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Studies on Genetic Variability of Sucker Head, *Garra gotyla* (Fam: Cyprinidae) using Microsatellite Markers

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ABSTRACT

Keywords

Garra gotyla, Genetic variation, Null alleles, microsatellite, Polymorphism Information Content.

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Garra gotyla, commonly known as sucker head fish belongs to family Cyprinidae and distributed all along the mountain regions of India, Pakistan, Bangladesh and Myanmar. Different geographically isolated populations of the species were assessed using few microsatellite markers for future conservation and strategic breeding plan. Three wild populations of *G. gotyla* were analysed for genetic variability using 20 microsatellite markers. The mean observed heterozygosity ranged from 0.11 to 0.85. The mean no of alleles ranged from 3-11 with an average number of alleles per locus was 5.7 and were found to be in Hardy-Weinberg equilibrium (HWE) ($p > 0.05$). Polymorphism Information Content (PIC) ranged from 0.42 to 0.87. Overall Fst value of all the samples combined over all the microsatellite loci was found to be 0.173. The present genetic status of these three populations was also discussed for future application in genetic improvement of the species.

Introduction

Garra gotyla, commonly known as sucker head fish belongs to family Cyprinidae. It has a broad geographical distribution all along the Himalayas, Chota-Nagpur plateau, the Vindhya-Satpura mountains of the Indian peninsula; Pakistan, Bangladesh, and upper Burma. The importance of the fish has been mentioned as minor commercial by Talwar and Jhingran, (1991). The wide distribution implies the broad environmental adaptation of the species, but there is a very limited study of physiology, genetics, breeding and other aspects of the species. There is a reduction in the genetic resources of natural fish populations due to various human activities, over utilization of fish stocks and pollution and listed as least concern in the IUCN red list of threatened species (version 2016-1). Conservation of this fragile genetic diversity is imperative to maintain ecological as well as the socio-economic harmony of developing country like India. Assessment of the genetic diversity present within a species is a

prerequisite for future sustainable breeding efforts. Molecular marker offers a practical and useful tool to investigate the genetic diversity of such fragile fish populations. Microsatellites or simple sequence repeats (SSRs) consist of short (1-6 base pair, bp) tandem arrays (Tautz and Renz, 1984; Tautz, 1989). Microsatellites are present in both exonic and interonic regions of the genome and are characterized by their high degree of length polymorphism. There is no sure evidence of such polymorphism though most acceptable hypothesis is slippage events during DNA replication (Schlotterer & Tautz 1992). These microsatellite loci seemed to be highly abundant and dispersed throughout the genome. In the present study, we used the avaiable microsatellite markers (Matura *et al*., 2012) to assess the genetic variaion and structure of three geographically isolated populations of *Garra gotyla.*

Materials and Methods

Sample collection and DNA isolation

A total of 30 samples of *Garra gotyla* were collected from three different riverine sources each *viz.* GGPR– Gola River, Ranibagh (29°, 18’, 02’’N 79°, 32’, 02’’E); GGPK– Kosi River, Ramnagar (29°, 24’, 56’’N 79°,08’,01”E) and GGPA– Alaknanda River, Srinagar Garhwal (30°, 13’, 11”N78°, 46’, 47”E) Uttarakhand. Fin tissues were collected by non-invasive technique (Wasko *et al*., 2003) followed by proper antimicrobial treatment of fishes before their release to the habitat. Total genomic DNA was isolated from the fin tissue of the sampled individuals using standard proteinase K digestion followed by extraction with phenol:chloroform and precipitation with ethanol following Sambrook *et al*., (2001). The quality of the isolated genomic DNA was checked on 0.8% agarose gel. After quantification DNA was subsequently diluted to a concentration of 50 ng/ml and stored at -20 °C until further use.

