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Assessment of genetic diversity through SSR markers in *Labeo rohita* and *Labeo gonius* from two reservoirs of Uttarakhand

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Abstract
The present study deals with the assessment of genetic diversity using microsatellite marker in the fish *Labeo gonius* from Nanak Sagar and Dhaura reservoirs of Uttarakhand having different morpho-edhaphic features and self- recruiting populations of this fish. These reservoirs are distantly located and distinctly separated without any connection having negligible possibility of gene exchange with each other. Total 12 microsatellite primers were selected and by using software Primer-BLAST and Primer-3 and all the designed microsatellite primers were screened in all 100 DNA samples of fish collected from both the reservoirs. Screened 12 microsatellite loci were successfully amplified. After PCR amplification of microsatellite loci and performing native PAGE using amplified DNA samples as above, POP GENE Version 1.32 was used to calculate Nei's observed heterozygosity, expected heterozygosity, Nei's genetic diversity, Fixation index (Fis) and Shannon's information index (SI) and genetic variability indices viz. Gene flow(Nm), the coefficients of genetic differentiation (Fst & Gst) and Nei's genetic distance. Genetic diversity and level of heterozygosity in *L. gonius* were found to be maximum (Hi=0.6770 and Ho=0.5046)) from Nanak Sagar reservoir followed by genetic diversity in *L.gonius* from Dhaura reservoir (Hi=0.5732andHo=0.4901) and genetic diversity in *L. rohita* (Hi=0.561 and Ho=0.4894) from Nanak Sagar reservoir. *L.rohita* from Dhaura reservoir shows minimum genetic diversity value (Hi=0.4894and Ho=0.4226). Genetic diversity values are lower in Dhaura reservoir which might be due to its reduced effective population size associated with the small size of reservoir, extensive fishing pressure and drying up of major portion in summer season. These reservoirs are distantly located and have no connection with each other indicating negligible gene exchange with each other which is responsible for weak sub structuring of *L.rohita and L.gonius* stocks. Overall Gst value (0.1601) recorded for *L. gonius* suggested the possibility of less gene exchange among the two stocks and indicated that 16.01% variation was attributable to interstock divergence, while 83.99% to individual differences within the stocks.

Keywords: Genetic characterization, heterozygosity microsatellites, primers, *Labeo gonius*

Introduction
Fishes form a highly successful group of animals comprising more than 30,700 species inhabiting all seas, rivers, lakes, canals, dams, brackish water, estuaries and all places wherever is water. Almost 25% of global vertebrate diversity is accounted for by fish and India is the home for more than 11.72% of global fish biodiversity. Economically, fishes constitute a very important group of animals as a rich source of protein, liver oil and omega fatty acids. Fisheries sector plays a very important role in social and economic development by providing employment and nutritional security for the greater part of population of the country. Indian fisheries constitute about 5.17% of agriculture GDP and 0.9 % of the net GDP with total fish production of 10.79 million metric tonnes of which of 3.58 million metric tonnes from marine fisheries and 7.21 million metric tonnes from inland sector, out of which 6.489 million metric tonnes is from aquaculture (DAHDF, 2017). Use of molecular markers, especially microsatellites, has been recognized to have great potential in revolutionizing the genetic management of fishery stocks for controlling the level of inbreeding and loss of genetic diversity through its ability to detect genetic uniqueness of individuals, populations or species (Lakra *et al*., 2007) [10]. Microsatellites or simple sequence repeats (SSRs) are short tandem repeat motifs (1-6 bases) with high level of allelic polymorphism and co-dominant inheritance.The fish population that are selected in the present investigation for applying molecular genetic marker (Microsatellites) were obtained

