Identification of a new repetitive element in the sex chromosomes of \textit{Leporinus elongatus} (Teleostei: Characiformes: Anostomidae): new insights into the sex chromosomes of \textit{Leporinus}

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Abstract. \textit{Leporinus elongatus} represents an interesting model for studies on chromosome evolution since it possesses a conspicuous ZZ/ZW sex chromosome system that has been characterized mainly by basic cytogenetic techniques. In the present study we describe a dispersed repetitive element (named LeSpeI) related to the sex chromosomes of \textit{L. elongatus}. Females revealed clusters of LeSpeI on the long arm of the W chromosome and in the acrocentric NOR-bearing chromosome pair. In males, the signal was restricted to the pericentromeric region of the NOR-bearing chromosomes. Considering the results obtained in the present study using FISH, NOR and C-banding, together with findings from previous studies, it can be inferred that the sex chromosome system of \textit{L. elongatus} is still undergoing an evolutionary process. The data suggest novelties in relation to the sex chromosomes of the genus \textit{Leporinus} with the description of a multiple sex chromosome system involving the NOR-bearing chromosomes. Therefore, it is hypothesized that the simple ZW chromosome system previously described for \textit{L. elongatus} rather is a multiple Z\textsubscript{1}Z\textsubscript{1}Z\textsubscript{2}Z\textsubscript{2}/Z\textsubscript{1}W\textsubscript{1}Z\textsubscript{2}W\textsubscript{2} system.

The family Anostomidae is distributed from Central to South America and comprises 138 described species, divided into twelve genera (Abramites, Anostomoides, Anostomus, Gnathodolus, Laemolyta, Leporellus, Leporinus, Pseudanos, Rhytiodus, Sartor, Schizodon and Synaptolaemus) (Garavello and Britski, 2003). Among anostomids, the genus \textit{Leporinus} contains the highest number of species (87 valid species) (Garavello and Britski, 2003). All species studied cytogenetically present 54 biarmed chromosomes, and the karyotypes of most species carry a single NOR-bearing pair. In spite of conserved chromosome formulae within the genus, a conspicuous ZZ/ZW sex chromosome system has been reported in seven species (\textit{L. conirostris}, \textit{L. elongatus}, \textit{L. aff. elongatus}, \textit{L. macrocephalus}, \textit{L. obtusidens}, \textit{L. reinhardtii} and \textit{L. trifasciatus}), while the remaining cogeneric species studied cytogenetically (about 40 species) lack differentiated sex chromosomes (Galetti et al., 1995). In the ZW system of female \textit{Leporinus}, the typical W chromosome is large, subtelocentric, and almost fully heterochromatic. In contrast, in the Z chromosome present in both sexes only the distal third of the long arm is heterochromatic. An initial heterochromatinization could be the
first step in the differentiation of these sex chromosomes (Galetti and Foresti, 1986). The morphological similarity of chromosomes Z and W among all ZW Leperinus suggests a common origin for these chromosomes (Galetti et al., 1995). On the other hand, a novel ZW sex chromosome system morphologically differentiated from the typical ZW system previously detected in these seven species, was described in Leperinus sp. (Venere et al., 2004).

Repetitive DNA sequences have been extensively exploit-
ed as chromosome markers, being useful in studies of spe-
cies evolution, identification of chromosome rearrange-
ments, sex identification and applied genetics. A study in-
volving molecular cytogenetics of sex chromosomes of Le
erinus elongatus was performed by Nakayama et al. (1994) and two sex-specific sequences were isolated, cloned and used to investigate the structure and variability of sex chromosomes in the species. The authors identified three atypical W chromosomes and proposed a model involving the formation of three new W chromosomes (W W1 , W W2 and W W3 ) and three new Z chromosomes (Z Z1 , Z Z2 and Z Z3 ) in female meiosis. These chromosomes would then associate prefer-
entially with normal Z male chromosomes, giving rise to six new genotypes (ZW W1 , ZW W2 , ZW W3 , ZW W2 , ZW W2 and ZW W2 ). In that study, only individuals with ZW W1 , ZW W2 and ZW W3 constitutions were observed, and the authors suggest that such a result may be related to limited fish sampling, inviability of offspring and sterility causing their absence in a population of spawning adults, or may have been recognized cytologi-
cally as a W.

Since the sex chromosomes of fishes are enriched with repetitive DNA sequences (Martins, 2006), the investiga-
tion of the chromosomal organization of repetitive sequenc-
es could provide new insights in the origin and evolution of sex chromosomes in the fish genome. In the present study, the investigation of repetitive DNA in the genome of L. elon-
gatus allowed the identification of a new repetitive element located in the sex chromosomes of this species. The mo-
olecular organization and chromosomal location of this re-
petitive sequence revealed some novelties about the sex chromosomess of this species.

