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Genetic diversity analysis of *Labeo gonius* (Hamilton, 1822) in three different reservoirs of Uttarakhand by using allozyme marker

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The present population genetic study was carried out to identify basic genetic structure of *Labeo gonius* from three different sized reservoirs *viz*., Dhaura, Baigul and Nanak Sagar reservoir of Uttarakhand by applying allozyme markers. The allozyme analysis was conducted for 18 enzymes but only 10 enzymes showed their presence with scorable activity. All ten allozymes yielded overall 19 scorable loci, out of which 11 loci were found to be polymorphic in the population with a percentage of 57.89. Genetic diversity parameters, that is number of polymorphic loci, the mean number of alleles per locus, observed (H_O) and expected heterozygosity (H_e), fixation index (F_{IS}) and genic differentiation were estimated by using software GENEPOP Ver.3.4 indicating high level of genetic diversity in stocks of all three reservoirs of *L. gonius* being more heterozygous in Nanak sagar followed by Baigul and Dhaura reservoirs. F_{ST} for overall populations ranging from 0.01 to 0.18 indicate moderate genetic differentiation as per Wright's criteria. The allozyme analysis in *L. gonius* showed that these populations are moderately differentiated from each other and likely to be associated with small gene exchange which is responsible for weak sub-structuring of stocks in the three reservoirs.

Key words: Allozyme, *Labeo gonius*, genetic diversity, population genetics.

INTRODUCTION

From genetic management perspectives, the aim of natural fisheries management should be to conserve intra-specific genetic diversity for which description of the genetic diversity of the concerned species is a pre-requisite for understanding the status and management requirements of the fish genetic resources. The study of genetic variation in fishes has proven to be valuable in aquaculture and fisheries management, for identification of stocks, selective breeding programmes, restoration of ecology and estimating contributions to stock mixtures. Generally, individuals with greater genetic variability have higher growth rates, developmental stability, viability, fecundity and resistance to environmental stress and diseases (Carvalho, 1993).

A proper knowledge of the genetic make-up and variability of the fish stocks will help in the management and conservation of endangered species apart from improvement of stocks of cultivable species. There is an urgent need to alert the fishing communities about the importance of fish resources conservation and integrating stakeholders and particularly local communities in all stages of project planning and implementation (Lakra et al., 2007). Initial molecular genetics studies during 1960s involved proteins such as hemoglobin and transferin but

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attentions quickly turned to enzymatic proteins (allozyme/isozyme variation) on which most subsequent studies have been based. From that time, fish geneticists have been using protein electrophoresis as their primary tool to characterize population-level genetic variation in various fish species (Waples, 1990). Isozymes that are the products of different alleles at the same locus are termed allozymes. Allozyme electrophoresis denotes the technique for identifying genetic variation at the level of enzymes, which are directly encoded by DNA. The allelic variants give rise to protein variants called allozymes that differ slightly in electric charges. Allozymes are co-dominant Mendelian characters (both alleles are individually expressed in a heterozygous individual), and characters are passed from parent to off-spring in a predictable manner (May, 2003). Being co-dominant marker, they are useful for assessing genetic variability and genetic identification of species (Dobrovolov et al., 2012; Sujatha et al., 2011).

Stock identification of several species has been carried out using allozyme technique (Shaklee et al., 1990; Ferguson et al., 1995). Allozymes were also found to be helpful in generating species-specific profiles and resolving taxonomic ambiguities in several species (Rognon et al., 1998; Gopalkrishnan et al., 1997; Pouyaud et al., 2000). The simplicity, speed, relatively low cost, little specialized equipment requirement (Ward and Grewe, 1995) and general applicability of the technique make this one of the commonly studied forms of molecular variation. The fish, *Labeo gonius* widely distributed in water bodies of North India, Asom and Odisha and along the East coast up to the Krishna River in India, is a dominant fish species in different reservoirs of Uttarakhand. It spawns during the South-West monsoon during July to August. Three reservoirs: Dhaura (1200 ha), Baigul (2693 ha) and Nanak sagar (4262 ha) with different morpho-edaphic features have self recruiting populations of *L. gonius* without stocking from outside. These reservoirs have different patterns of change in water volume, sedimentation and water abstraction, catchment area degradation due to siltation and water levels during summer followed by breeding season, which makes fishery activities and effective population size of different fishes in these reservoirs highly vulnerable.

