Physical chromosome mapping of repetitive DNA sequences in Nile tilapia *Oreochromis niloticus*: Evidences for a differential distribution of repetitive elements in the sex chromosomes

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Abstract

Repetitive DNAs have been extensively applied as physical chromosome markers on comparative studies, identification of chromosome rearrangements and sex chromosomes, chromosome evolution analysis, and applied genetics. Here we report the characterization of repetitive DNA sequences from the Nile tilapia (*Oreochromis niloticus*) genome by construction and screening of plasmid library enriched with repetitive DNAs, analysis of a BAC-based physical map, and hybridization to chromosomes. The physical mapping of BACs enriched with repetitive sequences and C<sub>T</sub>-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences) to chromosomes using FISH showed a predominant distribution of repetitive elements in the centromeric and telomeric regions and along the entire length of the largest chromosome pair (X and Y sex chromosomes) of the species. The distribution of repetitive DNAs differed significantly between the p arm of X and Y chromosomes. These findings suggest that repetitive DNAs have had an important role in the differentiation of sex chromosomes.

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1. Introduction

In most genomes, repeated sequences comprise a large portion of the DNA content of the cells. For example, 95% of the onion genome is composed of repeated sequences (Flavell et al., 1994), and at least 50% or much more in humans (The Genome International Sequencing Consortium, 2001). During eukaryotic evolution, genes and genome segments evidently have duplicated (Pennisi, 2001) leading to an increase of the DNA content. The variation in genome size of different eukaryotes is often attributed to differences in the amount of repeated DNA sequences (Cavalier-Smith, 1985; Brenner et al., 1993). Recent advances in studies concerning non-coding repetitive DNA sequences have shown that such sequences are extremely important in the structural and functional organization of the genome (Grady et al., 1992; Schueler et al., 2001). Among completely sequenced genomes the repetitive areas remain as gaps due to the difficulty to correctly identify their position and array in the genome. Even chromosomes reportedly “sequenced to completion” such as chromosome 22 of humans have multiple gaps in the pericentromeric regions (Dunham et al., 1999). A complete understanding of the relationship between chromosome structure and function requires the understanding of the repetitive segments. Also, the integration of DNA sequences with physical chromosome mapping of repetitive DNAs can provide a better landscape of the genome, not yet clearly defined even in the completely sequenced genomes. The repetitive DNA sequences can also provide good chromosome markers useful in studies of species evolution, identification of chromosome rearrangements, sex identification and applied genetics.

In this study, we have isolated and characterized several classes of repetitive DNAs in the tilapiine Nile tilapia (*Oreochromis niloticus*) genome. Members of the tilapiine tribe of cichlid fishes have gain an increasing scientific interest because of their rapid adaptive radiation leading to an extensive ecological diversity, and because of their enormous importance to tropical and subtropical aquaculture, especially the Nile tilapia, *O. niloticus* (Pullin, 1991). Although a genetic linkage map for *O. niloticus* based on polymorphic DNA markers has

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recently been published (Lee et al., 2005), limited molecular genetic information is currently available. Physical mapping of DNA sequences on the chromosomes of *O. niloticus* represents a powerful way to understand the differentiation of cichlid genomes during the evolution. Except for the largest chromosome pair, the remaining chromosomes in the Nile tilapia karyotype are nearly identical in morphology and size, which makes difficult the identification of particular karyotypic elements. The integration of a physical and a genetic map has been hindered due to the absence of specific chromosome markers for this species. To further our understanding of the organization of the *O. niloticus* genome, and to assist in the construction of a physical chromosome map for this species, we have searched its genome for repetitive DNA sequences and generated a chromosomal map for the species.

