RESEARCH PAPER

Mobile Genetic Elements 3:6, e27460; November/December 2013; © 2013 Landes Bioscience

Chromosome mapping of retrotransposable elements *Rex1* and *Rex3* in *Leporinus* Spix, 1829 species (Characiformes: Anostomidae) and its relationships among heterochromatic segments and W sex chromosome

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Keywords: transposable elements, mobile DNA, repetitive sequences, sex chromosome, FISH

The family Anostomidae is an interesting model for studies of repetitive elements, mainly because of the presence of high numbers of heterochromatic segments related to a peculiar system of female heterogamety, which is restricted to a few species of *Leporinus* genus. Thus, cytogenetic mapping of the retrotransposable elements *Rex1, Rex3,* and *Rex6* was performed in six *Leporinus* species, to elucidate the genomic organization of this genus. The sequencing of the *Rex1* and *Rex3* elements detected different base pair compositions in these elements among species, whereas the *Rex6* element was not identified in the genomes of these species. FISH analysis using *Rex1* detected different distribution patterns, *L. elongatus, L. macrocephalus,* and *L. obtusidens* had clusters in the terminal regions, whereas the signals were dispersed throughout all of the chromosomes with some signals in the terminal position in other species. The *Rex3* signals were found mainly in the terminal positions in all the chromosomes of all species. The W chromosomes of *L. elongatus*, *L. macrocephalus*, and *L. obtusidens* contained the Rex1 and *Rex3* signal in an interstitial position. These results suggest the emergence of different activity levels for these elements during the evolution of the species analyzed. Despite the conserved karyotype macrostructure species *Leporinus* often discussed, our results show some variation in hybridization patterns, particularly between the species with specific patterns in their sex chromosomes and species without this differentiated system.

Introduction

Large portions of eukaryote genomes comprise repetitive DNA sequences. These sequences may be related to specific functions and structures, such as rDNA synthesis, segregation of centromeric regions, and the protection of telomeric regions. These sequences may have been produced by changes in the genome, which affected evolutionary trajectories and led to the production of regulatory and coding segments.[1,2] Repetitive sequences can be organized into two classes: tandem repeats, such as satellites, minisatellites, and microsatellites; and repeats dispersed throughout the genome as transposable elements.[3] Transposable elements can move around the genome in two ways: sequence excision mediated by a transposase enzyme (transposons) or via intermediate RNA produced by reverse transcriptase (retrotransposons).[4]

Transposable elements have important roles in genomic diversity and evolution,[5] and these elements may affect the evolutionary trajectory of the host organism in different ways, such as causing changes in gene functions via insertions and inducing chromosome rearrangements.[6] From a cytogenetic perspective, these elements may accumulate in specific sites in chromosomes, such as terminal, pericentromeric, and other heterochromatic regions where the recombination rate is reduced,[7] or in small euchromatic segments, such as those found in *Drosophila melanogaster* chromosomes.[8] Thus, these elements may be associated with mechanisms that lead to speciation events,[4] which confirms that these elements have very important roles in the genomic and biological diversity of vertebrates.

The retrotransposable elements in the *Rex* family have been studied widely in some fish groups, including Cichlidae,[9-14] Tetraodontidae,[15-17] Nototheniidae, Artedidraconidae,

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Submitted: 10/02/2013; Revised: 11/28/2013; Accepted: 12/08/2013
Citation: de Borba RS, da Silva EL, Parise-Maltempi PP. Chromosome mapping of retrotransposable elements *Rex1* and *Rex3* in *Leporinus* Spix, 1829 species (Characiformes: Anostomidae) and its relationships among heterochromatic segments and W sex chromosome. Mobile Genetic Elements 2013; 3:e27460; http://dx.doi.org/10.4161/mge.27460

Table 1. Base pare number and similarity levels of *Rex1* and *Rex3* in *Leporinus* species

