Research Article

Genetic Diversity Analysis of *Labeo rohita* (Hamilton, 1822) and Common carp (*Cyprinus carpio* var. *communis*) from Swapan private hatchery located in Dineshpur in Udham Singh Nagar district of Uttrakhand 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 swapan private hatchery in Dineshpur 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 0.430 to 0.493 with mean value of 0.467 for *Labeo rohita* across all loci from hatchery whereas the average expected gene diversity ranged from 0.489 to 0.522 with mean value of 0.505 for *Cyprinus carpio* var. *communis* across all loci from hatchery. The observed and expected heterozygosity ranged from 0.2620 to 0.3798 and 0.2824 to 0.3860 respectively for *Labeo rohita* from hempur hatchery. The mean value of observed heterozygosity was 0.3143 and that of expected heterozygosity was 0.3426. Mean Fis values were found to be 0.187 at all loci for rohu in hatchery and the Mean Fis values were found to be 0.175 at all loci in *Cyprinus carpio* var. *communis*. The observed and expected heterozygosity ranged from 0.2999 to 0.4170 and 0.3288 to 0.4082 respectively for *Cyprinus carpio* var. *communis* with mean value of observed heterozygosity was 0.3586 and expected heterozygosity was 0.3932.

Mean values for Shannon's information index for all microsatellite loci were 1.1205 for *Labeo rohita* and 1.1437 for *Cyprinus carpio* var. *communis* population. Slightly more level of observed heterozygosity in *Cyprinus carpio* 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*

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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] and [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 and 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 swapan private hatchery in Dineshpur 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 kidney tissue using phenol choloroform procedure [18], with minor modifications.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/)[19] 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:

$$\text{PIC} = 1 - 1/n \sum_{i=1}^{n} Pij$$

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 [20], 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 Swapan hatchery

Twelve microsatellite primers were successfully amplified and showed polymorphism (**Table 1**). Total 53 numbers of alleles scored in hatchery stock. Number of alleles per locus ranges from 3 to 6 with mean value of 4.41 per locus. A total of 5 SSR loci were scored by the primer PL-01. The product size ranged from 0.13 Kb to 0.23 Kb and the PIC value and average expected gene diversity of the primer were 0.51 and 0.486 respectively. A total number of 5 SSR loci were scored by the primer PL-02 and all the loci were polymorphic (**Tables 2** and **3**). The product size ranged from 0.16 Kb to 0.32 Kb and the PIC value and average expected gene diversity of the primer were 0.51 and 0.465 respectively. The totals of 4 SSR loci were scored for the primer PL-03 with product size ranged from 0.19 to 0.30 Kb. and the PIC value and average expected gene diversity of the primer were 0.55 and 0.459 respectively. The total of 5 SSR loci was scored for the primer PL-08 (Tables 2 and 3). The product size ranged from 0.26 to 0.34 Kb and the average expected gene diversity and PIC value of the primer were 0.54 and 0.451 respectively. Total numbers of 3 SSR loci were scored by the primer PL-10. The product size ranged from 0.28Kb to 0.49 Kb and the average expected gene diversity and PIC value of the primer were 0.49 and 0.430respectively (Tables 2 and 3). 6 SSR loci were scored by the primer PL-11which and the product size was 0.32-0.48 Kb and the expected genetic diversity and PIC value of the primer 0.55 and 0.485respectively (Tables 2 and 3). 3 SSR loci with product size ranged 0.29 Kb to 0.45 Kb was scored for the primer PL-13. The average expected gene diversity and PIC value were 0.49 and 0.459 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 0.484 respectively and product size ranged from 0.15 to 0.27 kb (Tables 2 and 3). 4 SSR loci were scored by the primer PL-15 and the average expected gene diversity and PIC value of the primer were 0.50 and 0.442 respectively and product size ranged from 0.19 to 0.42kb (Tables 2 and 3). 4 SSR loci were scored by the primer PL-16 and the average expected gene diversity and PIC value of the primer were 0.471 and 0.50 respectively. Product size ranged from 0.15 to 0.39 kb. 3 SSR loci were scored by the primer PL-17 and the average expected gene diversity and PIC value of the primer were 0.49 and 0.487 respectively and product size ranged from 0.18 to 0.31 kb (Tables 2 and 3).6 SSR loci were scored by the primer PL-20 and the average expected gene diversity and PIC value of the primer were 0.55 and 0.493 respectively and product size ranged from 0.23 to 0.36 kb (Tables 2 and 3).

