Determiniation of Sex Markers by Amplified Fragment Length Polymorphism (AFLP) in Killifish Aphanius anatoliae anatoliae (Leidenfrost, 1912)

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ABSTRACT

Identification of sex in fishes is a difficult task, thus genetic and ecological factors involved in sex determination. Therefore, sex specific genetic markers can be developed with using molecular techniques. In this study, sex markers of Anatolian endemic Aphanius anatoliae anatoliae [1] is tried to identify with using AFLP (Amplified Fragment Length Polymorphism) technique. A total of 15 different primer combinations were analysed with using a sex-typed pool strategy for 18 individuals of two populations from Turkey. Three of clearly appeared bands were polymorphic between populations. Observed different bands were found for the males of Tuz Gölü populations while they were found for the females of Güneşli populations. The results of the study showed that the AFLP technique has a potential of solving sex identification problems of A. a. anatoliae and it is obvious that sex associated markers can be developed with AFLP technique. Between the populations of A. a. anatoliae, sex independent polymorphic AFLP band profiles were observed, thus we suggest to study larger numbers of populations. From the results of this study, it is recommended to use larger number of primer combinations for the identification of sex-specific marker of A. a. anatoliae to obtain precise results.

Keywords: Sex Marker, Sex Determination, AFLP, Aphanius anatoliae anatoliae, Fish

1 INTRODUCTION

Fishes are the most abundant vertebrates on Earth, showing a diversity of species (27 977 species) unmatched by other classes [2], [3]. The group exhibits a large variety of mechanisms of sexual determination. Sexual determination refers to the event that compromises a bipotential gonad to develop as an ovary or a testicle [4]. The processes of sexual determination and differentiation in fish are highly plastic, even on top of a given genetic background, as it were, certain environmental factors, such as pH, temperature, and social conditions, may wield a strong influence [5], [6], [7], [8].

Sex identification is an important issue for studies including ecology, behavior, conservation and genetics of many species for especially fish species [3]. Genetic sex determination (GSD) and environmental sex determination (ESD) models are available for the identification of sex in fishes.

Genetic sexual determination (GSD) may involve monogenic and polygenic systems, with factors localized on autosomes or on sex chromosomes [9]. In polygenic systems, sex is determined by the accumulative genetic action of all factors involved in its determination, and not by a single genetic locus [10]. In monogenic systems, sex is determined by a gene located on a certain chromosome and genes on other chromosomes have little effect [8].

Many species of teleosts do not present morphologically differentiated sex chromosomes. However, some species have heteromorphic sex chromosomes which can be identified by fluorescence in situ hybridization (FISH) and cytogenetic studies. Fishes have both XX/XY and ZZ/ZW heterogametic modes (XY, male, ZW, female) of sex determination. Though the medaka (Oryzias) follows an XX/XY heterogametic mode of sex determination, the X and Y chromosomes are homomorphic, unlike in mammals [8], [11].

Most fish species exhibit reduced sexual dimorphism and usually lack heteromorphic sex chromosomes [12]. Sexing is thus difficult and alternative techniques to those based on karyotyping are required for the determination of sex. DNA-based tests can solve this problem but sex specific markers need to initially be isolated [13], [14].

DNA markers provide useful tools for examining sex linkage in fish since DNA structure is not anticipated to change with altered physiology or environments. Moreover, examination of DNA sequence organization on sex chromosomes can provide useful insights into the evolutionary processes that are operating to influence sex-chromosome structure, and ultimately, can yield information on the conservation (or lack thereof) of sex-determination processes among species [9].

Sex-specific DNA sequences have been identified in several fish species using different assays, including RAPDs, RFLPs, AFLPs and microsatellite markers [13], [14], [15], [16].

The center of diversity of the killifish genus Aphanius [17] is in Turkey [18]. Of the 19 described species in the genus, six species and four subspecies occur in Anatolia. Sex determination markers were not identified for Aphanius species that are lack in heteromorphic sex chromosomes [19], [20], [21], [22].
In this article, we focus on the use of the amplified fragment length polymorphism (AFLP) technique [23] for the identification of sex-specific markers in Aphanius anatolica anatolica [1] based on pooled DNA samples from known male and female individuals which were previously phenotypically sexed.

2 MATERIAL AND METHODS

2.1 Genomic DNA Isolation

Total of 18 Aphanius anatolica anatolica fishes were collected from two populations. Four male and 4 female were collected from Güneşli (G), Eşmekaya region of Turkey. Five male and 5 female were collected from south of Tuz Gölü (T) (Fig. 1). Fish samples were stored in 95 % ethanol immediately after collection for DNA analysis.

![Fig. 1. Sample locations (G=Güneşli, T=Tuz Gölü).](Image)

Genomic DNA was isolated from muscle tissue with a standard proteinase K, phenol-chloroform method [24]. Approximately 100 mg muscle tissue was digest in 500 µL STE buffer (0.1 M NaCl, 0.05 M Tris and 0.01 M EDTA, pH 8), 20 µL proteinase K (10 mg/ mL) and 50 µL SDS (10 %). Following, DNA was extracted by a standard phenol-chloroform procedure and precipitated with absolute ethanol. Precipitated DNA was dissolved in distilled water and quantified at wavelength of 260 nm by a spectrophotometer.

Pooled DNA samples each population were prepared using 100 ng/ µL of genomic DNA from 4 (Güneşli, 4 ♂ and 4 ♀) to 5 (Tuz Gölü, 5 ♂ and 5 ♀) males and females to do 2 pools/each sex (n= 4-5 fish/pool). Pooled DNA were initially analyzed using a total of 15 different primer combinations.

