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Representational difference analysis for detection of sex-specific DNA markers from the Nile tilapia (*Oreochromis niloticus*)

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Abstract. Sex-specific DNA markers are useful for identification of male and female fish even in the early stage of life which has many practical implications. Representational difference analysis (RDA) technique was applied to develop sex-specific DNA markers for the Nile tilapia *Oreochromis niloticus*. Genomic DNA of YY male fish, XY male fish and XX female fish was restricted with BglII. We used oligonucleotide adaptors RBgl, JBgl and NBgl, RBam12/24 and RBgl12/24 adaptors and RBam24 and JBgl24 primers for preparing the amplicons from the genomic DNA. Three RDA experiments were performed: (1) YY tester with XX driver, (2) XX tester with YY driver and (3) XY tester with XX driver. A total of 146 RDA products from the male and female genomic DNA were cloned and sequenced from which 81 pairs of primers were designed and applied on three different genotypes to test specificity of the primers. One primer set designed from female RDA-derived clones (XX1_8F/R) yielded PCR products only in the tester female but not in the driver male. Another primer set designed male RDA-derived clones (YY1_14F/R) amplified one band in the male but two bands in the female DNA. However, the RDA analysis failed to detect unique fragments from the male and female genome in *O. niloticus*. Therefore, we suggest that the sex chromosomes are very weakly differentiated in tilapia.
Key words: *Oreochromis niloticus*, DNA markers, PCR, RDA

Introduction

The Nile tilapia (*Oreochromis niloticus*) is the fourth most contributing farmed fish species to global aquaculture production and it is described as the most important aquaculture species of the 21st century (Shelton 2002). The fish is being farmed in about 85 countries worldwide and about 98% of tilapia produced in these countries is grown outside their original habitats (Shelton 2002). However, the prolific breeding characteristics of tilapia is considered as the major constraint of its aquaculture. Since male tilapia grows faster than the female counterpart (Mair *et al*. 1995), all-male populations of tilapia are usually preferred over mixed sex populations. Direct hormonal sex reversal using androgen hormones is widely recognized as a commercial method for producing monosex male population of tilapia though it is not accepted in many countries due to environmental and consumer concerns. Sex chromosome specific markers could help generate all-male population of tilapia without direct administration of hormones.

Sex determination and differentiation are key events in the development of either testis or ovary in vertebrates. In mammals, SRY/*Sry* (sex determining region of Y chromosome) is the Y chromosome gene responsible for initiating testicular development (Sinclair *et al*. 1990,

Koopman *et al*. 1991). Fishes display a variety of mechanisms for sex determination and sex differentiation (reviewed in Devlin and Nagahama 2002). Primary sex determination in most species is genetic but sex determination of fishes is notably plastic and is determined by both genetic and environmental factors in many species (Baroiller *et al*. 1999). *O. niloticus* is thought to exhibit a predominantly monofactorial genotypic system of sex determination with heterogametic (XY) male (Müller-Belecke and Hörstgen-Schwark 1995) but sex determination in this species is sometimes influenced by autosomal loci as well as by high temperature (Baroiller *et al*. 1995). Sex chromosomes in tilapia are not morphologically identifiable and YY individuals are viable and fertile in one hand and sex-reversed XX male can act as fertile male on the other indicating a poor degree of differentiation between X and Y chromosome. Synaptonemal complex analysis identified pairing anomalies in a terminal portion of chromosome 1 in XY males but not in XX females and YY males, suggesting that this pair corresponded to the sex chromosome (Carrasco *et al*. 1999).