Detection of microsatellite polymorphism

Twenty eight microsatellite loci that revealed high polymorphisms (Matura *et al.*, 2012) were selected for genetic variability in different geographically isolated populations in the present study. All the loci were were amplified in a 10µl PCR reaction containing 50ng of genomic DNA, 200µM of dNTPs, 0.5 µM of each primer, 1.5mM $MgCl_2$, 10mM Tris(pH 9.0), 50mM KCl, 0.01% Gelatin and 0.5U of Taq DNA Polymerase (Thermo Fisher Scientific, Wilmington, US). The PCR was performed in a Gene Amp PCR system 9700 thermocycler (Applied Biosystem) set at cycling conditons as follows; 94°C for 4 min; followed by 34 cycles of 94°C for 30s, 50–64°C for 35s (depending on the primer pairs; Table 1), 72°C for 1 min; and a final extension for 10 min at 72°C. Amplified products were dried on a vacuum concentrator (Eppendorf, Hamburg, DE), mixed with 2µl of formamide loading dye, heat denatured at 95°C for 7 min and then subjected to electrophoresis on 8% denaturing polyacrylamide gel with 7.5 M urea and 1x TBE. Separation was performed in a vertical gel electrophoresis (Hoefer, Holliston, MA) which were further visualized by Ethidium bromide staining. The size of alleles was estimated according to ϕ ×174 DNA/Hinf I marker (Thermo Fisher Scientific, Wilmington, US).

Statistical Analysis of genetic data

Genetic polymorphism within 3 populations was measured by the number of alleles (Na), observed (Ho) and expected heterozygosity (He), the effective number of alleles (Ne) and Shannon’s diversity index (I) using GDA v.1.1 (Lewis and Zaykin, 2008). The

probability of scoring errors, DNA polymerase slippage (stutter band), allele dropout and presence of null allele (due to non-amplified locus or homozygote excess) were calculated using Micro-Checker v.2.2 (Van-Oosterhout *et al*., 2004). Polymorphism information content (PIC) was calculated using CERVUS v.3.0 (Kalinowski *et al*., 2007). Fis (inbreeding coefficient), Fst as well as Rst for each locus were calculated using FSTAT v.2.9 (Goudet 1995). Genotypic linkage disequilibrium, deviation from Hardy-Weinberg equilibrium and population differentiation was computed using GENEPOP v.4.0 (Rousset, 2008).

Finally, we used two approaches to determine which of the population may have undergone significant size reduction. Firstly by depicting the extent of distortion of allele frequency mode shift (from typical 'L-shaped' distribution) and secondly by calculating the excess of observed heterozygosity using Wilcoxon signed rank test using BOTTLENECK v.1.2.02 (Piry *et al*., 1999). Heterozygote excess was tested using two models of mutation-drift equilibrium i.e. infinite alleles model (I.A.M.) and stepwise mutation model (S.M.M.) using 1000 replicates of the dataset.

Results and Discussion

Out of 28 primers 20 primer pairs were successfully amplified in genomic DNA. Other loci were either non-specific or failed to amplify in a different range of temperatures. Few of the loci also showed null alleles frequency based on Brookfield1 estimate from Micro-checker software (Van-Oosterhout *et al*., 2004) and were excluded from the study. Therefore, out of 28 markers, we used 20 highly polymorphic primer pairs for population studies.

The mean no of alleles ranged from 3-11 with an average number of alleles per locus 5.7. All loci were in Hardy-Weinberg equilibrium (HWE). The allele frequencies did not deviate significantly from the Hardy– Weinberg expectation at any locus in all the sample sets ($p > 0.05$). Polymorphism Information Content (PIC) ranged from 0.42 to 0.87, with all the 20 loci revealing moderate to high polymorphism (0.40-0.82). The mean PIC value over all loci was found to be 0.70. The highest PIC value was found for locus GGM 027 with 0.87. The expected heterozygosity (H_E) ranged from 0.53 to 0.88 and observed heterozygosity from 0.13 to 0.85. No heterozygote excess was observed for any population. No linkage disequilibrium was detected for any pairwise combination of loci. The Summary statistics for genetic variation at 20 microsatellite loci which were used to study the genetic variation across 3 populations of *Garra gotyla* is shown in Table 2.

