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RESEARCH PAPER

Microsatellite DNA Marker Analysis of Genetic Variation in Wild and Hatchery Populations of Caspian Kutum (*Rutilus kutum*)

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Abstract

Rutilus kutum, is a commercially important species inhabiting the Caspian Sea. As the wild stocks of Caspian kutum have decreased in recent years, restocking the wild populations is implemented through releasing the hatchery produced individuals into the nature. Here, 10 microsatellite loci were applied to investigate the genetic diversity of hatchery and wild populations of *R. kutum* in the southeastern parts of the Caspian Sea. Totally 105 alleles were detected, with some of them being unique to each population. The number of alleles ranged from 4 to 16. The mean values of the observed and expected heterozygosity were 0.772 and 0.797, respectively. No significant differences (P>0.05) in genetic diversity were observed between the wild and hatchery populations. In most cases, significant genetic disequilibrium was detected after Bonferroni correction, mainly due to the heterozygote deficiency. Low genetic differentiation and close genetic relationship were detected among the studied populations. Our results are anticipated to provide essential data in establishing more efficient strategies for appropriate management and conservation of Caspian kutum populations during restocking programs.

Keywords: Caspian kutum, *Rutilus kutum*, microsatellite loci, population genetics, restocking.

Introduction

Caspian kutum, *Rutilus kutum*, belonging to the family Cyprinidae, is one of the most highly valuable bony fishin habiting the Caspian Sea (Afraei Bandpei, Mashhor, Abdolmaleki, & El-Sayed, 2010) .This fish is considered as one of the Arctic species entered the Caspian Sea after the ice age and became native to it (Adeli, 1993). The *R. kutum* is distributed in the Caspian Sea basin from the Terek River in northern coasts to the Atrak River in southern parts of the sea. However, this species is rarely found in the northern regions of the Caspian Sea (Shariati, 1992) and the main stocks are located in the southern coasts. During recent years, the wild stocks of Caspian kutum have been considerably depleted due to the various reasons including overexploitation, industrial and agricultural sewage influx, sand mining and consequently unfavorable conditions of natural spawning (Khanipour&Valipour, 2010).To promote the wild populations recovery, Iranian fisheries organization has proceeded to restock through the supportive breeding program since the late 1986s when the *R. kutum* catch dropped sharply. In supportive breeding, a fraction of the wild individuals are captured and brought into captivity for reproduction and their offspring are released into the natural habitat (Wang & Ryman, 2001). In this regard, at the Sijeval Bony Fishes Breeding Center in Golestan province, the produced offspring (approximate weight of 1-2 g) through the artificial reproduction ofthe breeders originated from the river of Gorganroud have been released annually in to the Gorganroud and Qaresou rivers during the months of June and July. More than 30 years have passed from the beginning of the massive release of Caspian kutum larvae into the Caspian Sea and natural spawning has decreased during this period. Now, a substantial portion of Caspian kutum stocks in the southern basin of the Caspian Sea is resulted from artificial reproduction (Khanipour &Valipour, 2010).

Although the artificial reproduction is considered as a common strategy in restocking programs, there is still uncertainty about the effectiveness of this method and its potential effects on the wild populations (Araki, Cooper, & Blouin, 2007; Berntson, Carmichael, Flesher, Ward, & Moran, 2011). In fact, the genetic quality of hatchery-produced larvae may affect the efficiency of hatchery-release program. In this regard, an appropriate level of genetic diversity is essential for the success of such programs. Hatchery-produced populations are usually

considered to exhibit a decreased level of genetic variability, generally because of the low effective population size which is mainly caused by the use of limited number of breeders in artificial reproduction (Loukovitis *et al.*, 2014). The release of hatchery-produced individuals with decreased genetic diversity into the nature may possibly have adverse effects on the wild populations including loss of genetic diversity and consequent breakdown of adaptation (Laikre, Schwartz, Waples, Ryman, & Group, 2010). Such adverse effects of hatchery release programson the wild populations have been previously reported for the species *Oncorhynchus mykiss* (rainbow trout) (Araki, Cooper, &Blouin, 2009; Christie, Marine, French, &Blouin, 2012). An explicit picture of genetic structure and variation of hatchery fish compared to the wild populations is crucial before carrying out restocking programs.

Unfortunately, despite that restocking programs have been widely implemented for *R. kutum* populations during recent years, there is no molecular information on the hatchery-produced populations of Caspian kutum. However, there are few studies on the wild populations of *R. kutum* in some regions of the Caspian Sea (Rezaei, Shabani, Shabanpour, &Kashiri, 2011; Abdolhay*et al.*, 2012; Rezvani Gilkolaei, Kavan, & Safari, 2012).

Molecular markers are considered as an efficient tool to assess the genetic variation within and among fish populations (Okumus&Ciftci, 2003; Abdul-Muneer, 2014). Microsatellites are one of the most widely used markers because of some characteristics such as the high level of polymorphism and mendelian inheritance (Georgescu *et al.*, 2014). Therefore, here, we used 10 microsatellite loci to assess the genetic variation of Caspian kutum populations. In the present study, given the importance of knowledge on genetic structure of species under conservation programs, the genetic diversity of *R. kutum* population used in restocking program was evaluated and compared to the wild populations. The results from this study are anticipated to provide essential data to establish more efficient strategies for appropriate genetic conservation and management of *R. kutum* populations during restocking programs.

Materials and Methods

Samples

We collected four samples of *R. kutum*from southeast parts of Caspian Sea: GorganBay (GB), Qaresouriver (QR), Gorganroud River (GR) and Gomishan wetland (GW) (Figure 1) during March and April 2015. Each sample consisted of 30 individuals. Similarly, a further 30hatchery-produced individuals used for restocking program, were obtained from Sijeval Bony Fishes Breeding Center, Golestan province, Iran. This facility has been restocking since 1980s and the breeders originate from Gorganroudriver (Khanipour&Valipour, 2010). Approximately, 1×1 cm of caudal fin was excised from each specimen and placed separately in 1.5-mL tube containing 96% ethanol. All the prepared samples were brought back to the laboratory and preserved at 4^0C for subsequent DNA extraction.

