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Genetic analysis of spring and autumn races of Caspian Sea kutum (*Rutilus frisii kutum*) using microsatellite markers

FEREIDOON CHAKMEHDOUZ GHASEMI[1*], MOHAMAD POURKAZEMI[1], ABBASALI ZAMINI[2], MAHTAB YARMOHAMMADI[1], SHAHROUZ BARADARAN NOVEIRI[1], MOHAMMAD HASANZADEH SABER[1], SOHRAB REZVANI[3] AND LEILA AZIZZADEH[1]

[1] *International Sturgeon Research institute, Rasht, Iran, P.O.Box: 41635-3464*
[2] *Islamic Azad University, Lahijan branch, Faculty of fisheries and Natural Sciences, Iran*
[3] *Iranian Fisheries Research Organization, Tehran, P.O.Box: 14155-6116*

The kutum (*Rutilus frisii kutum*) is one of the economically valuable species of Caspian Sea. Two different races of this fish exist in the Caspian Sea, the spring race and the autumn race. The aims of this study were to analysis the population genetic structure and genetic diversity among and between populations of these races based on microsatellite markers. For this purpose, 100 samples of adult kutum from two regions of Caspian Sea (Anzali Wetland and Shiroud River) were collected. DNA was extracted and 31 pair microsatellite primers were used for PCR and 10 made polymorphic patterns. In this study 191 alleles were observed totally. The maximum numbers of alleles (18) were found in two loci (Ca1 and Ca3) and the minimum number of alleles (2) was found in MFW1 locus. The differences between both races were not statistically significant (P>0.05), neither for average number of alleles per locus nor for observed heterozygosities. The calculated Fst and Rst between two races was 0.056 and 0.15 which shows that the genetic difference was significant (P< 0.01). Spring race in MFW1 locus and autumn race in Lid1 locus were at Hardy-Weinberg equation but not in other loci in both races. The genetic distance was 0.407 which indicating the greatest genetic distance between the two studied races. The data generated in this study showed that the spring and autumn races of kutum in two region of southern part of Caspian Sea are two independent populations.

Key words: Caspian Sea kutum, *Rutilus frisii kutum,* Microsatellite, Genetic structure, Population genetic

INTRODUCTION

The Caspian Sea kutum (*Rutilus frisii kutum*) is a valuable and economic species in southern basin of Caspian Sea. This fish is endemic in the Caspian Sea which that tributes from Volga bight to Miankale Creek Bay, Black Sea and Azov Sea and their rivers (Derzhavin, 1934). The main distribution area of the fish is the South-West Caspian Sea (Vicinity of Bandar Anzali and Ghezel Chai Bay) and also reported from eastern shore (Brackish regions of Atrak river). Over 50% catch of bony fishes in south part of Caspian Sea belongs to this species, catch rate of this fish was over 17000 tons in 2008 (Abdolmaleki & Ghaninezhad, 2008). Derzhavin (1934) reported a fresh water population of this taxon. Spring race of this fish composed over 98% of catch rate. In recent years, autumn stocks gradually recede due to deterioration of its spawning grounds, overfishing etc (Valipour & Khanipour, 2007).

*CORRESPONDING AUTHOR

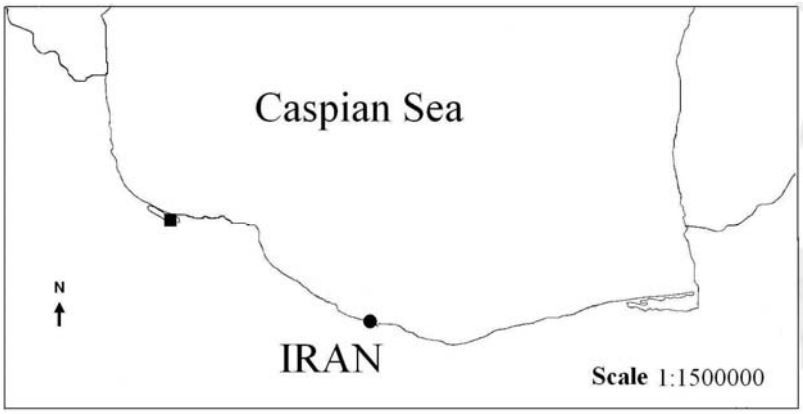

FIG.1.- Sampling regions map: Anzali Wetland (■) and Shiroud river (●)

