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Genetic diversity analysis of common carp (*Cyprinus carpio* var. *Communis*) and *Labeo rohita* (hamilton, 1822) collected from hatchery by using microsatellite markers

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Abstract
The present study deals with genetic diversity analysis of *Cyprinus carpio* var. *communis* and *Labeo rohita* (Hamilton, 1822) collected from hatchery stock through microsatellite marker. Total 20 microsatellite primers were designed by using software Primer-BLAST and Primer-3. A total of 12 microsatellite loci were successfully amplified. After performing native PAGE using amplified 50 DNA samples each, POP GENE Version 1.32 was used to calculate microsatellite variation. The average expected Nei's genetic diversity ranged from 0.328 to 0.529 with mean value of 0.458 for *Labeo rohita* across all loci from hatchery whereas the average expected gene diversity ranged from 0.392 to 0.537 with mean value of 0.483 for *Cyprinus carpio* var. *communis* across all loci from hatchery. The observed and expected heterozygosity ranged from 0.2237 to 0.3326 and 0.2786 to 0.3763 respectively for *Labeo rohita* from hatchery. The mean value of observed heterozygosity was 0.2864 and that of expected heterozygosity was 0.3238. Mean Fis value were found to be 0.193 at all loci in hatchery whereas for common carp the observed and expected heterozygosity ranged from 0.2659 to 0.3910 and 0.3145 to 0.4129. The mean value of observed heterozygosity was 0.3350 and that of expected heterozygosity was 0.3731. Mean Fis values for common carp were found to be 0.184 at all loci in hatchery. Mean values for Shannon's information index for all microsatellite loci were 1.1091 for rohu and 1.1320 for *Cyprinus carpio* from hatchery stock. Slightly more level of observed heterozygosity in *Cyprinus carpio* var. *communis* than *Labeo rohita* from hatchery might be due to presence of more differentiated stocks. Lesser value of observed heterozygosity in *Labeo rohita* from hatchery than *Cyprinus carpio* var. *communis* might be possibly due increase in incidents of inbreeding in successive generations owing to lack of regular germplasm exchange of appropriate genetic diversity. The microsatellite analysis showed that *Cyprinus carpio* var. *communis* of hatchery is more genetically diversed and genetically differentiated as compared to *Labeo rohita.*

Keywords: Genetic Diversity, Microsatellites, Primers, *Labeo rohita*, *Cyprinus carpio*

Introduction
The inter-specific genetic divergence established through species specific diagnostic molecular markers provides precise knowledge on phylogenetic relationships and also resolve taxonomic ambiguities [1, 2, 3, 4]. With the rapid expansion of aquaculture, the knowledge of gene pool of individual candidate species has become a necessity during breeding program. Because, this can help to elucidate the genetic differences among wild populations, assess genetic variation within captive stocks, and determine the genetic impacts of aquaculture on wild populations, thereby promoting sustainable aquaculture. Indian Aquaculture is fastly growing sector. It has grown over six and half fold in the last two decades. Freshwater aquaculture contributes over 73.8 million tones of the total aquaculture production [5]. *Labeo rohita*, popularly known as rohu is a widely cultured species in the whole Indian subcontinent. Common carp (*Cyprinus carpio*) is one of the extensively cultured and highly domesticated aquaculture fish species in the world. Common carp, an exotic fish species brought from Bangkok (Thailand) in 1956. It has become an integral part of fish culture system in India. It belongs to Cyprinidae, the largest family among freshwater teleosts. It has a farming history of several hundred years in Europe and about 4,000 years in China. In India, Common carp is an extremely important aquaculture species especially in north eastern and southern parts of the country. It has significant contribution in inland fish production.