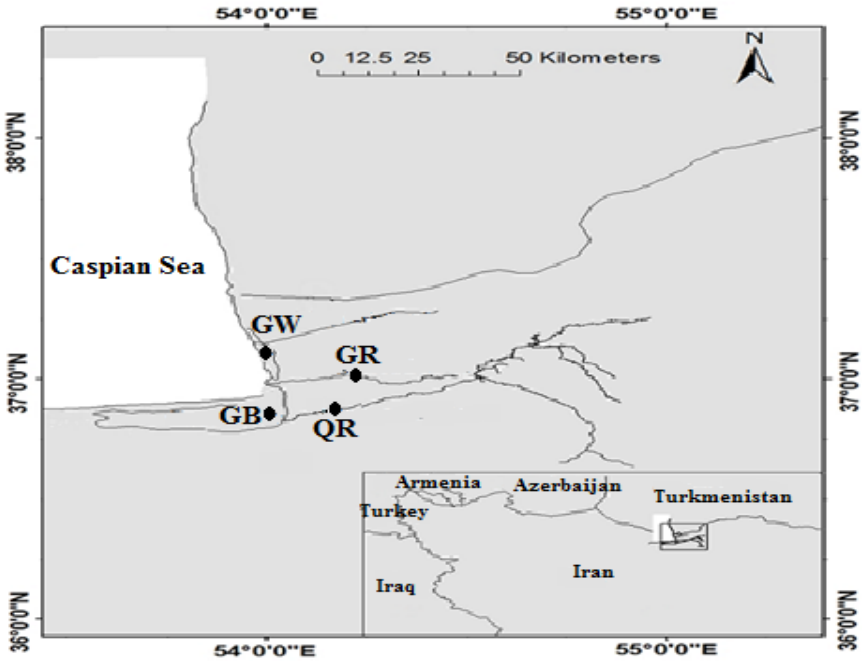

Figure 1. Sampling locations of *R. kutum* populations.Solid circles indicate sampling sites.

Extraction of Genomic DNA and Microsatellite Loci Amplification

The genomic DNA was extracted from fin tissues using Gene All Tissue and Tissue plus sv mini kit (GeneAll, Korea) according to the manufacturer's protocol. The quality and quantity of the extracted DNA were assessed by 1% agarose gel electrophoresis and a Biophotometer Spectrophotometer (Eppendorf, Germany), respectively. The genomic DNA was stored at -20^{0}C until PCR reactions.

10pairs of microsatellite primers, CypG3, CypG24, CypG27, CypG30 (Baerwald& May, 2004), Rru2, Rru4, Lid1 (Barinova, Yadrenkina, Nakajima, & Taniguchi, 2004), Z21908 (ZFIN, 2003), Ca1 and Ca3 (Dimsoski, Toth, & Bagley, 2000) (Table 1),were selected to investigate the genetic diversity between *R. kutum* populations based on the studies by Hamilton and Tayler (2008)and Rezaei *et al.* (2011).PCR reactions were performed using a thermal cycler system (Bio-RAD MJ Mini Thermal Cycler, USA) with a reaction volume of 12.5 µl. The thermal cycling conditions were as follows: 3 min at 94^{0}C, followed by 35 cycles of 30 s at the annealing temperatures given in Table 1, 1 min at 72^{0}C and 3 min at 72^{0}C (Rezaei *et al.*, 2011).

The PCR products were separated using electrophoresis in 10% polyacrilamide gel for 4 h. The gels were stained using silver nitrate method (Benbouza, Jacquemin, Baudoin, &Mergeai, 2006) and visualized under UV light on a gel documentation system (Gel Doc XR, Bio-Rad, USA).The length of microsatellite alleles was determined by Gel Pro Analyzer 3.9 software.

Statistical Analysis

The number of alleles (N_A), effective number of alleles (N_E), allele ranges (S), observed and expected heterozygosity ($H_O$and H_E, respectively), number of effective migrants (N_M) among populations, unbiased genetic distance (GD) and genetic identity (GI) based on Nei (1978) were determined using GenAlex 6.3 software (Peakall&Smouse, 2006). This software was also used to test deviation from Hardy-Weinberg

Table 1. 10 microsatellite markersapplied for genetic diversity and structure analysis of *R. kutum* populations

Locus (Reference)	GenBank accession no.	Primer sequence (5'-3')	Allele ranges	No. of alleles	Polymorphism information content (PIC)	Annealing (^{0}C)
CypG3 (Baerwald& May, 2004)	AY439122	F:AGT AGG TTT CCC AGC ATC ATT GT R:GAC TGG ACG CCT CTA CTT TCA TA	156-228	13	0.84	59
CypG24 (Baerwald& May, 2004)	AY439142	F:CTG CCG CAT CAG AGA TAA ACA CTT R: TGG CGG TAA GGG TAG ACC AC	156-208	14	0.81	58
CypG27 (Baerwald& May, 2004)	AY439145	F:AAG GTA TTC TCC AGC ATT TAT R:GAG CCA CCT GGA GAC ATT ACT	244-304	12	0.78	49
CypG30 (Baerwald& May, 2004)	AY439148	F:GAA AAA CCC TGA GAA ATT CAA AAG A R:GGA CAG GTA AAT GGA TGA GGA GAT A	176-256	12	0.83	52
Rru2 (Barinova*et al.*, 2004)	AB112738	F:TTC CAG CTC AAC TCT AAA GA R:GCA CCA TGC AGT AAC AAT	108-140	9	0.69	46
Rru4 (Barinova*et al.*, 2004)	AB112740	F:TAA GCA GTG ACC AGA ATC CA R:CAA AGC CTC AAA AGC ACA A	180-228	7	0.67	54
Z21908 (ZFIN, 2003)	G40277	F:ATT GAT TAG GTC ATT GCC CG R:AGG AGT CAT CGC TGG TGA GT	156-176	5	0.64	59
Ca1 (Dimsoski et al., 2000)	AF277573	F:AAG ACG ATG CTG GAT GTT TAC R:CTA TAG CTT ATC CCG GCA GTA	100-128	8	0.72	55
Ca3 (Dimsoski et al., 2000)	AF277575	F:GGA CAG TGA GGG ACG CAG AC R:TCT AGC CCC CAA ATT TTA CGG	232-320	17	0.87	52
Lid1 (Barinova*et al.*, 2004)	AB112732	F:TAA AAC ACA TCC AGG CAG ATT R:GGA GAC GTT ACG AGA GGT GAG	216-252	8	0.74	51

equilibrium (HWE).The significance values for multiple tests were adjusted by sequential Bonferroni correction (Rice, 1989). Linkage disequilibrium was investigated using linkd is method implemented in GENETIX (Belkhir, Borsa, Goudet, &Bonhomme, 1999). MICRO-CHECKER software (Oosterhout, Hutchinson, Wills, & Shipley, 2004) was used to detect possible null alleles, scoring errors and large allele dropouts. Number of unique alleles (U), polymorphism information content (PIC) and frequency of null alleles were determined using CERVUS 3.03 (Kalinowski, Taper, & Marshall, 2007). The differences in genetic diversity parameters between population swere assessed using nonparametric analysis (Wilcoxon, 1945). The possibility of recent bottlenecks was assessed through a two-phase model (TPM) implemented in the BOTTLENECK program 1.2.02 (Piry, Luikart, &Cornuet, 1999). Under the TPM, a model of 95% single-step mutations,5% multi-step mutations and a variance between multiple steps of 12 was utilized as suggested by Piry*et al.* (1999) for microsatellite data. Significance of heterozygote deficit or excess was checked through Wilcoxon sign-rank test with 5000 iterations. The BOTTLENECK program was also used to testa mode-shift away from an L-shaped distribution of allele frequencies. Using the mode-shift test, the distortion of the allele frequency distributions can be detected in recently bottlenecked populations (Luikart, Allendorf, Cornuet, & Sherwin, 1998).

