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Original Article

Genetic characterization of *Garra rufa* (Heckel, 1843) populations in Tigris Basin, Iran using microsatellite markers

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Abstract: The isolation-by-distance theory states that the genetic differentiation between individuals raised by increasing geographic distance. Therefore, this study tested this hypothesis for *Garra rufa*, a freshwater fish species of Iranian inland waters, from six rivers located at the different distances in Tirgis basin. For this purpose, eight variable microsatellite loci were applied to identify geographic-based population structure of *G. rufa*. From 240 fish of six populations, 102 alleles were found with a mean number of 11.625 to 13.250 alleles. Heterozygosity was ranged 0.567-0.638 in six studied populations. Moreover, a significant deviations from Hardy-Weinberg were found in the studied populations. Unweight pair group analysis indicated that the six studied populations could be divided into four major clusters. The results revealed a fairly high level of genetic variation in the microsatellite loci within six studied populations. Wright's fixation index (Fst) ranged between 0.013-0.044 indicating little genetic differentiation between populations. Within this range, however, we found a strong positive relation between Fst and geographical distance lending support to the isolation-by-distance theory.

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Introduction

Genetic diversity is one of the important indicators of ecological condition in aquatic ecosystems and has been considered as a useful and powerful tool for evaluation and management of biological communities (Avise, 2000). It is one of three levels of biodiversity, proposed by the global conservation organization for the stocks conservation program (Lucentini et al., 2009). Since higher genetic diversity may lead to an increase in the survival rate of natural populations (Zoller et al., 1999), maintaining genetic diversity in these populations is crucial for conservation biologists.

Of the common DNA markers used to study genetic diversity at the molecular levels, microsatellite markers are especially informative (Chen et al., 2008). Because of unique features such as high polymorphism, high scope in genome, and high mutation rates, the microsatellite markers have been widely applied in population genetic studies (Li et al., 2009). Microsatellite markers have been identified in the genomes of many species, and widely used in relation to species with an economic value (Wang et al., 2012). The application of these markers includes aspects of evolutionary biology, population genetics, ecology and pedigree identification in populations (Cui et al., 2005; Cruz et al., 2005; Maremi et al., 2005).

There are 257 fish species in the inland waters of Iran (Joulade-Roudbar et al., 2015), which mostly belong to the family Cyprinidae. *Garra rufa* is the member of Cyprinidae and occurs in the river basins of the Northern and Central Middle East (Keivany et al., 2015; Mousavi-Sabet and Eagderi, 2016). This species has been utilized in psoriasis treatment (Ündar et al., 1990) and are preyed by piscivorous fishes such as *Anguilla anguilla* and *Clarias gariepinus* in their habitats (Yalçin-Özdilek, 2007).

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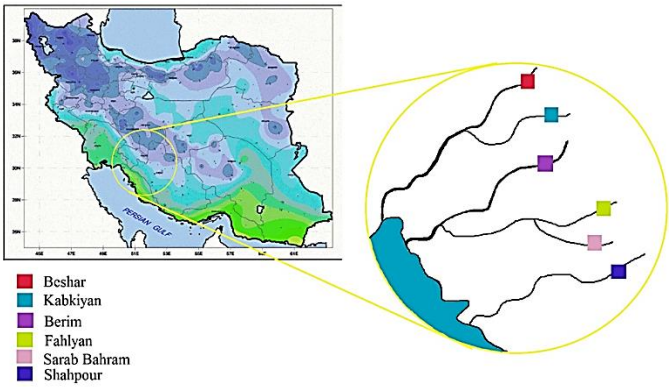

Figure 1. Sampling sites of Garr rufa from Tirgis Basin in Iran (1- Kabkiyan River, 2-Berim River, 3-Fahlyan River, 4-Beshar River, 5-Sarab Bahram River, and 6-Shahpour River).

Despite the ecological importance of *G. rufa*, the studies on its genetic structure and demography remain rudimentary.

