The Determination of Sex-Linked Molecular Markers with Random Amplified Polymorphic DNA (RAPD) Technique in *Aphanius danfordii* (Cyprinodontidae) Species

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Abstract: The sex determination system (SDS) has been widely investigated as a major interest in fish species for aquaculture. The molecular marker techniques have been introduced to be an effective tool for both the identification of sex-specific genetic markers and sex control [1] . With this aim, sex-specific DNA markers are useful for hatchery management. Sex identification for the fish at early ages can reduce broodstock rearing costs. In the present study, *Aphanius danfordii* (Cyprinodontidae) species was used for investigating the determination of sex-specific DNA markers by using RAPD technique. Firstly genomic DNA was isolated from total fifty male and female individuals of *Aphanius danfordii* species. Later twenty random PCR primers were used and RAPD reaction was optimized for each RAPD primer. The RAPD products were seperated on agarose gels and obtained the band profiles. It did not show any sex-specific genetic polymorphism between male and female fish samples to use as a sex-linked genetic marker.

Key Words: *Aphanius danfordii*, RAPD, sex-specific markers

Aphanius danfordii (Cyprinodontidae) Türünde Rasgele Artırılmış Polimorfik DNA (RAPD) Tekniğiyle Cinsiyete Özgü Moleküler Markerlerin Belirlenmesi

Özet: Su ürünleri yetiştiriciliği açısından, balık türlerindeki cinsiyet belirleme sistemleri temel bir ilgi alanı olarak geniş çapta araştırılmaktadır. Moleküler marker teknikleri, hem cinsiyetin kontrolü hem de cinsiyete özgü genetik markerlerin belirlenmesinde çok etkili bir araç olarak gösterilmekte olup eşeye özgü DNA markerleri, balık yetiştiriciliği için yararlı olmaktadır [1]. Balıklarda erken dönemlerde cinsiyetin belirlenmesi, balık çiftliklerindeki yavru yetiştirme maaliyetlerini düşürebilir. Bu çalışmada, RAPD tekniği kullanılarak *Aphanius danfordii* (Cyprinodontidae) türünde eşeye özgü DNA markerleri araştırılmıştır. İlk olarak *Aphanius danfordii* türüne ait toplam 50 erkek ve dişi bireyden genomik DNA izole edilmiş ve 20 rasgele PCR primeri kullanılarak, RAPD reaksiyonu optimize edilmiştir. PCR ürünleri agaroz jelde ayrıştırılarak band profillerinin elde edildiği bu çalışmada eşeye bağlı genetik marker olarak kullanılabilecek, erkek ve dişi bireyler arasında herhangi bir eşeye özgü genetik polimorfizm gözlenmemiştir..

Anahtar sözcükler: *Aphanius danfordii*, RAPD, cinsiyete özgü genetik marker.

1. Introduction

Fish species have characteristic diversity of their morphological, ecological and behavioural traits, and furthermore different sex determination systems (SDS). In a small group of fish species, morphologically differentiated sex chromosomes have been reported [2] but usually different sex determination systems have been observed in fishes [3, 4]. These various sex determination systems can be classified as a genetic sex determination system (GSD) with a simple genetic mono-factorial system, XX/XY or ZZ/ZW and different polyfactorial systems or environmental sex determination (ESD). Temperature is the basic environmental factor influencing the sex ratio in fish (i.e. temperature dependent sex determination-TSD) [5]. The ESD system has been firstly observed in the Atlantic silverside, *Menidia menidia* [6] and later documented in a numerous of species from different families [4, 7] and one of the most common studied fish group is Nile tilapia. According to Bezault et al. (2007) the Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) is likely the best studied model species showing a complex system of sex determination [7, 8] , combining both genetic and environmental factors. The genetic sex determination system (GSD) is shaped on a predominant mono-factorial genotypic system with male heterozygous gametes

(XX/XY) [9-12] and the influence of minor genetic factor(s) independent of (autosomal) and/or epistatic to the major sex determinant [7, 10, 13-17]. The ESD influence is shaped with the temperature effect on sex differentiation with a functional masculinization of female genotypes at high temperature (over 32-34 ^{0}C) [8, 18-20]. Also some sex-linked markers have recently been identified in the Nile tilapia [21-24].

