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

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Abstract. *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 Le*Spe*I) related to the sex chromosomes of *L. elongatus*. Females revealed clusters of Le*Spe*I 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 *L. elongatus* is still undergoing an evolutionary process. The data suggest novelties in relation to the sex chromosomes of the genus *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 *L. elongatus* rather is a multiple $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ system.

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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 *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 *(L. conirostris, L. elongatus, L.* aff. *elongatus, L. macrocephalus, L. obtusidens, L. reinhardti* and *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 *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

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first step in the differentiation of these sex chromosomes (Galetti and Foresti, 1986). The morphological similarity of chromosomes Z and W among all ZW *Leporinus* 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 *Leporinus* sp. (Venere et al., 2004).

Repetitive DNA sequences have been extensively exploited as chromosome markers, being useful in studies of species evolution, identification of chromosome rearrangements, sex identification and applied genetics. A study involving molecular cytogenetics of sex chromosomes of *Leporinus 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_1, W_2 and W_3) and three new Z chromosomes (Z_1, Z_2 and Z_3) in female meiosis. These chromosomes would then associate preferentially with normal Z male chromosomes, giving rise to six new genotypes (ZW_1, ZW_2, ZW_3, ZZ_1, ZZ_2 and ZZ_3). In that study, only individuals with ZW_1, ZW_2 and ZW_3 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 cytologically as a W.

Since the sex chromosomes of fishes are enriched with repetitive DNA sequences (Martins, 2006), the investigation of the chromosomal organization of repetitive sequences 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. elongatus* allowed the identification of a new repetitive element located in the sex chromosomes of this species. The molecular organization and chromosomal location of this repetitive sequence revealed some novelties about the sex chromosomes of this species.

Materials and methods

Chromosomal and genomic DNA preparation

Wild specimens of *Leporinus elongatus* (five males and eight females) were collected in the Mogi-Guaçu River, Pirassununga, São Paulo, Brazil. Mitotic chromosomes were obtained and stained according 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 enzyme digestion of the genomic DNA of *L. elongatus* with different restriction endonucleases. The endonuclease *Spe*I 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

Positives clones were isolated and their sequence determined using DYEnamicTM ET Terminator Cycle Sequencing (Amersham Biosciences) and ABI 377 automated DNA sequencer (Applied Biosystems). Nucleotide sequences were subjected to BLASTN (Altschul et al., 1997) search at the National Center for Biotechnology Information (NCBI), web site (http://www.ncbi.nlm.nih.gov/blast), and the sequence alignment was performed using Clustal X (Thompson et al., 1997) and manually checked. Analyses employing the genetic p-distance model were conduced using the software MEGA version 3.1 (Kumar et al., 2004).

Genomic organization

The genomic organization of the isolated repetitive fragment was determined using Southern blot hybridization with aliquots of 10 μg of genomic DNA from males and females. Genomic DNA was digested with different restriction enzymes (*Sac*I, *Mbo*I, *Spe*I and *Msp*I), separated by electrophoresis in 1.5% agarose gel, and the DNA fragments were transferred onto a Hybond N+ nylon membrane (Amersham Biosciences) by capillarity. Clones bearing the isolated repetitive fragment were used as probes and hybridized under conditions of high stringency, using the ECL direct nucleic acid labeling and detection systems Kit (Amersham Biosciences), according to the manufacturer's specifications.

Fluorescent in situ hybridization (FISH)

For fluorescent in situ hybridization (FISH), the repetitive probe was labeled with the Bionick Labeling System Kit (Invitrogen), following the manufacturer's specifications. The slides were incubated in 2× SSC and 100 μg/ml RNase at 37°C for 90 min and dehydrated in an ethanol series (75%, 80%, 95%). Chromosomal DNA was denatured by immersing slides for 5 min at 70°C in 70% formamide, 2× SSC, and then dehydrated in an ethanol series (70%, 85%, 100%). The hybridization solution was composed of 50% formamide in 2× SSC, 10% dextran sulfate and 1.5 μg/ml biotinylated DNA probe. After 10 min of denaturation at 95°C, 20 μl of hybridization solution was applied to each slide under a coverslip. Hybridization was detected with fluorescein-labeled avidin and the signal was enhanced by incubation with anti-avidin antibody followed by the application of fluoresceinated avidin. Chromosomes were counterstained with propidium iodide/antifade solution.

