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ORIGINAL PAPER

5S rDNA variation and its phylogenetic inference in the genus *Leporinus* (Characiformes: Anostomidae)

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Abstract 5S rDNA sequences have proven to be valuable as genetic markers to distinguish closely related species and also in the understanding of the dynamic of repetitive sequences in the genomes. In the aim to contribute to the knowledge of the evolutionary history of *Leporinus* (Anostomidae) and also to contribute to the understanding of the 5S rDNA sequences organization in the fish genome, analyses of 5S rDNA sequences were conducted in seven species of this genus. The 5S rRNA gene sequence was highly conserved among *Leporinus* species, whereas NTS exhibit high levels of variations related to insertions, deletions, microrepeats, and base substitutions. The phylogenetic analysis of the 5S rDNA sequences clustered the species into two clades that are in agreement with cytogenetic and morphological data.

Keywords Fish · *Leporinus* · NTS · Phylogeny · 5S rDNA

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Introduction

The family Anostomidae is distributed from Central to South America and comprehends 138 described species distributed in 12 genera (*Abramites*, *Anostomoides*, *Anostomus*, *Gnathodolus*, *Laemolyta*, *Leporellus*, *Leporinus*, *Pseudanos*, *Rhytiodus*, *Sartor*, *Schizodon* and *Synaptolaemus*), (Garavello and Britski 2003). Some species of the genera *Leporinus* and *Schizodon* are exploited in commercial and subsistence fisheries as an important food item (Garavello and Bristski 2003). Furthermore, relatively small colored species have been appreciated for aquarium activities.

Among anostomid genera, *Leporinus* contains the highest number of species (87 valid species) (Garavello and Britski 2003). All species cytogenetically studied have 54 biarmed chromosomes and most of the species has only one chromosome pair that harbors the nucleolar organizer regions (NORs). In spite of the conserved chromosome formulae for the genus, there is a conspicuous ZZ/ZW sex chromosome system that was described for seven species (*L. conirostris*, *L. elongatus*, *L.* aff. *elongatus*, *L. macrocephalus*, *L. obtusidens*, *L. reinhardti* and *L. trifasciatus*), while the remaining species of the genus that have been cytogenetically studied (around 40 species) have no differentiated sex chromosomes (Galetti et al. 1995). On the other hand, a novel ZW sex chromosome system, morphologically differentiated from the typical ZW system previously detected for the seven species, was described for *Leporinus* sp. (Venere et al. 2004).

Previous data suggest that 5S rDNA sequences might be of considerable value as genetic markers for identification of species, subspecies, populations, strains, and hybrids in fishes, specially farmed trout and salmon

(Pendás et al. 1995; Carrera et al. 2000). The multigene family that codes for the 5S rRNA consists of a highly conserved coding sequence of 120 base pair (bp), which is separated from each transcriptional unit by a variable non-transcribed spacer (NTS). Variations in the NTS owing to insertions/deletions, minirepeats and pseudogenes have been frequently characterized in several organisms, and these variations might represent valuable markers for population and/or species characterization (Martins and Wasko 2004). In the characiform *Leporinus*, two classes of 5S rDNA, one consisting of monomeric repeat units around 200 bp (designated 5S rDNA type I) and another one with monomers of 920 bp (designated 5S rDNA type II) were identified (Martins and Galetti 2001). Each of these 5S rDNA classes is characterized by distinct NTS sequences and was clustered in distinct chromosome pairs.

The present study examines the variation of the 5S rDNA type I sequences from seven species of the genus *Leporinus*. The 5S rDNA type I was chosen for the present analysis since it can be easily amplified by PCR, cloned and sequenced. The obtained results suggest that 5S rDNA sequences are valuable molecular markers to access the evolutionary history among closely related species.

Materials and methods

Eight species, including seven *Leporinus* (Anostomidae) and one *Steindachnerina* (Curimatidae), included as outgroup, were analyzed (Table 1). DNA was extracted from liver and fins (Wasko et al. 2003), and PCR amplifications of the 5S rDNA were carried out according to Martins and Galetti (2001) using the primers 5SA (5′TAC GCC CGA TCT CGT CCG ATC3′) and 5SB (5′CAG GCT GGT ATG GCC GTA AGC3′).

