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Assessing Genetic Differentiation in Geographic Populations of *Labeo calbasu* Using Allozyme Markers

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Abstract The population structure of *Labeo calbasu* from 11 rivers belonging to the Indus, Ganges, Bhima, Mahanadi, and Godavari basins was investigated using allozyme marker systems. Seven out of 20 allozyme loci (35%) were polymorphic ($P < 0.99$). Both probability and score tests indicated significant deviation of genotype proportions from Hardy–Weinberg expectations at two loci, *XDH** (Mahanadi, Bhima, and Godavari) and *G6PDH** (Mahanadi). A pairwise genetic homogeneity test and F_{ST} values indicated a low-to-moderate level (0.0515) of genetic structuring in the wild population of *L. calbasu*. AMOVA analysis also indicated moderate differentiation among the samples from different river basins. Analysis for genetic bottleneck was performed under the infinite allele model. The study revealed nine genetic stocks of *L. calbasu* from the natural population across Indian rivers. Evidence of genetic bottlenecks in some rivers was also revealed.

Keywords *Labeo calbasu* · Allozyme · Electrophoresis · Genetic differentiation

Introduction

Labeo calbasu (Hamilton-Buchanan 1822) is one of four Indian major carps inhabiting deep pools of rivers, natural lakes, and man-made ponds. *L. calbasu* supports important fishery resources in rivers and reservoirs of the Indo-Gangetic

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plain, as well as some rivers from Peninsular India, such as the Mahanadi, Godavari, and Krishna. *L. calbasu* is one of the constituents of the compatible-complex of major carp species used in Indian polyculture. Various favorable attributes, such as growth potential, response to induced breeding methods, high consumer preference, as well as high market price, make this fish attractive to aquaculturists (Chondar 1999). Over the years, there has been a distinct reduction in its population, evident from catches from open waters (Chaudhary and Jugal 2003; Mahapatra 2003), owing to several factors, such as construction of check dams across many river stretches and overexploitation of wild populations.

From a genetic perspective, the aim of natural fishery management should be to conserve intraspecific genetic diversity. Genetic variation is an important feature for imparting capability to adapt to changing environmental conditions and is vital for long-term survival of a population or species (Ferguson 1995). Natural genetic resources form the basis for selection of founder stocks for stock improvement programs. Therefore, it is evident that data from stock structure assessment can be vital in scientific planning of the breeding programs aimed at conserving and maintaining natural genetic diversity. Genetic studies on this species have been limited to identification of polymorphic microsatellite DNA markers (Singh et al. 2008) and allozyme markers (Lal et al. 2007). RAPD markers, however, have been utilized for species differentiation (Barman et al. 2003) and for genetic diversity analysis of wild and farmed *L. calbasu* of Bangladesh (Mostafa et al. 2009).

Allozyme markers are proven tools for determining genetic variation and drawing inferences on population structure in fishes and shellfishes and to unearth population-level evolution for a variety of vertebrates (Ward et al. 1994). This marker system provides an independent estimate of the level of variation within a population without an extensive morphological and quantitative account (Menezes et al. 1993; Haniffa et al. 2007). These markers have been successfully used to determine stock structure of several fish species (Lal et al. 2004; Salini et al. 2004; Chauhan et al. 2007). The present study aims to identify polymorphic allozyme loci suitable for assessing and documenting the genetic variation and stock structure of wild *L. calbasu* accross its native distribution in India.

Materials and Methods

Fish Sample Collection

Between December 2000 and December 2007, 406 *L. calbasu* tissue samples were obtained from commercial catches from rivers including the Satluj, Ganga, Ghaghra, Brahmaputra, Mahanadi, Godavari, and Krishna. Sampling sites were selected to cover genetic variation across a wide geographic distribution range of the species (Fig. 1; Table 1). The river Satluj is a part of the Indus river basin. Other locations, except the Godavari, Krishna, and Mahanadi rivers, are distant tributaries of the Ganga river basin (ECAFE 1966). Specimen weight range was 0.15–11.5 kg. The sampling was performed at the site of collection. The liver tissue was

