *et al*., 2004; Vicari *et al*., 2008c; Noleto *et al*., 2009a). In *C. lanei*, the major rDNA sites are located on the sex chromosomes (Noleto *et al*., 2009a), whereas these sites in *C. gomesi* and *C. alipioi* are on independent chromosomes (Centofante *et al*., 2001, 2003; Vicari *et al*., 2008c). As the chromosomes in these species emerged from a homologous ancestral pair, structural events have acted on both the differentiation of the Z and W chromosomes and the dispersion of rDNA in the genomes of these species.

In *C. gomesi*, the W chromosome, which is completely heterochromatic, was microdissected and amplified using DOP-PCR. The amplified DNA was used as a probe for FISH in the chromosomes in *C. gomesi* and *C. lanei*. The W chromosome was completely labelled in *C. gomesi*, along with a small homologous site near the long arm of the Z chromosome and small terminal sites in some chromosomes [Fig. 7(a)]. In *C. lanei*, the W probe was detected in the short arms of the Z and W chromosomes and in several terminal sites in other chromosome pairs [Fig. 7(b)]. Although still partial, the results demonstrate that the sex chromosomes of different species of *Characidium* have regions with similar DNA sequences, indicating a possible common origin.

**Parodontidae family**

The Parodontidae family has serious problems regarding phylogenetic grouping. For example, the genus *Apareiodon* was recently considered as a junior synonym of *Parodon* (Ingenito, 2008). Vicari *et al.* (2006a) considered the hypothesis that species with differentiated ZZ/ZW sex chromosomes, two from the genus *Parodon* (*P. hilarii* and *P. moreirai*) and three from *Apareiodon* (*A. vladii*, *A. ibitiansis* and *Apareiodon* sp.) form a more closely related group in relation to homomorphic species. The results of the analysis of the pPh2004 satellite DNA in *P. hilarii* (described earlier) indeed support this notion. In the continuity of these studies, metaphases in *Apareiodon* sp. were submitted to C-banding for the identification of the W sex chromosome, with subsequent microdissection and amplification using DOP-PCR. With this method, only satellite DNA sequences that compose the W chromosome were amplified, as only the heterochromatic blocks remain intact after C-banding. The results of FISH in the chromosomes of *Apareiodon* sp. and
Fig. 8. Sex chromosome diversification in the Parodontidae fishes: (a) metaphase of the female Apareiodon sp.; and (b) metaphase of the female Parodon hilarii. In both cases, the localization on the chromosomes of the W chromosome probe (red sites) obtained using the microdissection of C-banding W chromosome of Apareiodon sp. is showed. (c) Hypothetical derivation of the W chromosome from an ancestral chromosome similar to the Z chromosome (protosex pair). Bar = 10 μm.

P. hilarii using the probe of the heterochromatic regions of the W chromosome of Apareiodon sp. revealed a sequence identity (Fig. 8), once again corroborating a possible common origin and phylogenetic proximity between these species. Similar analyses with other Parodontidae species, with or without differentiated sex chromosomes, will provide important information about the differentiation of the W chromosome and the evolutionary relationship among these species.

**Percomorpha group**

Among the Percomorpha, species from the order Tetraodontiformes exhibit broad karyotype variability, with diploid numbers ranging from 28 to 52 chromosomes (Brum & Galetti, 1997). Another genetic characteristic of special interest in Tetraodontiformes is the small amount of DNA in some of its representatives. Numerous studies have shown that pufferfishes (Tetraodontidae) have the smallest genome of all invertebrates, ranging from 0.7 to 1.0 pg (c. 400 Mb) per haploid content (Hinegardner & Rosen, 1972; Brenner et al., 1993; Noleto et al., 2009b). Members of the Diodontidae family, a sister group to Tetraodontidae, have an average genome that is two-fold larger (c. 800 Mb). Even with the smallest genome, Tetraodontidae, however, have a similar set of genes to other vertebrates, which are densely organized in 6 kb per gene, supposedly due to the reduction in the number and length of the introns (Venkatesh et al., 2000; Elmerot et al., 2002). The obtainment of whole satellite DNA in Tetraodontidae and Diodontidae species using re-association kinetics based on the CoT – 1 DNA and its in situ localization using FISH has revealed important qualitative differences regarding the amount of heterochromatin between Sphoeroides spengleri (Bloch) (Tetraodontidae) and Chilomycterus spinosus (L.) (Diodontidae). In S. spengleri, the amount of satellite DNA is lower than C. spinosus (Fig. 9).
Fig. 9. Localization of the satellites DNAs obtained through the Cot–1 DNA method using FISH in Tetraodontiformes (green sites): (a) metaphase of the Sphoeroides spengleri (Tetraodontidae); and (b) metaphase of the Chilomycterus spinosus (Diodontidae). In S. spengleri, the amount of satellite DNA is lower than C. spinosus. Bar = 10 μm.

The results demonstrate that losses in heterochromatic regions may also lead to differences in the amount of DNA between species of Tetraodontiformes, although deletions in the length or the total loss of introns are the mechanisms most associated with the contraction of their genomes (Elgar et al., 1996; Loh et al., 2008).

Although still scarce, the results available on the different classes of satellite DNA investigated thus far allow important inferences regarding heterochromatic chromosome domains, the origin of B chromosomes and the diversification of sex chromosomes as well as considerations of a phylogenetic nature, dispersion and biogeography of natural populations in diverse groups of fishes. Thus, these studies demonstrated the enormous potential that the investigation of satellite DNA offers to improve the knowledge on karyotype differentiation and the evolution of Neotropical ichthyofauna and, very likely, in this group of vertebrates as a whole.

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