Specie	Retrotransposable element	Base pair	Similarity level
L. elongatus	*Rex1*	498	79%, *Pterophyllum scalare* (JX576338.1)
	Rex3	411	88%, *Dissostichus mawsoni* (AY331110.1)
L. friderici	*Rex1*	482	82%, *Pterophyllum scalare* (JX576338.1)
	Rex3	429	83%, *Cichla monoculus* (KF131684.1)
L. lacustris	*Rex1*	483	82%, *Pterophyllum scalare* (JX576338.1)
	Rex3	426	88%, *Dissostichus mawsoni* (AY331110.1)
L. obtusidens	*Rex1*	477	82%, *Pterophyllum scalare* (JX576338.1)
	Rex3	426	88%, *Notothenia coriiceps* (AY331103.1)
L. macrocephalus	*Rex1*	500	80%, *Pterophyllum scalare* (JX576338.1)
	Rex3	429	88%, *Dissostichus mawsoni* (AY331110.1)
L. striatus	*Rex1*	456	83%, *Pterophyllum scalare* (JX576338.1)
	Rex3	426	88%, *Dissostichus mawsoni* (AY331110.1)

Bathydraconidae, Bovichtidae,[18] and Loricariidae.[19] The *Rex* family elements have a high diversity of chromosome locations in previously studied fish species, and it has been hypothesized that they may have roles in genome differentiation and evolution.[9-17,20] However, the characteristics and dynamics of these fish retroelements, which occur mainly in the order Characiformes (a large fish order), still need to be elucidated.

The family Anostomidae is considered to be an interesting model for studies of repetitive elements. Cytogenetic studies of several species in this family have detected a conserved karyotypic structure in the 41 species that have already been karyotyped. The diploid number of 54 comprises metacentric and submetacentric chromosomes, and the karyotypes of most species carry a single NOR bearing pair.[21-24] The genus *Leporinus* has been well studied and 25 of the 87 species in this genus have been karyotyped. The presence of a peculiar sex chromosome system with female heterogamety is restricted to a few species in this genus, i.e., *L. elongatus*, *L. macrocephalus, L. obtusidens, L. reinhardtii, L. silvetrii, L. conirostris,* and *L. trifasciatus*.[25] Recent studies of *Leporinus* have isolated, characterized, and correlated repetitive elements in these species to study the evolution of sex chromosomes, as well as to discriminate hybrid species.[26-32] Other studies have shown that repetitive sequences such as 5S rDNA may have different distributions among *Leporinus* species with or without heteromorphic sex chromosomes.[33]

The high heterochromatic content in the chromosomes of *Leporinus* species, especially the sex chromosomes, demonstrates the presence of high numbers of repetitive sequences in the genomes of these species.[28-32,34] In the present study, cytogenetic mapping of the retrotransposable elements *Rex1*, *Rex3,* and *Rex6* was performed in the chromosomes of *Leporinus* species to elucidate the activity and dispersion levels of these elements in genome of this genus. This analysis determined whether these elements are related to the heterochromatic regions of autosomes and sex chromosomes and whether their distributions are compatible with the hypothetical maintenance of a stable macrostructure in the karyotype of this genus.

Results

Isolation of *Rex1*, *Rex3*, and *Rex6* elements, and sequence analysis

The amplification and sequencing of *Rex1* and *Rex3* elements detected sequences with different base pair numbers among *Leporinus* species. The sequence alignment detected a high similarity among the sequences that corresponded to the *Rex1* element, where the scores ranged from 97.93% to 100.0%. However, the *Rex3* sequences were more variable among species, with scores ranging from 48.12% to 100.0%. The similarity levels of *Rex1* with others sequences in Gen Bank were 79–83% and *Rex3* were 83–88%. The amplified segments corresponded to the coding domains of the reverse transcriptase gene. The base pair numbers and similarity levels for each species are shown in **Table 1**.

Sequencing of the amplified fragments generated using the *Rex6* primer set in different PCR conditions, showed that these fragments did not correspond to the *Rex6* element. The sequences contained 1019 base pairs and their maximum shared identity was with fragments of a chromosome 13 clone from *Mus musculus* (accession number: AC034285.6).

