Organization of 5S rDNA in species of the fish *Leporinus*: two different genomic locations are characterized by distinct nontranscribed spacers

Cesar Martins and Pedro Manoel Galetti, Jr.

Abstract: To address understanding the organization of the 5S rRNA multigene family in the fish genome, the nucleotide sequence and organization array of 5S rDNA were investigated in the genus *Leporinus*, a representative freshwater fish group of South American fauna. PCR, subgenomic library screening, genomic blotting, fluorescence in situ hybridization, and DNA sequencing were employed in this study. Two arrays of 5S rDNA were identified for all species investigated, one consisting of monomeric repeat units of around 200 bp and another one with monomers of 900 bp. These 5S rDNA arrays were characterized by distinct NTS sequences (designated NTS-I and NTS-II for the 200- and 900-bp monomers, respectively); however, their coding sequences were nearly identical. The 5S rRNA genes were clustered in two chromosome loci, a major one corresponding to the NTS-I sites and a minor one corresponding to the NTS-II sites. The NTS-I sequence was variable among *Leporinus* spp., whereas the NTS-II was conserved among them and even in the related genus *Schizodon*. The distinct 5S rDNA arrays might characterize two 5S rRNA gene subfamilies that have been evolving independently in the genome.

Key words: 5S rDNA, 5S rRNA gene, nontranscribed spacer, *Leporinus*, fish.

Résumé : Afin de mieux connaître l'organisation de la famille multigénique codant pour l'ARNr 5S chez les poissons, la séquence nucléotidique et l'organisation de l'ADNr 5S ont été étudiées chez le genre *Leporinus*, un poisson d'eau douce représentatif de la faune sud-américaine. La PCR, le criblage de banques sub-génomiques, le buvardage génomique, l'hybridation in situ en fluorescence et le séquençage ont été employés dans le cadre de ce travail. Deux blocs d'ADNr 5S ont été identifiés chez toutes les espèces à l'étude, l'un formé d'un monomère de 200 pb et l'autre d'un monomère de 900 pb. Ces blocs d'ADNr 5S se distinguaient au niveau de la taille de leurs NTS, appelés NTS-I et NTS-II (associés respectivement aux monomères de 200 et de 900 pb), tandis que les régions codantes étaient pratiquement identiques. Les gènes codant pour les ARNr 5S étaient situés à deux locus chromosomiques, un locus majeur comprenant des régions NTS-I et un site mineur avec des NTS-II. La séquence NTS-I était variable au sein des espèces du genre *Leporinus* tandis que celle de l'espaceur NTS-II était conservée chez ces mêmes espèces et même chez le genre apparenté *Schizodon*. La présence de deux blocs distincts d'ADNr 5S pourrait suggérer deux sous-familles des gènes codant pour l'ARNr 5S, lesquelles auraient évolué indépendamment dans le génome.

Mots clés : ADNr 5S, gène codant pour l'ARNr 5S, espaceur non-transcrit, *Leporinus*, poisson.

[Traduit par la Rédaction]

Introduction

In higher eukaryotes, tandem arrays of rRNA genes are organized in two distinct multigene families composed of hundreds to thousands of copies. One class is represented by the 45S rDNA, which codes for the 18S, 5.8S, and 28S rRNAs and the second by the 5S rDNA, which codes for the 5S rRNA. 5S rDNA repeats consist of 120 bp long coding sequences separated from each other by nontranscribed spacers (NTSs). It has been demonstrated that the coding sequences are highly conserved, even among nonrelated taxa, whereas the NTS evolves more rapidly (Long and David 1980).

The organization of 5S rRNA genes is known in several eukaryotes organisms, revealing that these genes can be found interspersed with other multicopy genes, such as histone genes, 45S rDNA (in most cases), and repeated trans-spliced leader sequences (Drouin and Moniz de Sá 1995). In some fungi, the 5S rRNA genes are dispersed throughout the genome, instead of being organized in tandem (Mao et al. 1982; Belkhiri et al. 1992). Among vertebrates, 5S rDNA variants related to pseudogenes and NTS variations have been reported for several species (Little and Braaten 1989; Leah et al. 1990; Frederiksen et al. 1997). Moreover, a dual 5S rRNA gene system (regulated differently in somatic and oocyte cells) has been described in vertebrates, including fish and amphibians (Komiya et al.

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1986). In fish, little is known about the chromosomal localization (Fujiwara et al. 1998; Martins and Galetti 1999) and molecular organization of these genes (Pendás et al. 1994; Móran et al. 1996; Sajdak et al. 1998).

In this study, the nucleotide sequences and molecular organization of the 5S rDNA were investigated in the genus *Leporinus*, a representative freshwater fish group of South American fauna, providing new findings about the organization and evolution of this multigene family.