To keep the production cost to minimum, hatchery owners in India maintain limited number of broods to minimize effective breeding numbers [6]. Knowledge of the genetic diversity of this species is important to support management and conservation programs which will subsequently help in sustainable production of this species. *Labeo rohita* is extensively cultivated as a part of polyculture system and major source of its seed for aquaculture is from hatchery breeding; moreover inbreeding is reported in Indian hatcheries [7]. In this species, quantification of genetic variability of wild stocks is essential for supporting programmes on management of fishery stocks, domestication and genetic up gradation. DNA markers, mostly microsatellite markers are excellent tool to evaluate genetic variation of populations. Owing to their advantages such as high level of polymorphism, co dominance, even distribution in the genome and easy analysis using PCR, microsatellites are the markers of choice for resolving genetic diversity and phylogenetic relationship in a wide range of taxonomic group [8]. Use of DNA markers in population genetic studies of rohu is limited to allozyme [9] and mtDNA [10]. Microsatellite markers have been developed for selected Indian fish species such as rohu [11, 12], catla [13], chitala [14] and mrigala [15]. Documentation of natural genetic variability of *L. rohita* is necessary not only for stock based management and conservation but also for genetic improvement programmes. Knowledge of genetic diversity in Indian major carps is considered significant for planning conservation of wild populations [16, 17] which are facing multiple threats and consequently decline of populations. The aim of the present study was to assess genetic variation among hatchery stock populations of *L. rohita and Cyprinus carpio* using microsatellite DNA markers.

Materials and Methods

Collection of samples and isolation of genomic DNA

Kidney tissue samples were collected from each individual (n=50) of *L. rohita and Cyprinus carpio* var. *communis* from Pantnagar fish hatchery located in district Udham Singh Nagar of Uttarakhand state and stored at -80^0 c in deep freezer for further analysis. DNA was isolated from the dissected kidney tissue through DNA isolation kit purchased (BANGLORE GENEI). Total twenty microsatellite primers were designed 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/)(18) 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 each 50 DNA samples of both fishes from captivity.

Amplification of microsatellite loci and analysis of microsatellite data

All the microsatellite primers were screened in each 50 DNA samples of both *L. rohita and Cyprinus carpio* var. *communis* collected from hatchery. A total of 12 microsatellite loci were successfully amplified and were produced clear and polymorphic bands from hatchery populations of *L. rohita* and *Cyprinus carpio* var. *communis*. 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. Polymorphic information content (PIC) of individual primer was estimated using the formula: PIC = 1- 1/n $\sum_{ij}^{n} Pij$ 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 (19) 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.

Results

Primers amplification results of *Labeo rohita* collected from hatchery

Twelve microsatellite primers were successfully amplified and showed polymorphism (Table-1). Total 52 numbers of alleles scored in hatchery stock. Number of alleles per locus ranges from 3 to 5 with mean value of 4.33 per locus. A total of 4 SSR loci were scored by the primer PL-01. The product size ranged from 0.11 Kb to 0.24 Kb and the PIC value and average expected gene diversity of the primer were 0.52 and 0.473 respectively. A total number of 3 SSR loci were scored by the primer PL-02 and all the loci were polymorphic (Table-3 & 4). The product size ranged from 0.13 Kb to 0.31 Kb and the PIC value and average expected gene diversity of the primer were 0.48 and 0.528 respectively. The totals of 5 SSR loci were scored for the primer PL-03 with product size ranged from 0.20 to 0.43 Kb. and the PIC value and average expected gene diversity of the primer were 0.56 and 0.474 respectively. The total of 5 SSR loci was scored for the primer PL-08 (Table-3 & 4). The product size ranged from 0.27 to 0.36 Kb and the average expected gene diversity and PIC value of the primer were 0.56 and 0.369 respectively. Total numbers of 4 SSR loci were scored by the primer PL-10. The product size ranged from 0.28 Kb to 0.53 Kb and the average expected gene diversity and PIC value of the primer were 0.52 and 0.418 respectively (Table- 3& 4). 5 SSR loci were scored by the primer PL-11which and the product size was 0.30-0.44 Kb and the expected genetic diversity and PIC value of the primer 0.56 and 0.497 respectively (Table-3&4). 4 SSR loci with product size ranged 0.29 Kb to 0.47 Kb was scored for the primer PL-13. The average expected gene diversity and PIC value were 0.52 and 0.529 respectively. 5 SSR loci were scored by the primer PL-14 and the average expected gene diversity and PIC value of the primer were 0.54 and 0.452 respectively and product size ranged from 0.16 to 0.24 kb (Table-3 & 4). 5 SSR loci were scored by the primer PL-15 and the average expected gene diversity and PIC value of the primer were 0.56 and 0.511 respectively and product size ranged from 0.19 to 0.43kb (Table-3 & 4). 3 SSR loci were scored by the primer PL-16 and the average expected gene diversity and PIC value of the primer were 0.328 and 0.48 respectively. Product size ranged from 0.15 to 0.40 kb. 4 SSR loci were scored by the primer PL-17 and the average expected gene diversity and PIC value of the primer were 0.52 and 0.439 respectively and product size ranged from 0.18 to 0.30 kb (Table-3 & 4). 5 SSR loci were scored by the primer PL-20 and the average expected gene diversity

