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Chromosomal Variability among Allopatric Populations of Erythrinidae Fish *Hoplias malabaricus*: Mapping of Three Classes of Repetitive DNAs

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Key Words
Fish · Karyotype evolution · 5S rDNA · 5S*Hin*dIII satellite DNA · 18S rDNA

Abstract
Karyotype and chromosomal characteristics from 3 allopatric populations of *Hoplias malabaricus*, cytogenetically the most studied Erythrinidae taxon, were investigated using different staining techniques (C-, Ag-, and CMA_3 banding) as well as fluorescent in situ hybridization (FISH) to detect 18S rDNA, 5S rDNA, and 5S*Hin*dIII satellite DNA sites. The isolation, cloning and characterization of an 18S rDNA probe from *H. malabaricus* genome were also performed for the first time in order to develop a more specific probe. The 3 populations, named PR, CR, and DR, showed identical karyotypes, with 2n = 42 chromosomes composed of 11 m pairs and 10 sm pairs, without heteromorphic sex chromosomes, which characterize the populations as belonging to karyomorph A. In all populations C-positive heterochromatin was situated in the centromeric/pericentromeric regions of the chromosomes, as well as in the telomeric region of several pairs. A conspicuous proximal heterochromatic block on the long arm of pair No. 16 was the only GC-rich segment in the karyotypes. 5S*Hin*dIII satellite DNA was always mapped in the centromeric region of several chromosomes. The 18S rDNA sites were situated on the telomeric or centromeric regions, whereas the 5S rDNA showed an interstitial or proximal location in some pairs. Several chromosomes bearing these repetitive DNA sequences were shared by the 3 populations, alongside with some exclusive chromosomal markers. In this sense, population CR was the most differentiated one, including a syntenic condition for the 18S and 5S rDNA probes, as confirmed by double FISH. Thus, despite their inclusion in the same major karyotypic group, the distinct populations cannot be considered an absolute evolutionary unit, as evidenced by their inner chromosomal differentiations.
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Erythrinidae fish (Characiformes) have a wide geographic distribution throughout South America, and are found in the main hydrographic basins of Brazil. Three genera, *Hoplias*, *Hoplerythrinus*, and *Erythrinus*, are recognized in this family. *Hoplias* is the most widely distributed, occurring in a variety of latitudes, including Surinam, Argentina, and Uruguay. *H. malabaricus*, cytogenetically the most studied taxon, shows a conspicuous karyotypic diversification, with seven karyomorphs (A to G) currently identified, which are easily distinguishable in regards to shape, size, or chromosome number

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Table 1. Collection sites of *Hoplias malabaricus* karyomorph A

Locality	Population	m	f
Descalvado (SP) – Pântano River	PR	8	6
Santo. Antônio do Leverger (MT) – Cuiabá River basin	CR	5	6
Parque Florestal do Rio Doce (MG) – Doce River basin	DR	2	1

Abbreviations: m and f = number of males and females analyzed, respectively; SP = São Paulo, MT = Mato Grosso, and MG = Minas Gerais Brazilian States.

and the presence of sex chromosome systems, suggesting the occurrence of distinct species [Bertollo et al., 1986, 2000].

Karyomorph A is characterized by 2n = 42 meta-submetacentric chromosomes without heteromorphic sex chromosomes, being distributed from northern to southern Brazil, reaching to Uruguay and Argentina [Bertollo et al., 2000]. Two previous studies have shown that some populations from this karyomorph can differ concerning the distribution of heterochromatic and NOR sites (nucleolar organizer regions), most likely due to a geographical isolation [Born and Bertollo, 2001; Vicari et al., 2003].

This study presents a comparative chromosomal analysis among 3 allopatric populations of karyomorph A, using distinct staining methods (C-banding, $AgNO_3$, and Chromomycin A_3) and fluorescent in situ hybridization (FISH) with repetitive DNA probes (5S rDNA, 18S rDNA, and 5S*Hin*dIII satellite DNA). This approach provided an in-depth karyotype characterization, highlighted by the distribution of the repetitive DNA sequences in the chromosomes.

