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Genetic diversity of Indian Major Carp, *Labeo calbasu* (Hamilton, 1822) populations inferred from microsatellite loci

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ABSTRACT

Genetic variation was assessed in 367 samples of *Labeo calbasu* from twelve riverine locations across India using nine polymorphic microsatellite loci. The mean value of the observed heterozygosity (Hobs.) after correction for null allele ranged from 0.50 (loci *R3** and *Lr38** in Satluj) to 1.00 (*Lr29** in Bhagirathi) whereas mean number of alleles per locus ranged from 4.66 (Narmada) to 10.11 (Ghaghra). The genetic data corrected for possible null alleles, analyzed with score test did not reveal significant deviation from HW equilibrium at any locus in any population after probability level was adjusted for Bonferroni corrections. Pair wise F_{st} and allelic homogeneity tests indicated distinct population structure in wild *L. calbasu*. F_{st} for all the samples combined over all loci was found to be 0.035 suggesting that 3.5% of the total variation was due to genetic differentiation. Analysis of Molecular Variance (AMOVA) indicated that most of the genetic variability was within populations from the same geographical locations.

1. Introduction

Labeo calbasu (Hamilton, 1822) commonly known, as 'kalabons' is primarily a riverine fish, but also well established in natural lakes and in several artificial reservoirs and ponds. It is also called 'tank fish' for its extensive utilization in stocking tanks for culture. The fish is native inhabitant of some South Asian countries ranging from Bangladesh, Myanmar, Nepal, and India up to Pakistan (Reddy, 2005). In India, the fish is common in catches from the rivers of Indo-Gangetic plains, besides some rivers from peninsular India such as Mahanadi, Godavari and Krishna supporting important proportion of capture fishery resources of India. *L. calbasu* is considered as good table fish for its less intermuscular bones. Due to faster growth rate, high consumer preferences, as well as high market price, this fish is of interest for aquaculturists. However, there has been a distinct reduction in its population over the years (Chondar, 1999; Chaudhary and Jugal, 2003; Mahapatra, 2003).

Conservation of genetic stocks is important as the genetic diversity is the outcome of thousands of years of evolutionary process (Swaminathan, 1984). The genetic variability data based on molecular markers could be a crucial input to identify the evolutionary significant units within the wild populations of a species and useful for scientific planning of the breeding programs for genetic improvement. Besides, this may also be applicable in adopting effective management strategies as well as registration of fish genetic stocks. Microsatellites are proven tool for assessing genetic diversity and has been used

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extensively in variety of vertebrates (Chistiakov et al., 2006). These are co-dominant, short tandem repeats ranging from 1 to 6 base pairs and flanked by unique DNA sequences that make non-coding part of genome (Liu and Cordes, 2004).

Despite high commercial significance of *L. calbasu,* there is no information available on the pattern and distribution of genetic variation in the natural populations of the species. The DNA based genetic studies in *L. calbasu* have been limited to identification of microsatellite markers where 14 loci were found polymorphic (Singh et al., 2008). In the present study, microsatellite markers were used to assess the pattern of distribution of genetic variability in wild *L. calbasu* populations across its natural range of distribution in India.

2. Material and methods

2.1. Fish samples

Blood, liver and muscle samples from *L. calbasu* individuals ($n = 367$) were collected from commercial catches from different river systems located in different geographical areas of India i.e. Ganga, Ghaghra, Brahmaputra, Satluj, Bhagirathi, Gomti, Narmada, Mahanadi, Tons, Bihad, Krishna and Godavari. The latitude and longitudes of all the collection sites are illustrated in Table 1. Collections were done at actual fishing sites during June 2001 to December 2007 and the riverine locations were chosen to cover geographically distant populations of *L. calbasu* (Fig. 1a and b). The blood samples were collected through caudal puncture and were fixed in 95% ethanol in 1:5 ratios and stored at 4 °C till use. Genomic DNA was extracted from blood through a protocol modified from Ruzzante et al. (1996), using phenol-chloroform method. The modifications made in the original protocol included change in amount (500 μl) and composition (0.5M Tris HCl, 0.5M $Na_2EDTA.2H_2O$) of the lysis buffer, (incubation time 37 °C for 16 h) and precipitation (with absolute alcohol).

2.2. Genotyping

Nine polymorphic microsatellite loci identified in *L. calbasu* and found suitable for genetic diversity analysis (Singh et al., 2008) were used for genotyping purpose in the present study. PCR products were resolved through vertical non-denaturing polyacrylamide (19:1 acrylamide : bisacrylamide) gels electrophoresis (size 10 × 10.5 cm, Amersham Biosciences Ltd.). A non-denaturing electrophoresis system has been found to provide the same resolution as that obtained with denaturing acrylamide gels and silver staining with the additional advantage of ease of use for analyzing large sample sizes (Wang et al., 2003). Moreover, Bovo et al. (1999), had already demonstrated that non denaturing electrophoresis is not responsible for spurious or multiple bands in microsatellite analysis.

2.3. Data analysis

Individual fish genotypes at all nine microsatellite loci were determined. The data were then 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 heterogenous were later combined (twelve sets) for further analysis for estimating

Table 1
Sample size, location, and year of collections of *L. calbasu* from different rivers in India.

River system	River	Location	Location (lat. and long.)	Year of collection	Sample size (*N*)
The Indus	Satluj	Heri ke patan, Punjab	31°13′N, 75°12′E	June, 2001	13[a]
The Ganges	Ganga	Bijnore, UP	29°23′N, 79°11′E	May, 2002	52[b]
	Bhagirathi	Farraka, WB	24°5′N, 88°06′E	June, 2001	16[c]
	Gomti	Sultanpur, UP	26°16′N, 82°4′E	June, 2001	20[d]
	Ghaghra	Ajjaipur, UP	27°34′N, 80°41′E	Dec., 2000 June 2001 Jan., 2002	12[e]
	Gerua	Katarniya ghat,UP	32°19′N, 75°30′E	Dec., 2000	24[e]
	Sharda	Nanaksagar, UT		Dec, 2007	11[e]
		Begul		Dec, 2007	05[e]
	Bihad	Rewa, MP	24°31′N, 81°17′E	April 2004	43[f]
	Tons		24°38′N, 81°25′E		15[g]
	Brahmputra	Kalangpar	26°11′N, 91°47′E	Jan, 2001	15[h]
		Assam		Dec, 2007	05[h]
The Narmada		Bhopal	23°14′N, 77°23′E	Jan, 2005	16[i]
The Mahanadi	Mahanadi	Sambhalpur	23°14′N, 77°23′E		45[j]
		Daspur/Goyalbank			04[j]
The Krishna	Bhima	Pune	18°31′N, 73°50′E		18[k]
The Godavari	Godavari	Adilabad	18°57′N, 79°06′E	Oct, 2002	53[l]
		Manthini			
				Total	**367**

lat. – Latitude; long. – Longitude.
Superscripts indicate multiple data sets within rivers or neighboring localities that were pooled after testing for absence of heterogeneity.

