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Genetic variation of *Garra rufa* fish in Kermanshah and Bushehr provinces, Iran, using SSR microsatellite markers

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A B S T R A C T

Six highly variable microsatellite loci were used to investigate the genetic diversity and population structure of the *Garra rufa* in Kermanshah and Bushehr provinces, Iran. All of the 6 microsatellite loci screened in this study showed polymorphism. A total of 90 individual fish from 3 populations were genotyped and 60 alleles were observed in all loci. The number of alleles per locus ranged from 6 to14. The average allelic number of these polymorphic markers was 10. The averages of observed (Ho) and expected heterozygosity (He) was 0.529 and 0.826, respectively. The genetic distance values ranged between 0.235-0.570. The UPGMA dendrogram based on genetic distance resulted in three clusters: Gamasiab population alone was classified as one and the other two populations as the second cluster. This study revealed a fairly high level of genetic variation in the microsatellite loci within the three populations, and identified distinct population groups of *Garra rufa*. This study gains significance for the analysis of the populations' genetic diversity as well as the management of this important fish resource.

Key words: *Garra rufa*, Genetic diversity, Population, Kermanshah, Bushehr

INTRODUCTION

The freshwater fish family, Cyprinidae, is the most important fish family in the rivers of Iran. Garra rufa belongs to the Cyprinidae family. It is native to the west and south west of Iran, eastern Iraq and the south east of Turkey. This species of freshwater Garra is found mainly in pounds, ditches, swamps and rivers. Among the Iranian inland fishes, *Garra rufa* is one of the important ecological species native to Iran [1]. The extension of microsatellite markers is of significance when determining the *Garra rufa* population's genetic diversity as well as future conservation studies of this endemic species. The genetic characterization of the populations authorizes the evaluation of genetic variability, an essential element in working out stock improvement and genetic conservation programs. Therefore, it is imperative that the current level

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of genetic diversity and differentiation within and between populations of this particular species be studied as a basis of conservation and sustainable management recommendations. Molecular markers have provided the opportunity to access this variability as they provide information on every region of the genome, irrespective of the level of gene expression. Microsatellites are currently the most preferred molecular markers due to their co-dominant essence and the options they provide for performing analysis using PCR. These markers have added new dimensions to the field of fishery and molecular ecology since many populations are subject to bottleneck, genetic diversity, and genetic drift and therefore represent little variation that cannot be discovered by other markers [2-4]

Although the flanking sequence of the microsatellites is usually specific to and/or conserved for the particular species, markers expanded from one species can sometimes reinforce markers in another closely related species. Microsatellite markers have been expanded from the genome of several Garra species and used for the study of the population's genetic structure [5]. Since microsatellite markers have not yet been isolated from the *Garra rufa*, the markers developed by Matura et al [5] from another Garra species, have been used in the present study, in which the genetic structure of three wild *Garra rufa* populations from different geographical distributions, latitudes and altitudes of Iran, were analysed with six microsatellite markers to provide molecular information and to develop and utilize wild *Garra rufa* populations.

MATERIALS AND METHODS

Two populations, 30 samples each, were collected from the Gamasiab and Sepidbarg rivers, Kermanshah province, Iran, and another population, again with 30 samples, was collected from the Dalaki river, Bushehr province, Iran. Total genomic DNA was extracted using traditional proteinase K and phenol-chloroform extraction protocols as described by Ma et al. [6]. The DNA was adjusted to a concentration of 100 ng/ml and stored at -20 °C until use.

Primer sets for six *Garra gotyla* microsatellite loci were selected according to the heterozygosity and number of alleles [5]. PCR amplification reactions were carried out in a 25 ml reaction volume containing 1.0 U of Extaq DNA polymerase, 10X PCR buffer, 0.2 mM dNTP mix, 1.5 mM MgCl2, 100 ng DNA template, and 0.4 µM of each primer. Thermal cycling conditions for each locus were as follows: 5 min at 94°C, followed by 35 cycles of 94°C for 30s, annealing temperature for 30s, and an extension temperature of 72°C for 1 min, and then a final extension of 72°C for 10 min.