The indices of inbreeding (F_{IS}) and genetic differentiation (F_{ST}; Weir &Cockerham, 1984) were analyzed by FSTAT 2.9.3 software (Goudet, 2001). The differentiation index of R_{ST} (Slatkin, 1995) was also determined by RSTCALC (Goodman, 1997). The F_{ST} and GD indices (Cavalli-Sforza & Edwards, 1967) were analyzed after INA and ENA correction by FREENA (Chapuis&Estoup, 2007). ARLEQUIN 3.1 (Excoffier, Laval, & Schneider, 2005) was applied for running analysis of molecular variance (AMOVA) to determine the partitioning of genetic variation between and within populations. The significance levels were obtained by 1000 permutations. Dendrogram of UPGMA based on Nei's GD was constructed by POPULATIONS program 1.2.30 (Langella, 2002). The bootstrap values were determined via 10000 replicate across loci. The dendrogram was visualized by TREEVIEW 1.6.6 software (Page, 1996). The patterns of the population structure were assessed using the Bayesian clustering approach in STRUCTURE 2.3 (Pritchard, Stephens, & Donnelly, 2000). A Markov chain Monte Carlo procedure was used and 10 independent runs of each K were implemented with 1×10^6 iterations after a burn-in period of 1×10^5 iterations for 1-8 populations. The most likely number of populations (K) was determined according to the procedure explained by Evanno, Regnaut, and Goud et (2005).

Results

Genetic Variation

Four wild populations and one hatchery population of *R. kutum* were screened for genetic variability at 10 microsatellite loci. All the loci amplified were polymorphic in the five populations. The number of alleles per locus ranged from 5 (Z21908) to 17 (Ca3), totally 105 alleles, 10.5 on an average (Table 1). The amplified fragments ranged from 100 to 320 bp in length (Table 1). MICRO-CHECKER indicated that none of the loci were affected by large allele drop-outs and stuttering errors but then ull alleles were observed at the Lid1, CypG3, CypG27 and CypG30 with the frequencies of 0.223, 0.236, 0.178 and 0.254, respectively. However, as no significant changes in our results were observed after re-performing the analysis when excluding these three loci, all 10 loci were retained in our study. The investigation of pairwise linkage disequilibrium showed that all the applied loci were in linkage equilibrium after Bonferroni correction (P>0.05). The polymorphism information content ranged from 0.64 for Z21908 to 0.87 for Ca3 (Table1).

The measures of genetic variation for five populations of *R. kutum* are presented in Table 2. In all populations, the expected and observed heterozygosity values ranged between 0.653-0.937 (mean H_E: 0.792) and 0.241-1.00 (mean H_O: 0.765), respectively. The number of alleles and effective number of alleles ranged from 4 to 16 and 3.02 to 15.94, respectively. Although, the GW population displayed higher genetic diversity (N: 9.2, H_O: 0.79, H_E: 0.809) compared to the other wild populations (N_A: 8.8, H_O: 0.786, H_E: 0.802 for GB; N_A: 8.1, H_O: 0.753, H_E: 0.782 for GR and N_A: 8.3, H_O: 0.762, H_E: 0.797 for QR), no significant differences in the average measures were noticed among the wild populations (Wilcoxon: $P>0.05$).When comparing the wild and hatchery populations, a little lower level of allelic diversity was noticed in the hatchery population (N_A: 7.3) compared to the wild populations (mean N_A: 8.6) (Wilcoxon: P>0.05). The H_E and H_O of hatchery samples tended to be lower in comparison to the wild populations (H_E and H_O: 0.771 and 0.734, respectively for hatchery population versus 0.797 and 0.772 for wild populations) (Wilcoxon: $P>0.05$). 97unique alleles were found for the studied populations: 24, 28, 17, 19 and 9 unique alleles for the GB, GW, GR, QR and hatchery populations, respectively(Table 2).However, despite the differences in genetic variation parameters, no statistically significant differences in genetic diversity were noticed among the wild and hatchery populations (Wilcoxon: $P>0.05$).

The HWE analysis indicated significant genetic disequilibrium after adjusting the P values across all loci by the sequential Bonferroni correction for multiple observations (Table 2). However, 10 cases

were in genetic equilibrium after Bonferroni correction: Ca1 for all the samples, CypG24 for the GW, GR, QR and Hatchery populations and Ca3 for GR population. Heterozygote deficiency was revealed by positive F_{IS} values in the studied populations (F_{IS}: 0.025, 0.022, 0.39, 0.046 and 0.049 for the GW, GB, GR, QR and Hatchery populations, respectively) (Table 2). The observed deficiency was significant at Lid1, CypG3 and CypG30 for all populations and CypG27 for QR and Hatchery populations ($P<0.05$). Heterozygosity excess was also detected at some loci but being only significant at Rru4 and Z21908. Under the TPM, bottleneck signatures were detected in the studied populations. The GR, QR and Hatchery populations showed a mode-shift in frequency of alleles potentially corresponding to a genetic bottleneck in these populations (Table 3).