and PIC value of the primer were 0.56 and 0.485 respectively and product size ranged from 0.21 to 0.34 kb (Table-3 & 4).

Primers amplification results of *Cyprinus carpio* var. *communis* collected from hatchery

In *Cyprinus carpio* twelve microsatellite primers were successfully amplified and showed polymorphism (Table-1). Total 60 numbers of alleles scored and Number of alleles per locus ranges from 3 to 7 with mean value of 5 per locus. A total of 5 SSR loci were scored by the primer PL-01. The product size ranged from 0.13Kb to 0.27 Kb and the PIC value and average expected gene diversity of the primer were 0.54 and 0.481 respectively (Table-2 &5). A total number of 3 SSR loci were scored by the primer PL-02. The product size ranged from 0.12 Kb to 0.32 Kb and the PIC value and average expected gene diversity of the primer were 0.48 and 0.497 (Table-2 & 5) respectively. Six SSR polymorphic loci were scored for the primer PL-03 with product size ranged from 0.19-0.32 Kb and the PIC value and average expected gene diversity of the primer were 0.55 and 0.483 respectively. The total of 5 SSR polymorphic loci was scored for the primer PL-08 The product size ranged from 0.20 Kb to 0.43 Kb and the average expected gene diversity and PIC value of the primer were 0.56 and 0.421 (Table-2 & 5) respectively. Total numbers of 5 SSR loci were scored by the primer PL-10. The product size ranged from 0.21 Kb to 0.49 Kb and the average expected gene diversity and PIC value of the primer were 0.56 and 0.464 respectively (Table-2 & 5). 6 SSR loci were scored by the primer PL-11 and the product size was 0.20-0.34 Kb. PIC value and the expected genetic diversity was 0.60 and 0.521 respectively. 7 SSR loci with product size ranged 0.25 Kb to 0.46 Kb was scored for the primer PL-13. The average expected gene diversity and PIC value were 0.62 and 0.537(Table-2 & 5) respectively. 6 SSR loci were scored by the primer PL-14 and the average expected gene diversity and PIC value of the primer were 0.60 and 0.494 respectively and product size ranged from 0.14 to 0.30 kb (Table-2 & 5). 4 polymorphic SSR loci were scored by the primer PL-15 and the average expected gene diversity and PIC value of the primer were 0.54 and 0.532 respectively and product size ranged from 0.18 to 0.49 kb (Table-2 & 5). 4 SSR loci were scored by the primer PL-16 and the average expected gene diversity and PIC value of the primer were 0.54 and 0.392 respectively. Product size ranged from 0.20 to 0.46 kb. 3 SSR loci were scored by the primer PL-17 and the average expected gene diversity and PIC value of the primer were 0.48 and 0.487 respectively and product size ranged from 0.16 to 0.34 kb. 6 SSR loci were scored by the primer PL-20 and the average expected gene diversity and PIC value of the primer were 0.60 and 0.498 respectively and product size ranged from 0.19 to 0.39 kb (Table-2 & 5)