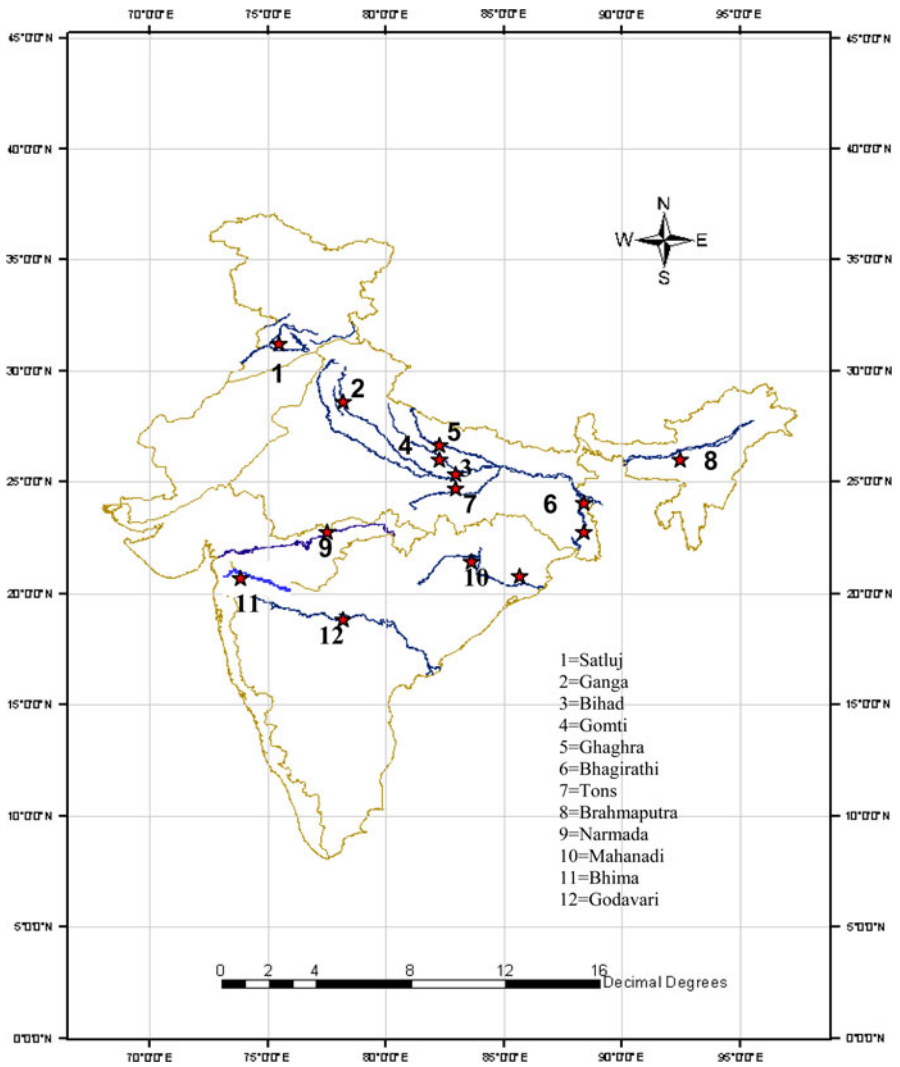

Fig. 1 Sampling stations (*stars*) across different river basins for population structure study of *Labeo calbasu*

immediately frozen in liquid nitrogen (−196°C) and transported to the laboratory for storage at −80°C till analysis.

Allozyme Analysis

Electrophoretic conditions were optimized to score allelic variation, with good resolution and band intensity. For the allozyme studies, muscle samples did not provide any additional loci or better resolution at any locus compared with that observed in liver. Therefore, the liver was chosen as the optimum tissue.

Table 1 Collection of *Labeo calbasu* samples from rivers in India

River system	River	Location	Latitude, Longitude	Year of collection	Sample size
Indus	Satluj	Heri ke patan, Punjab	31°13′N, 75°12′E	June 2001	11[a]
Ganges	Banganga	Laksar, UP	29°58′N, 77°23′E	May 2002	17[b]
	Ganga	Bijnore, UP	29°23′N, 79°11′E	May 2002	20[b]
	Yamuna	Allahabad, UP	25°28′N, 81°54′E	Apr 2004	09[b]
	Bhagirathi	Farraka, WB	24°5′N, 88°06′E	June 2001	15[c]
				July 2002	
		Nabadeep, WB	23°24′N, 88°23′E	Jan 2001	08[c]
	Gomti	Sultanpur, UP	26°16′N, 82°4′E	June 2001	19[d]
	Ghaghra	Ajjaipur, UP	27°34′N, 80°41′E	Dec 2000	12[e]
				June 2001	
				Jan 2002	
	Gerua	Katarniya ghat, UP	32°19′N, 75°30′E	Dec 2000	24[e]
	Sharda	Nanaksagar, UT		Dec 2007	11[e]
		Begul		Dec 2007	07[e]
	Bihad	Rewa, MP	24°31′N, 81°17′E	Apr 2004	76[f]
	Tons		24°38′N, 81°25′E		30[g]
	Brahmaputra	Kalangpar, Assam	26°11′N, 91°47′E	Jan 2001	07[h]
				Dec 2007	13[h]
Mahanadi	Mahanadi	Sambhalpur	23°14′N, 77°23′E		64[i]
		Daspur/Goyalbank			05[i]
Krishna	Bhima	Pune	18°31′N, 73°50′E		17[j]
Godavari	Godavari	Adilabad Manthini	18°57′N, 79°06′E	Oct 2002	41[k]
Total					406

Superscripts indicate multiple data sets within rivers or neighboring localities that were pooled after testing for absence of heterogeneity

Electrophoresis running conditions were described in a previous study (Lal et al. 2007).

Frozen liver samples (~100 mg) were homogenized in 250 mg/ml of extraction buffer (0.17 M sucrose, 0.2 EDTA, 0.2 Tris–HCL, pH 7.0). Homogenized samples were centrifuged for an hour at 10,000 rpm at 4°C, and the supernatant was recentrifuged for 20 min. Allelic variation was investigated using 7% polyacrylamide gel electrophoresis (8 × 10 cm SE 250; GE Healthcare, USA) for 45–145 min using Tris–borate EDTA buffer (TBE). Electrophoresis was carried out at constant 150 V at 4°C. Histochemical staining procedures outlined by Whitmore (1990) were used to visualize alleles. Loci and alleles were designated following the nomenclature system of Shaklee et al. (1990). In total, 26 enzyme systems were examined, and 17 enzymes yielding scorable activity were further analyzed to score loci and alleles (Table 2). Individual fish genotype data were determined for each locus. The most common allele was assigned 100, and other alleles were designated corresponding to the most common allele. A locus was considered polymorphic if the frequency of the most common allele was ≤0.99 (Hartl and Clark 1997).

