Genetica (2006) 127:243–252
DOI 10.1007/s10709-005-4013-8
© Springer 2006

Genomic organization and evolution of the 5S ribosomal DNA in Tilapiini fishes

F.A. Alves-Costa, A.P. Wasko, C. Oliveira, F. Foresti & C. Martins*
*Instituto de Biociências, Departamento de Morfologia, UNESP – Universidade Estadual Paulista, CEP 18618-000, Botucatu, SP, Brazil; *Author for correspondence (Phone: +55-14-38116264; E-mail: cmartins@ibb.unesp.br)*

Received 30 June 2005; Accepted 12 October 2005

Key words: Cichlidae, fish, NTS, *Oreochromis*, 5S rDNA, *Tilapia*, tilapia

Summary

5S rDNA sequences present an intense dynamism and have proved to be valuable as genetic markers to distinguish closed related species and also in the understanding of the evolutionary dynamic of repetitive sequences in the genomes. In order to identify patterns of 5S rDNA organization and their evolution in the genome of fish species, such genomic segment was investigated in the tilapias *Oreochromis niloticus* and *Tilapia rendalli*, and in the hybrid *O. urolepis hornorum* × *O. mossambicus*. A dual 5S rDNA system was identified in the three analyzed tilapia samples. Although each 5S rDNA class was conserved among the three samples, a distinct 5S rDNA genome organization pattern could be evidenced for each sample. The presence of a dual 5S rDNA system seems to be a general trait among non-related teleost fish orders, suggesting that evolutionary events of duplication have occurred before the divergence of the main groups of teleost fishes.

Introduction

The nuclear genes that code the ribosomal RNA (rRNA) molecules are organized in a head-to-tail tandem pattern distributed in two multigene families in the genome: one of them is represented by the 45S rDNA which consists of a transcriptional unit that codes for the 18S, 5.8S and 28S rRNAs, and an intergenic non-transcribed spacer (IGS); the second family codes for the 5S rRNA and consists of a highly conserved coding sequence of 120 base pairs (bp) separated by a variable non-transcribed spacer (NTS). The genomic organization of the 5S rRNA genes is known on several eukaryotes organisms. The accumulating data demonstrate that 5S RNA genes are highly conserved, even among non-related taxa, both with respect to length and nucleotide sequence, whereas, the NTS evolves more rapidly. Some fungi species can have 5S genes within the 18–28S rDNA repeat, whereas others present 5S genes dispersed throughout their genome (Belkhiri, Buchko & Klassen, 1992). In some eukaryotes representing non-related taxonomic groups, the 5S rRNA genes can be found interspersed with other multicopy genes, such as histone genes, 45S rDNA (most of the cases) and repeated trans-spliced leader sequences (Drouin & Moniz de Sá, 1995). However, in most eukaryotes, the 5S rRNA genes are normally detected in distinct areas of the genome, organized as one or more tandemly repeated clusters.

The structural and functional organization of 5S rRNA genes has been mostly described for plants (Hanson et al., 1996; Amarasinghe & Carson, 1998; among others), mammals (Leah et al., 1990; Suzuki, Moriwaki & Sakurai, 1994) and some amphibian (del Pino et al., 1992). In vertebrates, 5S rDNA variants related to either pseudogenes or NTS variations have been reported for several species (Leah et al., 1990; Suzuki, Moriwaki & Sakurai, 1994; Frederiksen et al., 1997). Such NTS variations have been useful on

evolutionary studies and can characterize species- or population-specific markers (Suzuki, Moriwaki & Sakurai, 1994; Pendás et al., 1995). Although several authors have considered the usefulness of 5S rDNA sequences as phylogenetic and/or population markers (Suzuki, Moriwaki & Sakurai, 1994; Pendás et al., 1995; Baker, Hedderson & Dransfield, 2000), special attention must be exercised, mainly in phylogenetic interpretations, once the 5S rDNA family might show a complex organization with the presence of paralogous sequences in the genome (Martins & Galetti, 2001a). A dual 5S rRNA gene system, differently regulated in somatic and oocyte cells, was described for vertebrates, including fish and amphibians (Komiya, Hasegawa & Takemura, 1986). In *Xenopus laevis*, for example, the oocyte unit is about 750 bp and it includes the 120 bp gene, a non-transcribed spacer and a pseudogene, while the somatic unit has approximately 880 bp and does not contain pseudogenes. The presence of a dual genomic 5S rDNA class has been documented for several groups of teleost fish suggesting that such pattern could be a common characteristic among fishes.

Cichlids of the tribe Tilapiini have received increasing scientific interest due to its importance to the tropical and subtropical aquaculture and its rapid adaptive radiation, which led to an extensive ecological diversity (Pullin, 1991). Among the tilapiine, *Oreochromis niloticus*, *Tilapia rendalli*, *Oreochromis mossambicus*, and their hybrids, represent groups of great aquaculture importance. In order to address the understanding of the dynamics and evolution of 5S rDNA tandemly repetitive sequences in the genome of fishes, the present paper discuss the nucleotide sequence and genome organization of 5S rDNA in *O. niloticus*, *T. rendalli* and in the hybrid *O. urolepis hornorum* × *O. mossambicus*. The obtained results on 5S rDNA in the tilapiine and the reviewed data on 5S rDNA strongly support that a dual 5S rDNA system seems to be of general occurrence in teleost fish genome.

