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# Failure of PCR-RAPD technique to differentiate sex in Mahisefied (*Rutilus frisii kutum)* from the South Caspian Sea

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## ABSTRACT
In order to identify the sex marker in Mahisefied, *Rutilus frisii kutum*, samples from 5 male and 5 female fish were collected from the south Caspian Sea. Polymerase chain reaction random amplified polymorphic DNA (PCR-RAPD) was performed using 124 primer sets. All bands were numbered using 1 and 0 scores corresponding to the presence or absence of bands, respectively and data were analyzed using RAPDPLOT program. Results indicated that 44 sets of primers did not show any flanking site and produced no bands, while the remaining 80 produced sharp and visible bands on polyacrylamid gel. In total, 1600 bands were scored. However, none of the bands corresponded to either the male or female fish. According to the results it has been concluded that RAPD technique failed to detect sex and cannot be considered as a robust molecular tool for sex differentiation in the studied fish. The reason may be the absence of sex chromosomes in this species or that the genes corresponding to sex differentiation are spread on different autosomal chromosomes with interaction of some environmental factors.

**Keywords**: *Mahisefid, Rutilus frisii kutum , Random Amplified Polymorphic DNA, sex determination.*

## INTRODUCTION
The Caspian Sea, covering an area of 373300 km$^2$, is the world's largest enclosed lake which is home to some 120 species and subspecies of fish. Mahisefid, *Rutilus frisii kutum* is a semi anadromous teleost which migrates into the Kura and Terek Rivers and Ghazel Aghach Bay in the Azerbaijan Republic and to the Iranian coasts for spawning. It is also distributed in the northern littorals of the Black Sea (Razavi Sayyad 1990).

This fish is considered as one of the highly commercially important and indigenous species of the southern Caspian Sea region which comprise 60% of the income of Cooperative Fishermen (Razavi Sayyad, 1990). Overfishing, water regulation for agricultural purposes, dams and bridge construction, influx of various pollutants into the rivers and the destruction of spawning sites have led to the reduction of its stocks (Razavi Sayyad, 1990) (Azari Takami, 1984). Hence, due to the importance of this species, the Iranian Fisheries Organization has taken measures to restore its natural stocks and each year more than 250 million fries are reared and released into the rivers entering the Caspian Sea (Abdolmaleki & Ghani Nejad, 2007).

Up to now, fish sex markers have been identified using various methods among which phenotype markers in guppy (*Poecilia reticulate*) such as Bcp and Rdt markers of the chromosome Y (Fernando & Phang, 1998), protein markers, HEX and sSOD-1 sites in rainbow trout (*Oncorhynchus mykiss*) (Allendorf et al., 1994), cytogenetic and karyotype markers of fish and observing heteromorphic sex chromosomes in medaka (*Oryzias laptupes*) (Matsuda et al., 1998) can be mentioned. (Devlin & Nagahama, 2002) used gynogenesis to calculate the sex ratio of progenies obtained from different crosses and chromosome manipulations. Sex determination was conducted using the positional cloning method in platyfish (*Xiphophoorus maculatus*) (Feroschauer et al., 2005), DNA Hybridization in Rainbow trout and Coho salmon (*Oncorhyncus kisutch*) (Iturra et al., 2001). The amplified fragment length polymorphism (AFLP) method, introduced two Y-chromosome sex

markers in three-spined stickleback (*Gasterostevs aculea*) (Griffiths et al., 2000). The random amplified polymorphic DNA (RAPD) technique was used to develop markers to differentiate sex in many fish species (Welsh & McCelland, 1990; Williams et al., 1990) such as in Nile tilapia (*Oreochromis niloticus*) (Bardakci, 2000), rainbow trout (Iturra et al., 1998), African catfish (*Clarias gariepinus*) (Kovacs et al., 2001), Atlantic salmon (*Salmo salar*) (MacGowen et al., 1998), blowfish (*Tetradon nigroviridis*) (Li et al., 2002) and *Huso huso* (Keyvanshokouh et al., 2004).