An understanding of genetic diversity in aquatic organisms can be very helpful in the conservation of their stocks. Genetic diversity is important to both natural and cultural populations because it provides the necessary spectrum of genotypes for adaptive response to changing conditions and heterozygous individuals usually are superior to less heterozygous individuals in many economically important characteristics like growth, fertility and disease resistance (Beardmore *et al.*, 1997). Potential applications in aquaculture include monitoring changes in genetic variation as a consequence of different breeding strategies, the investigation of interactions between wild and cultural populations, parentage assignment and estimation of relatedness between potential breeding pairs (Cross, 2000; Cross *et al.*, 2005; Davis and Hetzel, 2000; Liu and Cordes, 2004; Norris *et al.*, 1999).

Microsatellite markers are routinely used to investigate the genetic structuring of natural populations. F_{ST} and R_{ST} factors have a great importance to determine the differentiation between populations using microsatellite markers. Fst is a measure of population differentiation based on genetic polymorphism data, such as Single nucleotide polymorphisms (SNPs) or microsatellites. This statistic compares the genetic variability within and between populations and is frequently used in the field of population genetics. Rst is a parameter defined as the correlation of allele sizes (rather than allelic states) between genes sampled within populations. (Ballox and Lugan-Moulin, 2002). Microsatellites have been found suitable for a variety of applications in fisheries and aquaculture research, particularly where genetic differentiation within and between populations may be limited.

In recent years, a large number of genetic studies have been done on wild cyprinid species throughout the world, using a variety of molecular markers (Mesquita *et al.*, 2001; Tibbets *et al.*, 2001; Mock and Miller, 2005). Among the molecular markers available in population genetics, microsatellite markers have been found useful for detecting high levels of polymorphism and rare alleles due to their high variability, abundance, neutrality, co-dominance and unambiguous scoring of alleles (Weber & May, 1989; Keyvanshokooh *et al.*, 2007).

Despite the biodiversity and commercial importance of Caspian kutum, unfortunately there is no any study available on its genetic and population structure. The aim of present study was to determine the genetic population differentiation between two spring and autumn races of this species in southern part of the Caspian Sea.

MATERIAL AND METHODS

SAMPLE COLLECTION

Totally 100 samples of adult *Rutilus frisii kutum* were collected from 2 regions including 50 samples from Shirood river (spring race) during the March-April period of 2008 and 50 samples from Anzali

TABLE 1. - Characteristics of 10 polymorphic microsatellite loci of *Rutilus frisii kutum* used in this study

Locus	Primer (5/-3/)	Size (bp)	Annealing temp. (oC)	Reference
Ca1	F- AAGACGATGCTGGATGTTTAC R- CTATAGCTTATCCCGGCAGTA	104–148	59	Dimsoski *et al.*, 2000
Ca3	F- GGACAGTGAGGGACGCAGAC R- TCTAGCCCCCAAATTTTACGG	224-300	61	//
Ca5	F- TTGAGTGGATGGTGCTTGTA R- GCATTGCCAAAAGTTACCTAA	164-204	60	//
Ca12	F- GTGAAGCATGGCATAGCACA R- CAGGAAAGTGCCAGCATACAC	168-200	61	//
Lco1	F- ACGGGACAATTTGGATGTTTTAT R- GGGGCAGCATACAAGAGACAAC	248-300	58	Turner *et al.*, 2004
Lco3	F- AAACAGGCAGGACACAAAGG R- GCAGGAGCGAAACCATAAAT	248-300	60	//
Lco5	F- TTACACAGCCAAGACTATGT R- CAAGTGATTTTGCTTACTGC	160-174	58	//
Lid1	F- TAAAACACATCCAGGCAGATT R- GGAGAGGTTACGAGAGGTGAG	224-280	60	Barinova *et al.*, 2004
Rru2	F- TTCCAGCTCAACTCTAAAGA R- GCACCATGCAGTAACAAT	104-152	53	//
MFW1	F- GTCCAGACTGTCATCAGGAG R- CAGGTGTACACTGAGTCACGC	74-76	61	Crooijmans *et al.*, 1997

Wetland (autumn race) during the September-October of the same year (Fig. 1). For each sample, 2-3g caudal fin tissue was collected and kept in absolute ethanol and transferred to the genetics department of International Sturgeon Research Institute, Rasht, Iran.