Several microsatellite and amplified fragment length polymorphism (AFLP) markers linked to the sex chromosomes have been identified (Lee *et al*. 2003, Ezaz *et al*. 2004) in *O. niloticus*. Their sex linkage was however, dependent on the family analyzed, indicating the differential influence of additional genetic and environmental sex-determining factors. Sex-specific DNA sequences have been identified in several fish species using different molecular marker techniques including Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), AFLP and microsatellite (Griffiths *et al*. 2000, Devlin *et al*. 2001, Kovacs *et al*. 2001, Brunelli and Thorgaard 2004, Felip *et al*. 2005). Representational difference analysis (RDA) followed by subtractive hybridization has been found to isolate differences in products from two closely related genomes (Lísitsyn *et al*. 1993). In this technique, one DNA population (the driver) is hybridized in excess against a second population (the tester) to remove common hybridizing sequences. The driver sequences are subtracted from the tester sequences to enrich the difference product unique for the tester sequence. Thus RDA allows the cloning and sequencing of fine genome differences between two highly similar genomes and further provides exact sequence information about these differences. RDA technique has been found effective in developing sex chromosome specific markers (Donnison *et al*. 1996, Navin *et al*. 1996, Fujisawa *et al*. 2001).Therefore, the objective of the study was to search the genome of the Nile tilapia through representational difference analysis for sex specific markers. We hypothesize that there is unique regions in the X and Y chromosome due to accumulation of insertions which could be enriched through subtractive hybridization that will allow development of sex-specific DNA markers.

Materials and Methods

***The experimental fish*:** A stock of the Nile tilapia, *O. niloticus* originally collected from the Lake Manzala, Egypt had been maintained in the tropical aquaria of the University of Goettingen. Normal XX female, gynogenetically produced YY male *O. niloticus* (Müller-Belecke and Hörstgen-Schwark 2007) and normal XY male were used in this study. Genomic DNA was isolated from the fin clips of the sampled fish following standard Proteinase-K/SDS digestion, Phenol/Chloroform/Isoamylalcohol (IAA) extraction and alcohol precipitation method (Sambrook *et al*. 1989). The concentration of DNA was estimated by using a NanodropND-1000 Spectrophotometer (PEQ Lab Biotechnologie GmbH).

***Representational difference analysis*:** The Representational difference analysis (RDA) procedure was essentially as described by Lisitsyn *et al.* (1993) and detailed in Lisitsyn and Wigler (1995) with some modifications. We used the restriction enzyme BglII with corresponding oligonucleotide adaptors RBgl, JBgl and NBgl. RBgl12/24 adaptors and JBgl24 primers were used for preparing the amplicons from the female and male fish DNA. Three RDA experiments were then performed: (1) YY tester with XX driver, (2) XX tester with YY driver and (3) XY tester with XX driver. The tester amplicons were ligated to the J-adaptor for the first and third rounds, and with the N-adaptor for the second round of RDA. A mixture of the tester and driver was resuspended in 8-μl of EE buffer [30mM N-(2-hydroxyethyl) piperazine-N-(3-propanesulfonic acid) and 3mM EDTA (pH=8.0)] and over-laid with 35 μl of mineral oil. After denaturation at 98°C for 5 min2 μl of pre-warmed 5M NaCl was added and the hybridization was continued at 67°C for 40 hr. Forty μg of the driver DNA was hybridized with 500 ng, 50 ng and 100 pg for the first, second and third round of subtractive hybridization respectively. Difference products obtained after the second and third round of RDA were dispensed on agarose gel and the prominent bands were excised and purified using QIAGEN gel extraction kit. The fragments were cloned into pGEM-T vector (Promega) and sequenced using vector-situated M13 primers. The identity of the sequences was searched by using the BLAST program of the National Center for Biotechnology Information (NCBI)(Altschul *et al.* 1997). Primers were designed from the sequence and used for amplification of male and female-specific markers.

Results

***Isolation of DNA fragments specific to the male or female by RDA*:** No distinct bands were observed in the male and female amplicons after the first round of subtractive hybridization and selective amplification. We observed distinct bands in both male and female tester after the second and third rounds (Figs. 1 and 2). These differences between the male and female RDA products reflect the potential enrichment of DNA fragments theoretically characteristic of the male or the female genomes and therefore was used for further investigation. After the second round of subtractive hybridization three bands (XX1, XX2, XX3) from the XX female tester and two bands (YY1 and YY2) from the YY male tester were excised and purified (Fig. 3). The Third round of subtractive hybridization was also continued and two bands (XX4 and XX5) from the XX female tester and 3 bands (YY3, YY4, and YY5) from the YY male tester were excised after this round (Fig. 4).

Another trial of RDA was conducted with equal amount of DNA from 15 XX female and 15 XY males, and three YY males pooled separately and used as driver and tester. After second round of subtractive hybridization and selective amplification six bands were excised and used for further analysis (Fig. 5).