Isolation by distance (IBD) model has been extensively used to study spatial patterns of genetic variation in natural populations (e.g., Crispo and Hendry, 2005; Storfer et al., 2010). According to the IBD theory, the genetic distance between individuals or populations will increase with decreasing in gene flow as a result of increasing geographic distance between them (Wright, 1943; Rousset, 1997). This restriction of gene flow can finally result in speciation events (Coyne and Orr, 2004). Although IBD has been detected in a range of species groups, including fishes, plants, oysters, beetles, and mammals across both small and large geographical scales (Angers and Bernatchez, 1998; Launey et al., 2002; Bockelmann et al., 2003; Peakall et al., 2003; Buonaccorsi et al., 2004; Oleksa et al., 2012), some studies have not supported this theory (e.g., Peterson, 1995; Peter and Slatkin, 2013). Here, we tested IBD hypothesis by comparing genetic structure of six populations of *G. rufa* from six rivers located in different distances from each other in the Tirgis basin of Iran.

Materials and Methods

During October and November 2011, a total of 240 samples (40 specimens per population) were collected using cast net from six rivers belonging to three river drainages, including Kabkiyan River (30°51'N, 51°19'E; Population 1), Berim River (30°19'N, 51°15'E; Population 2), Fahlyan River (30°11'N, 51°31'E; Population 3), Beshar River (30°26'N, 51°46'E; Population 4); Sarab Bahram River (30°05'N, 51°26'E; Population 5), and Shahpour River (29°45'N, 51°33'E; Population 6) (Fig. 1). The average distance (± SD) between sampling sites was 98±44 Km (range 30-180) (Table 1).

Table 1. Sampling locations and distance between sampled rivers (Km).

	Pop2	Pop3	Pop4	Pop5	Pop6
Pop1	130	125	30	150	180
Pop2	-	80	100	90	100
Pop3	-	-	90	30	90
Pop4	-	-	-	90	150
Pop5	-	-	-	-	40
Pop6	-	-	-	-	-

A small piece of the pelvic and pectoral fins of the specimens were removed and fixed in 95% ethanol and the fish were released. Total genomic DNA was extracted and stored at -20°C from tissues using the traditional proteinase-K digestion and standard phenol/chloroform techniques based on Hillis et al. (1996). The extracted DNA was analyzed by electrophoreses via a 0.6% agarose gel containing 5 μg ml^{-1} ethidium bromide.

According to Crooijmans et al. (1997) and Matura et al. (2012), eight microsatellite loci, comprising MFW17, GGM002, GGM007, GGM023, GGM024, GGM027, GGM034 and GGM045 were used (Table 2). PCR amplification was performed in total reaction volume of 25 ml containing 2.0 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 mM each primer, 1 U Taq DNA polymerase, 1 X PCR buffer, approximately 100 ng template DNA and deionized water. Initial denaturation was achieved at 94°C for 3 min followed by 30 cycles of denaturation in 30 seconds at 94°C, 30 seconds at the respective annealing temperatures, and extension to 72°C for 1 min. The final step was extended for 3 min at 72°C. PCR products were separated using 8% polyacrylamide gels stained with silver nitrate (Bassam et al., 1991). The observed number of alleles (Na), the effective number of alleles (Ne), observed heterozygosity

Table 2. Characteristics of the used microsatellite loci in this study.