Genetic Relationship

Genetic differentiation among the *R. kutum* populations was assessed using F_{ST} and R_{ST}. The global F_{ST}, including all loci, was 0.018 ($P<0.01$). However, the F_{ST} value was estimated to be 0.016 after correction for possible null alleles. Estimating the genetic distance of Cavalli-Sforza and Edwards (1967), also did not display any significant difference before and after INA correction (Table 4), indicating no obvious effect of null alleles on our results. The average R_{ST} was estimated to be 0.039, with

Table 2. Genetic diversity indices at 10 microsatellite loci of *R.kutum* populations

		CypG3	CypG24	CypG27	CypG30	Rru2	Rru4	Z21908	Ca1	Ca3	Lid1	Mean
GB	N_A	10	13	9	9	9	4	5	8	13	8	8.8
	N_E	5.79	6.83	7.61	4.65	5.19	3.31	3.40	4.54	15.94	4.65	6.19
	H_O	0.546	1.00	0.638	0.347	0.916	1.00	1.00	0.927	0.924	0.562	0.786
	H_E	0.824	0.851	0.867	0.784	0.806	0.691	0.702	0.774	0.937	0.784	0.802
	U	1	4	1	3	3	0	2	4	3	3	2.4
	F_{IS}	0.354	-0.162	0.274	0.568	-0.125	-0.425	-0.406	-0.173	0.031	0.29	0.023
	HWE	***	**	**	***	***	***	***	ns	**	***	
GW	N_A	11	14	11	9	8	5	4	7	16	7	9.2
	N_E	5.45	8.11	7.28	5.82	4.68	3.48	3.54	4.76	15.92	5.21	6.42
	H_O	0.563	1.00	0.642	0.326	0.927	1.00	1.00	0.938	0.936	0.568	0.79
	H_E	0.812	0.875	0.861	0.826	0.782	0.703	0.712	0.785	0.937	0.806	0.809
	U	2	5	3	3	2	1	1	3	6	2	2.8
	F_{IS}	0.324	-0.121	0.277	0.614	-0.172	-0.406	-0.386	-0.174	0.03	0.313	0.030
	HWE	***	ns	*	***	***	***	***	ns	**	***	
GR	N_A	10	12	9	8	7	4	4	7	13	7	8.1
	N_E	4.71	6.61	5.76	5.19	4.6	3.02	3.51	4	12.04	4.32	5.38
	H_O	0.471	1.00	0.605	0.273	0.857	1.00	1.00	0.881	0.876	0.573	0.753
	H_E	0.784	0.846	0.822	0.806	0.776	0.653	0.712	0.743	0.916	0.762	0.782
	U	1	3	1	2	1	0	1	3	3	2	1.7
	F_{IS}	0.408	-0.171	0.241	0.671	-0.086	-0.521	-0.386	-0.156	0.071	0.267	0.034
	HWE	***	ns	**	***	**	***	***	ns	ns	***	
QR	N_A	10	12	9	7	8	4	4	8	14	7	8.3
	N_E	5.21	7.6	6.95	4.52	5.64	3.15	3.34	4.52	13.11	5.02	5.91
	H_O	0.486	1.00	0.612	0.304	0.871	1.00	1.00	0.914	0.892	0.542	0.762
	H_E	0.804	0.866	0.853	0.772	0.819	0.677	0.689	0.777	0.923	0.793	0.797
	U	1	3	1	1	2	0	1	4	4	2	1.9
	F_{IS}	0.408	-0.139	0.306	0.617	-0.037	-0.453	-0.432	-0.154	0.062	0.289	0.047
	HWE	***	ns	***	***	***	***	***	ns	*	***	
Hatchery	N_A	10	11	8	7	7	4	4	6	10	6	7.3
	N_E	4	6.53	5.41	4.08	4.96	3.08	3.41	3.89	9.18	5.03	4.96
	H_O	0.493	1.00	0.522	0.241	0.850	1.00	1.00	0.887	0.853	0.494	0.734
	H_E	0.743	0.844	0.816	0.746	0.793	0.657	0.694	0.734	0.890	0.795	0.771
	U	1	2	0	1	1	0	1	2	0	1	0.9
	F_{IS}	0.347	-0.165	0.372	0.685	-0.057	-0.512	-0.423	-0.182	0.072	0.389	0.053
	HWE	***	ns	***	***	***	***	***	ns	*	***	

Significant levels of F_{IS} are shown in bold; HWE = the probability test of Hardy-Weinberg equilibrium after correction with sequential test of Bonferroni (Rice, 1989); ns = not significant; * $P\leq0.005$; ** $P\leq0.001$; *** $P\leq0.0001$

Table 3. Results from the BOTTLENECK tests for *R. kutum* populations

Population	Allele frequency distribution	Probability (Wilcoxon sign-rank test under TPM)
GB	L-shaped	0.003
GW	L-shaped	0.004
GR	Mode-shift	0.002
QR	Mode-shift	0.001
Hatchery	Mode-shift	0.001

L-shaped: normal L-shaped allele frequency distribution; TPM: two-phase mutation model.

Table 4. Genetic relationships between the *R. kutum* populations

Population pair	F_{ST}	R_{ST}	N_M	Nei's GD	Nei's GI	Cavalli-Sforza and Edwards GD	Cavalli-Sforza and Edwards GD with INA correction
GW vs. GR	0.020**	0.042	12.28	0.227	0.795	0.264	0.259
GW vs. QR	0.024**	0.046	10.18	0.289	0.732	0.776	0.773
GW vs. GB	0.023***	0.044	10.6	0.271	0.757	0.329	0.321
GR vs. QR	0.014*	0.027	17.64	0.137	0.875	0.182	0.178
GR vs. GB	0.019**	0.039	12.92	0.207	0.817	0.241	0.232
QR vs. GB	0.016**	0.034	15.42	0.168	0.848	0.209	0.198
Hatchery vs. GW	0.022**	0.043	11.12	0.259	0.760	0.302	0.290
Hatchery vs. GR	0.010*	0.022	24.73	0.086	0.923	0.130	0.124
Hatchery vs. QR	0.013**	0.025	19.00	0.118	0.904	0.189	0.181
Hatchery vs. GB	0.020**	0.040	12.27	0.236	0.786	0.281	0.278

Significant values of F_{ST} are marked with *. * P<0.05; ** P<0.01; *** P<0.001

somewhat higher level compared to the F_{ST} (Table 4). However, these two indices were in accordance with each other so that the highest and lowest differentiation levels were observed among the GW with QR populations (F_{ST}: 0.024, R_{ST}: 0.046) and GR with hatchery populations, respectively (Table 4). The AMOVA for 10 loci revealed low level of variation between individuals within populations (2.96%; P=0.022) (Table 5). In addition, 91.85% (P=0.010) and 5.19% (P=0.000) of the variance were observed within and between populations, respectively (Table 5).

Considerable values of N_M index were detected among the studied populations with the average N_M of 14.62. The lowest N_M was observed among the GW with GR. Unbiased genetic identity and distance (Nei, 1978) among all samples are shown in Table 4. The lowest GI (0.732) and the highest GD (0.289) were detected between the GW with QR populations while the highest GI (0.923) and the lowest GD (0.086) were between the GR with Hatchery populations. Population relationships depicted in UPGMA cluster based on the Nei's genetic distance revealed that GR and Hatchery were clustered with each other at first, and then with QR and GW was the most distant population (Figure 2).