Materials and methods

Animals, DNA preparation, PCR, and cloning

Four species of *Leporinus* (Anostomidae, Characiformes) were the main focus of this study: *Leporinus elongatus*, *L. obtusidens*, *L. friderici*, and *L.* cf. *elongatus* (awaiting better taxonomic identification). All species were caught in the Mogi-Guaçu River (Sao Paulo, Brazil), except *L.* cf. *elongatus*, which was collected in the São Francisco River (Minas Gerais, Brazil). Other species of the same taxonomic family (*L. reinhardti*, *L. piau*, *L. desmotes*, *L. conirostris*, and *Schizodon borelli*) were also analyzed. DNA was extracted from kidney-cell suspensions, which had been previously fixed for chromosome preparations, and liver tissue, according to Sambrook et al. (1989). PCR amplifications of 5S rDNA were performed using primers A (5′-TACGCCCGATCTCGTCCGATC-3′) and B (5′-CAGGCTGGTATGGCCGTAAGC-3′), which were designed from the 5S rRNA sequence of rainbow trout (Komiya and Takemura 1979) to amplify the 5S rDNA repeat unit. A standard PCR reaction was performed using 150 pmoles of each primer, 20 ng of genomic DNA, 1× Taq buffer, 200 μM of each dNTP, and 2 U of Taq polymerase in a final reaction volume of 50 μL. Cycling times were as follows: 5 min at 94°C (denaturation); 35 cycles of 1 min at 95°C, 30 s at 63°C, and 1–3 min at 72°C; and a final 7 min at 72°C (extension). The PCR-amplified products were visualized in 10% polyacrylamide and 1% agarose gels. The PCR-generated 5S rDNA fragments were cloned in the plasmids pBSIIKS (Pharmacia Biotech), pGEM-T, or pGEM-T Easy (Promega), and used to transform *Escherichia coli* competent cell strain DH5α.

Southern-blot hybridization

The genomic organization of 5S rDNA was determined by Southern blot hybridization. Genomic DNA (10 μg) was partially to completely digested with *Hin*dIII, submitted to gel electrophoresis in 1% agarose, and Southern-transferred to Hybond-N nylon membrane (Southern 1975). The immobilized DNA was probed with four different sequences: (1) the whole monomer of type I 5S rDNA (5S rRNA gene + NTS) of *L. elongatus*; (2) the 5S rRNA gene-free NTS-I of *L. obtusidens*; (3) the 5S rRNA gene-free NTS-I of *L. friderici*; and (4) the 5S rRNA gene-free NTS-II of *L. friderici*. Double-stranded DNA probes 1 and 4 were cleaved from the recombinant plasmids where they were cloned. As the NTS-I was small (60 bp in *L. obtusidens* and 80 bp in *L. friderici*) and difficult to isolate from the recombinant plasmids, single-stranded DNA probes 2 and 3 were designed from the obtained sequences and artificially constructed. For a better understanding of the different probes used, see Results. The probes were denatured at 100°C, covalently labelled with the enzyme horseradish peroxidase, and hybridized for 12 h to filter-immobilized target DNA in a hybridization buffer (6 M urea – 50% formamide – 0.5 M NaCI) at 42°C. After hybridization, the filters were washed in 6 M urea – 0.4% SDS – 0.5× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate) buffer at 42°C. Hybridized DNA was detected by enhanced chemiluminescence (ECL direct nucleic acid labeling and detection system; Amersham Life Science).

Subgenomic library construction and screening

Genomic DNA samples (10 μg) of *Leporinus* spp. were digested to completion with *Hin*dIII and then electrophoresed in a 1% low melting temperature agarose gel. Agarose slices containing DNA fragments of 800–1100 bp were excised from the gel; the DNA was purified by centrifugation in filter paper funnels, ligated with the *Hin*dIII-digested vector pUC18, and used to transform *E. coli* (strain DH5α) competent cells. One thousand recombinant clones were recovered, replated, lifted onto Hybond-N nylon membranes, and screened with the type I 5S rDNA probe (probe 1) obtained from *L. elongatus*. Probe labelling, hybridization, and detection were conducted as described in the previous section. Positive colonies and their recombinant purified plasmids were screened in the presence of positive and negative controls, to confirm clones for 5S rRNA gene sequence.

Sequencing and sequence analysis

The clones obtained from the 5S rDNA PCR products and screening of the subgenomic library constructions were sequenced on an ABI Prism 377 DNA sequencer (Perkin Elmer) with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The sequences were aligned, analyzed, and the percent sequence similarity determined using ClustalW (Thompson et al. 1994). Nucleic acid sequences were subjected to BLASTN (Altschul et al. 1990) searches at the National Center for Biotechnology Information (NCBI) Web site (http://www.ncbi.nlm.nih.gov/blast).