from two majors reservoirs of Uttarakhand viz. Dhaura (1200 ha) and Nanak Sagar (4662 ha). These reservoirs are mainly used for irrigation purpose and fisheries activity is secondary. The fish Fauna of these reservoirs mainly comprises of various species of catfishes, major carps and minor carps. *L.gonius*, a minor carp contributing maximum in catches of these reservoirs followed by *L.rohita* was selected in the present study. *L. gonius* contributes maximum in commercial catches from tarai region reservoirs of Uttarakhand and enjoys a good market value @Rs.80-100/kg as a food fish in the state. *L.gonius* catch may decline in future due to overexploitation of natural resources, habitat destruction, loss of feeding and breeding grounds due to siltation, gratuitous destruction of spawners, destructive fishing practices, aquatic pollution etc. In view of the above facts and reasons the present study was taken up for getting information on stock structure of *L.gonius* in above reservoirs which will be useful for long term sustainable production of this species in them and this species has a larger scope as an alternative candidate species in carp aquaculture system.In 2003, the production of *L. rohita* ranked 7th among the carps cultured in Asia (FAO, 2005) [7]. Owing to its high market value, excellent meat quality and flavour, *L. rohita* is being targeted for genetic improvement in order to increase its production efficiency. This necessitates the proper evaluation of genetic stock that will greatly assist in creating base population which can help in elucidating the genetic differences among wild populations, assess genetic variation within cultured stocks and discover the genetic impacts of aquaculture on wild populations.

Materials and Methods

Collection of samples and isolation of genomic DNA

Kidney tissue samples was collected from each individual (n=50) of *L. rohita* and *L.gonius* from Nanak Sagar and Dhaura reservoir and stored at -86^0 c in deep freezer for further analysis. DNA was isolated from the dissected kidney tissue through DNA isolation kit purchased (BANGLORE GENEI).Total twelve cross amplified microsatellite primers was selected and accordingly rearranged by using software Primer-BLAST and Primer-3. To amplify the repeat regions, primers were designed using the web based tool Primer3 (http://primer3.sourceforge.net/)(Rozen & Skaletsky 2000) [15] to amplify a PCR product of approximately 120-150 bp, with an optimum Ta of 55 °C and a minimum GC content of 40-70%. All the microsatellite primers were screened in 50 DNA samples of fishes from both the reservoirs.

Amplification of microsatellite loci and analysis of microsatellite data

All the microsatellite primers were screened in each 50 DNA samples of fishes collected from both the reservoirs. A total of 12 microsatellite loci (Table-1) were successfully amplified and were produced clear polymorphic bands from both reservoir populations of *L. rohita and L.gonius.* PCR amplification of microsatellite loci were performed in a 25 µl reaction mixture, which included 1X PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl), 0.2 mM of each dNTP, 2.0 mM of MgCl2, 5 p mol of each primer, 1.5 U Taq DNA polymerase and 25–50 ng of template DNA. Initial denaturation at 94 degree Celsius for 3 minutes followed by 30 cycles of 94 degree Celsius for 30 seconds, locus specific annealing temperatures for 60 seconds and 72 degree Celsius for 90 seconds and a final elongation of 1 cycle at 72 °C for 8 min and stored at 4 °C. Amplified products were mixed with 2(µl) of gel loading dye and then separated on 6% denaturing poly acrylamide gel with 1x TBE on PAGE Gel along with standard marker Φ X 174/ Hinf I marker at constant power supply of 25 volts for 2 hrs. Gel was then stained with Etbr. Polymorphic information content (PIC) of individual primer was estimated using the formula: PIC = 1- 1/nc Where Pij is the frequency of jth allele. After performing native PAGE using amplified 50 DNA samples each from both the populations, POP GENE Version 3.4 (Raymond and Rousset, 1998) was used to calculate Nei's observed heterozygosity (Ho), expected heterozygosity (He) and Fixation index (Fis). Nei's average expected gene diversity (Hi) was calculated from the banding pattern of every primer. Individual genotypes were scored using the Gene Mapper (version 4.0; Applied Biosystems) with a size standard and an internal control for allele calling; each allele was coded according to its size in nucleotide base pairs (bp). A panel that included all of the alleles detected in the 50 individuals was created for each locus. Possible null alleles and genotyping errors caused by stuttering and/or large-allele dropout were tested using MICRO-CHECKER (1000 randomizations) (Van Oosterhout *et al*., 2004). Scoring and human error were estimated by duplicate analyses. The polymorphic information content (PIC) calculated by using the CERVUS version 3.03 (Kalinowski *et al*., 2007) [9].