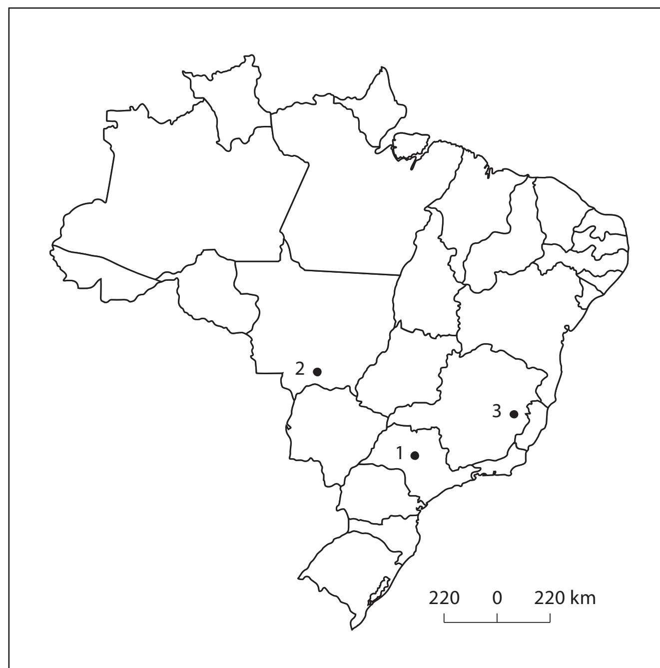

Fig. 1. Collection sites of *Hoplias malabaricus* karyomorph A from Brazil. 1 = Pântano River (population PR), São Paulo State; 2 = Cuiabá River basin (population CR), Mato Grosso State; 3 = Doce River basin (population DR), Minas Gerais State.

Material and Methods

Specimens and Chromosome Preparation

Samples of *Hoplias malabaricus* were obtained from distinct Brazilian hydrographic basins. Three populations were analyzed, named PR (Pântano River), CR (Cuiabá River) and DR (Doce River), as specified in table 1 and figure 1. The specimens were deposited in the fish collection of the Cytogenetic Laboratory, Departamento de Genética e Evolução, Universidade Federal de São Carlos. Mitotic chromosomes were obtained from cell suspensions of the anterior kidney, using the conventional air-drying method [Bertollo et al., 1978].

Chromosome Staining

In addition to the standard Giemsa method, chromosomes were analyzed after silver nitrate staining [Howell and Black, 1980] in order to visualize the nucleolar organizing regions (Ag-NORs). C-banding was also employed to detect the C-positive heterochromatin [Sumner, 1972] and Chromomycin A_3 (CMA_3) staining to identify the GC-rich regions on the chromosomes [Sola et al., 1992].

Probes

Three repetitive DNA sequences isolated from the genome of *H. malabaricus* were used as probes, namely the first one contained a 5S rDNA repeat consisting of a 120-base pair (bp) copy of the 5S rRNA encoding gene and a 200-bp copy of the non-transcribed spacer (NTS), the second one contained a satellite 5S*Hin*dIII-DNA repeat consisting of a 360-bp copy with a 95-bp segment similar to the 5S rRNA encoding gene and a 265-bp segment similar to the NTS of the first probe. The 5S rDNA and the satellite 5S*Hin*dIII-DNA probes were previously cloned into plasmid vectors and propagated in *Escherichia coli* DH5α [Martins et al.,

2006]. The third probe contained an 18S rDNA repeat consisting of a 1,400-bp copy of the 18S rRNA gene and was obtained via PCR from nuclear DNA of *H. malabaricus* using the primers 18SF (5′-CCGAGGACCTCACTAAACCA-3′) and 18SR (5′-CCGCTTTGGTGACTCTTGAT-3′), which were designed from the complete 18S rRNA gene of the catfish *Ictalurus punctatus* (GenBank accession entry AF021880). The PCR-amplified products (approximately 1,400-bp) were visualized in a 1% agarose gel. The DNA band was isolated from the gel, purified with the Sephaglas Band Prep Kit (Pharmacia Biotech), ligated with the plasmid pGEM-T (Promega), and the plasmid was used to transform DH5α *E. coli* competent cells (Invitrogen).

The 18S rRNA gene clones were sequenced on an ABI Prism 377 DNA sequencer (Perkin-Elmer) with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Nucleotide sequences were subjected to Blastn [Altschul et al., 1990] searches at the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast) for identification of any similarities of the isolated sequences to the sequences deposited in the GenBank databases.

Chromosome Hybridization and Karyotype Analysis

Fluorescent in situ hybridization (FISH) was performed according to Pinkel et al. [1986]. The probes were labeled with biotin-14-dATP by nick translation, following the manufacturer's instructions (Bionick Labeling System, Invitrogen). The detection and amplification of hybridization signals were performed using conjugated avidin-FITC and anti-avidin biotin (Sigma). Chromosomes were counterstained with propidium iodide (50 μg/ml) and analyzed with an Olympus BX50 epifluorescence microscope. Chromosomal images were captured with the use of CoolSNAP-Pro software (Media Cybernetic). For double FISH, the 18S rDNA probe was labeled with biotin, as previously described, and the 5S rDNA probe with the FluoroRed dUTP-rhodamine conjugate (Roche), according to the manufacturer's specifications. The chromosomes were counterstained with DAPI (0.3 μg/ml).

Approximately 30 metaphases were analyzed per specimen to determine the diploid chromosome number and karyotype structure. The chromosomes were classified as metacentric (m) or submetacentric (sm), according to arm ratios [Levan et al., 1964].