Fluorescence in situ hybridization (FISH)

Mitotic chromosomes were prepared from anterior kidney cells (Bertollo et al. 1978) and submitted to FISH. The same four probes employed in the Southern-blot hybridizations were used for FISH and were labelled as follows: the double-stranded DNA probes (probes 1 and 4) were labelled by nick translation with biotin-14-dATP (BioNick labelling system; GibcoBRL); the single-stranded DNA synthetic probes (probes 2 and 3) were labelled by random primer with biotin-14-dCTP (BioPrime DNA labelling system; GibcoBRL). Metaphase chromosome slides were incubated with RNase (40 μg/mL) for 1.5 h at 37°C. After denaturation of the chromosomal DNA in 70% formamide – 2× SSC for 5 min at 70°C, 40 μL of the hybridization mixture (100 ng of denatured probe, 50% formamide, 10 mg dextran sulfate/mL, 2× SSC) was dropped on slides and the hybridization performed overnight at 37°C. Hybridization washes included 50% formamide in 2× SSC at 42°C and 2× and 4× SSC at room temperature. Detection of hybridized probes was carried out with avidin–FITC (fluorescein isothiocyanate) conjugate (Sigma) followed by two rounds of signal amplification. After each step of the amplification, the slides were washed in a blocking buffer (1.26% $NaHCO_3$, 0.018% sodium citrate, 0.0386% Triton X-100 – 1% nonfat dried milk). Chromosomes were counterstained with propidium iodide, and the slides were mounted in antifade (Vector).

Results

PCR amplification with 5S primers A and B produced DNA fragments of approximately 200 bp for all species of *Leporinus* studied. These DNA fragments were cloned, sequenced, and compared with the DDBJ–EMBL–GenBank database, resulting in the identification of one unit of the 5S rDNA tandem array (5S rRNA gene + NTS) of approximately 200 bp in *L. elongatus*, *L. obtusidens*, and *L.* cf. *elongatus* and one of 220 bp in *L. friderici*. The primers used were designed to amplify the incomplete 5S rRNA gene (118 bp) and the complete NTS. At least five clone sequences from each species were aligned and a consensus

Fig. 1. PCR-generated 5S rDNA sequences of *Leporinus* spp. (*a*) Monomeric units from *L. obtusidens* (lo), *L. elongatus* (le), *L.* cf. *elongatus* (lte), and *L. friderici* (lf). (*b*) A dimeric unit from *L. obtusidens*. The 5S rRNA gene coding sequence is in boldface type and the primer homology regions are underlined. Dots indicate sequence identity and dashes represent indels. The restriction site for *Hin*dIII (AAGCTT) is underlined and in italics. The sequences are deposited in GenBank under the accession numbers AF284728–AF284747.

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(a)
          +24
lo  5′ TACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGGGCCGGGCCTGGTTAGTACTTGGATGGGAGACCGCCTGGGAATA
le     ..............................................................................
lte    ..............................................................................
lf     ...........................................................G..................

          5S rRNA gene ←→ NTS
lo     CCAGGTGCTGTAAGCTTTTTTGTTTT------------------GAAACAAAGTGCCTTTAA-ACTGGACTTATATATAG
le     ..................G.....------------------TG.............C.-.........C.......
lte    ..................G.....------------------.G.--.............-.T...........G...
lf     ................-..GA....CAAGCCGTACTCAAAGGGA..G.............T.AC...........AC.

                                                 +1
lo     GCA-ATTGATATAAAGGACCTGTACAGGCCCTGAGCTTTCGCTTACGGCCATACCAGCCTG 3′ 201
le     ...-.......................A...C.............................. 201
lte    ...-.......................................................... 199
lf     ...C.AAA.........GA....TTT......C...C....................... 220