RAPD technique was first introduced in the 1990s (Welsh & McCelland, 1990; Williams et al., 1990). Some of the advantages of this technique are its high speed, initial information on the DNA sequences to design a primer, the use of radioactive, small amount of DNA and using various primers for different species. The disadvantages of the technique include weak reproducibility of the bands, scoring of the produced bands, extreme sensitivity to pollution and its dominant inheritance (Welsh & McCelland, 1990). RAPD has various applications in aquatics, including; development of sex markers for identification (Iturra et al., 1998), gene maps and cold resistance markers in fish (Sun & Liang, 2003). Other applications of RAPD techniques are in species identification (Callejas & Ochando, 2001), population, phylogenetic and systematic studies (Congiu et al., 2002), (Callejas & Ochando, 2002). The RAPD markers were also used for diagnosing diseases and identifying fish pathogen (Ravelo et al., 2002).

Up to the present, nine sex determination systems have been reported in fish (Tave, 1993); however, despite the fisheries, economic and biodiversity importance of the Caspian Sea Mahisefied, no studies have been conducted on sex determination in this species. Since all stocks are mainly restored through artificial breeding and fry release, and with regard to a change in the sex ratio and decreased length of male breeders, it is necessary to determine the sex mechanism for this valuable species to answer the question of whether the sharp decrease in the number of male brooders in some rivers is caused by inbreeding or by environmental factors, or a combination of both. The objective of the present study was to identify and separate the DNA-based molecular marker in order to determine sex of the Caspian Sea Mahisefied.

## MATERIALS AND METHODS

Adult Mahisefied, *Rutilus frisii kutum* were obtained from Shahid Ansari Breeding and Culture Center in Rasht, Iran during the breeding season. Caudal fins clips of 5 male and 5 female fish were sampled. About 2-3 g of the soft fin tissue was cut with scissors and stored in 1.5 ml tubes containing 96 % alcohol.

To extract DNA, all samples were transferred to the Laboratory of Molecular Genetics in Sturgeon International Research Institute (Rasht, Guilan, Iran). DNA was extracted by ammonium acetate method (McQuowen et al., 2000). Quality and quantity of the DNA were assessed by spectrophotometer (Nanodrop, model ND1000) at 260-280 nm wavelengths and an electrophoresis on 1% agarose gel. The concentration of DNA samples was set at 50 ng/ $\mu$ L using redistilled water.

PCR experiments were done using 124 random 10-nucleotide primers. Sequences of the primers can be observed in Keyvanshoukouh, et al. (2004). In each PCR reaction with a final volume of 25 µL, 5 u/µ *Taq* DNA polymerase (Cinagen, Iran), 50 mM of $MgCl_2$, 10X PCR buffer, 10 mM of dNTP mix, redistilled water and 50 ng of DNA were used. The thermal cycles for the PCR were set as 94 $^{o}$C for 4 min (primary denaturation), 94 $^{o}$C for 1 min (cyclic denaturation), 33 $^{o}$C for 0.5 min (annealing), 72 $^{o}$C for 2 min (extension) and 72 $^{o}$C for 10 min (final extension). To evaluate the PCR product and to have an accurate separation of the produced DNA bands, 6% polyacrylamide gel and silver nitrate staining was used (Pourkazemi, 1996).

Following electrophoresis, polyacrylamide gels were stained by silver nitrate. Multiplied DNA bands were scored as 1 and 0 for the presence and absence of the bands, respectively. In order to compare the genetic identity and differences between male and female fish based on the counted bands, RAPDPLOT software was used. The statistical analysis was done by the RAPDBIOSYS program.

**RESULTS**

In all DNA samples the absorption at 260 and 280 nm wavelengths revealed their ratio was more than 1.7 and less than 1.9, and they were suitable for conducting RAPD studies. Also, the observation of the clarity of the produced bands on gel showed that DNA samples were both qualitatively and quantitatively acceptable to be used in PCR experiments (Figure 1).

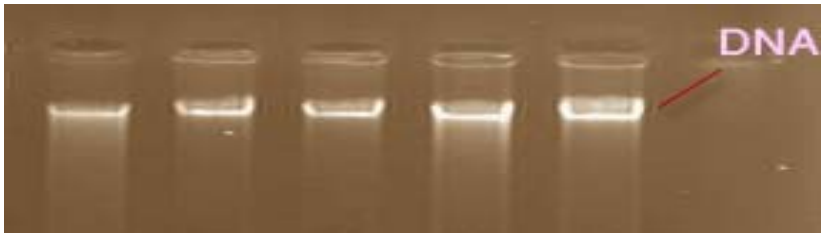


**Fig 1.** DNA electrophoresis of Mahisefied on 1% agarose gel.

Out of 124 primers used, 44 did not produce any bands. The remaining primers amplified distinctive bands with approximate sizes between 100 and 2500 base pairs (Figures 2 and 3). Overall, 1600 bands were counted. Each primer produced 13 bands on an average and primers No.30 and D8 produced the lowest (2) and highest number of DNA bands (30), respectively.