Results

Karyotypes, C-Banding and Chromomycin A_3 Staining

The 3 populations showed identical karyotypes, invariably composed of 2n = 42 chromosomes for both sexes, with 11 m pairs and 10 sm pairs, without morphologically differentiated sex chromosomes (fig. 2). C-positive heterochromatic bands were observed in the centromeric/pericentromeric region of all chromosomes, as well as in the telomeric region of several pairs in the karyotype (fig. 2). In all populations, a more conspicuous proximal heterochromatic block, situated on the long arms of pair No. 16, was the only observed GC-rich segment (fig. 3).

Nucleotide Sequence

Nucleotide sequences were determined for rDNA clones obtained from *H. malabaricus*, which confirmed that the PCR-isolated DNA fragment corresponded to copies of the 18S rRNA genes. This sequence was deposited in GenBank under the accession number FJ577367.

*Physical Chromosome Mapping of 5S*HindIII*-DNA, 5S rDNA, and 18S rDNA Sequences, and Ag-NOR Staining*

5S*Hind*III-DNA sequences were mapped in the centromeric region of several chromosomes. Specimens of the populations PR and DR presented 18 sites distributed in 3 m pairs (Nos. 1, 5, and 6) and in 6 sm pairs (Nos. 12, 13, 14, 18, 19, and 21). The specimens of population CR presented a total of 22 sites, 18 of them coinciding with the distribution observed in populations PR and DR, while the remaining 4 sites were located on the m pair No. 10 and the sm pair No. 20 (figs. 4, 6).

FISH utilizing the 5S rDNA probe demonstrated that the m pair No. 10 harbors an interstitial site on the long arms, which is shared between populations PR and DR. However, an exclusive cluster is present in a proximal location on the short arms of sm pair No. 13 of population PR, and in an interstitial location on the long arms of chromosome No. 16 of population CR (figs. 4, 6).

The 18S rDNA sites were located on the centromeric and telomeric regions or in both telomeres (bitelomeric sites). The 3 populations shared the m pair No. 5 with bitelomeric sites, and the sm pair No. 16 with proximal sites on the long arms. On the other hand, the sm pairs Nos. 18 and 21, which present signals in telomeres of the long arms, were only shared by populations PR and CR, and populations PR and DR, respectively (figs. 5, 6). In addition, double FISH analysis demonstrated that population CR presented the exclusive syntenic location of the 18S and 5S rDNA loci on the sm No. 16 (fig. 3). All Ag-NORs detected were coincident with the 18S rDNA sites (fig. 5).

Discussion

Several *H. malabaricus* populations have been cytogenetically analyzed throughout the geographic distribution of this species. Until now, 7 karyomorphs (A–G) were easily discriminated, 3 of them (B, D and G) with distinct sex chromosome systems [Bertollo et al., 1983, 1997a, b; Dergam and Bertollo, 1990; Bertollo and Mestriner, 1998; Born and Bertollo, 2000), appearing to characterize species-specific karyotypes.