The present investigation was carried out to assess the status of genetic variability and its relation with effective population size of *L. gonius* from three reservoirs located in the Tarai region of Uttarakhand (India) with the objective of devising better management practices for its sustainable optimized production in these reservoirs.

MATERIALS AND METHODS

Thirty live specimens of the fish, *L. gonius* were collected from commercial catches of all the three reservoirs. Liver tissue samples were dissected out by using sterilized scissors and forceps and stored at -86°C in deep freezer for further analysis. Protein was extracted by homogenization of liver tissues (250 mg) in a glass homogenizer under chilled conditions in 0.01 M Tris-HCl buffer of pH 6.8 having 1 mM ethylene di-amine tetra acetic acid (EDTA). Homogenized samples were centrifuged at 10000 rpm for 30 min at 4°C in a refrigerated centrifuge to remove the debris. Supernatant was pipetted out and used for allozyme analysis. Vertical polyacrylamide gel electrophoresis was used for the separation of allozymes at different enzyme loci. Gel consisted of 3.9% acrylamide and 3.36% bis-acrylamide and electrophoresis was performed at constant voltage of 50 V for 30 min and then 110 V till indicator dye (Bromophenol Blue) approached the bottom of the gel. The bands of each enzyme were revealed by incubating the gels in the dark at 37°C in the presence of substrate specific staining solution until sharp bands were visualized. The visualization of allozymes on gel was performed by using staining recipe described by Shaw and Prasad (1970) and Shaklee et al. (1990) with certain modifications. The locus and allele designations were done following the standardized genetic nomenclature for protein coding loci (Shaklee et al., 1990).

Genetic data analysis

The protein profiles generated for samples from all three stocks were compared and different parameters were estimated to assess the interpopulation and intrapopulation genetic variation. Standard parameters that is number of polymorphic loci (P), the mean number of alleles per locus (A), observed heterozygosity per locus (H_0), expected heterozygosity per locus (He), the coefficient of genetic differentiation (F_{ST}) and fixation index (F_{IS}) were estimated by using GENEPOP Version 3.4 (Raymond and Rousset, 1998). Deviations at each locus from Hardy-Weinberg equilibrium were tested by Markov chain method of exact probability test and the P values were corrected by using the Bonferroni correction (Rice, 1989).

RESULTS

The allozyme analysis was conducted for detecting 18 enzymes but only 10 enzymes showed their presence with scorable activity in *L. gonius*. All ten allozymes yielded overall 19 scorable loci in all three populations of *L. gonius* from Dhaura, Baigul and Nanak sagar reservoir. The enzymes analyzed for electrophoretic pattern, E.C. number and abbreviation of enzymes used for study of genetic variation are shown in Table 1. The enzymes with scorable activity were aldehyde oxidase (AO), esterase (EST), glucose 6-phosphate dehydrogenase (G_6PDH), glucose phosphate isomerase (GPI), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), malic enzyme (ME), phosphoglucomutase (PGM), superoxide dismutase (SOD) and xanthine dehydrogenase (XDH). These enzymes showed consistent phenotypic variations and were therefore useful for genetic analysis. They were coded by 19 putative loci (Table 2). LDH exhibited maximum number of loci, that is, 4 (LDH-1*, LDH-2*, LDH-3*, LDH-4*) followed by esterase with 3 loci (EST-1*, EST-2*, EST-3*). Two loci each were present in G_6PDH, GPI, MDH and XDH while all other enzymes (AO, ME, PGM and SOD) had only one locus each (Table 2). A total of 26 alleles were detected

Table 1. Name of enzymes with their enzyme commission number (E.C.) used in allozyme analysis in *L. gonius*.