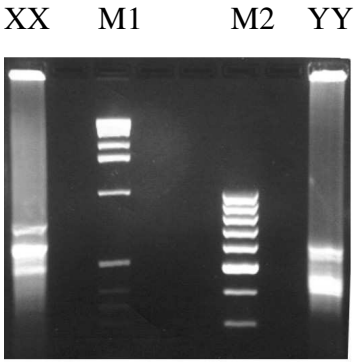
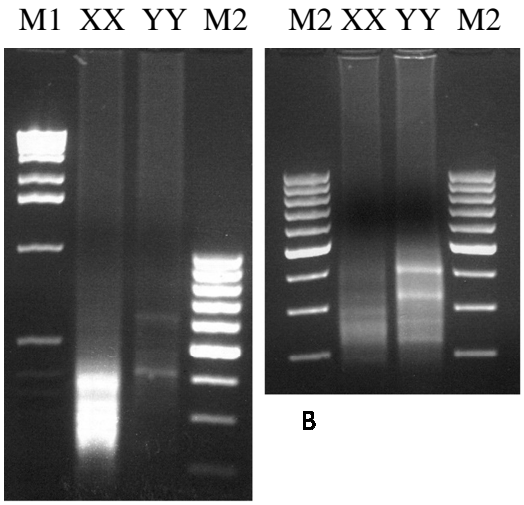


Fig. 1. Agarose gel electropherogram after second round of subtractive hybridization and selective amplification. XX: Female tester, YY: male tester. M1: 1kb and M2 100bp DNA ladder.



Figs. 2A and B. Difference product of the 3rdround hybridization. XX: Female and YY: Male difference product respectively. M1:1kb DNA ladder, M2: 100bp DNA ladder.

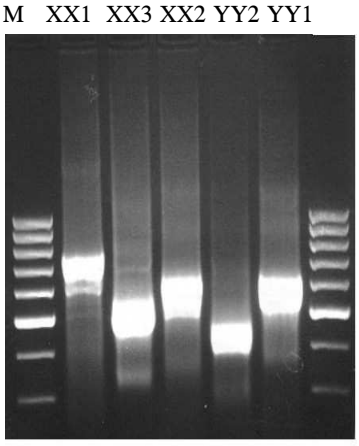


Fig. 3. PCR amplification of the 2nd round difference products after gel-extraction of different bands. XX1 XX2, XX3: selected female band-1, band-2 and band-3, and YY1 and YY2: selected male band -1 and band-2 respectively. M: 100bp DNA ladder.

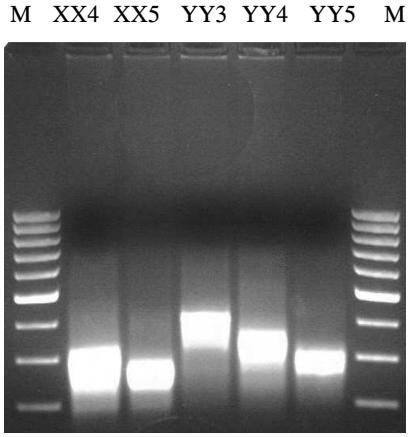


Fig. 4. PCR amplification of gel-extracted bands of the 3rd round hybridization difference products. XX4 and XX5, YY3 YY4, YY5: selected female band-4 and band-5 and male band 3, 4 and 5 respectively. M: 100bp DNA ladder.

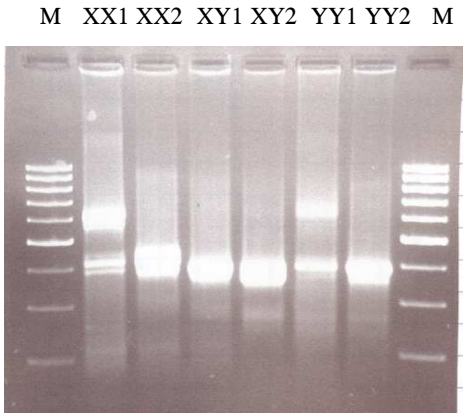


Fig. 5. RDA products generated from three different types of tester: XX, XY and YY testers. XX: Female, XY: Male, YY: Male, M: 100 bp DNA

The RDA products collected as bands from the male and female genomic DNA were cloned into the pGEM-T cloning vector and sequenced. The number of fragments sequenced and primer-pairs designed and tested on male and female fish are shown in Table I.