Table 1: Primer-BLAST microsatellite primers for *L. rohita* and *L. gonius*

Code	Primer Sequence(5'-3')	Annealing Temp	Annealing Time
Lr-01	F-GAAAGCTGCTCGTCCTTGAA R-GAAAGCTGCTCGTCCTTGAA	57 ^{0}C	1min 30 sec
Lr-02	F-GGGTGTGGGAGAGAAAGAGAG R-GGAGTCTGACAAATGCAGCAAG	62 ^{0}C	1min 30 sec
Lr-03	F-TCTCAGTGGGTGTCATTACCTG R-CCCATCAAACCATCTCTCTAGC	52 ^{0}C	1min
Lr-08	F-CTGACACTCTTATCTCGCTGCC R-GACCTGAGCAAACAAACCTCAT	53 ^{0}C	1min 30 sec
Lr-10	F-TCTCTCTTTGTCTTTCCCCTTG R-CACAAGCCACTGTTTAGCTTCA	64 ^{0}C	1min
Lr-11	F-CAAATCTGTGAACATGCAAGC R-CCTAGTCCCACTCTAGTCAGCA	57 ^{0}C	1 min 30 sec
Lr-13	F-AGATAAGACCCTTCTTCCTCGG R-TTTATTAGGGAGCGTCGAGTG	62 ^{0}C	1min 30 sec
Lr-14	F-CTGTTGGTGACTGTAGGGTGAA R-GAGAACTCGGTTTGAACATGC	58 ^{0}C	1min
Lr-15	F-ACAGTAATCTTGTGTCTGTCTCTCTC R-GTCTAAACGTGTCTGAGCTGTG	55 ^{0}C	1 min 30 sec

Lr-16	F-TGAATGTTTCCAGTCACCACAT R-GTAATGCAGCGGAGAATAAACC	57 ^{0}C	1min
Lr-17	F-ACAATTCCTGTGTCAACTGTGC R-TACCGTCTCAGTCTCTTTTCGG	57 ^{0}C	1min 30 sec
Lr-20	F-ATAGTCGAAATTGGTCCTCTGC R- CAATACCATGACTGAAGTGCC	55 ^{0}C	1min 30 sec

Results

Genetic divergence in *L. gonius* from Nanak Sagar and Dhaura reservoirs

Observations related with genetic divergence in *L. gonius* stocks from both reservoirs are presented in Table-2. Genetic differentiation (P-value) for *L. gonius* across all loci among different population pairs in Nanak Sagar and Dhaura reservoirs was found to be 0.0184. Values of Gene flow (Nm) and Nei's genetic distance among reservoir populations of *L. gonius* were found to be 1.312 and 0.2134 respectively. Values of coefficients of genetic differentiation (Fst & Gst) observed were 0.093 and 0.1632 respectively for overall population of *L. gonius*. Total genetic diversity in overall population (Ht) and within sample genetic diversity (Hs) was 0.5274 and 0.4430, respectively. The observed (na) and effective number of alleles (ne) in *L. gonius* from Nanak Sagar reservoir were found to be 4.9805 and 4.7762 respectively and for Dhaura reservoir these values were 4.8126 and 4.5531 respectively.

Table 2: Genetic variability indices in *L. gonius* from Nanak Sagar and Dhaura reservoirs

Parameters	Values	
Coefficient of genetic differentiation (Fst)	0.093	
Estimation of Gene flow (Nm)	1.312	
Total genetic diversity in population (Ht)	0.5274	
Within sample genetic diversity (Hs)	0.4430	
Coefficient of genetic differentiation (Gst)	0.1601	
Nei's genetic distance P-Value	0.2134 0.0184*	
	Nanak Sagar Reservoir	Dhaura Reservoir
Observed number of alleles (na)	4.9805	4.8126
Effective number of alleles (ne)	4.7762	4.5531

*Significant at $P<0.05$

Genetic divergence in *L. rohita* between Nanak Sagar and Dhaura reservoirs

Observations related with genetic divergence in *L. rohita* stocks from both reservoirs are presented in Table-3. Genetic differentiation (P-value) for *L. rohita* across all loci among different population pairs in Nanak Sagar and Dhaura reservoirs was found to be 0.0177. Values of Gene flow (Nm) and Nei's genetic distance among reservoir populations of *L. rohita* were found to be 1.427 and 0.2096 respectively. Values of coefficients of genetic differentiation (Fst & Gst) observed were 0.081 and 0.1490 respectively for overall population of *L. rohita*. Total genetic diversity in overall population (Ht) and within sample genetic diversity (Hs) was 0.4996 and 0.4256, respectively. The observed (na) and effective number of alleles (ne) in *L. rohita* from Nanak Sagar reservoir were found to be 4.8974 and 4.7156 respectively and for Dhaura reservoir these values were 4.7952 and 4.4886 respectively.