Chromosome mapping of the *Rex1* element

Fluorescent in situ hybridization analysis (FISH) using the PCR fragments of the *Rex1* element as a probe detected different mapping patterns in the study species. *L. elongatus* (**Fig. 1A**), *L. macrocephalus* (**Fig. 1B**), and *L. obtusidens* (**Fig. 1C**) had isolated clusters in the terminal regions of most chromosomes, although the W chromosome contained signals in the interstitial region of the long arm. *L. elongatus* had an interstitial cluster in the W1 chromosome (**Fig. 1A**). In *L. friderici* (**Fig. 1D**), *L. lacustris* (**Fig. 1E**), and *L. striatus* (**Fig. 1F**), the majority of the clusters

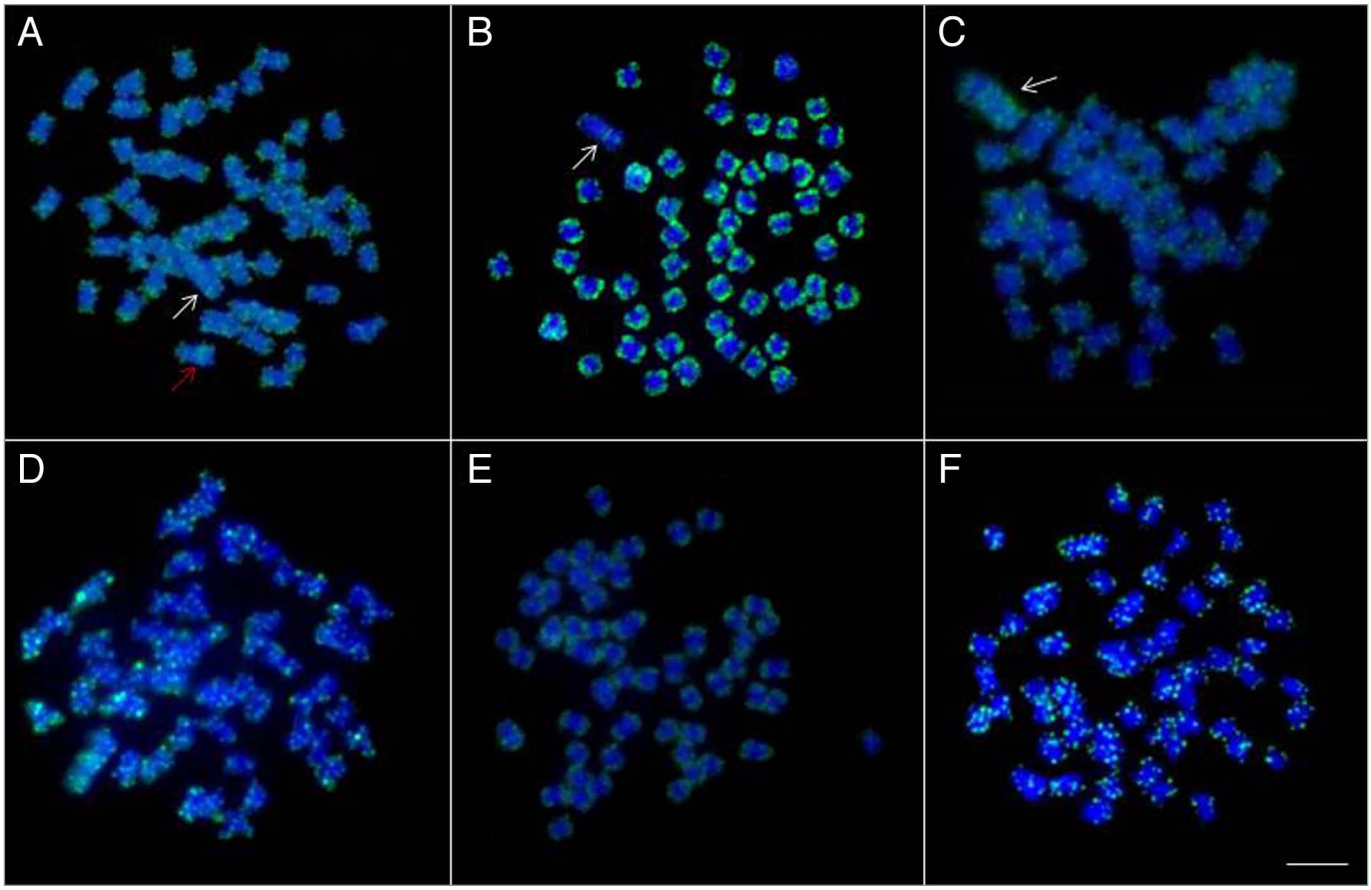

Figure 1. Chromosome mapping of *Rex1* element in metaphases of female specimens of (**A**) *Leporinus elongatus*, (**B**) *L. macrocephalus*, (**C**) *L. obtusidens*, (**D**) *L. friderici*, (**E**) *L. lacustris*, and (**F**) *L. striatus*. The arrows indicate the W chromosome, red arrow indicate W1 chromosome. Scale bar: 10 μm.

were dispersed throughout all of the chromosomes and some of the signals were in terminal positions in male and female specimens.

Chromosome mapping of the *Rex3* element

The results obtained using the PCR fragments of *Rex3* as a probe detected the same mapping pattern in all species (**Fig. 2**). The clusters were isolated in the terminal regions of all chromosomes and some clusters were dispersed in the short and long arms of male and female specimens. In *L. elongatus*, *L. macrocephalus,* and *L. obtusidens,* the W chromosome had signals in terminal positions and the interstitial regions of the long arms.

Discussion

