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Comparative chromosome mapping of 5S rDNA and 5S*Hin*dIII repetitive sequences in Erythrinidae fishes (Characiformes) with emphasis on the *Hoplias malabaricus* 'species complex'

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Abstract. Chromosomal localization of 5S rDNA and 5S*Hin*dIII repetitive sequences was carried out in several representatives of the Erythrinidae family, namely in karyomorphs A, D, and F of *Hoplias malabaricus,* and in *H. lacerdae*, *Hoplerythrinus unitaeniatus* and *Erythrinus erythrinus*. The 5S rDNA mapped interstitially in two chromosome pairs in karyomorph A and in one chromosome pair in karyomorphs D and F and in *H. lacerdae*. The 5S*Hin*dIII repetitive DNA mapped to the centromeric region of several chromosomes (18 to 22 chromosomes) with variations related to the different karyomorphs of *H. malabaricus*. On the other hand, no signal was detected in the chromosomes of *H. lacerdae*, *H. unitaeniatus* and *E. erythrinus*, suggesting that the 5S*Hin*dIII-DNA sequences have originated or were lost after the divergence of *H. malabaricus* from the other erythrinid species. The chromosome distribution of 5S rDNA and 5S*Hin*dIII-DNA sequences contributes to a better understanding of the mechanisms of karyotype differentiation among the Erythrinidae members.

The Erythrinidae family includes Neotropical fishes with a wide distribution in South America with three recognized genera, *Hoplias*, *Hoplerythrinus* and *Erythrinus* (Britski et al., 1986). *Hoplias* is the most widespread genus within the family and *Hoplias malabaricus*, in particular, exhibits significant morphological and genetic variation of much interest to evolutionary biologists. Although usually considered to be a single nominal species, the taxonomy of *H. malabaricus* is still not well understood (Oyakawa, 1990). Growing evidence has pointed to the karyotypic diversity of *H. malabaricus*, showing interpopulational differences in the diploid chromosome number and morphology, as well as in the presence of sex chromosome systems. The cytogenetic diversity of *H. malabaricus* allowed the characterization of seven karyotype variants (karyomorphs) referred to as 'cytotypes' in several previous publications (Bertollo et al., 2000, for review) (Table 1). The available karyotype data for *H. malabaricus* have led to the assumption that this fish represents a 'species complex' or 'catch-all' taxon that requires taxonomic revision. The diversity of karyotypes also makes *H. malabaricus* an important model for understanding mechanisms of karyotype differentiation.

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Table 1. Karyomorphs previously identified in *H. malabaricus* according to Bertollo et al. (2000)

Karyomorphs	Chromosome number	Presence of sex chromosomes	Geographic occurrence
Karyomorph A	2n = 42	–	Northern to southern Brazil, Uruguay and northern Argentina
Karyomorph B	2n = 42	XX/XY	Vale do Rio Doce, Minas Gerais State, Brazil, and Iguaçu River (Paraná State, Brazil)
Karyomorph C	2n = 40	–	Northern Brazil to northern Argentina
Karyomorph D	2n = 40 (♀) 2n = 39 (♂)	$X_1X_1X_2X_2/X_1X_2Y$	Upper Paraná hydrographic basin in Brazil
Karyomorph E	2n = 42	–	Trombetas River, Pará State, Brazil
Karyomorph F	2n = 40	–	Surinam to southeastern Brazil
Karyomorph G	2n = 40 (♀) 2n = 41 (♂)	XX/XY_1Y_2	Amazonian rivers

Table 2. Characteristics of the Erythrinidae species analyzed including karyomorphs, sample sizes (N) and localities in Brazil

Species	Karyomorphs	N	Locality
Hoplias malabaricus	A	2♀; 3♂	Araquá river, Botucatu, SP
Hoplias malabaricus	D	1♀; 1♂	Mogi-Guaçu River, Pirassununga, SP
Hoplias malabaricus	F	1♀	São Francisco River, Três Marias, MG
Hoplias lacerdae		1♂	UNESP fish culture facility, Jaboticabal, SP
Hoplerythrinus unitaeniatus		1♂	Paraná River, Bataguassu, MS
Erythrinus erythrinus		1♀; 1♂	Lagoons, Natal, RN

The 5S rDNA sequences have been investigated in several teleost fishes and proved to be good chromosomal markers and to be of considerable value for understanding the evolutionary dynamics of repetitive multigene families in the genome (Martins and Wasko, 2004). Besides the 'true' members of the rDNA multigene family, dispersed or clustered copies of rDNA were detected in several eukaryotic genomes, including yeast (Childs et al., 1981), animals (Emerson and Roeder, 1984; Leah et al., 1990; Lohe and Roberts, 1990), and plants (Falquet et al., 1997). These elements have been characterized mainly as non-coding, small-unit tandem repeats of variable copy number. In *H. malabaricus,* a class of repetitive DNAs, named 5S*Hin*dIII-DNA, that share similarities to 'true' 5S rDNA repeats, have previously been described (Martins et al., 2006). The interesting feature of the variant 5S*Hin*dIII-DNA is the high abundance of copy number, the tandem array, and their pericentromeric locations.