Material and methods

Fish samples, DNA extraction and PCR

Three tilapia samples – *Oreochromis niloticus* (172 animals), *Tilapia rendalli* (5 animals), and the hybrid *O. urolepis hornorum* × *O. mossambicus* (8 animals) – obtained from Brazilian fish farms, were analyzed in the present paper. *Oreochromis niloticus* specimens were sampled from four different strains that are commonly farmed in Brazil: CESP, Pernambuco and Santa Catarina strains were obtained from one original stock introduced in Brazil in 1971 from Ivory Coast, West Africa (Lovshin, 2000); Chitralada strain was originated from the Nile River, Alexandria, Egypt in the decade of 1940 and maintained in Japan and Thailand up to 1996 when it was introduced in Brazil (Zimmermann, 1999). Genomic DNA was extracted from fin tissues, according to the method detailed in Wasko et al. (2003). A set of primers, 5SA (5′-TAC GCC CGA TCT CGT CCG ATC-3′) and 5SB (5′-CAG GCT GGT ATG GCC GTA AGC-3′), designed from the 5S rRNA sequence of rainbow trout (Komiya & Takemura, 1979), was used to amplify 5S rDNA repeats containing putative coding regions for the 5S rRNA and also the NTS. Based in the nucleotide sequences of the PCR fragments obtained using the primers 5SA and 5SB, the set of primers NTSIA (5′-CTG CTG CTG TCT CTG TTT GTG-3′) and NTSIB (5′-TGT ATG CTG AAG CTA AAG CTG-3′) was designed to amplify a specific segment of the NTS of the 5S rDNA type I subfamily of *O. niloticus*. PCR-amplifications were carried out in a PTC-200^{TM} Programmable Thermal Controller (MJ Research, INC) using a total volume of 50 µl containing 20–100 ng of genomic template DNA, 150 pmol of each primer, 1.25 mM of each dNTP, PCR buffer (Biotools) containing 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Biotools DNA polymerase). The optimum cycling times were obtained as follows: 5 min at 94°C; 35 cycles of 1 min at 95°C, 30 s at 63°C, and 1 min at 72°C, and a final 5 min extension at 72°C. The 5S rDNA–PCR products were resolved in 1% agarose and 6% polyacrylamide gels and visualized after ethidium bromide or silver nitrate staining, respectively.

Cloning, sequencing and nucleotide sequence analysis

The PCR products were inserted in the plasmids pCR2.1 (Invitrogen) and pGEM-T (Promega), which were used to transform competent cells of *Escherichia coli* strain DH5α according to

Sambrook and Russel (2001). Positive recombinant clones were recovered and stored in 75% glycerol at −80°C. The positive clones were sequenced on an ABI Prism 377 automatic DNA sequencer (Perking-Elmer) with the kit DYEnamic™ ET Terminator Cycle Sequencing (GE Healthcare Bio-Sciences), following the manufacturer instructions. 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), and the sequence alignment was performed using the computer program Clustal W (Thompson, Higgins & Gibson, 1994) and checked by hands. Neighbor-Joining (NJ) phylogenetic analysis employing the Kimura-2-parameter genetic distance model (Kimura, 1980) were conduced using the software MEGA 2.1 (Kumar et al., 2001). Bootstrap re-sampling (Felsenstein, 1985) was applied to assess support for individual nodes using 1000 replicates.

Southern blot hybridization

Genomic DNA samples (8 µg) of *Oreochromis niloticus*, *Tilapia rendalli*, and the hybrid *O. urolepis hornorum* × *O. mossambicus* were totally digested with the restriction endonucleases *Sac*I, *Hin*dIII, *Pvu*II, or *Pst*I. The digestion products were subjected to 1% agarose gel electrophoresis and Southern-transferred to a Hybond-N nylon membrane (Sambrook & Russel, 2001). The hybridization of the filter-immobilized DNA was performed using as probes clones containing repeat units of the 5S rDNA of *O. niloticus*. The hybridization labeling and detection steps were performed with the kit ECL-Direct Nucleic Acid Labeling and Detection System (GE Healthcare Biosciences), following the manufacturer instructions.

Results

5S rDNA nucleotide sequences and organization in the tilapia genomes

PCR amplification of the genomic DNA of *Oreochromis niloticus*, *Tilapia rendalli* and the hybrid *O. urolepis hornorum* × *O. mossambicus*, with the set of primers 5SA and 5SB, generated just one band of approximately 500 bp. No size differences in the amplified fragments were detected under agarose gel electrophoresis. The 5S rDNA PCR products obtained with the primers 5SA and 5SB were cloned and a total of 12 clones for *O. niloticus*, nine clones for *T. rendalli* and nine clones for the hybrid were sequenced (Table 1). The GenBank accession numbers for the nucleotide sequences are AY945232–AY945261. Sequences ranged in size from 475 to 505 bp including a 120 bp coding region (5S rRNA gene) and a variable NTS (Table 1). It is of note the occurrence of two distinct 5S rDNA sequence types or subfamilies, hereafter referred to as 5S rDNA type I and 5S rDNA type II classes that were detected in the three analyzed tilapia samples (Figure 1).

Five phylogenetic analysis were conduced in the present study: (i) with the whole 5S rDNA

Table 1. 5S rDNA sequence data of the analyzed tilapia samples

	5S rDNA type I			5S rDNA type II			5S rDNA type I and type II
	NC	SL	GD	NC	SL	GD	GD
5S rRNA gene							
O. niloticus	08	120	0.024 ± 0.007	04	120	0.013 ± 0.007	0.029 ± 0.008
T. rendalli	03	120	0.023 ± 0.011	06	120	0.025 ± 0.009	0.033 ± 0.009
Hybrid	08	120	0.027 ± 0.008	01	120	0.000 ± 0.000	0.039 ± 0.009
NTS							
O. niloticus	08	355–360	0.022 ± 0.004	04	379–380	0.055 ± 0.008	0.516 ± 0.048
T. rendalli	03	356–368	0.120 ± 0.015	06	378–385	0.068 ± 0.008	0.609 ± 0.065
Hybrid	08	361–377	0.069 ± 0.009	01	384	0.000 ± 0.000	0.295 ± 0.031