(b)
5′ TACGCCCGATCTCGTCCGATCTCGGAAGCTAAGCAGGGCCGGGCCTGGTTAGTACTTGNATGGGANACCGCCTGGGAATACCAG
GTGCTGTAAGCTTTTTGGTTTTTGAACAAAGTGCCTTTAAATTGGACTTATATATAGGCAATTGATATAAAGGACCTGTACAGGACC
TCAGCTTTCGCTTACGGCCATACCGCCTTGAGCGCGCCAGATCTCGTCTGATCTCGGAAGCTANGCAGGGCCGGGCCTGGTTAGNAC
TTGNATGGGAGACCGCCTGGNAATACCAGGTGCTGTAAGCTTTTGGTTTTTGAACAAAGTGCCTTTAAATTGGATTTATATATAGG
CCATTGATATCAAGGACCTGTACAGGACCTCATCTTTCGCTTACGGCCATACCAGCCTG 3′ 404
```

sequence was produced (Fig. 1*a*). The longer sequence of *L. friderici* is the result of an insertion–deletion of 18 bp in the NTS of the species analyzed. The complete 5S rRNA gene sequence of *L. obtusidens* was obtained by amplifying the dimeric 5S rDNA unit using longer PCR elongation times before cloning (Fig. 1*b*).

To examine the organization of the 5S rDNA in *Leporinus* spp., genomic DNA was digested with *Hin*dIII, which only cleaves once in the 5S rRNA gene of the species studied (Fig. 1), and hybridized with a 5S rRNA gene probe (probe 1). Complete digestions showed the existence of two classes of 5S rDNA units in all species of *Leporinus* analyzed: a small one of approximately 200 bp (corresponding to the PCR products) and a large one with monomers of 900 bp (Fig. 2). Hereinafter these are referred to as type I and type II 5S rDNA, respectively. *Leporinus* cf. *elongatus* showed a third unit of around 850 bp. Evidence that these 5S rDNA units were organized in tandem arrays was demonstrated by the detection of a ladder of exact integers of 200 and 900 bp after Southern-blot hybridization using genomic DNA partially digested with *Hin*dIII (Fig. 2). The screening of subgenomic libraries to search for the type II 5S rDNA unit (900 bp monomer) yielded two positive clones that contained the complete sequence of the 5S rRNA gene (120 bp) and its NTS (776 bp) only in *L. friderici* (Fig. 3).

Comparative analysis of the NTSs of the type I 5S rDNA unit (designated NTS-I) showed several base substitutions and insertions–deletions among species. The NTS-I sequences of *Leporinus obtusidens*, *L. elongatus*, and *L.* cf. *elongatus* were closely related (average similarity of 92.3%), and the NTS-I sequence of *L. friderici* showed a mean similarity of 67.3% to them (Table 1). In contrast, when the NTS-I sequences from the four species of *Leporinus* were compared with the NTS sequence typical of the type II 5S

Fig. 2. Genomic organization of the 5S rDNA of *L. friderici* (*a*), *L. elongatus* (*b*), *L. obtusidens* (*c*), and *L.* cf. *elongatus* (*d*), determined by Southern-blot hybridization to a 5S rRNA gene probe from *L. elongatus*. Aliquots (10 µg) of genomic DNA (lanes I, II, and III) were digested with 30 U of the restriction endonuclease *Hin*dIII. Lanes I and II are partial digestions (10 and 30 min, respectively) and lane III is a complete digestion. Molecular weight markers (kb) are shown on the left.

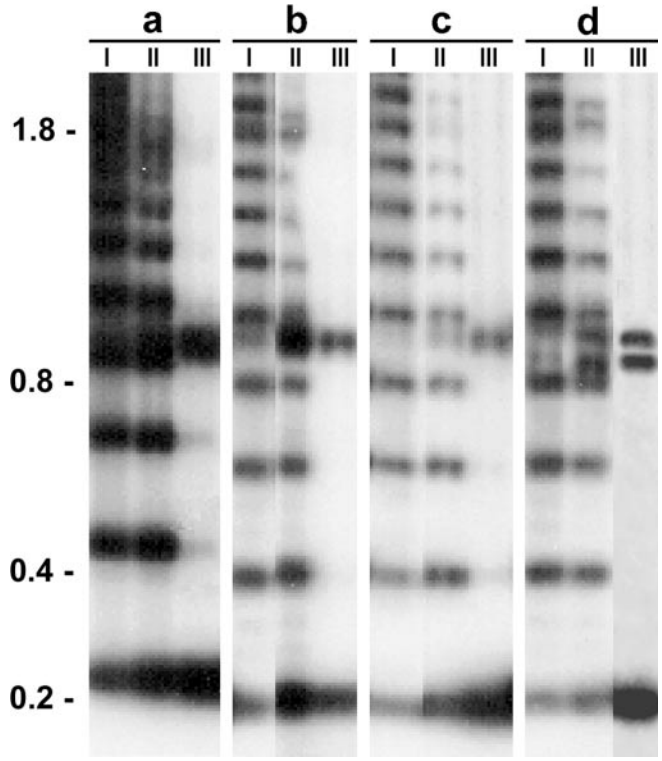

Fig. 3. 5S rDNA type II sequence from *L. friderici*. The 5S rRNA gene coding sequence is in boldface type. The *Hin*dIII-restriction flanking sites are in italics. The sequence is deposited in GenBank under the accession number AF284748.

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5S rRNA gene ←→  NTS                                                              81
      5′ AGCTTTTTGGTTTTGGGGGGCCCCTCTCTTGGGCCGGGTTGTTTAACCCACCCTGGCTTTTTTTTGGGCCGCCCTCATTTT
                                                                                 172
GCCCCCTTAGTCAGGGGGGGTTCTTTTCCCGGGGCGGGACCCCTGGGGGGAAATGGGGCCTTGTACGCCCTTCCCCCAACTTATTTTTTCC
                                                                                 263
CCAGCAGCAAAGGGAATCCGTGGTTGAAGATCCGCCCGGACATACGATGGAAAGAAAACGNTTAAAGTTGCAGCTCAACACTGGATCGNTC
                                                                                 354
CTCTTCCTTTGTAGGACCCCATATTGGTGTAGGATTCAGGTGCTTTGAAGAAGTAGCAATGATTGAATTCTGACATTTGGGGCGATAGTGA
                                                                                 445
ACCACAGAACCCGTGTACCGGACAGAGCTCATAGTTTTTTGTTTGTTTGTTTGTTAACCCAGCAGTGTATTAGAAAGGGAGGTTTTTTGTT
                                                                                 536
GCTCTACAAAGCCTATAAATGACACCTCTGGGTATTCTCTTTTAATCGCTGCATAAGCATACAGTCTATGGCGCTCTAAGACAGGTGGTAA
                                                                                 627
GGTTGAGTGGGACAAAGTCCATCATATTCCTATTAAGCTGTTTATTCCACAATACAGTCTTTCATCTTTGATTCCGTCTTTGAAACACGGT
                                                                                 718
GAGGTCTTTGGGGTGAGGGCTTTTATTATGATAAAATCATGTCGGCCTGTCAGAGATCTGTGTTGCTTTTGCAGCAGCAGTGTTCTTTTCG
                                                        NTS ←→ 5S rRNA gene      809
TTTTCTAATGGGCTCTAAATGAGAAACTGGACGATAAATAGTCTTATGCGGACAGGGTCACCCGCTTACGGCCACACCACCCCGAGGACGC
                                                                               896
CCGATCTCGTCTGATCTCGGAAGCTAAGCAGGGTCGGGCCTGGTTAGTACTTAGATGGGAGACTGCCTAGGAATACCAGGTGCTGTA 3′
```