Materials and methods

Chromosomal and genomic DNA preparation

Wild specimens of Leperinus elongatus (five males and eight fe-
males) were collected in the Mogi-Guaçu River, Pirassununga, São Paulo, Brazil. Mitotic chromosomes were obtained and stained accord-
ing to Foresti et al. (1993). Genomic DNA was extracted from liver and blood by standard methods (Sambrook and Russel, 2001).

A search for repetitive DNAs was conducted using restriction en-
zyme digestion of the genomic DNA of L. elongatus with different re-
striction endonucleases. The endonuclease Spl revealed a conspicuous band of about 650 bp. This DNA band was isolated from the gel, cloned into pMOS Blue plasmid vector (Amersham Biosciences), and used for transformation in E. coli DH5α competent cells. Positive clones were identified and stored at –75°C for further analysis.

Sequencing and sequence analysis

Positive clones were isolated and their sequence determined using DYEnamicTM ET Terminator Cycle Sequencing (Amersham Biosci-
The chromosomal distribution of the LeSpeI element was determined by FISH in metaphase spreads of *L. elongatus* (Fig. 3). Two strong blocks and one small signal of LeSpeI were observed on the long arm of the W chromosome in females. Positive blocks of LeSpeI were also seen at the pericentromeric region and dispersed on the long arm of a medium-sized acrocentric chromosome while the signal was restricted to the pericentromeric position in its homolog. This acrocentric pair was identified as pair 2 (Z₂W₂). In males, the signal was restricted to the pericentromeric region of both members of pair 2 (Z₁Z₁). All eight females and five males analyzed by FISH showed the same results. Ag-NOR staining showed that pair 2 also represented the NOR-bearing chromosomes in this species, since Ag-NOR marks were seen on long arms of both chromosomes in male and female cells (Fig. 3). C-banding analysis in the same individuals showed that the long arm of the W₁ chromosome is entirely heterochromatic (Fig. 3). The supposed Z₂W₂ pair of females also revealed a distinct pattern of C-bands between homologs, where a conspicuous interstitial band was present in just one homologous element (Fig. 3). C-banding on male metaphase spreads showed a remarkable band on the long arms of a single chromosome pair, identified as Z₁.

**Discussion**

The digestion of *L. elongatus* DNA with the enzyme SpeI showed the presence of distinct bands in agarose gels as observed in other species after genomic DNA digestion with restriction endonucleases (Jesus et al., 2003; Azevedo et al., 2005). The observed bands indicate the presence of highly repetitive sequences that were cleaved by SpeI. Comparisons of the sequences to nucleic acid sequences available on databases revealed no similarities to any known DNA sequence.

Analysis of membrane immobilized genomic DNA of *L. elongatus* hybridized to clones carrying the LeSpeI element indicates that LeSpeI is a highly repetitive element, predominantly dispersed throughout the genome. Although

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**Fig. 1.** Alignment of nucleotide sequences of three cloned SpeI repetitive fragments of *Leperinus elongatus*. Dots indicate sequence identity, hyphens represent indels. The LeSpeI sequences were deposited in GenBank under the accession numbers EF107659, EF107660, and EF107661.

**Fig. 2.** Southern blot of the genomic DNA samples of *L. elongatus* males (M) and females (F) after digestion with the restriction enzymes *SacI*, *MboI*, *MspI* and *SpeI* and hybridization to the LeSpeI repetitive probe. The lanes on the right digested with SpeI were hybridized under conditions of higher stringency. The arrowhead indicates the 450-bp band detected by SpeI. L, molecular size marker in kilobases.
the copy number of LeSpeI was not determined in males and females, the membrane hybridization results suggest a higher number of copies of LeSpeI in females. This result was confirmed by chromosome hybridization.

The dispersed repetitive pattern of the LeSpeI element suggests that this sequence could belong to a dispersed transposable element (TE) class (Oliveira et al., 1999; Bodvarsdottir and Ananthawat-Jonsson, 2003; Ziegler et al., 2003). Although the Southern blot hybridization using LeSpeI generated bands between 450 and 3,000 bp, as observed for most DNA-based TEs (Tafalla et al., 2006), it was not possible to identify characteristics of TE in the isolated LeSpeI fragments.

Fluorescent in situ hybridization showed that LeSpeI sequences are arranged in a peculiar pattern within chromosomes of L. elongatus labeling three subtelocentric chromosomes in females and two subtelocentric chromosomes in males. In females, this sequence is distributed in three clusters—two large blocks, one small block—over the long arm of the W chromosome. The other two chromosomes presenting intense LeSpeI hybridization signals in female metaphases were a middle-sized chromosome with three signals, two located in the long arm and one in the short arm, and a middle-sized chromosome bearing a single cluster on the short arm. These latter chromosomes, despite of the difference in the distribution pattern of LeSpeI sequences, are homologous, corresponding to the NOR-bearing pair, as previously observed by Galetti et al. (1984). In male metaphases, the LeSpeI sequences are only present clustered on the short arms of the NOR-bearing pair.