The sex control is very important phenomenon especially in teleost fish, due to one of sex grows much faster than the other in many teleost fish. For example, in tilapia species, the males grow faster and therefore they have a greater value. Thus, monosex stocks have been developed in various fish species like chinook salmon [25, 26] , Nile tilapia [27] and with this purpose, sex identification for the fish at early ages can reduce broodstock rearing costs. For this respect, sex-specific DNA markers are useful for hatchery management nowadays. The molecular marker technique is presented to be an effective tool for both the identification of sex-specific genetic markers and sex control [28, 29]. The sex-linked molecular markers in teleosts are available in many reports. For example, male-specific RAPD markers were isolated and used for genetic sex determination in salmonids [4, 30, 31]. Also male specific RAPD markers were isolated from African catfish, *Clarias gariepinus* [32], and sex linked AFLP and microsatellite markers were identified in Nile tilapia, *Oreochromis niloticus* [21, 33]. Due to advances in molecular biology techniques, too many highly informative DNA markers have been developed for the identification of genetic polymorphism. The random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers [34-39].

The aim of the present study was to investigate the sex-linked specific genetic markers between male and female individuals of *Aphanius danfordii* (Boulenger, 1890) species by using random amplified polymorphic DNA (RAPD) technique.

2. Material and Methods

2.1. Sample Collection

In this study *Aphanius danfordii* species, spreading in Turkey was used. A total of fifty specimens with 25 female and 25 male samples of *Aphanius danfordii* species were collected from a small lake called Karpuzatan in Kayseri. Most of the samples

were caught by fishing net and electro-fishing tools, and preserved in 95% (v/v) ethanol for the analysis. Phenotypic sex was determined by verifying the presence of eggs or sperm using catheterization.

2.2. Laboratory protocol

Total genomic DNA was extracted either from muscle tissue at the bottom of dorsal fin or alternatively from pectoral fin tissue of specimens. Approximately 100 mg tissue was treated with 25 µl proteinase K (10 mg/ml) and 50 µl SDS (10%) in 500 µl STE buffer (0.1 M NaCl, 0.05 M Tris and 0.01 M Na_2EDTA pH: 8.0) for over night at 37 ^{o}C. After incubation, DNA was isolated by two steps of phenol-chloroform- isoamyl alcohol (25:24:1), followed by precipitation with cold absolute ethanol. Precipitated DNA was re-suspended in 1mM TE (Tris-EDTA) buffer and quantified at wavelength of 260 nm by a spectrophotometer.

Isolated DNA samples from female individuals were collected in a test tube to get a mix DNA solution and the same process was also made for the male samples. RAPD analysis was performed on polymerase chain reaction (PCR)-amplified segments with twenty RAPD primer. The primers' name and base sequence were given in Table 1. Each PCR reaction was carried out in 25 µl volume containing 200 µM each deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 0.2 mM each primer, approximately 500 ng template DNA, 1x PCR reaction buffer (75 mM Tris-HCI pH 8.8 at 25 ^{o}C, 20 mM $(NH_4)_2SO_4$, 0.01% Tween20, MBI Fermentas), 2.5 mM $MgCl_2$ and 2 units *Taq* polymerase (MBI Fermentas). The PCR conditions for the 35-cycle amplification reaction was as follows: initial denaturation at 94 ^{o}C for 3 min, denaturation at 94 ^{o}C for 30 s, annealing at 45 ^{o}C for 30 s, extension at 72 ^{o}C for 1,5 min and final extension at 72 ^{o}C for 5 min.

Table 1. The base sequences and names of RAPD primers and observed band profiles of each RAPD result.