Most investigations in the *H. malabaricus* species complex were conducted using basic cytogenetic analyses and few studies applied the molecular cytogenetic approach. In this paper, we provide data on comparative chromosome mapping of 5S rDNA and 5S*Hin*dIII-DNA repetitive sequences in several representatives of Erythrinidae with emphasis on the *H. malabaricus* species complex.

Material and methods

Animals and mitotic chromosome preparations

Chromosome preparations were obtained from animals specified in Table 2. Mitotic chromosomes were prepared from anterior kidney cells with in vivo colchicine treatment (Bertollo et al., 1978).

Chromosome hybridization, probes and karyotype analysis

Mitotic chromosome spreads were submitted to fluorescent in situ hybridization (FISH) (Pinkel et al., 1986) using two repetitive tandem-arrayed DNA sequences previously isolated from the genome of karyomorph A of *H. malabaricus* and cloned in plasmids (Martins et al., 2006). The first probe contained a 5S rDNA repeat copy and included 120 base pairs (bp) of the 5S rRNA encoding gene and 200 bp of the non-transcribed spacer (NTS). The second probe contained a copy of the repetitive satellite 5S*Hin*dIII-DNA sequence with 360 bp composed of a 95-bp segment with similarity to the 5S rRNA gene of the first probe, and a 265-bp segment similar to the NTS of the first probe (Martins et al., 2006) (Fig. 1). The probes were labeled by nick translation with biotin-14-dATP (Bionick labeling system-Invitrogen). The metaphase chromosome slides were incubated with RNase (40 μg/ml) for 1.5 h at 37°C. After denaturation of chromosomal DNA in 70% formamide, 2× SSC for 4 min at 70°C, hybridization mixtures containing 100 ng of denatured probe, 10 mg/ml dextran sulfate, 2× SSC, and 50% formamide in a final volume of 30 μl were dropped on the slides and the hybridization was performed overnight at 37°C in a 2× SSC moist chamber. Post-hybridization washes were carried out at 37°C in 2× SSC, 50% formamide for 15 min, followed by a second wash in 2× SSC for 15 min, and a final wash at room temperature in 4× SSC for 15 min. Detection of hybridized probes was carried out with 0.07% avidin-FITC conjugate (Sigma) in C buffer (0.1 M $NaHCO_3$, 0.15 M NaCl) for 1 h followed by two rounds of signal amplification using 2.5% anti-avidin biotin conjugate (Sigma) in blocking buffer (1.26% $NaHCO_3$, 0.018% sodium citrate, 0.0386% triton and 1% non-fat dried milk) for

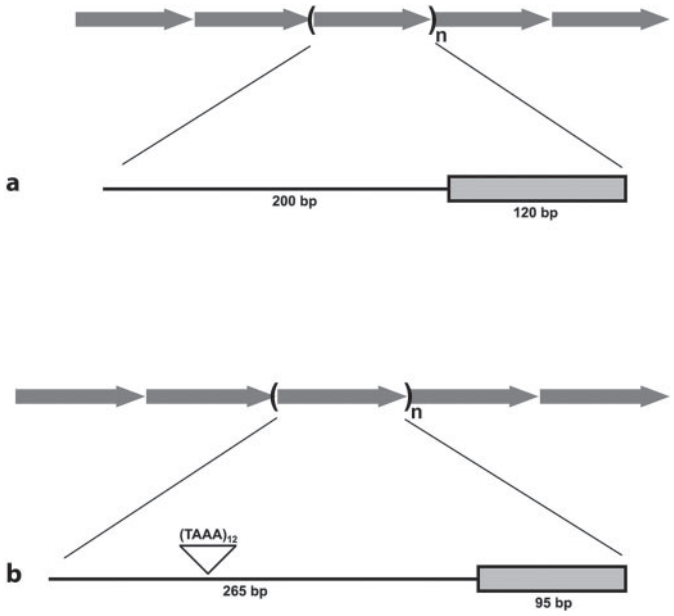

Fig. 1. Schematic representation of the true 5S rDNA (**a**) and the variant 5S rDNA (5S*Hin*dIII-DNA) repeats (**b**) isolated in *Hoplias malabaricus* according to Martins et al. (2006). The boxes indicate segments with similarity to the coding region of the 5S rDNA and the horizontal lines the NTSs. The sizes of the NTS and the 5S rDNA coding region, and the TAAA microsatellite are indicated. Large arrows represent the repetitive pattern of both sequence classes.