Genetic variation in the three populations was assessed by the following standard measures. The number of alleles per locus (Na), number of effective alleles (Ne), observed heterozygosity (Ho) and expected heterozygosity (He) for each population at each locus and fixation index (Fis) were all calculated directly from microsatellite phenotypes using the program GeneAlex version 6.5 [7] (Table 1). Deviation from the Hardy–Weinberg equilibrium of each locus for each population was tested by the Markov chain method of exact probability test using Genepop 3.1 [8]. The program Arlequin 3.0 [9] was used to calculate pairwise Fst values and test their significance by bootstrapping analysis (1000 replicates) to evaluate genetic differentiation between populations. Distance matrices were then used to construct UPGMA dendrogram using Neighbor in Phylip 3.5 [10].

Table 1. Characteristics of *Garra rufa* microsatellite loci used in this study.

Microsatellite Loci	Primer sequence	N	Size (bps)	Anneal (°C)
GGM014	F: 3'TGATGCATTATGGGAACAGG5' R: 3'TCATCAATACTTCAGAAACGAAAT5'	6	100-132	54
GGM015	F: 3'TGCAGTTCTGACCTGAATGAG5' R: 3'TTGTGGGACCTAATCGATTTTT5'	13	220-292	55
GGM021	F: 3'TCCTAAGAATTTTTGGCATAAAAGA5' R: 3'AAATGGAACTTTCAGCATAATAAAC5'	9	184-248	54
GGM024	F: 3'TCCCTCTTTTTGCTCTCAGG5' R: 3'TAGGTGAACAAATGGCATGG5'	14	128-212	54
GGM034	F: 3'CGCGCAAGTTTCTTTCAGTT5' R: 3'GCTGTGAGACAAGCCTAAACC5'	8	160-208	56
GGM044	F: 3'GGACGACGTTCACAGCAGTA5' R: 3'CAAGCCAACAGCAAATTCAA5'	10	144-220	52

N: Number of alleles

RESULTS

All 6 loci were polymorphic and diverse in all populations (Table 1). Allele frequencies at all loci in all populations are shown in Table 2. A total of 60 alleles, ranging in size from 100 to 292 bp, were found at the 6 loci. The number of alleles ranged from 6 (GGM014) to 14 (GGM024). The mean of the alleles was 10. The average observed heterozygosity was lowest (0.19) in the Gamasiab population in GGM021 and highest (0.905) in the Dalaki population in GGM044. The average observed heterozygosity showed similar trends in the 3 populations (Table 2). Of the 18 HWE tests, 5 were significant and associated with heterozygote deficiency. A greater proportion of significant HWE tests occurred at GGM014 and GGM024 (2 of 3) than at GGM044 (1 of 3). Gametal correlation coefficient (F_{ST}), also known as coefficient of inbreeding, and Gene Flow (Nm) were computed to estimate the differences between populations. Pairwise F_{ST} analyses revealed no significant genetic heterogeneity among populations. The three studied populations were well differentiated (F_{st}=0.022–0.063). The average of F_{ST} was 0.043, and the average of Nm was 7.276 (Table 3). This indicated that Nm might be the significant factor between populations. The results of the analysis of molecular variance (AMOVA) showed that 96% of the variation was observed within populations while only 4% was observed between populations (Figure 1). Based on the genetic distance, the UPGMA dendrogram displayed three major clusters (Figure 2).

Table 2. Genetic variability of six microsatellite loci in three populations for *Garra rufa* Iran.

Location		GGM014	GGM015	GGM021	GGM024	GGM034	GGM044
Sepidbarg	N_a	5	14	8	15	7	10
	N_e	3.556	8.481	5.765	10.756	4.618	7.289
	H_o	0.571	0.429	0.333	0.857	0.333	0.619
	H_e	0.719	0.882	0.827	0.907	0.783	0.863
	F_{IS}	0.205	0.514	0.597	0.055	0.575	0.283
	P_{HW}	**	***	**	ns	***	ns
Gamasiab	N_a	7	14	9	16	8	11
	N_e	4.18	9.000	5.188	10.256	6.168	8.243
	H_o	0.714	0.333	0.190	0.810	0.429	0.762
	H_e	0.761	0.889	0.807	0.902	0.838	0.879
	F_{IS}	0.061	0.625	0.764	0.103	0.488	0.133
	P_{HW}	ns	***	***	ns	***	**
Dalaki	N_a	6	11	11	11	9	8
	N_e	2.706	8.321	6.945	5.690	9.000	5.04
	H_o	0.524	0.381	0.381	0.667	0.286	0.905
	H_e	0.630	0.880	0.856	0.824	0.824	0.802
	F_{IS}	0.169	0567	0.555	0.191	0.653	-0.129
	P_{HW}	ns	**	***	***	***	***