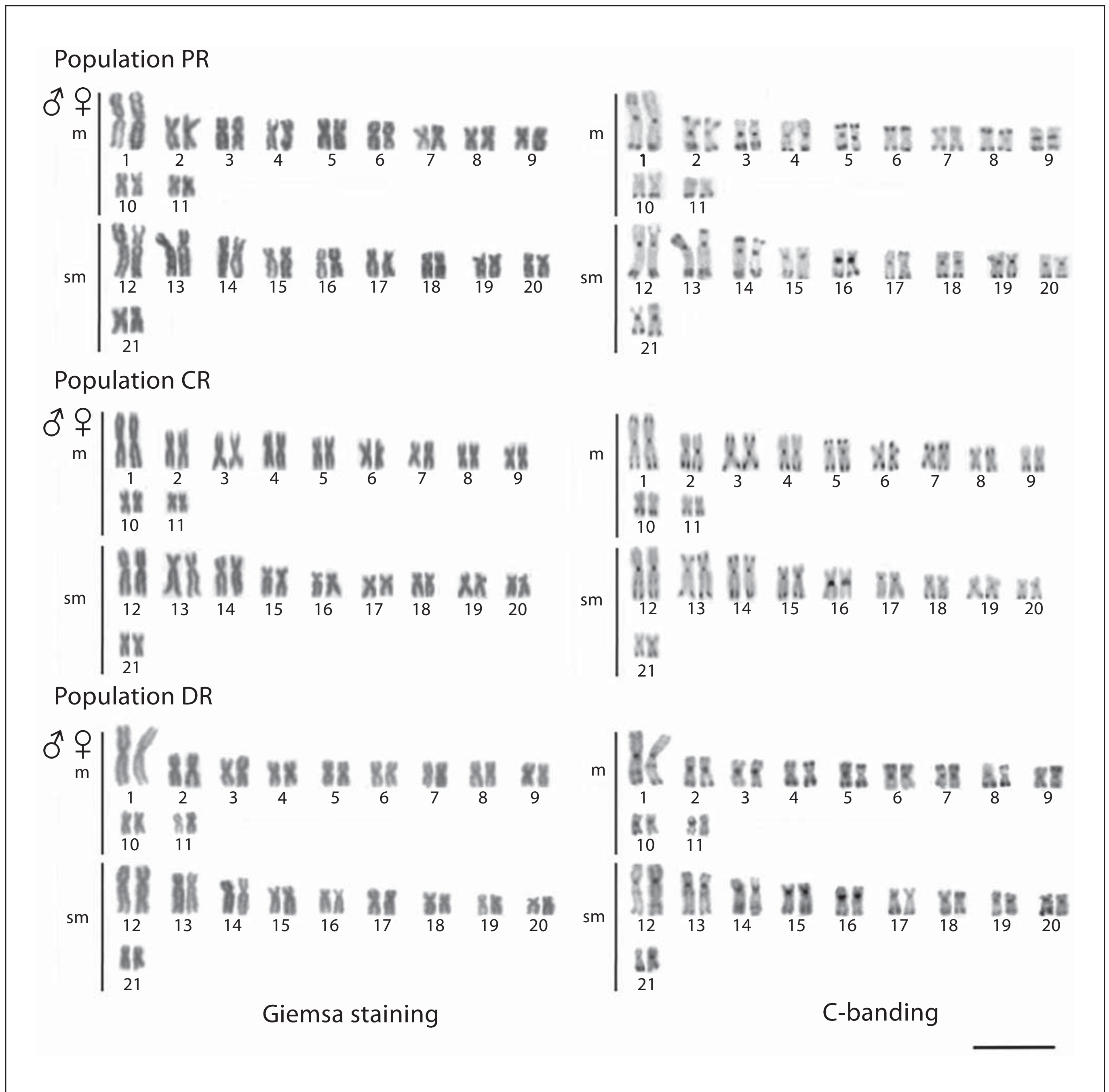

Fig. 2. Karyotypes of *Hoplias malabaricus* populations PR, CR, and DR arranged from sequentially Giemsa-stained (left) and C-banded chromosomes (right). Bar = 5 μm.

The 3 populations analyzed belong to karyomorph A, exhibiting a diploid number of 2n = 42 and a karyotype with 22 m and 20 sm chromosomes, without heteromorphic sex chromosomes. This same karyotype composition indicates a close relationship among the populations, which is also supported by the similar heterochromatin distribution in the karyotypes that exhibits no conspicuous differences. Indeed, the C-positive heterochromatic bands were always located in the centromeric/pericentromeric region of all chromosomes and in

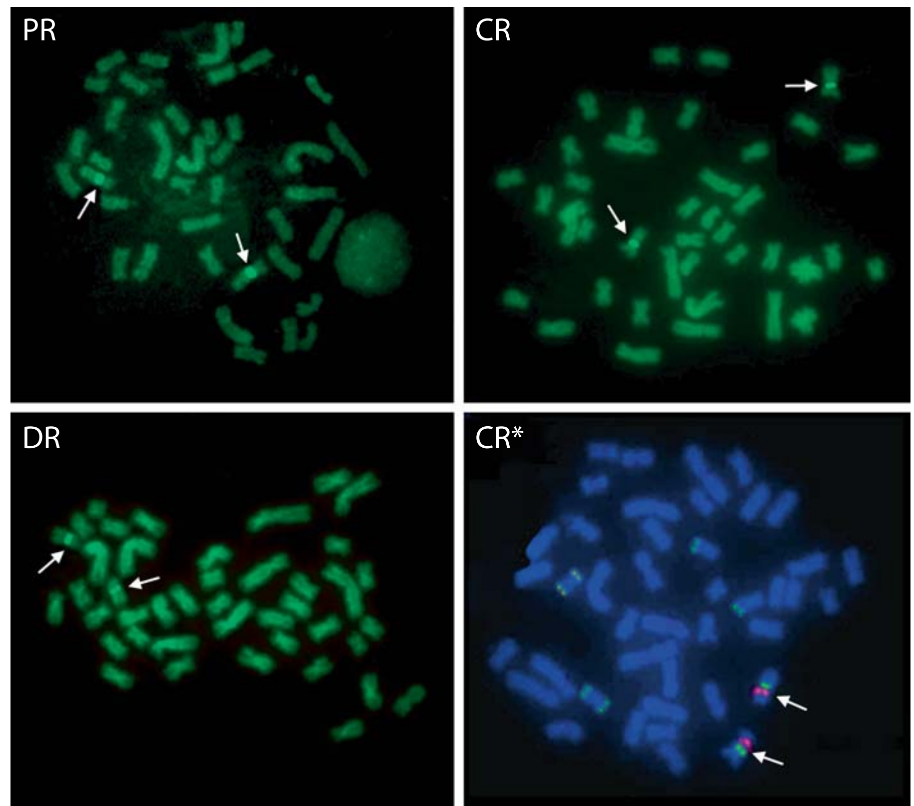

Fig. 3. Metaphases from *Hoplias malabaricus* populations PR, CR, and DR after Chromomycin A3 staining. The arrows indicate the bright fluorescent bands which correspond to GC-rich DNA segments located on the chromosome pair No. 16. CR* refers to population CR after double FISH, showing the syntenic location of the 5S rDNA (red) and 18S rDNA (green) sites on the 16th chromosome pair (arrows).