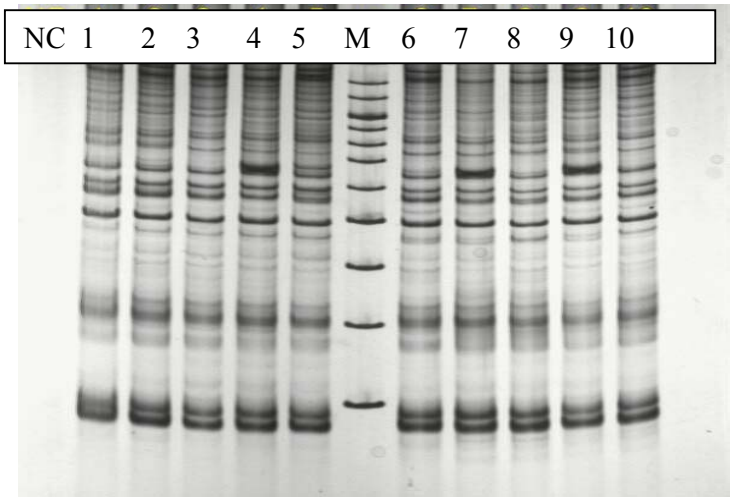


**Fig 2.** PCR product, columns 1-5 are male and 6-10 are female samples; M: molecular marker (100 base pairs). NC is negative control.

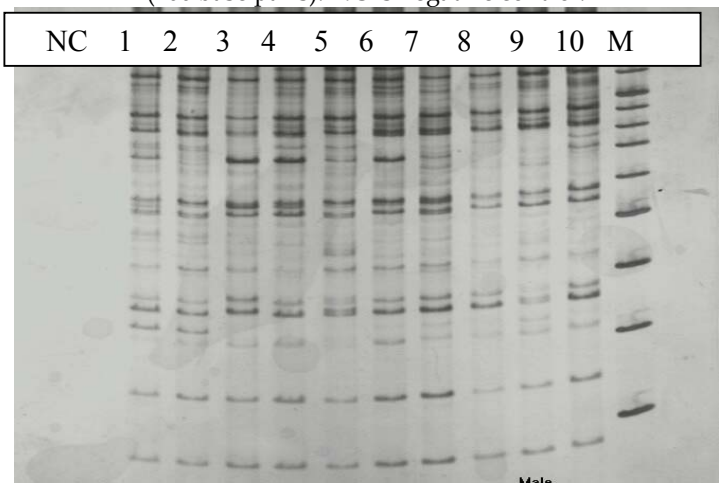


**Fig 3.** PCR product using N15 primer, lane 1-5 are male and 6-10 female samples. M: molecular marker (100 base pairs).NC is negative control.

Except for primer No. 54, band patterns of all studied primers were identical in both sexes and this primer was the only one which produced a band with an approximate size of 350 base pairs only in the female DNA. However, this was not confirmed by the following replicates of the experiment (Figure 4).