Na, number of observed alleles; Ne, number of effective 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 3. Number of migrant and Fst index of six microsatellite loci in three populations for *Garra rufa* in Iran.

Loci	GGM014	GGM015	GGM021	GGM024	GGM034	GGM044
N_m	2.440	10.891	4.525	6.429	8.987	10.384
F_{ST}	0.093	0.022	0.052	0.037	0.027	0.024

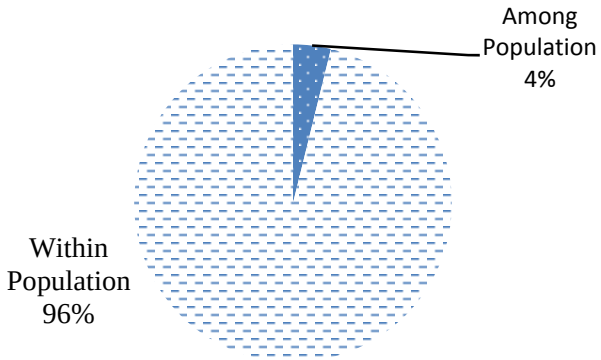

Figure 1. Distribution of genetic diversity, based on FST

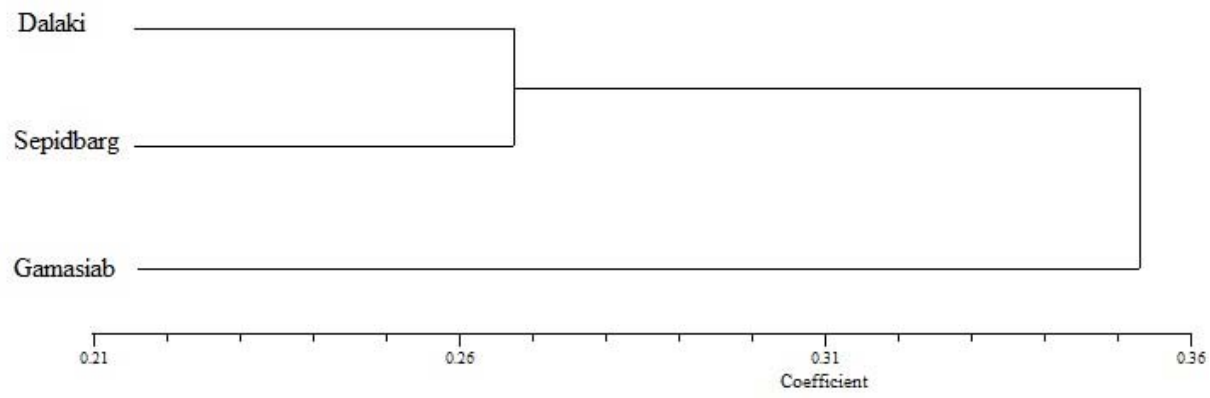

Figure 2. UPGMA dendrogram of *Garra rufa* populations in Iran (Kermanshah and Bushehr Provinces) based on a matrix of Genetic distance.

DISCUSSION

Biological population variation is an important factor regarding evaluating species resources. Based on this factor, species adapt to various environments. It is a precondition to make persistent use of the species resource and to keep the highest level of genetic variation. A precise estimation of population structure and genetic distances from microsatellite data is dependent on the sample size, number of loci, number of alleles, and range in allele size [3]. Population genetic structures of a fish species is subject to change over time by biological and physical factors including overexploitation, contamination of native gene pools through introgression, inbreeding, bottleneck effect, environmental pollution, habitat degradation, hydrological manipulations and mutation at various levels. Microsatellite markers have been proved to be effective and efficient in detecting very low levels of genetic change induced by the aforementioned factors. Microsatellite markers developed from one species sometimes cross-amplify microsatellite loci in closely related species. Matura et al [5], for example, reported amplification of the *Garra gotyla* in India. In the present study, we used the microsatellite markers developed from *Garra gotyla* by Matura et al [5] to analyze the *Garra rufa's* population genetic structure. This is the first study of genetic variation using microsatellite markers on the *Garra rufa* in Kermanshah and Bushehr provinces. Analyses of six microsatellite loci revealed low levels of genetic variation between populations (expressed in number of alleles and heterozygosity) during study.