the telomeric region of some pairs, in addition to multiple Ag-NOR sites. These heterochromatic and NOR characteristics also fit the general patterns that have been documented for other populations or karyomorphs of *H. malabaricus* [Dergam and Bertollo, 1990; Haaf et al., 1993; Bertollo, 1996; Bertollo et al., 1997a, b; Born and Bertollo, 2000; Vicari et al., 2003, 2005]. As a whole, all of the aforementioned similarities indicate that the 3 populations belong to the same karyomorph group in close proximity, despite the distinct river basins of origin.

Among Erythrinidae fishes, the repetitive DNA class named 5S*Hin*dIII-DNA, which shares similarities to 5S rDNA 'true' repeats, was previously isolated and characterized from the *H. malabaricus* genome [Martins et al., 2006]. This sequence is located in the centromeric region of several chromosomes and not shared by other Erythrinidae genera, being exclusive to this species [Ferreira et al., 2007]. In this study, the physical mapping of this repetitive sequence demonstrated that 3 m and 6 sm chromosome pairs were shared among the populations, strengthening the karyotype relationships. However, the population CR was differentiated from the populations PR and DR by the presence of 2 additional sites, one located on an m pair and the other on an sm pair. The exclusive 5S*Hin*dIII-DNA sites for the population CR are of particular significance in regards to karyotype differentiation, since the 3 populations belong to the same karyomorph and the satellite DNA is specific for *H. malabaricus.* Thus, chromosomal differentiations related to this highly specific DNA have already been fixed among these populations.

H. malabaricus harbors multiple nucleolar organizer regions that are usually located in the telomeric regions, as also observed in the populations now analyzed, except for the chromosome pair No. 16, with NORs occurring in a proximal location on the long arms. Major rDNA clusters of fish and amphibians can be also identified by CMA_3 fluorochrome, which binds to the GC-rich rDNA chromatin of these groups [Mayr et al., 1985; Amemiya and Gold, 1986; Schmid and Guttenbach, 1988]. Coincidently, the chromosome pair No. 16 was the only one to exhibit a GC-rich DNA region in the karyotype, coinciding with the NOR site and the conspicuous C-positive heterochromatic band. This chromosome pair appears to be a fixed characteristic in *H. malabaricus,* which has already been characterized as polymorphic in some other