2. Materials and methods

2.1. Construction and screening of enriched repetitive DNA libraries

Nile tilapia genomic DNA was extracted according to standard phenol–chloroform procedures (Sambrook and Russel, 2001). An enriched library with repetitive sequences was constructed based on the renaturation kinetics of C<sub>0.7</sub>-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences) (Zwick et al., 1997). DNA samples (200 μL of 100–500 ng/μL genomic DNA in 0.3 M NaCl) were autoclaved for 30 min at 1.4 atm and 120 °C and the fragmented DNA was sized by electrophoresis in a 1% agarose gel. Expected DNA size fragments should range between 100 and 1000 base pairs (bp). Samples of 50 μL of DNA fragments were denatured at 95 °C for 10 min, ice cold for 10 s, and transferred to a 65 °C water bath for reannealing. After 1 min of reannealing, the samples were incubated at 37 °C for 8 min with 1 U of S1 nuclease to permit digestion of single-stranded sequences. The samples were immediately frozen in liquid nitrogen and submitted to DNA extraction with phenol–chloroform. The DNA fragments obtained were used as probes for fluorescent *in situ* hybridization (FISH) or inserted in the plasmid vector of the Kit *pMOS-Blue* (GE Healthcare Life Sciences) and cloned by nick translation with biotin-14-dATP (Bionick labeling system-Invitrogen). The metaphase chromosome slides were incubated with RNase (40 μg/ml) for 1.5 h at 37 °C. After the chromosomal DNA denaturation in 70% formamide/2 × SSC for 4 min at 70 °C, hybridization mixtures containing 100 ng of denatured probe, 10 mg/ml dextran sulfate, 2 × SSC, and 50% formamide in a final volume of 30 μl, were dropped on the slides and the hybridization was performed overnight at 37 °C in a 2 × SSC moist chamber. Post-hybridization washes were carried out at 37 °C in 2 × SSC/50% formamide for 15 min, followed by a second wash in 2 × SSC for 15 min, and a final wash at room temperature in 4 × SSC for 15 min. Detection of hybridized probes was carried out with 0.07% avidin-FITC conjugate (Sigma) in C buffer (0.1 M NaHCO<sub>3</sub>/0.15 M NaCl) for 1 h followed by two rounds of signal amplification using 2.5% anti-avidin biotin conjugate (Sigma) in blocking buffer (1.26% NaHCO<sub>3</sub>, 0.018% sodium citrate, 0.0386% triton and 1% non-fat dried milk) for 30 min. Each treatment with anti-avidin biotin results in the isolation of repetitive DNAs used in this work was modified from the protocol to isolate C<sub>0</sub>-1 DNA (Zwick et al., 1997) and proved to be highly valuable as a tool to isolate repetitive sequences.

2.2. Screening Nile tilapia bacterial artificial chromosome (BAC) libraries for repetitive sequences

A physical map of the Nile tilapia genome based on restriction fingerprints of more than 35,000 large-insert BAC clones (Katagiri et al., 2005) was searched for BACs containing highly repetitive DNA sequences. This physical map generated a total of 3621 contigs and the number of clones per contig varied from 2 to >200 (Table 1) (Katagiri et al., 2005). The resulting contigs of the physical map were visualized using the online FPC (FingerPrinted Contigs) viewer at HCFS (Hubbard Center for Genome Studies, University of New Hampshire, USA, web site http://www.hcgs.unh.edu/fpc/image.php). BAC candidates likely to contain highly repetitive sequences were fragmented by autoclaving or restriction enzymes and were submitted to subcloning and sequencing.

2.3. Sequencing and sequence analysis

The positive clones were sequenced on an ABI Prism 377 DNA sequencer (Perkin-Elmer) using the Kit DYEEnamic ET Terminator Cycle Sequencing (Amersham Biosciences). The sequences were subjected to Blastn (Altschul et al., 1990) searches at the National Center for Biotechnology Information (NCBI), website (http://www.ncbi.nlm.nih.gov/blast) to check for any similarity of the isolated sequences to the sequences deposited in the GenBank databases.

