Asian Fisheries Science **22** (2009): 359-391
ISSN: 0116-6514
E-ISSN: 2073-3720
https://doi.org/10.33997/j.afs.2009.22.2.001

Asian Fisheries Society, Selangor, Malaysia

Low Genetic Differentiation in the Populations of the Malabar Carp *Labeo dussumieri* as revealed by Allozymes, Microsatellites and RAPD.

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Abstract

The population structure of *Labeo dussumieri*, an endangered and endemic cyprinid from three riverine locations in the Western Ghats, India was investigated using allozyme, microsatellite and RAPD markers. *L. dussumieri* samples were obtained from Meenachil, Manimala and Pamba River basins, Kerala. Fourteen (46.7%) out of 30 allozyme loci, seven microsatellite loci and 12 RAPD Operon decamers gave polymorphic pattern. Six allozyme loci (*AAT-2*, EST-4*, GLDH*, GPI-2*, G_6PDH** and *LDH-2**) and three microsatellite loci (*LdussG1*, *MFW19* and *Bgon22*) exhibited consistent significant deviation from Hardy-Weinberg Equilibrium expectations in different populations after probability level ($P<0.05$) was adjusted for sequential Bonferroni correction. All the three marker types demonstrated concordant results and various estimates revealed genetic variability within the subpopulations but surprisingly low level ($\theta = 0.0034$ to 0.0081) of genetic differentiation among *L. dussumieri* from different river samples. AMOVA analysis also indicated low differentiation among subpopulations. No evidence for a recent genetic bottleneck was observed in *L. dussumieri* populations based on allozyme and microsatellite data set analysis. Meenachil, Manimala and Pamba Rivers open in to the southern end of Vembanad Lake in Kerala and are connected to each other in the lower reaches through an extensive network of natural canals. Common ancestry in the prehistoric period; and possible mixing of fish populations resulting in high gene flow across the rivers through the lake and interconnecting canals could have been responsible for the lack of significant allelic heterogeneity among the *L. dussumieri* populations. The stocks from the three rivers do not require different management strategies and for propagation assisted river ranching programme of this species, large effective breeding population can be developed by mixing individuals from three rivers.

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Introduction

Labeo dussumieri (Valenciennes, 1842) a cultivable food fish, popularly known as 'Malabar Labeo'; or 'thooli' or 'pullan' (in Malayalam) or 'hiri kanaya' (in Sinhalese) and belonging to the family Cyprinidae is endemic to the west flowing rivers originating from the southern part of Western Ghats, India and lowlands of Sri Lanka (Day 1878; Silas, 1953; Smith and Jiffry, 1986; Talwar and Jhingran, 1991; Pethiyagoda, 1991; Jayaram and Dhas, 2000). In India, the species is one of the highly esteemed food fishes and commands a higher price as compared to the Indian major carps, especially in Kerala State (Padmakumar et al., 2004). To date, stock assessment of the species have not been made in different rivers, hence there is no information about the current exploitable potential of Malabar Labeo. In recent years, there has been a massive hunt for the species from the wild in India and its occurrence became sparse in the rivers (Kurup, 1990, 1998). Similarly, Pethiyagoda (1991) recorded decline of this species in Sri Lanka because of competition with the exotic tilapia. The workshop on Conservation Assessment Management Plan (CAMP) to evaluate the status of freshwater fishes of India, held in 1997 categorized this species as 'endangered' based on IUCN criteria due to restricted distribution, loss of habitat, over-exploitation, destructive fishing practises and trade (CAMP, 1998; Ponniah and Gopalakrishnan, 2000). Hence, *L. dussumieri* was short-listed for taking up stock-specific, propagation assisted rehabilitation programme in rivers where it is naturally distributed. In connection with this, captive breeding and milt cryopreservation techniques have been developed in this species by the National Bureau of Fish Genetic Resources (NBFGR) in collaboration with the Regional Agricultural Research Station (RARS) of the Kerala Agricultural University, Kumarakom, Kerala (Gopalakrishnan et al., 2000; Padmakumar et al., 2004). In order to devise adequate conservation and management strategies for an endangered species, it is important to investigate its population history, geographical partitioning throughout its natural distributional range; and distribution of genetic diversity within and among populations through genetically controlled markers. This can also help in scientific planning of propagation assisted rehabilitation programmes and monitoring their impact on natural gene pool. Natural genetic resources also form the basis for selection of founder stocks for stock improvement programmes. Kurup (1990) studied the biology, bionomics and other related aspects of *L. dussumieri*. Genetic studies on this species have been limited to karyotyping (Nagpure et al., 2003). The present work is a part of the integrated plan covering different aspects including captive breeding, development of sperm cryopreservation protocols, documenting life history traits and information on genetic markers as well as stock structure of *L. dussumieri* in Indian rivers.