30 min. Each treatment with anti-avidin biotin conjugate was followed by a treatment with avidin-FITC. The treatments with avidin-FITC and anti-avidin-biotin were conducted in a 2× SSC moist chamber at 37°C. After each amplification step, the slides were washed three times for 5 min each in blocking buffer at 42°C. The post-hybridization washes were conducted in a shaker (150 rpm). Chromosomes were counterstained with propidium iodide (PI) (0.2%) diluted in antifade (Vector).

Hybridized chromosomes were analyzed using a Zeiss Axiophot photomicroscope, the images captured with a digital camera and processed with Adobe Photoshop v. 7.0 software. The karyotypes were arranged according to Bertollo et al. (2000).

Results

The karyotype analyses of the Erythrinidae species agreed with previous karyotype descriptions of the representatives of the family (Bertollo et al., 2000, 2004; Diniz and Bertollo, 2003). In *H. malabaricus,* the 5S rDNA was clustered interstitially in a large submetacentric chromosome pair (pair No. 3) and in a small-sized metacentric pair (No. 14) in karyomorph A (Fig. 2a); in the interstitial region of only one metacentric pair (No. 5) in both sexes of karyomorph D (Fig. 2b, c); and in the pericentromeric region of a medium-sized metacentric pair (No. 9) in karyomorph F (Fig. 2d). In *H. lacerdae* (Fig. 3) the 5S rDNA clusters were located in the pericentromeric region of a medium-sized metacentric chromosome pair (No. 14).

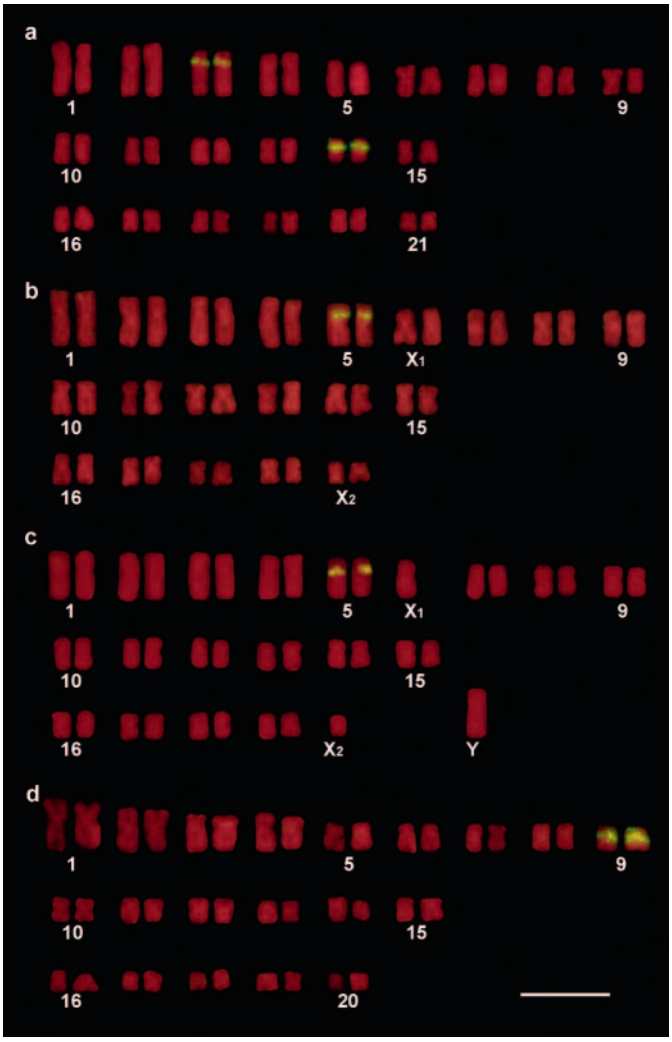

Fig. 2. Karyotypes of *H. malabaricus* forms A (**a**), D female (**b**), D male (**c**), and F (**d**) arranged from chromosomes probed with 5S rDNA (yellow signals) and PI counterstained. Bar = 5 μm.