Bayesian analysis of population structure indicated two genetic clusters(K=2) to which *R. kutum* individuals can be assigned (Figure 3).The studied populations are admixed with a significant proportion of individuals being almost equally assigned to both clusters, red and green (Figure 3). However, individuals deriving from the GW population displayed a lower level of ad mixture- all of them were attached with higher probability (0.8) to the green cluster (Figure 3).

Discussions

Analysis of Population Genetic Variation

Genetic diversity is one of the most fundamental levels of biodiversity which is important for sustainability of many species (Vellend & Geber, 2005). In the present study, no significant differences in the number of alleles, effective number of alleles, observed and expected heterozygosity were observed among the wild populations of *R. kutum*. The genetic diversity noticed in our study was higher than that reported by Rezvani Gilkolaei *et al.* (2012) for wild populations of Caspian kutum in the southwest of Caspian Sea (Anzali wetland and Khoshkroud River) (N_A: 8.6 vs. 5.5, H_O: 0.772 vs. 0.527 and H_E: 0.797 vs. 0.678). The difference in applied loci, various sample size and sampling sites may be the possible explanations for such difference. In another study by

Table 5. Analysis of molecular variance (AMOVA) for *R. kutum* populations

Variation	Sum of squares	Degrees of freedom	Variation (%)	Probability
Between populations	29.815	4	5.19	0.000
Between individuals within populations	520.541	138	2.96	0.022
Within individuals	495.829	115	91.85	0.010

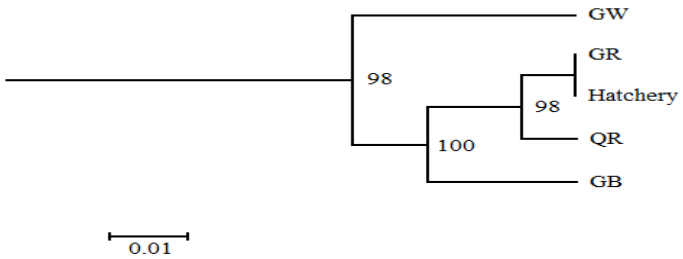

Figure 2. UPGMA dendrogram based on genetic distance deriving from analysis of 10 microsatellite loci for the wild and hatchery populations of *R. kutum*.The bootstrap values are shown.

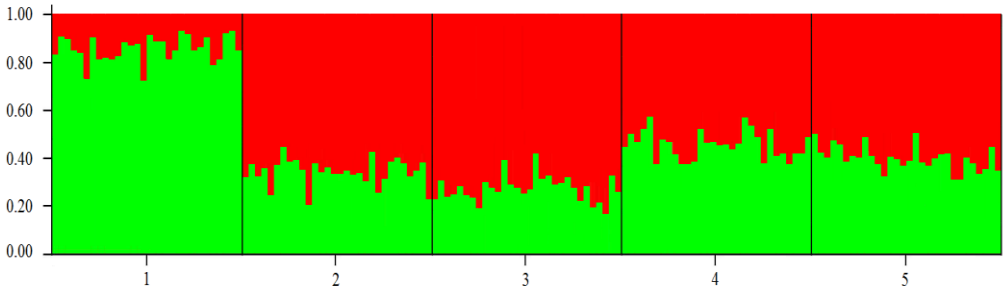

Figure 3. STRUCTURE bar plot based on analysis of 10 microsatellite loci for 5 populations of *R. kutum*. On the *x* axis, each vertical line indicates an individual within a population. The *y* axis represents the probability of individual assignment to 2 of identified genetic clusters (K=2, red and green). 1: GW; 2: QR; 3: GB; 4: GR; 5: Hatchery.

Abdolhay*et al.* (2012) using RFLP, low genetic variability was observed for wild populations of *R. kutum* in the south of Caspian Sea (Sefidroud, Lamir, Shiroud and Tajan rivers). In addition to the mentioned possible reasons, microsatellites are more efficient to detect genetic variation in species than RFLP (Shaw, Turan, Wright, O'Connell, &Carvalho, 1999), which may explain the lower concordance among the RFLP data and our results. However, our results were in accordance to the study by Rezaei *et al.* (2011) who reported the number of alleles, expected and observed heterozygosity 8.29, 0.793 and 0.766, respectively for three wild populations (Goharbaran, Tajan and Qaresou) of Caspian kutum using the same microsatellite loci. While the observed and expected heterozygosity noticed in the present study were higher than those reported in 13 other freshwater (H_O: 0.46 and H_E: 0.54) and 7 anadromous species (H_O and H_E: 0.68), the mean number of alleles was lower than that of freshwater and anadromous fishes (9.1 and 10.8, respectively) (DeWoody&Avise, 2000). Allendorf (1986) declared that reduction in the observed number of alleles without any significant changes in the heterozygosity may be the sign of genetic bottleneck. In our study, according to the results from mutation-drift equilibrium, the signs of population reduction were observed. As a result, the heterozygosity in these populations was high while the allelic diversity was at a low level. The recent population reduction observed in the *R. kutum* populations could be mainly related to the human activities including overfishing, increased level of pollution in the Caspian Sea and destruction of natural habitat. In this regard, artificial reproduction for stock enhancement programs can also be mentioned as a possible influential factor. Results from comparing the wild and hatchery populations of *R. kutum* revealed that no significant differences in genetic variation parameters were found among the wild and hatchery populations (P>0.05).Despite non significant differences, a reduction in the number of alleles and unique alleles were noticed in the Hatchery population (N_A: 7.3 and U: 9 for the Hatchery population vs. mean N_A: 8.6 and U: 22 for the wild populations) (Table 2),with a slightly lower level of observed and expected heterozygosity. In this regard,

genetic variation reduction is a common phenomenon in hatchery populations and high rate of releasing the hatchery-produced individuals with reduced genetic variation might have adverse effects on genetic diversity and adapted gene pools of the wild populations (Thanh, Liu, Zhao, Zhang, & Liu, 2015). More than 30 years have passed from the beginning of the restocking program for Caspian kutum populations by yearly releasing the hatchery-produced individuals from the wild-captured breeders. Unfortunately, there is no genetic information on *R. kutum* populations before the beginning of restocking program to determine definitely whether the restocking program have had any impact on genetic variation of wild populations or not. However, our results indicated that the genetic diversity of hatchery population was somewhat lower than those observed in the wild populations. There is no dispute that low level of genetic variation can lead to decrease in population persistence and fitness by inbreeding depression (Market *et al.*, 2010) which arises from the loss of heterozygosity and deleterious alleles expression (Neff *et al.*, 2011). In our study, heterozygote deficiency was revealed by the positive F_{IS} values across the populations, suggesting that inbreeding events may have occurred in the studied populations of *R. kutum*. Therefore, although the observed differences were not statistically significant, even the slightly lower level of genetic diversity observed in hatchery-produced individuals should not be ignored since the high rate of releasing the hatchery-produced individuals into the nature can lead to negative effects on the wild populations over time.