Identification of genetic markers with scorable alleles is prerequisite to generate stock structure data of any species (Ferguson et al., 1995). Genetic methods have great potential to distinguish distinct populations or stocks of fish species that cannot be

identified by morphological and meristic characters (Cadrin et al., 2005). Allozyme and microsatellite markers have been used independently or collectively to document genetic diversity and to draw inference about population structure in finfishes and shellfishes in natural environments (Muneer et al., 2009; Chauhan et al., 2007; Salini et al., 2004) and to unearth population level evolution in variety of vertebrates (Chistiakov et al., 2006). The development of species-specific microsatellite primers for PCR amplification of alleles can be expensive and time consuming, as it involves construction of genomic libraries, screening of clones with microsatellite sequences and designing microsatellite primers (Scribner et al., 1996). However, the flanking sequences of microsatellite loci are highly conserved among related taxa so that primers developed for one species can be used to amplify homologous loci in related species (Gopalakrishnan et al., 2004; Zardoya et al., 1996). Successful amplification of homologous microsatellite loci has been demonstrated in many cyprinid fishes (Lal et al., 2004; Gopalakrishnan et al., 2004; Mohindra et al., 2001). RAPD methodology which involves DNA amplification using arbitrary short sequences has also proved to be useful in discriminating species and in detecting genetic variation in cultures and natural populations of fishes (Muneer et al., 2008; Nagarajan et al., 2006). In the present study, allozymes, microsatellites and RAPD markers were used to investigate the genetic structure of three geographically isolated riverine populations of *L. dussumieri* in the Western Ghat Biodiversity Hotspot region of India. The work was taken up to assess genetic variation and to understand the scale of population structure of *L. dussumieri* across its natural distribution range with an ultimate objective to support the breeding and restocking programme of this species in Indian rivers for conservation purpose.