Table 2 Polymorphic loci detected in *Labeo calbasu*

Allozyme	E.C. No.	Locus	Alleles
Acid phosphatase	3.1.3.2	*ACP**	ns
Adenylate kinase	2.7.4.3	*AK**	100
Alcohol dehydrogenase	1.1.1.1	*ADH**	ns
Aldolase	4.1.2.32	*ALDO**	100
Alkaline phosphatase	3.1.3.1	*ALP**	ns
Aspartate amino transferase	2.6.1.1	*AAT**	100
Creatine kinase	2.7.3.2	*CK**	100
Esterase	3.1.1.1	*EST 1**	092, 100, 107, 116
		*EST 2**	100, 105
		*EST 3**	100, 105
Fructose 1,6 bi phosphatase	3.1.3.11	*FBP**	100
Glutamate dehydrogenase	1.4.1.3	*GDH**	ns
Glucose-6-phosphate dehydrogenase	1.1.1.49	*G6PDH**	074, 086, 100, 115
Glucose phosphate isomerase	5.3.1.9	*GPI**	084, 100
Glucose dehydrogenase	1.1.1.47	*GLDH**	100
α-Glycerophosphate dehydrogenase	1.4.1.3	*GPDH2**	84, 100
Hexokinase	2.7.1.1	*HK**	100
Isocitrate dehydrogenase	1.1.1.42	*ICD**	100
Lactate dehydrogenase	1.1.1.27	*LDH**	ns
Malate dehydrogenase	1.1.1.37	*MDH**	100
Malic enzyme	1.1.1.40	*ME**	100
Octonol dehydrogenase	1.1.1.73	*ODH**	100
Phosphogluconate dehydrogenase	1.1.1.44	*PGDH**	ns
Phosphogluco mutase	5.4.2.2	*PGM**	ns
Pyruvate kinase	2.7.1.40	*PK**	ns
Superoxide dismutase	1.15.1.1	*SOD1**	100
		*SOD 2**	100
Xanthine dehydrogenase	1.1.1.204	*XDH**	094, 100, 103

ns No scorable activity

Data Analysis

The individual fish genotype at each allozyme locus was determined, and the data were analyzed for homogeneity between data sets for collections at different times and neighboring localities within each river. Data sets within each river or neighboring tributaries that were not heterogeneous ($P > 0.05$) were combined for further analysis to estimate genetic variation and differentiation parameters. Allelic frequencies and heterozygosities (observed and expected) values were calculated using Genetix version 4.05 software (Belkhir et al. 1997). In the present study, the options available in Genepop version 3.3 (Raymond and Rousset 1995a) were used to determine conformity to Hardy–Weinberg expectations of genotype frequencies. The powerful score test was employed to calculate the probability, against the

specific alternate hypothesis of heterozygote deficiency or excess, as indicated by positive or negative F_{IS} value. The significance level of probabilities was estimated through sequential Bonferroni adjustment of critical level of 0.05 (Lessios 1992). Probability values obtained from the test to determine linkage disequilibrium between pairs of allozyme loci in each sample were also calculated. A genotype differentiation test was performed on genotype tables (Raymond and Rousset 1995b). The null hypothesis tested was that the genotype distribution was identical across all populations. The Genetix version 4.05 software (Belkhir et al. 1997) was used to estimate F statistics (Wright 1951), computed as the estimators Θ, F, and f of Weir and Cockerham (1984). The probability of Θ significantly deviating from zero was calculated using 1000 bootstraps. The hierarchical analysis was carried out using analysis of molecular variance (AMOVA) in Arlequin 2000 (Excoffier and Schneider 2005). This software yields information of population structure at different levels, within population, among subpopulations within river basins, and among river basins. The bottleneck test uses genetic data from a contemporary population to test for the presence of heterozygosity excess, such as would be expected following a bottleneck event (Cornuet and Luikart 1996; Luikart and Cornuet 1998). The expectations of heterozygote excess at mutation-drift equilibrium differs depending on the type of mutation model selected. The infinite allele model based on the assumption that all new alleles arise de novo, with an infinite number of potential alleles was used to analyse genetic bottleneck.

Results

Genetic Variability

The products of 17 enzyme coding loci were detected that provided scorable results for population genetic analysis (Table 2). The loci *EST-1**, *EST-2**, *EST-3**, *GPI**, *GPDH2**, *XDH**, and *G6PDH** were polymorphic in *L. calbasu*. No significant genotype heterogeneity was observed among the multiple data sets (collections at different time intervals and neighboring locations) within the Ganga (Banganga, Ganga, and Yamuna), Bhagirathi, and Ghaghra (Ghaghra, Gerua, and Sharda) rivers. After the genotypic data from the multiple data sets within each river were combined, 11 data sets were available for analysis of genetic variation and differentiation (Satluj, Ganga, Bhagirathi, Gomti, Ghaghra, Brahmaputra, Bihad, Tons, Mahanadi, Bhima, and Godavari). Allele frequencies at the polymorphic allozyme loci are presented for the 11 localities in Table 3. In the Mahanadi samples, the private allele *EST1*107* was detected (frequency 0.0074).

The screening of 406 individual samples resolved 33 alleles for 20 allozyme loci. The number of alleles at the seven polymorphic loci ranged from 2 to 5. Loci *EST-2**, *EST-3**, *GPI**, and *GPDH-2** exhibited 2 alleles each, and locus *XDH** had 3 alleles. Loci *G6PDH** and *EST-1** were found to have 4 and 5 alleles, respectively, in *L. calbasu*.

Genetic variation (observed and expected heterozygosity) was analyzed for *L. calbasu* from the 11 collection sites, at 7 polymorphic allozyme loci (Table 4).

