REVIEW PAPER

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 C_0t-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 5S*Hin*dIII-DNA satellite DNA, which is specific to *Hoplias malabaricus* of the Erythrinidae family, has an exclusively centromeric location. The As51 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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some species of Neotropical Tetraodontiformes. These topics are discussed in the present review, showing the importance of satellite DNA analysis in the differentiation and karyotype evolution of Actinopterygii.

Key words: B chromosomes; genome evolution; karyotype diversification; repetitive DNA; sex chromosomes.

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.*, 2009*a*). 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.*, 2008*a*; Kantek *et al.*, 2009*a*), determine likely origins of supernumerary chromosomes (Mestriner *et al.*,

2000; Jesus *et al*., 2003; Ziegler *et al*., 2003; Artoni *et al*., 2006*a*) 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
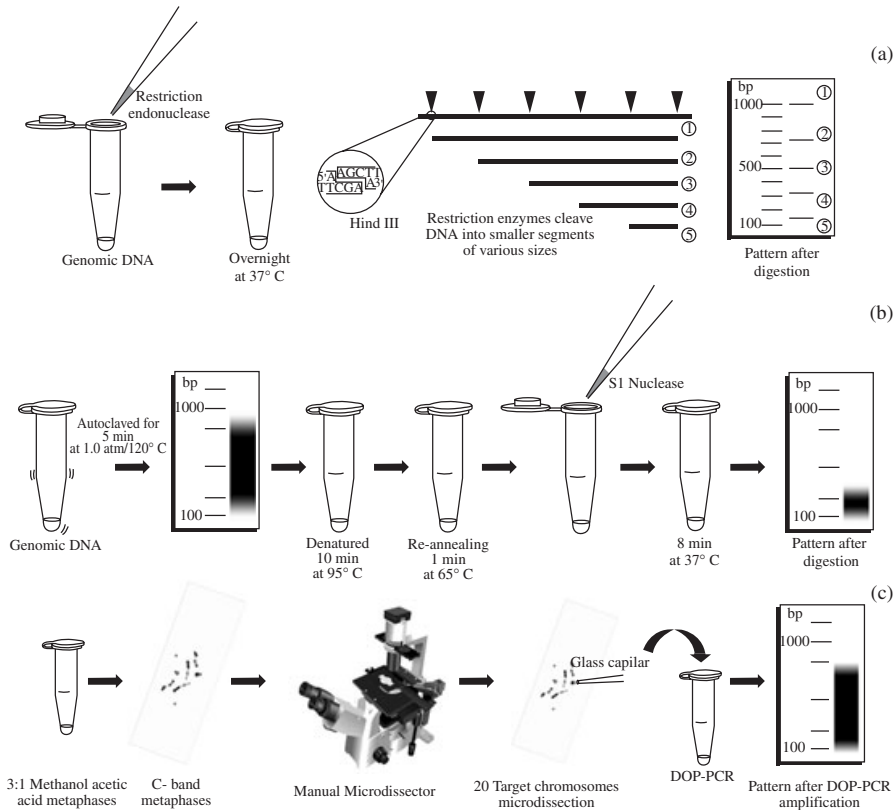
Fig. 1. Representative schematic of the methods for obtaining and isolating satellites DNAs: in (a) isolation of repetitive DNAs through genomic restriction; (b) isolation of repetitive DNAs using re-association kinetics based on the C_0t-1 DNA method and (c) isolation of repetitive DNAs through chromosome 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 $1\times$ 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 $\times$ 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′ ccg 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 MgCl$_2$, 400 μM dNTPs, 2 μM DOP primer and 2 U *Taq* DNA polymerase under the following conditions: (1×) 94° C 3 min; (35×) 90° C 1 min and 30 s, 52° C 1 min and 30 s, 72° C 1 min and 30 s, and (1×) 72° 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 MgCl$_2$, 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 ACTINOPTERYGII

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 5SHindIII-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, *Hoplerythrinus* Gill, *Erythrinus* 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 *Hin*dIII 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 *Hin*dIII on karyomorph A in *H. malabaricus* ($2n = 42$ chromosomes, with

