RESEARCH ARTICLE

Chromosomal localization of rDNA genes and genomic organization of 5S rDNA in *Oreochromis mossambicus*, *O. urolepis hornorum* and their hybrid

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Abstract

In this study, classical and molecular cytogenetic analyses were performed in tilapia fishes, *Oreochromis mossambicus* (XX/XY sex determination system), *O. urolepis hornorum* (WZ/ZZ sex determination system) and their hybrid by crossing *O. mossambicus* female × *O. u. hornorum* male. An identical karyotype ((2*n* = 44, NF (total number of chromosomal arms) = 50) was obtained from three examined tilapia samples. Genomic organization analysis of 5S rDNA revealed two different types of 5S rDNA sequences, 5S type I and 5S type II. Moreover, fluorescence *in situ* hybridization (FISH) with 5S rDNA probes showed six positive fluorescence signals on six chromosomes of all the analysed metaphases from the three tilapia samples. Subsequently, 45S rDNA probes were also prepared, and six positive fluorescence signals were observed on three chromosome pairs in all analysed metaphases of the three tilapia samples. The correlation between 45 rDNA localization and nucleolar organizer regions (NORs) was confirmed by silver nitrate staining in tilapia fishes. Further, different chromosomal localizations of 5S rDNA and 45S rDNA were verified by two different colour FISH probes. Briefly, the current data provide an insights for hybridization projects and breeding improvement of tilapias.

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Introduction

Tilapia is an important fishery resource, some species, such as *Oreochromis niloticus, O. mossambicus, O. aureus*, and their hybrids have become important aquaculture fishes that are now distributed worldwide in tropical and subtropical regions (Martins *et al*. 2004). Around the world, aquaculture industries have rapidly increased the production of tilapia from 0.6 million tons in 1994 to 2.6 million tons in 2007 (Kevin 2008). Because tilapia males grow much faster than the females, most of the breeding programmes have been designed to produce male populations by crossing related species, such as *O. mossambicus* females with *O. urolepis hornorum* males, and *O. niloticus* females with *O. aureus* males (Hickling 1960; Fishelson 1966), or by sex-reversed broodstocks (Tave 1995). The extreme ecological diversity and rapid adaptive radiation of tilapia fishes have led them to be considered as a promising model system for understanding the genetic basis of vertebrate speciation and evolution (Kocher 2004).

Chromosome banding techniques and fluorescent *in situ* hybridization (FISH) have been demonstrated to be useful cytogenetic methods for chromosome identification, which has allowed further resolution of similar size chromosomes (Martins and Galetti 2001; Yi *et al*. 2003; Affonso and Pedro 2005; Zhu *et al*. 2006; Zhu and Gui 2007; Morescalchi *et al*. 2008; Mazzuchelli and Martins 2009). In fish, the 5S rDNA multi-gene family consists of a highly conserved coding sequence of 120 bp forming arrays of hundreds to thousands

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of tandem copies that are separated from each other by variable non-transcribed spacers (NTSs) (Long and David 1980). Their fluorescent location on the chromosomes can be easily detected because of the numerous copies of these highly conserved families of repeated sequences. Studies on 5S rDNA organization could provide useful data for understanding genomic organization and molecular evolution of repetitive sequences, and also provide genetic markers for the identification of species, subspecies, population, strain, or hybrids (Martins *et al*. 2004). Although 5S rDNA repeats have been characterized in some tilapiine cichlid fish, present data are mostly focused on the fish *O. niloticus* (Martins *et al*. 2002, 2004).

Orecochromis mossambicus and *O. u. hornorum* have different sex determination systems of XX-female / XY-male and WZ-female / ZZ-male, respectively, and their hybrid (*O. mossambicus* female × *O. u. hornorum* male) has shown high percentage of males with a high growth rate (Hickling 1960; Yang *et al*. 2008). Cytogenetic studies revealed that the haploid genome of *O. mossambicus* and *O. u. hornorum* consists of 22 chromosomes and conventional karyotype was reported in *O. mossambicus* (Fukuoka and Muramoto 1975; Thompson 1981; Chen and Chen 1983). In this study, we performed karyotype analysis, cloned and analysed the repeated sequences and genomic organization of 5S rDNA, localized 5S rDNA and 45S rDNA chromosomal sites, revealed the correlation of 45S rDNA chromosomal locations with nucleolar organizer regions (NORs) in *O. mossambicus, O. u. hornorum* and their hybrids.