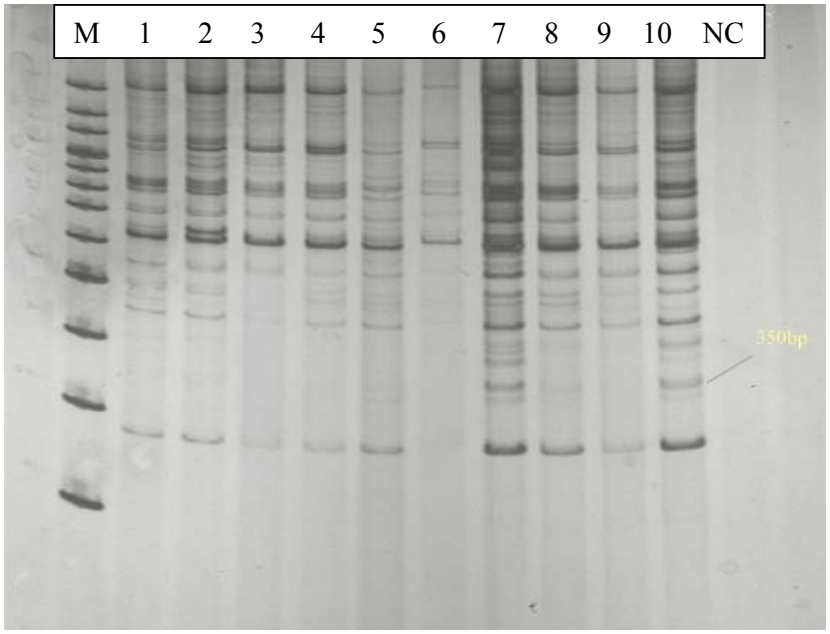


**Fig 4.** PCR product of the Caspian Sea Mahisefied using primer 54, column 1-5: male and 6-10: female fish; M: molecular marker (100 base pairs). NC is negative control.

Based on the identical counted bands in the male and female Mahisefied, RAPDPLOT statistical analysis showed no differentiation limits for the separation of two sexes.

## DISCUSSION

DNA markers provide useful information for evaluating sex linkage map in aquatics because gene and DNA structures do not change with various physiological or environmental conditions. Moreover, they provide a deep and effective insight into the evaluation of organisms, so that sex determination mechanisms can be specified in the species. Generally, teleosts are among fish for which a variety of sex mechanisms exist (e.g. from hermaphrodite to gonochorism or even those influenced by environmental and genetic factors) (Schartl et al., 2004).

Sex determination is quite different in various organisms. In fish, 9 sex determination systems have been identified (Tave, 1993). Although more than 1700 species have been cytogenetically studied, differentiated sex chromosomes have been reported only in the karyotype of 176 fish species (or 10.4% of samples) (Delvin and Nagahama, 2002).

In the present study, evaluation of the PCR product showed that extracted DNA samples were of good quality and the ammonium acetate method was a suitable procedure to extract DNA in RAPD experiments. Totally, 124 primers were used of which 80 primers produced clear bands and 44 others did not produce any. This could be due to various reasons such as unsuitable reaction conditions. With consideration of the fact that reaction conditions were favorable. In addition, it could be due to the lack of flanking sites in 44 primers. With the development of sex determination techniques in a number of teleosts, it would be expected to develop sex determination DNA markers for *R. frisii kutum* using the RAPD technique. However, counting about 1600 DNA bands of PCR product revealed that none of the scored bands were relevant to the molecular sex marker of the fish.

Li et al. (2002), Jaillon et al. (2004) and Roest Crollius and Weissenbach (2005) using sequenced genome of two species,(*Takifugu rubripes* and *Tetradon*

*nigroviridis*), claimed that no sex determination system existed in these species; although recent information suggests the existence of a chromosomal sex determination mechanism by heterogametic material in *T. rubripes* (Cui et al., 2006).

Davidson and MacGowen (1998) studied banding patterns of Atlantic salmon (*Salmo salar*) using 200 different primers and no sex differences were observed. Furthermore, Keyvanshokouh et al. (2004) did not find any differences between male and female *Huso huso* specimens after using 300 different primers and counting about 4146 DNA bands. Wurtz et al. (2006) studied male and female genomes of four sturgeon species by different techniques including the RAPD, but identified no sex markers.

Absence of molecular marker linked to sex determination in *Rutilus frisii* may be due to the shortcomings of RAPD techniques the most important of which are hypersensitivities to pollution, difficult scoring of the produced bands on the gel, dominance of markers, ambiguity of identifying the allele system, unspecified position of RAPD markers on genetic maps and unclear consanguinity and similarities of bands which have similar migrations on the gel (Ref.?). In fact, all of these shortcomings cause the application of RAPD techniques to be limited in certain cases. On the other hand, RAPD has been used in more than 100 species and there are various references which have been able to specify sex markers in different species. For instance, in Nile Tilapia (*Oreochromis niloticus*) (Bardakci, 2000), catfish (*Clarius gariepinus)* (Kovacs et al., 2000), rainbow trout (*Oncorhyncus mykiss*) (Iturra et al., 1998) and guppy (*Poecilia reticulate*) RAPD has been able to successfully identify and introduce sex molecular markers. Moreover, several Y chromosome-linked RAPD ? markers have been identified in different mammalian and avian species (Wardell et al., 1993). Levin et al. (1993) identified thirteen Z chromosome-linked RAPD markers in chicken using 298 primers. Horng et al. (2006) identified a female-specific 732 bp sequence in pigeons using 120 of the primers. Therefore, it can be concluded that despite the mentioned shortcomings and complications, the RAPD technique is capable of identifying and introducing molecular markers in some but not all species.