Microsatellite Variation and Gene diversity analysis

After performing native PAGE using amplified 50 DNA samples as above, POP GENE Version 1.32 was used to calculate Nei's observed heterozygosity, expected heterozygosity, Nei's genetic diversity and Fixation index (Fis). Average expected gene diversity was calculated from the banding pattern of every primer. The average expected Nei's genetic diversity ranged from 0.328 to 0.529 with mean value of 0.458 for *Labeo rohita* across all loci from hatchery whereas the average expected gene diversity ranged from 0.392 to 0.537 with mean value of 0.484 for *Cyprinus carpio* var. *communis* across all loci from hatchery. The observed and expected heterozygosity ranged from 0.2237 to 0.3326 and 0.2786 to 0.3763 respectively for *Labeo rohita* from hatchery (Table 4 & 5). The mean value of observed heterozygosity was 0.2864 and that of expected heterozygosity was 0.3238. Mean Fis value for rohu were found to be 0.193 at all loci in hatchery Mean Fis values for common carp were found to be 0.184 at all loci in hatchery. The observed and expected heterozygosity ranged from 0.2659 to 0.3910 and 0.3145 to 0.4129 respectively for *Cyprinus carpio* var. *communis* with mean value of observed heterozygosity was 0.3350 and expected heterozygosity was 0.3731. Mean values for shannon's information index for all microsatellite loci were 1.1091 for *Labeo rohita* and 1.1320 for *Cyprinus carpio* population (Table 4 & 5).

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

Locus	Primer Sequence(5'-3')	Annealing Temp	Annealing Time
Lr-01	F-GAAAGCTGCTCGTCCTTGAA R-GAAAGCTGCTCGTCCTTGAA	53 ^{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

Table 2: Screened primer amplification results of *Cyprinus carpio* var. *communis* collected from hatchery

Locus	Amplified Product (Kb)	Number of alleles	(PIC)
PL- 01	0.13-0.27	5	0.54
PL-02	0.12-0.32	3	0.48
PL-03	0.19-0.32	6	0.55
PL-08	0.20-0.43	5	0.56
PL-10	0.21-0.49	5	0.56
PL-11	0.20-0.34	6	0.60
PL-13	0.25-0.46	7	0.62
PL-14	0.14-0.30	6	0.60
PL-15	0.18-0.49	4	0.54
PL-16	0.20-0.46	4	0.54
PL-17	0.16-0.34	3	0.48
PL-20	0.19-0.39	6	0.60

Table 3: Screened primer amplification results of *Labeo rohita* collected from hatchery stock

Locus	Amplified Product (Kb)	Number of alleles	PIC
PL-01	0.11-0.24	4	0.52
PL-02	0.13-0.31	3	0.48
PL-03	0.20-0.33	5	0.56
PL-08	0.27-0.36	5	0.56
PL-10	0.28-0.53	4	0.52
PL-11	0.30-0.44	5	0.56
PL-13	0.29-0.47	4	0.52
PL-14	0.16-0.24	5	0.54
PL-15	0.19-0.43	5	0.56
PL-16	0.15-0.40	3	0.48
PL-17	0.18-0.30	4	0.52
PL-20	0.21-0.34	5	0.56

Table 4: Genetic Diversity of *L. rohita* from hatchery based through Microsatellite markers.

Locus	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Nei's genetic Diversity (Hi)	Shanon's Information Index	Fixation Index Fis
PL-01	0.2682	0.2885	0.473	1.1522	0.162
PL-02	0.2981	0.3042	0.528	1.1121	0.208
PL-03	0.3326	0.3763	0.474	1.0972	0.289
PL-08	0.2549	0.3119	0.369	1.1020	0.291
PL-10	0.2237	0.2786	0.418	1.0124	0.259
PL-11	0.2646	0.3127	0.497	1.2149	0.188
PL-13	0.3015	0.3269	0.529	1.0975	0.154
PL-14	0.2988	0.3420	0.452	1.0556	0.179
PL-15	0.3004	0.3119	0.511	1.1061	0.132
PL-16	0.2817	0.3438	0.328	1.2239	0.161
PL-17	0.3114	0.3329	0.439	1.1241	0.141
PL-20	0.3018	0.3569	0.485	1.0118	0.153
Mean	0.2864	0.3238	0.4585	1.1091	0.193

Table 5: Genetic Diversity of *Cyprinus carpio* var. *communis* from hatchery through Microsatellite markers.