Materials and methods

Genomic DNA extraction

In the present study we analysed three tilapia samples: *O. mossambicus* (six males and eight females), *O. u. hornorum* (nine males and nine females), and their hybrid *O. mossambicus* female × *O. u. hornorum* male (16 males), weight ranging from 200 to 260 g, were obtained from the Gaoyao Aquaculture Germplasm Conservation Station, Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences. Genomic DNA was extracted from whole blood according to the standard phenol–chloroform procedures (Sambrook and Russell 2001).

Isolation of 5S rDNA and 45S rDNA sequences

One pair of primer (P_1, 5′-AATACGCCCGATCTCGTCCGA-3′ and P_2, 5′- CAGGCTGGTATGGCCGTAAGC-3′) was designed and synthesized to amplify the 5S rDNA repeats directly from genomic DNA by PCR, following the method described by Morescalchi *et al*. (2008). The primer pair P_1 and P_2 was used to amplify the region between the 3′ end of one 5S gene unit and the 5′ end of the following unit. Therefore, each amplified fragment was composed of one 5S gene unit and the NTS. The PCR amplification of the partial sequences of 45S rDNA was carried out using primers P_3 (5′-AGCATATGCTTGTCTCAAAG-3′) and P_4 (5′-ACGACTTTTACTTCCTCTAG-3′) designed according to the 45S rDNA conservative regions of *Cyprinus carpio* (GenBank accession number: AF133089.2). The amplification reaction mixture (25 μL) consisted of 2 ng of genomic DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.1 μM each dNTP, 0.2 μM each primer, and 0.5 unit of *Taq* polymerase (Advanced Biotechnology, Epsom, UK). The temperature profile for 5S rDNA was: initial denaturation step at 94°C for 4 min, followed by 25 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with the final extension step at 72°C for 10 min. For 45S rDNA, each cycle consisted of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Amplification products were separated on a 1% agarose gel using TBE buffer. The DNA fragments purified by the BioStar Grass milk DNA Purification Kit (BioStar International, Toronto, USA), cloned in the pMD19-T cloning vector (Promega, Madison, USA), sequenced by Big DyeTM terminators and analysed on an ABI-3730 auto-sequencer (Applied Biosystems, Foster City, USA).

Sequence analysis

BLAST searching of sequences was performed on the web server of the National Center for Biotechnology Information http://www.ncbi.nih.gov/BLAST. The sequences were aligned with software Vector NTI suite 8.0 program (Invitrogen, Carlsbad, USA).

Chromosome preparation and silver staining

Chromosome preparations were obtained from cephalic kidney by the method of kidney cell-phytohemagglutinin (PHA) culture *in vivo* as described by Gui (1999) and were submitted to the Giemsa staining, Ag-NOR staining (Yu *et al*. 1989) and FISH (Zhu *et al*. 2006; Zhu and Gui 2007).

Fluorescence in situ hybridization (FISH)

Chromosomal localization of 5S rDNA and 45S rDNA were performed by FISH according to the method described by Zhu *et al*. (2006). In brief, the purified PCR product of rDNA labelled with Dig-11-dUTP by nick translation kit (Roche, Mannheim, Germany) were used as probes. After treating with RNase A, the slides with chromosome metaphase spreads were denatured in 70% deionized formamide/2× SSC for 2 min at 70°C, dehydrated in a 70, 90 and 100% ethanol series for 3 min each and air dried. One hundred nanograms of labelled probes were denatured for 10 min in boiling water and then were placed on the slides carrying denatured metaphase chromosomes under a 24×50 mm^2 coverslip. Hybridization was carried out overnight at 37°C in a moist chamber. The slides were then washed for 10 min each in 2× SSC, 2× SSC with 50% formamide, 0.1× SSC with 0.1% Tween 20, pH 7.0, and then 3×5 min at room temperature with 1× PBS. After a series of post-hybridization washes were performed, the spectrum signals were achieved with 50 μL of FITC-conjugated antidigoxigenin antibody

from sheep (Roche, Mannheim, Germany) at 5 μg/mL. Chromosomes were counterstained with DAPI (4.6-diamino-2-phenylindole, 0.5 μg/mL) in antifade solution, which produced adequate bands for chromosome identification. Moreover, 5S rDNA and 45S rDNA FISH were simultaneously performed using biotin-labelled 5S rDNA probe and digoxigenated 45S rDNA probe, and detected via CY3 and fluorescein isothiocyanate (FITC), respectively.