2.4. Chromosome *in situ* hybridization

Mitotic chromosomes were prepared from anterior kidney cells with *in vivo* colchicine treatment (Bertollo et al., 1978) and were submitted to FISH (Pinkel et al., 1986) using BAC-clones of contigs C2, C4 and C5 (Table 3), the C<sub>0.7</sub>-1 DNA generated by the renaturation kinetics and the clone Oml1013-5.4 containing the SATA satellite DNA family (Table 2). The probes were labeled by nick translation with biotin-14-dATP (Bionick labeling system-Invitrogen). The metaphase chromosome slides were incubated with RNase (40 μg/ml) for 1.5 h at 37 °C. After the chromosomal DNA denaturation in 70% formamide/2 × SSC for 4 min at 70 °C, hybridization mixtures containing 100 ng of denatured probe, 10 mg/ml dextran sulfate, 2 × SSC, and 50% formamide in a final volume of 30 μl, were dropped on the slides and the hybridization was performed overnight at 37 °C in a 2 × SSC moist chamber. Post-hybridization washes were carried out at 37 °C in 2 × SSC/50% formamide for 15 min, followed by a second wash in 2 × SSC for 15 min, and a final wash at room temperature in 4 × SSC for 15 min. Detection of hybridized probes was carried out with 0.07% avidin-FITC conjugate (Sigma) in C buffer (0.1 M NaHCO<sub>3</sub>/0.15 M NaCl) for 1 h followed by two rounds of signal amplification using 2.5% anti-avidin biotin conjugate (Sigma) in blocking buffer (1.26% NaHCO<sub>3</sub>, 0.018% sodium citrate, 0.0386% triton and 1% non-fat dried milk) for 30 min.
The reassociation kinetics of repetitive DNAs since their copies match each other more frequently during reassociation. The reassociation kinetics of repetitive DNAs during reaction. The first DNA molecules to reassociate are highly repetitive DNAs containing highly repetitive sequences. Eleven percent of the screening using genomic DNA as a probe to identify clones containing highly repetitive DNA segments were recovered from the library of C0-1 DNA, dot-blotted on nylon membranes and submitted to screening using genomic DNA as a probe to identify clones containing highly repetitive sequences. Eleven percent of the clones showed strong positive signals after hybridization and were subject to sequencing analysis. Table 2 summarizes the characteristics of the isolated sequences. All sequences obtained through the method based on the DNA renaturation kinetics demonstrated that it was possible to identify different types of tandem and dispersed repetitive elements. Reassociation between complementary sequences of DNA occurs by base pairing in a reversal of the denaturation process and reflects the variation and copy number of sequences that are present in the reaction. The first DNA molecules to reassociate are highly repetitive DNAs since their copies match each other more frequently during reassociation. The reassociation kinetics of the genome represents an interesting strategy to isolate repetitive DNAs and has been used to isolate C0-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences) that is required for the suppression or blocking of highly repetitive DNAs during in situ hybridization of nucleic acids (Zwick et al., 1997). Although some isolated sequences were previously characterized in the genome of O. niloticus, new repetitive elements were identified in the Nile tilapia genome, and are promising markers for chromosome mapping purposes.

3.2. Screening Nile tilapia BAC libraries for repetitive sequences

Another way to search the tilapia genome for repetitive sequences was realized through the access of a restriction fingerprint map of more than 35,000 BAC clones available at the HCGS for O. niloticus (Katagiri et al., 2005). This physical map was constructed to help accelerate positional cloning and to facilitate the long-range assembly of a tilapia genome sequence (Katagiri et al., 2005).

The 35,000 BAC clones of the Nile tilapia assembled from 3621 contigs and a total of 3127 questionable clones (Q’s) were identified (Katagiri et al., 2005) (Table 3). The number of these questionable clones was strongly correlated with the number of clones in the contig (more clones in the contig means more questionable clones), which indicates that these clones represent BACs containing repetitive sequences. Through analyses of Q values, 18 BACs from 3 contigs (named C2, C4 and C5), with a large number of clones, were selected to investigate their DNA sequence content (Table 3). The selected BACs, candidates to contain repetitive sequences, were fragmented by autoclaving or restriction enzymes and were submitted to subcloning and sequencing. Fourteen clones were recovered and sequenced for BACs from contigs C2, C4 and C5 showing the presence of several classes of tandem and dispersed repetitive elements (Table 4).
Several subcloned DNA fragments of BACs from contigs C2 and C4 showed similarity to the KLR (Killer cell-Like Receptor) gene complex of the Nile tilapia (Kikuno et al., 2004) (Table 4). The KLR gene complex presents genes and pseudogenes organized in 18 loci that are distributed in approximately 500 kilobases of the genome (Kikuno et al., 2004). The isolated DNA fragments were dispersed between the pseudogene KLR2 and KLR3. Our results, associated with the BAC restriction map (Katagiri et al., 2005), suggest that the isolated sequences are not only dispersed among the KLR gene copies, but also are repeated among other regions of the Nile tilapia genome. The other isolated DNA fragments were related mainly to transposon elements, satellite DNAs and non-identified repetitive sequences. The results obtained in this work are in agreement with the restriction map of the entire BAC library of Nile tilapia by Katagiri et al. (2005) that suggested that the BACs from contigs C2, C4 and C5 were supposed to contain highly repetitive DNA sequences. The presence of several classes of repetitive sequences in the BACs of the investigated contigs reinforces that the physical map developed by Katagiri et al. (2005) is an informative tool to get basic genomic information about the species.

3.3. Physical chromosome mapping of repetitive DNAs

The Nile tilapia possess 2n = 44 chromosomes, including one submetacentric (sm) pair (pair no. 7) and 21 subtelo (st) to acrocentric (a) pairs (Fig. 1). Basic karyotype information regarding chromosome number and morphology and the heterochromatin distribution for the karyotype of this species were described by Majumdar and McAndrew (1986). Although a construction of a physical chromosome map for O. niloticus was already attempt (Martins et al., 2004), the unambiguous identification of chromosomal elements in the karyotype is still limited. Except easily identifiable chromosome pairs Nos. 1 and 2, most chromosomes are of similar size and morphology, hindering their identification among all the karyotype elements. In this way, DNA markers that identify specific chromosomes of the Nile tilapia are required.