Microsatellite Loci	Gene Bank Accession no.	Primer sequence	N	Size (bps)	Anneal (°C)
MFW17	MFW17	F: CTCAACTACAGAGAAATTTCATC R: GAAATGGTACATGACCTCAAG	9	112 - 232	46
GGM002	HQ288485	F:CACTTTGTCCTTGCCATTGA R:CTCAACACCGTGGACTCTCA	25	200 - 344	55
GGM007	HQ288490	F:GCTGTGCTGACTGGCACTT R:CAAACCAACATTTCATCAAAAA	11	232 - 300	52
GGM023	HQ288506	F:TCACCATCCACTGAAGACCA R:GAAATATGTAACGTCATTAATTGTGTG	9	96 - 136	53
GGM024	HQ288507	F:TCCCTCTTTTTGCTCTCAGG R:TAGGTGAACAAATGGCATGG	14	128 - 208	52
GGM027	HQ288510	F:TCGGTGCACCCCTAGTAAAC R:CCAAGTGTGTGTTTGGATGG	12	188 - 252	54
GGM034	HQ288517	F:CGCGCAAGTTTCTTTCAGTT R:GCTGTGAGACAAGCCTAAACC	11	128 - 184	56
GGM045	JF268662	F:TCTCATGGGTCTCTGGGTTC R:TGTGCAGAAAGGCTGTTGAG	11	152 - 200	53

N: number of alleles

(Ho), expected heterozygosity (He), number of migrant (Nm), Nei's genetic distance, Wright's fixation index (Fst), genetic identity, inbreeding coefficient (FIS) and Hardy–Weinberg equilibrium (HWE) were calculated by Genealex (ver.6.5) Software (Peakall and Smouse, 2012). The Unweighted pair group method with arithmetic mean (UPGMA) diagram based on the matrix of genetic distances between populations was produced by NTSYS (ver.2.2) (Smýkal et al., 2008). The null allele frequency was estimated using MICRO-CHECKER (ver.2.2.3) software (Van-Oosterhout et al., 2004). The Hardy-Weinberg equilibrium tests were adjusted using the sequential Bonferroni correction (Rice, 1989). Analysis of molecular variance (AMOVA) was calculated using ARLEQUIN (ver. 3.1) to evaluate genetic diversity (Excoffier et al., 2005). AMOVA is a suitable approach to determine the population structure and genetic differentiation between populations (Grassi et al., 2004). Effective population size reductions were evaluated using BOTTLENECK (ver. 1.2.02) (Cornuet and Luikart, 1996). The distance matrix was then used to construct a UPGMA dendrogram using the software PopGene (ver.1.31) (Yeh et al., 1999). This program examines discrepancy between the observed heterozygosity and expected heterozygosity based on the observed number of alleles using two- tails model (S.M.M.).

Results

In 240 individuals of the six studied populations, 102 alleles were observed. The average number of alleles per locus was 12.750. The highest (0.638) and lowest (0.567) average heterozygosity was found in the populations 1 and 2, respectively, whereas the highest (0.876) and lowest (0.840) expected heterozygosity (He) were found in the populations 2 and 6, respectively (Table 3).

Thirty-seven out of 48 loci showed consistent significant deviations from Hardy-Weinberg Equilibrium expectations in populations after the probability level (P<0.05) (Table 3). Locus GGM002 and GGM024 in the populations 1, 3, 5 and 6; GGM002 and GGM045 in the population 2, and GGM024 in the population 4 were within HWE. Fixation index (FIS), a measure of heterozygote deficiency or over-plus (inbreeding co-efficient), was often larger than zero, showing a deficiency of heterozygotes in most of loci in all populations (Table 3). According to FIS values, there were no significant differences between regions. There were

Table 3. Genetic variability of eight microsatellite loci in six studied populations for *Garra rufa*.

