

## Nucleotide sequence of 5S rDNA and localization of the ribosomal RNA genes to metaphase chromosomes of the Tilapiine cichlid fish, *Oreochromis niloticus*

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In this study, we report the cloning and nucleotide sequence of PCR-generated 5S rDNA from the Tilapiine cichlid fish, *Oreochromis niloticus*. Two types of 5S rDNA were detected that differed by insertions and/or deletions and base substitutions within the non-transcribed spacer (NTS). Two 5S rDNA loci were observed by fluorescent in situ hybridization (FISH) in metaphase spreads of tilapia chromosomes. FISH using an 18S rDNA probe and silver nitrate sequential staining of 5S-FISH slides showed three 18S rDNA loci that are not syntenic to the 5S rDNA loci.

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In higher eukaryotes, ribosomal RNA (rRNA) genes are organized as two distinct multigene families comprised of tandemly-arrayed repeats composed of hundreds to thousands of copies. One class 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 (NTS). Multiple copies of this array correspond to the nucleolar organizer regions (NORs). The other class codes for the 5S rRNA and consists of a highly conserved coding sequence of 120 base pairs (bp) which is separated from each transcriptional unit by a NTS. Unlike the 45S rDNA, the 5S rDNA is not normally associated with nucleoli formation (reviewed in LONG and DAVID 1980). Although the 5S rDNA coding sequence is highly conserved, even between non-related species, variations in the NTS owing to insertions/deletions, minirepeats and pseudogenes have been frequently characterized in several organisms (NELSON and HONDA 1985; LEAH et al. 1990; SAJDAK et al. 1998). This variation in the NTS of 5S rDNA has been useful for evolutionary studies and served as species- or population-specific markers (SUZUKI et al. 1994; PENDÁS et al. 1995).

The arrangement of 5S rDNA genes has been extensively studied in plants and animals (VITELLI et al. 1982; FUKUI et al. 1994; LOMHOLT et al. 1995; PRADO et al. 1996; ADACCHI et al. 1997) and has yielded information about the evolution of this gene cluster as well as the species. Ribosomal RNA genes

can be either clustered at a single locus or found at multiple loci. In some eukaryotes, the 5S rRNA genes can be found interspersed with other multicopy genes, such as histone genes (ANDREWS et al. 1987) or 45S rDNA (DROUIN 1999). However, in most eukaryotes, the 5S rDNA is normally detected in areas of the genome distinct from the 45S rDNA and histone gene clusters (DROUIN AND MONIZ DE SA 1995).

In some organisms, the 5S rDNA genes are located on a single chromosome pair, while NORs are often present on multiple chromosomes (SUZUKI et al. 1996; MAKINEM et al. 1997). In amphibians (SCHMID et al. 1987; LUCCHINI et al. 1993) and some fish species (FUJIWARA et al. 1998; MURAKAMI and FUJITANI 1998), the 5S rRNA genes may be located on several chromosomes. Moreover, NOR and 5S rDNA loci may assume a syntenical organization in the same chromosome (PENDÁS et al. 1994; MÓRAN et al. 1996) or can be detected in different chromosome pairs (MARTÍNEZ et al. 1996; SAJDAK et al. 1998; MARTINS and GALETTI 1999). Although chromosomal localization of 5S rDNA has been reported in several fish species, data are limited, primarily, to species of the Salmoniformes (PENDÁS et al. 1994; MÓRAN et al. 1996; FUJIWARA et al. 1998; SAJDAK et al. 1998) and Cypriniformes (MURAKAMI and FUJITANI 1998).

The tilapiine tribe of cichlid fishes has received increasing scientific interest because of their rapid adaptive radiation has led to extensive ecological diversity, and their enormous importance to tropical and subtropical aquaculture, especially the Nile

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tilapia, *Oreochromis niloticus* (PULLIN 1991). Although a genetic map for *O. niloticus* based on polymorphic DNA markers has recently been published (KOCHER et al. 1998), limited molecular cytogenetic data are available for this species (OLIVEIRA et al. 1999). To further our understanding of the organization of the tilapiine genome, and to assist in the construction of a physical map for this species, the nucleotide sequence and the chromosome distribution of the 5S rDNA and its relation to the large rDNA genes were investigated.