Table 3 Allele frequencies of seven polymorphic allozyme loci in *Labeo calbasu* from 11 riverine locations

Locus Allele	Population (number of specimens)										
	Satluj (11)	Ganga (46)	Bhagirathi (23)	Gomti (19)	Ghaghra (54)	Brahmaputra (20)	Bihad (76)	Tons (30)	Mahanadi (69)	Bhima (17)	Godavari (41)
*EST 1**											
92	0.0000	0.0128	0.0217	0.0000	0.0000	0.0000	0.0000	0.0000	0.0294	0.0000	0.0125
100	0.9545	0.8333	0.7826	0.7895	0.8333	0.8500	0.7887	0.7833	0.7721	0.8438	0.8125
107	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0074	0.0000	0.0000
116	0.0455	0.0897	0.1522	0.0263	0.1389	0.0000	0.0000	0.0833	0.0221	0.1250	0.0750
126	00.0000	0.0641	0.0435	0.1842	0.0278	0.1500	0.2113	0.1333	0.1691	0.0313	0.1000
*EST 2**											
100	0.0455	0.4359	0.4348	0.3611	0.3796	0.2000	0.4225	0.4333	0.7319	0.4118	0.6500
105	0.9545	0.5641	0.5652	0.6389	0.6204	0.8000	0.5775	0.5667	0.2681	0.5882	0.3500
*EST 3**											
100	0.6818	0.5513	0.5435	0.6944	0.5673	0.5000	0.5986	0.5333	0.5000	0.6765	0.5139
105	0.3182	0.4487	0.4565	0.3056	0.4327	0.5000	0.4014	0.4667	0.5000	0.3235	0.4861
*GPI**											
100	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.9762	1.0000	0.9091
114	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0238	0.0000	0.0909
*GPDH 2**											
84	0.0500	0.1860	0.0870	0.1389	0.1667	0.1944	0.3359	0.2391	0.0926	0.3214	0.1607
100	0.9500	0.8140	0.9130	0.8611	0.8333	0.8056	0.6641	0.7609	0.9074	0.6786	0.8393
*XDH**											
94	0.0455	0.1667	0.3235	0.1579	0.1569	0.0250	0.1210	0.3500	0.4590	0.5313	0.4028
100	0.4091	0.6481	0.6176	0.7368	0.5686	0.7750	0.6694	0.4000	0.4016	0.4375	0.4722
103	0.5455	0.1852	0.0588	0.1053	0.2745	0.2000	0.2097	0.2500	0.1393	0.0313	0.1250

Table 3 continued

Locus Allele	Population (number of specimens)										
	Satluj (11)	Ganga (46)	Bhagirathi (23)	Gomti (19)	Ghaghra (54)	Brahmaputra (20)	Bihad (76)	Tons (30)	Mahanadi (69)	Bhima (17)	Godavari (41)
*G6PDH**											
74	0.0455	0.0441	0.1429	0.2105	0.0660	0.1389	0.0616	0.0667	0.1000	0.1471	0.0758
86	0.1818	0.2794	0.4762	0.1842	0.2830	0.1667	0.1644	0.1333	0.5583	0.2353	0.2879
100	0.5455	0.4412	0.3571	0.4737	0.3774	0.3333	0.3767	0.3667	0.2250	0.5000	0.3939
115	0.2273	0.2353	0.0238	0.1316	0.2736	0.3611	0.3973	0.4333	0.1167	0.1176	0.2424

Table 4 Genetic variation for seven polymorphic allozyme loci in *Labeo calbasu* from 11 river locations

Locus Parameter	River										
	Satluj	Ganga	Bhagirathi	Gomti	Ghaghra	Brahmaputra	Bihad	Tons	Mahanadi	Bhima	Godavari
*EST1**											
H_e	0.0868	0.2932	0.362	0.3421	0.2855	0.2550	0.3333	0.3617	0.3739	0.2715	0.3241
H_o	0.0909	0.3333	0.3478	0.4211	0.3333	0.3000	0.3380	0.2333	0.3529	0.3125	0.2750
P_{HW}	–	1.0000	0.7126	1.0000	0.7411	1.0000	1.0000	0.0302*	0.1249	1.0000	0.2585
F_{IS}	0.0135	0.0279	0.0108	0.0261	0.0384	0.0209	0.0189	−0.0199	0.0012	0.0187	−0.0033
P_{sc}	–	1.0000	0.4264	1.0000	1.0000	1.0000	0.6489	0.9954	0.3772	1.0000	0.9641
*EST-2**											
H_e	0.0868	0.4918	0.4915	0.4614	0.4710	0.3200	0.4880	0.4911	0.3925	0.4844	0.4550
H_o	0.0909	0.7179	0.6957	0.3889	0.5741	0.4000	0.6197	0.6667	0.3043	0.5882	0.3500
P_{HW}	–	0.0083*	0.0927	0.6122	0.1534	0.5484	0.0308*	0.0766	0.0676	0.6244	0.1666
F_{IS}	−0.1338	−0.0933	−0.1141	−0.1482	−0.1192	−0.1289	−0.1000	−0.1130	−0.1994	−0.1297	−0.1763
P_{sc}	–	0.0057	0.0684	0.9065	0.1034	0.4193	0.0231	0.0648	0.9859	0.3995	0.9708
*EST3**											
H_e	0.4339	0.4947	0.4962	0.4244	0.4909	0.5000	0.4806	0.4978	0.5000	0.4377	0.4996
H_o	0.6364	0.4359	0.5652	0.1667	0.4423	0.4737	0.5775	0.6667	0.2667	0.4118	0.5278
P_{HW}	0.4799	0.5196	0.6842	0.0152	0.5713	1.0000	0.1377	0.1387	<0.0003**	1.0000	1.0000
F_{IS}	0.0693	0.0477	0.0681	0.0312	0.0481	0.0554	0.1142	0.0903	−0.0257	0.0551	0.0674
P_{sc}	1.0000	0.3073	0.8391	0.0152	0.3068	0.5448	0.9720	0.9852	1.0000	0.5634	0.7225