Population Number		MFW17	GGM002	GGM007	GGM023	GGM024	GGM027	GGM034	GGM045
Pop 1	Na	10	23	12	8	13	13	10	9
	Ne	5.939	17.231	9.011	6.644	9.446	8.711	4.795	5.407
	Ho	0.393	1.000	0.357	0.750	0.929	0.321	0.643	0.714
	He	0.832	0.942	0.889	0.849	0.894	0.885	0.791	0.815
	FIS	0.528	- 0.062	0.598	0.117	- 0.039	0.637	0.188	0.124
	PHw	***	ns	***	***	ns	***	***	**
Pop 2	Na	7	24	9	8	15	8	11	11
	Ne	4.159	18.892	5.851	5.091	12.346	4.709	7.502	5.244
	Ho	0.250	0.964	0.107	0.500	0.857	0.429	0.714	0.714
	He	0.760	0.947	0.829	0.804	0.919	0.788	0.867	0.809
	FIS	0.671	- 0.018	0.871	0.378	0.067	0.456	0.176	0.117
	PHw	***	ns	***	***	**	***	***	ns
Pop 3	Na	11	22	13	8	15	15	10	9
	Ne	9.064	16.860	7.127	3.655	10.116	9.924	7.362	5.580
	Ho	0.714	1.000	0.250	0.357	0.929	0.357	0.857	0.464
	He	0.890	0.941	0.860	0.726	0.901	0.899	0.864	0.821
	FIS	0.197	- 0.063	0.709	0.508	- 0.030	0.603	0.008	0.434
	PHw	***	ns	***	***	ns	***	***	**
Pop 4	Na	7	25	10	9	11	11	13	11
	Ne	4.780	18.667	6.701	4.284	8.760	5.521	9.503	8.859
	Ho	0.000	1.000	0.143	0.714	0.857	0.393	0.643	0.786
	He	0.791	0.946	0.851	0.767	0.886	0.819	0.895	0.887
	FIS	1.000	- 0.057	0.832	0.068	0.032	0.520	0.282	0.114
	PHw	***	**	***	***	ns	***	***	***
Pop 5	Na	10	30	12	8	14	10	10	12
	Ne	5.297	24.889	7.362	5.209	11.281	6.426	6.348	5.620
	Ho	0.321	1.000	0.393	0.571	0.964	0.197	0.750	0.464
	He	0.811	0.960	0.864	0.808	0.911	0.844	0.842	0.822
	FIS	0.604	- 0.042	0.545	0.293	- 0.058	0.789	0.110	0.435
	PHw	***	ns	***	***	ns	***	***	***
Pop 6	Na	9	23	11	10	17	15	10	11
	Ne	6.426	16.505	7.575	6.759	11.701	9.800	5.502	7.840
	Ho	0.393	1.000	0.250	0.714	0.857	0.357	0.429	0.929
	He	0.844	0.939	0.868	0.852	0.915	0.898	0.818	0.872
	FIS	0.535	- 0.064	0.712	0.162	0.063	0.602	0.476	- 0.064
	PHw	***	ns	***	***	ns	***	***	***

Na, number of observed alleles; Ne, Effective number of alleles; Ho, observed heterozygosity; He, Expected heterozygosity; Fis, fixation indices; PHW, Hardy-Weinberg probability test (*P< 0.05, **P<0.01,***P<0.001, n.s., non-significant).

Table 4. Analysis of the possibility of a recent bottleneck under two tails for H excess or deficiency.

	Wilcoxon test S.M.M	Mode-shift
Pop 1	0.8432	**No**
Pop 2	0.7421	**No**
Pop 3	0.5468	**No**
Pop 4	0.7421	**No**
Pop 5	0.1953	**No**
Pop 6	0.7426	**No**

no significant indications of a recent reduction in the effective population size according to the S.M.M test in any of the populations and there was no evidence of any genetic bottlenecks (Table 4). Examination of genotyping errors revealed no evidence for large allelic dropout or stutter-band scoring at any of the eight loci. At the loci GGM007, GGM023, GGM027 and MFW17, null alleles might have appeared. For removing possible bias in results we repeated our analysis, excluding loci that showed null alleles in all populations. Since the results remained the same, therefore we retained all loci for the analysis.

Table 5. Pairwise Fst between six studied populations of *Garra rufa* based on eight microsatellite loci.

	Pop 1	Pop 2	Pop 3	Pop 4	Pop 5	Pop 6
Pop 1	-	0.035**	0.033**	0.013**	0.039**	0.044**
Pop 2		-	0.023**	0.031**	0.025**	0.027**
Pop 3			-	0.027**	0.013**	0.030**
Pop 4				-	0.025**	0.040**
Pop 5					-	0.023**
Pop 6						-

**P<0.01

Table 6. Pairwise Population Nm Values Based on Fst Values between six studied populations.