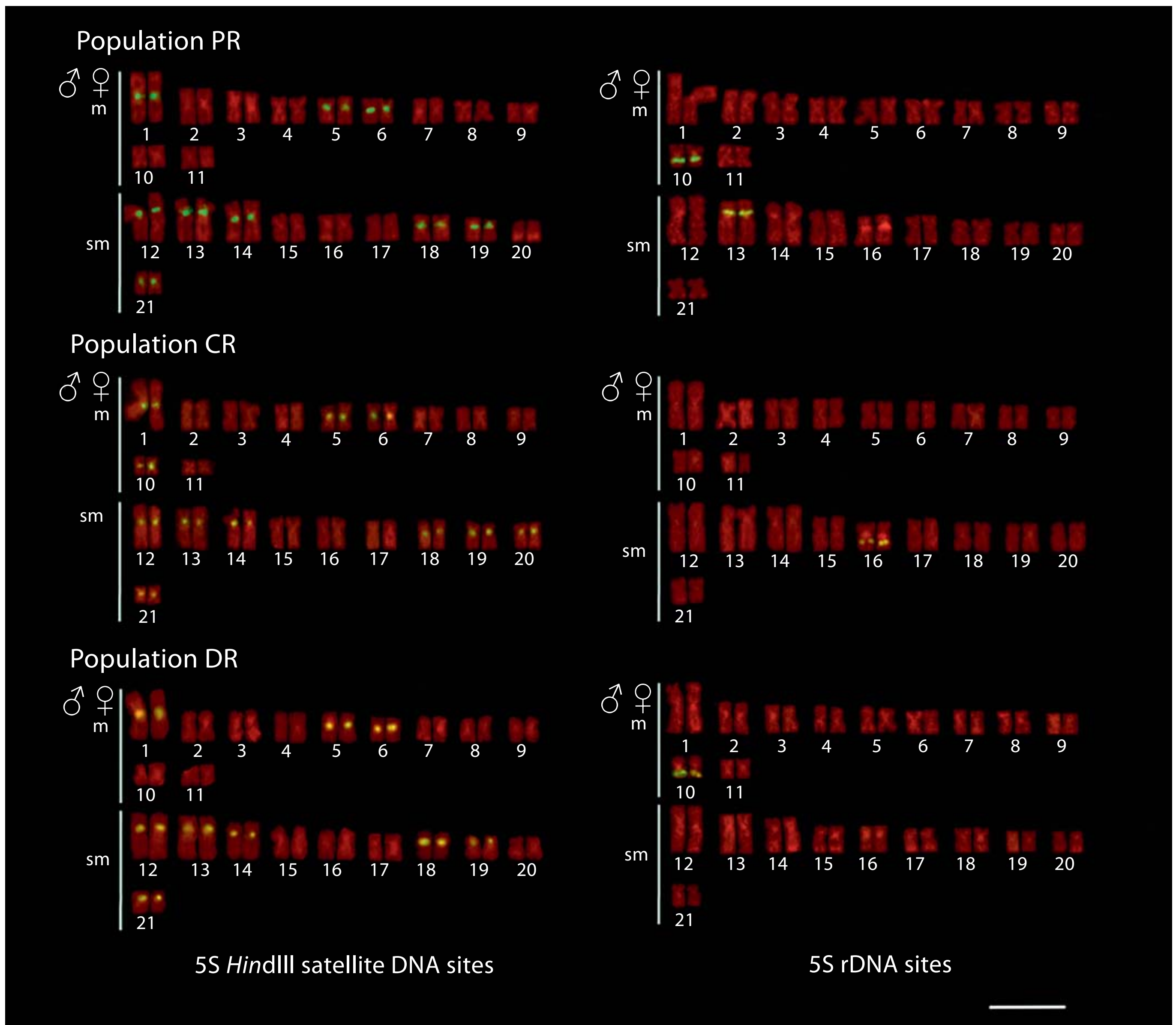

Fig. 4. Karyotypes of the *Hoplias malabaricus* populations PR, CR, and DR arranged from chromosomes probed with 5S*Hin*dIII-DNA and 5S rDNA (yellow signals) counterstained with propidium iodide. Bar = 5 μm.

karyomorph A populations, due to variations in the GC-rich heterochromatin amount and 18S rDNA clusters [Vicari et al., 2005]. In addition to the 5S*Hin*dIII-DNA, the 18S rDNA sequences strengthened the karyotypic relationships among populations PR, CR and DR. The majority of chromosomes bearing these sites were generally shared among the populations, highlighting the m pair No. 5, which harbors bitelomeric NORs. However, partially shared chromosomes were also observed, since the sm marker pairs Nos. 18 and 21 were only identified in populations PR and CR, and in populations PR and DR, respectively. Sequential and comparative analysis revealed that, in general, there was a perfect correlation between the number and location of 18S rDNA and Ag-NOR sites, except for population DR, indicating that some 18S rDNA sites were not active in the precedent interphase [Miller et al., 1976].

