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Original Article

# Microsatellite loci to determine population structure of *Garra rufa* (Heckel, 1843) in the Khuzestan Province (Iran)

**Ali Shabani, Ghasem Askari***

[1] Department of Fisheries, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran.

**Abstract:** Genetic diversity of *Garra rufa* was studied using 6 polymorphic microsatellite DNA loci. The specimens of *G. rufa* were collected from the Kheirabad and Maroon rivers. Despite high importance of this species, there is no genetic information about its population structure. A total of 133 alleles were detected at the 6 loci across the two populations. The Kheirabad population exhibited a lower genetic variation (Ho=0.429 and He=0.850) than the Maroon one. The average numbers of observed alleles in the Kheirabad and Maroon populations were 11.8 and 10.3, respectively. The genetic similarity and distance between the two populations were 0.721 and 0.326, respectively. It seems that Maroon population live under better conditions in contrast to the Kheirabad one. Diminution of genetic variation within examined populations decreases its adaptation to environmental alterations. Based on the results of this study, we can identify two different *Garra rufa* populations in the Khuzestan Province.

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## Introduction

There are about 200 fish species in the inland waters of Iran, which generally belong to three families: Cyprinidae, Balitoridae, and Cobitidae (Abedi et al., 2011). The cyprinid species exhibit a wide range of geographical distribution, life histories, and reproductive styles (Winfield and Nelson, 1991). The family Cyprinidae, with about 220 genera and about 2420 species, is the largest family of freshwater fishes and, with the possible exception of Gobiidae, the largest family of vertebrates (Nelson, 2006). The members of the genus *Garra* Hamilton-Buchanan, 1822 are found throughout the southwest Asia and from Africa to Southeast Asia, and are predominantly adapted to live in swift-flowing waters, streams, and lakes (Krupp and Schneider, 1989). Among the Iranian inland fishes, *Garra rufa* is one of the important biological species that is native to the Tigris basin. It has a small size and no economic importance. Some of the common names of this fish used in Iran are Gel-cheragh, Gel-khorak, Mahi-e-sang lis, and Shirbot. *Garra rufa* has a wide dispersion, but there is little information on its biology in Iran.

Microsatellite DNA markers are utilized in the assessment of genetic variation and population differentiation studies for a variety of vertebrates, including aquatic organisms (O'Connell and Wright, 1997; Neff and Gross, 2001; Askari et al., 2013). Their high level of polymorphism and co-dominant inheritance pattern makes them markers of choice for population genetic studies. Microsatellites which occur in non-coding parts of DNA have conserved flanking sequences (Ellegren, 2004), and there exists the potential for using microsatellite PCR primers developed for one species to characterize loci in other related species (Moore et al., 1991; Zardoya et

* Corresponding author: Ghasem Askari
E-mail address: Askarighasem82@gmail.com

al., 1996; Galbusera et al., 2007) or unrelated species across families.

Microsatellite and mitochondrial DNA haplotype markers became the methods of choice for many fish studies. Taxonomy and systematic have undoubtedly benefited from DNA sequencing technology (Hillis et al., 1996). Therefore, the principal objectives of this study were to assess the intra- and inter-population genetic variations and genetic differentiation in two populations of *Garra rufa* inhabiting the Khuzestan province, using the microsatellite DNA markers developed by Matura et al. (2012).

## Materials and methods

The tissue samples for DNA extraction were collected using the fin clipping procedure of Lourie et al. (1999 a, b). The specimens used in this study, were collected from the Kheirabad and Maroon rivers of Iran between September and October 2012. Fin tissues were collected from 80 fish from Kheirabad and Maroon rivers (Fig. 1), and then stored in 96% ethanol for subsequent DNA extraction and amplification. Genomic DNA was extracted from fin clips using the Phenol-Chloroform procedure described by Hillis and Moritz (1990). The quality and concentration of DNA from samples were assessed through 1% agarose gel electrophoresis.