	Pop 1	**Pop 2**	**Pop 3**	**Pop 4**	**Pop 5**	**Pop 6**
Pop 1	0.000					
Pop 2	12.386	0.000				
Pop 3	9.831	13.888	0.000			
Pop 4	6.192	7.422	5.949	0.000		
Pop 5	9.157	9.748	10.460	6.802	0.000	
Pop 6	19.106	7.691	10.788	5.385	9.043	0.000

Table 7. Nei genetic distance (above diagonal) and genetic identity (below diagonal) on the studied *Garra rufa* populations

	Pop 1	**Pop 2**	**Pop 3**	**Pop 4**	**Pop 5**	**Pop 6**
Pop 1	-	0.272	0.316	0.404	0.354	0.208
Pop 2	0.762	-	0.260	0.355	0.348	0.374
Pop 3	0.729	0.771	-	0.426	0.330	0.293
Pop 4	0.668	0.701	0.653	-	0.402	0.452
Pop 5	0.702	0.706	0.719	0.669	-	0.351
Pop 6	0.812	0.688	0.746	0.636	0.704	-

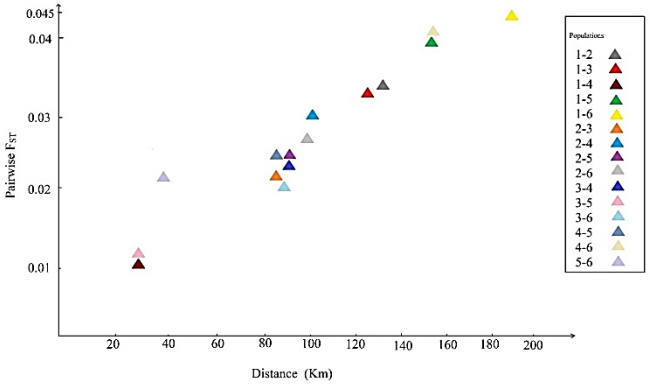

Figure 2. UPGMA dendrogram based on Nei's genetic distance, summarizing the data on differentiation between six studied populations of *Garra rufa*, according to microsatellite DNA marker analysis.

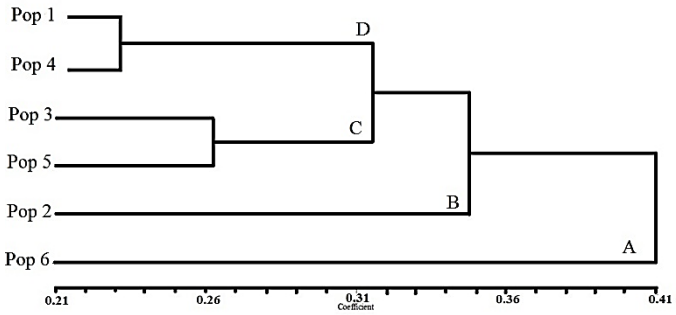

Figure 6. The relationship between geographical distance and genetic differentiation between six studied populations of *Garra rufa*

The average level of genetic differentiation between populations, as indicated by FST, was 0.039. There was a significant relationship between genetic divergence (Pair-wise Fst) and geographical distance (r^2 = 0.93, P<0.001, Fig. 2). Pair-wise FST estimates between population pairs differed significantly (P<0.01) from zero for all the pairs in populations (Table 5). Analysis of molecular variance (AMOVA) showed that 97% of the observed variation was found within populations and only 3% of the variation was observed between populations. The results revealed high levels of gene flow (Nm) between populations (Table 6).

On the basis of Nei's (1978) genetic distance values (Table 7), an UPGMA dendrogram was created displaying four major clusters (Fig. 3). Most of the similarity was observed between populations 1 and 4 in cluster D and between populations 3 and

5 in cluster C. The UPGMA revealed a distinct population structure for sixth population of *G. rufa* in Tigris Basin (Fig. 3).