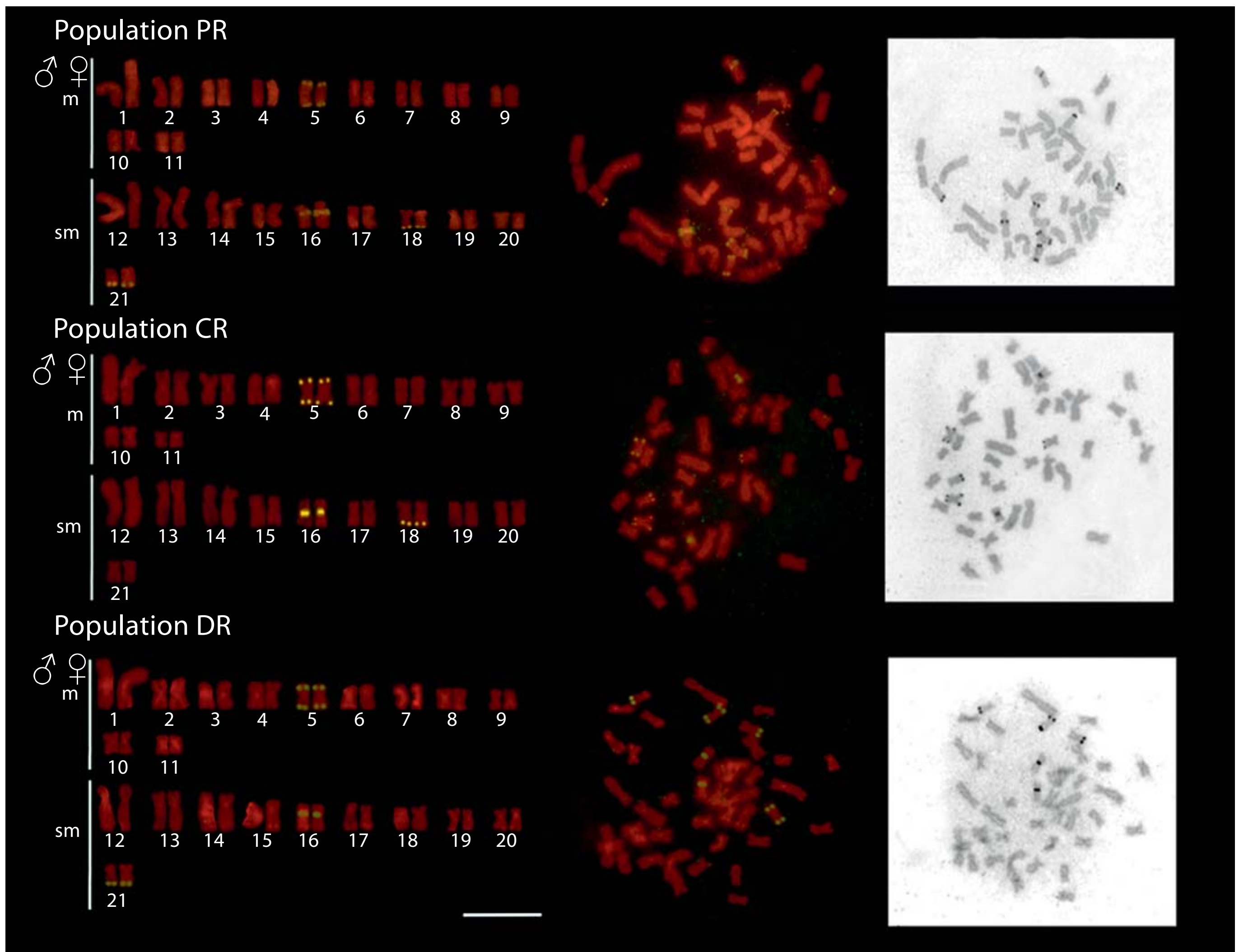

Fig. 5. Karyotypes of the *Hoplias malabaricus* populations PR, CR, and DR arranged from chromosomes probed with 18S rDNA (yellow signals) counterstained with propidium iodide, and sequentially analyzed for Ag-NORs. Note the general correspondence between the number and location of the 18S rDNA and Ag-NOR sites. Bar = 5 μm.

The 5S rRNA genes typically occur in an interstitial position of the chromosomes for almost all fish species, as well as in mammals and amphibians [Vitelli et al., 1982; Schmid et al., 1987; Lucchini et al., 1993; Mellink et al., 1996; Mäkinem et al., 1997], indicating that such pattern seems to be pervasive. Martins and Galetti Jr. [1999] suggested that this interstitially-nested distribution could even represent some advantage related to the organization of these genes in the vertebrate genome. Accordingly, all 5S rDNA sites were also interstitially located in the populations PR, CR and DR, although evidencing differentiations concerning the number and location of these sites. Indeed, only population PR exhibited 2 chromosome pairs containing the 5S rDNA genes, sharing 1 pair with population DR. The other sites were exclusive for each population.

Martins and Galetti Jr. [2000] proposed that the localization of the 5S and 18S rDNA sites in different chromosomes, as observed for the majority of vertebrates, could allow for these loci to evolve independently, since their divergent functional dynamics requires a physical distance. The divergent location of NOR and 5S rDNA loci seems to be the most common situation observed in fish, as well as in other vertebrates [Lucchini et al., 1993; Su-

