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Population structure of *Rutilus frisii kutum* in Iranian Coastline of the Caspian Sea using microsatellite markers

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Abstract

Kutum is considered as one of the anadramous species of the Caspian Sea. Due to continuous population decline of the fish since 1975, Iranian fisheries organization started to restock this species. Nowadays, there is growing concern over the effects of restocking on natural populations. For this purpose, population structure and genetic variation of this species in the Iranian coastline of Caspian Sea were investigated by using microsatellite markers. The results showed that the average genetic variation of kutum in the southern coast of the Caspian Sea is lower than those reported for the most anadramous fish. After applying the Hardy-Weinberg equilibrium (HWE) test, all populations were found to be significantly deviated from HWE. IN spite of high number of migration between the Cheshmekileh and Gharasu populations, there is 3 different populations of Cheshmekileh, Gharasu, and Sefidrud in the Caspian Sea. The reported results could be of interest to management and restocking programs of this species in Caspian Sea.

Keywords: *Rutilus frisii kutum*, Population structure, Microsatellite

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1. Introduction

Rutilius frissi kutum is regarded as one of the valuable species, and is mainly distributed along the south and southwest of the Caspian Sea from Atrek river located in Caucasus region (Western coasts of the central Caspian region) into the southern coasts of Turkmenistan (Valipour and Khanipour, 2008). Due to continuous population decline of the fish since 1975, the Iranian fisheries organization started the enhancement programs of this species by producing and releasing more than 200 million of fingerlings every year (Abdolhay *et al.,* 2011). Although stock enhancement programs have been successful in many countries such as Japan, the United State of America, Norway, and Iran, there is growing concern over the genetic effects of these activities on its natural populations (Russell *et al.,* 2011) such as reduced fitness or increased susceptibility to disease. Since genetic variation promotes adaptation to changing environmental conditions and heterozygous individuals are usually superior to less heterozygous individuals in many economically important characteristics like growth, fertility, and disease resistance (Beardmore *et al.,* 1997), conservation of resources is an essential component of species management programs (O'Connell and Wright, 1997). An effective strategy for the conservation of a particular species should be determined by information on its genetic structure (Franklin, 1980). Microsatellites are highly variable nuclear genetic markers and have been found suitable for a variety of applications in fisheries and aquaculture research including monitoring change in genetic variation as a consequence of different breeding strategies, investigation of interactions between wild and cultured populations, parentage assignment, and estimation of relatedness between potential breeding pairs (Gonzalez *et al.,* 2011). The objective of this study was to compare the levels of genetic variation of *kutum* samples in Iranian coastline based on microsatellites.

2. Materials and methods

2.1. Fish Sampling and DNA Extraction

The fish were caught from three different locations of the Iranian coastline (Sefidrud, Cheshmekileh, and Gharasu) (Figure 1). Fin tissue samples were taken from 30 fish from each river and stored in 96% ethanol for subsequent DNA extraction and amplification. Genomic DNA was extracted from a piece of fin clips using the phenol-chloroform procedure described by Hillis and Moritz (1990). The quality and concentration of DNA from samples were assessed by 1% agarose gel electrophoresis, and then the samples were stored at -20˚C until use.

2.2. PCR amplification and Electrophoresis

Six microsatellite loci were analyzed: Ca1, Ca2, and Ca3 (Dimsoski *et al.,* 2000), Lco1, Lco2, and Lco3 (Turner *et al.,* 2004). Gen Bank Accession numbers are AF277573, AF277574, AF277576, AY318777, AY318778, and AY318779

respectively. The polymerase chain reaction (PCR) conditions, especially the annealing temperatures were optimized for the seven microsatellite loci as necessary to produce scorable amplification products. Annealing temperatures were 55ºC for Ca1, 58ºC for Ca_2, and 61ºC for Ca_3, 60ºC for Lco1, 62ºC for Lco_2, and 53ºC for Lco3. Amplification was performed in PCR system (Gradient Eppendorf) using a 25-µl reaction mixture. Each PCR reaction (final volume 25 µl) was composed of 5 µl of 10X reaction buffer, dNTPs 10 mM, $MgCl_2$ 50 mM, primer 20 pmol, genomic DNA 100ng, and 1.5-2 unit of Taq polymerase. The temperature profile consisted of a 3-min initial denaturation at 94˚C, followed by 30 cycles of 30 s at 94˚C, 30 s at the respective annealing temperature, and 1 min at 72˚C ending with 5 min at 72˚C. PCR products were separated on 8% polyacrylamide gels stained with silver nitrate.