Images were acquired using Leica inverted DMIRE2 epifluorescence microscope equipped with a charge-coupled device (CCD) camera and a Leica LCS SP2 confocal image system (Leica, Heidelberg, Germany). DAPI, FITC and Cy3 fluorescences were detected using appropriate filter sets (DAPI filter cube: excitation 340/40 nm, emission 430/50 nm; FITC filter cube: excitation 480/20 nm, emission 510/30 nm; Cy3 filter cube: excitation 545/20 nm, emission 580/70 nm). Captured images were coloured and overlapped in Adobe Photoshop 7.0. At least 15 metaphases were analysed for each sample.

Results

Karyotype characterization of the three tilapia samples

The mitotic karyotypes were determined by the analysis of 20 metaphase spreads from each individual. The karyotypes of *O. mossambicus*, *O. u. hornorum* and their hybrid are all composed of 22 pairs ($2n$ = 44; NF (total number of chromosomal arms) = 50) chromosomes, in which three chromosome pairs were submetacentric, 12 chromosome pairs were subtelocentric and seven chromosome pairs were telocentric. The first telocentric pair (pair 1) is the largest chromosome, and is twice as large as the second large chromosome (pair 2). In comparison with the karyotypes of the males and females of the three samples, no morphologically distinguishable sex chromosomes were observed in each samples, and there were no visible differences between parental and hybrid individuals. Figure 1 shows the representative metaphase spread and the corresponding karyotypes from the hybrid.

Genomic organization and chromosomal localization of 5S rDNA sequences

PCR amplification of 5S rDNA of *O. mossambicus*, *O. u. hornorum* and their hybrid, with the set of primers P_1 and P_2 generated one band of about 500 bp. The PCR products were cloned and a total of 11 clones for *O. mossambicus*, 12 for *O. u. hornorum* and 11 for the hybrid were sequenced. The sequences ranging in size from 476 to 493 bp include a 99-bp 5S rDNA encoding region, 356–373-bp NTS region and 21-bp 5S rDNA encoding region. Similarly data reported by Alves-Costa *et al*. (2006), two types of 5S rDNA sequences, referred to as 5S rDNA type I (nine clones for *O. mossambicus*, eight clones for *O. u. hornorum* and ten clones for the hybrid) and type II (two clones for *O. mossambicus*, four clones for *O. u. hornorum* and one clone for the hybrid), were also detected among these clones of 5S rDNA in the analysed tilapia samples. As shown in figure 2A, their identity between type I (492 bp) and type II (501 bp) is 55.9%. Moreover, we aligned and compared the nucleotide sequences of six representative type I 5S rDNA fragments from each analysed tilapia samples. As shown in figure 2B, their identities range from 94% to 100%, and the typical type I promoter elements for the RNA polymerase III is located at the position from +29 to +76 bp, which includes three conserved sequences, A-box (AGCTAAGCAGGGTCG), intermediate element (GCCTGGT) and C-box (TGGATGGGAGACCGCCTG) in the 5S rDNA encoding regions. One TATA-box (GTAATAAAAGG) exists at 3′ of the non-transcribed spacer, and one cluster of five T residues is present at 5′ of the non-transcribed spacer, which could serve as sites of RNA polymerase termination. No other regulatory elements were found in these sequences.

Subsequently, the 5S rDNA fluorescent probes were prepared from the above cloned 5S rDNA repeated sequences, and chromosome localization was performed by FISH on metaphase chromosomes of the three samples of tilapia, *O. mossambicus*, *O. u. hornorum*, and their hybrid. As shown in figure 3, six chromosomes display characteristic green

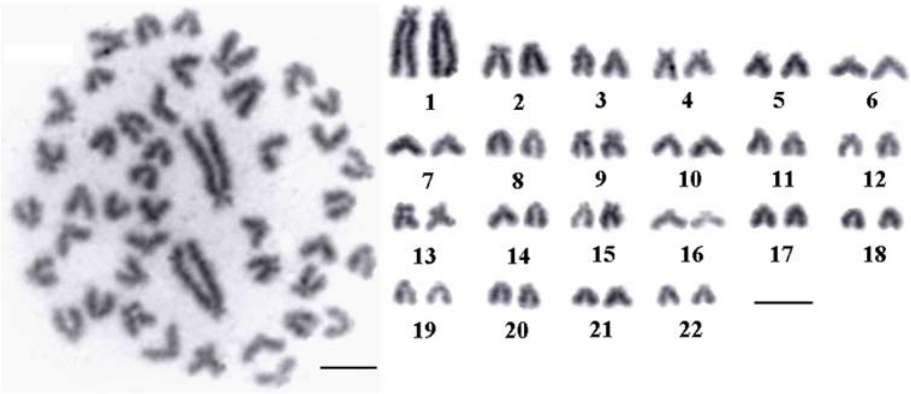

Figure 1. (A) The representative giemsa-stained metaphase spread and (B) the corresponding karyotype in the hybrid of *Oreochromis mossambicus* female × *Oreochromis urolepis hornorum* male. Scale bar = 10 μm.