NC, Number of clones; SL, Sequence length; GD, Genetic distance.

```
Type I   GCTTACGGCC ATACCAGCCT GNNTACGCCC GATCTCGTCC GATCTCGGAA GCTAAGCAGG GTCGTGCCTG GTCAGTACTT GGATGGGAGA
Type II  .......... .......... .......... .......... .......... ..C....... ....G..... .......... ...C......

Type I   CCGCCTGGGA ATACCAGGTG CTGTAAGCTT TT----TTCA -CACAAAAAA CTGCAGGGGG CGCTGCTGCT GTCTCTGTTT GTGCTTTGCT
Type II.......... .A.......C A......... ..GCAC.... A....C...- -..-.----- --...T.T-A .ATAT..AAA -C.AA.CTGA

Type I   CCATATTCTT CTCTGTTTGC TTGCATTGTG TGTGCGCGTG TGTGTGTGTT TTAC-TAAGT CTCTGAACAC GTTTTCC-AT CCAGTGTTGA
Type II GACAC.CT.. TAGCTCA.TT .C-T.A.A-. G...-----. .TA...A..C ..G.GC..-- ---A..G.T. T.CCA.TG.. GT...AA..T

Type I   AACATTTTTT CCTACGGTAA AACCTTTCAC TTCAACTTTA CCTTCAGCA- TAGAAAGCTG TTTACAGTAA -CACA-TCA- CTGATCTTGA
Type II C.A...C..C AAGCGTTAT. TCAA.-.... .GT.CA.GC. -.AGAGCACC .GT.GCTG.T ...-GT.C.. A...TCA..A ...T.TA.--

Type I   GATCGGAATA GTATGCAGC- TGGCTGTTAA AATCATCGTA TTTATTT-AA GTAAC----- ---AAAACAA ACTGAAGAAG A---CCT-CA
Type II ..CGT....T --T......A .TAAGTA.GC .T.ATAAC.G .-GC.G.C.T ..GC.GGTA- -TT....A.. .GGA---.G. .AGAAACTA.

Type I   AGATATAAGT CT-GCGAAAT GCTCCTGAAG CAG-TAAATA AAGGGGTGTT TGTCGTCCTC GCTGTGCCTC ---------- ------- 491
Type II TC.ATATCC. TCACTCT..A TTGTT.TTTA A.TCAC.... ..T....TAC A..ATCTACA TGAACC.TGT TATCGAGGAA GCCTCTC 498
```

Figure 1. Sequence alignment of two 5S rDNA sequences of *O. niloticus* representing the 5S rDNA type I (Type I) and the 5S rDNA type II (Type II) identified in the analyzed tilapiines. The 5S rRNA gene coding sequence is in boldface type and the TG microsatellite is indicated in gray shading. Dots indicate sequence identity and hyphens represent insertions/deletions.

sequences; (ii) with the 5S rRNA coding region only; (iii) with the NTS only; (iv) with the NTS I (NTS of the 5S rDNA type I) and; (v) with the NTS II (NTS of the 5S rDNA type II). The analysis of the whole 5S rDNA sequences and using just the NTSs reflected the existence of the two subfamilies of sequences (Figure 2a and c). On the other hand, the analysis of just the 5S rRNA gene did not reflect the two classes clearly discriminated by the NTS variations. The two 5S rDNA classes coexist within the tilapia samples and were not differentiated between samples (Figure 2a and c). Although the 5S rRNA coding region was quite conserved among the three tilapia samples and between the two 5S rDNA subfamilies (Figures 1 and 2b), it was possible to detect two important base substitutions in the 5S rRNA gene: (i) one base substitution in the position +53 that discriminates the 5S rRNA gene of the 5S rDNA type I and type II; (ii) one base substitution in the position +110 that discriminates *O. niloticus* from *T. rendalli* and the hybrid in both 5S rDNA subfamilies (Table 2). On the other hand, the NTSs were highly differentiated between the two subfamilies and quite conserved within the same subfamily (Figures 1 and 2c; Table 1). Analysis of the NTS I among the samples showed a mean genetic distance of 0.066 ± 0.007, whereas the NTS II had a mean genetic distance of 0.079 ± 0.008. No consistent similarity was detected between the NTS I and the NTS II and they were separated in different branches of the phylogenetic tree with a bootstrap value of 100.

In order to examine the genome organization of the 5S rDNA type I and type II sequences identified in the tilapia samples, restriction enzyme digestion, Southern blot and membrane hybridization experiments were performed (Figure 3). The genomic DNA of each tilapia sample was totally digested by the enzymes *Sac*I, *Hin*dIII, *Pst*I and *Pvu*II and membrane-immobilized. The employed enzymes were determined on the basis of the 5S rDNA hybridization patterns previously described for *O. niloticus* (Martins et al., 2002). The membrane was probed to a mix of two clones representing the 5S rDNA type I and the 5S rDNA type II classes of *O. niloticus*. The *Sac*I digestion demonstrated the presence of 1.4 kb repeat units of 5S rDNA for all analyzed tilapia samples. The enzyme *Hin*dIII produced one band around 0.7 kb for *T. rendalli* and several bands for *O. niloticus* and for the hybrid. One of these bands of *O. niloticus* (around 1 kb) was absent in the hybrid and in *T. rendalli*. The enzyme *Pvu*II detected three bands for the hybrid and *O. niloticus* and did not evidence bands for *T. rendalli*. The enzyme *Pst*I evidenced only one band for *T. rendalli* and several bands for the hybrid and *O. niloticus*. The treatment with *Hin*dIII, *Pvu*II and *Pst*I enzymes generate one band around 0.5 kb that was shared by the hybrid and *O. niloticus* but was not detected in *T. rendalli*. Contrary to the nucleotide sequencing data, the membrane hybridization results strongly support the discrimination among the two tilapia species and the hybrid.