Table 1. Matrix of the percentage of sequence similarity between the nontranscribed spacer (NTS) of the type I 5S rDNA unit (NTS-I) and the NTS of the type II 5S rDNA unit (NTS-II) from the four species of *Leporinus* investigated.

	NTS-I				NTS-II
	L. obtusidens	*L. elongatus*	*L.* cf. *elongatus*	*L. friderici*	*L. friderici*
NTS-I					
L. obtusidens	—				
L. elongatus	91	—			
L. cf. *elongatus*	95	91	—		
L. friderici	69	66	67	—	
NTS-II					
L. friderici	20	24	13	14	—

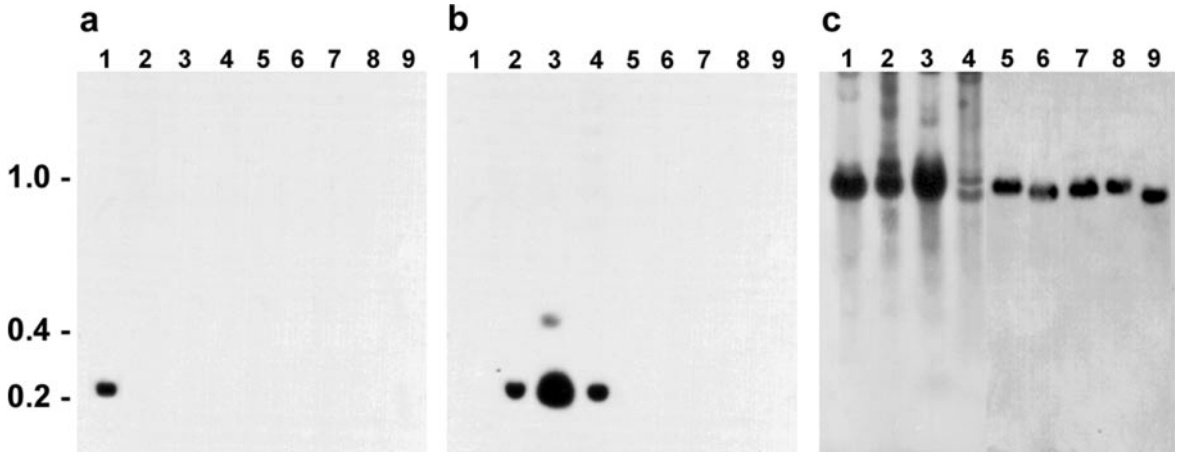
Table 2. Matrix of the percentage of sequence similarity among the 5S rRNA genes (type I 5S rRNA gene unit (5S-I) and type II 5S rRNA gene unit (5S-II)) from the four species of *Leporinus* investigated.

	5S-I				5S-II
	L. obtusidens	*L. elongatus*	*L.* cf. *elongatus*	*L. friderici*	*L. friderici*
5S-I					
L. obtusidens	—				
L. elongatus	100	—			
L. cf. *elongatus*	100	100	—		
L. friderici	98	98	98	—	
5S-II					
L. friderici	94	94	94	93	—

rDNA unit (designated NTS-II) from *L. friderici*, a weak relationship could be established between them (average similarity of 17.75%) (Table 1). Comparative analysis of 5S rRNA genes from the type I and type II 5S rDNA units was performed among the species of *Leporinus* (Table 2). The type I 5S rRNA gene had a high similarity (similarity of 100%) among *L. obtusidens*, *L. elongatus*, and *L.* cf. *elongatus* and was slightly different in *L. friderici*, which showed 98% similarity with the other species of *Leporinus*. Comparison of the type II 5S rRNA gene from *L. friderici* with the type I 5S rRNA gene from *L. friderici* and the other *Leporinus* spp. showed an average similarity of 93.75% (Table 2).