Considering the presence of two exclusive chromosomes in females, they should be regarded as W1, identified by its morphology and accumulation of heterochromatin and repetitive sequences, and W2, identified by the presence of two clusters of LeSpeI sequences over the long arms. Therefore, we propose here the hypothesis that the simple ZW chromosome system previously described for L. elongatus (Galetti et al., 1981) is rather a multiple Z1Z2Z3Z4W1W2 system. The occurrence of Z1Z2Z3Z4/Z1W1Z2W2 is described here for the first time in fish (Devlin and Nagahama, 2002). The occurrence of such an unusual sex chromosome system was not expected since males would be able to produce only a single type of spermatozoa (Z1Z2), but the females would be able to produce four types of oocytes: Z1Z2, Z1W2, Z2W1, and W1W2. Evaluation of the exact segregation pattern of these gametes will be possible by analysis of the meiotic pairing, a study which our research group will soon be undertaking.

The presence of LeSpeI elements may represent a recent sequence accumulation in the L. elongatus genome, directly or indirectly, favoring the divergence of sex chromosomes. Nakayama et al. (1994) isolated two sequences related to sex chromosomes of L. elongatus: L/H115415, which hybridized with both Z and W chromosomes and L/H1154146 that was part of a W-specific chromosomal region. These sequences lack any similarity to the LeSpeI element when analyzed in GenBank. According to the authors, these sequences apparently have no biological significance, since they are not part of a coding sequence and do not show a clear similarity to any known sequences. Nevertheless, unlike the LeSpeI sequence, they would be related to the divergence of sex chromosomes. The results of both studies are in total agreement with a 'Muller's ratchet'-like process, involving the accumulation of elements, free from genetic exchange, and thus leading to morphologically distinct chromosomes, which, in turn, would fix the heterogametic sex determination (Charlesworth, 1991). On the other hand, we cannot address any discussion in relation to gene loss in the sex chromosomes of L. elongatus.

Amongst individuals analyzed by Nakayama et al. (1994), there were three individuals cytologically classified as ZW
that did not hybridize with the W-specific L'46 sequence. These individuals were classified as ZW males, but they could not be regarded as ordinary genetic males, since they bore a W chromosome. The interest of these three cases lies in the fact that they can be interpreted through single genetic exchanges among four regions from sex chromosomes in female meiosis, giving rise to the three atypical W chromosomes. C-banding results suggest that the size difference between Z and W chromosomes of *L. elongatus* is basically due to an increased size of the long arms in the W chromosome, which is strongly heterochromatic, mainly at its distal region (Galetti and Foresti, 1986). These cytogenetic studies have also shown that the long arms of W chromosomes are composed of heterochromatic segments interspersed with euchromatin. Such information is compatible with our molecular data. Our results also demonstrate that the heterochromatin located on a portion of the long arms of the W chromosome, and that on the NOR-bearing pair share a common nature.

Therefore, combining our results obtained by FISH, NOR and C-banding with data provided by Nakayama et al. (1994), comprising three putatively distinct W chromosomes, we can infer that the sex chromosome system of *L. elongatus* is still undergoing an evolutionary process, not fixed within the population. We conclude that it is probably a multiple Z1Z1Z2Z2/Z1W1Z2W2 sex chromosome system, where the NOR-bearing pair, formerly defined as pair 2, would represent the pair Z2, carrying ribosomal cistrons.

Other examples of sex-linked repeat sequences have been observed in fish. Nanda et al. (1990) reported the presence of a single-sequence repeat on Y chromosomes of poeciliid species, although not all strains have been found to carry these sequences on their sex chromosomes (Nanda et al., 1992; Hornaday et al., 1994). Fish have also been examined for sex-linkage of simple sequence repeat classes including Bkm sequences, which are considered sex-linked in many other organisms (Lloyd et al., 1989; Wachtel et al., 1991). However, only an Antarctic ice fish species presented evidence of Y-specificity of Bkm and another satellite sequence (Capriglione et al., 1994). Reed and Phillips (1995) reported a sex-linked repeat sequence in the lake trout, which appears to be associated with a Q-band in the distal heterochromatin on the X chromosome. Moreover, Moran et al. (1996) described the X-linkage of 5S rDNA in the rainbow trout.

Multiple sex chromosome systems have already been reported in other Neotropical fish species (Centofante et al., 2002) and, although other systems might also be found in fish, most species lack differentiated sex chromosomes. The diversity of sex determining mechanisms in fish, along with the absence of differentiated sex chromosomes in most species, indicates that this animal group would be an ideal model for studies about the evolutionary process of sex determination in vertebrates (Venere et al., 2004). We believe that the isolation and characterization of other repetitive sequences from the *L. elongatus* genome would represent a promising step toward understanding the origin of sex chromosomes within the genus *Leporinus*, and, consequently, of fish and other vertebrates as well.

References


Martins C: Chromosomes and repetitive DNAs: a contribution to the knowledge of fish genome; in Pisano E, Ozouf-Costaz C, Foresti F, Kapoor BG (eds): Fish Cytogenetics, pp 421–453 (Science Publisher Inc., Enfield 2006).