Primer code	Primer sequence (5'-3')	Band profiles on agarose gels
OPC-02	GTGAGGCGTC	Both females and males have four bands
OPA-08	GTGACGTAGG	Both females and males have two bands
OPA-05	AGGGGTCTTG	Both females and males have a single band
OPA-04	AATCGGGCTG	Both females and males have three bands

OPA-19	CAAACGTCGG	Both females and males have two bands
OPA-16	AGCCAGCGAA	Both females and males have a single band
OPA-14	TCTGTGCTGG	Both females and males have a single band
OPA-10	GTGATCGCAG	Both females and males have a single band
OPA-01	CAGGCCCTTC	Both females and males have three bands
OPB-05	TGCGCCCTTC	Both females and males have a single band
OPA-03	AGTGAGCCAC	Both females and males have three bands
OPA-07	GAAACGGGTG	Both females and males have a single band
OPA-11	CAATCGCCGT	Both females and males have four bands
OPA-12	TCGGCGATAG	No band
OPA-13	CAGCACCCAC	Both females and males have four bands
OPA-15	TTCCGAACCC	Both females and males have two bands
OPA-20	GTTGCGATCC	Both females and males have three bands
OPB-08	GTCCACACGG	Both females and males have two bands
OPC-03	GGGGGTGTTT	No band
OPC-04	CCGCATCTAC	Both females and males have three bands

The PCR products were analysed by electrophoresis using a %1,5 agarose gel in 1x TBE buffer. Hind III DNA ladders (MBI, Fermentas) were run along with the samples of PCR products as a DNA size marker. Gels were run at 90 mA for 2h and stained with ethidium bromide solution. RAPD banding patterns on gels were visualised using UV system and recorded by polaroid camera.