## MATERIALS AND METHODS

Ten adult specimens (5 females and 5 males) of *O. niloticus* were obtained from the tilapia culture facility at Dalhousie University. DNA was extracted from the liver according to SAMBROOK et al. (1989) and PCR amplifications of the 5S rDNA were performed as described by PENDÁS et al. (1994). Primers A (5'-TACGCCCCGATCTCGTCCGATC-3') and B (5'-CAGGCTGGTATGGCCGTAAGC-3') were designed from the 5S rRNA sequence of rainbow trout (KOMIYA and TAKEMURA 1979) to amplify the 5S genes and their NTS. The PCR-amplified products were cloned in the plasmid pGEM-T (Promega) and used to transform competent cells of the *E. coli* strain, DH5 $\alpha$  (Gibco Life Technologies). Clones were sequenced on a LICOR 4200 automatic sequencer with the T7 Sequenase 7-deza-dGTP sequencing kit (Amersham) and the alignment of sequences was performed 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 (USA), website (<http://www.ncbi.nlm.nih.gov/blast>).

The genomic organization of 5S rDNA was determined by Southern blot-hybridization. Genomic DNA (10  $\mu$ g) was completely digested with *Hind*III or *Rsa*I at 37°C overnight or partially digested for 10 or 30 min with *Hind*III. DNA digests were subjected to gel-electrophoresis in 1% agarose and transferred to Hybon-N nylon membrane (Amersham) according to SAMBROOK et al. (1989). Inserts of the 5S clones were randomly labelled with [ $\alpha$ -<sup>32</sup>P]-dCTP using the Random Primers DNA Labelling System (Gibco Life Technologies). Hybridization of the filter-immobilized DNA was according to SAMBROOK et al. (1989).

Mitotic chromosomes were obtained from anterior kidney cells as described by BERTOLLO et al. (1978). Fluorescent in situ hybridization (FISH) was conducted using an 18S rDNA fragment from tilapia (supplied by Dr Greg Booton) and PCR-generated 5S rDNA obtained in the present study as probes that

were labelled by nick translation with biotin-dATP (Bionick™ Labelling System-Gibco Life Technologies). Prior to hybridization, the chromosome slides were incubated in RNase (40  $\mu$ g/ml in 2  $\times$  SSC) at 37°C for 1.5 h, dehydrated in an ethanol series, denatured in 70% formamide at 70°C for five minutes and dehydrated once in an ice cold ethanol series. After 10 min denaturation in boiling water, a hybridization mixture with 130 ng of probe, 50% formamide, 10% dextran sulfate and 2  $\times$  SSC, was applied to each slide under a glass coverslip. Hybridization was performed overnight in a moist chamber at 37°C. The slides were then washed in 50% formamide at 37°C (15 min), twice in 2  $\times$  SSC (15 min) and twice in 4  $\times$  SSC (5 min). Avidin-fluorescein isothiocyanate (FITC) conjugate (Oncor) was added to the slides and incubated at 37°C for 45 min and then washed in block buffer (0.15 M NaHCO<sub>3</sub>, 6 mM sodium citrate, 0.13% Tween 20 and 1% non-fat dried milk) at 42°C. The hybridization signal was enhanced using biotinylated anti-avidin antibody (Oncor) and FITC treatments. Chromosomes were counterstained with propidium iodide/antifade solution (Oncor). Sequential silver nitrate (Ag-) staining (HOWELL and BLACK 1980) was performed after rinsing the 5S FISH slides in tap water followed by dehydration in an alcohol series.

## RESULTS AND DISCUSSION

PCR amplification of 5S rDNA in *O. niloticus* generated one band of approximately 500 bp. DNA sequences were determined for 10 positive clones (six from one fish and four from another) and a consensus sequence was produced based on their alignment (Fig. 1), which enabled us to identify a highly conserved region that corresponds to the 5S rRNA gene (ALTSCHUL et al. 1990). The 5S rDNA NTS, however, were highly variable. Based on nucleotide sequence, two distinct 5S rDNA units were detected for tilapia, characterized by insertions/deletions and base substitutions, mainly in the NTS. A type I 5S rDNA is represented by clones 2.4, 4.3, 4.2, 4.5, 2.8, 2.9 and 4.8, while the type II 5S rDNA is represented by clones 2.1, 4.1 and 4.7 (Fig. 1). 5S rDNA spacer variations have been well characterized in several plants of agricultural interest and are very useful as genetic markers to distinguish closely-related species, subspecies, lines and hybrids (GOTTLÖB-MCHUGH et al. 1990; ZANKE et al. 1995; CRONN et al. 1996; DANNA et al. 1996). Among animals, considerable variation in the sequence and length of 5S rDNA NTS has been described for mouse (SUZUKI et al. 1994), *Salmo salar* and *Salmo trutta* (PENDÁS et al. 1995), showing that the 5S rDNA length polymor-