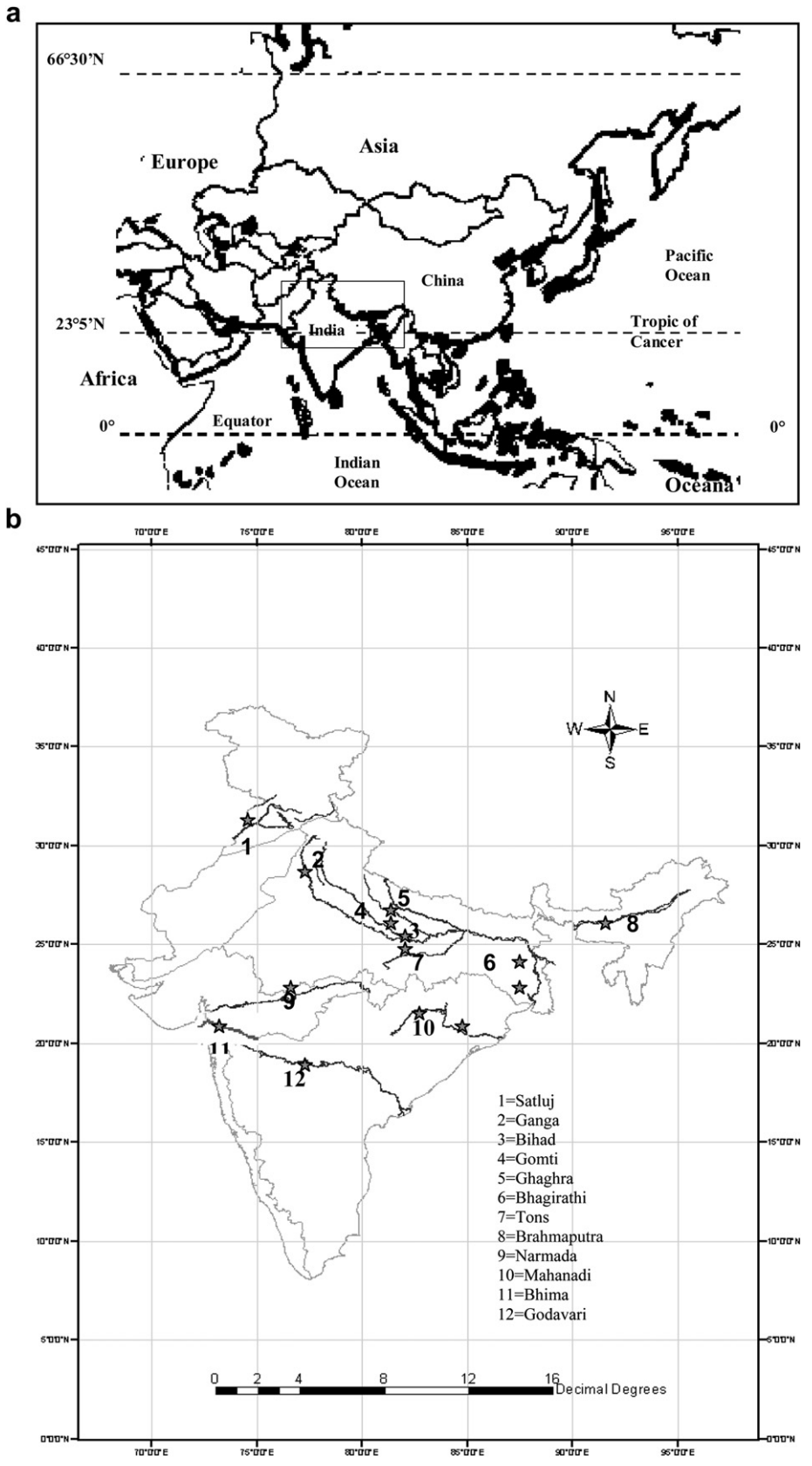

Fig. 1. (a) General map of the region, study area is located within the box. (b) Locations of sample collections across different river basins for population structure study of *Labeo calbasu*.

genetic variation and differentiation parameters. A locus was considered polymorphic, if the frequency of the most common allele was less than or equal to 0.99 (Hartl and Clark, 1997). Allele frequencies and heterozygosity (observed and expected) values were calculated using Genetix version 4.05 software (Belkhir et al., 1997). Tests for conformity to Hardy–Weinberg expectations (probability test) and linkage disequilibrium were undertaken in Genepop ver. 3.3d software (Raymond and Rousset, 1995a). Genetic heterogeneity of all populations and pair wise localities were determined using an exact test that assumes random samples of genotypes (Genepop ver. 3.3d, Genotype differentiation test) (Raymond and Rousset, 1995b). This test is performed on genotype tables and possible non independence of alleles within genotypes does not affect test validity (Goudet et al., 1996). The null hypothesis tested was, that the genotype distribution was identical across all populations. Fixation indices based on an infinite allele model (Kimura and Crow, 1964) and a stepwise mutation model (Kimura and Ohta, 1978) were estimated to determine the extent of population subdivision among samples. For the former, Genetix ver. 4.05 software was used to estimate *F*-statistics (Wright, 1951) computed as estimators θ, *F* and *f* of Weir and Cockerham (1984). Probability of θ significantly deviating from zero was calculated using 1000 bootstraps. Under the SMM, model, estimates of Rst (Slatkin, 1995) was made using the Genepop ver. 3.3d software. To correct for multiple simultaneous comparisons, sequential Bonferroni corrections were applied using a global significance level of 0.05 (Lessios, 1992). Microsatellite genotype data were checked for possible null alleles using the software FreeNA (Chapuis and Estoup, 2007) and all genetic analyses were done before and after corrections of genotype data for null alleles. The hierarchical analysis was carried out using analysis of molecular variance (AMOVA) in the Arlequin 2000 package (Excoffier et al., 2005). AMOVA estimations were done at three levels of the population structure. within population, among subpopulations within river basins and among river basins.