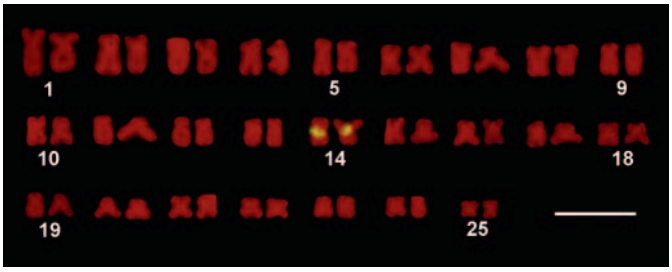

Fig. 3. Karyotype of *H. lacerdae* representative of the 'lacerdae group' arranged from chromosomes probed with 5S rDNA (yellow signals) and PI counterstained. Bar = 5 μm.

The 5S*Hin*dIII-DNA repetitive sequence was mapped to the centromeric region of several chromosomes (18 to 22 chromosomes) with variations in the different karyomorphs of *H. malabaricus* (Fig. 4). On the other hand, no positive

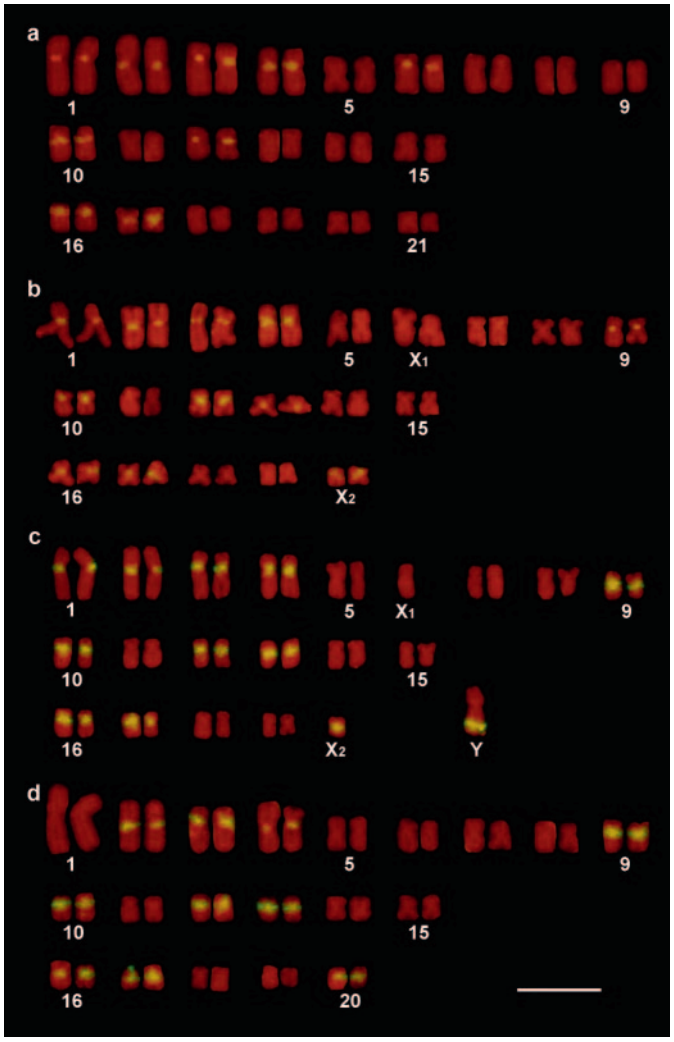

Fig. 4. Karyotypes of *H. malabaricus* forms A (**a**), D female (**b**), D male (**c**), and F (**d**) arranged from chromosomes probed with 5S*Hin*dIII-DNA repetitive sequence (yellow signals) and PI counterstained. Bar = 5 μm.

hybridization was observed in the chromosomes of *H. lacerdae, H. unitaeniatus* or *E. erythrinus*. The number of chromosomes labeled by the 5S*Hin*dIII-DNA was characteristic for each of the karyomorphs analyzed: karyomorph A had 18 labeled chromosomes; karyomorph D displayed 22 labeled chromosomes for male and female; and karyomorph F had 20 labeled chromosomes (Fig. 4). Some chromosomes, such as the pairs Nos. 1 to 4, can be easily recognized in the different karyomorphs. Chromosome pairs 1 to 4 were labeled in karyomorphs A and D and the pairs 2 to 4 were labeled in karyomorph F. No labeling was detected in the large chromosome No. 1, which is a distinct feature of karyomorph F. Several 5S*Hin*dIII-DNA labeled chromosomes (pairs 2, 3, 4, 10, 12, 16 and 17) were conserved among the different karyomorphs (Fig. 4). One of the most interesting features of the chromosomal distribution of 5S*Hin*dIII-DNA sequences in *H. malabaricus* was its presence in the sex chromosomes of karyomorph D (Fig. 4b, c).