Table 1 Primer-BLAST designed microsatellite primers for *L. rohita* and *Cyprinus carpio* var. *communis*

Locus	Primer Sequence(5'-3')	Annealing Temp	Annealing Time
Lr-01	F-GAAAGCTGCTCGTCCTTGAA	53 ^{0}C	1min 30 sec
	R-GAAAGCTGCTCGTCCTTGAA		
Lr-02	F-GGGTGTGGGAGAGAAAGAGAG	62 ^{0}C	1min 30 sec
	R-GGAGTCTGACAAATGCAGCAAG		
Lr-03	F-TCTCAGTGGGTGTCATTACCTG	52 ^{0}C	1min
	R-CCCATCAAACCATCTCTCTAGC		
Lr-08	F-CTGACACTCTTATCTCGCTGCC	53 ^{0}C	1min 30 sec
	R-GACCTGAGCAAACAAACCTCAT		
Lr-10	F-TCTCTCTTTGTCTTTCCCCTTG	64 ^{0}C	1min
	R-CACAAGCCACTGTTTAGCTTCA		
Lr-11	F-CAAATCTGTGAACATGCAAGC	57 ^{0}C	1 min 30 sec
	R-CCTAGTCCCACTCTAGTCAGCA		
Lr-13	F-AGATAAGACCCTTCTTCCTCGG	62 ^{0}C	1min 30 sec
	R-TTTATTAGGGAGCGTCGAGTG		
Lr-14	F-CTGTTGGTGACTGTAGGGTGAA	58 ^{0}C	1min
	R-GAGAACTCGGTTTGAACATGC		
Lr-15	F-ACAGTAATCTTGTGTCTGTCTCTCTC	55 ^{0}C	1 min 30 sec
	R-GTCTAAACGTGTCTGAGCTGTG		
Lr-16	F-TGAATGTTTCCAGTCACCACAT	57 ^{0}C	1min
	R-GTAATGCAGCGGAGAATAAACC		
Lr-17	F-ACAATTCCTGTGTCAACTGTGC	57 ^{0}C	1min 30 sec
	R-TACCGTCTCAGTCTCTTTTCGG		
Lr-20	F-ATAGTCGAAATTGGTCCTCTGC	55 ^{0}C	1min 30 sec
	R- CAATACCATGACTGAAGTGCC		

Table 2 Genetic Diversity of *Cyprinus carpio* var. *communis* from Swapan 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	0.4084	0.4248	0.498	1.1939	0.157
PL-02	0.4170	0.4282	0.496	1.1278	0.212
PL-03	0.4120	0.4279	0.498	1.1465	0.198
PL-08	0.3585	0.4022	0.497	1.1422	0.208
PL-10	0.2999	0.3288	0.499	1.0523	0.207
PL-11	0.3390	0.3845	0.519	1.2450	0.206
PL-13	0.3502	0.4133	0.522	1.1041	0.127
PL-14	0.3539	0.4005	0.516	1.0950	0.148
PL-15	0.3514	0.3894	0.499	1.1399	0.156
PL-16	0.3207	0.3699	0.489	1.2577	0.166
PL-17	0.3480	0.3755	0.512	1.1613	0.143
PL-20	0.3448	0.3735	0.515	1.0588	0.170
Mean	0.3586	0.3932	0.505	1.1437	0.175

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

Locus	Amplified Product (Kb)	Number of alleles	PIC
PL- 01	0.13-0.23	5	0.51
PL-02	0.16-0.32	5	0.51
PL-03	0.19-0.30	4	0.55
PL-08	0.26-0.34	5	0.54
PL-10	0.28-0.49	3	0.49
PL-11	0.32-0.48	6	0.55
PL-13	0.29-0.45	3	0.49
PL-14	0.15-0.27	5	0.54
PL-15	0.19-0.42	4	0.50
PL-16	0.15-0.39	4	0.50
PL-17	0.18-0.31	3	0.49
PL-20	0.23-0.36	6	0.55

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

In *Cyprinus carpio* var. *communis* twelve microsatellite primers were successfully amplified and showed polymorphism (Table 1). Total 56 numbers of alleles scored and Number of alleles per locus ranges from 3 to 7 with mean value of 4.66 per locus. A total of 5 SSR loci were scored by the primer PL-01. The product size ranged from 0.13Kb to 0.30 Kb and the PIC value and average expected gene diversity of the primer were 0.54 and 0.498 respectively (**Tables 4** and **5**). A total number of 3 SSR loci were scored by the primer PL-02. The product size ranged from 0.14 Kb to 0.34 Kb and the PIC value and average expected gene diversity of the primer were 0.48 and 0.496 (Tables 4 and 5) respectively. Six SSR polymorphic loci were scored for the primer PL-03 with product size ranged from 0.17-0.31 Kb and the PIC value and average expected gene diversity of the primer were 0.55 and 0.498 respectively. The total of 5 SSR polymorphic loci was scored for the primer PL-08 The product size ranged from 0.20 Kb to 0.41 Kb and the average expected gene diversity and PIC value of the primer were 0.54 and 0.497 (Tables 4 and 5) respectively. Total numbers of 4 SSR loci were scored by the primer PL-10. The product size ranged from 0.23 Kb to 0.46 Kb and the average expected gene diversity and PIC value of the primer were 0.54and 0.499 respectively (Tables 4 and 5). 6 SSR loci were scored by the primer PL-11 and the product size was 0.22-0.36 Kb. PIC value and the expected genetic diversity was 0.55 and 0.519 respectively. 7 SSR loci with product size ranged 0.26 Kb to 0.43Kb was scored for the primer PL-13. The average expected gene diversity and PIC value were 0.62 and 0.522 (Tables 4 and 5) 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 0.516 respectively and product size ranged from 0.16 to 0.31kb (Tables 4 and 5). 5 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 0.499 respectively and product size ranged from 0.18 to 0.47 kb (Tables 4 and 5). 3 SSR loci were scored by the primer PL-16 and the average expected gene diversity and PIC value of the primer were