DNA EXTRACTION AND MICROSATELLITE ANALYSIS

Approximately 30-50 mg of caudal fin tissue was cut into small pieces with scissors. Total genomic DNA was extracted by standard SDS proteinase-K digestion, phenol: chloroform: isoamylalcohol extraction and ethanol precipitation (Hillis *et al.*, 1996). The concentration of DNA were measured in 260 and 280 nm using spectrophetometr method by Nanodrop (ND 1000 model) and the quality of DNA were assessed by 1% agarose gel electrophoresis and stored at -20 ^{oC} until use.

In this study 31 microsatellite loci: Z1, Z3, Z5, Z7, Z9 (Shimoda *et al.*, 1999), Ca1, Ca3, Ca5, Ca12, Ca17 (Dimsoski *et al.*, 2000), Lco1, Lco3, Lco4, Lco5, Lco7, Lco8 (Turner *et al.*, 2004), CypG3, CypG9, CypG24, CypG27, CypG30 (Baerwald & May, 2004), Lid1, Lid2, Lid8, Lid11, Rru2, Rru3, Rru4 (Barinova *et al.*, 2004), MFW1 (Crooijmans *et al.*, 1997), Ppro132 (Bessert & Orti, 2003) and Rhca20 (Girard & Angers, 2006) were analysed which 10 produced polymorphic patterns. The PCR conditions, especially the annealing temperatures, were optimized for 10 microsatellite loci to produce scorable amplification products (Table 1).

PCR was performed in a 20 µl reaction volume containing 100 ng of template DNA, 15 pmol of each primer, 200 µm of $dNTP_s$, 0.5 unit of *taq* DNA polymerase, 1.5 mM $Mgcl_2$ and 10X reaction buffer (2 µl). PCR reactions were performed with an eppendorf thermal cycler (Mastercycler ep gradient, 96 plus, eppendorf, Germany) under the following conditions: initial denaturation of 5 min at 94 ^{oC} followed by 30 cycle of 30 s denaturation at 94 ^{oC}, 30 s at the respective annealing temperature, and 30 s extension at 72 ^{oC}, ending with 5 min at 72 ^{oC} as the elongation period (Keyvanshokooh *et al.*, 2007).

TABLE 2.-Variability of 10 microsatellite loci in two races of kutum (A, number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; P, *P*-values of X^2 tests for Hardy-Weinberg equilibrium)

Locus	Parameters	Spring race Shirood river	Autumn race Anzali Wetland
Ca1	A	18	15
	H_o	0.957	0.958
	H_e	0.904	0.873
	P	0.001	0.000
Ca3	A	18	16
	H_o	0.723	0.750
	H_e	0.907	0.910
	P	0.000	0.003
Ca5	A	8	11
	H_o	0.340	0.438
	H_e	0.771	0.864
	P	0.000	0.000
Ca12	A	9	9
	H_o	0.745	0.604
	H_e	0.833	0.832
	P	0.013	0.004
Lco1	A	6	5
	H_o	0.340	0.333
	H_e	0.749	0.573
	P	0.000	0.005
Lco3	A	8	10
	H_o	0.553	0.417
	H_e	0.800	0.794
	P	0.000	0.000
Lco5	A	10	5
	H_o	0.638	0.458
	H_e	0.732	0.492
	P	0.000	0.000
Lid1	A	7	12
	H_o	0.957	0.896
	H_e	0.754	0.856
	P	0.001	0.238
MFW1	A	2	2
	H_o	0.298	0.125
	H_e	0.335	0.187
	P	0.447	0.022
Rru2	A	10	9
	H_o	1.00	0.972
	H_e	0.862	0.802
	P	0.001	0.000
Average number of alleles per locus		9.5 ± 4.99	9.3 ± 4.45
Average H_o		0.655 ± 0.26	0.557 ± 0.26
Average H_e		0.765 ± 0.16	0.718 ± 0.23

The PCR products were separated on 6% polyacrylamide gels using a Hoefer gel electrophoresis system (Pharmacia-Biotech, USA). Gel run carried out at 120 V until the loading buffer reached the bottom of the plate. DNA fragments were stained with silver nitrate protocol. The recorded microsatellite genotypes were used as the input data for the GENALEX software (Peakall & Smouse, 2006) in order to calculate allele and genotype frequencies, observed heterozygosity (Ho),

expected heterozygosity (He) and to test for deviations from Hardy-Weinberg equilibrium. Genetic distance between two races was estimated using Nei standard genetic distance index (Nei, 1972). Genetic differentiation between races was also evaluated by the calculation of pairwise estimates of Fst and Rst values. All calculations were conducted using the GENALEX version 6.