Results

Restriction digestion of genomic DNA of *L. elongatus* with *Spe*I revealed the presence of a band of approximately 650 bp after electrophoresis in agarose gel. The 650-bp band was excised from the gel, cloned and the sequence partially determined for three clones (Fig. 1). The analysis of the sequenced segment of the clones showed that the sequences were very similar with a mean genetic distance of 0.138 and an AT content of 52.5%. No identity between the isolated sequences and any sequence deposited in the nucleotide sequence databases was detected. The isolated sequences were named Le*Spe*I family.

The organization of the cloned sequences along the genome of *L. elongatus* was analyzed using Southern blot. The probe was labeled and hybridized onto the genomic DNA, previously cleaved with the restriction enzymes *Sac*I, *Mbo*I, *Msp*I and *Spe*I. The results indicated that Le*Spe*I sequence is predominately dispersed and not arranged in tandem arrays in the genome (Fig. 2). Most restriction generated bands were found between 450 and 3,000 bp. A comparative analysis of membrane hybridization using female and male DNAs revealed that bands of 650 and 450 bp were always faint in males when compared to females (Fig. 2). Using higher stringency conditions in the membrane hybridiza-

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LeSpeI1.3 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ATTGGAAAGT AAACGTCACA
LeSpeI2.2 CTCAGTTGGC CTACAAGTTT TGTTCNAGTG CAGTCCAAAC TTCAATGGGC ATTGTGATTC AATAATTTTT .......... ..........
LeSpeI2.6 ---------- ---------- ------TCAT GGTGCAATCA AACGTAATAG GCATGTTATC AATA---TTT .......... ......-...

LeSpeI1.3 TTGAATGAAA ACAAGT-CGA AATGAAATCA GGAGTAA-CC AGCGAACTCT GAACGAACAC GGAGCGAGCG AACGCGGAAC AAACAAGGAG
LeSpeI2.2 .......... ......T... ........-. .......A.. .......... .......... .......... .......... ..........
LeSpeI2.6 .-........ ......-... ........-. .......-.. .......G.. .......... .......... .......... G.G..CC...

LeSpeI1.3 CGAGCGAACA CCGAGCAAGA GAACGTCGGG CGAACACTGA GCGAGCGAAC GCAGAACGAG CACCGAGCGA GCGAACATCG GGCGAACACC
LeSpeI2.2 .......... .......... .......... .......... .......... .......... .......... .......... ..........
LeSpeI2.6 T......G.G TG..A.G..C ACGGAG..A. ...G...C.. ..A.AG.... .TC.GG...A ...T....-- ---------. A......G..

LeSpeI1.3 GAGTGAGCAA GCACCGAGTG AGCGAACGCG AAACGAGCAC CGAGCGAGCG CGCGGCGAAC AAACAAGGAG CGAGCGAGCG CGAAAAAATC
LeSpeI2.2 .......... .......... .......... .......... .......... .......... .......... .......... ..........
LeSpeI2.6 ...G...... .......... .......... G......... .......... A..------- ---------- ---------- .....C....

LeSpeI1.3 AAGGA-GCGA GCGAGCAAGT GCACTA-GCC CACCTCCCCA AAAAAA---C ATTTTTCTAA AAGTCATAAT -GACTGTGAT GTCATAGTAA
LeSpeI2.2 .....-.... .......... ......-... .......... ......---. T......... .......... T......... ..........
LeSpeI2.6 .....-.... .......... ......A... .......... ......AAA. ...C...... .......... -....A.A.. ...G..T...