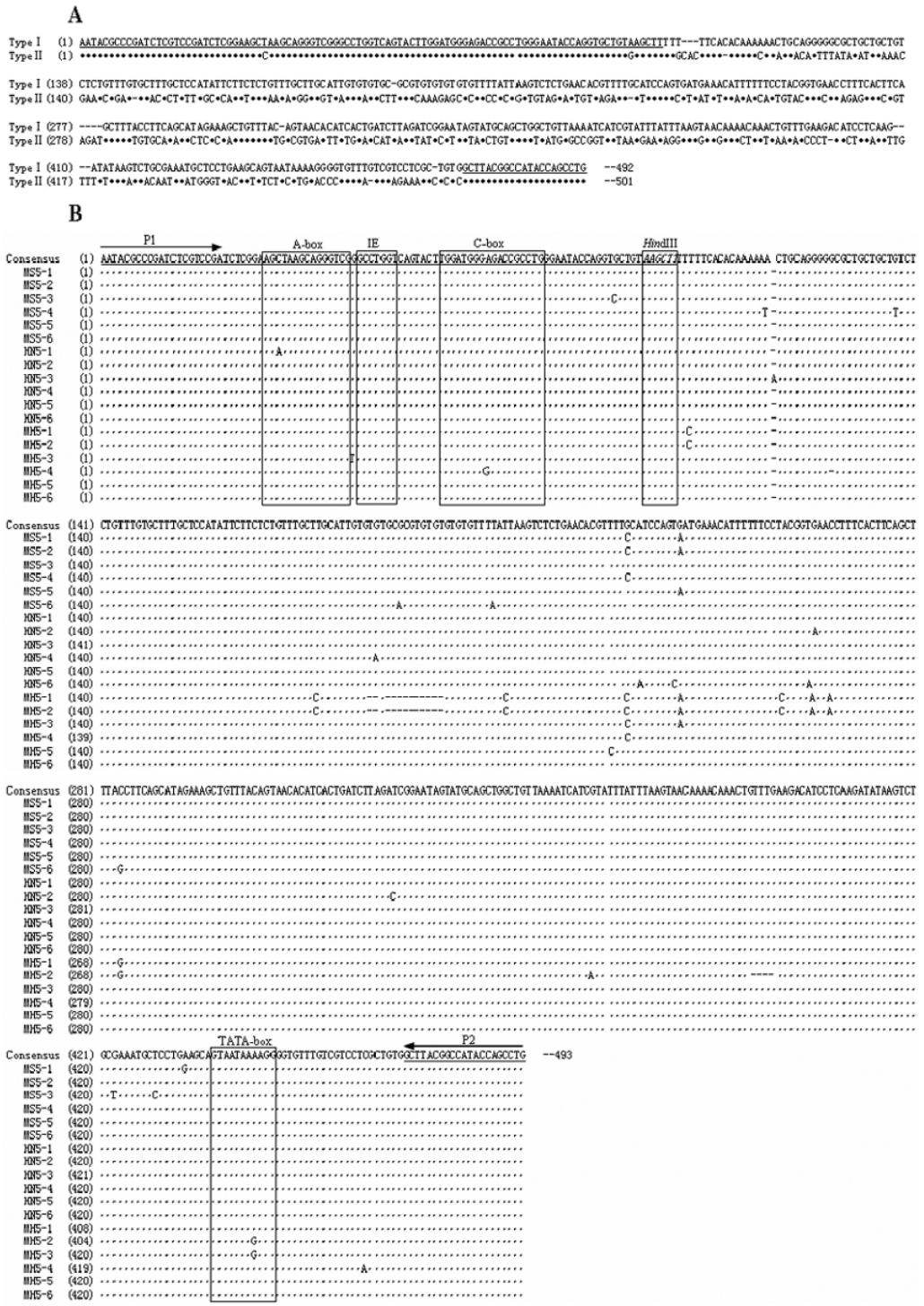

Figure 2. (A) Sequence alignment of two types of 5S rDNA sequences of *O. u. hornorum* representing the 5S rDNA type I (type I) and the 5S rDNA type II (type II) (GenBank accession numbers GU075925) identified in the analysed tilapias. The 5S rRNA gene coding sequence is underlined. Dots indicate sequence identity and hyphens represent insertions/deletions. (B) Nucleotide sequence alignment of six sequenced 5S rDNA fragments (type I) in the three analysed tilapia samples. The 5S rRNA gene coding region is underlined, and the *Hind*III recognition sites (AAGCTT) are indicated by boxes and italics. The regulatory sequences (A-box (AGCTAAGCAGGGTCG), intermediate element (GCCTGGT) and C-box (TGGATGGGAGACCGCCTG) and TATA-box (GTAATAAAAGG)) are included in boxes. The PCR primers (P_1, P_2) are shown by arrows. MS, *O. mossambicus*; HN, *O. u. hornorum*; MH, *O. mossambicus* female × *O. urolepis hornorum* male. The sequences have been submitted to GenBank under accession numbers from GU075907 to GU075924. Dots indicate sequence identity and hyphens represent insertions/deletions.

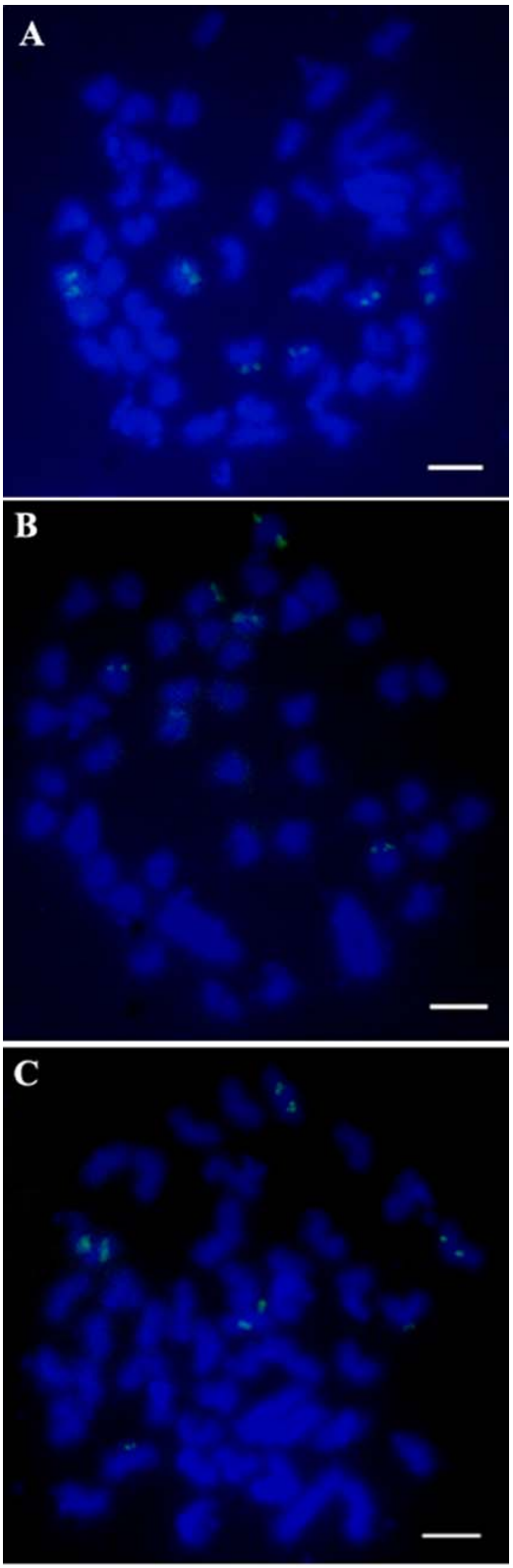

Figure 3. (A) The FISH-stained metaphases of *O. mossambicus*, (B) *O. u. hornorum* and (C) the hybrid of *O. mossambicus* female × *O. u. hornorum* male with 5S rDNA probes. The metaphase was counterstained blue fluorescence with DAPI to visualize chromosome morphology. Arrows show the positive green signals. Scale bar = 10 μm.

fluorescence signal in all analysed metaphases of *O. mossambicus* (figure 3A), *O. u. hornorum* (figure 3B), and their hybrid (figure 3C), in which four fluorescence signals exist in the interstitial position of long arm of two pairs of subteloacrocentric chromosomes, and two locates on the short arm terminal of one subteloacrocentric chromosome pair.