2.2 AFLP Reaction

AFLP analysis was carried out as described by [23] with some modifications. DNA samples were cutted with EcoRI and MseI (Fermentas, MBI) restriction enzymes. The oligonucleotid adaptors were ligated to cutted to the ends of DNA fragments to generate template DNA for amplification. In preamplification step, the genomic DNAs were amplified with two AFLP primers (EcoRI-A and MseI-C) both having a single selective nucleotide. PCR (Stuart, UK) amplifications were performed 26 cycles with the following cycle profile: a 30 s DNA denaturation step at 94 ℃, a 30 s annealing step (see below) and a 2 min extension step at 72 ℃. The annealing temperature in the first cycle was 60 ℃, was subsequently reduced each cycle by 0.7 ℃ for the next 12 cycles and was continued with a 30 s DNA denaturation step at 94 ℃, a 30 s annealing step at 56 ℃ and a 2 min extension step at 72 ℃ for the next 23 cycle.

For the selective amplification, primer combinations used were as follows:

- E-AGC-M-CGC (a1)
- E-AGC-M-CGA (a2)
- E-AGC-M-CAC (a3)
- E-AGC-M-CGT (a4)
- E-AGC-M-CTA (a5)
- E-ATC-M-CGC (b1)
- E-ATC-M-CAC (b2)
- E-ATC-M-CTA (b5)
- E-AAT-M-CTG (c4)
- E-AAT-M-CTG (c3)
- E-AAT-M-CTA (c5)

AFLP-PCR products were denatured at 90 ℃ for 4 min. and separated on 6 % denaturing polyacrilamide gels and visualized with silver staining. AFLP markers were analyzed manually based on the presence/absence of DNA fragments.

3 RESULTS AND DISCUSSION

The AFLP analysis involves three steps: (1) restriction of the genomic DNA and ligation of oligonucleotide adapters, (2) selective amplification of sets of restriction fragments, and (3) gel analysis of the amplified fragments [23]. [The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA.] As demonstrated in this document, the numbering for sections upper case Arabic numerals, then upper case Arabic numerals, separated by periods. Initial paragraphs after the section title are not indented. Only the initial, introductory paragraph has a drop cap.

AFLP is relatively cheap, easy, fast and reliable method to generate hundreds of informative genetic markers [23], [25], [26]. Pre-knowledge about DNA sequence is not required for AFLP method. Many different DNA regions can be screened simultaneously with this technique. AFLP markers are useful tools for identification of near relations like twin species and hybrid species, structure and diversity of population at the levels of species and subspecies. For a wide range of taxa, including plants, fungi, animals and bacteria, AFLP markers have been used to uncover cryptic genetic variation of strains, or closely related species, which had been impossible to resolve with morphological or other molecular systematic characters [27], [28], [29], [30], [31], [32], [33], [34], [35], [36], [37]. At the same time it is showed that AFLP is a useful toll for the development of sex markers for both animals ([38], [39], [40], [41], [42], [14], [43] and plants ([44], [45], [46].

In this study, 18 individuals, 9 male and 9 female from two different populations of Aphanius anatolica anatolica species were from Turkey collected. Sex associated markers were examined with using AFLP technique by 15 different primer combinations. AFLP-PCR products were separated on 6 % denaturing polyacrilamide gels and analyzed manually based on the presence/absence of DNA fragments. The 20 bands from produced 36 bands were appeared clearly. For three
primer combinations (\(a_4\), \(a_5\) and \(c_5\)) of these clearly bands, sex-independent polymorphic bands were observed for the populations. The observed different fragments are in the range of 350-400 bp. For primer combinations of \(a_4\), \(a_5\) and \(c_5\), observed different bands were found for the males of Tuz Gölü populations while they were found for the females of Güneşli populations (Fig. 2).

![Fig. 2. AFLP band profiles of male and female individuals (G: Güneşli, T: Tuz Gölü).](image)

The small number of primer combinations (\(n=15\)) used in this study seems to be disadvantage, but sex specific markers had been developed for some studies with conducting relatively small numbers of primer combinations (\((46), n=16\); \((43), n=64\); \((47), n=64\)) and besides many studies had failed to identify sex specific markers with using higher primer combinations (\((48), n=570\); \((49), n=128-396\); \((14), n=486\)). On the other hand, according to some researchers [16]; [50]; [14], the success of the identification of sex-specific cichic fish markers has depended largely on the presence of a sex chromosome or non-chromosomal genetic sex-determining mechanisms [39] in the target species. The failure to identify sex-associated markers in the present study may be arises from the absence of sex chromosomes for Aphanius a. anatolica. Another suggestion of failure to develop sex markers is suggested by [49], according to [49], in order to explain the failure of sex-marker identification, a sex determining system based on a gene dosage effect has to be considered. Based on these facts, to obtain precise results, for future investigations, we recommend to use the number of primer combinations for the identification of sex-specific marker of Aphanius a. anatolica.

4 Conclusion

In this study, sex dependent markers were not developed as insufficient numbers of individuals or primer combinations were used. It is noteworthy that the inter-population sex-independent polymorphic bands were different between females and males of same populations. Sex markers can be developed with conducting AFLP-PCR technique. Further studies with AFLP technique, sex dependent markers can be developed with using many more primer combinations, populations and individuals. Consequently, this study will be a guide to researcher for the choice of primers and primer combinations.

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specific marker Aquaculture, 258, 685-688.