LeSpeI1.3 AATGTTAAAG TT-GATAAAT TT--GGGATT TGCTAAGTTT CCAG-TTTCA GCCGCCTTTT T-TGCCAATT AAACATGA [445]
LeSpeI2.2 .......... ..T....... ..TG...... ......A... ....G..... ....G.A.A. .A.....N.. ........ [520]
LeSpeI2.6 ....CC..TT AAAC..G..A G.---ATT.. A.A.C.A.AC AA.TT...T. AATAA.CN.. .A.TGTGGNA CT..TG.. [449]
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Fig. 1. Alignment of nucleotide sequences of three cloned *Spe*I repetitive fragments of *Leporinus elongatus*. Dots indicate sequence identity, hyphens represent indels. The Le*Spe*I sequences were deposited in GenBank under the accession numbers EF107659, EF107660, and EF107661.

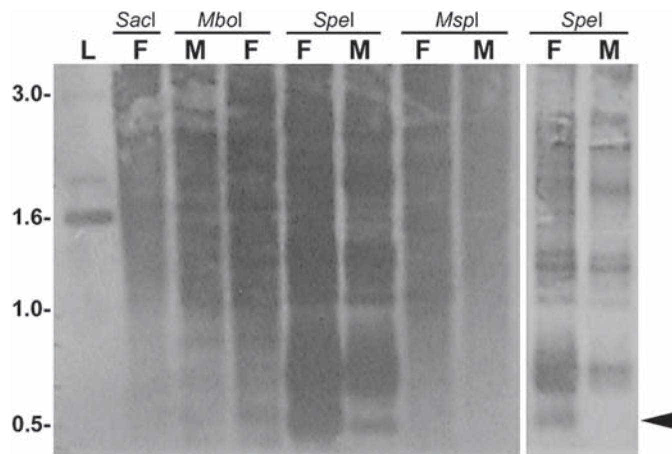

Fig. 2. Southern blot of the genomic DNA samples of *L. elongatus* males (M) and females (F) after digestion with the restriction enzymes *Sac*I, *Mbo*I, *Msp*I and *Spe*I and hybridization to the Le*Spe*I repetitive probe. The lanes on the right digested with *Spe*I were hybridized under conditions of higher stringency. The arrowhead indicates the 450-bp band detected by *Spe*I. L, molecular size marker in kilobases.

tion the 450-bp band disappeared from the male genome (Fig. 2).

The chromosomal distribution of the Le*Spe*I element was determined by FISH in metaphase spreads of *L. elongatus* (Fig. 3). Two strong blocks and one small signal of Le*Spe*I were observed on the long arm of the W chromosome in females. Positive blocks of Le*Spe*I 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 (Z_2W_2). In males, the signal was restricted to the pericentromeric region of both members of pair 2 (Z_2Z_2). 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 W_1 chromosome is entirely heterochromatic (Fig. 3). The supposed Z_2W_2 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 Z_1.

Discussion

The digestion of *L. elongatus* DNA with the enzyme *Spe*I 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 *Spe*I. 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 Le*Spe*I element indicates that Le*Spe*I is a highly repetitive element, predominantly dispersed throughout the genome. Although