Discussion

Chromosomal organization of 5S rDNA in Erythrinidae

The chromosomal distribution of 5S rDNA in the karyotypes of Erythrinidae species reflects high levels of variation with the pronounced karyotype diversity discovered for the family. Previous analyses on the chromosomal localization of 5S rDNA were conducted in karyomorph B of *H. malabaricus* (Born and Bertollo, 2000) and mapped it in an interstitial position in a small-sized metacentric chromosome pair. Karyomorphs A and B were considered to be close since they share similar karyotype structures and it was suggested that karyomorph B can be derived from karyomorph A (Bertollo et al., 2000). The small metacentric chromosome that harbors the 5S rDNA in karyomorphs A and B seems to be a homologous chromosome. On the other hand, the large submetacentric chromosome pair No. 3 that also contains a 5S rDNA cluster in karyomorph A was not observed in karyomorph B, but it is quite similar in size and morphology to the chromosome pair that harbors the 5S rDNA in karyomorph D. The presence of this large submetacentric chromosome bearing a 5S rDNA cluster seems to be exclusive to karyomorphs A and D, since it was not observed in karyomorphs B or F, or in other Erythrinidae species. Although there are significant differences in diploid chromosome number and presence/absence of sex chromosomes among karyomorphs A, B and D, these karyomorphs showed similar karyotype structure of the previously listed four chromosomal pairs which are larger than the remaining ones that gradually decreased in size (Bertollo et al., 2000). On the contrary, karyomorph F has a distinct feature: the presence of a large metacentric pair (pair No. 1), which constitutes the largest chromosome yet found in the karyotypes of *H. malabaricus* species. Bertollo et al. (2000) recognized two major karyotype groups in *H. malabaricus*: the karyomorphs A, B, C and D, and the karyomorphs E, F and G. The chromosomal distribution of 5S rDNA is consistent with this grouping. Although the karyomorphs of *H. malabaricus* can be identified by gross differences of the karyotypes, the chromosomal distribution of 5S rDNA also differs among the karyomorphs. The same applies if we consider other representatives of the Erythrinidae family, such as *H. lacerdae*, with the presence of 5S rDNA clusters located in the pericentromeric region of a medium-sized metacentric chromosome pair. The presence of pericentromeric clusters of 5S rDNA could likely constitute a plesiomorphic condition for the genus *Hoplias*, since in *Hoplerythrinus unitaeniatus* the 5S rDNA was also located on the pericentromeric region of two to five meta- and submetacentric chromosomes (Diniz and Bertollo, 2003, 2006).

Data on the chromosomal location of 5S rDNA in more than 70 fish species from distinct groups, such as Acipenseriformes, Anguilliformes, Cypriniformes, Characiformes, Salmoniformes, Perciformes, and Tetraodontiformes have shown a conservative chromosomal distribution pattern (for review see Martins and Wasko, 2004). For some groups, the chromosomal location of 5S rDNA is conserved even

among the members of major taxa. This was detected for several species of *Leporinus* and *Schizodon* (Anostomidae) (Martins and Galetti, 1999, 2000, 2001), *Parodon* (Parodontidae) (Vicente et al., 2001) and *Prochilodus argenteus* (Prochilodontidae) (Hatanaka and Galetti, 2004), showing that the 5S rDNA sites seem to be in orthologous chromosomes in these families. This characteristic was observed even for species with high rates of chromosomal changes such as characid fishes of the genus *Astyanax* (Almeida-Toledo et al., 2002; Mantovani et al., 2005). Contrary to the rather conserved location of 5S rDNA among other fishes, we observed variable chromosomal locations of the 5S rDNA for the *Hoplias* species.