3. Results

In this study, 20 female and 20 male individuals of *A. danfordii* species were used and 20 random oligonucleotid primers were tried to define sex-linked genetic polymorphism. Finding a polymorphism between male and female fish samples was aimed by mixing all isolated DNA from male individuals in one tube and female samples in a different tube. Except for just two RAPD primers (OPC-03, OPA-12), the other primers were worked successfully (Table 1). And after RAPD reaction, whole band profiles of RAPD primers were obtained. They did not show any different band pattern between male and female individuals. The band pattern were the same and given fig. 1 and fig. 2.
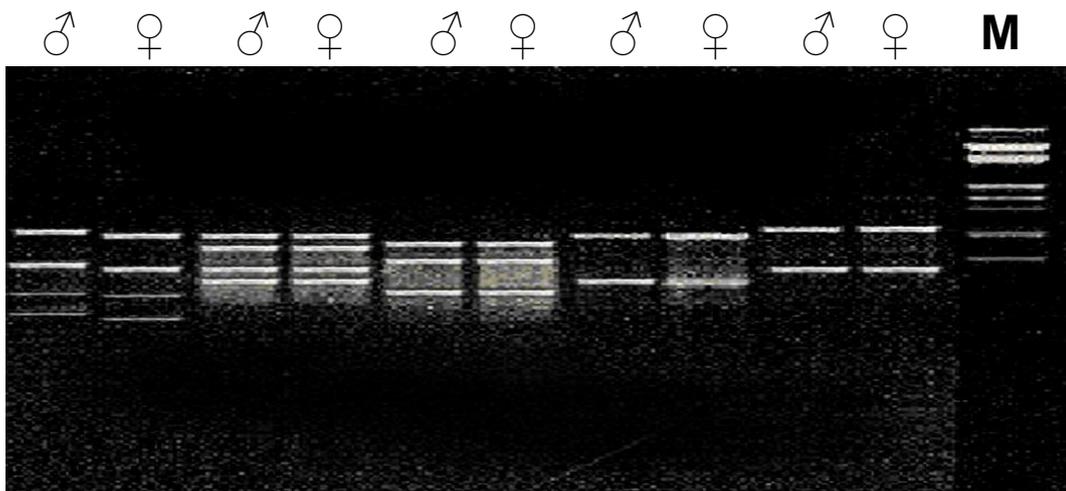
Fig. 1. RAPD band profiles of male and female individuals. Primers are given in the following order: OPA-11, OPA-13, OPA-03, OPB-08, OPA-15 and the last column is λ DNA cut with Hind III marker. All the primers have two sample as female and male individuals.
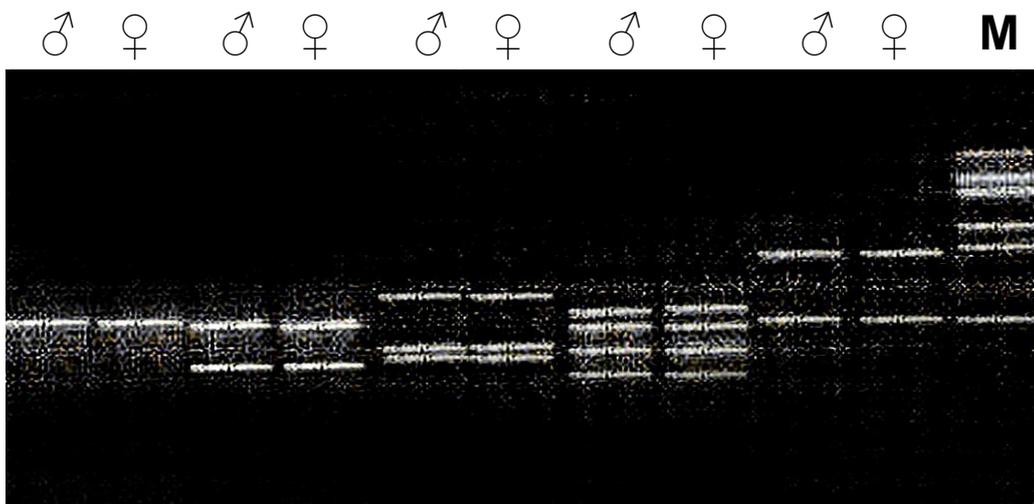
Fig. 2. RAPD band profiles of male and female individuals. Primers are given in the following order: OPA-05, OPA-08, OPA-04, OPC-02, OPA-19 and the last column is λ DNA cut with Hind III marker. All the primers have two sample as female and male individuals.

4. Discussion

According to the result of this study, there was no sex-linked polymorphism between the male and female individuals of *A. danfordii* species. So it is considered that there is no sex-linked genetic marker in this species to identify the male and female individuals according to twenty RAPD primers used in this research. But it is also clear that to say the certain consequence, twenty RAPD primers and fifty specimens are not enough especially in RAPD techniques. Obtaining a marker linked to a gene or genomic

region through RAPD analysis depends to a little bit chance. For example, although Levin et al. [40] obtained 13 Z-linked RAPD markers in chickens using only 298 primers, Hormaza et al. [41] found a single female specific RAPD marker in *Pistacia vera* using 700 primers [42]. Moreover, the possibility 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 [42]. On the other hand, the simplicity and applicability of the RAPD technique have captivated many scientists' interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterisation of the genome of the species in study. Just only a single oligonucleotide of random sequence is used and no prior knowledge of the genome subjected to analysis is required. At an appropriate annealing temperature during the thermal cycle, oligonucleotide primers of random sequence bind several priming sites on the complementary sequences in the template genomic DNA and produce DNA products if these priming sites are within an amplifiable. Therefore, amplification products from the same alleles in a heterozygote differ in length and will be detected as presence and absence of bands in the RAPD profile. The main problem for RAPD technique is band intensity on electrophoresis gels. This reproducibility problem is usually the case for bands with lower intensity. The reason for bands with high or lower intensity is still not known. Perhaps some primers do not perfectly match the priming sequence, amplification in some cycles might not occur, and therefore bands remain fainter [34].

Acknowledgements

5. References

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