The analysis of *Rex1* and *Rex3* generated sequences of 456–500 base pairs and 411–429 base pairs, respectively, which were similar to these sequences in other fish groups from the Antarctic,[18] and cichlid fishes.[10] This was confirmed by the BLASTn analysis where the *Rex1* and *Rex3* sequences shared high similarity with those of other fish groups, which demonstrates that these elements are fairly well conserved in different families. Previous researchers have suggested that the *Rex6* element may have been lost or diverged greatly, depending on the rate of host evolution. The loss of Rex6 elements has been reported in Antarctic fishes,[18] and may have occurred in *Leporinus* species because none were identified in the species analyzed in the present study.

The chromosomal mapping of retrotransposable elements in fish has demonstrated their remarkable diversity.[20] In the present study, the *Leporinus* species had different hybridization signals with dispersed clusters and signals in the terminal regions. These patterns were similar to those observed in the Bryconinae,[35] Erythrinidae,[36] Sternopygidae,[37] Artedidraconidae,[18] Bathydraconidae,[18] Bovichtidae,[18] Channichthyidae,[18] Nototheniidae,[18] Loricariidae,[19] Pimelodidae,[38] and Tetraodontidae.[17] Species in the family Cichlidae are exceptional because the *Rex* elements are frequently distributed in the pericentromeric regions.[9-14]

In particular, *Rex1* had different chromosome locations in *Leporinus* species. *Leporinus elongatus, L. macrocephalus,* and *L. obtusidens* had isolated signals in terminal positions in all chromosomes. In other species, there was hybridization with clusters in the short and long arms of all chromosomes. It is known that the distribution patterns of transposable elements are nonrandom and are related to the specific characteristics of different sites in the genome.[5] Specific distribution patterns have also been observed in *Drosophila*, where some euchromatic regions have been replaced by retrotransposable elements.[39] Furthermore, a correlation between chromosome rearrangement