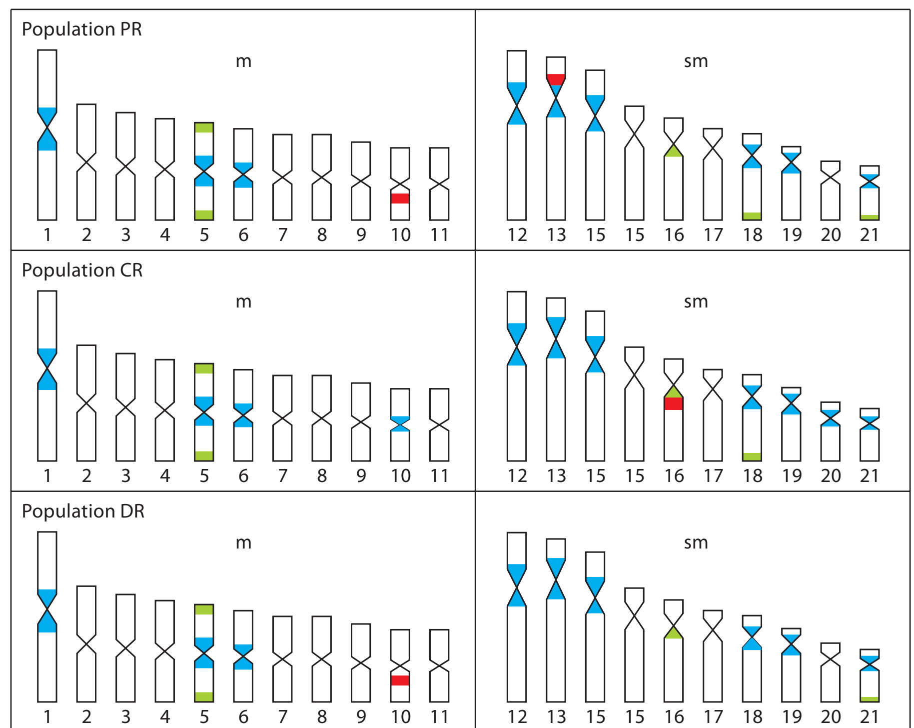

Fig. 6. Representative idiograms of the *Hoplias malabaricus* populations PR, CR, and DR. The 5S*Hin*dIII-DNA, 18S rDNA and 5S rDNA sites are indicated in blue, green and red, respectively.

zuki et al., 1996]. However, double FISH with 18S and 5S rDNA probes exhibited a syntenic condition for these clusters, located in the sm pair No. 16 of population CR. Although the co-localization of both rRNA genes was already found in some fish groups, such as Siluriformes [Pendás et al., 1994; Móran et al., 1996; Rossi and Gornung, 2005], Cypriniformes [Inafuku et al., 2000; Borón et al., 2006], and Characiformes [Jesus et al., 2003; Hatanaka and Galetti Jr., 2004; Mantovani et al., 2005; Diniz et al., 2008], the population CR is the first one to demonstrate this syntenic condition in *H. malabaricus.* Thus, this characteristic represents another key differential feature among the 3 populations analyzed. Therefore, this condition cannot be conclusively determined as a shared characteristic by populations of this karyomorph or other karyomorphs, or as an exclusive chromosomal marker for the population from the Cuiabá River basin (population CR).

The results obtained with different repetitive DNA markers (5S*Hin*dIII, 18S rDNA and 5S rDNA) corroborated the similarities among the 3 populations analyzed, reinforcing their relatedness. However, although the physical mapping of the 3 repetitive sequences demonstrated several shared chromosomes, other ones were exclusive to a specific population, probably as a consequence of a restricted gene flow. These populations belong to distinct hydrographic basins and are largely isolated by hundreds of kilometers (fig. 1), which were probably fundamental factors for the fixed chromosome differentiations. In this sense, population CR emerges as the most differentiated, with 4 exclusive chromosomal markers in chromosome pair Nos. 10, 16, 20, and 21. The killifish species *Chromaphyosemion bivittatum* exhibits a low degree of inter-populational haplotype differentiation, which agrees with the low morphological divergence. However, a karyotypic variability is observed within and among populations, which might indicate fast chromosomal evolution [Völker et al., 2007]. It also seems to be the case for *H. malabaricus.* Although preserving a similar karyotype, it is evident that distinct karyomorph A populations present inner chromosomal differentiations. As previously indicated, such divergences can manifest in structural terms by C-bands and NOR patterns, as well as in functional terms by differential expression of the NORs [Vicari et al., 2005]. The present insights on this diversity concerning the repetitive DNAs reinforce the view that distinct populations of karyomorph A cannot be considered an evolutionary unit, despite their inclusion in the same major karyotypic group.

Repetitive DNAs, such as the satellite 5S*Hin*dIII-DNA, were long considered to be junk DNA with no clearly identified function, which was reinforced by indications that these sequences were not transcribed in eukaryotes [Doolittle and Sapienza, 1980; Orgel and Crick, 1980]. However, accumulated data from eukaryotic species of diverse taxonomic origins have challenged this view over the past few years [Bonaccorsi and Lohe, 1991], supporting a major role of repetitive DNAs in the structural and functional evolution of genes and genomes in a variety of organisms [Biémont and Vieira, 2006]. Although evolutionary mechanisms have avoided major changes in the karyotypes of different populations of a specific *H. malabaricus* karyomorph, these genomes are in continuous evolution, as demonstrated by the observed minor chromosomal variations. In this way, the repetitive fraction of the genome (as here exemplified by the rDNAs and 5S*Hin*dIII satellite) seems to escape the selective pressure that acts in the non-repetitive segments, thus representing good evolutionary markers to detect recent events of evolution. In addition, the accumulation of repetitive sequences in specific genomic areas can cause chromosomal rearrangements through chromosome breakage, deletions, inversions, and amplifications [Lim and Simmons, 1994; Dimitri et al., 1997]. The investigation of other repetitive DNA families in the Erythrinidae fish model will yield a greater understanding of basal evolutionary mechanisms involved in the generation of the complex genomic structure of fishes.

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