To detect whether the *L. calbasu* populations have experienced a recent reduction in the effective population size or a genetic bottleneck, two different approaches were followed. In the first approach based on heterozygosity excess, Wilcoxon test were employed under Two-phase mutation model (SMM and IAM). The second approach was the graphical representation of the mode-shift indicator (Luikart and Cornuet, 1998). These analyses were conducted using Bottleneck v1.2.02 software (Cornuet and Luikart, 1996).

3. Results

3.1. Genetic diversity within samples

A total of 367 individual samples of *L. calbasu* from twelve riverine sites (Table 1, Fig. 1a and b) were genotyped for 9 polymorphic microsatellite loci and 106 alleles were detected. The allele frequencies at these loci (*MFW-11**, *R1**, *R3**, *R12**, *Lr28**, *Lr29**, *Lr38**, *Lro23** and *Lro25**) exhibited considerable variation in all the populations and some loci also had private alleles (Table 2). The total number of alleles at each locus ranged from 8 to 17 (mean 11.7). Heterozygosity values, observed (Hobs.) and expected (Hexp.), for *L. calbasu* from 12 collection sites, at each polymorphic microsatellite locus are given in Table 3. The mean values for observed heterozygosity following null allele corrections ranged from 0.5 (loci *R3** and *Lr38** in river Satluj) to 1.00 (*Lr29** in Bhagirathi) where as mean number of alleles per locus ranged from 4.66 (Narmada) to 10.11 (Ghaghra).

3.2. HWE and linkage disequilibrium

No test for linkage disequilibrium was found statistically significant for any pair of loci across all the populations after probability levels were adjusted for Bonferroni corrections. The results confirmed that the microsatellite loci under analysis were genetically independent. Probability tests showed significant deviation from Hardy–Weinberg equilibrium at locus *Lr38** (Ganga and Bhagirathi), locus *Lro23** (Ghaghra, Ganga, Bihad and Mahanadi), locus *Lr29** (Ghaghra) after the probability level ($P = 0.05$) was adjusted for sequential Bonferroni corrections (Table 4). However, score test did not confirm significant deviation from Hardy–Weinberg equilibrium expectations for these locality samples except Ganga and Ghaghra. At these loci, significant ($P < 0.05$) heterozygote deficiency or excess was evident from the positive and negative F_{is} value, respectively.

The estimates for the null alleles (FreeNA) indicated their presence at some loci in a few riverine samples e.g. locus *Lro23** (Satluj, Ganga and Bhagirathi), *R1** (Ghaghra, Tons and Brahmaputra) and *Lr38** (Ganga, Bhagirathi and Brahmaputra).

Table 2
List of microsatellite private alleles in *Labeo calbasu*.

Locus	Allele (bp)	Frequency	Collection site
*R1**	102	0.100000	Brahmaputra
*Lro23**	186	0.125000	Tons
*Lro23**	214	0.065789	Bihad
*Lro23**	210	0.045455	Satluj
*Lro25**	092	0.038462	Ghaghra
*Lr29**	160	0.010638	Ganga

bp – base pairs.

Table 3
Heterozygosity values and mean number of alleles (Na) before (row above) and after (row below) correction for null alleles in *L. calbasu*.

S. no	Locus	Satluj	Gomti	Ghaghra	Ganga	Bhagirathi	Narmada	Bihad	Tons	Godavari	Mahanadi	Brahmaputra	Bhima
1.	*R1**												
	Hobs.	0.5000	0.5556	0.6170	0.7381	0.6667	0.7143	0.4750	0.3846	0.6800	0.6667	0.3000	0.5294
		0.8333	0.6667	0.7872	0.7619	0.8333	0.7143	0.5500	0.9231	0.7400	0.6667	0.7500	0.6471
	Hexp.	0.7326	0.6559	0.7537	0.7202	0.7847	0.7653	0.5297	0.7899	0.7342	0.6916	0.6787	0.6246
		0.7743	0.6914	0.7836	0.7208	0.8090	0.7653	0.5650	0.8107	0.7504	0.6916	0.7538	0.6678
