The Use of Random Amplified Polymorphic DNA (RAPD) Markers in Sex Discrimination in Nile Tilapia, Oreochromis niloticus (Pisces: Cichlidae)

Fevzi BARDAKCI
School of Biological Sciences, University of Wales, Singleton Park, Swansea SA2 8PP, U.K.

Received: 23.02.1999

Abstract: Random amplified polymorphic DNA (RAPD) markers were successfully used in discrimination of sexes in Nile tilapia fish (Oreochromis niloticus) using linear discriminant function analysis. The results provide support for the view that major genetical sex determining factors exist in tilapia.

Key Words: RAPD, DNA, sex discrimination, tilapia, Oreochromis

Introduction

Amongst vertebrates, fishes contain the greatest variability in sex determination mechanisms including monofactorial, polyfactorial and environmental control (1). In most cases, genes located on the heteromorphic sex chromosomes play the main role in the expression of sex determination in fishes (2). On the other hand, morphological differentiation of sex chromosomes is not apparent in most fish species studied, thus has been demonstrated cytogenetically in few fish species (3-5). The most recent approach to studying the mechanism of sex determination in fishes is to develop sex-specific molecular markers. So far sex-specific markers have only been developed in the guppy, Poecilia reticulata (6), chinook salmon, Oncorhyncus tshawytscha (7), coho salmon, O. kisutch (8) and Leporinus elongatus (9).

Studies of the sex determination mechanism in tilapia are primarily based on the sex ratio of offspring obtained from inter- and intra-specific crosses, crosses between sex reversed parents and after chromosome manipulations leading to polyploid, gynogenetic and androgenetic individuals, as well as cytogenetic methods, and several hypotheses, including monofactorial, polyfactorial, autosomal and environmental sex determination, have been proposed (10-12).
addition, several studies were carried out to identify heteromorphic sex chromosomes, but without success (13-15). Hybridization with known sex-linked nucleic acid sequences and subtractive hybridization have also so far failed in the detection of sex-specific DNA markers in tilapia (16).

The objective of the present study was to use the Random Amplified Polymorphic DNA (RAPD) method (17) to develop sex-linked RAPD markers and to use them in discriminating between sexes of Oreochromis niloticus niloticus.

Materials and Methods

The manzala strain used in this study is known to be relatively inbred in comparison with other strains of this species (18). Three different genotypes (referred to as XX female, XY male and YY male) were used. These were chosen on the basis of the known sex ratios obtained in progeny tests (19). YY male fish generally produce 100% male offspring in crosses with XX females and XY sex-reversed females. These three genotypes are referred to for convenience as genotypic sexes. This does not imply that specific sex chromosomes exist in this species. All YY males and XY females were from the crosses, (1) XY sex-reversed female x YY male and (2) XY male x XX female, respectively. The male parent of cross (2) was an offspring from cross (1). XY males used in the RAPD experiment were progenies from both crosses.

The isolation of DNA from fin tissue samples, and conditions for the amplification of RAPD markers and non-denaturing polyacrylamide gel electrophoresis are described by Bardakci and Skibinski (18). Although RAPD markers were resolved mainly using 5% non-denaturing polyacrylamide gel electrophoresis, most of them were also separated on 1.5% agarose gels in 1xTAE buffer (0.4M tris, 5mM sodium acetate and 1mM disodium EDTA pH 8.2) visualized with ethidium bromide in order to simplify the profile of RAPD bands.

Results

Initially, bulk segregant analysis (20) was used to search for sex-specific markers with a total of 140 10-mer RAPD primers (OPA, OPB, OPC, OPD, OPE, OPF and OPG, each containing 20 primers) from Operon Tech. Alameda, CA. Comparison of amplification of pooled DNA samples from three genotypic sexes failed to show any reproducible and clear cut RAPD markers occurring in one sex alone. Therefore, the first three sets of primers (sets OPA, OPB and OPC) were tested on four individuals of each genotypic sex separately. Of these primers, 21 primers produced polymorphism between sexes. A total of 173 bands were examined and each primer included in the analysis produced 8 bands on average. Of these, 46 bands were polymorphic between individuals tested.