NTS I variation in O. niloticus

Besides the nucleotide variation characterized among the NTS sequences, it was also detected

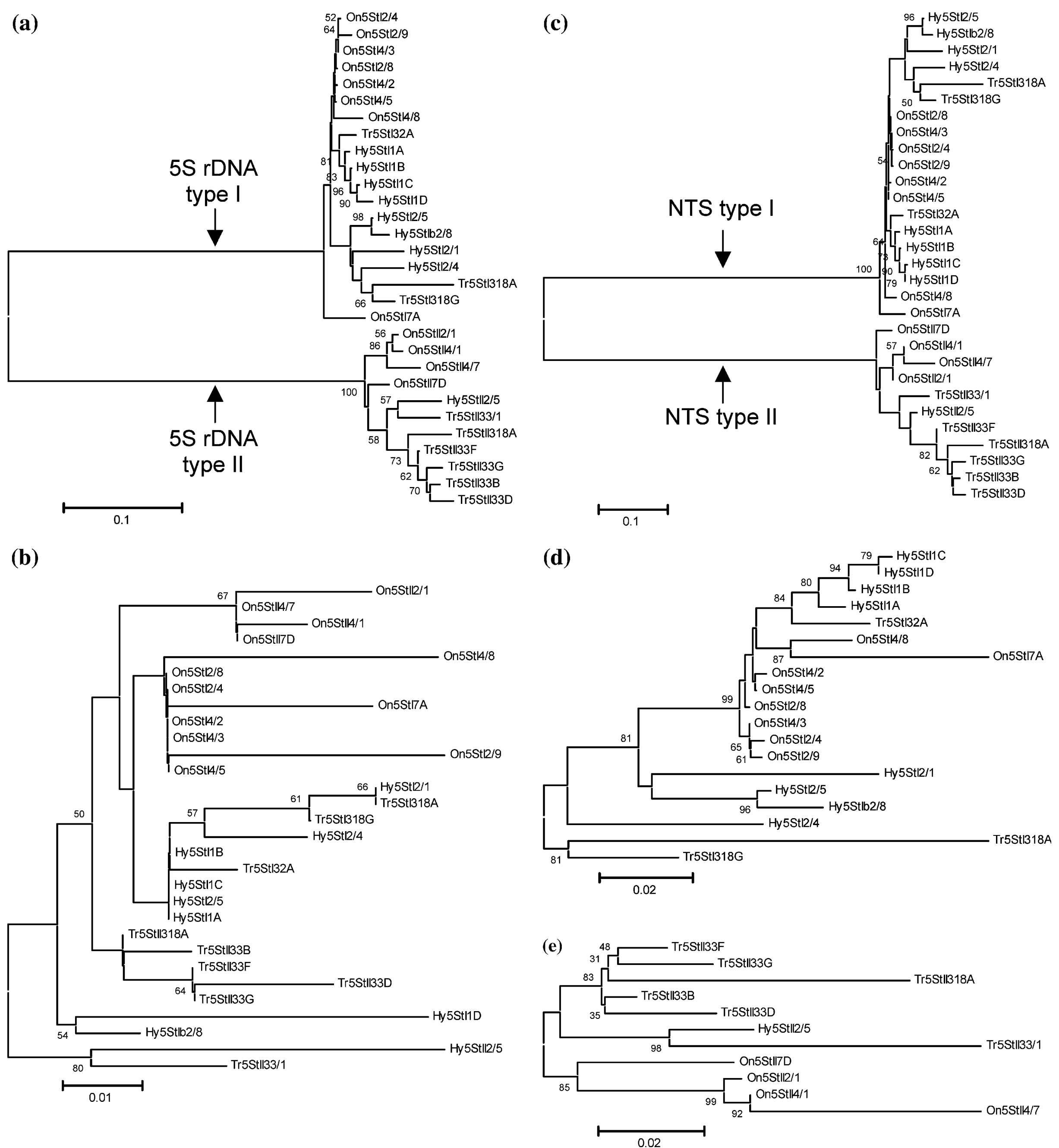

Figure 2. Neighbor-joining trees based on the 5S rDNA sequences of *O. niloticus* (On), *T. rendalli* (Tr) and the hybrid *O. urolepis hornorum* × *O. mossambicus* (Hy). Phylogenetic analysis were conduced with the whole 5S rDNA sequences (a), with the 5S rRNA coding region only (b), with the NTS only (c), with the NTS I (d) and with the NTS II (e). The numbers at each node indicate the percentage recovery (>50%) of the particular node (1000 bootstrap replicates). The 5S rDNA type I and 5S rDNA type II subclasses are indicated.

the presence of a TG microsatellite repeat in the NTS I that showed variation among the analyzed tilapia samples (Figure 1). In order to investigate the potential of the TG microsatellite repeat as a genetic marker to access variability, PCR with the primers NTSIA and NTSIB was performed to amplify a specific segment of the NTS of the 5S rDNA type I of the Nile tilapia including the TG

Table 2. Diagnostic nucleotide differences in the 5S rRNA gene among the analyzed tilapia samples

	5S rDNA type I			5S rDNA type II		
Nucleotide position	53	84	110	53	84	110
O. niloticus	T	T	C	C	C	C
T. rendalli	T	T	G	C	T	G
Hybrid	T	T	G	C	T	G

microrepeat region. The PCR generated two bands of around 155 and 165 bp that were cloned and the nucleotide sequence was determined for 54 clones. The GenBank accession numbers for these NTS I sequences are AY763289–AY763351. The detected size variation (155 and 165 bp) was related just to the presence/absence of the TG repeat. Although the low bootstrap values detected did not support evidence for the presence of variant types of NTS I in *O. niloticus*, the phylogenetic analysis of the 54 analyzed NTS I sequences suggests the presence of NTS I subclasses characterized by variations in the TG microrepeat (Figure 4).