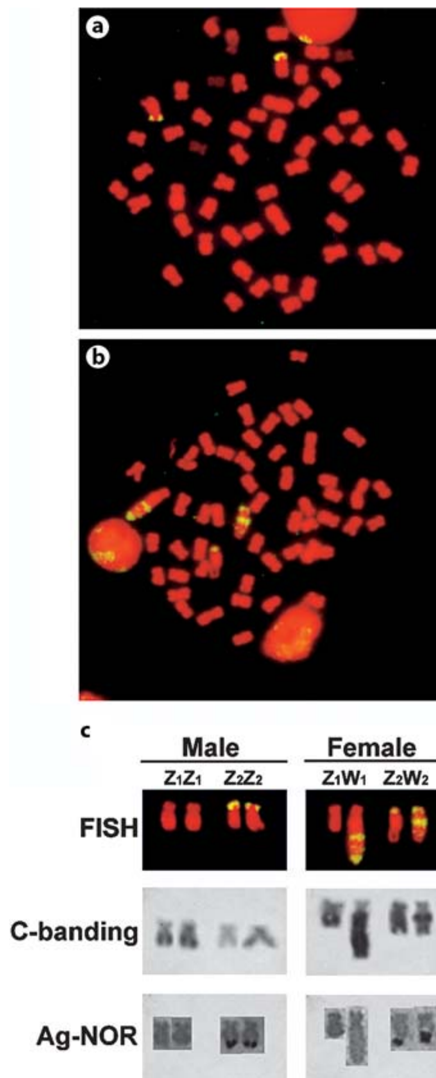

Fig. 3. Fluorescent in situ hybridization of the biotinylated Le*Spe*I repetitive element in the chromosomes of *Leporinus elongatus*. (**a**) Male metaphase spread showing the presence of the Le*Spe*I repetitive family in two chromosomes. (**b**) Female metaphase spread showing the presence of Le*Spe*I in three chromosomes. (**c**) Results from FISH, C-banding and Ag-NOR staining of the respective Z and W chromosomes.

the copy number of Le*Spe*I was not determined in males and females, the membrane hybridization results suggest a higher number of copies of Le*Spe*I in females. This result was confirmed by chromosome hybridization.

The dispersed repetitive pattern of the Le*Spe*I element suggests that this sequence could belong to a dispersed transposable element (TE) class (Oliveira et al., 1999; Bodvarsdottir and Anamthawat-Jonsson, 2003; Ziegler et al., 2003). Although the Southern blot hybridization using Le*Spe*I 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 Le*Spe*I fragments.

Fluorescent in situ hybridization showed that Le*Spe*I 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 Le*Spe*I 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 Le*Spe*I sequences, are homologous, corresponding to the NOR-bearing pair, as previously observed by Galetti et al. (1984). In male metaphases, the Le*Spe*I 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 W_1, identified by its morphology and accumulation of heterochromatin and repetitive sequences, and W_2, identified by the presence of two clusters of Le*Spe*I 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 $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ system. The occurrence of $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ 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 (Z_1Z_2), but the females would be able to produce four types of oocytes: Z_1Z_2, Z_1W_2, Z_2W_1, and W_1W_2. 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 Le*Spe*I 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′5, which hybridized with both Z and W chromosomes and L′46 that was part of a W-specific chromosomal region. These sequences lack any similarity to the Le*Spe*I 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 Le*Spe*I 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 $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ sex chromosome system, where the NOR-bearing pair, formerly defined as pair 2, would represent the pair Z_2, 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

Altschul SF, Madden TL, Schaffer Z, Zhang JH, Zhang Z, et al: Gapped BLAST and PSI-BLAST; a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402 (1997).

Azevedo MFC, Oliveira C, Martins C, Wasko AP, Foresti F: Isolation and characterization of a satellite DNA family in *Achirus lineatus* (Teleostei: Pleuronectiformes: Achiridae). Genetica 125:205–210 (2005).

Bodvarsdottir SK, Anamthawat-Jonsson K: Isolation, characterization and analysis of *Leymus*-specific DNA sequences. Genome 46:673–682 (2003).

Capriglione T, Morescalchi A, Olmo E, Rocco L, Stingo V, Manzo S: Satellite DNAs, heterochromatin and sex chromosomes in *Chionodraco hamatus* (Channichthyidae, Perciformes). Polar Biol 14:285–290 (1994).

Centofante L, Bertollo LAC, Moreira-Filho O: A ZZ/ZW sex chromosome system in a new species of the genus *Parodon* (Parodontidae). Caryologia 55:139–150 (2002).

Charlesworth B: The evolution of sex chromosomes. Science 251:1030–1033 (1991).

Devlin RH, Nagahama Y: Sex determination and sex differentiation in fish: an overview of genetic, physiological and environmental influences. Aquaculture 208:191–364 (2002).

Foresti F, Oliveira C, Almeida-Toledo LF: A method for chromosome preparations from large specimens of fishes using in vitro short treatment with colchicines. Experientia 49:810–813 (1993).

Galetti PM, Foresti F: Evolution of the ZZ/ZW-system in *Leporinus* (Pisces, Anostomidae). The role of constitutive heterochromatin. Cytogenet Cell Genet 43:43–46 (1986).

Galetti PM Jr, Foresti F, Bertollo LAC, Moreira Filho O: Heteromorphic sex chromosomes in three species of the genus *Leporinus* (Pisces, Anostomidae). Cytogenet Cell Genet 29:138–142 (1981).