TABLE I. Chromosomal characteristics of *Hoplias malabaricus* karyomorphs

Karyomorph	$2n$	Sex chromosome system	Localities
A	42	Not differentiated	From northern to southern Brazil, Uruguay and northern Argentina
B	42	♀:XX	Vale do Rio Doce (São Francisco basin) and upper Iguaçu river
		♂:XY	
C	40	Not differentiated	From northern Brazil to north-eastern Argentina
D	39/40	♀: $X_1X_1X_2X_2$	Upper Paraná hydrographic basin
		♂: X_1X_2Y	
E	42	♀: unknown	Trombetas River (PA State), Brazil
F	40	Not differentiated	From Surinan to south-eastern Brazil
G	40/41	♀:XX	Amazonian rivers
		♂:XY_1Y_2	

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 5S*Hin*dIII-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 5S*Hin*dIII-DNA, and unequal exchanges, transposition, RNA-mediated transposition and gene conversion could be the mechanisms acting on the diversification of 5S*Hin*dIII-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 5S*Hin*dIII-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 5S*Hin*dIII-DNA sites between these species (Ferreira *et al.*, 2007). Although 18 5S*Hin*dIII-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 5S*Hin*dIII-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 5S*Hin*dIII-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, 5S*Hin*dIII-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
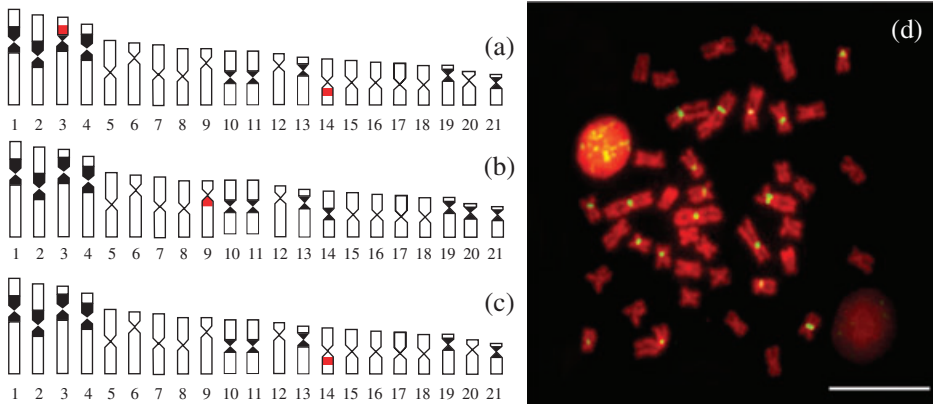
FIG. 2. Representative idiograms of three populations [(a)–(c)] belonging to karyomorph A of *Hoplias malabaricus* showing differentiations concerning the distribution of 5S*Hin*dIII-DNA (black) and 5S rDNA (red) sites on the chromosomes. (d) Metaphase plate of a population showing eighteen 5S*Hin*dIII-DNA 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*Hin*dIII-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 phenotype 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*., 2008*b*).

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*., 2008*a*; Kantek *et al*., 2009*a*), 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*., 2006*b*), 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*. (2008*a*) 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*., 2008*a*). 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*., 2008*a*).

Recently, Kantek *et al*. (2009*a*) 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*., 2006*b*; Domingues *et al*., 2007; Vicari *et al*., 2008*a, b*; Ferreira Neto *et al*., 2009; Kantek *et al*., 2009*b*), 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*., 2009*a*).
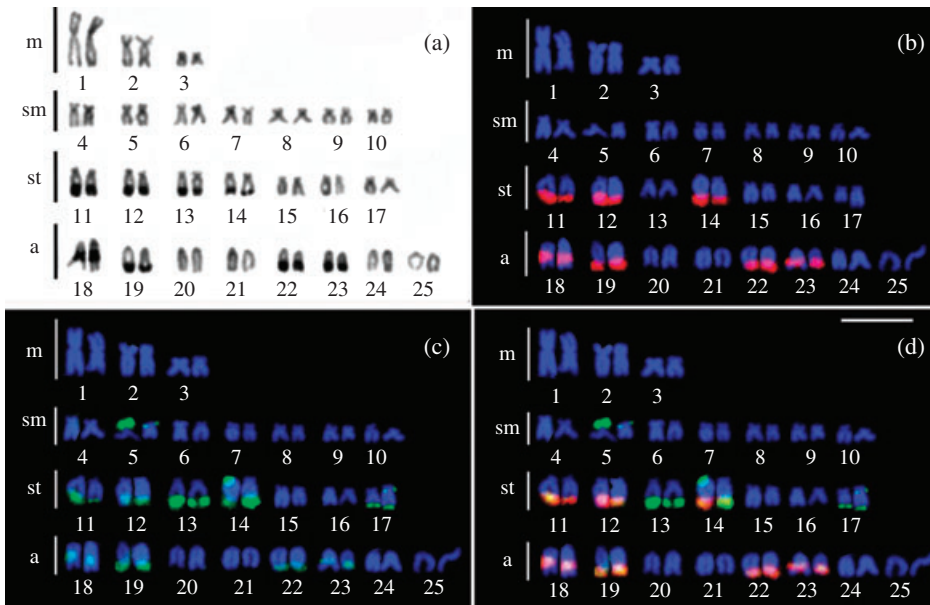
FIG. 3. Male karyotypes of *Astyanax janeiroensis*: (a) C-banded chromosomes; (b) FISH with As51 satellite DNA probe showing 14 distinct sites (red); (c) mapping of the 18S rDNA by FISH showing 22 sites on the chromosomes (green); (d) double FISH with 18S rDNA and As51 probes showing a co-localization 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.*, 2009*a*). 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 lngenito & Buckub, 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, 2000*a*) 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_1W_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, 2000*a*; Centofante *et al.*, 2002; Vicente *et al.*, 2003; Rosa *et al.*, 2006; Vicari *et al.*, 2006*a*; Bellafronte *et al.*, 2009).