Chromosomal location of 45S rDNA and its correlation with nucleolar organizer regions

From total tilapia genomic DNA, one band of 1780-bp 45S rDNA fragment was amplified by PCR and verified by the partial sequencing. Then, the amplified product was prepared as probe, and used to chromosome localization by FISH on metaphase chromosomes of the three samples of tilapia. As shown in figure 4, six positive green fluorescence signals are obviously localized to telomeric area of three chromosome pairs in all analysed metaphases of *O. mossambicus* (figure 4A), *O. u. hornorum* (figure 4B), and their hybrid (figure 4C). Moreover, we detected NORs on metaphase chromosomes of the three tilapia samples by Ag-NORs staining (Zhu and Gui 2007). Significantly, six similar size silver-stained NORs were observed on the six identical chromosomes with 45S rDNA positive signals in all analysed metaphases of *O. mossambicus* (figure 4D), *O. u. hornorum* (figure 4E), and their hybrid (figure 4F). Therefore, the correlation between 45 rDNA localization and the NORs was revealed in the tilapia fishes.

Confirmation of different chromosomal localizations of 5S rDNA and 45S rDNA

In order to determine whether the 5S rDNA and 45S rDNA genes are localized to the same or different chromosomes, two different colour FISH probes of 5S rDNA-labelled with biotin (stained by CY3, red) and 45S rDNA-labelled with digoxigenin (stained by FITC, green) were prepared, and used to detect the same metaphase spreads. As shown in figure 5, two different colour signals appear on 12 different chromosomes in the representative metaphase of *O. u. hornorum*. The 5S rDNA red fluorescence is present on six chromosomes, and the 45S rDNA green fluorescence appears on six other different chromosomes. The data confirm that the 5S rDNA and 45S rDNA genes are distributed on different chromosomes and the distinct chromosome localization can be used to demarcate six different chromosome pairs in all tilapia karyotypes.

Discussion

In this study, we analysed the karyotypes of *O. mossambicus*, *O. u. hornorum* and their hybrid, and observed that all of them consist of 44 chromosomes, and have the same karyotypic formula (NF = 50). No differences were observed between male and female karyotypes. The identical karyotypes among different species and their hybrids had been reported in the majority of *Oreochromis* species