Table I. Summary of the RDA and other techniques applied for development of sex specific markers from *O. niloticus*

Tester genotype	Nos. of fragments sequenced	Nos. of pairs of primers designed and tested	Significant similarity with known genes from the gene bank
YY	73	39	*O. niloticus* KLR genes (AY495714.1)
XX	65	34	*Astatotilapia burtoni* clone BAC 20D21 platelet-derived growth factor receptor beta b (DQ386647.1), *O. niloticus* MHC class IA peudogenes (AB270897.1), *Platichthys flesus* isolate pGEM46 transposon Tc1 (DQ778467.1), *Pleuronectes platessa* Tc1 transposon (AJ303068.1)
XY	8	8	
Total	146	81	

Sequence analysis of the RDA-derived DNA fragments: A total of 146 clones were sequenced from the three types of tester, YY, XX, and XY (Table I). The SEQMAN program (Swindell and Plasterer 1997) aligned the sequences of the fragments, into three major groups. Most fragments from a particular tester were grouped together. For example, in one contig of 31 sequences, all, except two, were from YY tester. In another contig of 15 sequences, all came from the XX tester. However, in one contig, sequences from both male and female testers grouped together. In this group, some differences in the form of single nucleotide polymorphisms (SNPs) were observed. There were also some sequences which did not align in any group, rather stay singly.

Most of the nucleotide sequences of the male derived RDA clones showed no significant similarity to known gene sequences however, the female-derived RDA clones showed high homology with the *Astatotilapiaburtoni* platelet-derived growth factor/colony stimulating factor genes (Table I). The other genes which were found to share homology with sequence of the isolated fragments include (1) *Oreochromis niloticus* MHC class IA antigen pseudogenes, (2) *Platichthysflesus* isolate pGEM46 transposon Tc1-like, complete sequence (3) *Pleuronectes plates* Tc1 transposon, tn5gene (4) *Xenopustropicalis* Tc1-like transposon, *Salmo salar* DTSsa10 Tc1-like DNA transposon, complete sequence and *O. niloticus* KLR pseudogenes (Table I).

Sex-specific primer design and PCR amplification: Some of the clones were found to be identical and thus only one pair of primers were designed from this set. For identification of male-specific RDA clones, 39 primer sets for the YY male (tester) RDA-derived clones, and for identification of female-specific clones, 34 primer sets from XX-female (tester) RDA-derived clones were designed. Eight primer sets were designed to identify male specific markers from XY-male tester. PCR test amplifications were performed on male and female genomic DNA using each of these primer sets to determine whether the RDA-derived DNA fragments were indeed specific to either the male or female used for the RDA. One primer set for female RDA-derived clones (XX1_8F/R) (Table II) yielded PCR products only in the tester female but not in the driver male (Fig 6). One primer set for male RDA-derived clones (YY1_14F/R) (Table II) amplified one band in the male but two bands in the female DNA (Fig 7).

Table II. Primer sequences derived from XX female and YY male for sex-specific amplification of DNA fragment

Primer	Source	Sequence (5-3)	Results
XX1-8F	XX Female	GGG TAA CAC AGG TGA CAC TGA	Three bands in case of female, no band in case of male
XX1-8R	XX Female	CAA TGA AAA GGT CAA AGG TCA	
YY1-14F	YY Male	GAT TGG GCT CCA CCT TAA CA	A single band in case of male but two bands in case of female
YY1-14R	YY Male	ATC CTG GCA CTC AAG CAG TT	

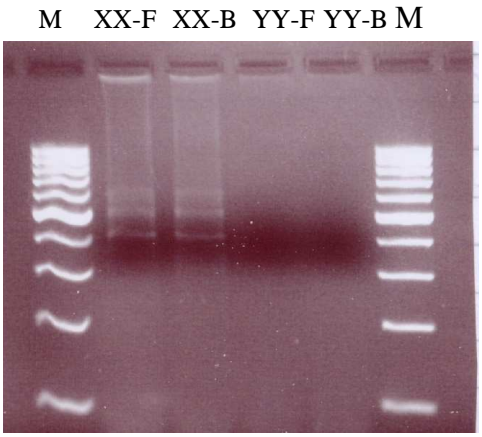


Fig. 6. Amplification of female and male DNA with female-specific marker (XX1_8F/R) primers. XX-F: Female fin, XX-B: Female blood, YY-F: Male fin, YY-B: Male blood M: 100bp DNA ladder.