2.	*R12**												
	Hobs.	0.5455	0.7000	0.7674	0.8723	0.7500	0.7500	0.6897	0.6923	0.8039	0.7778	0.6842	0.5833
		1.0000	0.8000	0.8837	0.8723	0.8333	0.7500	0.7931	0.6923	0.8824	0.8611	0.7368	0.8333
	Hexp.	0.7810	0.8075	0.8448	0.8694	0.7986	0.5313	0.7836	0.7071	0.8403	0.8364	0.7562	0.7917
		0.8099	0.8287	0.8548	0.8694	0.8021	0.5313	0.8044	0.7071	0.8470	0.8453	0.7770	0.8264
3.	*Lr28**												
	Hobs.	0.5000	0.7895	0.6957	0.7021	0.6429	0.3333	0.8286	0.7333	0.8043	0.8378	0.7895	0.6875
		0.80000	0.8421	0.8261	0.7872	0.8571	0.8889	0.8286	0.8000	0.8043	0.8919	0.7895	0.6875
	Hexp.	0.6850	0.8698	0.8251	0.7526	0.8240	0.8025	0.7824	0.7711	0.8119	0.8364	0.7562	0.6973
		0.7400	0.8726	0.8429	0.7675	0.8444	0.8086	0.7824	0.7756	0.8119	0.8393	0.7562	0.6973
4.	*Lr38**												
	Hobs.	0.5000	0.6842	0.7500	0.6939	0.3846	0.4444	0.7442	0.7143	0.7708	0.7959	0.6000	0.7500
		0.5000	0.7895	0.7955	0.8571	0.8462	0.8889	0.8372	0.7143	0.8333	0.8163	0.8500	0.7500
	Hexp.	0.6875	0.8324	0.8293	0.8517	0.7899	0.7593	0.8167	0.7168	0.8442	0.8186	0.8025	0.8611
		0.6875	0.8518	0.8386	0.8713	0.8254	0.8025	0.8302	0.7168	0.8548	0.8219	0.8263	0.8611
5.	*Lro23**												
	Hobs.	0.4545	0.8750	0.7609	0.5870	0.5385	0.6250	0.7895	0.7500	0.7568	0.7347	0.8750	0.6667
		0.7273	0.8750	0.8913	0.8043	0.7692	0.6250	0.7895	0.7500	0.7568	0.7347	0.8750	0.8333
	Hexp.	0.7149	0.8047	0.8542	0.7658	0.7781	0.5938	0.8206	0.7578	0.8053	0.7472	0.8262	0.7882
		0.7562	0.8047	0.8625	0.7999	0.8195	0.5938	0.8206	0.7578	0.8053	0.7472	0.8262	0.7917
6.	*Lro25**												
	Hobs.	0.6667	0.8824	0.8205	0.7209	0.6923	0.7778	0.7027	1.0000	0.8333	0.8293	0.7368	0.5714
		0.6667	0.8824	0.8974	0.9070	0.8462	0.7778	0.7838	1.0000	0.8333	0.8780	0.7368	0.7857
	Hexp.	0.7037	0.8304	0.8918	0.8734	0.8728	0.8086	0.7812	0.8050	0.8965	0.8730	0.8310	0.7704
		0.7037	0.8304	0.9004	0.8875	0.8905	0.8086	0.7988	0.8050	0.8965	0.8751	0.8310	0.8112
7.	*Lr29**												
	Hobs.	0.5000	0.7000	0.7209	0.8936	0.8462	0.2500	0.7429	0.8462	0.7600	0.8182	0.8421	0.7500
		0.8750	0.7000	0.8837	0.8936	1.0000	0.7500	0.8286	0.8462	0.8600	0.8182	0.8421	0.7500
	Hexp.	0.7500	0.8100	0.8326	0.8282	0.8669	0.6563	0.8514	0.7485	0.8370	0.8257	0.8310	0.8359
		0.8047	0.8100	0.8464	0.8282	0.8698	0.7500	0.8616	0.7485	0.8486	0.8257	0.8310	0.8359
8.	*R-3**												
	Hobs.	0.5000	0.7500	0.6250	0.6939	0.4615	0.1250	0.6842	1.0000	0.7500	0.7292	0.7895	0.7647
		0.5000	0.7500	0.8333	0.6939	0.6154	0.1250	0.7895	1.0000	0.8654	0.7292	0.7895	0.7647
	Hexp.	0.6000	0.7825	0.7648	0.6868	0.6509	0.1172	0.7199	0.8160	0.7949	0.7461	0.7742	0.7682
		0.6000	0.7825	0.7884	0.6868	0.7041	0.1172	0.7375	0.8160	0.8082	0.7461	0.7742	0.7682
9.	*MFW11**												
	Hobs.	0.6667	0.5789	0.7317	0.7381	0.4615	0.0000	0.7353	0.4667	0.8039	0.7955	0.7000	0.5556
		0.6667	0.6842	0.7317	0.7381	0.8462	0.6667	0.7353	0.7333	0.8039	0.7955	0.7000	0.5556
	Hexp.	0.6420	0.6482	0.7356	0.7208	0.7278	0.4444	0.7470	0.6578	0.8049	0.7828	0.7113	0.6528
		0.6420	0.6773	0.7356	0.7208	0.7929	0.6111	0.7470	0.7200	0.8049	0.7828	0.7113	0.6528
Over all loci													
	Hobs.	0.5370	0.7239	0.7210	0.7378	0.6049	0.4466	0.7102	0.7319	0.7737	0.7761	0.7019	0.6510
		0.7299	0.7766	0.8367	0.8128	0.8274	0.6874	0.7706	0.8288	0.8199	0.7991	0.7855	0.7341
	Hexp.	0.6996	0.7824	0.8147	0.7854	0.7882	0.6087	0.7592	0.7522	0.8188	0.7953	0.7742	0.7545
		0.7243	0.7944	0.8281	0.7947	0.8175	0.6432	0.7720	0.7619	0.8253	0.7972	0.7874	0.7680
	Na	5.1111	7.3333	9.2222	9.1111	7.0000	4.2222	6.6667	6.1111	8.4444	7.7778	7.1111	6.4444
		5.6667	7.8889	10.1111	9.6667	8.0000	4.6667	7.3333	6.4444	9.0000	8.2222	7.4444	6.8889