Locus	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Nei's genetic Diversity (Hi)	Shanon's Information Index	Fixation Index Fis
PL-01	0.3824	0.4015	0.481	1.1747	0.155
PL-02	0.3910	0.4129	0.497	1.1286	0.197
PL-03	0.3876	0.3986	0.483	1.1273	0.278
PL-08	0.3341	0.3879	0.421	1.1280	0.284
PL-10	0.2659	0.3145	0.464	1.0381	0.252
PL-11	0.3050	0.3652	0.521	1.2347	0.177
PL-13	0.3512	0.3940	0.537	1.0961	0.149
PL-14	0.3149	0.3812	0.494	1.0858	0.169
PL-15	0.3224	0.3654	0.532	1.1257	0.122
PL-16	0.3117	0.3459	0.392	1.2435	0.153
PL-17	0.3329	0.3562	0.487	1.1521	0.133
PL-20	0.3219	0.3542	0.498	1.0496	0.146
Mean	0.3350	0.3731	0.4839	1.1320	0.184

Discussion

In the present study microsatellite marker was employed to reveal genetic variability in *Labeo rohita* and *Cyprinus carpio* var. *communis* collected from captivity. The microsatellite heterozygosity values were high in *Cyprinus carpio* but lower in *Labeo rohita and is supported* by findings of [20] that the effects of inbreeding and genetic drift of hatchery operations contributed to the reduction of genetic diversity of natural stocks of salmonid species. A significant deficiency of heterozygotes was observed in some of the loci in the present study on the basis of H-W comparisons. Null alleles, alleles that are not amplified due to mutation in primer site may contribute to an excess of homozygotes [21]. The presence of null alleles and/or the inability to separate closely sized alleles due to presence of stutter bands in the microsatellites used might lead to reducing measures of heterozygosity. Several evolutionary forces like random genetic drift, migration, mutation and their mutual interactions act on the wild populations and influence the pattern of genetic differentiation [22]. Random genetic drift tends to cause genetic differentiation, after subpopulations are fragmented and gene flow between them is either reduced or absent. Microsatellite loci generally show considerable evolutionary conservation, suggesting that primers developed for any one species may often be useful across a wide range of taxa. However, one drawback of heterologous primers is that

mutations in the flanking sequences, to which PCR primers are designed to anneal, can result in non-amplifying PCR null alleles [23, 24]. Null alleles produce an apparent heterozygote deficiency in a sample due to mis-scoring of heterozygotes as homozygotes. Heterozygote deficiency can also reflect various biological processes such as inbreeding, Wahlund effects and selection [25]. The FST indicates the proportion of genetic variation that could be attributed to the genetic differentiation processes between the co-specifics from two localities [26]. In the present study, q [27] has been used to compute the partitioning of genetic variation. The sample size in the present study was 50 individuals in each population. Therefore, estimates of population differentiation obtained are unlikely to be confounded by small sample sizes. The overall FST for all samples combined was found to be 0.053. Thus, approximately 5.3 % of genetic variation was found to be caused by genetic differentiation in L. rohita, indicating low level of genetic differentiation. This pattern of variation corresponds to that obtained in other Indian freshwater fishes [28, 29] Different physiological and reproduction pattern along with frequency of germplasm exchange might be the factor associated with these two species being somewhat genetically differentiated.

Conclusion

The present study provides useful insights into genetic diversity of rohu and common carp, an important aquaculture species in India. Our data show that rohu samples collected from hatcery show less level of genetic diversity as compared to samples of common carp collected from same hatchery and this might be due to less chances of inbreeding among common carp population which is associated with the fact of presence of diversed common carp germplasm as compared to that of rohu. Despite the popularity of microsatellites in the study of population genetics, their development requires substantial time, financial as well as technical resources. Cross-species amplification is an alternative strategy to extend the utilization of microsatellites across related species. The results of this study would be a valuable piece of information for genetic improvement and conservation of this species**.**

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