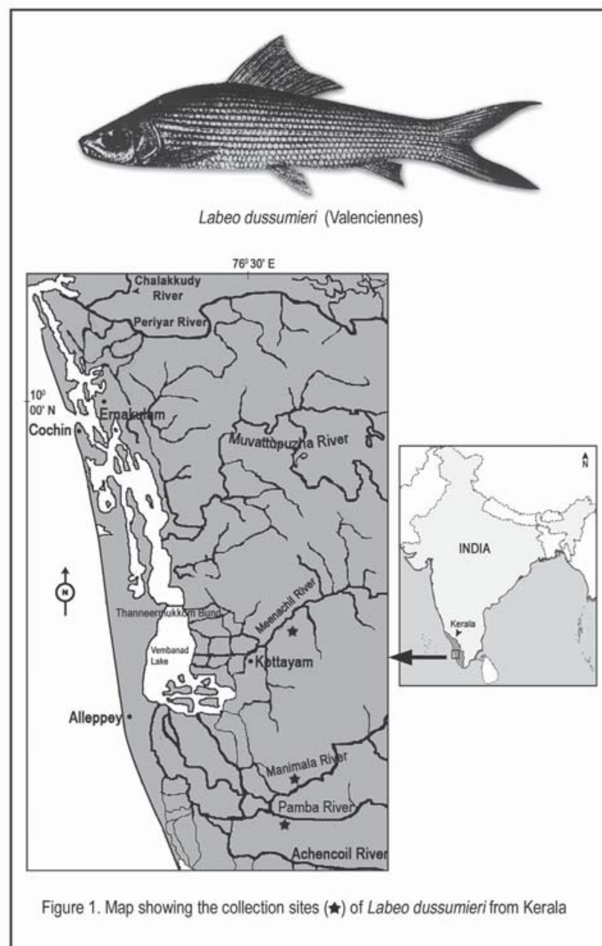

Figure 1. Map showing the collection sites (★) of *Labeo dussumieri* from Kerala

Materials and Methods

Sampling

A total of 198 specimens (66 each from three rivers) were collected during July 2003 to October 2006 from commercial riverine catches from three different rivers (Meenachil, Manimala and Pamba) in southern Kerala, India (Fig. 1; Table 1). The weight of the specimens ranged from 400 to 1100 grams. Sampling procedures were performed at actual site of collection. Liver tissue samples were taken and frozen immediately in liquid nitrogen ($^{-}196°C$) for allozyme analysis. Blood samples for DNA extraction were collected from the caudal vein using heparinized syringes, fixed in 95% ethanol in 1:5 (blood: ethanol) and transported to the laboratory on ice and stored at 4°C until used for genomic DNA extraction. Liver samples transported to the laboratory were stored at $^{-}80°C$ until analysis.

Table 1. Sample size, location and sampling period of *Labeo dussumieri* from three riverine locations in Kerala, India.

River system	Collection Site	Sampling Date	No. of specimens	Total samples (N)
Meenachil River	Cheepunkal, Kidangoor 09⁰ 41' 19" N 76⁰ 38' 30" E	16.07.2003	10 [a]	66
		12.09.2004	16 [a]	
		15.11.2004	15 [a]	
		11.07.2005	15 [a]	
		06.10.2006	10 [a]	
Manimala River	Kavumbhagom, Tiruvalla 09⁰ 22' 04" N 76⁰ 35' 24" E	04.02.2005	07 [b]	66
		14.04.2005	28 [b]	
		07.04.2006	11 [b]	
		01.06.2006	03 [b]	
		16.07.2006	17 [b]	
Pamba River	Parumala, Chengannoor 09⁰ 19' 19" N 76⁰ 32' 12" E	04.07.2005	32 [c]	66
		27.02.2006	20 [c]	
		19.06.2006	14 [c]	

Common superscripts indicate the multiple data sets within rivers that were pooled after testing for absence of heterogeneity.

Allozyme analysis

Frozen liver samples (approximately 100mg) were homogenized in 250 mg/ml extraction buffer (0.17 M Sucrose, 0.2 M EDTA, 0.2 M Tris-HCl, pH 7.0). Homogenized samples were centrifuged for an hour at 12,000 rpm at 4°C and the supernatant was recentrifuged for 30 min. Allelic variation was investigated using 7% polyacrylamide gel electrophoresis. Electrophoresis was carried out at constant voltage 150 V at 4°C. A total 23 enzyme systems were examined and 15 enzymes yielded scorable activity (Table 2). Histochemical staining procedures outlined by Whitmore (1990) were used to visualize different alleles. Loci and alleles were designated following the nomenclature system of Shaklee et al. (1990a, b).

Table 2. Names of enzyme loci, enzyme commission (E.C.) number and observed alleles for allozyme analysis in *Labeo dussumieri*.