Enzymes used	Abbreviation	E.C. Number	Structure
Acid phosphatase	ACP	3.1.3.2	Dimer
Adenylate kinase	AK	2.7.4.3	Monomer
Alcohol dehydrogenase	ADH	1.1.1.1	Dimer
Aldehyde oxidase	AO	1.2.3.1	Dimer
Creatine kinase	CK	2.7.3.2	Dimer
Esterase	EST	3.1.1.-	Monomer
Fumerase	FUM	4.2.1.2	Tetramer
Glutamate dehydrogenase	GDH	1.4.1.3	Hexamer
Glucose6phosphate dehydrogenase	G6PDH	1.1.1.49	Dimer
Glucose phosphate isomerase	GPI	5.3.1.9	Dimer
Lactate dehydrogenase	LDH	1.1.1.27	Tetramer
Malate dehydrogenase	MDH	1.1.1.37	Dimer
Malic enzyme	ME	1.1.1.40	Tetramer
Octanol dehydrogenase	ODH	1.1.1.73	Dimer
Phosphoglucomutase	PGM	5.4.2.2	Monomer
Pyruvate kinase	PK	2.7.1.40	Tetramer
Superoxide dismutase	SOD	1.15.1.1	Dimer
Xanthine dehydrogenase	XDH	1.1.1.204	Dimer

Table 2. The name of enzyme loci, number of loci and observed alleles for allozyme analysis in *L. gonius*. The enzymes mark 'ns' did not yield any scorable activity.

Enzymes used	Number of loci	Locus	Alleles	Mono-/polymorphic
Acid phosphatase	ns	ACP*	ns	ns
Adenylate kinase	ns	AK*	ns	ns
Alcohol dehydrogenase	ns	ADH*	ns	ns
Aldehyde oxidase	1	AO*	A,B	Polymorphic
Creatine kinase	ns	CK*	ns	ns
Esterase	3	EST-1*	A	Monomorphic
		EST-2*	A,B,C	Polymorphic
		EST-3*	B	Monomorphic
Fumerase	ns	FUM*	ns	ns
Glutamate dehydrogenase	ns	GDH*	ns	ns
Glucose6phosphate dehydrogenase	2	G6PDH -1*	A, B	Polymorphic
		G6PDH -2*	A.B,C	Polymorphic
Glucose phosphate isomerase	2	GPI–1*	A	Monomorphic
		GPI-2*	A,B	Polymorphic
Lactate dehydrogenase	4	LDH -1*	A	Monomorphic
		LDH-2*	A	Monomorphic
		LDH-3*	A	Monomorphic
		LDH -4*	A,B,C,D	Polymorphic
Malate dehydrogenase	2	MDH – 1*	A	Monomorphic
		MDH -2*	A,B	Polymorphic
Malic enzyme	1	ME*	A,B	Polymorphic
Octanol dehydrogenase	ns	ODH*	ns	ns

Table 2. Continued

Phosphoglucomutase	1	PGM*	A,B	Polymorphic
Pyruvate kinase	ns	PK*	ns	ns
Superoxide dismutase	1	SOD*	A,B	Polymorphic
Xanthine dehydrogenase	2	XDH-1*	A	Monomorphic
		XDH-2*	A,B	Polymorphic

from 11 polymorphic loci of ten enzyme systems out of which LDH-4* contained maximum of four alleles, EST-2* and G_6PDH-2* contained three alleles and the others had two alleles. Scorable activity of the following enzymes could not be detected in *L. gonius*: acid phosphatase (ACP), adenylate kinase (AK), alcohol dehydrogenase (ADH), creatine kinase (CK), fumerase (FUM), glutamate dehydrogenase (GDH), octonol dehydrogenase (ODH) and pyruvate kinase (PK).