Table 3: Genetic variability indices in *L. rohita* from Nanak Sagar and Dhaura reservoirs

Parameters	Values	
Coefficient of genetic differentiation (Fst)	0.079	
Estimation of Gene flow (Nm)	1.427	
Total genetic diversity in population (Ht)	0.4996	
Within sample genetic diversity (Hs)	0.4256	
Coefficient of genetic differentiation (Gst)	0.1490	
Nei's genetic distance P-Value	0.2096 0.0177*	
	Nanak Sagar Reservoir	Dhaura Reservoir
Observed number of alleles (na)	4.8974	4.7952
Effective number of alleles (ne)	4.7156	4.4886

*Significant at $P<0.05$

Pattern of genetic variation both within and between the populations reservoirs

Genetic diversity as well as level of heterozygosity of *L. gonius* in the present study was found to be maximum (Hi=0.6770and Ho=0.5046)) from Nanak Sagar reservoir followed by genetic diversity in *L gonius* from Dhaura reservoir (Hi=0.5732andHo=0.4901) followed by genetic diversity in *L. rohita* (Hi=0.561 and Ho=0.4894) from Nanak sagar reservoir and *L.rohita* from Dhaura reservoir shows minimum genetic diversity value (Hi=0.4894and Ho=0.4226) mentioned in Table 4. Genetic diversity values are lower in Dhaura reservoir which might be due to its small population size associated with the small size of reservoir, extensive fishing pressure and drying up of major portion in summer. This reservoir is also subjected to siltation because of deforestation in catchment area as well as conversion of water area into marshy land cause habitat destruction which ultimately affect the population size of fish and subsequently their genetic polymorphism. Among the two reservoirs, Nanak sagar is the largest one with great volume of water.

Although, this reservoir was also subjected to drying of water in summer season from last few years it retains water during monsoon. Therefore, it does not affect population size. Random mating takes place in the reservoir resulted in heterozygous population.

Table 4: Genetic diversity indices of *L. rohita* and *L. gonius* in two reservoirs

Parameter	*L. rohita L gonius*	
	Both Reservoir	**Both Reservoir**
Avg. H_o	0.456	0.495
Avg. H_e	0.493	0.517
Avg. H_i	0.582	0.625
Avg. PIC	0.59	0.65
Avg. n_a	4.85	4.895
Avg. n_e	4.60	4.66
Avg. no. of alleles/locus	5.79	6.9