Enzymes	E. C. Number	No. of loci	Locus	Alleles	Monomorphic/ Polymorphic
Aspartate amino transferase	2.6.1.1	2	*AAT-1**	*100*	Monomorphic
			*AAT-2**	*100,108, 117*	Polymorphic
Creatine kinase	2.7.3.2.	2	*CK-1**	*100*	Monomorphic
			*CK-2**	*100*	Monomorphic
Esterase	3.1.1.-	7	*EST-1**	*086, 100*	Polymorphic
			*EST-2**	*100*	Monomorphic
			*EST-3**	*100*	Monomorphic
			*EST-4**	*096, 100*	Polymorphic
			*EST-5**	*100*	Monomorphic
			*EST-6**	*100*	Monomorphic
			*EST-7**	*100*	Monomorphic
Glucose dehydrogenase	1.1.1.47	1	*GLDH**	*086, 100*	Polymorphic
Glucose phosphate isomerase	5.3.1.9	2	*GPI-1**	*100*	Monomorphic
			*GPI-2**	*088, 100*	Polymorphic
Glucose-6-phosphate dehydrogenase	1.1.1.49	1	G_6PDH^*	*095, 100, 120*	Polymorphic
α-Glycerophosphate dehydrogenase	1.1.1.8	2	$\alpha G_3PDH\text{-}1^*$	*100*	Monomorphic
			$\alpha G_3PDH\text{-}2^*$	*080, 100*	Polymorphic
Glyceraldehyde-3-Phosphate dehydrogenase	1.2.1.12	1	*GAPDH**	*090,100*	Polymorphic
Lactate dehydrogenase	1.1.1.27	3	*LDH-1**	*100*	Monomorphic
			*LDH-2**	*077, 100, 150*	Polymorphic
			*LDH-3**	*100*	Monomorphic
Malate dehydrogenase	1.1.1.37	2	*MDH-1**	*100*	Monomorphic
			*MDH-2**	*100*	Monomorphic
Malic enzyme	1.1.1.40	1	*ME**	*100*	Monomorphic
Octonol dehydrogenase	1.1.1.73	1	*ODH**	*085, 100, 115*	Polymorphic
Phosphogluco mutase	5.4.2.2	2	*PGM-1**	*075, 100*	Polymorphic
			*PGM-2**	*078, 100*	Polymorphic
Superoxide dismutase	1.15.1.1	2	*SOD-1**	*100*	Monomorphic
			*SOD-2**	*100, 125*	Polymorphic
Xanthine dehydrogenase	1.1.1.204	1	*XDH**	*100, 108*	Polymorphic

Table 3. Characteristics of polymorphic microsatellite loci in *Labeo dussumieri*.

Sl. No.	Species & Reference	Locus	Primer sequence (5' → 3')	Repeat motif	Ta (°C)	Repeat motif	Ta (°C)	No. of alleles#	NCBI GenBanl Accessic Number
	Resource species					***Labeo dussumieri***			
1	*Catla catla* (Naish and Skibinski, 1998)	*LdussG1*	F: AGCAGGTTGAT CATTTCTCC R: TGCTGTGTTTCAAATGTTCC	(GATA)n --- (CCA)n	61	(GATA)n	54	4	AF51793
2	*Cyprinus carpio* (Crooijmans *et al*., 1997)	*MFW19*	F: GAATCCTCCATCATGCAAAC R: CAAACTCCACATTGTGCC	(CA)n	55	(GGA)n	52	5	DQ7800
		MFW26	F: CCCTGAGATAGAAACCACTG R: CACCATGCTTGGATGCAAAAG	(CA)n	55	(CA)n	57	7	EU2728
3	*Barbodes gonionotus* (Kamonrat *et al*., 2002)	*Bgon22*	F: TCTTGTTGATCACACGGACG R: ACAGATGGGGAAAGAGAGCA	(CCT)n	55	(CCT)n	55	5	EU2728
4	*Barbus barbus* (Chenuil *et al*., 1999)	*Barb37*	F: AAATACGCTCTCCTCATTAC R: GTACAAAAGCAAAAATAAATTA	(ATTT)n	50	(AAAT)n	50	4	DQ7800
5	*Labeo rohita* (Das *et al*., 2005)	*R3-1**	F: TATTCACCCCAAATCCATTA R: GACCCTTGTGCATAAGACC	(TG)n	58	(TG)n	58	4	DQ7800
		R6	F: TATCCTGGCTGAAAACTTTG R: [illegible]	(TG)n	56	(TG)n	56	5	DQ7800

*Primer sequence of *R3* given by Das *et al*. (2005) was modified using PRIMER3 in the present study.