Polymorphic enzyme pattern

The banding pattern of aldehyde oxidase of *L. gonius* exhibited locus AO* with two alleles producing 3 genotypes viz. AA, AB and BB. The banding patterns of esterase showed three different zones designated as EST-1*, EST-2 and EST-3* according to their order of increasing mobility. The first and third locus were monomorphic, while the second locus EST-2* had 3 type of alleles (A, B and C). G_6PDH is one of the most thoroughly studied allozymes showing banding pattern of two different zones of enzymatic activity designated as G_6PDH-1* and G_6-PDH-2* according to their order of increasing mobility differences with two (A and B) and three alleles (A, B and C), respectively. The locus GPI-1* exhibited one allele (A) and GPI-2* exhibited two alleles (A and B) and presumed to be under the control of two independent loci. The banding pattern of LDH enzyme systems of *L. gonius* showed four different zones. The initial three locus (LDH-1*, LDH-2*, LDH-3*) had slight difference in their mobility with only one allele (A), while the fourth locus (LDH-4*) is a fast moving zone having 4 types of alleles (A, B, C and D). Liver extracts of *L. gonius* showed the presence of two loci of MDH *viz*. MDH-1* with only one allele and MDH-2* locus stained intensely showing polymorphic pattern. Presence of bands of ME in the gel indicated only one locus (ME*) exhibiting 2 alleles A and B and produced genotypes viz. A_4A_4, A_3B_1, A_2B_2, A_1B_3 and B_4B_4. PGM showed one zone of enzyme activity in all three populations of *L. gonius* with two alleles, A and B. Superoxide dismutase enzyme also showed only one zone of enzymatic activity on the gel in the form of bleached area with two types of alleles, A and B. From the banding pattern of XDH, it was inferred that it is controlled by 2 different loci viz. XDH*-1 (slow moving zone) with only one allele (A) and XDH-2* (fast moving zone). The first locus (XDH*-1) was monomorphic, while locus 2 (XDH*-2) had three types of alleles: A, B and C.

Genetic diversity analysis

The observed heterozygosity ranged from 0.316 in Dhaura population to 0.501 in Nanak sagar population. The expected heterozygosity was 0.405 in Dhaura population followed by 0.442 in Baigul population and 0.539 in Nanak sagar reservoir (Table 3). The probability test showed that the observed allele frequencies significantly deviated ($P < 0.05$) from that expected under Hardy-Weinberg equilibrium in most of the loci in Dhaura population as compared to Baigul and Nanak sagar where few loci deviated after the Bonferroni corrections. These significant deviations from Hardy-Weinberg equilibrium were produced at AO*, MDH-2*, ME*, LDH-4*, PGM-2* and XDH- 2* in Dhaura population, GPI*, LDH-4*, SOD* and XDH-2* in Baigul population and ME* in Nanak sagar population (Table 3). The F_1s value for each locus ranged from -0.2632 to +0.2121 in overall population. The mean F_Is for Dhaura population was 0.226 followed by Baigul population (0.146) and Nanak sagar population (0.134) (Table 3). The coefficient of genetic differentiation (F_{ST}) ranged from 0.01 at G_6PDH-1* and G_6PDH-2* to 0.18 at SOD* with a mean of 0.097 indicating that about 9.7% of the total genetic variation exists among populations due to population differentiation (Table 4). Genic differentiation between different population pairs showed maximum value between Dhaura and Nanak sagar population, while least differentiation was observed between Baigul and Dhaura population (Table 5).

DISCUSSION

The genetic variability in the three natural populations of *L. gonius* was evidenced using ten allozymes, out of a total of 18 enzymes used due to random phenomenon of their phenotypic expression in the species. Haniffa et al. (2007) selected only 16 enzymes out of a total of 18 enzyme systems used on the basis of consistent phenotypic variation in liver samples of *Channa punctatus*. All enzymes produced a total of 19 loci, out of which 11 were polymorphic and they were used for the population genetic analysis of the *L. gonius*. Salini et al. (2004) used

Table 3. Summary of genetic variation and heterozygosity statistics of ten allozymes in *L. gonius*.