Hobs. – observed heterozygosity, Hexp. – expected heterozygosity, Na – number of alleles per locus.

Table 4
P_{HW}, and P_{score} values for eight polymorphic microsatellite loci in *Labeo dero* from nine riverine sites.

S. no	Locus	Satluj	Gomti	Ghaghra	Ganga	Bhagir	Narmada	Bihad	Tons	Godav	Mahan	Bputra	Bhima
1.	*R1**												
	P_{HW}	0.0788	0.2209	0.0010**	0.0957	0.4128	0.0262*	0.2344	0.0016**	0.0152*	0.0255*	0.0000**	0.4363
		0.8746	0.5580	0.1095	0.1394	0.9120	0.0276*	0.5609	0.8182	0.0580	0.0250*	0.1684	0.8459
	Fis	+0.356	+0.147	+0.192	−0.013	+0.193	+0.143	+0.116	+0.542	+0.084	+0.048	+0.575	+0.182
		−0.033	+0.064	+0.006	−0.045	+0.013	+0.143	+0.039	−0.099	+0.024	+0.048	+0.031	+0.061
	P_{score}	0.0082*	0.1550	0.0031*	0.8830	0.1367	0.3022	0.0882	0.0007*	0.1346	0.3358	0.0024*	0.1456
		0.4817	0.3566	0.7106	0.2951	0.5629	0.3001	0.1893	0.3313	0.2776	0.3469	0.5070	0.3704
2.	*R12**												
	P_{HW}	0.0324*	0.1570	0.3040	0.0000**	0.0862	1.0000	0.0935	0.1225	0.0000**	0.0020**	0.0077*	0.0017**
		0.6331	0.4172	0.8626	0.0000**	0.1894	1.0000	0.4669	0.1241	0.0009*	0.0143*	0.0341*	0.0715
	Fis	+0.344	+0.158	+0.103	+0.007	+0.104	−0.286	+0.137	+0.061	+0.053	+0.084	+0.122	+0.303
		−0.189	+0.060	−0.022	+0.007	+0.005	−0.286	+0.032	+0.061	−0.032	−0.005	+0.079	+0.035
	P_{score}	0.0048*	0.1177	0.0000**	0.3501	0.1135	0.5714	0.1063	0.1596	0.0230*	0.0177*	0.1693	0.0340*
		0.1623	0.2888	0.3974	0.3430	0.6479	0.5714	0.4072	0.1553	0.5564	0.6941	0.1967	0.3167
3.	*Lr28**												
	P_{HW}	0.1765	0.2320	0.0771	0.0926	0.0067*	0.0018**	0.0673	0.0665	0.2201	0.0145*	0.6826	0.0359*
		0.9587	0.3840	0.6985	0.3847	0.2408	0.9922	0.0705	0.1190	0.2175	0.0546	0.6814	0.0366*
	Fis	+0.318	+0.119	+0.168	+0.078	+0.255	+0.622	−0.044	+0.083	+0.020	+0.012	−0.017	+0.046
		−0.029	+0.062	+0.031	−0.015	+0.022	−0.041	−0.044	+0.003	+0.020	−0.049	−0.017	+0.046
	P_{score}	0.0219*	0.0767	0.0099*	0.0049*	0.0188*	0.0001**	0.2678	0.1212	0.3678	0.0291*	0.4292	0.2173
		0.5220	0.2841	0.1944	0.5300	0.3171	0.5978	0.2696	0.4756	0.3654	0.3249	0.4363	0.2146
4.	*Lr38**												
	P_{HW}	0.3143	0.0097*	0.0842	0.0006**	0.0011**	0.1023	0.3846	0.2847	0.0327*	0.0269*	0.0547	0.0629
		0.3143	0.1151	0.2517	0.1099	0.7108	0.9940	0.7609	0.2847	0.1140	0.0437*	0.8385	0.0597
	Fis	+0.400	+0.204	+0.107	+0.195	+0.542	+0.462	+0.100	+0.041	+0.097	+0.038	+0.276	+0.172
		+0.400	+0.100	+0.063	+0.027	+0.015	−0.049	+0.003	+0.041	+0.036	+0.017	−0.003	+0.172
	P_{score}	0.2571	0.1001	0.1308	0.0078*	0.0028*	0.0133*	0.0510	0.2887	0.1209	0.1641	0.0041*	0.1468
		0.2571	0.2482	0.2054	0.3855	0.4709	0.4251	0.4452	0.2887	0.3386	0.2343	0.5732	0.1372
5.	*Lro23**												
	P_{HW}	0.1725	0.1426	0.0018**	0.0026**	0.0192*	1.0000	0.0040**	0.2869	0.0530	0.0000**	0.1705	0.3209
		0.9102	0.1442	0.0776	0.4896	0.4157	1.0000	0.0035*	0.2866	0.0497*	0.0000**	0.1708	0.7972
	Fis	+0.405	−0.055	+0.120	+0.244	+0.344	−0.018	+0.051	+0.077	+0.074	+0.027	−0.027	+0.196
		+0.086	−0.055	−0.022	+0.005	+0.101	+0.014	+0.051	+0.077	+0.074	+0.027	−0.027	−0.009
	P_{score}	0.0034*	0.4274	0.0008*	0.0000**	0.0214*	0.6614	0.1386	0.4967	0.2365	0.6682	0.4824	0.0069*
		0.3247	0.4494	0.5472	0.7136	0.2229	0.6370	0.1416	0.4905	0.2474	0.6665	0.4866	0.5982
6.	*Lro25**												
	P_{HW}	0.1354	0.4830	0.1081	0.0311*	0.0121*	0.0067*	0.0975	0.3368	0.0203*	0.3869	0.6006	0.0952
		0.1540	0.4748	0.5063	0.8368	0.2431	0.0072*	0.4141	0.3263	0.0167*	0.6775	0.5798	0.7299
	Fis	+0.111	−0.032	+0.093	+0.186	+0.245	+0.097	+0.114	−0.192	+0.082	+0.062	+0.140	+0.293
		+0.111	−0.032	+0.016	−0.010	+0.090	+0.097	+0.032	−0.192	+0.082	+0.009	+0.140	+0.068
	P_{score}	0.4007	0.6530	0.1117	0.0044*	0.0273*	0.4237	0.1786	0.1398	0.0802	0.0350*	0.1250	0.0713
		0.3943	0.6605	0.5652	0.4498	0.1310	0.4114	0.4016	0.1425	0.0843	0.2941	0.1329	0.4367
7.	*Lr29**												
	P_{HW}	0.0174*	0.3134	0.0002**	0.0485*	0.0327*	0.0286*	0.1468	0.6479	0.0202*	0.3487	0.0121*	0.5038
		0.6739	0.3348	0.0254	0.0468	0.2107	0.6600	0.6131	0.6468	0.2353	0.3570	0.0097	0.5135
	Fis	+0.391	+0.187	+0.146	−0.068	+0.064	+0.700	+0.142	−0.091	+0.102	+0.021	+0.014	+0.135
		−0.021	+0.187	−0.032	−0.068	−0.110	+0.143	+0.053	−0.091	−0.003	+0.021	+0.014	+0.135
	P_{score}	0.0352*	0.2163	0.0006*	0.7165	0.0292*	0.0286*	0.0373*	0.3791	0.0074*	0.2416	0.0962	0.0948
		0.5699	0.2099	0.3896	0.7145	0.2962	0.4876	0.3436	0.3709	0.6876	0.2437	0.0883	0.0963
8.	*R-3**												
	P_{HW}	0.1123	0.0235	0.0000**	0.1691	0.1675	–	0.0199	0.0021**	0.0016**	0.7325	0.0131	0.4266
		0.1073	0.0232*	0.0319*	0.1616	0.7306	–	0.1428	0.0028*	0.0223*	0.7222	0.0139*	0.4055
	Fis	+0.217	+0.067	+0.193	−0.000	+0.327	–	+0.063	−0.184	+0.066	+0.033	+0.007	+0.035
		+0.217	+0.067	−0.046	−0.000	+0.165	–	−0.057	−0.184	−0.061	+0.033	+0.007	+0.035
	P_{score}	0.2551	0.2683	0.0000**	0.3186	0.1198	–	0.0049*	0.1419	0.0086*	0.3162	0.5666	0.3823
		0.2465	0.2697	0.2621	0.3265	0.2723	–	0.2494	0.1368	0.1638	0.3110	0.5691	0.3781
9.	*MFW11**												
	P_{HW}	0.5919	0.0642	0.0300*	0.0284*	0.0154 *	0.2000	0.0412*	0.0122*	0.0160*	0.0000**	0.0644	0.1471
		0.5919	0.2370	0.0301*	0.0297*	0.5944	1.0000	0.0415*	0.3057	0.0165	0.0000**	0.0644	0.1557
	Fis	+0.020	+0.133	+0.018	−0.012	+0.400	+1.0000	+0.031	+0.322	+0.011	−0.005	+0.041	+0.177
		+0.020	+0.017	+0.018	−0.012	−0.027	+0.111	+0.031	+0.016	+0.011	−0.005	+0.041	+0.177

Table 4 (*continued*)

S. no	Locus	Satluj	Gomti	Ghaghra	Ganga	Bhagir	Narmada	Bihad	Tons	Godav	Mahan	Bputra	Bhima
	P_{score}	0.5524	0.1265	0.4070	0.3279	0.0325*	0.2000	0.2051	0.0168*	0.4592	0.8628	0.4114	0.2459
		0.5524	0.6443	0.4018	0.3160	0.5104	0.6000	0.2006	0.6647	0.4703	0.8630	0.4114	0.2407

Before (row above) and after (row below) correction for null allele; PHW: probability value of significant deviation from Hardy–Weinberg equilibrium, Fis: fixation index, Pscore: probability value of significant heterozygosity deficiency.
*Significant ($P < 0.05$).
**After Bonferroni correction ($P < 0.0040$) for PHW without null correction; After Bonferroni correction ($P < 0.0001$) for PHW with null correction.