Based on the investigations performed on sex determination and population studies using RAPD technique, the number of primers used was between 4 and 300. For example, Bardakci (2000) in Nile Tilapia, *Oreochromis niloticus* and Callijas et al. (2002) in eight species of the genus Barbus (Cyprinidae) used 6, 140 and 10 primers, respectively. In the present study, 124 primers were used which itself is considered among studies a large numbers of primers and is an indication of the sufficient and required number of them that were used to identify sex markers in Mahi Sefied. Theoretically, one of the reasons for the failure of this technique to differentiate sex in Mahi Sefied could be the absence of a genetic sex determination system and the involvement of an environmental sex determination system in the studied species (Li et al., 2002). In such cases, several environmental factors including temperature, light duration, pH, salinity and other physical variables may determine an individual's sex (Baroillera & D'cotta, 2001). With consideration of the above-mentioned, lack of a molecular marker or not identifying a genetic sex determination system in the Caspian Sea Mahisefied cannot be precisely attributed to the absence of a sex determination system or a sex marker in this species. To make a precise suggestion on the issue, conducting accurate and comprehensive studies to determine the species' sex from genetic, physiological and/or environmental points of view is necessary.

With regard to the observation of identical DNA banding patterns in both the males and females, it seems that probably there are no sex chromosomes in Mahi sefied and even if they exist, there may be in very few restricted points. Another probability is that sex-controlling genes in this fish may be located on different chromosomes and intervene together in the sex determination process. With the results of the present study taken into account, it is suggested that in case of applying the RAPD technique, more primers should be used to identify sex markers in Mahisefied and that according

to the success of Griffith et al. (2002) in determining the sex of three-spined stickleback (*Gasterostevs aculea*) and that of Jian Zhou et al. (2006) in introducing a molecular marker for *Takifugu rubripes*, using other molecular methods such as AFLP could be suggested.

Moreover, artificial reproduction and counting the sex ratio in 20-50 family crosses (Devlin & Nagahama, 2002) is highly recommended for future studies on sex determination in this valuable species.

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# عدم تعیین تمایزجنسیت ماهی سفید (*Rutilus frisii kutum*) جنوب دریای خزر با استفاده از روش PCR-RAPD

**محمد پورکاظمی، ساره رضی کاظمی**

**چکیده**

بمنظور شناسایی نشانگر جنسیت در ماهی سفید، نمونه ها از ۵ عدد ماهی نر و ۵ عدد ماهی ماده از سواحل جنوبی دریای خزر جمع آوری گردید. واکنش زنجیرهای پلیمراز به روش تصادفی RAPD و استفاده از ۱۲۴ پرایمر، باندهای چند شکلی DNA را تکثیر نمود. تمام باندها با روش یک و صفر بمنزله وجود و یا عدم وجود باند شمارش و رتبه بندی شدند و سپس داده ها با برنامه RAPDPLOT مورد تجزیه و تحلیل قرار گرفتند.

نتایج نشان داد که چهل و چهار پرایمر، هیچگونه پهلو گیری با DNA رشته مادری نداشته و باندی تولید نکرد. در حالیکه هشتاد پرایمر دیگر باندهای قابل رویت و قوی تولید نمودند که بر روی ژل آکریلامید بخوبی قابل مشاهده و شمارش بود. درمجموع ۱۶۰۰ باندDNA شمارش شد، اما هیچیک از باندها بصورت اختصاصی به جنس نر و یا ماده تعلق نداشت. بنابر این نتیجه‌گیری شد که روش RAPD، نتوانست جنسیت نر و ماده را در این گونه تفکیک نموده و این روش نمی‌تواند بعنوان یک روش مولکولی مناسب برای تعیین تمایز جنسی در ماهی سفید مورد استفاده قرار گیرد. عدم یافتن نشانگر مولکولی تمایز جنسیت، ممکن است یا بدلیل فقدان کروموزوم جنسی در گونه مورد مطالعه باشد و یا جنسیت این گونه توسط ژنهایی که برروی کروموزومهای غیر جنسی پخش شده اند همراه با دخالت اثرات محیطی تعیین می گردد.