Table 4 continued

Locus Parameter	River										
	Satluj	Ganga	Bhagirathi	Gomti	Ghaghra	Brahmaputra	Bihad	Tons	Mahanadi	Bhima	Godavari
*GPI**											
H_e	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0465	0.0000	0.1653
H_o	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0159	0.0000	0.0606
P_{HW}	–	–	–	–	–	–	–	–	0.0240*	–	0.0109*
F_{IS}	0.6499	0.6507	0.6501	0.6501	0.6509	0.6501	0.6515	0.6503	0.6425	0.6500	0.6665
P_{sc}	–	–	–	–	–	–	–	–	0.0240	–	0.0109
*GPDH2**											
H_e	0.0950	0.3029	0.1588	0.2392	0.2778	0.3133	0.4462	0.3639	0.1680	0.4362	0.2698
H_o	0.1000	0.2791	0.1739	0.1667	0.2000	0.1667	0.2969	0.1304	0.1481	0.3571	0.2500
P_{HW}	–	0.6148	1.0000	0.2727	0.0807	0.0850	0.0104*	0.0052*	0.3672	0.5548	0.5332
F_{IS}	0.2753	0.3006	0.2862	0.2706	0.2709	0.2602	0.2447	0.2380	0.2884	0.2774	0.2881
P_{sc}	–	0.4350	1.0000	0.2727	0.0807	0.0850	0.0071	0.0052	0.3672	0.4064	0.5332
*XDH**											
H_e	0.5331	0.5178	0.5104	0.4211	0.5767	0.3587	0.4934	0.6550	0.6086	0.5254	0.5992
H_o	0.5455	0.5556	0.5882	0.3158	0.4510	0.2000	0.4032	0.7000	0.2951	0.1250	0.2500
P_{HW}	1.0000	1.0000	1.0000	0.1587	0.1131	0.0232*	0.0142*	0.7949	<0.0001**	<0.0004**	0.0000*
F_{IS}	0.2932	0.3116	0.3042	0.2852	0.2953	0.2776	0.3029	0.3234	0.2276	0.2616	0.2455
P_{sc}	0.6037	0.7000	0.7980	0.0639	0.0123	0.0232	0.0118	0.6737	<0.0001**	<0.0004**	<0.0001**

Table 4 continued

Locus / Parameter	River										
	Satluj	Ganga	Bhagirathi	Gomti	Ghaghra	Brahmaputra	Bihad	Tons	Mahanadi	Bhima	Godavari
*G6PDH**											
H_e	0.6157	0.6700	0.6247	0.6801	0.6983	0.7114	0.6695	0.6556	0.6140	0.6592	0.6974
H_o	0.5455	0.6471	0.3333	0.5789	0.6415	0.5000	0.6164	0.5667	0.4167	0.3529	0.4242
P_{HW}	0.1171	<0.0004**	0.0057*	0.0036*	0.0000*	0.0061*	<0.0001**	<0.0014**	0.0000**	<0.0004**	0.0000*
F_{IS}	0.2160	0.2314	0.1989	0.2167	0.2364	0.2085	0.2463	0.2199	0.1944	0.2012	0.1947
P_{sc}	0.1649	0.0151	0.0010	0.0244	0.0060	0.0555	0.4564	0.0634	<0.0003**	0.0009	0.0027
Over all loci											
H_e	0.0926	0.1385	0.1322	0.1284	0.1400	0.1229	0.1455	0.1513	0.1352	0.1074	0.1505
H_o	0.1005	0.1484	0.1352	0.1019	0.1321	0.1020	0.1426	0.1455	0.0900	0.1074	0.1069
$P_{0.95}$	0.2000	0.3000	0.3000	0.3000	0.3000	0.3000	0.3000	0.3000	0.3000	0.3000	0.3500
$P_{0.99}$	0.3000	0.3000	0.3000	0.3000	0.3000	0.3000	0.3000	0.3000	0.3500	0.3000	0.3500
A_n	1.4500	1.5500	1.5500	1.5000	1.5000	1.4500	1.4500	1.5000	1.6500	1.5000	1.6000

H_o observed heterozygosity, H_e expected heterozygosity, P_{HW} probability value of significant deviation from Hardy–Weinberg equilibrium, F_{IS} fixation index, P_{sc} probability value of significant heterozygosity deficiency

* Significant ($P < 0.05$), ** significant after Bonferroni adjustment

Mean observed heterozygosity over all loci ranged from 0.090 (Mahanadi) to 0.148 (Ganga), and the mean number of alleles per locus was from 1.450 (Satluj, Brahmaputra, and Bihad) to 1.650 (Mahanadi).