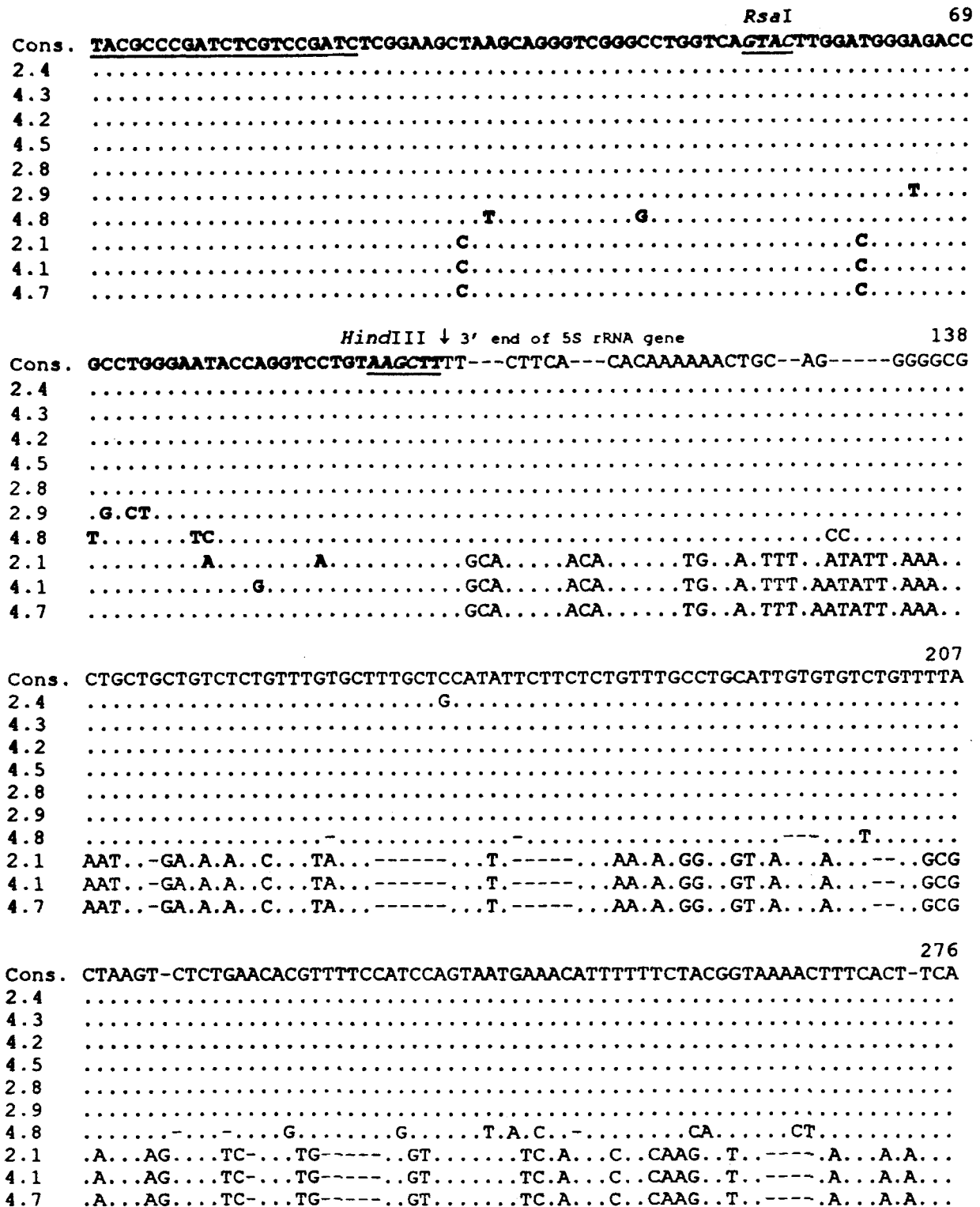


Fig. 1. Sequence alignment of 5S rDNA clones of *O. niloticus*. The coding sequence of 5S rDNA is in boldface type and the primers used to amplify the 5S rDNA are underlined. Dots indicate sequence identity and hyphens represent gaps. Restriction sites used in this study are in italics, underlined and indicated by the enzymes *HindIII* and *RsaI*. The sequence of each clone is deposited in GenBank under accession numbers: AF176349, AF176497-AF176505.