Heterozygosity is an important index for assessing population variation at the genetic level. According to H_o is easily influenced by sample sizes, while H_e can better reflect genetic diversity. The genetic diversity value 0.630 based on observed and expected heterozygosities (0.4894 and 0.5151) from Nanak Sagar and 0.5340 based on observed and expected heterozygosities (0.4226 and 0.4716) from Dhaura reservoir in *L. rohita* indicated that its stock in Nanak Sagar reservoir exhibited better genetic diversity than Dhaura stock as these reservoirs are distantly located and distinctly separated without any connection hence having negligible possibility of gene exchange with each other and this also might be responsible for varied sub- structuring of the stocks of *L. rohita.* The values of Nei's genetic diversity (0.630 and 0.534) in *L. rohita* through microsatellite marker are found to be higher than that to the Nei's genetic diversity values (0.3980 and 0.2243) in *L. gonius* through RAPD marker from Nanak Sagar and Dhaura reservoir (Tewari *et al*., 2013 b) [19]. The present observations are well correlated with the findings of Sahoo *et al.* (2014) where high level of genetic diversity in the populations of peninsular *L. rohita* was detected by eleven microsatellite loci as evident from high level of heterozygosity (0.500-0.847). Nei's genetic diversity range (0.478 to 0.749) in *L. rohita* (over all loci) was found to be in similar range (0.679 to 0.874) reported in *T. tambroides* through microsatellite marker by Overall Gst value (0.1490) calculated for *L. rohita* suggested the possibility of less gene exchange among the two stocks and indicated that 14.90% variation was attributable to interstock divergence, while 85.10% to individual differences within the stocks. The moderate level genetic differentiation on the basis of calculated value of coefficient of genetic differentiation across all loci (Fst=0.079) was present due to different population size as Dhaura reservoir have smaller population size than Nanak Sagar reservoir. This pattern of variation corresponds to that obtained in other Indian fresh water fishes through microsatellites reported by Fst value in present findings (Fst=0.079) corresponds to that obtained in other Indian freshwater fishes by in *L. dero* (Fst=0.019) and by Gopalakrishnan *et al*. (2009) [8] in *L.dussumieri* (Fst=0.041) and by Singh *et al*. (2012) [18] in *L. calbasu* and Sahoo *et al*. (2014) in *L. rohita* reported low Fst value of 0.035 and 0.032 respectively whereas Appleyard and Mather, (2002) [1] reported high F_{ST} values (0.501 to 0.598) in two species of *Oreochromis* indicating there was little evidence of introgression between these species. The values of Nei's genetic diversity (0.6770 and 0.5732) in *L. gonius* through microsatellite markers were found to be higher than values (0.3980 and 0.2243) through RAPD marker observed by (Tewari *et al*., 2013 b) [19] in *L. gonius* from Nanak Sagar and Dhaura reservoirs. Genetic diversity values based on co-dominant microsatellite markers are more accurate and preferable as compared to allozyme and dominant RAPD markers as these markers are highly polymorphic, capable of detecting small genetic differences (even single nucleotide base change variation) which is essential in studies on the genetic variability of the populations (Balloux and Lugonae, 2002) [4]. The mean values of observed heterozygosity in *L. gonius* from both the reservoirs were found to be comparable with the mean value of observed heterozygosity (0.46) reported for some other freshwater fishes (DeWoody and Avise, 2000) [6]. The observed heterozygosity range (over all loci) in *L. gonius* are found to be comparable with the observed heterozygosity range (0.0000 to 0.9000) reported in *Tor putitora* using seven microsatellites loci developed from *Catla catla* and *Barbus barbus* by Mohindra *et al.* (2004) [13]. Same observations (heterozygosity range- 0.10–1.00 and 0.500 to 0.870) were reported for other cyprinid fishes like silver carp and bighead carp by Tong *et al*. (2002) [21]. However, the observed heterozygosity range in *L. gonius* was found to be higher than in *Cirrhinus mrigala* (0.247 to 0.333) reported from different rivers by Lal *et al.* (2011) [5].The mean values of observed and expected heterozygosity in *L. gonius* from Nanak Sagar population using microsatellite marker was found to be comparable with the mean values (0.501 and 0.539) of observed and expected heterozygosity using allozymes marker in *L. gonius* from Nanak Sagar population (Tewari *et al*., 2013a) [20] .Small differences between observed values of population genetic diversity (Hs=0.4430) and total genetic diversity (Ht=0.5274) indicated moderate genetic differentiation among *L. gonius* stocks. Nei's genetic diversity results in *L. gonius* in present findings are well correlated with the observations made by Singh *et al*. (2015) [17] where five microsatellite loci was used to study the genetic diversity and characterization of different strains of common carp *C. carpio* L. and reported mean observed heterozygosity ranged (0.45 and 0.62) while expected heterozygosity ranged (0.32–0.68).On the basis of calculated (na=4.9805 and 4.8126) number of alleles and effective (ne=4.7762 and 4.5531) number of alleles in Nanak Sagar and Dhaura reservoirs it is indicated that significant genetic variation is there within stocks of *L. gonius* and *L.rohita* in both reservoirs.

Conclusion

On the basis of the observed values of Average Nei's Genetic Diversity, Observed and Expected heterozygosities, Fis values and Shannon's information index and cluster analysis obtained by applying microsatellite marker technique the present study indicated that populations of both *L.gonius* and *L. rohita* from Nanak Sagar reservoir represented better germplasm status as compared to Dhaura reservoir. Wide geographical location, different hydro-biological conditions, different habitat and no connectivity between these two water resources and low or absence of gene flow between the populations may be the possible reasons to make reservoir and populations differentiated. Despite the popularity of microsatellites in the study of population genetics, their development requires substantial time, financial as well as technical resources. Based on the present study it may be concluded that for sustaining diversity in hatcheries, regular and reliable germplasm exchange of hatchery broodstock from different sources is necessary and fish stock of

reservoirs with known genetic divergence can serve as an important source.

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