Galetti PM, Foresti F, Bertollo LAC, Moreira-Filho O: Characterization of eight species of Anostomidae (Cypriniformes) fish on the basis of nucleolar organizer region. Caryologia 37:401–406 (1984).

Galetti PM Jr, Lima NRW, Venere PC: A monophyletic ZW sex chromosome system in *Leporinus* (Anostomidae, Characiformes). Cytologia 60: 375–382 (1995).

Garavello JC, Britski HA: Family Anostomidae; in Reis RS, Kullander CJ, Ferrearis Jr (Org.): Check List of the Freshwater Fishes of South and Central America, 1st ed., Vol 1, pp 71–84 (Editora da PUCRS, Porto Alegre 2003).

Hornaday K, Alexander S, Breden F: Absence of repetitive DNA sequences associated with sex chromosomes in natural populations of the Trinidad guppy (*Poecilia reticulata*). J Mol Evol 39:431–433 (1994).

Jesus CM, Galetti PM, Valentini SR, Moreira-Filho O: Molecular characterization and chromosomal localization of two families of satellite DNA in *Prochilodus lineatus* (Pisces, Prochilodontidae), a species with B chromosomes. Genetics 118:25–32 (2003).

Kumar S, Tamura K, Nei M: MEGA 3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5:150–163 (2004).

Lloyd MA, Fields MJ, Thorgaard GH: Bkm minisatellite sequences are not sex associated but reveal DNA fingerprint polymorphisms in rainbow trout. Genome 32:865–868 (1989).

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).

Moran P, Martinez JL, Garcia-Vazquez E, Pendas AM: Sex chromosome linkage of 5S rDNA in rainbow trout (*Oncorhynchus mykiss*). Cytogenet Cell Genet 75:145–150 (1996).

Nakayama I, Foresti F, Tewari R, Schartl M, Chourrout D: Sex chromosome polymorphism and heterogametic males revealed by two cloned DNA probes in the ZW/ZZ fish *Leporinus elongatus*. Chromosoma 103:31–39 (1994).
Nanda I, Feichtinger W, Schmid M, Schroder JH, Zischler H, Epplen JC: Simple repetitive sequences are associated with differentiation of the sex chromosomes in the guppy fish. J Mol Evol 30:456–462 (1990).
Nanda I, Schartl M, Feichtinger W, Epplen JT, Schmid M: Early stages of sex chromosome differentiation in fish as analysed by simple repetitive DNA sequences. Chromosoma 101:301–310 (1992).
Oliveira C, Chew JSK, Porto-Foresti F, Dobson M, Wright JMA: LINE2 repetitive DNA sequence from the cichlid fish, *Oreochromis niloticus*: sequence analysis and chromosomal distribution. Chromosoma 108:457–468 (1999).
Reed KM, Phillips RB: Molecular characterization and cytogenetic analysis of highly repeated DNAs of lake trout, *Salvelinus namaycush*. Chromosoma 104:242–251 (1995).
Sambrook J, Russel DW: Molecular Cloning. A Laboratory Manual. Third Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor 2001).
Tafalla C, Estepa A, Coll JM: Fish transposons and their potential use in aquaculture. J Biotech 123:397–412 (2006).
Thompson JD, Gibson TJ, Plewniak F, Higgins DG: The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25: 4876–4882 (1997).
Venere PC, Ferreira IA, Martins C, Galetti PM Jr: A novel ZZ/ZW sex chromosome system for the genus *Leporinus* (Pisces, Anostomidae, Characiformes). Genetica 121:75–80 (2004).
Wachtel S, Demas S, Tiersch T, Pechan P, Shapiro D: Bkm satellite DNA and *ZFY* in the coral reef fish *Anthias squamipinnis*. Genome 34:612–617 (1991).
Ziegler CG, Lamatsch DK, Steinlein C, Engel W, Schartl M, Schmid M: The giant B chromosome of the cyprinid fish *Alburnus alburnus* harbours a retrotransposon-derived repetitive DNA sequence. Chromosome Res 11:23–35 (2003).