2.3. Microsatellite Analysis

The presence of null alleles was tested using Microchecker version 2.2.3 (Van Oosterhout *et al.*, 2004). The recorded microsatellite genotypes were used as input data for the GENALEX software version 6 packages (Peakall and Smouse, 2006) to calculate allelic and genotypic frequencies, observed (H_o) and (H_e) expected heterozygosities, and to test the deviations from Hardy-Weinberg equilibrium. Genetic distance among populations was estimated from Nei standard genetic distance and genetic similarity index (Nei, 1972). Genetic differentiation among populations was evaluated by the calculation of pairwise estimates of Fst values ($Fst = {H_T - \hat{H}_e}/{H_T}$), number of migrant Nm ($Nm = \left[\left({1}/{Fst}\right) - 1\right]/4$ Wright, 1969; Slatkin, 1987) was also calculated using GeneAlex software (Peakall and Smouse, 2006).

3. Results

All the six employed microsatellite loci exhibited polymorphism (Table 1). The number of allele ranged from 2 at Ca1 in Cheshmekileh to 15 at Lco3 in Sefidrud. Among the studied populations, Gharasu population revealed higher allelic and genetic variation (A= 5.1, Ho=0.62 and He=0.67) than the two others (Table 2).

Significant deviation from Hardy–Weinberg Equilibrium (HWE) in most of the cases (7 loci*3 populations) indicated deficit in heterozygosity (Table 2). The population differentiation was found the highest between Cheshmekileh and Gharasu (*Fst*=0.144, P≤0.01) while the lowest (*Fst*=0.013, P≤0.01) was seen between Cheshmekileh and Sefidrud (Table 3). Principal coordinates analysis (PCA) (Figure 2) also revealed the difference among studied populations (Figure 2). The estimated number of migration (N_m) between the Cheshmekileh and Sefidrud across all the studied loci was the highest (N_m =19.5) while it was the

lowest ($N_{m=}$ 1.49) between Cheshmekileh and Gharasu (Table 3). AMOV Aanalysis with consideration of 1 sampling regions (Iranian coastline) and 3 sampling locations (Sefidrud, Cheshmekileh and Gharasu) revealed that almost all of the variances in data namely 77% (P≤0.01) were within locations and genetic variances among locations was23% (P≤0.01) (Table 4). Genetic distance (D) and genetic similarity index (I) among three populations are shown in Table 4. The highest genetic distance (D=0.95) was found between Cheshmekileh and Gharasu while the lowest one was (D=0.02) between Cheshmekileh and Sefidrud (Table 5). Mantel test indicated that the estimated standard genetic distance according to Nei (1972) is negatively correlated with Fst (Y= -3.469x + 9.64, R^2= 0.003) (Figure 3).

Tabel 2. Variability of six microsatellite loci in *Rutilus frisii kutum* populations from Iranian coastline (A, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; P P-values of x^2tests for Hardy-Weinberg equilibrium).

Locus	parameter	Sefidrud	Cheshmekileh	Gharasu
Lco3	*A*	15	12	7
	H_o	0.83	0.46	0.4
	He	0.92	0.858	0.744
	P	0.000***	0.000***	0.000***
Lco1	*A*	12	10	6
	H_o	0.31	0.46	0.367
	He	0.7	0.774	0.765
	P	0.000***	0.000***	0.000***
Lco2	*A*	3	3	6
	H_o	0.367	0.3	0.9
	He	0.316	0.259	0.75
	P	0.68 ns	0.81 ns	0.000***
Ca1	*A*	4	2	4
	H_o	0.16	0.06	0.6
	He	0.5	0.42	0.57
	P	0.000**	0.000***	0.29 ns
Ca2	*A*	5	6	4
	H_o	0.3	0.46	0.5
	He	0.63	0.75	0.53
	P	0.000***	0.000***	0.000***
Ca3	*A*	6	6	4
	H_o	0.53	0.46	1
	He	0.78	0.75	0.64
	P	0.000***	0.000***	0.000***
Avarage number of allells per locus		7.5	6.5	5.16
Avarage H_o		0.46	0.372	0.62
Avarage *He*		0.65	0.64	0.67