Discussion

Structure and genetic diversity of *G. rufa* has been studied using eight microsatellite loci in six populations that showed an average FST of 0.039 (range 0.013-0.044) indicating little genetic differences between studied populations (Wright, 1987). In general, the fixation index can range from 0 to 1, where values close to 0 indicate that the populations are sharing their genetic material through high level of breeding and values close to 1 indicate that population do not share any alleles with one another (DeWoody and Avise, 2000). The population structure of freshwater organisms depends on the distribution of the river systems (Nagarajan et al., 2006). The previous studies showed that genetic structuring can happen even across short geographical distances (e.g., Angers and Bernatchez, 1998; Koskinen et al., 2001, 2002; Primmer et al., 2006) as similar to the results of the present study.

The positive significant relationship between genetic variation and geographical distance revealed that the genetic differentiation between studied populations is raised by increasing geographic distance. In addition, UPGMA dendrogram revealed that the six studied populations can be divided into four clusters. The populations 1 and 4 were in one group and were not significantly different (cluster D). Similarly, populations 3 and 5 were categorized in cluster C and the population 6 (cluster A) was a distinct population from others. These findings indicated that the majority of migration occurred between populations being located at about 30 Km from each other. Thus, our study lends support to IBD theory because *Garra* populations that lived in close spatial proximity were genetically similar. Similarly, using 17 microsatellites, Primmer et al. (2006) identified the relatively high level of genetic structuring and significant isolation-by-distance signal between Atlantic salmon, *Salmo salar* sampled from the tributaries and main stream of the Varzuga river system. In this study, the average waterway distance between sampling site was 60 Km (range 5-165 Km) and the level of genetic differentiation between sampling locations (FST values) ranged 0.006-0.07, with the global FST being 0.014.

Our results revealed a fairly high level of genetic variation in the microsatellite loci within six studied populations. Although no information is available on genetic diversity of *G. rufa* using microsatellite markers, Durna et al. (2010) applied RFLP markers in this fish species. Here, the mean observed and expected heterozygosity, and number of alleles per locus were 0.46, 0.56 and 9.1, respectively. The values obtained in this study are higher than those reported by Durna et al. (2010) and accordingly represent high genetic diversity of this species. The average observed heterozygosity among six regions was 0.567-0.638, higher than those generally reported in freshwater fish (DeWoody and Avise, 2000). The average observed heterozygosity across all populations was less than the expected average heterozygosity. The microsatellite data showed that allele diversity and heterozygosity levels are high in studied populations.

In genetic diversity investigations, allelic richness is higher than heterozygosity values and high allelic richness represents effective population size (Diz and Persa, 2009). According to AMOVA analysis, the mean observed number of alleles in the populations 1 to 6 was 12.250, 11.625, 12.875, 12.125, 13.250 and 13.250, respectively. The primer of GGM002 revealed the highest number of allele (25) compared to other primers. Mean number of alleles per locus was 12.75 across the 8 microsatellite loci which is generally higher than rates reported for freshwater fish (DeWoody and Avise, 2000). However, the finding is similar to those of Kanapen et al. (2006) in *Gobio gobio* as the average number of alleles per locus ranged from 2 to 13. Furthermore, Kim et al. (2007) reported a positive linear relationship between microsatellite length and number of alleles as well as the average number of

alleles 11.7 per locus in *Hemibarbus mylodon*. Many of the variation in polymorphism at microsatellite loci that exist between species can be ascribed to differences in their population biology and life history traits (Neff and Gross, 2001). This may be the reason for the observed differences in the number of alleles in the studied populations.