Significant deviation was detected in 4 of the 77 tests performed to assess conformity to Hardy–Weinberg expectations, after application of sequential Bonferroni corrections to probability levels ($P < 0.05$). Both probability and score tests indicated significant deviation ($P < 0.0004$) of genotype proportions from Hardy–Weinberg expectations at two loci *XDH** (Mahanadi, Bhima, and Godavari) and *G6PDH** (Mahanadi). The results exhibited significant ($P < 0.05$) deficiency of heterozygotes (+ve F_{IS}) at those loci and localities where significant deviation from Hardy–Weinberg equilibrium was observed. Significant deviation at the *EST 3** locus observed in Mahanadi was not confirmed by the score test, and the locus had a small excess of heterozygotes (F_{IS} −0.025). The F_{IS} value did not deviate from zero significantly at this locus. No test for linkage disequilibrium was statistically significant ($P < 0.05$) for any pair of allozyme loci within each of the sample sites or when all the samples were considered together.

Population Structure

An exact test for population differentiation was performed to assess allelic homogeneity in natural populations of *L. calbasu*. Pairwise comparisons of samples from all the localities over all loci indicated that 50 of the 55 possible pairs had significant genetic heterogeneity ($P < 0.05$), and after the sequential Bonferroni correction, 30 pairs exhibited significant heterogeneity ($P < 0.00183$) (Table 5). Locus wise examination revealed that 34 pairs of sample localities had significant probabilities for genotype heterogeneity, at least at one locus after sequential Bonferroni adjustment.

The mean F_{ST} value across all populations and all allozyme loci was 0.0515 (pairwise comparisons in Table 6). The river Satluj exhibited significant divergence from other rivers, and F_{ST} ranged from 0.0523 (Ghaghra) to 0.221 (Mahanadi). Significant genetic divergence was observed between the Ganga samples and those of the other rivers, except Bhagirathi, Gomti, Ghaghra and Brahmaputra. Bhagirathi samples showed significant divergence with the samples of Ghaghra (F_{ST} 0.0189), Brahmaputra (0.067), Bihad (0.065), Tons (0.051), and Mahanadi (0.037). There was evidence of weak-to-moderate genetic differentiation among the *L. calbasu* subpopulations sampled from different rivers.

Bottleneck Analysis

The genotype data of *L. calbasu* were analyzed to detect if the population had undergone any genetic bottlenecks in the recent past. Significant probabilities for excess heterozygosity, beyond that expected under mutation-drift equilibrium, indicated a reduction in population size in the sample. Under the infinite allele model of mutation, significant ($P < 0.05$) heterozygosity excess (Wilcoxon's test) was detected in the samples from the Ghaghra ($P = 0.016$), Brahmaputra (0.016), Bihad (0.008), Tons (0.008), and Godavari (0.0273).

Table 5 Exact test of allele homogeneity for all population pairs of *Labeo calbasu*

Serial no.	Population pair	Significant allelic homogeneity	*P* expect Over all loci
1.	Satluj & Ganga	*EST-2***, *XDH**	0.00135**
2.	Satluj & Bhagirathi	*EST-2***, *XDH***, *G6PDH**	0.00000**
3.	Satluj & Gomti	*EST-1**, *EST-2**, *XDH***	0.00098**
4.	Satluj & Ghaghra	*EST-2**, *XDH**	0.00993*
5.	Satluj & Brahmaputra	*EST-1**, *XDH**	0.00514*
6.	Satluj & Bihad	*EST-1**, *EST-2***, *GPDH-2**, *XDH**	0.00000**
7.	Satluj & Tons	*EST-2***, *XDH**	0.00010*
8.	Satluj & Mahanadi	*EST-2***, *XDH***, *G6PDH***	<0.0001**
9.	Satluj & Bhima	*EST-2**, *GPDH-2**, *XDH***	0.00001**
10.	Satluj & Godavari	*EST-2***, *XDH***	<0.0001**
11.	Ganga & Bhagirathi	*G6PDH***	0.03680*
12.	Ganga & Gomti	*G6PDH**	0.12982
13.	Ganga & Ghaghra		0.88691
14.	Ganga & Brahmaputra	*EST-2**	0.02384*
15.	Ganga & Bihad	*EST-1***, *GPDH-2**	0.00028**
16.	Ganga & Tons	*XDH**, *G6PDH**	0.15994
17.	Ganga & Mahanadi	*EST-1**, *EST-2***, *XDH***, *G6PDH***	0.00000**
18.	Ganga & Bhima	*XDH***	0.02018*
19.	Ganga & Godavari	*GPI**, *XDH**	0.00805*
20.	Bhagirathi & Gomti	*EST-1**, *XDH**	0.02902*
21.	Bhagirathi & Ghaghra	*G6PDH***	0.00351*
22.	Bhagirathi & Brahmaputra	*G6PDH***	0.00000**
23.	Bhagirathi & Bihad	*EST-1***, *GPDH-2***, *XDH**, *G6PDH***	<0.0001**
24.	Bhagirathi & Tons	*XDH**, *G6PDH***	0.00009**
25.	Bhagirathi & Mahanadi	*EST-1**, *EST-2***	0.00064**
26.	Bhagirathi & Bhima	*GPDH-2**	0.12001
27.	Bhagirathi & Godavari	*EST-2**, *G6PDH**	0.00911*
28.	Gomti & Ghaghra	*EST-1***, *G6PDH**	0.00445*
29.	Gomti & Brahmaputra		0.08862
30.	Gomti & Bihad	*GPDH-2**, *G6PDH**	0.00551*
31.	Gomti & Tons	*XDH**, *G6PDH**	0.00267*
32.	Gomti & Mahanadi	*EST-2***, *XDH***, *G6PDH***	0.00000**
33.	Gomti & Bhima	*XDH**	0.03584*
34.	Gomti & Godavari	*EST-2**, *XDH**	0.00173**
35.	Ghaghra & Brahmaputra	*EST-1***, *XDH**	0.00183**
36.	Ghaghra & Bihad	*EST-1***, *GPDH-2**	<0.0001**
37.	Ghaghra & Tons	*EST-1**, *XDH**	0.01545*
38.	Ghaghra & Mahanadi	*EST-1***, *EST-2***, *XDH***, *G6PDH***	<0.0001**
39.	Ghaghra & Bhima	*XDH***	0.00017**
40.	Ghaghra & Godavari	*EST-2***, *GPI**, *XDH***	0.00001**