Evidences of null alleles were found at locus *Lro25** (Ganga) where as at loci *Lr28**, *Lr29** and *R3** in Ghaghra. It is noteworthy here, that none of these nine loci showed evidences of null alleles in Rivers Gomti, Bihad, Godavari, Mahanadi and Bhima. The individual genotype data was corrected for the likely presence of null alleles at each locus and corrected genotype data were reanalyzed to compute the parameters of genetic variation and differentiation. After correction for null alleles, score test did not show significant probability for deviation from Hardy–Weinberg expectations at any of these loci in all the samples under study.

3.3. Genetic differentiation among samples

A total of 66 pair wise comparisons were performed and all possible pairs (Table 5) had significant genetic heterogeneity ($P < 0.05$), over all loci before and after corrections for null alleles. All these population pairs were found to display significant genotype heterogeneity at least at three loci.

Pair wise F_{st} values for population differentiation in 12 different collections of *L. calbasu* are given in Table 6. The mean F_{st} at all the loci across all collections was 0.035 which indicated that 3.5% of the total variability (between the populations) was due to inter-population differences. Fixation indices under SMM model (R_{st}) were found to be comparable with F_{st} values in pair wise comparisons of samples. AMOVA analysis revealed that as much as 98.77% of genetic variation was found within the populations whereas small variation was among the river basins (0.5%) and among populations within the groups (0.73%). After correction for null alleles, probabilities (Wilcoxon test) under Two-phase model (TPM) for excess of heterozygosity were significant, in *L. calbasu* samples from the rivers Ghaghra ($P = 0.0010$), Bihad ($P = 0.0010$), Tons ($P = 0.0244$), Godavari ($P = 0.0010$) and Brahmaputra ($P = 0.0186$) localities.

4. Discussion

The present study was aimed to determine genetic variation and population genetic structure of wild *L. calbasu* across the range of distribution. The range of observed heterozygosity (over all loci) from 0.4466–0.7761 was comparable to the range (0.10–1.00) reported for other cyprinid-fishes (Dimsoski et al., 2000; Tong et al., 2002; Bessert and Orti, 2003). The observed heterozygosity also agreed to the mean value reported (0.46 ± 0.34) for freshwater fishes (DeWoody and Avise, 2000).

The determination of inbreeding coefficient (F_{is}) through partitioning of genetic variability as suggested by Wright (1965) and Weir and Cockerham (1984) has been widely used to determine if the population has excess or deficit of heterozygotes. Before correction for the null alleles in microsatellite analysis, F_{is} values were found to be positive in different samples at loci that exhibited deviation from HW equilibrium. Two microsatellite loci (*R12** and *Lro23**) deviated significantly from HWE had +ve F_{is} values and a test of heterozygote deficiency confirmed that those populations had a significant deficiency of heterozygotes, while locus *MFW11** exhibited HW disequilibrium associated with marginal excess of heterozygotes ($F_{is} = -0.005$). The evidence of heterozygote deficiency could be due to several factors such as inbreeding, non-random mating, reduction in effective breeding populations and existence of the subpopulations or Wahlund effect (Garcia de Leon et al., 1997).

The partitioning of genetic variation F_{st} indicated the proportion of genetic variation that could be attributed to genetic differentiation processes between the con-specifics from two localities (Wright, 1965). The pattern of genetic differentiation in wild *L. calbasu* populations presented weak to moderate genetic differentiation between the rivers Satluj (Indus river system) and tributaries of Ganga river basin and moderate to high between the different river basins such as, Mahanadi, Godavari and Narmada. It was evident that some of the riverine populations from different basins exhibited high F_{st} values like 7.8–13%. However, it did not appear true for all the pairs of populations. Overall F_{st} for all samples combined was found to be 0.0354 revealing that approximately 3.5% of genetic variation could be caused by genetic differentiation in *L. calbasu* population indicating low level of differentiation overall populations. AMOVA analysis also confirmed low level of differentiation, as most of the genetic variation (98.77%) was due to variability within populations from the same geographical locations and only a small proportion could be attributed to the divergence between the populations.

Random genetic drift tends to cause genetic differentiation (Hartl and Clark, 1997) after subpopulations are fragmented and gene flow between them is either reduced or absent. Paleogeographical reconstructions clearly indicate the possibility that *L. calbasu* from different river basins sampled here, are likely to have evolved from common ancestral gene pool, like other major carp, *Cirrhinus mrigala* (Chauhan et al., 2007).

In Ganga river system, northern side tributaries like Gomti, Ghaghra and Ganga main channel (including Yamuna samples) did not exhibit any significant genetic divergence. The four associated rivers of the Ganges, viz. Ganga main channel, Gomti

Table 5
Fisher's exact test for allelic homogeneity for all the populations pairs of *L. calbasu*.