PCR reactions were carried out in a thermal cycler (PTC 200 gradient; M.J. Research, Watertown, MA, USA). PCR amplifications were done using six microsatellite loci analyzed: GGM014, GGM015, GGM021, GGM024, GGM034, GGM044 (Matura et al., 2012) (Table 1). The Polymerase Chain Reaction (PCR) conditions, especially the annealing temperatures, were optimized for the 6 microsatellite primers as necessary to produce amplification products. Amplification was performed in PCR system (Gradient Eppendorf) using a 25 µl total volume containing 5 µl of 10X reaction buffer, dNTPs 10 mM, $MgCl_2$ 50 mM, primer 20 pmol of each (Foward and Reverse) (Table 1), genomic DNA 100 ng and 1.5-2 unit of Taq polymerase. Initial denaturation was achieved at 94°C for 3 min


Figure 1. Map showing the sampling localities in the Kheirabad and Maroon Rivers

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

| Microsatellite Loci | Primer sequence | N | Size (bps) | Anneal (°C) |
|---|---|---|---|---|
| **GGM014** | F:TGATGCATTATGGGAACAGG<br>R:TCATCAATACTTCAGAAACGAAAT | 7 | 100-132 | 54 |
| **GGM015** | F:TGCAGTTCTGACCTGAATGAG<br>R: TTGTGGGACCTAATCGATTTTT | 11 | 220-292 | 55 |
| **GGM021** | F:TCCTAAGAATTTTTGGCATAAAAGA<br>R:AAATGGAACTTTCAGCATAATAAAC | 11 | 184-248 | 54 |
| **GGM024** | F:TCCCTCTTTTTGCTCTCAGG<br>R:TAGGTGAACAAATGGCATGG | 14 | 128-212 | 54 |
| **GGM034** | F:CGCGCAAGTTTCTTTCAGTT<br>R:GCTGTGAGACAAGCCTAAACC | 10 | 160-208 | 56 |
| **GGM044** | F:GGACGACGTTCACAGCAGTA<br>R:CAAGCCAACAGCAAATTCAA | 16 | 144-220 | 52 |

N: number of Allele

followed by 30 cycles of denaturation at 94°C for 30 seconds, 30 seconds at the respective annealing temperatures, and extension at 72°C for 1 minute, and an extension for 5 minutes at 72°C. PCR products were separated using 8% polyacrylamide gels stained with Silver Nitrate.

The presence of null alleles was tested using Microchecker version 2.2.3 (Van Oosterhout et al., 2004). The recorded microsatellite genotypes were applied as input data for the GeneAlex software version 6 (Peakall and Smouse, 2012) to calculate allelic and genotypic frequencies, observed (Ho) and (He), expected heterozygosity and to test for deviations from Hardy-Weinberg Equilibrium (HWE). For each marker allelic variation was estimated by the polymorphic information content (PIC) value first described by Botstein et al. (1980) and modified by Anderson et al. (1993).

## Results

Primer sequences and specific annealing temperature (Ta°C) of the resource species (Matura et al., 2012) and studied *Garra rufa* species are given in Table 1. The optimal annealing temperatures to get scorable bands in samples differed from that reported for the resource species. All primer pairs tested yielded successful amplification.