Southern hybridization using the 5S rRNA gene-free NTS-I probe obtained from *L. friderici* (probe 3) detected positive hybridization only in the genome of this species (Fig. 4*a*), whereas the 5S rRNA gene-free NTS-I probe from *L. obtusidens* (probe 2) hybridized, at least, with the genomes of *L. elongatus* and *L.* cf. *elongatus* (Fig. 4*b*). In addition, Southern hybridization detected homology between the 5S rRNA gene-free NTS-II from *L. friderici* (probe 4) and the genome of several other anostomid species, including *L. elongatus*, *L. obtusidens*, *L.* cf. *elongatus*, *L. reinhardti*, *L. piau*, *L. desmotes*, *L. conirostris*, and *Schizodon borelli* (Fig. 4*c*). The band pattern of the filter hybridization with the NTS-I and NTS-II probes was coinci-

Fig. 4. Southern-blot hybridization of *Hin*dIII-digested genomic DNA of several anostomids, using 5S rRNA gene-free NTS-I from *L. friderici* (*a*) and *L. obtusidens* (*b*) and 5S rRNA gene-free NTS-II from *L. friderici* (*c*) as probes. Lanes: 1, *Leporinus friderici*; 2, *L. elongatus*; 3, *L. obtusidens*; 4, *L.* cf. *elongatus*; 5, *L. reinhardti*; 6, *L. piau*; 7, *L. desmotes*; 8, *L. conirostris*; and 9, *Schizodon borelli*. Molecular weight markers (kb) are shown on the left.



dent with the organization of the 5S rRNA gene array shown in Fig. 2.

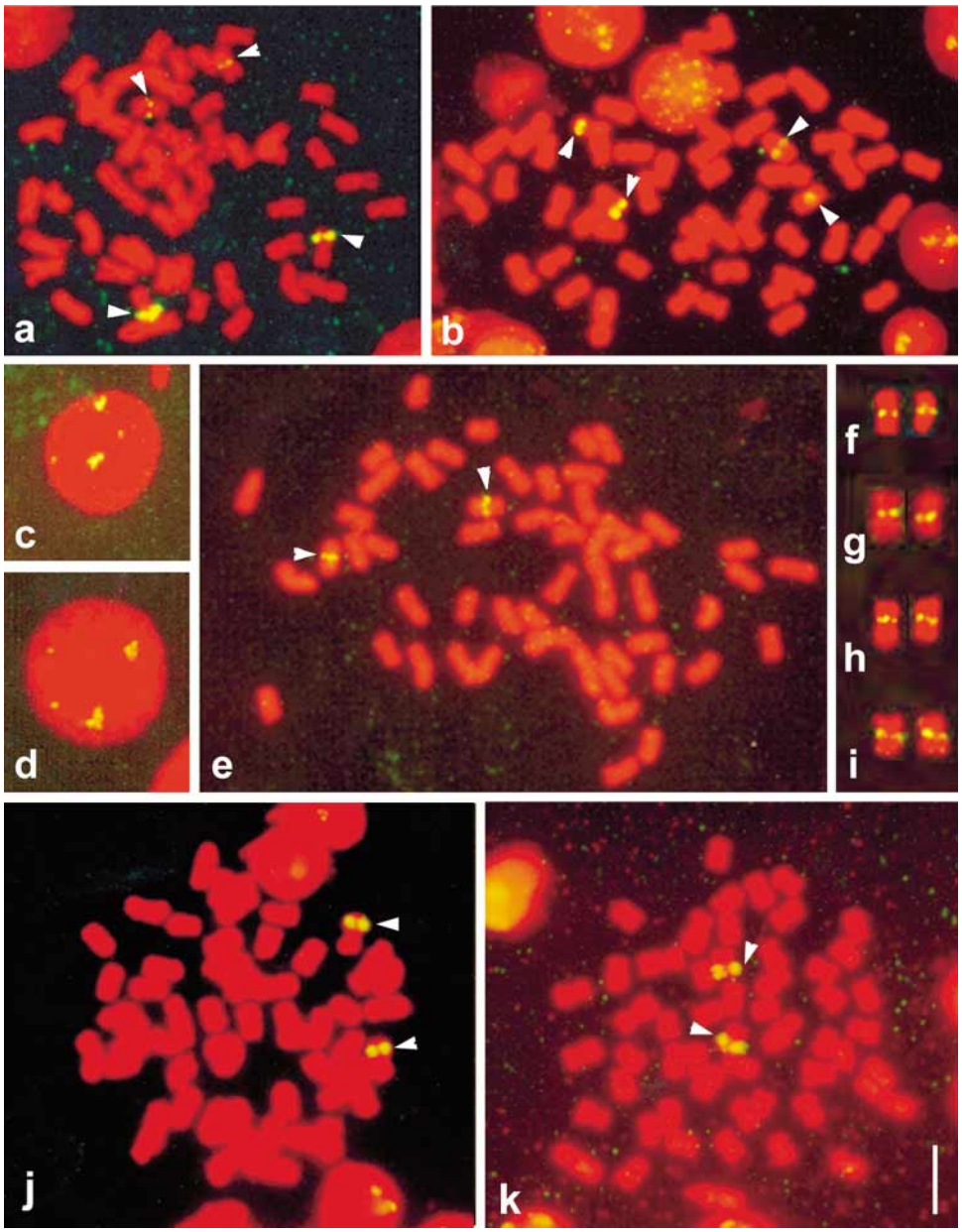
Chromosomal localization of the 5S rRNA genes was previously conducted in *L. elongatus*, *L. friderici*, and *L. obtusidens* (Martins and Galetti 1999), and two loci, one major and one minor, were described. Similar results were confirmed for *L.* cf. *elongatus* and *L. reinhardti* in the present study (Figs. 5*a* and 5*b*). The major and minor 5S rDNA loci were frequently visualized in distinct areas of the interphasic nuclei (Figs. 5*c* and 5*d*). The relation between the two chromosomal sites of the 5S rRNA genes and the two NTSs (NTS-I and NTS-II) was determined by chromosomal localization of both the 5S rRNA gene-free NTS-I (probes 2 and 3) and 5S rRNA gene-free NTS-II (probe 4) probes in different species of *Leporinus* (Figs. 5*e*–5*k*). The NTS-II was conserved in several *Leporinus* spp., as shown by Southern hybridization and FISH, and its chromosomal cluster was located near the centromeric region of the long arm of a medium-sized submetacentric chromosome pair (Figs. 5*e*–5*i*). The NTS-I chromosomal clusters were subterminally located in a small metacentric chromosome pair of *L. friderici* and *L. obtusidens* (Figs. 5*j* and 5*k*). The NTS-I and NTS-II chromosomal sites were coincident with the major and minor 5S rRNA gene clusters, respectively. The chromosomal hybridization showed that no mosaicism exists in the array of either 5S rDNA class.