Locus	Parameter	Dhaura	Baigul	Nanak Sagar
AO*	Ho	0.25	0.42	0.50
	He	0.38	0.45	0.54
	P_{Hw}	0.04*	1.00	1.00
	F_IS	+0.428	+0.111	-0.142
EST-3*	Ho	0.31	0.33	0.52
	He	0.38	0.41	0.56
	P_{Hw}	0.09	0.89	0.23
	F_IS	+0.212	-0.250	-0.333
G6PDH-1*	Ho	0.38	0.45	0.50
	He	0.42	0.44	0.55
	P_{Hw}	0.84	1.00	0.06
	F_IS	-0.212	+0.142	-0.111
G6PDH-2*	Ho	0.37	0.45	0.51
	He	0.42	0.44	0.53
	P_{Hw}	0.84	1.00	0.06
	F_IS	-0.209	+0.145	-0.101
GPI-2*	Ho	0.32	0.35	0.50
	He	0.44	0.46	0.51
	P_{Hw}	0.62	0.029*	0.63
	F_IS	+0.085	+0.142	-0.142
LDH-4*	Ho	0.25	0.32	0.52
	He	0.39	0.45	0.55
	P_{Hw}	0.04*	0.013*	0.16
	F_IS	+0.294	+0.135	-0.166
MDH-2*	Ho	0.39	0.40	0.50
	He	0.26	0.45	0.56
	P_{Hw}	0.04*	1.00	0.34
	F_IS	-0.538	+0.111	-0.081
ME*	Ho	0.29	0.38	0.45
	He	0.45	0.44	0.56
	P_{Hw}	0.03*	1.00	0.039*
	F_IS	-0.111	+0.142	-0.081
PGM-2*	Ho	0.32	0.39	0.49
	He	0.46	0.43	0.51
	P_{Hw}	0.04*	0.70	1.00
	F_IS	+0.135	-0.176	-0.290
SOD*	Ho	0.30	0.30	0.53
	He	0.42	0.43	0.55
	P_{Hw}	1.00	0.028*	1.00
	F_IS	+0.085	-0.142	-0.176
XDH-2*	Ho	0.30	0.35	0.50

Table 3. Continued.

He	0.44	0.47	0.51
P_{Hw}	0.01*	0.024*	0.62
$F_{I}S$	+0.179	-0.111	-0.142
Mean Ho	0.316	0.376	0.501
Mean He	0.405	0.442	0.539
Mean $F_{I}S$	0.226	0.146	0.134
Mean A	1.68	1.79	2.17

*Values are significant at p<0.05; Ho = observed heterozygosity; He = expected heterozygosity; P_{HW} = probability value of significant deviation from HWE; F_{Is} = fixation index,; A = number of alleles per locus.

Table 4. Fixation index (F_{IS}) and F_{ST} for overall population of *L. gonius* using F-statistics.

Locus name	Sample size	F_{IS}	F_{ST}
ALO*	90	+0.2121	0.08
EST-2*	90	-0.2632	0.12
G_6PDH-1*	90	-0.1538	0.01
G_6PDH-2*	90	-0.1541	0.01
GPI-2*	90	+0.0667	0.16
LDH-4*	90	0.1287	0.03
MDH-2*	90	-0.2121	0.08
ME*	90	+0.111	0.15
PGM*	90	+0.098	0.15
SOD*	90	+0.076	0.18
XDH-2*	90	+0.182	0.10

Table 5. Genic differentiation (P-value) for each population pair of *L. gonius* across all loci.

Population pair	P-value
Baigul and Dhaura	0.0192
Baigul and Nanak Sagar	0.0690
Dhaura and Nanak Sagar	0.0849

three polymorphic enzymes (5 loci) in Bangladesh populations of *Tenualosa ilisha* to detect genetic variation, while Lal et al. (2004) reported polymorphism in 13 out of 26 scorable loci in the same species from the river Ganges. Appleyard and Mather (2002) reported 25 polymorphic allozyme loci out of 50, helpful to screen differences in two stocks of *Oreochromis niloticus* and *Oreochromis mossambicus.* In the above studies, several polymorphic allozymes were common viz., EST, G_6PDH, GPI, LDH, PGM and SOD indicating their usefulness in characterization of genetic diversity and helpful in estimating the basic genetic structure. Percentage value of polymorphic loci across populations was 57.89% in *L. gonius* indicating genetic polymorphism in all three populations. Slightly lower percent of polymorphic loci (6.2 to 43.8%) was observed in *Cyprinus carpio* (Kohlmann et al., 2003) and 22 to 27% polymorphic loci in *Cirrhinus mrigala* (Chauhan et al., 2007) by allozyme analysis. However, Reusing et al. (2011) reported very low percent of polymorphic loci in a fresh water fish,