Table 5 continued

Serial no.	Population pair	Significant allelic homogeneity	*P* expect Over all loci
41.	Brahmaputra & Bihad	*EST-2**	0.04990*
42.	Brahmaputra & Tons	*EST-2**, *XDH***	0.00028**
43.	Brahmaputra & Mahanadi	*EST-2***, *GPI***, *XDH***, *G6PDH***	<0.0001**.
44.	Brahmaputra & Bhima	*EST-1**, *XDH***	<0.0001**
45.	Brahmaputra & Godavari	*EST-2***, *XDH***	<0.0001**
46.	Bihad & Tons	*EST-1***, *XDH***	0.00092**
47.	Bihad & Mahanadi	*EST-1**, *EST-2***, *GPDH-2***, *XDH***, *G6PDH***	<0.0001**
48.	Bihad & Bhima	*EST-1***, *XDH***, *G6PDH**	0.00000**
49.	Bihad & Godavari	*EST-1***, *EST-2***, *GPI***, *GPDH-2**, *XDH***	0.00000**
50.	Tons & Mahanadi	*EST-2***, *GPDH-2**, *G6PDH***	<0.0001**
51.	Tons & Bhima	*XDH**, *G6PDH**	0.01863*
52.	Tons & Godavari	*EST-2**, *GPI**	0.02023*
53.	Mahanadi & Bhima	*EST-1**, *EST-2***, *GPDH-2**, *G6PDH**	0.00000**
54.	Mahanadi & Godavari	*G6PDH***	0.00757*
55.	Bhima & Godavari	*EST-2**	0.01913*

* Significant ($P < 0.05$), ** significant after Bonferroni adjustment

Discussion

The present study reports the distribution and pattern of genetic variation in a natural population of *L. calbasu* estimated from allozyme markers. Observed heterozygosity values were relatively high (0.090–0.148) compared with the values described for many freshwater fish species (Ward et al. 1994; −0.046) and the range (0.05–0.07) for teleost species (Nevo 1978). For fresh water fish species with wide distribution, however, higher observed heterozygosity is not uncommon. In another Indian major carp, *Cirrhinus mrigala*, Chauhan et al. (2007) reported a range of 0.105–0.135 for observed heterozygosity. Among European cyprinids, the common, widespread roach *Rutilus rutilus* (L.) exhibits a high degree of variability (H_e 0.097–0.124; Bouvet et al. 1991), as did *Leuciscus cephalus* from the Central European Drainages (0.074–0.113; Hanfling and Brandl 1998).

Allele frequencies for the allozyme loci confirmed the Hardy–Weinberg expectation, except at *XDH** (Mahanadi, Bhima, and Godavari) and *G6PDH** (Mahanadi), after the probability level ($P = 0.05$) was adjusted for Bonferroni corrections. To avoid Type I errors, Lessios (1992) suggested application of standard or sequential Bonferroni corrections to critical probability values ($P = 0.05$). The score test was used in this study. It is more powerful than the probability test for Hardy–Weinberg conformity tests. Both probability and score test demonstrated concordant results for these loci and were tested against specific alternate hypotheses of heterozygote deficiency. The deviation indicated by a probability test at the *EST3** locus in the Mahanadi samples was not considered, as it was not confirmed when tested against the specific alternate hypothesis of excess

Table 6 Pairwise F_{ST} values on the basis of allozymes in *Labeo calbasu*

Population	Ganga	Bhagirathi	Gomti	Ghaghra	Brahmaputra	Bihad	Tons	Mahanadi	Bhima	Godavari
Satluj	0.08176*	0.13150*	0.08258*	0.05228*	0.06547*	0.10038*	0.08896*	0.22065*	0.12658*	0.14966*
Ganga		0.01187	0.00407	−0.00670	0.01907	0.01145*	0.01490*	0.07245*	0.02863*	0.01871*
Bhagirathi			0.01959	0.01886*	0.06728*	0.06526*	0.05103*	0.03699*	0.02492	0.01972
Gomti				0.01379	0.01469	0.02037*	0.04009*	0.10695*	0.02645	0.04629*
Ghaghra					0.01693	0.01886*	0.01122*	0.08407*	0.03554*	0.03150*
Brahmaputra						0.01626	0.04199*	0.16104*	0.08648*	0.08867*
Bihad							0.01531*	0.11395*	0.04902*	0.04892*
Tons								0.07619*	0.01990	0.01534
Mahanadi									0.07766*	0.01456*
Bhima										0.02072

* Significant probability

heterozygosity through the score test. Excess heterozygosity indicated by the F_{IS} value was also not found to be significant. The inbreeding coefficient (F_{IS}) is used to determine if the population has an excess or deficit of heterozygotes (Wright 1965). The loci and samples exhibiting significant deviation from Hardy–Weinberg expectation had significant deficiency of heterozygotes indicated by positive F_{IS} values. The heterozygote deficit as represented in the results could be due to several factors, such as inbreeding, nonrandom mating, reduction in effective breeding population, and existence of subpopulations, or the Wahlund effect (Garcia de Leon et al. 1997). In natural populations, concordance to the Hardy–Weinberg principle means that the allele frequencies are stable from one generation to the next. This is subject to fulfillment of certain assumptions, like sexual reproduction, random mating, and large effective breeding population with equal sex ratio (Hartl and Clark 1997). This also implies that interactive evolutionary forces like migration, mutation, and selection have a negligible effect or no effect on population allele frequency. Nonconformity to Hardy–Weinberg equilibrium indicates violation of these assumptions in natural populations.