345

Cons. GCTTTAGCTTCAGCATAGAA-----AGCTGTTTA----CAGTAACACATCACTGATCTTAGATCGGAAT  
 2.4 .....  
 4.3 .....  
 4.2 .....  
 4.5 .....  
 2.8 .....  
 2.9 .....  
 4.8 .....  
 2.1 -. .C. .CA.G. -. .G. .C.CCTGT. .A. . . . .TTGTG. .AAC. .T. .CA. . . . .- .T.AT. .CGT. . . . .  
 4.1 -. .C. .CA.G. -. .G. .C.CCTGT. .A. . . . .TTGTG. .AAC. .T. .CA. . . . .- .T.AT. .CGT. . . . .  
 4.7 -. .C. .CA.G. -. .G. .C.CCTGT. .A. . . . .TTGTG. .AAC. .T. .CA. . . . .- .T.AT. .CGT. . . . .

414

Cons. AGTATGCAGCTGGCTGTTAA--AATCATCATATTTATT---TAA-----GTAACAAAACAAACTG--  
 2.4 .....  
 4.3 .....  
 4.2 .....G.....T.....  
 4.5 .....G.....TT  
 2.8 .....G.....  
 2.9 .....C.....  
 4.8 .....TT  
 2.1 --.T.....--A....GT.TG...T...AC.G.GCTG.C.TGTGCCG...TT....-...AG.AA  
 4.1 --.T.....--A....GT.TG...T...AC.G.GCTG.C.TGTGCCG...TT....-...AG.AA  
 4.7 --.T.....--A..T.TG.TG...-...AC.G.GCTG.C.TGTGCCG...TT....-...AG.AA

483

Cons. --AAGACA-TCCTCAAGATA--TAAGTCTGCGAAATGCTCCTGAAG--CAGTAATAAAAGGGGT--GT  
 2.4 .....G.....  
 4.3 .....G.....  
 4.2 .....  
 4.5 TG.....  
 2.8 .....G.....  
 2.9 .....G.....  
 4.8 TG.....  
 2.1 GG....A.C.AA....T..CCT.C.C...--A..T..T.TT.T..AT...C-.....T...T.ACA..  
 4.1 GG....A.C.AA....T..CCT.C.C...--A..T..T.TT.T..AT...C-.....T...T.ACA..  
 4.7 GG....A.C.AA....T..CCT.C.C...--A..T..G.TT.T..ATT..C-...-...TA....ACA..

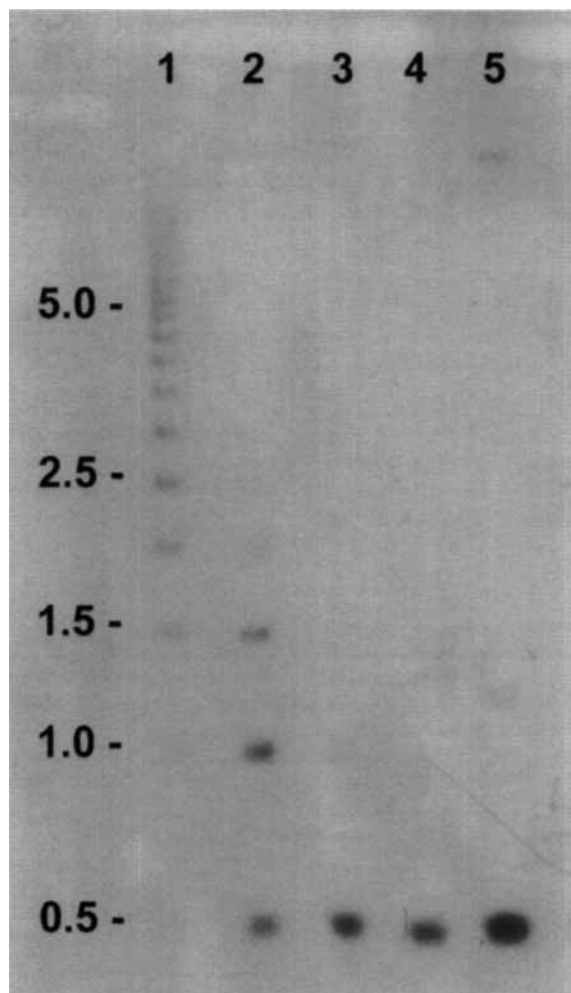
↓ 5' end of 5S rRNA gene 539

Cons. TTGTC-----GTCCTCG-----CTGTGGGCTTACGGCCATAACCAGCCTG  
 2.4 .....  
 4.3 .....  
 4.2 .....  
 4.5 .....  
 2.8 .....  
 2.9 .....  
 4.8 .....  
 2.1 A.C.ACATGAACCCT..TA....AGGAAGC..C.C.....  
 4.1 A.C.ACATGAACCCT..TA....AGGAAGC..C.....  
 4.7 A.C.CTAGGAACCCGT.TA..TCAGGAAGC.--C.....