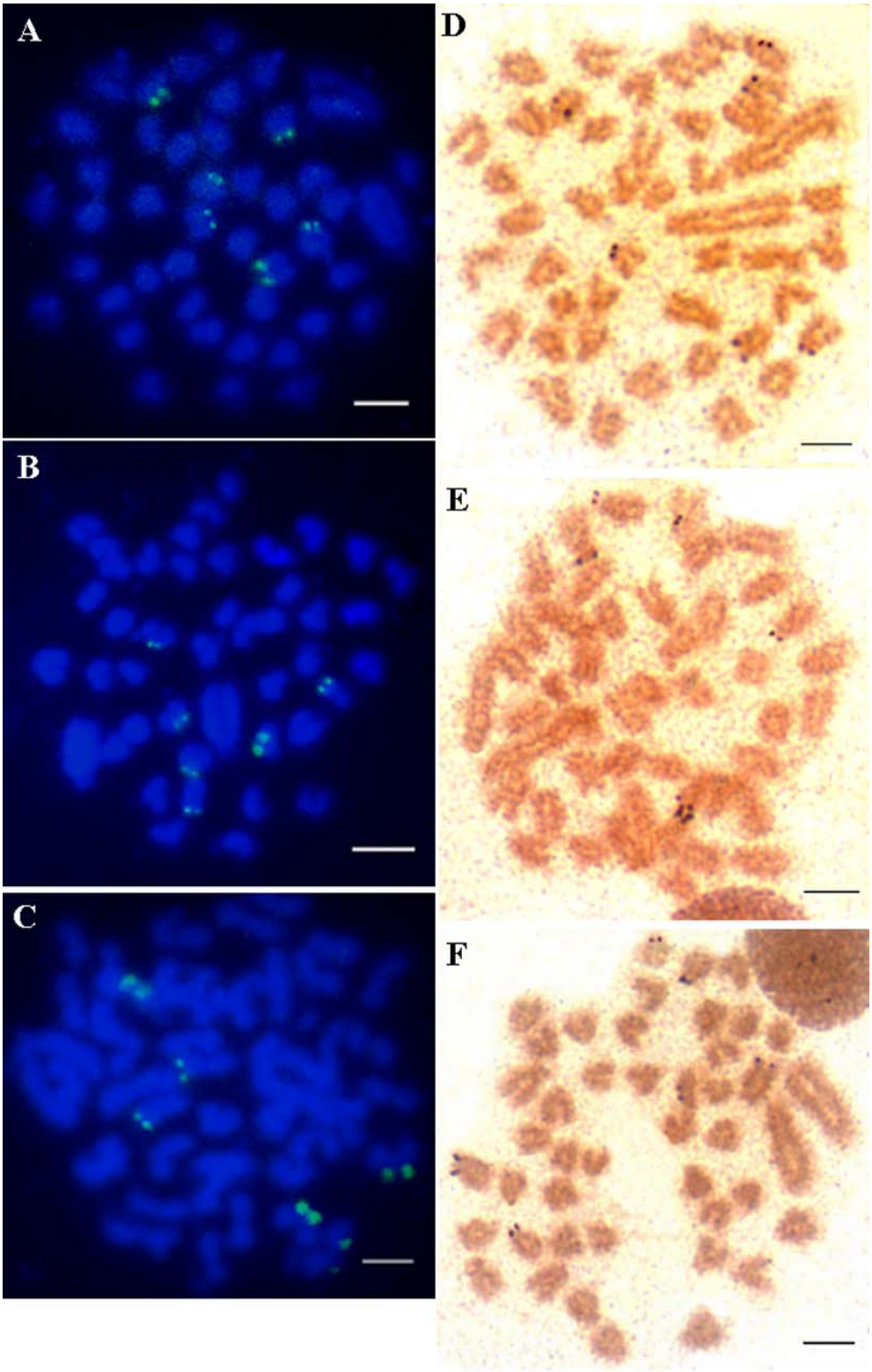

Figure 4. (A–C) The FISH-stained and (D–E) silver-stained (A, D) metaphases of *O. mossambicus*, (B, E) *O. u. hornorum* and (C, F) the hybrid of *O. mossambicus* female × *O. u. hornorum* male with 45S rDNA probes and Ag-NORs staining. The metaphase was counterstained blue fluorescence with DAPI to visualize chromosome morphology. Arrows show the positive green 45S rDNA signals and Ag-NOR signals. Scale bar = 10 μm.

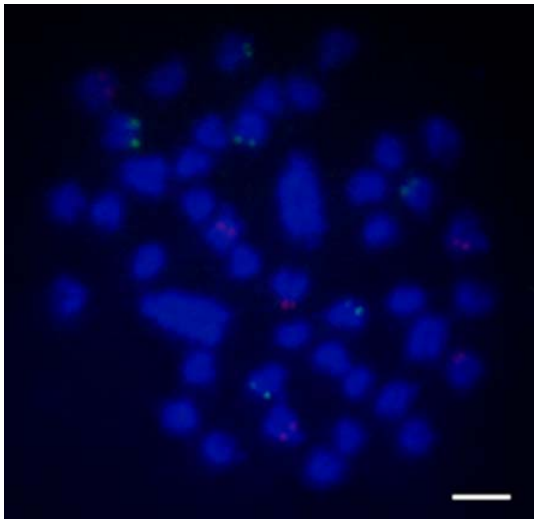

Figure 5. The two different colour FISH-stained metaphase from *O. u. hornorum* with 5S rDNA probes (red) and 45S rDNA probes (green). Arrows indicate the 5S rDNA red signals, and arrowheads show the 45S rDNA green signals, respectively. The metaphase was counterstained blue fluorescence with DAPI to visualize chromosome morphology. Scale bar = 10 μm.

($2n$ = 44) (Hinegardner and Rosen 1972; Kornfield *et al*. 1979; Vervoort 1980; Thompson 1981; Chen and Chen 1983; Gao 1986; Wang *et al*. 1990; Liu and Yu 1991). A similar finding has been observed in natural hybrids resulting from crosses between *Cichla monoculus* and *C. temensis* (Brinn *et al*. 2004). Significantly, the highly conservative chromosomal evolution as regards to chromosome number, structure and morphology is in contrast to the high species diversity in tilapia fishes (Feldberg *et al*. 2003). In the past, some karyotypic formula variants were described in the species *O. mossambicus* (Fukuoka and Muramoto 1975; Thompson 1981; Chen and Chen 1983), but they might have resulted from artifactual differences of chromosome preparation techniques.