0.48 and 0.489 respectively. Product size ranged from 0.23 to 0.49 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 0.512 respectively and product size ranged from 0.18 to 0.31 kb. 4 SSR loci were scored by the primer PL-20 and the average expected gene diversity and PIC value of the primer were 0.54 and 0.515 respectively and product size ranged from 0.20 to 0.42 kb (Tables 4 and 5).

Table 4 Screened primer amplification results of *Cyprinus carpio* var. *communis* collected from Swapan hatchery

Locus	Amplified Product (Kb)	Number of alleles	(PIC)
PL- 01	0.13-0.30	5	0.54
PL-02	0.14-0.34	3	0.48
PL-03	0.17-0.31	6	0.55
PL-08	0.20-0.41	5	0.54
PL-10	0.23-0.46	4	0.54
PL-11	0.22-0.36	6	0.55
PL-13	0.26-0.43	7	0.62
PL-14	0.16-0.31	5	0.54
PL-15	0.18-0.47	5	0.54
PL-16	0.23-0.49	3	0.48
PL-17	0.18-0.31	3	0.48
PL-20	0.20-0.42	4	0.54

Table 5 Genetic Diversity of *L. rohita* from Swapan hatchery based on Microsatellite markers

Locus	Observed Heterozygosity (Ho)	Expected Heterozygosity (He)	Nei's genetic diversity (Hi)	Shanon's Information Index	Fixation Index Fis
PL- 01	0.3798	0.3860	0.486	1.1652	0.171
PL-02	0.3584	0.3631	0.465	1.1181	0.201
PL-03	0.3750	0.3824	0.459	1.1188	0.282
PL-08	0.3135	0.3488	0.451	1.1160	0.284
PL-10	0.2633	0.2824	0.430	1.0256	0.250
PL-11	0.2620	0.3347	0.485	1.2247	0.185
PL-13	0.3244	0.3635	0.459	1.0846	0.151
PL-14	0.2975	0.3307	0.484	1.0755	0.169
PL-15	0.2898	0.3309	0.442	1.1130	0.124
PL-16	0.2991	0.3104	0.471	1.2350	0.158
PL-17	0.3203	0.3547	0.487	1.1376	0.125
PL-20	0.2893	0.3237	0.493	1.0381	0.150
Mean	0.3143	0.3426	0.4676	1.1205	0.187

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 0.430 to 0.493 with mean value of 0.467 for *Labeo rohita* across all loci from hatchery whereas the average expected gene diversity ranged from 0.489 to 0.522 with mean value of 0.505 for *Cyprinus carpio* var. *communis* across all loci from hatchery. The observed and expected heterozygosity ranged from 0.2620 to 0.3798 and 0.2824 to 0.3860 respectively for *Labeo rohita* from hempur hatchery (Tables 3 and 5). The mean value of observed heterozygosity was 0.3143 and that of expected heterozygosity was 0.3426. Fis values were found to be positive at all loci in hatchery with mean value of 0.187. The Mean Fis values were found to be 0.175 at all loci in *Cyprinus carpio* var. *communis*. The observed and expected heterozygosity ranged from 0.2999 to 0.4170 and 0.3288 to 0.4082 respectively for *Cyprinus carpio* var. *communis* with mean value of observed heterozygosity was 0.3586 and expected heterozygosity was 0.3932. Mean values for Shannon's information index for all microsatellite loci were 1.1205 for *Labeo rohita* and 1.1437 for *Cyprinus carpio* var. *communis* population (Tables 3 and 5).

Discussion

In the present study microsatellite marker was employed to reveal genetic variability in *Labeo rohita* and *Cyprinus carpio* var. *communis* collected from hatchery. The microsatellite heterozygosity values were high in *Cyprinus carpio* but lower in *Labeo rohita and are supported* by findings of [21], 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 [22]. 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 [23]. 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 [24, 25]. 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 [26]. The FST indicates the proportion of genetic variation that could be attributed to the genetic differentiation processes between the co-specifics from two localities [27]. In the present study, q [28], 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 [29, 30]. Different physiological and reproduction pattern along with frequency of germplasm exchange might be the factor associated with these two species being somewhat genetically differentiated.

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