RESULTS

Allele frequencies at all loci in both races are shown in table 2. Overall 191 alleles resulted at 10 microsatellite loci, the loci Ca1 and Ca3 presented the highest numbers of alleles (18) in spring race, while the locus MFW1 in both races was the lowest (2).

The average number of alleles per locus was 9.5 for spring race and 9.3 for autumn race. Average observed heterozygosities were 0.655 and 0.557 in spring race and autumn race, respectively. The differences between both races were not statistically significant ($P > 0.05$), neither for the average number of alleles per locus nor for observed heterozigosities. Except MFW1 locus in spring race and Lid1 locus in autumn race, the other loci in both races were significantly detected from Hardy-Weinberg equilibrium (Table 2). The Fst (0.056) and Rst (0.15) values from pairwise comparisons were significant ($P<0.01$), indicating that the populations of two races were divergent from each other. The genetic distance between the races computed by Nei (1972) was 0.407.

DISCUSSION

The long-term persistence of an endangered fish species can be investigated by allelic diversity, gene diversity; effective population size and population structure (Yue *et al*., 2004). Regarding to commercial importance of Caspian Sea kutum and its biodiversity, information about these populations is pivotal for their conservation and sustainable use. Unfortunatly, the knowledge on the molecular genetics and genetic structure of this species and its races are not clear and two races of this species are genetically studied here for the first time.

In this study we have employed 31 microsatellite loci to assess the genetic relationship among populations of two races of Caspian Sea kutum which 10 of them produce polymorphic patterns. According to the results, there is no significant differences between two races ($P>0.05$) neither for average number of alleles per locus nor for observed heterozygosities. High level of heterozygosity in this species can be attributed to use of many breeders in artificial breeding. Regarding of the fact that this species is annually breeding and released into the south Caspian Sea rivers and Anzali Wetland for restocking, regular monitoring of genetic variability among the offsprings is essential to avoid the loss of current polymorphism due to inbreeding and outbreeding problems.

The value of Fst and Rst is a useful measure of genetic differentiation among populations (Ballox and Lugan-Moulin, 2002). At present study the Fst and Rst values based on AMOVA test were significant between two races in two regions ($P<0.01$), although the lowest value of Fst can be considered as an a important genetic differences between populations (Wright, 1978; Hartl and Clark, 1997), suggesting that the two races of these areas are genetically differentiated and do not represent a single panmictic population. The genetic distance between populations was 0.407. Shaklee *et al* (1982) and Thorpe and Sol-Cava (1994) showed that genetic distance value based on (Nei, 1972) for conspecific populations averaged 0.5 (range: 0.002-0.07) and for congeneric species averaged 0.30 (range: 0.03-0.61). The distance value obtained in the present study (0.407) falls within the average value of congenerics, which indicates that the genetic difference among the studied populations of two races is pronounced ($P<0.01$).

Although significant deviations from Hardy-Weinberg equilibrium were found at more loci in two races other than MFW1 loci in spring race and Lid1 in autumn race, there were no significant differences in the average expected and observed heterozygosities among the races ($P>0.05$). The significant differences from Hardy-Weinberg equilibrium could be explained either by sample bias,

migration, artificial breeding or the presence of null alleles in these two races. In the presence of null alleles, heterozygotes possessing a null allele could be erroneously recorded as homozygotes for the variant allele leading to a deficiency of heterozygotes in the respective population.
The result of this study showed that populations of spring and autumn races of kutum in southern part of the Caspian Sea in two areas are genetically differentiated. This information can be applied for future genetic provement and assessment of this species in hatcheries and to design suitable management guidelines for autumn race artificial breeding activities. However, in order to have better conservational policy and restocking programs, further studies are recommended on determining other possible populations of this species in other regions of the Caspian Sea.

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