The diversity of 5S rDNA loci have been reported in the diploid genome of different plants and animals (Lomholt *et al*. 1995; Laura *et al*. 2003; Fujiwara *et al*. 2007; Morescalchi *et al*. 2008; Kwon and Kim 2009). The 5S rDNA is often located on a single chromosome pair in fish (Pendás *et al*. 1994; Fujiwara *et al*. 2007; Morescalchi *et al*. 2008; Nirchio *et al*. 2009) and in some mammals (Suzuki *et al*. 1996; Laura *et al*. 2003), probably representing a more ancient condition among animal groups. The occurrence of multiple 5S rDNA sites found in the present study, as well as in some other fish represents derived conditions in their evolutionary dynamic (Ferro *et al*. 2001; Martins *et al*. 2002, 2006; Zhu *et al*. 2006). The 5S rDNA sites are also present on multiple chromosomes in humans (Lomholt *et al*. 1995) and other mammals like *Rhinolophus hipposideros* (Puerma *et al*. 2008). In some vertebrates, including fish and amphibians, a dual 5S rDNA system has been reported, and different types of these genes were thought to be differently regulated in each cellular type (Komiya *et al*. 1986; Martins *et al*. 2002). In Tilapiini tribe *Oreochromis* and *Tilapia* species, the two classes of 5S rDNA were found to exist by analysis with the whole 5S rDNA sequences and just the NTSs sequence, but these sequences were not differentiated between the species (Alves-Costa *et al*. 2006). These types of multi-gene families usually evolve based on homogenization processes, and were governed by molecular drive and concerted evolution, resulting in a sequence similarity of the repeating units that is greater within rather than among species (Morescalchi *et al*. 2008). In this study, two types of 5S rDNA were observed in three types of samples. Each of the 5S rDNA copies, repeated in tandem, comprises of a highly conserved 120-nucleotide coding sequence, followed by NTS with an extensive sequence and length variation (figure 2B).

The number and location of the Ag-NORs have been used as a cytotaxonomic parameter. Our current research observed three pairs of Ag-NOR sites in all of the examined samples. The result appears to coincide with that reported previously in tilapia *O. niloticus* (Foresti *et al*. 1993; Martins *et al*. 2004) and cichlid fish *Crenicichla lepidota* (Martins *et al*. 1995). However, in some fishes, only one or two pairs of NORs were observed (Feldberg and Bertollo 1985; Martins *et al*. 1995; Brinn *et al*. 2004). In some cases, higher FISH signal numbers were detected by 45s rDNA probes than by silver nitrate staining, which is probably due to inactivation of rDNA genes by regulation processes (Kakagawa *et al*. 2007). The FISH experiments with 45S rDNA probes performed here shows that the fluorescence signals coincide with signals seen in the Ag-NOR-bearing chromosomes (figure 4). The data demonstrate that the 45S rDNA location corresponds to the NORs, indicating that the three types of samples do not possess additional NOR sites and that all NOR-cistrons are active.

The distinct chromosome localization of 5S rDNA and 45S rDNA genes in the currently examined tilapias were coincident with that observed in tilapia *O. niloticus* (Martins *et al*. 2002, 2004). A highly conserved chromosomal location of rDNA genes characterizes the karyotype evolution of this fish group (figure 5). The divergent locations of 5S and 45S rDNA loci seem to be the most common situation observed in most fish (Martins and Galetti 2001) and by far the most frequent distribution pattern observed in vertebrates (Suzuki *et al*. 1996). In contrast, Fujiwara *et al*. (2007) indicated that the 5S and 45S rDNA loci are linked on the same chromosome. Moreover, different size and fluorescence intensity of 5S rDNA and 45S rDNA were observed in both metaphases of parental species and the hybrids, and the size and fluorescence intensity can be used to discriminate the chromosome identity. Fish rDNAs are highly repeated sequences, and the size variation might be related to the changes in genomic sequence duplications and/or deletions and chromosome rearrangement (Yogeeswaran *et al*. 2005), suggesting that rapid chromosome rearrangements should exist in

the cichlid genomes. The chromosomal mapping of such sequences in other cichlids may provide important insights into the evolution of the cichlidae karyotype. The cytogenetic information obtained from this study may contribute to a better understanding of the dynamics of the interspecific hybridization process in fish, and provide support for hybridization projects and breeding improvement in tilapias.

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