Discussion

The 5S rRNA genes are organized in at least two arrays, differentiated by distinct NTS types, within each of the species of *Leporinus* analyzed. *Leporinus* cf. *elongatus* showed a third array (850 bp) that shared homology with the NTS-II, suggesting that it originated from the 900-bp unit. Unlike the 5S rRNA gene, polymorphisms are more frequent in the NTS, since this DNA segment represents a nontranscribed sequence that is not under strong selective pressure. The NTS-II was conserved among anostomids, whereas the NTS-I was diverse among *Leporinus* spp. and seems to evolve faster than the NTS-II. Searches at DDBJ–EMBL–GenBank identified low divergences between the 5S rRNA gene sequences described here and from those of other vertebrate species previously studied (average similarity of 88.8%). Comparison of the NTSs from selected organisms and the animals in this study, demonstrates that an NTS of least 80 bp, such as the NTS-I of *Leporinus* spp., can represent a minimum size for the organization of this rDNA in the genome, and seems to be the optimum condition for the organization and (or) activity of 5S rRNA genes. The 5S rRNA gene is transcribed by RNA polymerase III and contains an internal control region that functions as a promoter for the gene (Hallenberg et al. 1994). Moreover, it has recently been demonstrated that conserved sequences which influence the transcription levels of 5S rRNA genes of mammals can also be located in the NTS (Nederby-Nielsen et al. 1993; Suzuki et al. 1996). Although little is known about the NTS sequence among fishes, a TATA sequence has been observed upstream from the 5S rRNA gene in some species (Pendás et al. 1994; Sajdak et al.1998; Inafuku et al. 2000; Martins et al. 2000; Wasko et al. 2001) and may be involved in the transcription of these genes. Comparison of upstream sequences of 5S rRNA genes from *Leporinus* and those of other fishes identified an upstream TATA control element at the –22 to –32 position. The presence of control elements in the NTS-I and NTS-II of *Leporinus* spp. suggests that the 5S rRNA genes located in both arrays might be functional genes.