Table 1 Species analyzed

Species	Collection sites
Leporinus elongatus	Mogi-Guaçu river, Pirassununga, São Paulo, Brazil
Leporinus obtusidens	Mogi-Guaçu river, Pirassununga, São Paulo, Brazil
Leporinus friderici	Mogi-Guaçu river, Pirassununga, São Paulo, Brazil
Leporinus aff. *elongatus*	São Francisco river, Três Marias, Minas Gerais, Brazil
Leporinus macrocephalus	Paraguai river, Coxim, Mato Grosso do Sul, Brazil
Leporinus octofasciatus	Paranapanema river, Itatinga, São Paulo, Brazil
Leporinus sp.	Araguaia river, Barra do Garças, Mato Grosso, Brazil
Steindachnerina insculpta	Paranapanema river, Itatinga, São Paulo, Brazil

The PCR-generated 5S rDNA fragments were cloned in the plasmids pGEM-T (Promega), and used to transform a host *E. coli* strain DH5α. The clones obtained from the 5S rDNA PCR products were sequenced on the ABI Prism 377 (Perking-Elmer) automatic sequencer with a Dye Terminator Cycle Sequencing kit (Applied Biosystems Division, Perkin-Elmer), following the manufacturer instructions. Nucleic acid sequences were subjected to BLASTN (Altschul et al. 1990) searches at the National Center for Biotechnology Information (NCBI), through web site (http://www.ncbi.nlm.nih.gov/blast). The sequences were aligned with the software Clustal W (Thompson et al. 1994) as implemented in the program DAMBE (Xia and Xie 2001). Consensus sequences were produced manually in the software BioEdit (Hall 1999).

Maximum-parsimony (MP) based phylogenetic analyses were performed using the software PAUP* beta version 4.0b10 (Swofford 2002) with heuristic searches using random addition of sequences and the tree bisection and reconnection (TBR) algorithm. In all analysis the character-state optimization method employed was the accelerated transformation (ACCTRAN). Parsimony trees were generated using 1:1 transition (Ti)/transversion (Tv) ratio, considering gaps as either missing data or a fifth base. Bootstrap resampling (Felsenstein 1985) was applied to assess support for individual nodes using 10,000 replicates with 100,000 random additions and TBR branch swapping. Decay indexes (Bremer 1988) were calculated with SEPAL (Salisbury 2001). Maximum-likelihood (ML) based phylogenetic relationships were estimated using the software PAUP* beta version 4.0b10 (Swofford 2002). The genetic distance among sequences was estimated by Jukes–Cantor model (Jukes and Cantor 1969) incorporating rate variation (Γ) based on a hierarchical hypothesis test of alternative models implemented with Modeltest 3.7 (Posada and Crandall 1998). The Ti/Tv ratio, gamma shape parameter, and proportion of non-variant sites were estimated by maximum likelihood from a maximum parsimony tree. Gaps were considered as missing data. Bootstrap resampling was applied to assess support for individual nodes using 1000 replicates with 10,000 random additions and TBR branch swapping.

Results and discussion

PCR amplification of the 5S rDNA type I produced approximately 200 bp fragments for all *Leporinus*

species and 180 bp for *S. insculpta*. These PCR products were cloned, and several clones were sequenced for each species (Table 2). The sequences were deposited in the NCBI database under the accession numbers AF284728–AF284746 and DQ009524–DQ009532.

Analysis of the 5S rDNA sequences obtained results in the identification of one unit of the 5S rDNA tandem array (5S rRNA gene + NTS). The coding region of the 5S rRNA gene was quite conserved showing few base substitutions among the species. On the other hand, significant differences related to base substitutions and insertions/deletions were detected in the NTS regions (Fig. 1). The mean genetic distance among the 5S rDNA sequences of the *Leporinus* species was 0.133 ± 0.021, mainly owing to base substitutions and insertions/deletion in the NTS (Table 2). The NTS has proven to be very dynamic regions of the genome, because they are free to mutate and the variant forms that arise are neutral (or almost neutral) to natural selection, and can be fixed or lost, causing differences among related species and even into the same individual (Cronn et al. 1996). The high dynamism of the NTS can be visualized by the presence of a TA microsatellite in *L. macrocephaus* (Fig. 1), which might represent a potential genetic marker that can be explored and applied in the genetic analyses of this species. The high genetic variation of the NTS supports its use as a genetic marker for studies of populations that have experienced recent events of evolution.

Table 2 Genetic distances between 5S rDNA type I sequences of the species analyzed

Species	Clones	GenBank no.	Length of 5S rDNA sequence (bp)	Mean genetic distance
Leporinus elongatus	Le67.3	AF284728	201	0.030 ± 0.011
	Le67.5	AF284729	201	
	Le67.6	AF284730	201	
	Le67.9	AF284731	202	
Leporinus obtusidens	Lo52.1	AF284732	202	0.034 ± 0.010
	Lo52.2	AF284733	202	
	Lo52.5	AF284734	202	
	Lo52.6	AF284735	199	
	Lo52.7	AF284736	201	
Leporinus friderici	Lf152.1	AF284743	220	0.087 ± 0.017
	Lf152.4	AF284744	220	
	Lf152.5	AF284745	220	
	Lf153.3	AF284746	219	
Leporinus aff. *elongatus*	Lte61.5	AF284739	199	0.023 ± 0.007
	Lte61.6	AF284740	199	
	Lte61.7	AF284741	200	
	Lte61.8	AF284742	200	
	Lte181.1	AF284737	200	
	Lte186.8	AF284738	200	
Leporinus macrocephalus	Lm4	DQ009524	213	0.010 ± 0.006
	Lm5	DQ009525	214	
	Lm10	DQ009526	206	
Leporinus octofasciatus	Loc7	DQ009527	220	0.065 ± 0.016
	Loc8	DQ009528	219	
	Loc10	DQ009529	219	
Leporinus sp.	Lsp8	DQ009530	219	0.000 ± 0.000
	Lsp9	DQ009531	219	
Steindachnerina insculpta	Si3	DQ009532	178	–