		Significant allelic homogeneity	Overall loci P
1.	Satluj& Gomti	*Lro23**, *Lr29**, *R3**, *MFW11**	0.00000*
2.	Satluj & Ghaghra	*R12**, *Lro23**, *MFW11**	0.00024*
3.	Satluj & Ganga	*R1**, *R12***, *Lr29***, *R3**, *MFW11**	0.00000*
4.	Satluj & Bhagirath	*R12**, *Lro25**, *R3**, *MFW11**	0.00016*
5.	Satluj & Narmada	*R1**, *R12***, *Lr28**, *Lr38**, *Lr29**, *R3**	0.00000*
6.	Satluj & Bihad	*R1**, *R12**, *Lr28**, *Lr38**, *Lro23***, *R3**, *MFW11**	0.00000*
7.	Satluj & Tons	*R1**, *R12**, *Lr38***, *Lro23**, *Lro25**, *Lr29***, *MFW11**	0.00000*
8.	Satluj & Godavari	*R1**, *R12**, *Lr28**, *Lro23**, *Lro25**, *Lr29**, *MFW11**	0.00000*
9.	Satluj & Mahanadi	*R1**, *R12**, *Lr28**, *Lro23***, *Lr29***, *R3**, *MFW11***	<0.00001*
10.	Satluj & Bputra	*R1**, *R12**, *Lr38**, *Lro23**, *Lr29**, *R3**, *MFW11***	0.00000*
11.	Satluj & Bhima	*Lr28**, *Lr29**, *R3**, *MFW11**	0.00000*
12.	Gomti & Ghaghra	*Lro23**, *Lr29**, *R3**, *MFW11**	0.00000*
13.	Gomti & Ganga	*R12**, *Lr28**, *Lro23**, *Lro25**, *Lr29**, *R3**	0.00000*
14.	Gomti & Bhagirathi	*Lr38**, *Lro23**, *Lro25**, *Lr29**, *MFW11**	0.00000*
15.	Gomti & Narmada	*R1***, *Lr28**, *Lr29**, *R3***	<0.00001*
16.	Gomti & Bihad	*Lr28**, *Lr38**, *Lro23**, *Lro25**, *Lr29**	0.00000*
17.	Gomti & Tons	*R1**, *R12**, *Lr29***	0.00000*
18.	Gomti & Godavari	*Lr38**, *Lro23***, *Lro25**, *Lr29***, *R3**, *MFW11**	<0.00001*
19.	Gomti & Mahanadi	*R1**, *R12**, *Lr38**, *Lro23***, *Lro25**, *Lr29***, *R3**, *MFW11**	<0.00001*
20.	Gomti & Bputra	*R1**, *Lr28**, *Lr38**, *Lro23**, *Lro25**, *MFW11**	0.00001*
21.	Gomti & Bhima	*Lr28**, *Lr38**, *Lro25**, *Lr29**, *R3**	0.00012*
22.	Ghaghra & Ganga	*R12**, *Lr28**, *Lr38**, *Lro23**, *Lro25**, *Lr29***, *R3***	<0.00001*
23.	Ghaghra & Bhagirathi	*Lr38**, *R3**, *MFW11**	0.00006*
24.	Ghaghra & Narmada	*R1***, *Lr28***, *Lr38**, *R3**, *MFW11**	<0.00001*
25.	Ghaghra & Bihad	*R1**, *Lr38**, *Lro23***, *Lro25***, *R3**	<0.00001*
26.	Ghaghra & Tons	*Lr38**, *Lro23**, *Lr29**, *R3**, *MFW11**	0.00000*
27.	Ghaghra & Godavari	*R1**, *Lr28**, *Lr38**, *Lro23***, *Lro25**, *Lr29**, *R3***, *MFW11**	0.00000*
28.	Ghaghra & Mahanadi	*R1**, *Lro23***, *Lro25**, *Lr29***, *R3***, *MFW11**	<0.00001*
29.	Ghaghra & Bputra	*R1**, *Lr38**, *Lro23**, *Lro25**, *R3**, *MFW11**	0.00000*
30.	Ghaghra & Bhima	*Lr28**, *Lr38**, *Lro23**, *Lr29**, *R3**	0.00000*
31.	Ganga & Bhagirath	*R1**, *Lr28**, *Lr38**, *Lro23**, *Lr29**, *R3**, *MFW11**	0.00000*
32.	Ganga & Narmada	*R1***, *Lr28***, *Lro25**, *Lr29**, *R3***	<0.00001*
33.	Ganga & Bihad	*R1**, *R12***, *Lr38***, *Lro23***, *Lro25**, *Lr29**, *R3***, *MFW11**	<0.00001*
34.	Ganga & Tons	*R1***, *R12**, *Lr38**, *Lro23**, *Lr29***, *R3**	<0.00001*
35.	Ganga & Godavari	*R1**, *R12**, *Lr28***, *Lr38**, *Lro23***, *Lro25**, *Lr29***, *R3***	<0.00001*
36.	Ganga & Mahanadi	*R12**, *Lr28**, *Lr38***, *Lro23***, *Lro25**, *Lr29***, *R3***, *MFW11**	<0.00001*
37.	Ganga & Bputra	*R1**, *R12**, *Lr28**, *Lr38**, *Lro23**, *Lro25**, *R3**	0.00000*
38.	Ganga & Bhima	*R12**, *Lr28**, *Lr38**, *Lr29**, *R3***	0.00000*
39.	Bhagirath & Bhopal	*R1**, *Lr28**, *Lro23**, *Lro25**, *R3***	<0.00001*
40.	Bhagirath & Bihad	*R1***, *Lr28**, *Lr38***, *Lro23***, *Lro25***, *MFW11**	<0.00001*
41.	Bhagirath & Tons	*R1**, *R12**, *Lr38***, *Lro23**, *Lr29**	<0.00001*
42.	Bhagirat & Godavari	*Lr28**, *Lr38**, *Lro23**, *Lro25**, *R3***, *MFW11**	0.00000*
43.	Bhagirath & Mahanadi	*R1**, *Lr38***, *Lro23***, *Lro25**, *Lr29**, *R3***, *MFW11**	<0.00001*
44.	Bhagirath & Bputra	*R1**, *Lr28**, *Lr38**, *Lro23**, *R3**, *MFW11**	0.00000*
45.	Bhagirath & Bhima	*R1**, *Lr28**, *Lr38**, *Lro23**, *Lro25**, *R3***, *MFW11**	0.00000*
46.	Narmada & Bihad	*R1**, *Lr28***, *Lr38**, *Lro23**, *Lro25***, *Lr29**, *R3***	<0.00001*
47.	Narmada & Tons	*R1**, *Lr28**, *Lr38**, *R3**	0.00000*
48.	Narmada & Godavari	*R1***, *Lr28**, *Lro25**, *Lr29**, *R3**, *MFW11**	<0.00001*
49.	Narmada & Mahanadi	*R1***, *Lr28***, *Lr38**, *Lro23**, *Lr29**, *R3**, *MFW11**	<0.00001*
50.	Narmada & Bputra	*R1***, *Lr28***, *Lr38**, *Lr29**, *R3***, *MFW11**	<0.00001*
51.	Narmada & Bhima	*R1***, *Lr28***, *Lr38**, *Lro25**, *Lr29**, *R3**, *MFW11**	0.00000*
52.	Bihad & Tons	*R1***, *R12**, *Lr38**, *Lro23**, *Lro25***, *Lr29***, *MFW11**	<0.00001*
53.	Bihad & Godavari	*R1**, *R12**, *Lr28**, *Lr38***, *Lro23***, *Lro25***, *Lr29***, *R3***, *MFW11**	<0.00001*
54.	Bihad & Mahanadi	*R1**, *R12**, *Lr38**, *Lro23***, *Lro25**, *Lr29***, *R3***, *MFW11***	<0.00001*
55.	Bihad & Bputra	*R1***, *Lr28**, *Lr38**, *Lro23***, *Lro25**, *MFW11***	0.00000*
56.	Bihad & Bhima	*Lr28**, *Lr38***, *Lro23**, *Lro25**, *R3**, *MFW11**	0.00000*
57.	Tons & Godavari	*R1***, *R12**, *Lr28**, *Lr38**, *Lro23**, *Lr29***, *MFW11**	<0.00001*
58.	Tons & Mahanadi	*R1***, *R12**, *Lr38**, *Lro23***, *Lro25**, *Lr29**, *MFW11**	<0.00001*
59.	Tons & Bputra	*R1**, *R12**, *Lr38**, *Lro23**, *Lro25**, *Lr29**, *MFW11**	0.00000*
60.	Tons & Bhima	*R1**, *Lr28**, *Lr38**, *Lro25**, *Lr29***	0.00000*
61.	Godavari& Mahanadi	*R1***, *Lr28**, *Lr38***, *Lro23***, *Lro25***, *Lr29**, *R3**, *MFW11**	<0.00001*
62.	Godavari & Bputra	*R1***, *R12**, *Lr28**, *Lr38***, *Lro23***, *Lro25**, *Lr29**, *R3**	<0.00001*
63.	Godavari & Bhima	*R1**, *Lr28**, *Lr38***, *Lro23**, *Lro25**, *Lr29**	0.00000*
64.	Mahanadi & Bputra	*R1***, *R12**, *Lr38***, *Lro23***, *Lr29**, *R3**	<0.00001*
65.	Mahanadi & Bhima	*Lr28**, *Lr38***, *Lro23***, *Lr29**, *R3**	<0.00001*
66.	Bputra & Bhima	*Lr28**, *Lr38**, *Lro25**	0.00004*