The isolation of repetitive DNAs from C0t-1 DNA for chromosome mapping purpose represents a very efficient

### Table 4
Characteristics of isolated DNA sequences of BAC clones

<table>
<thead>
<tr>
<th>Clones</th>
<th>Size (bp)</th>
<th>GenBank entries</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>OnC2C12-3-9</td>
<td>390</td>
<td>EF438165</td>
<td>Repeated segments spread out within the KLR gene complex of O. niloticus (AY495714$^*$)</td>
</tr>
<tr>
<td>OnC2C12-3-10</td>
<td>435</td>
<td>EF438166</td>
<td>Penelope-like transposon of Tetraodon nigroviridis (AJ621377.1)$^<em>$ and retrotransposon Xena of Takifugu rubripes (AF355377.1)$^</em>$; different clones of Xenopus tropicalis (AC149900.2, AC147826.2 e AC148456.2)$^<em>$; repeated segments of Danio rerio genome (BX927285.6, BX088594.5, AL929210.4, BX546473.10 and AC149227.18)$^</em>$</td>
</tr>
<tr>
<td>OnC2C12-16</td>
<td>323</td>
<td>EF438170</td>
<td>Repeated segments spread out within the KLR gene complex of O. niloticus (AY495714$^*$)</td>
</tr>
<tr>
<td>OnC2C12-18</td>
<td>278</td>
<td>EF438171</td>
<td></td>
</tr>
<tr>
<td>OnC2C12-21</td>
<td>221</td>
<td>EF438172</td>
<td></td>
</tr>
<tr>
<td>OnC4D12-5</td>
<td>439</td>
<td>EF438173</td>
<td></td>
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<tr>
<td>OnC4E03-13</td>
<td>318</td>
<td>EF438174</td>
<td></td>
</tr>
<tr>
<td>OnC4E09-2</td>
<td>273</td>
<td>EF438175</td>
<td></td>
</tr>
<tr>
<td>OnC4E09-10</td>
<td>198</td>
<td>EF438176</td>
<td></td>
</tr>
<tr>
<td>OnC4E09-11</td>
<td>78</td>
<td>EF438177</td>
<td></td>
</tr>
<tr>
<td>OnC4E09-20</td>
<td>237</td>
<td>EF438178</td>
<td></td>
</tr>
</tbody>
</table>

$^*$ GenBank accession entries for the sequences at NCBI.
methodology for the identification of chromosome physical markers. The chromosome mapping of the clone On11013-5.4, containing the SATA satellite (Table 2), hybridized to all centromeres of the karyotype complement, in agreement to the previously described results for *O. niloticus* (Oliveira and Wright, 1998), allowing the correct identification of the centromeric region and of the correct arrangement of the chromosomes in the karyotype (Fig. 1a).

The chromosome hybridization of C$_0^{-}t$-1 and the BAC clones of contigs C2, C4 and C5 generated an interesting banding pattern along the chromosomes of *O. niloticus*. The repetitive DNAs were distributed in the centromeric and telomeric regions of most chromosomes and throughout the entire extension of chromosome No. 1 (Fig. 1). The chromosome pair No. 1 of the Nile tilapia represents the X and Y sex chromosomes (Foresti et al., 1993). Whole chromosome probes obtained by microdissection and DOP-PCR-amplification from the X and Y chromosomes of *O. niloticus* hybridized more intensely in the q arm, suggesting the presence of large number of repetitive elements in this chromosome region, with the accumulation of repetitive DNAs in the first chromosome pair of the karyotype followed by suppression of meiotic recombination, leading to loss of euchromatin segments and gains of heterochromatin segments and, consequently, to the genetic differentiation of the X and Y chromosomes. It appears that chromosomal sex differentiation in the Nile tilapia is in course and the accumulation of repetitive DNAs in the first chromosome pair of the karyotype followed by suppression of meiotic recombination, will lead to a clear differentiation between the X and Y chromosomes.