As three genotypic sexes were analyzed, the potential existed for the identification of both X and Y chromosome-linked RAPD markers. In the first instance, data were examined for bands that were unique to individuals carrying X or Y. Any marker linked to the Y would be present in both YY and XY males. Similarly, X markers were expected to be present in both XX females and XY males.
XY males. Results showed no single, reproducible RAPD band unique to individuals with X or Y. Therefore all gels were scored for presence (1) and absence (0) with the aim of using linear discriminant function analysis (SPSS Inc.) to identify a combination of bands that might discriminate between the genotypic sexes. RAPD fragment patterns of two primers are shown in Fig. 1a,b.

The results showed significant difference (P=0.004; Wilks' lambda=0.006) between individuals with XX and XY genotypic sex and individuals with YY genotypic sex. In addition the difference between individuals with and without Y was significant (P=0.004; Wilks' lambda=0.006). The individuals used for analysis were obtained from two closely related families (see above). The analysis failed to discriminate between families (P=0.676; Wilks' lambda=0.222) giving confidence that significant X versus Y related differences are not the result of family genetic differences unrelated to sex. When three genotypic sexes were compared in the same test, no significant discrimination between them was obtained (P=0.550; Wilks' lambda 0.252). Large values of Wilks' lambda suggest that group mean scores tend not to be different.

Results of canonical discriminant function analysis showed correct classification (100%) of all individuals within group defined Y and X. The significant discrimination between individuals belonging to three different genotypic sexes was the result of the intermediacy of XY - the differences in discriminant scores are highest between the YY and XY genotypic sexes. Discriminant function scores are given in Table 1.

![Figure 1. RAPD fragment patterns of primers (a) OPA02 and (b) OPA07 from three genotypic sexes of O. niloticus. M: Molecular weight marker.](image-url)
One of the important points arising from the present study was to select markers for effective separation of sexes. Stepwise discriminant function analysis was used to achieve this objective. As a result, 6 RAPD primers (OPA11, OPA02, OPB08, OPC14, OPC18 and OPC11) giving the best discrimination between individuals with and without Y were selected. Similarly, 5 RAPD primers (OPA02, OPA04, OPA11, OPB18 and OPA13) were selected that discriminated individuals with and without X.

Discussion

Obtaining a marker linked to a gene or genomic region through RAPD analysis depends to a large extent on chance because random sequences are used as PCR primers. For example, although Levin et al. (21) obtained 13 Z-linked RAPD markers in chickens using only 298 primers, Hormaza et al. (22) found a single female specific RAPD marker in pistachio, Pistacia vera using 700 primers. Moreover, the chance of any RAPD markers being linked to a gene or a genomic region of interest is dependent on genome size, type of gene or genomic region (dominant or codominant) and on the type of population used to generate markers. Genetic homogeneity between groups compared (apart from in the target genomic region) will increase...
the chance of detection of a marker linked to the target region. The subspecies used in this study is relatively inbred in comparison with other subspecies and strains of this species (18) and this should have favoured identification of markers linked to a segregating sex determining genomic region. One reason for the failure to obtain perfectly reproducible clear cut differences could be the complexity of the mechanism of sex determination in tilapia. For example, Mair et al. (23) obtained a small proportion of males from several *O. niloticus* gynogens. These males were progenies tested and found to be naturally sex-reversed females with XX genotypes. Several hypotheses have been proposed for sex determination in tilapia based on studies of interspecific hybridizations, chromosome set manipulations, sex inversion and intraspecific crosses (10-12). However, none of these hypotheses gives a satisfactory explanation for all data. A plausible model is that sex in tilapia is controlled by major sex determining factors with aberrant sex ratios being the result of minor sex modifying factors. Recently, Baroiller et al. (24) demonstrated the effect of high temperature on the sex ratio of *O. niloticus*. The result of the present study also give support to the hypothesis of the presence of a major sex determining factor in *O. niloticus*. This is simply because it has proved possible to discriminate between the X and Y groups. However, the observation that several RAPD bands are necessary for the perfect discrimination suggests that minor sex factors associated with RAPD markers might also be involved. Consequently, regarding sex as a quantitative trait appears to be the most hopeful approach in sex determination in tilapia.

**Acknowledgements**

I would like to thank D.O.F. Skibinski for his supervision during this study. I also thank G.C. Mair and colleagues at the Freshwater Aquaculture Centre (FAC), Central Luzon State University, Philippines for providing the fish samples used in this study. I am grateful for the support of a scholarship from Cumhuriyet University, Sivas, Turkey.

**References**

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