The results of the present study are comparable in variability to the report by DeWoody and Avise [11] regarding other freshwater fishes (Na: 9.1, He: 0.54), such as the *Paraschistura bampurensis* (Na: 13, He: 0.872, [3]) and *Oxynoemacheilus kiabii* (Na: 811.5, He: 0.850, [12]), which have small populations and a high gene flow. The Hardy–Weinberg disequilibrium is common in many fishes, but deviations to the equilibrium generally prevail over heterozygote deficits [3, 13-15] resulting from factors involving the fish's reproductive systems, presence of null alleles, and a Wahlund effect (reduction of heterozygosity in a population caused by subpopulation structure). In the *Garra rufa*, we found more deviations toward heterozygous excess, possibly a consequence of the mixture and reproduction of individuals of different

cohorts that could have different allelic composition. However, we could not find a clear tendency towards the Hardy–Weinberg disequilibrium in a specific loci or location, so there is a chance that those deviations from the equilibrium are a consequence of stochastic events in allelic frequency distributions. The average expected heterozygosity was highest in the Gamasiab (0.846) and lowest in the Dalaki populations (0.803). The average observed heterozygosity values of all the populations were lower than the corresponding expected heterozygosity values. The results of the observed heterozygosity were not consistent with the average number of alleles per locus; however, the expected heterozygosity values were consistent with the average number of alleles per locus in a population. For example, the average number of alleles per locus and the average expected heterozygosity value were both the highest in the Gamasiab population (Table 2). Violations of the Hardy-Weinberg assumptions can cause deviations from expectation. Reduction in size of a population is considered to be one of the few factors that might be responsible for deviations from this equilibrium. Small population size causes a random change in genotypic frequencies, particularly if the population is very small due to genetic drift. From the present study, it can be postulated that the Dalaki population deviated from the Hardy-Weinberg equilibrium.

These results and those from the AMOVA showed a slight genetic heterogeneity in spatial scales. However, Bayesian assignment analysis carried out using Structure software and an isolation-by-distance test, which correlates geographic distance with genetic distance, failed to detect any different genetic group in the entire data set. The values of F_{ST} in our study (0.022-0.093) are low compared to cichlid fish (0.04–0.2). In our study, no significant values of F_{ST} were found between the population and little genetic differentiation between populations was observed. Besides, *Garra rufa* have biological features that support the hypothesis, including large migratory activity and reproductive capacity [1]. The phylogenetic dendrogram (Figure 2) drawn from Nei's genetic distance also suggests the separation of the Gamasiab from the other two populations.

Analysis of the population’s genetic structure of threatened or commercially important fish species is essential to develop management strategy because a population can suffer severe genetic erosion (bottleneck, genetic drift, inbreeding, founder effect) without being detected by the traditional demographic monitoring approach [16]. The microsatellite technique has been found to be suitable for characterizing the genetic structure of the three populations of *Garra rufa*. The allele numbers and heterozygosity levels observed across the studied loci indicate the presence of a reasonably high level of genetic variability in the *Garra rufa* populations in Iran. This information should be taken into consideration for any genetic conservation. However, to reveal the detailed genetic structure of this important fish species in Iran, further research employing additional microsatellite loci to study large numbers of populations across the country is recommended. To characterize and distinguish *Garra rufa* populations, microsatellite loci should be preferred because of their generally higher variability and better performance, particularly if populations are within geographical regions [4]. The panel of six microsatellite loci used in the present study can be used for a relatively precise description of the genetic diversity and relationships of *Garra rufa* populations. However, the final number of loci used should depend on the number of populations scored and the expected degree of genetic divergence among populations.

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