BACs C4E09 and C5E01 hybridized in the whole extension of chromosome pair No. 1, except around the terminal two-thirds of the q arm of chromosome pair No. 1 (Oliveira et al., 1999). It has been suggested that the distribution of Ron-1, CiLINE2, and telomeric repeats (Chew et al., 2002) in an interstitial position in chromosome pair No. 1 is a consequence of two chromosome fusions thought to have originated the large chromosome pair of *O. niloticus* (Oliveira et al., 1999). The presence of repetitive elements was also evidenced in the sex chromosomes of the Antarctic fish *Chionodraco hamatus* where such elements were found interstitially in the p arm of the Y chromosome, which is supposed to be originated by tandem fusion (Capriglione et al., 2002; Ozouf-Costaz et al., 2004). Ohno (1967) proposed that during the differentiation and evolution of mammalian sex chromosomes, it is thought that proto-X and -Y chromosomes may have encountered various genetic alterations that possibly contributed to the suppression of X/Y meiotic recombination, leading to loss of euchromatin segments and gains of heterochromatin segments and, consequently, to the genetic differentiation of the X and Y chromosomes. It appears that chromosomal sex differentiation in the Nile tilapia is in course and the accumulation of repetitive DNAs in the first chromosome pair of the karyotype followed by suppression of meiotic recombination, will lead to a clear differentiation between the X and Y chromosomes.

Fig. 1. Distribution of repetitive DNAs in the chromosomes of the Nile tilapia *O. niloticus* revealed by the chromosome hybridization of SATA centromeric satellite (a), C$_0^{-}t$-1 DNA (b), BAC-clone C4E09 (c), and BAC-clone C5E01 (d). Scale bar = 8 µm.
centromeric area (Figs. 1c and d and 2), showing that both BACs do not contain SATA satellite sequences. The chromosome signals generated after hybridization of BAC clones allowed the identification of several chromosome elements in the karyotype: chromosome pairs Nos. 2, 3, 6, 7, 14 and 15 with the BAC C4E09, and chromosome pairs Nos. 2, 4, 7 and 11 with the BAC C5E01.

Quantitative analysis of chromosome hybridization of BACs C4E09 and C5E01 in males and females of *O. niloticus* permit the identification of differences in the repetitive DNA content between the X and Y chromosomes (Fig. 2). The hybridization signals differed significantly between the p arms of the X and Y sex chromosomes (Fig. 2). No significant differences were detected in relation to the q arms of these chromosomes. Previous cytological data based on synaptonemal complex analysis demonstrated that the genomic differences between the X and Y chromosomes of the Nile tilapia reside in the q arm of these sex chromosomes (Foresti et al., 1993). Cloning, sequencing and chromosome hybridization of microdissected DNA from the X and Y sex chromosomes of *O. niloticus* showed differences in the repetitive DNA content between the X and Y chromosomes, most of them in the q arm (Harvey et al., 2003). The comparative analysis between the sex chromosomes of males and females permitted to infer that the p arm of the X chromosome has a greater amount of repetitive sequences when compared to the Y chromosome.

Most repetitive DNAs identified among the C₀'-1 and the BAC sequences were related to transposable elements (Tables 2 and 4). This chromosomal distribution pattern coincides with heterochromatic regions identifiable onto chromosomes (Majumdar and McAndrew, 1986). The transposable elements are also situated in centromeric and telomeric regions and are not randomly distributed in the genome of the Notothenioidei Antarctic fishes (Capriglione et al., 2002; Ozouf-Costaz et al., 2004) as well as in the pufferfish *Tetraodon nigroviridis* (DaSilva et al., 2002; Bouneau et al., 2003; Fischer et al., 2004). This chromosomal distribution pattern of repetitive DNA is different from that observed in humans, where repeat sequences make up an important fraction of euchromatic DNA, and is more similar to the repetitive DNA distribution observed in *Drosophila melanogaster* and *Arabidopsis thaliana* (Fischer et al., 2004). This may indicate that transposable elements accumulate preferentially in some regions of the fish genomes.

Although in the last two decades cytogenetic studies have been carried out on a large number of fish species, such analyses were mainly directed to the knowledge of basic karyotype structure and few works have been conducted on the
organization of DNA sequences in chromosomes. The integration of DNA sequence data with chromosome mapping of repetitive DNAs can provide a better landscape of the genome, which is not yet clearly defined even in the wholly sequenced genomes. Also, the application of BAC clones that label several chromosomes in the karyotype of *O. niloticus* could be used to anchor a second probe labeling, making the chromosome map of the species more detailed. The chromosome mapping of BACs enriched by repetitive DNAs, C0 DNA and specific repetitive probes, proved to be valuable in generating a basic chromosome map for the species. The chromosome physical map of repetitive DNAs are of interest for whole genome sequencing programs, since repetitive DNAs represent a puzzle that is difficult to comprehend. A proposal for complete genome sequencing of the Nile tilapia *O. niloticus* is in progress (Kocher, 2005) and the repetitive chromosome physical map presented herein will be certainly welcome by the tilapia research community. Detailed knowledge of the genomes will be achieved only with the integration of different technologies involving complete genome sequencing, restriction mapping, linkage mapping and physical chromosome mapping.

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