Statistically significant values are marked with asterisks. ***≤0.001

Table 3. Multilocus N_m (above diagonal) and F_{st} values (below diagonal) between pairs of *Rutilus frisii kutum* population across all loci.

populations	Sefidrud	Cheshmekileh	Gharasu
Sefidrud	****	19.5	1.7
Cheshmekileh	0.013	****	1.49
Gharasu	0.127	0.144	****

Tabel 4. Genetic distance (D) (above diagonal) and genetic similarity (below diagonal) between pairs of *Rutilus frisii kutum* Populations.

populations	Sefidrud	Cheshmekileh	Gharasu
Sefidrud	****	0.02	0.82
Cheshmekileh	0.97	****	0.95
Gharasu	0.439	0.388	****

Table 5. Analysis of Molecular Variance (AMOVA) based on microsatellite data.

Source	df	SS	MS	%	Prob
Among populations	2	152.92	51.46	23	0.010
Within populations	87	443.83	5.1	77	0.010

Figure 1. Sampling locations of *Rutilus frisii kutum* population.

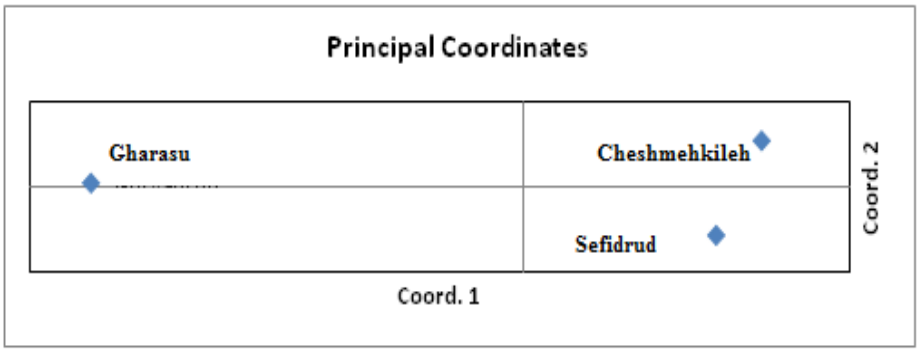

Figure 2. PCA (Principal Coordinates Analysis)

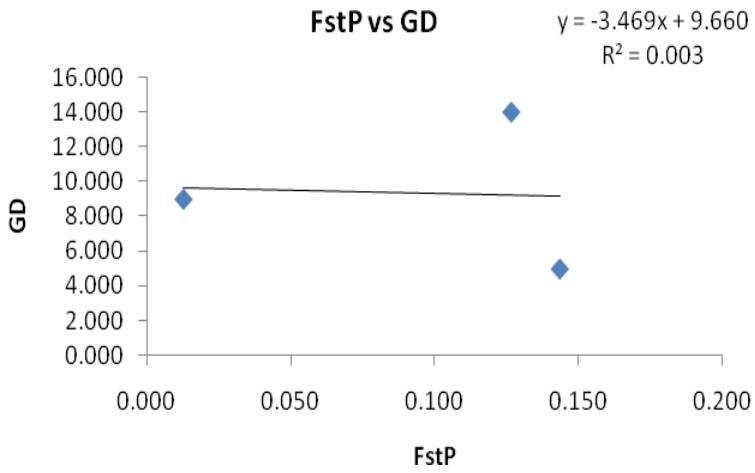

Figure 3. Correlation between Nei's genetic distance and *Fst* revealed by the Mantel test

4. Discussion and Conclusion

This Genetic diversity is important for ecological and evolutionary processes ranging from individual fitness to ecosystem function (Reed, 2009). Despite preparing the majority of kutum stocks from artificial propagation, the knowledge on the molecular genetics and genetic structure of this species is not extensive. Genetic variability estimates for kutum in southern coast of the Caspian Sea (heterozygosity 0.48; alleles per locus 6.4) for these microsatellite loci are lower than those reported for most anadromous fish (heterozygosity 0.68; alleles per locus 11.3) by DeWoody and Avise (2000). Loss of genetic variation of hatchery stocks is a common phenomenon which has been reported in many species [*Salmo trutta* (Was and Wenne, 2002); *Rutilus rutilus caspicus* (Keyvanshokoh *et al.*, 2007); *Abramis brama* (Ghasemi *et al.*, 2007); *Cyprinus carpio* (Thai *et al.*, 2007);