The test of heterozygosity excess was implemented to analyse the possibilities of any historical bottlenecks. The tests for heterozygosity excess were significant ($p < 0.01$) under both I.A.M. and S.M.M. mutation models. In bottleneck analysis we found limited evidences for recent reduction in population size, using stepwise mutation model. Although, the mode shift test did not detected any significant distortion of allele frequency but showed some deflection from normal "L" shaped distribution (Figure 1) which is a typical property of a population under little selection pressure.

Microsatellite markers show high polymorphisms within populations, therefore, they are most preferable for genetic diversity studies. However, selection of loci is also crucial for any population study. The validation of microsatellite makers originally developed by Matura *et al*., (2012) may also help to select highly

polymorphic loci in future population studies.

Twenty microsatellite loci were selected for characterization of three different geographically isolated populations of *Garra gotyla*. It is for the first time an attempt was made to evaluate the genetic structure of different populations of *G. gotyla*. PCR amplification of 20 loci was successfully achieved in 90 individuals. All the markers had high polymorphic information content (0.80) and mean allele number (5.65), indicating a high allelic variability.

The observed allele sizes of present study were comparable to the allele size observed by Matura *et al*., (2012) at respective loci. Thus the selection of markers had shown their ability to characterize the *Garra gotyla* stock in India.

Shabani and Askari (2013) studied the genetic diversity in *Garra rufa* using 6 microsatellite markers. The authors reported medium to high genetic diversity in two different populations of *G. rufa* which is similar to the present study. We also observed high allele numbers in *Garra gotyla* populations comparable to the study of Shabani and Askari (2013).

Kirchner *et al*., (2014) developed 19 microsatellite markers containing di- and tetranucleotide simple sequence repeats through NGS 454 sequencing to analyse the genetic variation among three different populations of *Garra barreimiae*. They observed a high genetic diversity among populations using those polymorphic markers.

Although the three populations have been collected from geographically isolated rivers but they all are associated to the Ganga River System. However the significant genetic variations among these populations are the indicator of substantial barriers among these populations. There may be physical barriers or their migratory space could be limited but the logical explanation requires more demographic studies on *Garra gotyla*. The GGPG and GGPK populations are closer to each other as predicted by Nei's Genetic distance (Figure 2). Geographically these sampling locations are closer to each other as compared to GGPA population. Hence geographical isolation is also playing a significant role in genetic variation among these populations. Wirth and Bernatchez (2001) found a significant correlation among genetic distance and geographical isolation of *Anguilla anguilla* (L.) populations which is comparable to the present findings.

In the present study Fst and Rst analysis revealed significant differences among three populations of *Garra gotyla* with overall Fst value of 0.173 and about 17 % of the genetic variation was gained due to inter-population differences. Overall Goodman Rst value was 0.46. In the present study, higher value of Rst than Fst was observed, which predicts a role of mutation rather than genetic drift in the differentiation of *Garra gotyla* populations.

On the basis of present study, the observed heterozygosity (Ho) in terms of average of all loci across the stocks was less in comparison to expected heterozygosity (He). This may be attributed to inbreeding effect (overall Fis was estimated to be 0.37; $p<0.001$); highest inbreeding coefficient was observed in GGPG population (0.447) while lowest was observed for GGPK (0.323). The average observed heterozygosity (Ho) was found to be 0.39 and expected heterozygosity (He) was 0.74 in the present study (Table 1).