Fig. 1. (continued)

phism may serve as a genetic marker for identification of species and hybrids. Even though a population analysis was not performed in the present work, the intra-individual NTS variation

detected in *O. niloticus* suggests that 5S rDNA polymorphism may provide a useful tool for species, populations and hybrids identification in this fish species.



**Fig. 2.** Genomic organization of the *O. niloticus* 5S rDNA clusters determined by Southern blot and hybridization. Aliquots of 10  $\mu$ g of genomic DNA were digested with the restriction endonucleases, *Hind*III (1,2,3) or *Rsa*I (4). 1 and 2 are partial digestion (10 and 30 minutes, respectively) and 3 and 4 are complete digestion (overnight). Molecular weight markers (Kb) are shown on the left.

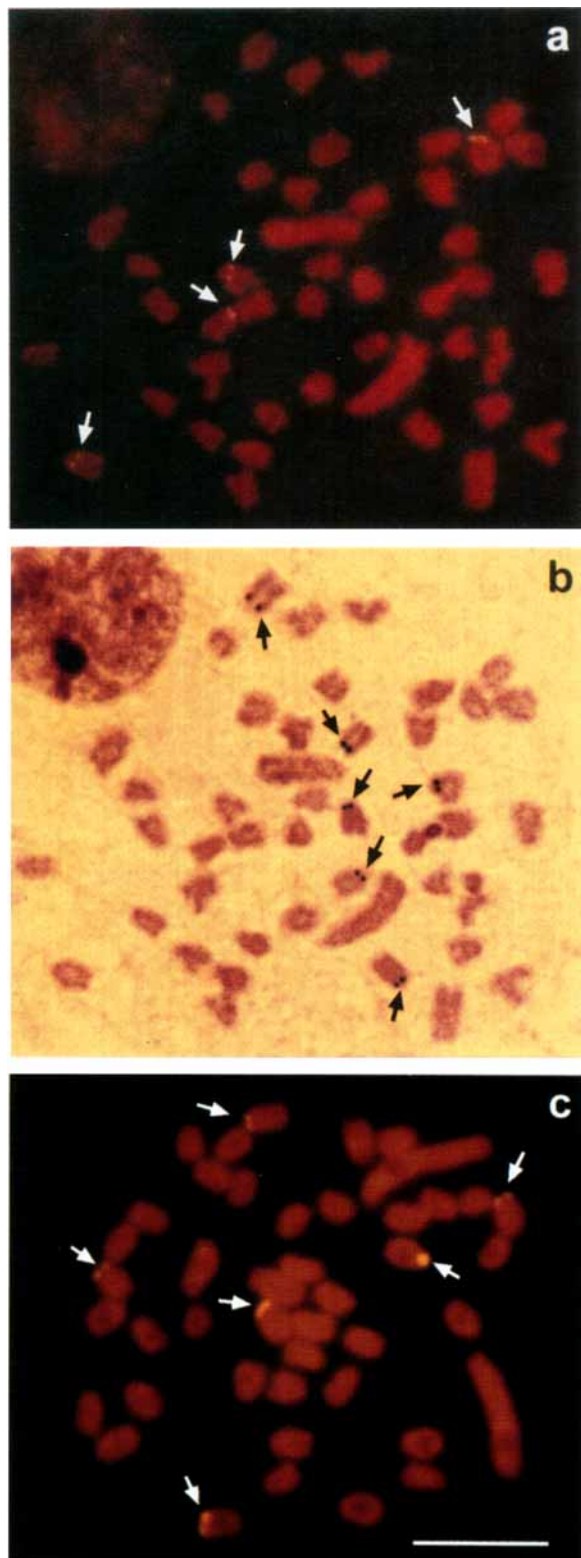
To determine if the genomic organization of the 5S rDNA was consistent with the PCR products and sequences obtained, we performed Southern blot hybridization experiments. Complete genomic DNA digestion with *Hind*III or *Rsa*I, restriction endonucleases that only cleave once in the 5S rDNA (Fig. 1), showed that the 5S rDNA genes are organized in monomers of approximately 500 bps (Fig. 2). Evidence that the tilapia 5S rDNA is organized in tandem-arrays was demonstrated by Southern blot-hybridization using genomic DNA partially digested with *Hind*III (Fig. 2), which enabled us to detect a ladder of bands of exact integers of the 500 bp 5S rDNA monomer. At least 16 multimers in the ladder were detected in the partial digestion. Additional evidence that the 5S rDNA exists as a tandem array