The presence or absence of the NTS I fragments (bands of 155 and 165 bp) showed a non-random distribution among the investigated Nile tilapia strains. Three patterns of NTS I bands were identified by acrylamide electrophoresis: the first one was characterized by the presence of two bands around 155 and 165 bp, named NTSIg1; the second one was characterized by the presence of just one band around 155 bp, named NTSIg2; and the last one was characterized just by one band around 165 bp, named NTSIg3 (Table 3). The NTSIg1 pattern was observed in 100% of the samples of the CESP strain and 94.12% of the Pernambuco and Santa Catarina strains. On the other hand, NTSIg1 had a low frequency in the Chitralada strain. In opposite, the NTSIg2 pattern could be identified in 83.33% of the Chitralada samples and in a low

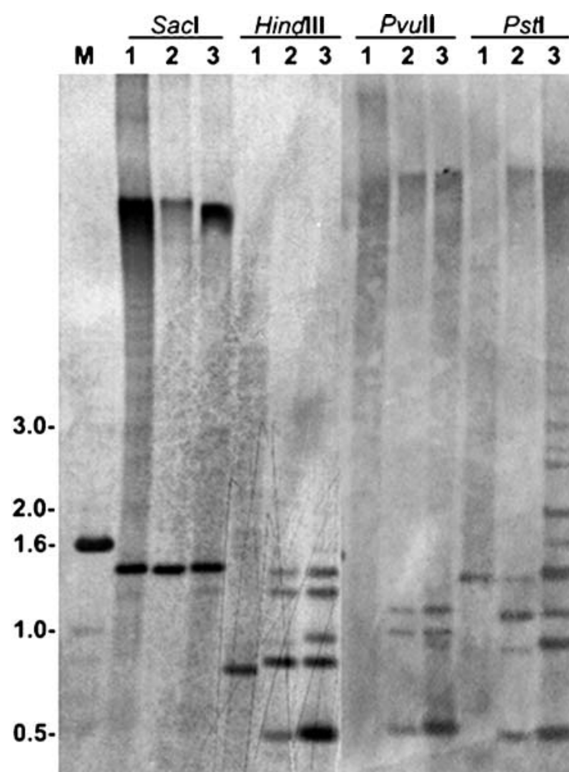

Figure 3. Results of enzyme digestion and Southern blot/hybridization experiments using 5S rDNA probes. Aliquots of 8 μg genomic DNA of *Tilapia rendalli* (1), *Oreochromis urolepis hornorum* × *Oreochromis mossambicus* hybrid (2) and *Oreochromis niloticus* (3) digested with 30 U of the restriction endonucleases *Sac*I, *Hin*dIII, *Pvu*II or *Pst*I. Molecular weight markers (kb) are shown on the left.

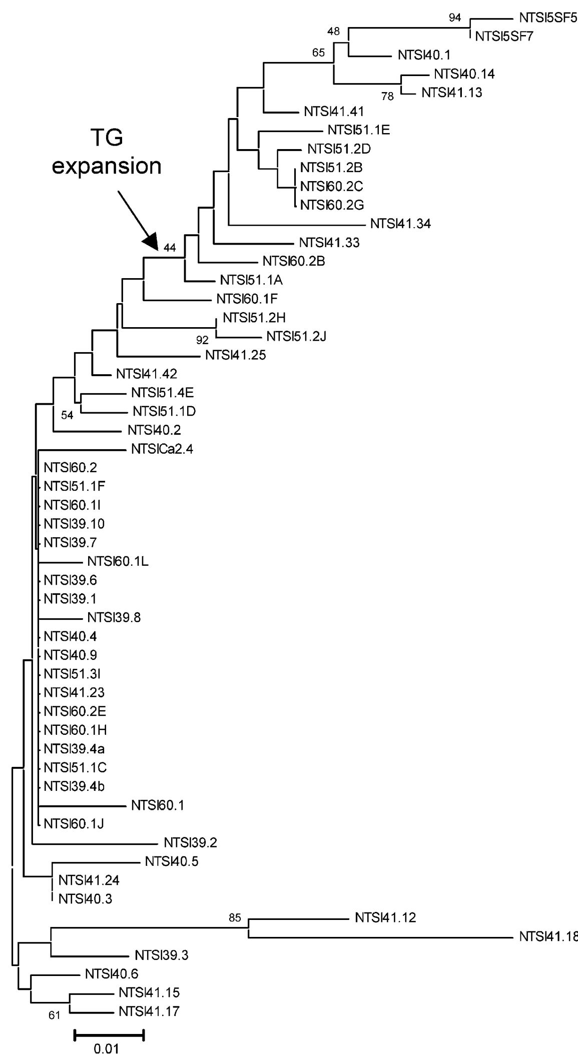

Figure 4. Neighbor-joining tree based on the NTS I sequences of *O. niloticus*. The numbers at each node indicate the percentage recovery (>40%) of the particular node (1000 bootstrap replicates). The TG microsatellite expansion is indicated.

frequency of the Pernambuco and Santa Catarina strains. An extremely low frequency could be observed for the NTSig3 pattern (Table 3).