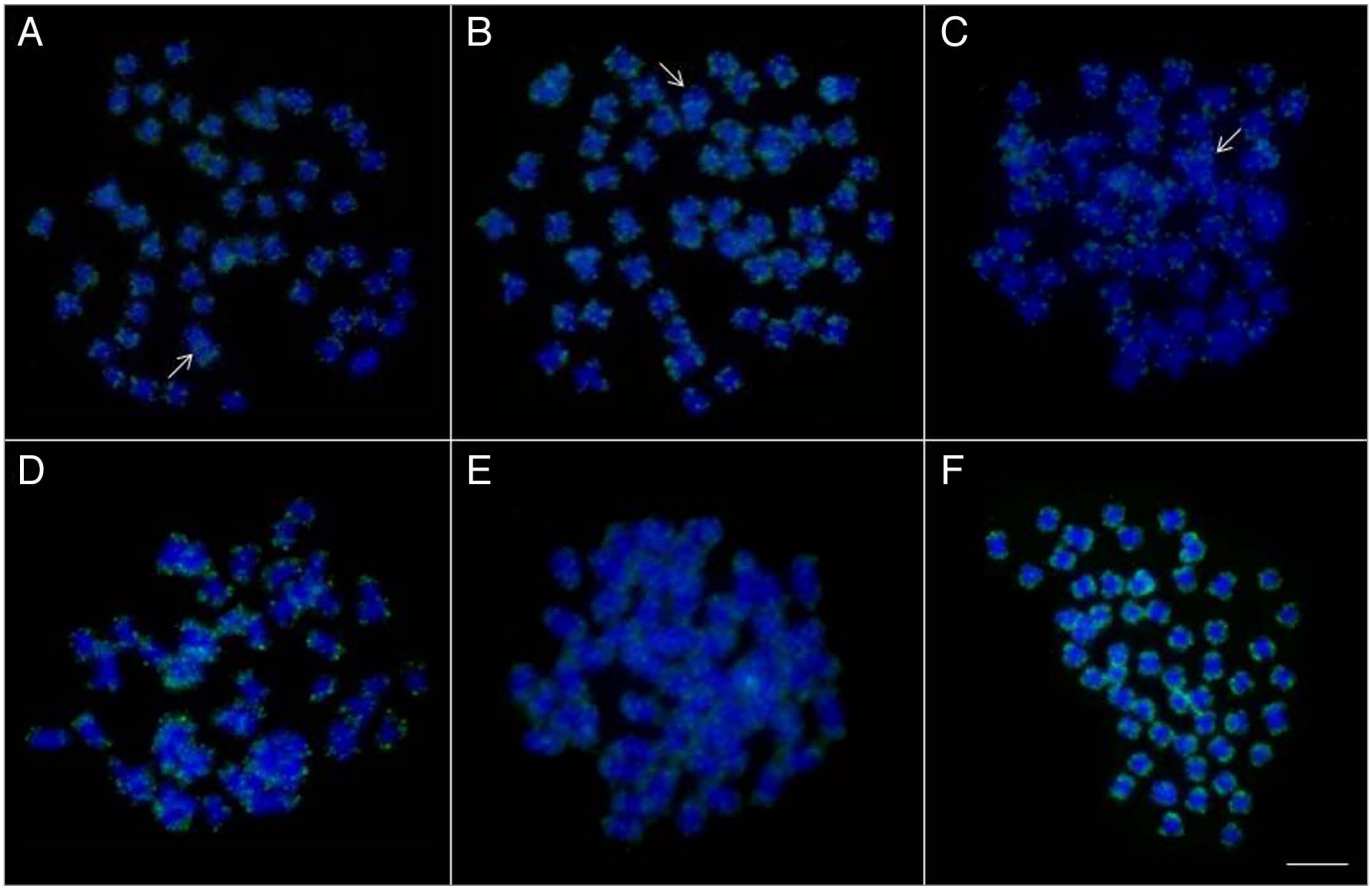

Figure 2. Chromosome mapping of *Rex3* element in metaphases of female specimens of (**A**) *Leporinus elongatus*, (**B**) *L. macrocephalus*, (**C**) *L. obtusidens*, (**D**) *L. friderici*, (**E**) *L. lacustris*, and (**F**) *L. striatus*. The arrows indicate the W chromosome. Scale bar: 10 μm.

and retrotransposon activity was reported by Ozouf-Costaz et al.[18] in notothenioid species. Thus, the differences in the *Rex1* hybridization sites in *Leporinus* species may be related to small rearrangements in specific regions of their genomes.