A total of 133 alleles were detected at the 6 loci and across two populations (the Kheirabad and Maroon Rivers) (Table 2). In the Kheirabad population, a total of 71 alleles were produced in microsatellite analysis across all samples. The number of allele per locus ranged from 5 to 17. In the Maroon population, a total of 62 alleles were detected in microsatellite analysis across all samples. The number of alleles at different microsatellite loci in Maroon population varied from 8 to 14 with an average value of 10.3. Primer GGM044 exhibited maximum allele number (14) compared to other primers. Considerable differences among 2 populations in the number of alleles were found at some of these loci (Table 2). The number of alleles in GGM024 ranged from 12 to 15 and GGM015 from 8 to 13 with a tendency to a reduction in the Maroon population. Allele sizes ranged from 100 to 292 bp across the microsatellite loci. The effective number of alleles varied from 3.58 for GGM015 to 10.02 for GGM024. In all populations, the effective number of alleles was lower than the observed number of alleles, except GGM014 loci in the Kheirabad population. The average of observed and expected heterozygosity ranged from 0.286 to 0.857 and from 0.721 to 0.902, respectively. The maximum and minimum numbers of the unique alleles were found at loci GGM044 (17) and loci GGM014 (5), respectively. In the Kheirabad population, the mean observed heterozygosity (Ho) and expected heterozygosity (He) values were 0.429 and 0.850. In the Maroon population, these values were 0.532 and 0.859, respectively.

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

| Location | | GGM014 | GGM015 | GGM021 | GGM024 | GGM034 | GGM044 |
|---|---|---|---|---|---|---|---|
| | $N_a$ | 5 | 13 | 11 | 15 | 10 | 17 |
| | $N_e$ | 7.17 | 3.58 | 7.87 | 10.02 | 6.89 | 9.00 |
| **Kheirabad** | $H_o$ | 0.381 | 0.381 | 0.333 | 0.810 | 0.286 | 0.381 |
| | $H_e$ | 0.721 | 0.861 | 0.873 | 0.900 | 0.855 | 0.889 |
| | $F_{IS}$ | 0.472 | 0.557 | 0.618 | 0.101 | 0.666 | 0.571 |
| | $P_{HW}$ | *** | *** | *** | *** | *** | *** |
| | $N_a$ | 8 | 8 | 11 | 12 | 9 | 14 |
| | $N_e$ | 6.48 | 6.48 | 7.41 | 7.73 | 5.65 | 10.25 |
| **Maroon** | $H_o$ | 0.429 | 0.381 | 0.524 | 0.857 | 0.286 | 0.714 |
| | $H_e$ | 0.846 | 0.846 | 0.865 | 0.871 | 0.823 | 0.902 |
| | $F_{IS}$ | 0.493 | 0.550 | 0.394 | 0.016 | 0.653 | 0.209 |
| | $P_{HW}$ | *** | *** | ns | ns | *** | ns |

Na, number of observed alleles; Ne, number of effective alleles; Ho, observed heterozygosity; He, expected heterozygosity; $F_{IS}$, fixation indices; $P_{HW}$, Hardy-Weinberg probability test (*$P$<0.05, **$P$<0.01,***$P$<0.001, n.s, non-significant).

Significant deviations from Hardy-Weinberg equilibrium (HWE) at the locus level are shown in Table 2. All six loci used in this study were tested for departure from HWE. Nine out of 12 (6 loci × 2 populations) possible tests for HWE were statistically significant ($P$<0.05). The population differentiation ($F_{ST}$) was modest whit $F_{ST}$ value between the Kheirabad and Maroon Rivers population was 0.022 and no significant. $R_{ST}$ value between the two populations was high (0.108) and significant. The estimated gene flow ($N_m$) value between the Kheirabad and Maroon rivers' populations across all the studied loci was 11.243 (Table 3). Genetic distances among the respective populations were small. The genetic distances and genetic similarity, as computed by Saitou and Nei (1987) between the Kheirabad and Maroon rivers' populations were 0.326 and 0.721, respectively and the Unweight pair group method with arithmetic mean (UPGMA) dendrogram, based on the genetic distance, showed that these two populations are distinctly two different branches. The $F_{IS}$ values ranged from 0.016 for the locus GGM024 to 0.666 for the locus GGM034 between two populations.