Table 3. Frequency of NTS I band patterns among the Nile tilapia strains

Strain	Number of analyzed fishes	Frequency of NTS I band patterns (%)		
		NTSIg1	NTSIg2	NTSIg3
CESP	36	100	0	0
Pernambuco	36	94.12	5.88	0
Santa Catarina	35	94.12	2.94	2.94
Chitralada	63	15	83.33	1.66

Discussion

5S rDNA variation in tilapia samples: a dual 5S rDNA system

Variations in the NTSs of the 5S rDNA owing to insertions/deletions, minirepeats and pseudogenes have been frequently characterized in several organisms (Suzuki, Sakurai & Matsuda, 1996; Baum & Bailey, 1997; Sadjak, Reed & Phillips, 1998; Martins et al., 2002). Particularly among fishes, variant types of 5S rDNA characterized by high differences in the NTS sequences were identified in species of Characiformes, Perciformes and Salmoniformes (for review Martins & Wasko, 2004). These variations have been useful for evolutionary studies and served as species- or population-specific markers for various organisms.

The present data permitted to identify two classes of 5S rDNA tandem repeats in the genome of the tilapia samples, which were clearly discriminated by their different NTS sequences. A dual system of 5S rDNA tandem repeats has been observed for several Characiformes (Martins & Galetti, 2001a, b; Wasko et al., 2001), Perciformes (Martins et al., 2000, 2002, present paper), Salmoniformes (Pendás et al., 1994; Morán et al., 1996; Sadjak, Reed & Phillips, 1998), Cypriniformes (Gornung et al., 2000) and Acipenseriformes (Robles et al., 2005), reinforcing the idea that two different 5S rDNA classes seem to be a common feature in the teleost fish genome. Although variant repeats of the 5S rDNA were reported for mammals (Hallemberg, Nederby-Nielsen & Frederiksen, 1994; Frederiksen et al., 1997), the presence of well-characterized subfamilies of 5S rDNA with distinct genome locations seems to be exclusive for fishes. A more detailed study on the 5S rDNA structure and organization in fish was conducted in several species of the South American genus *Leporinus*, which not only clearly evidenced and characterized a dual 5S rDNA system with distinct sized monomers, but also showed that these two different 5S rDNA classes were clustered in distinct chromosome pairs (Martins & Galetti, 2001a).

The discovery of several duplicate genes among teleosts has suggested that whole-chromosome duplication or independent gene duplication events have occurred in different teleost lineages (Venkatesh, 2003). As so, it seems possible that a duplication of the 5S rDNA cluster has occurred before the diversification of the teleost fish.

The membrane hybridization results suggested that the 5S rDNA sequences have a more complex organization pattern in the genus *Oreochromis* when compared to *Tilapia*. Data on 5S rDNA organization in the Nile tilapia genome demonstrated that such sequences present pseudogenes and inverted sequences (Martins et al., 2002). Previous analysis have demonstrated that the enzyme *Sac*I does not cleave the 5S rDNA type II sequence of *O. niloticus* (Martins et al., 2002). Similarly, the intense band of high molecular weight evidenced in the DNA samples of the three analyzed species digested by *Sac*I suggested that repeats of 5S rDNA were preserved from digestion and remain as large arrays of DNA. Although just 0.5 kb segments of the 5S rDNA type I of each of the analyzed species were cloned and sequenced, it is clear that this fragment is inserted in a large repeat unit of 1.4 kb that is conserved among the analyzed tilapia species. While the Southern blot hybridization evidenced a greater similarity between the genome organization of the Nile tilapia *Oreochromis niloticus* and the *O. urolepis hornorum* × *O. mossambicus* hybrid, the former species was discriminated from *T. rendalli* and the hybrid. It is interesting to note that while the nucleotide sequence analysis did not discriminate the species, the membrane hybridization showed different 5S rDNA patterns for each analyzed sample. Such results demonstrate that the organizational arrays of the 5S rDNA have evolved independently in the different genomes.

The 5S rDNA structure in the tilapia genomes hereby analyzed corroborates the complex organization of this repeated element in fishes. The presence of two types of tandem repeats in *Oreochromis* and *Tilapia* species seems to indicate that such variants have arisen before the divergence of the main groups in the Tilapiini tribe. The two classes of 5S rDNA were not differentiated between the species but were found to coexist in each one of the species.

NTS I variations among O. niloticus *strains*

In several studies of 5S rDNA sequences among vertebrates, like rat (Suzuki, Sakurai & Matsuda, 1996), mouse (Suzuki, Moriwak & Sakurai, 1994) and fish species (Martins & Wasko, 2004), it was possible to detect variant types of these tandem repeats, related to differences in the NTSs. One interesting feature of the NTS I of the analyzed tilapia samples was the presence of a TG microsatellite repeat. The occurrence of such tandem repeats in the 5S rDNA segment demonstrates the intense dynamism that rules the evolution of the NTSs. Microsatellite sequences, such the TG motifs observed in the NTS I of the Nile tilapia, were also found in the 5S rDNA repeats of several fishes as *Micropterus salmonides* (Deiana et al., 2000), *Danio rerio* (Gornung et al., 2000), and *Aulopus japonicus* (Ota, Tateno & Gojobori, 2003). NTS regions are free to mutate, because these variants are neutral (or almost neutral) to natural selection, and can be fixed or lost, causing differences among related species and even within the same individual. In contrast, most mutations in the 5S rRNA gene are selectively neutral just when it happens in a sub-critical proportion (Cronn et al., 1996). The TG microsatellite expansion/contraction was responsible for the identification of different genetic patterns, whose frequency varied among the different strains of Nile tilapia analyzed.

5S rDNA variation: is it a useful genetic marker?

Particularly among fishes, there are several examples of the applicability of the 5S rDNA as genetic markers. In the present work, the 5S rDNA PCR patterns and the nucleotide sequences of the three different analyzed tilapias did not differentiate the species. However, Southern blot hybridization showed different 5S rDNA patterns for each one of the analyzed tilapia, demonstrating that the genome organization of the sequences have evolved independently.