The distributions of *Rex3* elements were similar to those of *Rex1* in *L. elongatus*, *L. macrocephalus,* and *L. obtusidens*. These similar patterns suggest the compartmentalization of sequences in the terminal positions of the chromosomes in these species. Thus, is it likely that similar evolutionary mechanisms account for the distribution of these elements in *Leporinus* species and those in other fish families, such as Cichlidae,[10,11] and Nototheniidae.[18] In *L. friderici*, *L. lacustris*, *L. obtusidens,* and *L. striatus,* the *Rex3* elements were present as isolated signals in terminal positions in the short and long arms of chromosomes.

Many studies of Anostomidae species have highlighted the correlations between heterochromatic regions in chromosomes and repetitive sequences.[28,30-32] Many repetitive sequences have been isolated from heterochromatic segments, mainly in *Leporinus* species, such as those present in the W chromosome, pericentromeric positions, and NOR regions.[26-32] In the present study, retrotransposable elements in the *Rex* family were also abundant in other heterochromatic regions in *Leporinus* chromosomes, such as terminal positions and some interstitial segments. This distribution reflects the pattern expected for transposable elements distribution described by Kidwell,[7] related to a low rate of recombination. *L. friderici* and *L. striatus* possessed a few signals in the euchromatic regions of chromosomes. Other studies have suggested that transposable elements can accumulate in euchromatic segments, such as those found in *Drosophila melanogaster* where 3.8% of the euchromatic genome comprises transposable elements.[40]

The presence of specific signals for retrotransposable elements in sex chromosomes has been poorly reported in fish. Ozouf-Costaz et al.[18] identified *Rex3* element hybridization signals in an interstitial position in the short arm of the Y-chromosome of *Chionodraco hamatus* (Channichthyidae). The interstitial markers in this chromosome may indicate the activity of fusion rearrangement mechanisms during the differentiation of this family.[41] The interesting localizations of *Rex1* and *Rex3* elements in the W chromosomes of *Leporinus elongatus*, *L. macrocephalus,* and *L. obtusidens* may be related to the suppression of recombination during the sex chromosome differentiation process, due mainly to an increase in heterochromatic segments. Suppression is a critical component of this process and there is a natural bias toward the accumulation of repetitive elements.[3]

An increase in heterochromatic segments because of the differentiation of W chromosomes in *Leporinus* was proposed by Galetti and Foresti.[42] These researchers suggested that

the heterochromatin concentration had a specific role in the differentiation of sex chromosomes, thereby indicating a common origin of the chromosomal sex system found in different species of *Leporinus*. Recent studies have supported a hypothesis based on repetitive sequences related to the W chromosome,[28,30-32] where these sequences are related strictly to species with W chromosomes. Another study demonstrated synteny among the W chromosomes of *Leporinus* species, which supports the hypothesis of a common origin for this chromosome.[43] In the present study, the similar distribution patterns of the *Rex1* and *Rex3* elements in the sex chromosomes of three *Leporinus* species, i.e., *L. elongatus*, *L. macrocephalus,* and *L. obtusidens,* also supports the hypothesis of a common origin of the W chromosome in *Leporinus* species.

The karyotypic macrostructures of *Leporinus* species have been studied many times and a fairly high level of conservation has been reported,[25,27,34,44] but our results show variations in the hybridization signal patterns, particularly between species with sex chromosomes and species without this differentiated system. The retroelements analyzed in the present study reflected the same patterns observed in other repetitive elements,[28,30] reinforced that heterochromatic regions of the sex chromosomes had distinct differences among autosomes of the *Leporinus* species.