Until recently, the ZZ/ZW system was restricted to the genus *Parodon* (Moreira-Filho *et al.*, 1993; Jesus & Moreira-Filho, 2000*b*; 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.*, 2006*a*; 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
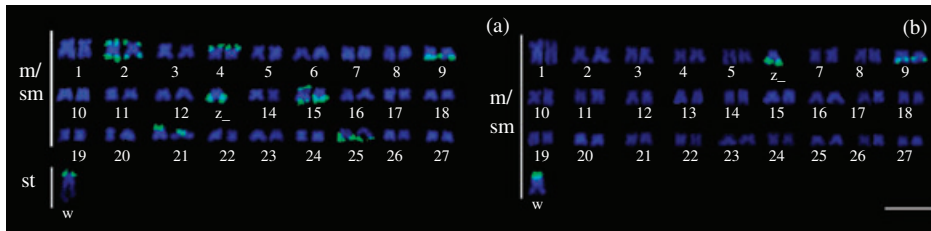
FIG. 4. Localization of the pPh2004 satellite DNA by FISH in the karyotypes of *Parodon hilarii* and *Parodon moreirai*. (a) Female of *P. hilarii* showing 15 autossomal pPh2004 sites plus one site terminal of long arm of the Z chromosome and one site terminal of short arm of the W chromosome; and (b) 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.*, 2006*a*). 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 supernu-merary microchromosomes (Pauls & Bertollo, 1990; Oliveira *et al*., 1997; Cavallaro *et al*., 2000; Maistro *et al*., 2000; Jesus *et al*., 2003; Artoni *et al*., 2006*a*; Vicari *et al*., 2006*b*). *Prochilodus* is an interesting model for the origin and evolution of B chro-mosomes. 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*., 2006*a*).