## Discussion

Genetic diversity is important for ecological and evolutionary processes ranging from individual fitness to ecosystem function. Heterozygosity serves as an indicator of evolutionary potential and is important in determining population dynamics as well as population viability (Reed, 2009). The result is consistent with earlier reports, suggesting the possibility of using primers interspecifically among teleosts (Gopalakrishnan et al., 2004). All of the loci were polymorphic and the genotypic distribution frequencies across all the loci were significantly different, suggesting genetic structuring among these two populations. Frequencies of alleles in the Kheirabad samples are higher from the Maroon samples, except at one locus (GGM014). It is likely that the Maroon population had originated from the Kheirabad, and that it had lost some alleles during the course of fisheries and environmental management. The losses of alleles and heterozygosity in the *Garra rufa* stocks may be intensified by bottlenecks and inbreeding.

Heterozygosity is an important measurement of population diversity at the genetic level and has drawn much attention from ecologists and aquaculturists (Xu et al., 2001). The results of the study indicated that the average number of alleles per locus and the observed heterozygosity in Kheirabad (0.429 and 0.850) and the average number of alleles per locus and the observed heterozygosity in the Maroon River were 0.532 and 0.839, respectively. In the current study, the observed heterozygosity for 6 microsatellite loci was lower than the expected heterozygosity in the two populations. However, the Kheirabad population showed the lowest genetic diversity among the two populations in terms of the average number of alleles and genotypes per locus, the number of unique alleles, and low-frequency alleles. The results of this study indicated that considerable heterozygosity excess was observed in intra-population based on allelic and genotypic frequencies. Significant deviations from Hardy-Weinberg expectations (HWE) were observed in the two populations. Genetic drift, inbreeding and divergent evolution are likely to be the causes for deviation from the H–W disequilibrium (Zolgharnein et al., 2011). Several hypotheses have been mentioned to explain the deviation from HWE, including inbreeding, intra-population structure (Wahlund effect), non-random sampling, selection against heterozygote, and fishing pressure (Abbas et al., 2010; Bergh and Getz, 1989; Castric et al., 2002; Ruzafa et al., 2006). These results are compatible

Table 3. Number of migrant and $F_{st}$ index of six microsatellite loci in two populations for *Garra rufa*.

| Loci | GGM014 | GGM015 | GGM021 | GGM024 | GGM034 | GGM044 |
|---|---|---|---|---|---|---|
| **$N_m$** | 6.009 | 12.754 | 10.646 | 13.702 | 10.725 | 13.621 |
| **$F_{ST}$** | 0.04 | 0.019 | 0.023 | 0.018 | 0.023 | 0.018 |

with a previous study that had been conducted for *Paraschistura bampurensis* (Askari and Shabani, 2013), and with studies on other types of fish (Salari Aliabadi et al., 2009; Bradshaw et al., 2007; Alam and Islam, 2005; Hansen and Mensberg, 1998).

The partitioning of variability of populations observed after F-statistics comparisons with total types of markers showed that most of the genetic variation is within populations. There was a low level of genetic differentiation among the two populations and significant $F_{ST}$ value of 0.022 ($P$<0.01). Based on analysis of molecular variance (AMOVA), $F_{ST}$ (0.022) was observed between the Kheirabad and Maroon rivers' populations ($N_m$=11.243). This issue represents the low differentiation between the two populations. According to Wright (1987), $F_{ST}$ value less than 0.05 indicates the low differentiation among communities. Li et al. (2007) noted that when $N_m$ > 1 and $N_m$ < 1, then genetic differentiation occurred due to number of migrant and gene flow, respectively; hence the results of this study revealed that number of migrant fish was the main reason for low genetics differentiation between the studied populations. It was demonstrated using UPGMA dendrogram, that there were two separate population groups in these rivers. Genetic structure of *Garra rufa* in these rivers was probably due to number of migrates which occurred during decades.

The results obtained from the present study show that at least 2 different populations of *Garra rufa* are found in the two rivers, which include the Kheirabad and Maroon rivers' populations. This information should be taken into account for any genetic conservation and stock improvement plan. However further study involving low numbers of populations covering all parts of the country with additional microsatellite loci is recommended to reveal detailed genetic structure of this important fish species in Iran.

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