Significant departures from Hardy-Weinberg equilibrium were detected in both wild and hatchery populations of *R. kutum* after sequential Bonferroni correction. Additionally, significant heterozygote deficiency was revealed by the positive F_{IS} values at some of the loci (Table 2) possibly resulting from several factors. In the current case, the presence of null alleles is suggested as a potential explanation for the observed deficiency. An, Hong, Lee, Park, and Kim (2010) and Diez-Del-Molino *et al.* (2013) also reported that the presence of null alleles is a likely explanation for heterozygote deficiency. The observed deficiency could have also arisen from homogenization of the populations due to stocking. In our study, low F_{ST} and considerable N_M were observed among the studied populations. Results from structure analysis also revealed some degree of admixture in the studied populations. In this regard, the observed heterozygote deficiency could also be caused by inbreeding depression, mainly due to the restocking practices. Moreover, despite the massive release of hatchery-produced individuals in stock enhancement program, the wild populations of Caspian kutum have experienced considerable reduction mainly because of overfishing and increased level of pollution. This phenomenon would cause a corresponding reduction in effective population size and subsequent increase in inbreeding depression (An *et al.*, 2013). However, only one factor cannot be considered to illustrate the heterozygote deficiency as the interaction of different factors may cause this phenomenon.

Genetic Relationship and Differentiation among Populations

The differentiation of the wild and hatchery-produced populations of Caspian kutum was determined using the F_{ST} and R_{ST} indices. The average F_{ST} of all loci was detected to be 0.018, indicating low level of differentiation as suggested by Wright (1978). However, as reported by Balloux and Lugon-Moulin (2002), even this low level of F_{ST} may indicate important genetic differentiation. The low F_{ST} value was previously detected between the other wild populations of *R. kutum* (Rezaei *et al.*, 2011) whereas Rezvani-Gilkolaei *et al.* (2012) reported a moderate level of genetic differentiation between the southwest populations of Caspian kutum, possibly related to the difference in geographical localities of sampling.Sefc, Payne, and Sorenson (2007) demonstrated that because of greater variance, R_{ST} is less dependable for detecting differentiation than F_{ST}. However, F_{ST} should be smaller under stepwise mutation model (Hardy, Charbonnel, Freville, & Heuertz, 2003). But when differentiation is independent on mutation model under short time separation of population and high level of migration between populations, the F_{ST} levels could be close to the R_{ST} ones (Slatkin, 1995). In our study, there were no significant differences between F_{ST} and R_{ST} values. The highest genetic differentiation was observed between the GW and QR populations which could be related to the higher geographical distance and lower migration among these two regions. The lowest differentiation among the wild populations was also noticed between GR and QR. The highest level of N_M was also among these two samples. The low differentiation observed among the GR and QR samples may be attributed to the restocking programs. The hatchery-produced larvae from the wild individuals of *R. kutum*are released yearly into the rivers of Qaresou and Gorganroud while the wild breeders are caught only from Gorganroud. In this regard, the lowest differentiation observed in the present study was among the GR and Hatchery populations. The result from Bayesian clustering analysis also indicated closer genetic relationship between GR and Hatchery populations, which was supported by the result of genetic distance. The UPGMA dendrogram based on genetic distance also showed that GR and Hatchery were the nearest groups. Therefore, in addition to the natural migration, the stock enhancement program by releasing the hatchery-produced individuals into the rivers without any attention to the parental catch place may be the important explanation for the high number of effective migrants and consequently low level of

genetic differentiation.

Conclusions and Management Implications

The main objective of this study was to obtain genetic information on the hatchery and wild populations of *R. kutum* for fisheries management and conservation programs. Some previous studies have reported some adverse genetic effects caused by releasing hatchery-produced individuals in restocking programs (Cheng *et al.*, 2011; Li *et al.*, 2016). Here, despite the high level of heterozygosity, the wild populations of *R. kutum* showed lower level of allelic diversity and a sign of genetic bottleneck. Furthermore, despite non significant differences, the genetic diversity of hatchery population was somewhat lower than that observed in the wild populations. Therefore, it is critical to establish the proper management strategies including the use of a maximum possible number of breeders for artificial reproduction and employing proper mating strategies to maintain the population genetic diversity and effectiveness of restocking program of *R. kutum*. In supportive breeding program of *R. kutum*, mass mating is the common approach to produce offspring. As this method has been removed from spawning guidelines for many hatcheries because of concerns about the probable adverse effects on genetic quality of offspring (Neff *et al*., 2011), other methods including single-pair or factorial mating can be investigated and employed for supportive breeding program of *R. kutum*. The results from genetic relations among the wild and hatchery populations of *R. kutum* may also provide some applied guidance for conservation strategies especially in the fields of hatchery release. In this regard, low genetic differentiation and close genetic relationship were observed among the wild populations especially between the GR and QR which may be related to the stock enhancement strategies. As releasing hatchery individuals without any attention to the parental catch place may lead to disturbance in the population structure of the wild populations, it is reasonable to release the hatchery-produced larvae into the places in which their breeders were caught. Taking into account the problems including overexploitation, increasing levels of pollution as well as high release of hatchery-produced individuals into the nature, monitoring the genetic variation of the *R. kutum* populations in particular the target populations in stock enhancement program is recommended in given time periods for more efficient management and detecting any probable changes in gene pools of the wild populations.

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References

Abdolhay, H.A., Khalijah, D.S., Rezvani Gilkolahi, S., Pourkazemi, M., Shapor, S.S.,&Javanmard, A. (2012). Genetic diversity of Mahisefid (*Rutilus frisiikutum* Kamensky 1901) in different rivers of the south Caspian Sea using PCR-RFLP. *Iranian Journal of Fisheries Sciences*, 11(2), 235-251.