Two satellite DNA families, denominated SATH1 and SATH2 (Jesus *et al*., 2003), were isolated through genomic DNA restriction in *P. lineatus* with the *Hin*dIII 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 forma-tion 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*., 2006*a*). 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*. (2006*a*) used SATH1 and SATH2 probes for mapping of these sites in a different population of *P. lineatus*. The data corrob-orated 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 differ-ent 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*., 2006*a*). 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
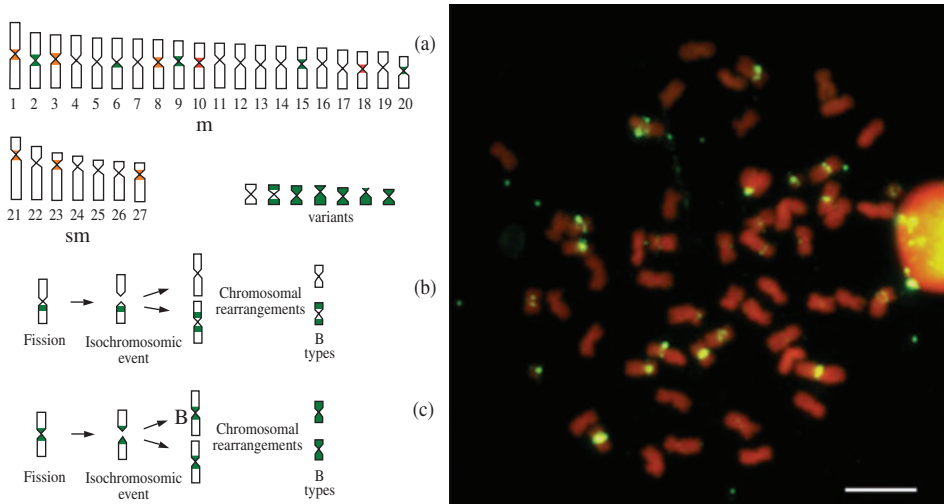
FIG. 5. Representative idiogram of *Prochilodus lineatus* populations: (a) showing the satellites DNAs SATH1 and SATH2 distribution on the chromosomes; in green SATH1 chromosomal domains, in red SATH2 chromosomal domains and, in orange chromosomal domains with co-localization of SATH1 and SATH2 repetitive DNAs. [(b) and (c)] Hyphotetical isochromosomal origin for the B chromosomes of *P. lineatus* from the A chromosomes bearing SATH1 DNA at pericentromeric region (b) or close to centromere (c); and, (d) metaphase of *P. lineatus* showing SATH1 satellite DNA sites distributed on the chromosomes. Bar = 10 μm.

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 hete-rochromatic B macrochromosome. The populations with a high frequency of this chromosome are those isolated by waterfalls at heights generally greater than 1920 m
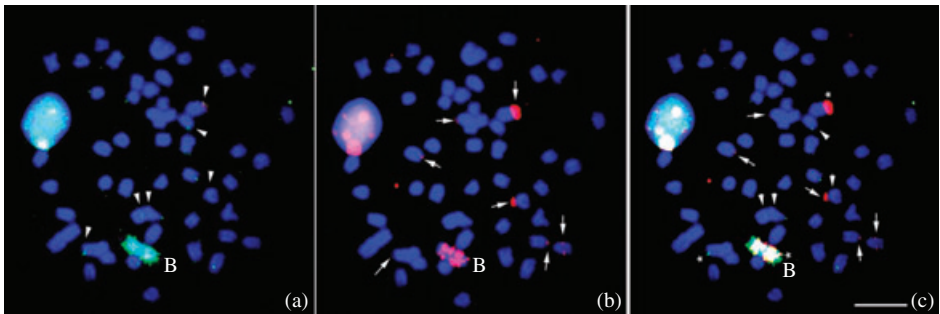
Fɪɢ. 6. Metaphasic plates of *Astyanax scabripinnis* from the Campos de Jordão population showing satellite
        DNAs on the A chromosomes and on the B macrochromosome. (a) Mapping of *A. scabripinnis* B
        chromosome probe. The letter 'B' represents the B chromosomes totally labelled and 'arrowheads'
        showing small sites with identity in A chromosomes. (b) FISH with As51 probe showing the localization
        of this DNA satellite on the A chromosomes (arrows) and in B chromosome at an equidistant position
        in both arms (B). (c) Double FISH with B chromosome and As51 probes showing a co-localization of
        these satellites DNAs in some A chromosomes and on the B chromosome (*). Bar = 10 μm.

above sea level (Néo *et al.*, 2000). Although located in apparently inhospitable envi-
ronments 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 vari-
ability 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 chromo-
somes 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 ori-
gin model proposed by Mestriner *et al.* (2000). Following up this issue, nucleotide
sequencing and a meiotic investigation associated with these probes will be car-
ried 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 heteromorphous ZZ/ZW sex chro-
mosome 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
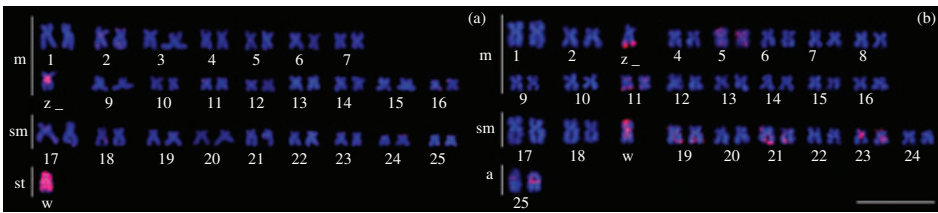W chromosomes may vary in shape and size (Vicari *et al.*, 2008*c*). Among the

Fig. 7. Karyotypes of female *Characidium gomesi* (a) and female *Characidium lanei* (b) showing the localization of the W chromosome probe obtained by microdissection of *C. gomesi* C-banded chromosome (red sites). Bar = 10 μm.