The chromosomal distribution of 5SHindIII-DNA repetitive sequence and karyotype differentiation in H. malabaricus

The chromosomal distribution of 5S*Hin*dIII-DNA repetitive sequences allowed discrimination among the different karyomorphs and corroborates previous data on the origin of the sex chromosomes in karyomorph D of *H. malabaricus*. The exclusive presence of 5S*Hin*dIII repetitive DNA in *H. malabaricus* shows that this repetitive DNA arose or was lost after divergence of the main groups of Erythrinidae and the divergence of *Hoplias* species. Since *H. malabaricus* is widely distributed throughout South America hydrographic systems, the origin of 5S*Hin*dIII sequences seems to have occurred before divergence and dispersion of the main lineages of *H. malabaricus* that produced the present karyomorphs. The present data suggest that the 5S*Hin*dIII repetitive sequences have accompanied the chromosome changes that led to the high diversity of karyotypes discovered in the species. Thus, the chromosome distribution of 5S*Hin*dIII-DNA is also consistent with the two major karyotype groups recognized for *H. malabaricus* (Bertollo et al., 2000). Several 5S*Hin*dIII-DNA-labeled chromosomes (pairs 2, 3, 4, 10, 12, 16 and 17) are conserved among the different karyomorphs and could be considered ancient chromosomes of *H. malabaricus*.

Repetitive DNAs are considered to be unstable regions of the genomes with high rates of evolution (Charlesworth et al., 1994). The magnitude of genome instability due to head-to-tail tandem repeats is often much higher than that due to dispersed repetitive elements (Gondo et al., 1998). Due to this hypervariability the tandem repeats are highly polymorphic and are considered good molecular markers for genotyping of individuals and populations (Jeffreys et al., 1985). The high evolutionary dynamics of repetitive DNA sequences makes them useful chromosome markers in studies of species evolution, identification of chromosome rearrangements, sex identification and applied genetics. Although the variability level of 5S*Hin*dIII-DNA sequences among the different karyomorphs of *H. malabaricus* was not investigated, we detected high rates of variability in the chromosomal organization of such sequences.

One interesting characteristic of the 5S*Hin*dIII-DNA repetitive family members is their centromeric location and high copy number. Centromeres have been recognized as evolutionarily dynamic regions of the genome (Eichler and Sankoff, 2003), and although they have been well investigated from animals to fungi, important points are still not understood (Henikoff et al., 2001). The centromeric regions are rich in repetitive DNAs, as seen in humans, mice, maize, fruit flies and yeast (Henikoff et al., 2001). If the 5S*Hin*dIII-DNA repeats may be conferring some structural or functional advantage to the chromosomes as a component of the centromeric DNA in *H. malabaricus*, it cannot be addressed yet. An extensive analysis of centromeric satellite DNAs of vertebrates showed the presence of typical short A-rich motifs (Viñas et al., 2004). The expanded TAAA motif in the 5S*Hin*dIII-DNA is similar to the short A-rich motifs that were previously identified in the centromeric satellite DNAs of different fish species (Wright, 1989; Denovan and Wright, 1990; Garrido-Ramos et al., 1995; Kato, 1999; Canapa et al., 2002; Viñas et al., 2004). This short motif also shows considerable similarity to other centromeric motifs found in humans (Vissel et al., 1992), mice (Wong and Rattner, 1988), and reptiles (Cremisi et al., 1988), suggesting that such sequences might play an important role in the structure and function of the *H. malabaricus* centromere. According to Martins et al. (2006), the 5S*Hin*dIII-DNA originated from true copies of 5S rDNA and was propagated and maintained in the centromeric region of most chromosomes due to a structural or functional advantage conferred to the nuclear genome.

A multiple sex chromosome system of the $X_1X_1X_2X_2/X_1X_2Y$ type is present in karyomorph D (Bertollo et al., 1983, 1997; Bertollo and Mestriner, 1998). The Y chromosome is one of the largest in the complement, while the X_1 and the putative X_2 are similar to chromosomes Nos. 6 and 20, respectively. Using several banding methods, it was demonstrated that this sex chromosome system originated from a translocation between two bi-armed chromosomes – chromosome No. 6 (X_1) and the probable chromosome No. 20 (X_2) – forming a single chromosome in males (chromosome Y) (Bertollo et al., 1997). This proposed mechanism for the origin of sex chromosomes in karyomorph D was later confirmed by meiotic analysis (Bertollo and Mestriner, 1998). The chromosome mapping of 5S*Hin*dIII-DNA sequences supports these previous studies. Indeed, the presence of 5S*Hin*dIII-DNA sequences in chromosome No. 20 (X_2) and in the long arm of chromosome Y proved that chromosome No. 20 fused to chromosome No. 6, producing chromosome Y.

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