Table.1 Characteristics of amplified microsatellite loci in *Garra gotyla*

Locus	Primer Sequence (5'-3')	Repeat motif	Ta	Na	H_o	H_e	PIC	Fis	Fst	Rst
GGM027	F-TCGGTGCACCCCTAGTAAAC; R-CCAAGTGTGTGTTTGGATGG	$(CA)_{15}$	54	10	0.85	0.88	0.87	-0.14	0.15	0.74
GGM015	F:TGCAGTTCTGACCTGAATGAG; R: TTGTGGGACCTAATCGATTTTT	$(GT)_{24}$	55	11	0.77	0.86	0.84	0.05	0.10	0.70
GGM028	F: GCACACAGCCTTAGTGCAAA; R:TTTCCAACTTCTGGTGTCGTG	$(CT)_9$ $(CA)_{24}$	56	8	0.28	0.84	0.82	0.60	0.17	0.73
GGM024	F:TCCCTCTTTTTGCTCTCAGG; R:TAGGTGAACAAATGGCATGG	$(CA)_{15}$	54	8	0.57	0.84	0.81	0.24	0.11	0.22
GGM021	F:TCCTAAGAATTTTTGGCATAAAAGA; R:AAATGGAACTTTCAGCATAATAAAC	$(CA)_{15}(TA)_{13}$	54	7	0.44	0.80	0.76	0.44	0.00	0.02
GGM0232F	F:TCACCATCCACTGAAGACCA; R:GAAATATGTAACGTCATTAATTGTGTG	$(CA)_{16}$	60	6	0.24	0.78	0.75	0.46	0.10	0.36
GGM007	F:GCTGTGCTGACTGGCACTT; R:CAAACCAACATTTCATCAAAAA	$(CAA)_2$n33$(GAACT)_2$	55	5	0.13	0.78	0.74	0.77	0.34	0.86
GGM045	F:TCTCATGGGTCTCTGGGTTC ; R:TGTGCAGAAAGGCTGTTGAG	$(GT)_{21}$	56	5	0.45	0.76	0.72	0.26	0.20	0.44
GGM018	F:GGTCGAGCTCGGCTTTCT; R:CTGGGCCTTTTACTGGACAA	$(GGA)_5$	55	6	0.35	0.75	0.71	0.28	0.36	0.64
GGM001	F:TGTTGTGCACTTTGTTTCCAA; R:TCTGGGTCCTTTCTGATGCT	$(GA)_8$	55	5	0.08	0.75	0.71	0.85	0.25	0.77
GGM013	F:CCTGGGCTTGAACCAATTAAA; R:CCCTTAGCTGAAGCACCTTTT	$(AG)_6$	56	5	0.93	0.72	0.66	-0.42	0.08	0.03
GGM023	F:GCTTTGTCAAGCCAGCCTAA; R:CCTGGTCTTCAGTGGATGGT	$(TA)_{14}(GT)_5$	56	5	0.33	0.72	0.66	0.63	0.15	0.30
GGM006	F:CCCTCATGCTCCGTTAGTGT; R:TCTTGGATGACATAGGGGTGA	$(AT)_3$n20$(AT)_3$	55	5	0.11	0.71	0.66	0.74	0.47	0.80
GGM002	F:CACTTTGTCCTTGCCATTGA; R:CTCAACACCGTGGACTCTCA	$(GT)_{17}$	54	5	0.33	0.71	0.66	0.28	0.41	0.49
GGM012	F:AAAACAGAAACATTTCAAACTG; R:AAAACAAATCAGCGCACCAT	$(AAC)_2$n10 $(GA)_5$	55	4	0.53	0.71	0.65	0.12	0.12	0.42
GGM10	F:CCTGCAGTCAGGCTATGACA; R:CCCATGCCATTAGGTTTTTG	$(AG)_6$	55	4	0.19	0.70	0.65	0.67	0.09	0.33
GGM08	F:TCTGGACCAGGAACGACTTC; R:GGCCAACACTCTTTCACACC	$(AT)_5$	58	4	0.25	0.69	0.62	0.62	0.07	0.11
GGM043	F:TTTAGCTGGGCGTTAAGTGG; R:CTCGCTCTGAATTGTTTCCA	$(CT)_8$	47	4	0.23	0.66	0.60	0.64	0.01	0.01
GGM011	F:GGTTTGCTGTTGTCTCCATCT; R:TGCGCTGCTACTTTTGAATG	$(GGT)_2$n17 $(AG)_6$	55	3	0.18	0.62	0.54	0.61	0.27	0.49
GGM016	F:CTTCACAGTCAGCGTCAGGA; R: TTGAAGCGCTTTCGGTTACT	$(AG)_6$	55	3	0.47	0.53	0.42	0.08	0.03	0.09
		Average over all loci		**5.7**	**0.39**	**0.74**	**0.70**	**0.37**	**0.17**	**0.43**

Ta - annealing temperature, $\mathbf{N_A}$ - mean number of alleles per locus, $\mathbf{H_e}$ - average expected heterozygosity per locus, $\mathbf{H_o}$ - average observed heterozygosity per locus, **PIC** –polymorphic information content; **Fis, Fst, Rst** –Allele frequency and allele size based correlation.