Cytogenetic data described for the anostomids genera *Leporinus*, *Leporellus*, *Schizodon* (Galetti et al. 1981), *Abramites*, *Anostomus*, and *Pseudanos* (Martins et al. 2000), show a common karyotype pattern with $2n = 54$ biarmed chromosomes. Chromosomal differentiation among *Leporinus* is mainly related to a ZW sex chromosome system described for seven species: *Leporinus conirostris*, *L. elongatus*, *L.* aff. *elongatus*, *L. macrocephalus*, *L. obtusidens*, *L. reinhardti*, and *L. trifasciatus* (for review, Galetti et al. 1995). The morphological similarity in the ZW chromosomes among these species of *Leporinus* suggests a common origin for these chromosomes (Galetti et al. 1995). Besides the presence of a ZW sex chromosome system, these species of *Leporinus* share some morphological characteristics as the presence of a common color pattern, large body sizes, and the same number of teeth, reinforcing the hypothesis that they may belong to a natural group. The present phylogenetic analyses of the 5S rDNA sequences allowed the identification of two groups for the *Leporinus* species: the first one (i) is composed of *L. elongatus*, *L.* aff. *elongatus*, *L. macrocephalus*, and *L. obtusidens* (Fig. 2). This data corroborate the monophyletic nature of *Leporinus* species with a ZW sex chromosome system. The second group (ii) identified in the phylogenetic analyses is composed of *Leporinus friderici*, *L. octofasciatus*, and *Leporinus* sp. (Fig. 2). The latter undescribed species of *Leporinus* has recently been reported to have a new ZW system (Venere et al. 2004). However, the Z and W chromosomes of this species are morphologically different from the typical Z and W chromosomes previously described for the seven species of *Leporinus* mentioned above. In the same way, *Leporinus* sp. shares the same morphological patterns with the species of *Leporinus* that do not have ZW sex chromosomes (Santos and Jégu 1989). These data reinforce the hypothesis that the atypical ZW chromosomes of *Leporinus* sp. could represent a de novo origin of sex chromosomes in the genus *Leporinus* (Venere et al. 2004).

Variations in the 5S rDNA NTS, including insertions/deletions, minirepeats and pseudogenes have been frequently characterized in plants, mammals, and