Abdul-moneer, P. M. (2014). Application of microsatellite markers in conservation genetics and fisheries management: Recent advances in population structure analysis and conservation Strategies. *Genetics Research International*, 2014, 1-11. http://dx.doi.org/10.1155/2014/691759

Adeli, Y. (1993). Caspian Sea. Anzali, Iran, Fisheries Research Center Press., 56 pp. (In Persian).

Afraei Bandpei, M.A., Mashhor, M., Abdolmaleki, S., & El-Sayed, A.F.M. (2010). Population dynamics of Caspian kutum, *Rutilus frisiikutum* (Cyprinidae) in southern Caspian Sea, Iran. *Cybium*, 34(3), 285-292.

Allendorf, F.W. (1986). Genetic drift and the loss of alleles versus heterozygosity. *Zoo Biology*, 5, 181–190. http://dx.doi.org/10.1002/zoo.1430050212

An, H.S., Hong, S.W., Lee, J.U., Park, J.Y., & Kim, K.K. (2010). Genetic diversity of wild and farmed black sea bream populations in Jeju. *Animal Cells and Systems*, 14(1), 37-44. http://dx.doi.org/10.1080/19768351003765020

An, H.S., Kim, E.M., Kang, H.W., Han, H.S., Lee, J.W., Park, J.Y., Myeong, J.I., &An, C.M. (2013). Comparative genetic diversity of wild and hatchery-produced populations of tongue sole (*Cynoglossus semilaevis*) using multiplex PCR assays with polymorphic microsatellite markers. *Genetics and Molecular Research*, 12(4), 6331-6343. http://dx.doi.org/10.4238/2013

Araki, H., Cooper, B., &Blouin,M.S. (2007). Genetic effects of captive breeding cause a rapid cumulative fitness decline in the wild. *Science*, 318, 100-103. http://dx.doi.org/10.1126/science.1145621

Araki, H., Cooper, B., &Blouin,M.S. (2009). Carry-over effect of captive breeding reduces reproductive fitness of wild-born descendants in the wild. *Biology Letters*, 5, 621–624. http://dx.doi.org/10.1098/rsbl.2009.0315

Baerwald, M.R.,& May, B. (2004) Characterization of microsatellite loci for five members of them in now family Cyprinidae found in the Sacramento-San Joaquin Delta and its tributaries. *Molecular Ecol*ogy, 4, 385-390.http://dx.doi.org/10.1111/j.1471-8286.2004.00661.x

Balloux, F., &Lugon-Moulin, N. (2002). The estimation of population differentiation with microsatellite markers. *Molecular Ecology*, 11, 155-165

Barinova, A., Yadrenkina, E., Nakajima, M. & Taniguchi, N. (2004) Identification and characterization of microsatellite DNA markers developed in Ide *Leuciscusidus* and Siberian roach *Rutilusrutilus*.*Molecular EcologyResources*, 4, 86-88. http://dx.doi.org/10.1046/j.1471-8286.2003.00577.x

Belkhir, K., Borsa, P., Goudet, J., &Bonhomme, F. (1999). Genetics: Software for windows for population genetics, Version 4.02. Montpellier, France, Laboratory Genome and Pollution, CNRS-UPR, University of Montpellier II. (In French).

Benbouza, H., Jacquemin, J.M., Baudoin, J.P., &Mergeai,

G. (2006).Optimization of the reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. *Biotechnology Agronomy Society and Environment*, 10, 77-81.

Berntson, E.A., Carmichael,R.W., Flesher, M.W.,Ward, E.J., & Moran,P. (2011). Diminished reproductive success of steelhead from a hatchery supplementation program (Little Sheep Creek, Imnaha Basin, Oregon). *Transactions of the American Fisheries Society*, 140(3), 685-698.http://dx.doi.org/10.1080/00028487.2011.584489

Cavalli-Sforza, L.L., &Edwards, A.W.F. (1967). Phylogenetic analysis: Models and estimation procedures. *American Journal of Human Genetics*, 19,233–257.

Chapuis, M.P.,&Estoup, A. (2007). Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution*, 24, 621-631.https://doi.org/10.1093/molbev/msl191

Cheng, F., Wu, Q., Liu, M., Radhakrishnan, K.V, Murphy, B.R., &Xie, S. (2011). Impacts of hatchery release on genetic structure of rock carp *Procyprisrabaudi*in the upper Yangtze River, China. *Fisheries Science*, 77, 765-771.

Christie, M.R., Marine, M.L.,French,R.A., &Blouin,M.S. (2012). Genetic adaptation to captivity can occur in a single generation. *Proceedings of the National Academy of Sciences*, 109(1),238-242. http://doi.org/ 10.1073/pnas.1111073109

DeWoody, J.A., &Avise, J.C. (2000). Microsatellite variation in Marin, freshwater and anadromous fishes compare with other animal. *Journal of Fish Biology*, 56, 461-473.http://doi.org/10.1111/j.1095-8649.2000.tb00748.x

Diez-Del-Molino, D., Carmona-Catot, G., Araguas, R.M., Vidal, O., Sanz, N., Garcia-Berthou, E., & Garcia-Marin, J.L. (2013). Gene Flow and Maintenance of Genetic Diversity in Invasive Mosquitofish (*Gambusiahol brooki*). *PLOS ONE*, 8(12), 1-11.http://doi.org/10.1371/journal.pone.0082501

Dimsoski, P., Toth, G.P.,& Bagley, M.J. (2000). Microsatellite characterization in central stoneroller *Campostoma anomalum* (Pisces: Cyprinidae). *Molecular Ecol*ogy, 9, 2155-2234.

Excoffier, L., Laval, G.,& Schneider, S. (2005).Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics online*, 1, 47-50.

Georgescu,S.E.,Burcea, A.,Florescu, I., Popa, O.G., Dudu, A., &Costach, M. (2014). Microsatellite Variation in Russian Sturgeon (*Acipenser gueldenstaedtii*) from Aquaculture. *Animal Science and Biotechnologies*, 47, 73-76.

Goodman, S.J. (1997). RST Calc: a collection of computer program for calculating estimates of genetic differentiation from microsatellite data and determining their significance. *Molecular Ecology*, 6, 881-885. http://doi.org/10.1111/j.1365-294X.1997.tb00143.x

Goudet, J. (2001).FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Retrieved from http://www2.unil.ch/popgen/softwares/fstat.htm

Hamilton, P.B.,& Tyler, C.R. (2008). Identification of microsatellite loci for parentage analysis in roach *Rutilus rutilus* and eight other cyprinidae fish by cross-species amplification, and a novel test for detecting hybrids between roach and other cyprinids. *Molecular Ecology Notes*, 8, 462-465. http://dx.doi.org/10.1111/j.1471-8286.2007.01994.x

Hardy, O.J., Charbonnel,N., Freville,H., &Heuertz,M. (2003).Microsatellite allele sizes: a simple test to assess their significance on genetic differentiation. *Genetics*, 163(4),1467–1482.