*Significant ($P < 0.05$), ** Significant after Bonferroni corrections ($P < 0.00015$).

Table 6
Pair wise comparison of F_{st} values in *Labeo calbasu* on the basis of microsatellite analysis.

	Gomti	Ghaghra	Ganga	Bhagirathi	Narmada	Bihad	Tons	Godavari	Mahanadi	Brahmaputra	Bhima
Satluj	0.04342	0.02418	0.05341	0.04281	0.12089	0.04487	0.09395	0.05852	0.06449	0.04877	0.05140
Gomti		0.01894	0.02566	0.03133	0.10087	0.02189	0.03108	0.02971	0.04001	0.02414	0.01674
Ghaghra			0.02334	0.01661	0.07840	0.02512	0.03306	0.01760	0.01718	0.02381	0.02384
Ganga				0.03830	0.12292	0.03409	0.04740	0.02903	0.04242	0.02563	0.02861
Bhagirathi					0.09876	0.04322	0.05814	0.02491	0.03553	0.03321	0.05128
Narmada						0.13119	0.06715	0.08506	0.08969	0.11554	0.10932
Bihad							0.05899	0.04368	0.03930	0.02765	0.02474
Tons								0.04491	0.05276	0.03661	0.05328
Godavari									0.01913	0.03402	0.02593
Mahanadi										0.03526	0.02992
Brahmaputra											0.02811

and Ghaghra appear to share a common gene pool of *L. calbasu*. The likely possibility of such sharing could be through connections associated with a common flood plain and 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 with southern side tributaries of Ganges, Tons and Bihad was higher than the northern side tributaries (5–6%). The inference is concordant to the findings reported in another Indian Major Carp, *C. mrigala*, where lack of genetic divergence in Ganga population was evident with the exception of Bhagirathi samples that had little but significant genetic differentiation (Chauhan et al., 2007). The significant genetic divergence between the river Tons and Bihad is of significant interest. The two rivers originate from central plateau and join before the Tons flows into the Ganga River. Significant genetic divergence (0.040) indicated the restricted gene flow between the populations in two associated rivers, possibly can happen due to geographical barriers. The geomorphological evidences suggest that the formation of gorges on Rewa plateau might have controlled evolution of drainages including rivers Tons and Bihad (Lakshmanan, 1972). The interrupting rocky substrata between the terrain of rivers Bihad and Tons could act as barrier in the movement of detritivorous fish like *L. calbasu*.

Low yet significant level of divergence was found between individuals from Brahmaputra and river Ganga including its tributaries (Tons). This is likely as Brahmaputra joins the main Ganga channel, known as river Padma (ECAFE, 1966) and there is possibility of constant gene flow. Likewise, significant genetic divergence was evident between the Brahmaputra and samples from Satluj, Mahanadi, Godavari and Bhima.

Low to moderate genetic divergence among wild *L. calbasu* populations of Satluj (Indus basin) and rivers of the Ganges could be the result from ongoing gene flow among populations via connectivity across common flood plains and changes in the courses of associated rivers. Common ancestry in the past and possible intermittent exchange of individuals among rivers such as Satluj and rivers of middle stretch of Ganga basin may explain the observed low to moderate (2–5%) levels of genetic differentiation among *calbasu* populations in these rivers as compared to lower reaches like Bhagirathi ($\sim$5%). Relatively higher level of genetic divergence between Satluj samples and the rivers such as Mahanadi, Bhima and Godavari was observed.

Narmada samples were significantly divergent from all the other river samples. The observed genetic divergence between the samples from three rivers (Mahanadi, Godavari and Bhima) and other populations clearly indicated the possibility of fragmentation of population. It is likely that the three populations might have fragmented out of a common ancestral gene pool.

In this study, data sets revealed higher R_{st} when compared to F_{st} in 46 out of 66 possible population pairs. This indicated that fragmentation has more bias towards allelic size rather than allelic identity.

This study provided conclusive evidence that *L. calbasu* in different rivers in India has distinct population substructure and is not a part of single gene pool. Comparison of genotype data (original and adjusted for possible null alleles) across the riverine basins, the population of *L. calbasu* in Satluj (Indus), Ganga, Bhima, Mahanadi, Godavari, Tons, Bhagirathi, Brahmaputra, Bihad and Narmada are genetically distinct. The estimates clearly pointed out that population in some of these rivers have undergone genetic bottleneck. These populations require specific measures such as stock specific propagation to maintain adequate genetic variability in nature. The significant genetic divergence even in some of the tributaries of Ganga, Bihad and Tons indicated the likelihood of finding more genetic stocks, particularly in associated rivers and tributaries of the Ganga system. This suggests that exploration need to be more focused on the tributaries of Ganga River system in future which is likely give more genetic divergence. The stock structure along with the technologies on sperm cryopreservation and captive breeding may prove to be an integrated package for *in situ* conservation of genetic diversity of natural populations of *L. calbasu.*

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