In wild populations, fish are often seen deviating from the Hardy-Weinberg equilibrium (HWE) (Lucentini et al., 2006; Yue et al., 2004). Based on the results, the studied populations deviated significantly from HWE at most of the microsatellite loci (37 out of 48 tests). Zhuo et al. (2012) and Quan et al. (2007) reported similar results for *Channa argus* and *Silurus soldatovi*, respectively. Factors such as inbreeding, intra-population structure (Wahlund effect), non-random sampling, fishing pressure, and fish migration have been reasons for deviation from HWE (Bergh and Getz, 1989; Garcia DeLeon et al., 1995; Castric et al., 2002; Shao et al., 2002; Ruzafa et al., 2006; Gopalakrishnan et al., 2009; Abbas et al., 2010). Non-significant deviation from HWE was reported by Israel et al. (2004) as a result of the presence of several stocks of green sturgeon that, in turn, showed the existence of one or more of the ovipositor population. Heterozygote deficits could have resulted in null alleles and real biological phenomena including mixing of differentiated wild populations (Mandal et al., 2012). The results indicated a null allele in all studied *Garra* populations. Examination of the genotyping errors revealed no evidence for large allelic dropout or stutter-band scoring at any of the eight loci. Deviation from Hardy-Weinberg expectations observed in the present study might be a result of non-specific primers usage, mistakes in reading alleles (Borrell et al., 2008), presence of migration, genetic drift (Bhassu et al., 2004), and the occurrence of null alleles in the populations.

As conclusion, this study revealed that the populations have a high genetic diversity and thus its ecological value requires adequate protection. The protection of *G.* rufa populations in Iran, as an important economic and ecological species, requires genetic monitoring to track changes in their genetic diversity. Therefore, the results of the present study could be useful as a reference to monitor any future genetic change created by environmental or anthropogenic factors. However, nature of stream habitats and susceptibility to barrier may lead to hierarchical genetic structure in freshwaters organisms. Hence, further studies are needed to separate the potential confounding impact that hierarchical structure and IBD have on one another for full understanding of genetic structure in *G. rufa* populations.

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چکیده فارسی

خصوصیات ژنتیکی جمعیت‌های ماهی گل چراغ (*Garra rufa*) در حوضه دجله با استفاده از نشانگرهای ریزماهواره

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چکیده:
بر اساس تئوری جدایی با فاصله، تمایز ژنتیکی بین افراد با افزایش فاصله جغرافیای افزایش می‌یابد. این فرضیه برای ماهی گل‌چراغ به‌عنوان ماهی آبهای داخلی ایران، در شش رودخانه مختلف از حوضه مورد ارزیابی قرار گرفت. برای این منظور تعداد هشت جایگاه ریز ماهواره جهت مطالعه ساختار ژنتیکی ماهی گل‌چراغ بر پایه پراکنش جغرافیای استفاده گردید. تعداد ۱۰۲ آلل در ۲۴۰ نمونه مشاهده گردید، میانگین آللی بین ۱۱/۶۳۵ تا ۱۳/۲۵۰ بود. میزان هتروزیگوسیتی در شش جمعیت مورد مطالعه در حدود ۰/۵۶۷-۰/۶۳۸ محاسبه گشت. به‌علاوه مشخص گردید که جمعیت‌های مورد مطالعه انحراف معنی‌داری از تعادل هاردی-واینبرگ دارند. تجزیه و تحلیل گروه جفت بدون وزن (UPGMA) نشان داد که شش جمعیت مطالعه شده می‌توانند در چهار کلاستر جداگانه قرار گیرند. نتایج سطح بالایی از تنوع ژنتیکی را در جایگاه‌های مورد استفاده در این تحقیق نشان می‌دهد. میزان Fst در دامنه ۰/۰۱۳-۰/۰۴۴ بود که بیانگر تمایز ژنتیکی پایین بین جمعیت‌های مورد مطالعه می‌باشد. بر اساس نتایج حاصل از این تحقیق مشخص گردید که رابطه ای بسیار قوی بین Fst و فاصله جغرافیای وجود دارد که نظریه جدایی با فاصله را تقویت می‌نماید.
کلمات کلیدی: تنوع ژنتیکی، ریز ماهواره، دکتر ماهی، حوضه دجله، ایران.