Kalinowski, S.T., Taper, M.L.& Marshall, T.C. (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology*,16, 1099-1106. http://dx.doi.org/10.1111/j.1365-294X.2007.03089.x

Khanipour, A.A., &Valipour, R. (2010). Kutum fish, Jewel of the Caspian Sea. Tehran, Iran, Iranian Fisheries OrganizationPress.,84 pp. (In Persian).

Laikre, L., Schwartz, M.K., Waples, R.S., Ryman, M., & Group, G.W. (2010). Compromising genetic diversity in the wild: unmonitored large-scale release of plants and animals. *Trends in Ecology and Evolution*, 25(9), 520-529. http://dx.doi.org/ 10.1016/j.tree.2010.06.013

Langella, O. (2002). Population genetic software, POPULATIONS 1.2.30. Retrieved from http://bioinformatics.org/~tryphon/populations

Li, X., Deng, Y., Yang, K., Gan, W., Zeng, R., Deng, L.,&Song, Z. (2016). Genetic diversity and structure analysis of *Percocypri spingi* (Cypriniformes: Cyprinidae): Implications for conservation and hatchery release in the Yalong river. *PLOS ONE*,11(12),1-18.https://doi.org/10.1371/journal.pone.0166769

Loukovitis, D., Ioannidi, B., Chatziplis, D., Kotoulas, G., Magoulas, A., &Tsigenopoulos, C.S. (2014). Loss of genetic variation in Greek hatchery populations of the European sea bass (*Dicentrarchus labrax* L.) as revealed by microsatellite DNA analysis. *Mediterranean Marine Science*, 16, 197-200.http://dx.doi.org/10.12681/mms.1033

Luikart, G., Allendorf, F.W., Cornuet, J.M., & Sherwin, W.B. (1998). Distortion of allele frequency distributions provides a test for recent population bottlenecks. *Journal of Heredity*, 89(3), 238-247.

Neff, B. D., Garner, S. R.,& Picher, T. E. (2011). Conservation and enhancement of wild fishpopulations: preserving genetic quality versus genetic diversity. *Canadian Journal of Fisheries and Aquatic Sciences*, 68, 1139-1154. https://doi.org/10.1139/f2011-029

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from small number of individuals. *Genetics*, 89,583-590.

Okumus, I.,&Ciftci, Y. (2003). Fish Population Genetics and Molecular Markers: II- Molecular Markers and Their Applications in Fisheries and Aquaculture. *Turkish Journal of Fisheries and Aquatic Sciences*, 3, 51-79.

Oosterhout, C.V., Hutchinson,W.F.,Wills, D.P.M.,& Shipley, P. (2004). MICRO-HECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4, 535-538. http://dx.doi.org/10.1111/j.1471-8286.2004.00684.x

Page, R.D.M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in Bioscience*, 12, 347-358.

Peakall, R.,&Smouse, P.E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for

teaching and research. *Molecular Ecology Notes*, 6, 288-295.http://dx.doi.org/10.1111/j.1471-8286.2005.01155.x

Piry, S., Luikart, G., &Cornuet, J.M. (1999). BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity*, 90(4), 502–503. http://doi.org/10.1093/jhered/90.4.502

Pritchard, J.K., Stephens, M., &Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*. 155(2), 945-959.

Rezaei, M., Shabani, A., Shabanpour, B., &Kashiri, H. (2011). Microsatellites reveal weak genetic differentiation between *Rutilus frisiikutum* (Kamenskii, 1901) populations south of the Caspian Sea. *Animal Biology*, 61, 469-483. http://dx.doi.org/10.1163/157075511X597584

Rezvani Gilkalaei, S., Kavan, S.L.,& Safari, R. (2012). A study of genetic structure of *Rutilus frisiikutum* in Anzali lagoon, using microsatellite markers. *Journal of Agricultural Science and Technology*, 14, 327-337.

Rice, W.R. (1989). Analyzing tables of statistical tests. *Evolution*, 43, 223-225.http://dx.doi.org/10.2307/2409177

Sefc, K.M., Payne, R.B., &Sorenson,M.D. (2007). Genetic differentiation after founder events: an evaluation of FST estimators with empirical and simulated data. *Evolutionary Ecology Research*, 9(1),21–39.

Shariati, A. (1992). Fishes of the Caspian Sea and its basin. Tehran, Iran, Fisheries Joint Stock Company Press., 171 pp. (In Persian).

Shaw, P.W., Turan, C., Wright, J. M., O'Connell, M., &Carvalho, G.R. (1999). Microsatellite DNA analysis of population structure in Atlantic herring (*Clupeaharengus*), with direct comparison to allozymeand mtDNA RFLP analyses. *Heredity*, 83, 490-499. http://dx.doi.org/10.1046/j.1365-2540.1999.00586.x

Slatkin, M. (1995). A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, 139(3), 457-462.

Thanh, H.N., Liu, Q.G., Zhao, L.J., Zhang, H., & Liu, J. (2015). Genetic diversity of the cultured giant freshwater prawn (*Macrobrachiumrosen bergii*) in China based on microsatellite markers. *Biochemical Systematics and Ecology*, 59, 144–154. https://doi.org/10.1016/j.bse.2014.12.023

Vellend, M., & Geber, M.A. (2005). Connections between species diversity and genetic Diversity. *Ecology Letters*, 8(7): 767-781.http://dx.doi.org/10.1111/j.1461-0248.2005.00775.x

Wang, J., & Ryman, N. (2001). Genetic effects of multiple generations of supportive breeding. *Conservation Biology*, 15(6): 1619-1631. http://dx.doi.org/10.1046/j.1523-1739.2001.00173.x

Weir, B.S.,&Cockerham, C.C. (1984). Estimating F-statistic for the analysis of population structure. *Evolution*, 38, 1358-1370.http://dx.doi.org/10.2307/2408641

Wilcoxon, F. (1945). Individual comparisons by ranking methods. *Biometrics Bulletin*,1, 80-83.https://doi.org/10.2307/3001968

Wright, S. (1978).Evolution and the Genetics of Populations, Vol. 4: Variability within and among natural populations. Chicago, USA, University of Chicago Press., 590 pp.

ZFIN. (2003). The Zebrafish information network. Retrieved from http://zfin.org