in the tilapia genome is supported by the PCR-amplification studies: PCR-amplification products could result only if at least two rDNA units are adjacent to each other.

The 5S rRNA gene is transcribed by RNA polymerase III and it contains an internal control region (ICR) which functions as a promoter for the gene (HALLENBERG et al. 1994). It has been recently demonstrated that the 5'-flanking sequence plays an important role in regulation of 5S rRNA gene expression in several mammals (NEDERBY-NIELSEN et al. 1993; SUZUKI et al. 1996). A TATA sequence has been observed in some fish species, upstream of the gene (FELGENHAUER et al. 1990; PENDÁS et al. 1994; SAJDAK et al. 1998) and a similar 5'-flanking sequence is present in the tilapia 5S rRNA genes (position 466 at the consensus sequence in Fig. 1). As found in the 5S rDNA of *Xenopus* (KORN 1982), a T-rich sequence was also identified at the 3'-end of the 5S rRNA coding sequence of *O. niloticus*, similar to the 5S rDNA termination signal found in a variety of other genes transcribed by RNA polymerase III (LITTLE and BRAATEN 1989).

Previous cytogenetic studies have shown that the haploid genome of *O. niloticus* consists of 22 chromosomes (KORNFELD et al. 1979; MAJUMBAR and MCANDREW 1986) and that the NOR regions are present in three chromosome pairs (FORESTI et al. 1993). Fluorescent in situ hybridization revealed 5S clusters located in the small arm of a submetacentric chromosome pair and interstitially in the long arm of another submetacentric pair (Fig. 3a). The possibility that small clusters of a few 5S genes at other loci may have gone undetected using the FISH protocol used here, however, can not be eliminated. Dispersed 5S genes have been described in other organisms (ROSENTHAL and DOERING 1983; NELSON and HONDA 1985). Although mammals are characterized by having only one 5S rRNA locus (LOMHOLT et al. 1995, 1996; SUZUKI et al. 1996; MAKINEM et al. 1997; CHRISTENSEN et al. 1998), multiple 5S rDNA loci have been detected in several fish species (MÓRAN et al. 1996; FUJIWARA et al. 1998; MURAKAMI and FUJITANI 1998; MARTINS and GALETTI 1999) and amphibians (VITELLI et al. 1982; SCHMID et al. 1987; LUCCHINI et al. 1993).

Sequential Ag-staining of 5S-FISH slides (Fig. 3b) and FISH analysis using biotinylated 18S probes (Fig. 3c) identified 6 positive sites (3' labeled-chromosome pairs) in tilapia chromosomes. The sequential Ag-staining showed that 5S rDNA loci and 18S rDNA loci are distributed on different chromosomes (Fig. 3b). Different chromosomal sites for NOR and 5S rDNA loci have already been reported for *Anguilla anguilla* (MARTÍNEZ et al. 1996), *S. trutta*



**Fig. 3a–c.** Fluorescent in situ hybridization (FISH) of chromosome metaphase spreads of tilapia to 5S (a) and 18S (c) rDNA probes. Sequential Ag-NOR of 5S-FISH is shown in b. Arrows indicate the 5S rDNA loci, the NORs and the 18S rDNA loci in a, b and c, respectively. Scale bar = 8  $\mu$ m.

(MÓRAN et al. 1996), *Coregonus artedti*, *Coregonus zenithicus* (SAJDAK et al. 1998), *Leporinus elongatus*, *Leporinus obtusidens* and *Leporinus friderici* (MARTINS and GALETTI 1999), an arrangement thus far most frequently observed in vertebrates (LUCCHINI et al. 1993; SUZUKI et al. 1996). For *S. salar* and *Oncorhynchus mykiss* (PENDÁS et al. 1994; MÓRAN et al. 1996), however, the 5S and 45S rDNA loci have been shown to be located on the same chromosome.

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