Neoplecostomus by using allozyme markers. The best estimate of genetic variation in natural population is the mean observed heterozygosity (H_O) per locus (Allendorf and Utter, 1979) which varies non-randomly between loci, populations and species. The H_O value in all three populations of *L. gonius* falls within the range reported by many authors in freshwater fishes (Kohlmann and Kersten, 1999; Lal et al., 2004, Singh et al., 2004; Salini et al., 2004). The observed heterozygosity (H_O) values obtained in *L. gonius* are approximately similar to expected values (H_e) in Nanak sagar population as compared to Baigul and Dhaura reservoir. The F_{IS} (fixation index) values were found to be negative at all loci in Nanak Sagar population, showing substantial presence of heterozygotes in population attributed to large effective population size with negligible chances of inbreeding. Deviation from the frequencies expected under Hardy-Weinberg equilibrium provide evidence that population are not in equilibrium which could be due to non-random mating or effect of other evolutionary forces like selection/migration or reduction in effective population size.

In the present study, significant deviations were found in AO*, LDH-4*, MDH-2*, ME*, PGM-2* and XDH-2* in Dhaura, GPI*, LDH-4*, SOD* and XDH-2* in Baigul population and ME* in Nanak sagar showed maximum number of homozygotes in Dhaura which are most probably due to the presence of limited number of brooders and/or unequal sex ratio in reservoir resulting from high fishing pressure on the population. Large areas of Dhaura get dried up in summer and fishes including *L. gonius* are extensively exploited from deeper isolated pockets holding them thus adversely affecting their effective population size available for breeding in the next season, resulting into genetic homozygosity. The problems of bottleneck, drift and inbreeding, closely associated with small populations have been correlated with effective population size (Ne) to population genetic structure of fishes (Ayappan, 2011) and similar observations have also been made in two mahseers, that is, *Tor khudree* and *Tor malabaricus* (Silas et al., 2004). The homozygotes excess is also confirmed by positive fixation index (F_{IS}) values (Table 3). Homozygote excess for allozyme has been reported quite commonly in many fish species (Engelbrecht and Mulder, 2000; Steenkemp et al., 2001). Several hypotheses have been mentioned to explain homozygote excess in fishes including inbreeding, population admixture or the presence of non-expressed alleles (Appleyard et al., 2001; Ward et al., 2003). The values of F_{ST} in present study showed moderate genetic differentiation with a mean value of 0.106 among all the three populations as indicated by Wright (1978) criteria. Rognon et al. (1998) reported a lower F_{ST} value (0.044) for populations of *C. gariepinus* with allozymes. Appleyard and Mather (2002) reported high F_{ST} values (0.501 to 0.598) in two species of *Oreochromis* indicating little evidence of introgression between these species. The moderate level of genetic differentiation despite these reservoirs being well isolated from each other and fed by separate unconnected rivers indicated that the stocks of *L. gonius* in all three reservoirs are not strongly sub-structured. Geographical isolation, limited dispersal and phylopatric behaviour of populations are also responsible for promoting genetic differentiation, particularly in freshwater habitats (Carvalho and Hauser, 1995).

Maximum genic differentiation among different population pairs was observed between Dhaura and Nanak sagar reservoir, as these reservoirs are distantly located from each other and have no connection with each other, indicating negligible gene exchange between the populations. As genetic differentiation increases with the increase in geographic distance, the observations pertaining to differentiation in stocks of *L. gonius* seem to be positively correlated with the geographical distances among the stocks.

Conclusion

The population genetic data generated by allozyme analysis indicated the presence of comparable genetic diversity in stocks of *L. gonius* of all three reservoirs with moderate genetic differentiation among them because of less gene exchange. Maximum genetic diversity observed in Nanak sagar population followed by Baigul and Dhaura reservoirs might be correlated with the effective population size due to variable morpho-edaphic features, hydrologic regime and anthropogenic activities. The stock structure data generated in the present study can provide an essential component for formulating meaningful stock management programme for *L. gonius* populations in all three reservoirs.

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