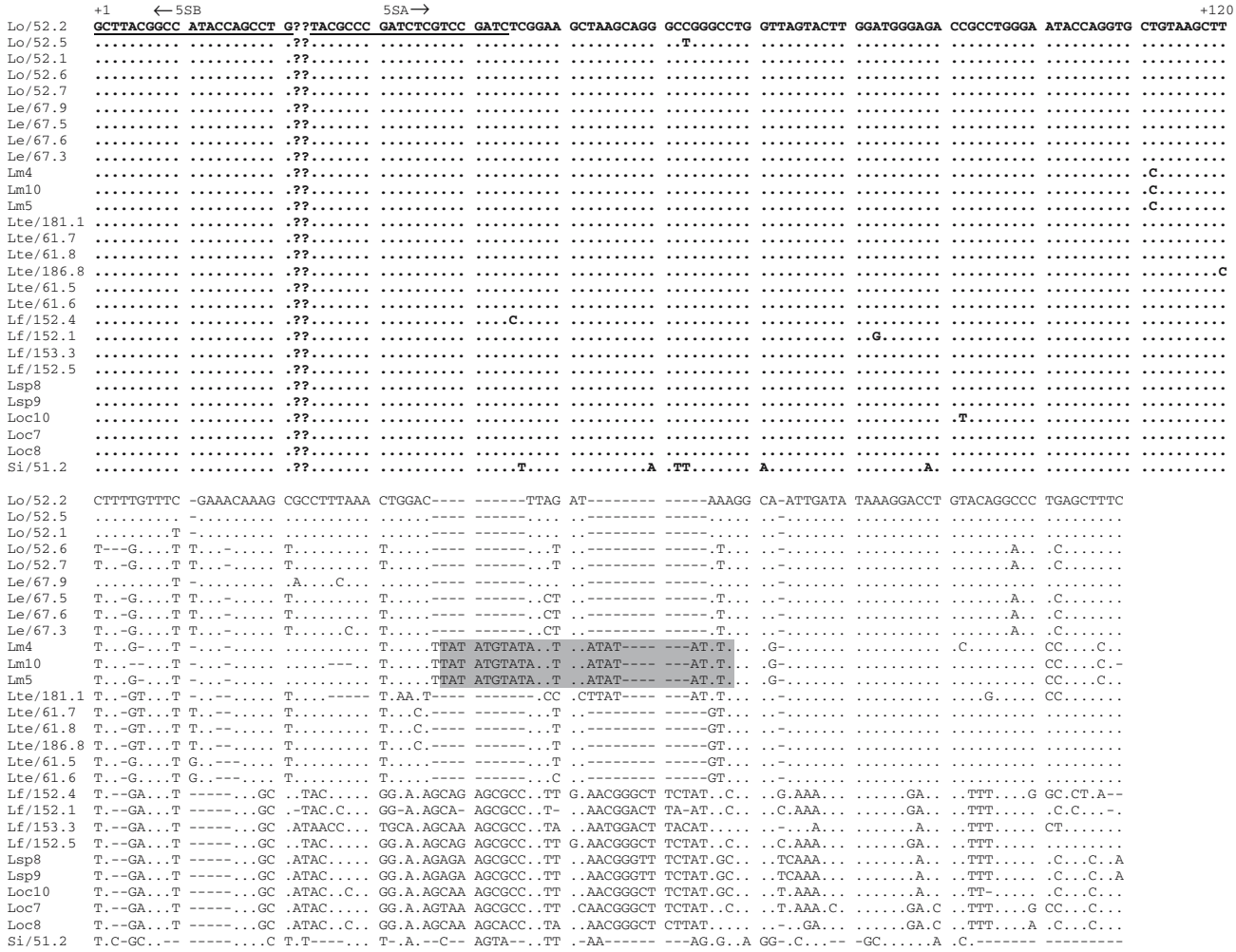

Fig. 1 Alignment of 5S rDNA type I sequences of *Leporinus*. The 5S rRNA gene coding sequence is in bold and the primer regions are underlined. Dots indicate sequence identity, hyphens represent indels and ? undefined nucleotides. Lo, *L. obtusidens*; Le, *L. elongatus*; Lm, *L. macrocephalus*; Lte, *L.* aff. *elongatus*; Lf, *L. friderici*; Lsp, *Leporinus* sp; Loc, *L. octofasciatus*, and Si, *S. insculpta*. The TA microsatellite of *L. macrocephalus* is in gray shading

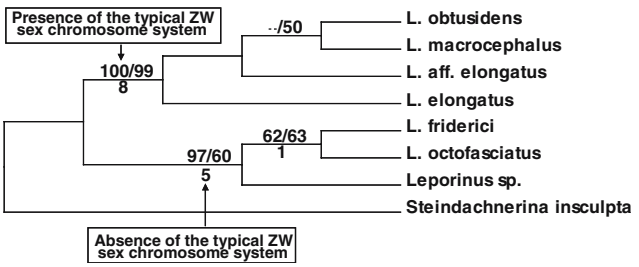

Fig. 2 Consensus MP tree produced when gaps were treated as missing data and the Ti/Tv ratio of 1:1 (TL = 104, CI = 0.9231, HI = 0.0769, RI = 0.8857). Numbers above branches are bootstrap values based on 1000 replicates employing the MP and the ML methods (MP/ML). Values below 50% are not shown. Numbers below branches represent Bremer support index values

fish and served as species- or population-specific markers useful for evolutionary studies (for review Martins and Wasko 2004). Particularly among fishes, the genome organization patterns of the 5S rDNA tandem repeats have been applied as efficient genetic markers for sex identification and inspection programs intended to access species, hybrids, or smoked products identity. In rainbow trout (*Oncorhynchus mykiss*), chromosome hybridization analyses on male and female metaphase spreads revealed a 5S rDNA chromosome sex-specific pattern (Morán et al. 1996). PCR amplified products clearly discriminate Atlantic salmon (*Salmo salar*), brown trout (*Salmo truta*), and their hybrids (Pendás et al. 1995) and also Neotropical fish species of the genus *Brycon* (Wasko et al. 2001). PCR was also applied in the identification of the flatfishes, *Solea solea* and *Reinhardtius hippoglossoides* (Cespedes et al. 1999) and also for the identification of smoked fillets of salmon, rainbow trout and bream (*Brama raii*) (Carrera et al. 2000).

The present data reinforce that 5S rDNA polymorphisms constitute important nuclear genetic markers, in agreement with cytogenetic and morphological data, for clarifying relationships between closely related species. Once the NTSs evolve fast, the analyses of NTS regions can function as species-population genetic marker, contributing to the knowledge of the biology of fish species in a broad range of aspects.

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