The tilapiine systematic is based on morphological, ecological, and behavioral parameters. However, species identification in this group may be very difficult, due to the considerable interpopulational variation detected within many species (Bardakci & Skibinski, 1994). Moreover, several

tilapiine species share similar morphological features and can be easily hybridized (Greenwood, 1991; Galls & Metz, 1998). These factors have caused several problems in species and sub-species identification, especially concerning to species of aquaculture importance. In this way, DNA markers that serve to species, sub-species, or strain identification in the tilapia group are of high value to fundamental and applied studies of these fishes. The identified nucleotide substitution in the position +110 of the 5S rRNA gene of *O. niloticus*, that represents a single nucleotide polymorphism (SNP), can be applied as a genetic marker for the discrimination of the Nile tilapia from the other tilapia species.

The frequency of the NTS I genotypes varied among the different analyzed strains of Nile tilapia. The NTSIg1 pattern was observed at a high frequency in CESP, Pernambuco, and Santa Catarina strains, while the NTSIg2 pattern was mainly observed in Chitralada strain. Interestingly, the CESP, Pernambuco and Santa Catarina strains of Nile tilapia were originated from the same original stock from Ivory Coast (West Africa) while the Chitralada strain was originated from the Nile River, Alexandria (Egypt) (Zimmermann, 1999; Lovshin, 2000). The NTS I variations among the 'Brazilian' Nile tilapia strains indicate that these sequences are promising markers to be used in the identification of worldwide stocks used for fish production. In the same way, the 5S rDNA repeat size-variation, as reported for several fish species including the three tilapia samples hereby analyzed, can also find application in the identification of species of economic importance.

The 5S rDNA polymorphisms constitute suitable genetic markers candidates to be applied in the assessment of identification of fish species, strains and hybrids of economic or ecological importance. Once the NTSs evolve rapidly, the PCR technology can also be easily applied to amplify specific NTS regions that function as species- or strain-specific genetic markers. These sequences also have been useful for evolutionary studies and have contributed to the knowledge of the biology of fish species in a broad range of aspects. The present data demonstrated the potential applicability of the 5S rDNA sequences as genetic markers for the differentiation of several fish species, with emphasis on the identification of tilapiine cichlid fishes, and for the establishment of relationship among these species. In the same way, the complex organization of the 5S rDNA in the tilapia species is also of considerable value to the understanding of the evolutionary dynamics of repetitive DNAs and multigene families in fish and other vertebrate genomes.

Acknowledgements

The authors are grateful to C.R. Moreira and A.M. Schaedler (SEAB/PR – Secretaria da Agricultura e do Abastecimento do Paraná), Fazenda Araucária, Londrina/PR, and Piscicultura Tole-Peixes, Toledo/PR, for the tilapia samples provided, and FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), for financial support.

References

Altschul, S.F., W. Gish, W. Miller, E.W. Myers & D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403–410.

Amarasinghe, V. & J.E. Carlson, 1998. Physical mapping and characterization of 5S rRNA genes in gouglas-fir. Am. Gen. Assoc. 89: 495–500.

Baker, W.J., T.A. Hedderson & J. Dransfield, 2000. Molecular phylogenetics of *Calamus* (Palmae) and related rattan genera based on 5S rDNA spacer sequence data. Mol. Phyl. Evol. 14: 218–231.

Bardakci, F. & D.O.F. Skibinski, 1994. Application of the RAPD technique in tilapia fish: species and subspecies identification. Heredity 73: 117–123.

Baum, B.R. & L.G. Bailey, 1997. The molecular diversity of the 5S rRNA gene in *Kengyilia alatavica* (Drobov) J. L. Yang, Yen & Baum (Poaceae: Triticeae): potential genomic assignment of different rDNA units. Genome 40: 215–228.

Belkhiri, A., J. Buchko & G.R. Klassen, 1992. The ribosomal RNA gene in *Pythium* species: two different genomic locations. Mol. Biol. Evol. 9: 1089–1102.

Cronn, R.C., X. Zhao, A.H. Paterson & J.F. Wendel, 1996. Polymorphism and concerted evolution in a tandemly repeated gene family: 5S ribosomal DNA in diploid and allopolyploid cottons. J. Mol. Evol. 42: 685–705.

Deiana, A.M., A. Cau, S. Salvadori, E. Coluccia, R. Cannas, A. Milia & J. Tagliavini, 2000. Major and 5S ribosomal sequences of the largemouth bass *Micropterus salmoides* Perciformes, Centrarchidae are localized in GC-rich regions of the genome. Chromosome Res. 8: 213–218.

Drouin, G. & M. de Moniz Sá, 1995. The concerted evolution of 5S ribosomal genes linked to the repeat units of other multigene families. Mol. Biol. Evol. 12: 481–493.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783–791.

Frederiksen, S., H. Cao, B. Lomholt, G. Levan & C. Hallemberg, 1997. The rat 5S rRNA bona fide gene repeat maps to chromosome 19q12 $\rightarrow$ qter and the pseudogene repeat maps to 12q12. Cytogenet. Cell Genet. 76: 101–106.

Galls, F. & J.A.J. Metz, 1998. Why are there so many cichlid species? Trends Ecol. Evol. 13: 1–2.

Gornung, E., S. De Innocentiis, F. Annesi & L. Sola, 2000. Zebrafish 5S rRNA genes map to the long arms of chromosome 3. Chromosome Res. 8: 362.

Greenwood, P.H., 1991. Speciation, pp. 86–102. Cichlid Fishes: Behavior, Ecology and Evolution edited by M.H.A. Keenleyside. Chapman & Hall, New York.

Hallemberg, C., J. Nederby-Nielsen & S. Frederiksen, 1994. Characterization of 5S rRNA genes from mouse. Gene 142: 291–295.