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.*, 2008*c*; Noleto *et al.*, 2009*a*). In *C. lanei*, the major rDNA sites are located on the sex chromosomes (Noleto *et al.*, 2009*a*), whereas these sites in *C. gomesi* and *C. alipioi* are on independent chromosomes (Centofante *et al.*, 2001, 2003; Vicari *et al.*, 2008*c*). 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.* (2006*a*) 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. ibitiensis* and *Apareiodon* sp.) form a more closely related group in relation to homomorphous 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
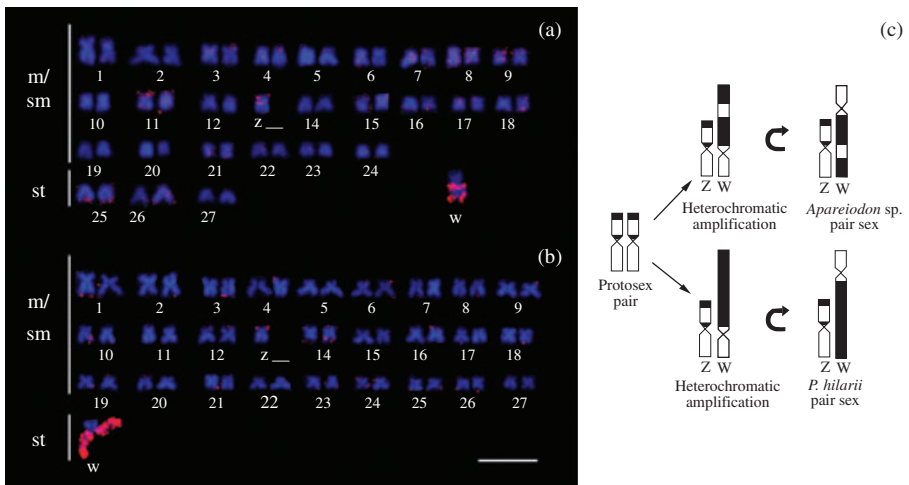
FIG. 8. Sex chromosome diversification in the Parodontidae fishes: (a) metaphase of the female *Apareiodon* sp.; and (b) metaphase of the female *Parodon hilarii*. In both cases, the localization on the chromosomes of the W chromosome probe (red sites) obtained using the microdissection of C-banding 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*., 2009*b*). 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 C_0t-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).
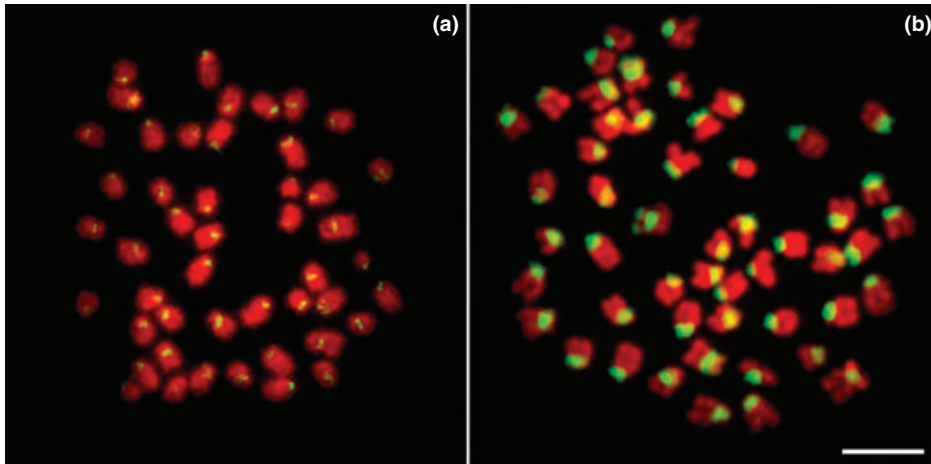
FIG. 9. Localization of the satellites DNAs obtained through the C_0t-1 DNA method using FISH in Tetraodontiformes (green sites): (a) metaphase of the *Sphoeroides spengleri* (Tetraodontidae); and (b) metaphase of the *Chilomycterus spinosus* (Diodontidae). In *S. spengleri*, the amount of satellite 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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