The type I 5S rRNA gene sequences were nearly identical (average similarity of 99%) among the different species of *Leporinus*. Some differences related to base substitutions were observed when gene copies from the type I and type II 5S rDNA from *L. friderici* were compared (average similarity of 93%). The similarity of the type I 5S rRNA gene sequences (Table 2) is greater among the different species, while comparison of type I and type II 5S rRNA gene sequences from *L. friderici* identified a lower level of similarity. It has been believed that multigene families evolve according to homogenization processes that are governed by molecular drive (Dover 1989) and concerted evolution (Elder and Turner 1995), resulting in a sequence similarity

Fig. 5. Localization of the 5S rRNA gene, 5S rRNA gene-free NTS-I, and 5S rRNA gene-free NTS-II sequences in *Leporinus* chromosomes, using FISH. The 5S rRNA gene probe was hybridized to metaphases of *L. reinhardti* (*a*) and *L.* cf. *elongatus* (*b*) and to interphase nuclei of *L. friderici* (*c* and *d*). The NTS-II probe was hybridized to chromosomes of *L. friderici* (*e*), *L. reinhardti* (*f*), *L. elongatus* (*g*), *L. obtusidens* (*h*), and *L.* cf. *elongatus* (*i*). In *f–i*, only the chromosomes bearing the NTS-II sites are shown. NTS-I probes from *L. friderici* and *L. obtusidens* were hybridized to chromosomes of these two species (*j* and *k*), respectively. Scale bar = 5 μm.



among the repeating units that is greater within than between species. According to the results observed for the organization of 5S rDNA in *Leporinus*, there is greater similarity among repeat units within the same cluster, even among different species, than among repeat units of different clusters, among which there is an accentuated variation.

Previous information on the chromosomal localization of the 5S rRNA genes in anostomids (Martins and Galetti 1999, 2000), as well as the results observed for *L. reinhardti* and *L.* cf. *elongatus* in the present study, has characterized two conserved loci, located interstitially in the chromosomes of apecies of *Leporinus* and *Schizodon*. This interstitial location for the 5S rRNA gene clusters has been observed for several other fishes, such as salmonids (Fujiwara et al. 1998), *Hoplias malabaricus* (Born and Bertollo 2000), *Oreochromis niloticus* (Martins et al. 2000), and the genus *Brycon* (Wasko et al. 2001). The same pattern of 5S rRNA gene chromosomal location has also been observed in mammals (for example, Lomholt et al. 1995; Mellink et al. 1996; Frederiksen et al. 1997; Mäkinem et al. 1997; Zijlstra et al. 1997) and amphibians (Vitelli et al. 1982; Schmid et al. 1987; Lucchini et al. 1993), suggesting that such chromo-

somal location and organization of the 5S rRNA genes is conserved in the vertebrate genome.

Although the chromosomal organization of the 5S rRNA genes has been conserved during karyotype diversification in anostomids, changes within the array of these genes have occurred. Several organisms have a unique rDNA variant on different chromosomes, and it has been demonstrated, at least for primates, that nonhomologous chromosome exchange may be a mechanisms for this homogenization (Williams and Strobeck 1985). The distinct 5S rDNA arrays detected in species of *Leporinus* indicated an absence of nonhomologous chromosome exchange between the chromosome pairs bearing the 5S rDNA clusters. This scenario supports the idea that individual chromosomes occupy specific territories in the nucleous (Lamond and Earnshaw 1998; Cremer et al. 2000) and that the chromosomes bearing the 5S rDNA clusters seem to be evolving in individual nuclear environments.

Two main families of 5S rRNA genes that are regulated differently in oocytes and somatic cells have been described in amphibians (reviewed by Krämer 1985) and fishes (Komiya et al. 1986). These 5S rDNAs differ slightly in the coding region and greatly in the NTS. The two 5S rDNA subfamilies observed in species of *Leporinus* could be related to this dual-gene system, which appears to represent paralogous copies of the 5S rRNA gene that have evolved in separate regions of the genome. Different 5S rDNA classes have been reported for several mammals (Hart and Folk 1982; Hallenberg et al. 1994; Frederiksen et al. 1997) and fishes. Information about 5S rDNA variants and genomic location has only been described for rats, in which bona fide 5S rDNA genes and pseudogenes map to different chromosomes (Frederiksen et al. 1997). In the tilapiine cichlid fish *Oreochromis niloticus*, two distinct 5S rDNA units have been characterized by distinct NTS types and base substitutions in the 5S rRNA gene (Martins et al. 2000). A similar situation has also been described for seven species of the genus *Brycon* (Wasko et al. 2001), *Salmo salar* (Pendás et al. 1994), *Oncorhynchus mykiss* (Móran et al. 1996), and the genus *Coregonus* (Sajdak et al. 1998). These previous studies in fishes have only described the occurrence of different classes of 5S rDNA. In the present work, we demonstrate that the two distinct arrays of 5S rDNA have different genomic locations that could represent the sites of two 5S rRNA gene subfamilies. The two 5S rDNA arrays observed here appear to be independent units of evolution that might express two different types of 5S rRNAs. Two arrays of 5S rDNA with distinct genome locations seem to be the rule for fishes.

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