The bottleneck analysis clearly pointed out that the *L. calbasu* natural population in some rivers has undergone genetic bottleneck or reduction in its effective breeding population. Gene diversity excess in allozyme loci was demonstrated under the infinite allele model for the samples from the Ghaghra, Brahmaputra, Bihad, Tons, and Godavari rivers. Populations that have experienced a recent reduction in their effective population size exhibit a correlative reduction of the allele numbers (k) and gene diversity at polymorphic loci (Cornuet and Luikart 1996). The reduction in allele numbers is faster than that of the gene diversity, and thus the observed gene diversity is higher than that expected under mutation-drift equilibrium for a few generations.

Pairwise genetic homogeneity tests and F_{ST} values clearly demonstrated genetic structuring of the wild *L. calbasu* populations from different rivers. Wright (1965) demonstrated the partitioning of genetic variation and suggested F_{ST} as a parameter that indicated the proportion of genetic variation, that can be attributed to genetic differentiation processes between the co-specifics from two localities. The pattern and distribution of genetic variation in the *L. calbasu* wild population depicted a weak-to-high level of genetic differentiation between the rivers studied. Ward et al. (1994) reviewed 49 freshwater fish species and observed an F_{ST} of 0–74%, with a mean of 22.2%. That study observed that 23 of the 49 species had genetic differentiation (F_{ST}) in the range 0–10% (Ward et al. 1994). Nevertheless, the mean F_{ST} value (0.0515) for all the samples combined indicated only a moderate level of genetic differentiation in the populations of *L. calbasu*. At least nine pairs, however, exhibited high F_{ST} values of more than 10%, with a maximum of 22% (Satluj–Mahanadi), belonging to different river basins. AMOVA (2-tier hierarchical) also confirmed that most of the genetic divergence was between rivers belonging to different river basins, rather than within a river basin. Of the 13.37% of genetic variation found between the populations, 10.77% was among the groups (river basins) and 2.60% within the groups.

In the Ganges river system, northern side tributaries like Gomti, Ghaghra and the Ganga main channel (including Yamuna samples) did not exhibit any significant

genetic divergence. Within the Ganges river system, allozyme analysis revealed low genetic differentiation (1.9%). The three associated rivers of the Ganges (the Ganga main channel, Gomti and Ghaghra) appear to share a common gene pool of *L. calbasu*. This might be possible via connections associated with a common floodplain and therefore likely dispersal of fish from the Ganga main channel to these tributaries. It was interesting that only Bhagirathi samples exhibited significant divergence from other localities in the Ganges. The divergence of Bhagirathi samples from the southern side tributaries of the Ganges (Tons and Bihad) was higher than that of the northern side tributaries (5–6%). The Bhagirathi–Hooghly drainage is the most western stretch of the Ganga delta. The river was the main channel of the Ganges until the river changed its course in the fifteenth century, leading to silting and disconnecting of the Bhagirathi–Hooghly (ECAFE 1966; Bhattacharya 1973). The river was rejuvenated via a feeder canal only in 1975. It is likely that the alteration of allele frequencies and genetic differentiation of *L. calbasu* populations in the Bhagirathi occurred during this period of restricted migration. Similar findings were seen in another Indian major carp, *Cirrhinus mrigala*, where the lack of genetic divergence in the Ganga population was evident with the exception of Bhagirathi samples that had small but significant genetic differentiation (Chauhan et al. 2007). The significant genetic divergence between the Tons and Bihad rivers is of peculiar interest. The two rivers originate from the central plateau and join before the Tons flows into the Ganga river system. Significant genetic divergence (0.04) indicated that lack of gene flow between the two populations could possibly be due to geographic barriers that might contribute to the genetic partitioning of the populations between the two tributaries. *Labeo calbasu* is a detritus bottom feeder (Chondar 1999), and the population exchange could be interrupted by the intermittent rocky and mountainous segments between the Tons and Bihad rivers (Lakshmanan 1970). The significant genetic divergence even in some of the tributaries of the Ganga, Bihad, and Tons indicates the likelihood of more genetic stocks, particularly in associated rivers and tributaries of the Ganga.

In spite of fragmentation, low-to-moderate genetic divergence among wild *L. calbasu* populations of the Satluj (Indus basin) and the rivers of the Ganges (Ganga basin) might be the result of possible gene flow among populations via connectivity across common floodplains and changes in the courses of associated rivers. Remote sensing and archeological evidence had already suggested that the seasonal Ghaggar river basin, located between the Indus and Ganga basins, was a remnant of the ancient perennial Saraswati river, with the present-day Satluj river (Indus river system) as its northwest tributary and the Yamuna river (Ganga river system) as the northeast tributary (Puri and Verma 1998; Lal 2002).

Data on partitioning of genetic variability detected by seven polymorphic allozyme loci suggested the existence of at least nine genetic stocks of *L. calbasu* across its native distribution in India. This study provides the first baseline information on pattern and distribution of genetic variation in the wild populations of *L. calbasu* and has applications in planning conservation and management strategies of the natural populations of *L. calbasu*.

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