Hanson, R.E., M.N. Islam-Faridi, E.A. Percival, C.F. Crane, Y. Ji, T.D. McKnight, D.M. Stelly & H.J. Price, 1996. Distribution of 5S and 18–28S rDNA loci in a tetraploid cotton (*Gossypium hirsutum* L.) and its putative diploid ancestors. Chromosoma 105: 55–61.

Kimura, M., 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111–120.

Komiya, H. & S. Takemura, 1979. Nucleotide sequence of 5S ribosomal RNA from rainbow trout (*Salmo gairdnerii*) liver. J. Biochem. 100: 369–374.

Komiya, H. & M. Hasegawa, 1986. Differentiation of oocyte – and somatic – type 5S rRNAs in animals. J. Biochem. 100: 369–374.

Kumar, S., K. Tamura, I.B. Jackobsen & M. Nei, 2001. MEGA2: molecular evolutionary genetics analysis sofware. Bioinformatics 17: 1244–1245.

Leah, R., S. Frederiksen, J. Engberg & P.D. Sorensen, 1990. Nucleotide sequence of a mouse 5S rRNA variant gene. Nucleic Acids Res. 18: 7441.

Lovshin, L.L., 2000. Tilapia culture in Brazil, pp. 133–140. A Tilapia culture in the Americas edited by A. Costa-Pierce & J.E. Rakocy. The World Aquaculture Society, Baton Rouge, Louisiana.

Martins, C., A.P. Wasko, C. Oliveira & J.M. Wright, 2000. Nucleotide sequence of 5S rDNA and localization of the ribosomal RNA genes to metaphase chromosomes of the tilapiine cichlid fish, *Oreochromis niloticus*. Hereditas 133: 39–46.

Martins, C. & P.M. Galetti Jr., 2001a. Organization of 5S rDNA in species of the fish *Leporinus:* two different genomic locations are characterized by distinct non-transcribed spacers. Genome 44: 903–910.

Martins, C. & P.M. Galetti Jr., 2001b. Two 5S rDNA arrays in Neotropical fish species: is it a general rule for fishes? Genetica 111: 439–446.

Martins, C. & A.P. Wasko, 2004. Organization and evolution of 5S ribosomal DNA in the fish genome, pp. 289–318. Focus on Genome Research edited by C.R. Williams. Nova Science Publishers, Hauppauge, NY.

Martins, C., A.P. Wasko, C. Oliveira, F. Porto-Foresti, P.P. Parise-Maltempi, J. Wright & F. Foresti, 2002. Dynamics of 5S rDNA in the tilápia (*Oreochromis niloticus*) genome: repeat units, inverted sequences, pseudogenes and chromosome loci. Cytogenet. Genome Res. 98: 78–85.

Morán, P., J.L. Martínez, E. Garcia-Vásquez & A.M. Pendás, 1996. Sex linkage of 5S rDNA in rainbow trout (*Oncorhynchus mykiss*). Cytogenet. Cell Genet. 75: 145–150.

Ota, K., Y. Tateno & T. Gojobori, 2003. Highly differentiated and conserved sex chromosome in fish species (*Aulopus japonicus*: Teleostei, Aulopidae). Gene 317: 187–193.

Pendás, A.M., P. Móran, J.P. Freije & E. Garcia-Vásquez, 1994. Chromosomal location and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rDNA. Cytogenet. Cell Genet. 67: 31–36.

Pendás, A.M., P. Morán, J.L. Martínez & E. Garcia-Vazquez, 1995. Applications of 5S rDNA in Atlantic salmon, brown trout, and in Atlantic salmon $\times$ brown trout hybrid. Mol. Ecol. 4: 275–276.

del Pino, E.M., C. Murphy, P.H. Masson & J.G. Gall, 1992. 5S rRNA-encoding genes of the marsupial frog *Gastrotheca riobambae*. Gene 111: 235–238.

Pullin, R.S.V., 1991. Cichlids in aquaculture, pp. 280–309. Cichlid Fishes: Behavior, Ecology and Evolution edited by M.H.A. Keenleyside. Chapman & Hall, New York.

Robles, F., R. De la Herran, A. Ludwig, C.R. Rejon, M.R. Rejon & M.A. Garrido-Ramos, 2005. Genomic organization and evolution of the 5S ribosomal DNA in the ancient fish sturgeon. Genome 48: 18–28.

Sadjak, S.L., K.M. Reed & R.B. Phillips, 1998. Intraindividual and interspecies variation in the 5S rDNA of coregonid fish. J. Mol. Evol. 46: 680–688.

Sambrook, J. & D.W. Russel, 2001. Molecular Cloning. A Laboratory Manual. Third Edition. Cold Spring Harbor Laboratory Press, New York.

Suzuki, H., K. Moriwaki & S. Sakurai, 1994. Sequences and evolutionary analysis of mouse 5S rDNAs. Mol. Biol. Evol. 11: 704–710.

Suzuki, H., S. Sakurai & Y. Matsuda, 1996. Rat 5S rDNA spacer sequences and chromosomal assignment of the genes to the extreme terminal region of chromosome 19. Cytogenet. Cell Genet. 72: 1–4.

Thompson, J.D., D.G. Higgins & T.J. Gibson, 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

Venkatesh, B., 2003. Evolution and diversity of fish genomes. Curr. Opin. Genet. Develop. 13: 588–592.

Wasko, A.P., C. Martins, J.M. Wright & P.M. Galetti Jr., 2001. Molecular organization of 5S rDNA in fishes of the genus *Brycon*. Genome 44: 893–902.

Wasko, A.P., C. Martins, C. Oliveira & F. Foresti, 2003. Non-destrutive genetic sampling in fish: an improved method for DNA extraction from fish fins and scales. Hereditas 138: 161–165.

Zimmermann, S., 1999. Incubação Artificial: técnica que permite a produção de tilápias do Nilo geneticamente superiores. Panor. Aqüic. mar./abr.: 69.