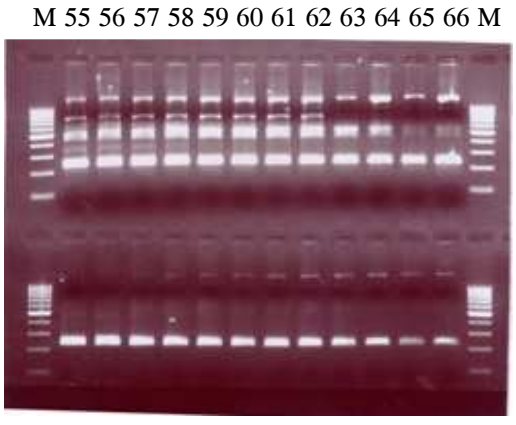


Fig. 7. A temperature gradient PCR amplification of male and female DNA with male-specific primers (YY1_14F/R). Upper panel: XX female template, Lower panel: YY male template. M: 100bp DNA ladder. The figures on each lane indicates the annealing temperature.

Discussion

RDA analysis has been successfully used to detect finer difference in closely related genomes (Lisitsyn 1993). This technique has also been successfully used to develop sex-specific markers (Donnison *et al.* 1996, Navin *et al.* 1996, Fujisawa *et al.* 2001).However, the RDA analysis in the present study failed to detect unique fragments from the male and female genome in *O. niloticus*. This study has raised a question if there is really differentiated X and Y chromosome with substantial sequence variation. Fluorescent In-situ hybridization (FISH) probes for the sex chromosomes, derived from transposable elements through degenerate oligonucleotide primer-polymerase chain reaction (DOP-PCR) after microdissection of chromosome 1 from XX and YY genotypes revealed modest signal difference between X and Y, suggesting an early stage of differentiation in *O. niloticus* (Griffin *et al.* 2002, Harvey *et al.* 2002). Avtalion *et al.* (1976) using polyacrylamide gel electrophoresis identified proteins that specifically expressed in male *O. niloticus*, which appeared to be sex limited, rather than sex-linked. Lee *et al.* (2003) searched for DNA markers linked to sex determining gene and identified a microsatellite locus that, according to them, was linked to the phenotypic sex with 95% accuracy in some but not all families. The alignment of the RDA clones towards the tester group indicates that the RDA analysis was able to accumulate some difference between the driver and the tester DNA. In one case, the marker amplified fragments only in the tester DNA but not in the driver DNA (Fig. 6). However, this difference, perhaps, did not come from a unique region of the X chromosome because when the markers were applied to 40 different non-related male and female fish, the pattern was not the same as was found in the original tester DNA (results not shown). Li et al (2002) failed to identify genomic sex markers in *Tetraodonni groviridis* using RAPD, AFLP and RDA techniques. Salvemini *et al.* (2014) identified putative Y-linked genes from *Ceratitis capitata* using subtractive and differential hybridization.

Theoretically the lack of sex-specific markers could be due to weak correlation between the genotypic and phenotypic sex resulting from autosomal modifier genes (Li *et al.* 2002) or from mixed genotypes in pools as a result of environmental sex determination (Baroiller *et al.* 1999). The second possibility however cannot be ruled out in our second experiment where DNAs were pooled before using as driver and tester (results not shown). However, the YY male used in the present study were previously tested for reliable production of all male progeny by crossing with normal XX female fish. So, the first assumption is not true for our first experiment involving a single YY male and a XX female. Therefore, in agreement with previous cytogenetic studies, we suggest that the sex chromosomes are very weakly differentiated in *O. niloticus*.

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