Orechromis niloticus (Nyingi *et al.,* 2009); *Ctenopharyngodon idella* (Liu *et al.,* 2009), and *Rutilus frissi kutum* (Shojaee *et al.,* 2009)]. This is mainly due to the reduction in the effective population size (Ne), inbreeding or combinations of these events (Falconer, 1998). As natural spawning grounds of this species have been destroyed due to pollution and its stock being decreased, annually, millions of fries (average weight 1 g) have been produced and released into the Caspian Sea by Iranian Fisheries Organization public hatcharies (Kavan *et al.,* 2009). This species is highly fecund (Absolute fecundity is averagely 74774 eggs) (Valipour and Khanipour, 2008) and the tendency of keeping a small number of broodstocks to reduce the cost of production coupled with mass spawning practices by many hatcheries would have promoted random genetic drift resulting in reduction in genetic diversity in hatchery stocks.

Significant deviations from HWE were detected in most of loci in studied populations ($P \leq 0.05$). Several possible alternatives such as null alleles and heterozygote deficiency may explain these observations. Where heterozygote deficiency were detected, such deviations would generally indicate that such factors as non-random mating, reduction in effective breeding population or selection pressure at a specific locus are the causes of the observed instance (Garcia de Leon *et al.,* 1997). By using microchecker, null alleles in some loci were found. This would have been likely that the primers had been designed for other species and failed to amplify some alleles in this species. Genetic structure of populations may vary considerably among species depending on relative importance of drift, gene flow, and selection (Slatkin, 1985) along with long-term historical events as postglacial recolonization from different glacial refuges (Taberlet *et al.,* 1998). Pairwise genetic differentiation (Fst) was used to assess genetic differentiation which is the acquisition of allele frequencies that differ among populations (Hartl and Clark 1997). First analysis showed significant genetic differentiation ($P \leq 0.01$) among populations; PCA test also, verified differentiation among them. The population differentiation between Sefidrud and that of two others were considerable.

The estimated F_{st} between Cheshmekileh and Sefidrud was about 0.013 which is lower than the estimated F_{st} (mean of 0.1) by reviewing 7 anadromous fish species (Ward *et al*. 1994) and (0.05) that Balloux, Lugon-Moulin (2002), and Tevfik Dorak (2005) were considered as a single population. Estimates of $Nm > 1$ suggest that gene flow among populations could be mentioned as one of the main factors in genetic variation (Li *et al*, 2007). Estimated gene flow also indicates lower levels of migration between Cheshmekileh and Gharasu (1.49). The lower value of *Nm* again reflects the higher value of F_{st}. AMOVA analysis of data also indicates significant genetic differentiation among sampled populations as well as sampled regions ($p \leq 0.01$). The most genetic variation was concerned among

individuals within the same population (77%) and genetic variation among populations was estimated (23%).

Table 1. Microsatellite Loci, GenBank acc no., Primer sequence of six microsatellite markers from *Rutilius frisii kutum*.

Microsatellite Loci	Gen Bank acc no.	Primer sequence
Lco3	AY318779	F:GCAGGAGCGAAACCATAAAT R:AAACAGGCAGGACACAAAGG
Lco1	AY318777	F:CACGGGACAATTTGGATGTTTTAT R:AGGGGGCAGCATACAAGAGACAAC
Lco2	AY318778	F:ATTTTTAGGAGTGATGTTCAGCAT R:CAAGTGTGTCATTGAGGAAGTGAG
Ca1	AF277573	F:AAGACGATGCTGGATGTTTAC R:CTATAGCTTATCCCGGCAGTA
Ca2	AF277574	F:GGACAGTGAGGGACGCAGAC R:TCTAGCCCCCAAATTTTACGG
Ca3	AF277575	F:TTGAGTGGATGGTGCTTGTA R:GCATTGCCAAAAGTTACCTAA

The lowest genetic distance was observed between the Cheshmekileh and the Sefidrud suggesting that they originated from a common ancestor. The genetic distance between populations in this research (0.6) falls within the range of (0.03-0.61) for congeneric (Shaklee *et al.*, 1982; Thorpe and Solé-Cava, 1994) species suggesting their genetic divergence.

In conclusion, this study provides us with useful insight into the genetic variability and differentiation of kutum populations in the Caspian Sea suggesting that there is need for development of management and conservation plans for different populations of this species in the sea.

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