Table.2 Summary statistics for genetic variation at 20 microsatellite loci used to study the genetic variation across 3 populations of *Garra gotyla*

Population →	GGPG					GGPK					GGPA				
Locus ↓	Ne	Ho	He	F	pHW	Na	Ho	He	F	pHW	Na	Ho	He	F	pHW
GGM001	4	0.00	0.54	1.00	0.000	2	0.00	0.51	1.00	0.000	3	0.25	0.63	1.00	0.000
GGM002	3	0.54	0.42	-0.27	0.313	3	0.17	0.17	-0.05	1.000	4	0.22	0.71	0.69	0.000
GGM006	3	0.13	0.57	0.77	0.000	3	0.14	0.46	0.70	0.000	1	0.00	0.00	0.00	-
GGM007	3	0.21	0.52	0.59	0.001	3	0.15	0.54	0.73	0.000	2	0.00	0.47	1.00	0.000
GGM08	4	0.12	0.68	0.83	0.000	3	0.22	0.48	0.55	0.001	4	0.38	0.70	0.45	0.000
GGM10	4	0.00	0.66	1.00	0.000	4	0.17	0.64	0.73	0.000	3	0.46	0.61	0.25	0.221
GGM011	2	0.00	0.40	1.00	0.000	3	0.32	0.49	0.35	0.001	3	0.22	0.47	0.53	0.000
GGM012	3	0.47	0.53	0.12	0.080	4	0.48	0.71	0.33	0.003	4	0.71	0.64	-0.13	0.300
GGM013	3	0.89	0.63	-0.43	0.016	5	1.00	0.72	-0.40	0.003	5	0.91	0.65	-0.43	0.029
GGM015	7	0.83	0.82	-0.01	0.000	6	0.80	0.82	0.03	0.837	5	0.63	0.72	0.13	0.372
GGM016	3	0.23	0.59	0.61	0.000	2	0.66	0.45	-0.47	0.013	2	0.54	0.51	-0.06	1.000
GGM018	3	0.03	0.24	0.86	0.000	5	0.34	0.54	0.36	0.005	4	0.68	0.69	0.02	0.004
GGM021	5	0.55	0.74	0.27	0.104	6	0.32	0.81	0.61	0.000	6	0.48	0.83	0.43	0.000
GGM023	5	0.21	0.58	0.64	0.000	3	0.35	0.79	0.57	0.000	5	0.18	0.62	0.71	0.072
GGM023.2F	5	0.32	0.68	0.53	0.000	5	0.17	0.47	0.64	0.000	5	0.56	0.77	0.27	0.000
GGM024	6	0.67	0.77	0.13	0.001	7	0.70	0.79	0.11	0.000	5	0.35	0.70	0.51	0.000
GGM027	7	0.85	0.77	-0.10	0.000	7	0.93	0.81	-0.15	0.000	5	0.80	0.69	-0.17	0.065
GGM028	4	0.21	0.60	0.66	0.000	6	0.44	0.71	0.38	0.000	5	0.21	0.78	0.74	0.000
GGM043.1F	4	0.07	0.66	0.89	0.000	3	0.19	0.58	0.68	0.000	4	0.46	0.71	0.36	0.006
GGM045	3	0.41	0.65	0.38	0.000	3	0.59	0.47	-0.27	0.336	4	0.36	0.70	0.49	0.000

Ne = No. of effective alleles; Ho= Observed heterozygosity; He = Expected Heterozygosity; F = fixation index; pHW= p value of HWE

Fig.1 Mode shift graph showing 'L-shaped' distribution and indicating the absence of bottleneck in *Garra gotyla* populations.