Material and Methods

Material

Fish material, chromosome preparation, and DNA extraction

Six *Leporinus* species were analyzed in this study: *L. elongatus* (Valenciennes, 1849) from Centro Nacional de Pesquisa e Conservação de Peixes Continentais (CEPTA), Pirassununga, São Paulo state; *L. obtusidens* (Valenciennes, 1847) from Leme fish farm, São Paulo state; and *L. macrocephalus* (Garavello and Britiski, 1988), *L. striatus* (Kner, 1858), *L. lacustris* (Amaral Campos, 1945), and *L. friderici* (Bloch, 1794) were collected from the Paraguay River basin, Mato Grosso State-Brazil. In total, 12 specimens were analyzed (one male and one female of each species). The chromosomal preparations were obtained using kidney cells, as described by Foresti et al.[45] Genomic DNA was extracted from the liver using the phenol-chloroform-isoamyl alcohol technique.[46] The specimens were collected in accordance with collection license issued by Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (19833-1), and the material was processed according to Colégio Brasileiro de Experimentação Animal (016/04—CEEA).

Methods

Isolation of Rex1, Rex3, *and* Rex6 *elements*

PCR of the *Rex1, Rex3,* and *Rex6* elements used a reaction mixture with a volume of 13.5 μL, which contained 6.25 μL of PCR Mix (Qiagen), 5.25 μL of Milli-Q water, 0.5 μL primer F (10 μM), 0.5 μL of primer R (10 μM), and 1.0 μL of template DNA (200 ng). PCR was performed in a thermocycler (Eppendorf Mastercycler) with the following cycling conditions: initial denaturation at 95 °C for 5 min, followed by 34 cycles at 95 °C for 40 s, annealing at 55 °C for 40 s, and chain elongation at 72 °C for 5 min, with a final extension at 72 °C for 5 min.

Specific primers were designed based on the partial sequences of *Rex1* in *L. elongatus,* which were obtained using the primer set *Rex1*F—5′-TTCTCCAGTG CCTTCAACACC and *Rex1*R—5′-TCCCTCAGCA GAAAGAGTCT GCTC.[47,48] This set was used in the PCR reaction and was subsequently sequenced by MacroGen, Korea. The sequence was used to design a specific primer for *Leporinus* with the tool OligoPerfect™ Designer (http://tools.invitrogen.com), which produced the following sequences: *Rex1*EL—F 5′-AGCAAGCTAG AGAGTGCTGG and *Rex1*EL—R 5′-ACAGAGCGTG TGTGTTGTCC. *Rex3* and *Rex6* amplification used the following primer sets: *Rex3*F—5′-CGGTGAYAAA GGGCAGCCCTG and *Rex3*R—5′-TGGCAGACNG GGGTGGTGGT, and *Rex6*F—5′-TAAAGCATAC ATGGAGCGCC AC and *Rex6*R—5′-GGTCCTCTAC CAGAGGCCTG GG.[47,48]

Cloning, sequencing, and sequence analysis

The amplified DNA was cloned with a Pgem-T kit (Promega) and used to transform competent *Escherichia coli* (Promega, Jm109) cells. Clones containing the digested DNA fragments were stored before nucleotide sequencing and used as probes in the chromosomal FISH experiments. The DNA fragments were purified using ExoSAP-IT enzyme and sequenced (MacroGen Inc). The sequences were edited using the Pregep4 program, aligned with CLUSTALW,[49] in DAMBE5,[50] and analyzed with the BLASTn tool,[51] via the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast) to determine the similarities among sequences.

FISH and chromosome analysis

The FISH analyses were based on the method of Pinkel et al.[52] with several modifications proposed by Silva et al.[31] where the retrotransposable elements *Rex1* and *Rex3* were used as the probes. The probes were labeled via nick translation with biotin-14-dATP (Invitrogen) to facilitate chromosomal mapping.

The hybridization signals were detected using appropriate antibody sets based on anti-avidin, which was followed by the application of avidin-FITC to enhance the signals of the biotin-labeled probes. The chromosomes were counterstained with DAPI, mounted with antifade solution, and observed using an Olympus BX51 microscope coupled to an Olympus digital camera (model D71). The chromosome images were captured using the DP Controller program.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors are grateful to Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP-2012/01437-0) for financial support, and to Bioedit® for helping with the English language. They also thank to Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) for providing the collection license (19833-1).

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