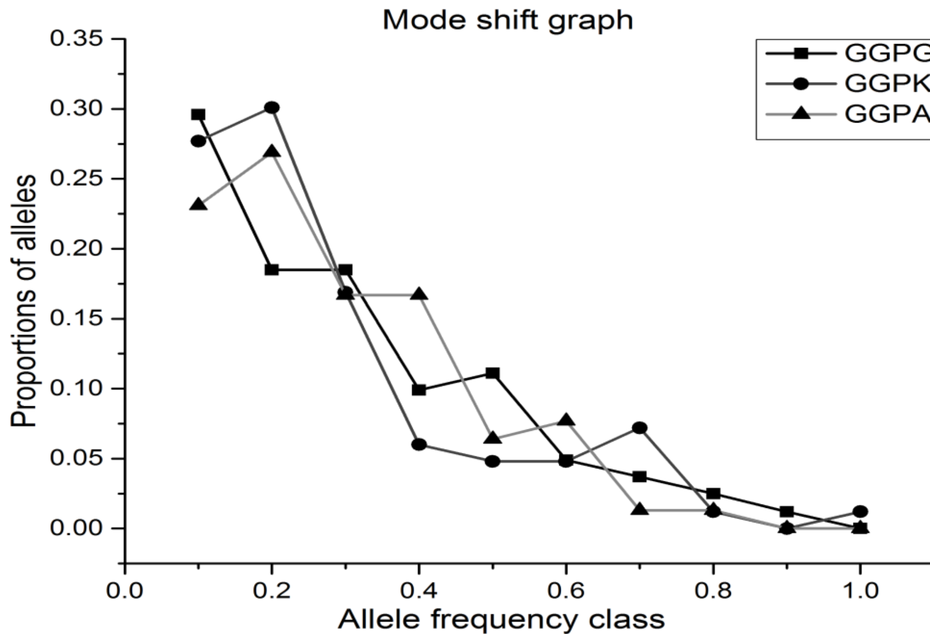
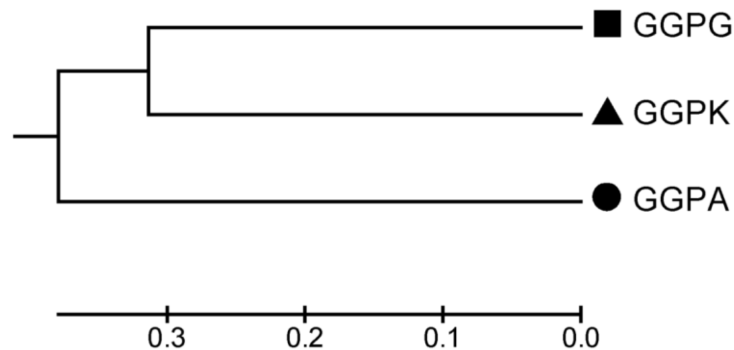


Fig.2 Dendrogram (UPGMA) constructed based on Nei's genetic distance among stocks. Genetic distance was computed using microsatellite genotype data in ARLEQUIN v.3.5.



All the populations of *Garra gotyla* showed nearly 'L-shaped curve' hence the populations are not in genetic bottleneck as observed in Wilcoxon test under BOTTLENECK v.1.2.02 (Figure 1). Garza and Williamson (2001) reported that number of alleles may decrease up to 2-3 alleles/locus in case of chronic bottleneck that last multiple generations. In the present study, the mean number of allele was 5.7 with a range of 3 to 11 across all the populations. This finding indicates significant allelic variability across the different populations hence the chances are less likely that stocks are under bottleneck.

In conclusion all the microsatellite markers were polymorphic and successfully amplified in three different populations of *Garra gotyla*. There is a substantial genetic variation among the three populations. Though the present stocks are having

considerable amount of genetic variability, but there is need of formulating management strategies so that genetic vigour can be increased using many pairings and equalization of family size to maximize effective population size and thereby the effects of drift. There is also a possibility for better genetic improvement of the existing populations by using individuals from different populations.

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