Microsatellite analysis

Genomic DNA was extracted from blood following a protocol modified from Ruzzante et al. (1996), using proteinase K, and phenol: chloroform. For microsatellite analysis to obtain polymorphic loci, 36 primers (microsatellite flanking regions) developed for cyprinid fishes *Catla catla* (Naish and Skibinski, 1998), *Cyprinus carpio* (Crooijmans et al., 1997), *Barbodes gonionotus* (Kamonrat et al., 2002; McConnell et al., 2001), *Barbus barbus* (Chenuil et al., 1999) *Labeo rohita* (Das et al., 2005), *Campostoma anomalum* (Dimsoski et al., 2000) and *Pimephales promelas* (Bessert and Orti, 2003) were examined for cross-priming in ten individuals of *L. dussumieri*. In this study, PCR amplification was performed in a 25 µl reaction mixture, that included 1 X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.01% gelatin), 0.2 mM of each dNTP, 2.0 mM of $MgCl_2$, 5 pmol of each primer, 1.5 U Taq DNA polymerase and 25-50 ng of template DNA. PCR (MJ Research PTC-200 thermal cycler) cycles were as follows (i) 1 cycle of denaturation of 94°C for 5 min, (ii) 25 cycles of denaturation at 94°C for 30 seconds relevant annealing temperature for 30 seconds, elongation at 72°C for 1 min, (iii) a final elongation of 1 cycle at 72°C for 4 min and stored at 4°C. PCR products were resolved through vertical non-denaturing polyacrylamide (19:1 acrylamide: bisacrylamide) gels electrophoresis (size 10.0 x 10.5 cm, Amersham Biosciences Ltd.). Electrophoresis was done with 1X TBE buffer for 5.30 hours at 10 v/cm at 4°C. Gel concentrations and annealing temperatures (Table 3) were optimized to obtain clear scorable allelic banding patterns. Amplified microsatellite loci were visualized via silver staining (silver staining kit, Amersham Biosciences, USA). Alleles were designated according to PCR product size, calculated relative to a molecular marker (pBR322 DNA/ *Msp*I digest) with Image master 1D Elite v3.01 (Amersham Biosciences, USA). 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) demonstrated that non-denaturing electrophoresis is not responsible for spurious or multiple bands in microsatellite analysis. Seven of the 36 loci screened (Table 3) gave clear scorable products with 4-7 alleles per locus. These seven polymorphic loci were finally analysed to confirm the occurrence of repeats through cloning and sequencing.The microsatellite products were run and eluted from 2% agarose gel, purified and ligated into TOPO vector (Invitrogen, Carlsbad, USA) and the ligation product was transformed into competent *Escherichia coli* DH5α cells. The transformed cells were cultured for 12-16hours at 37°C in LB media plate containing 50µg/mL ampicillin coated with 40mL X-gal (20mg/mL) and 4µL IPTG (200mg/mL). The transformants containing inserts were selected by blue/white screening (Sambrook et al., 1989). The recombinant plasmids were isolated by alkaline lysis method (Sambrook

et al., 1989) and were further purified through PEG precipitation for sequencing purpose. The sequencing was done in forward and reverse directions using ABI Prism Big Dye Terminator cycle sequencing ready reaction kit in the automated DNA sequencer ABI 3730 (Applied Biosystems, USA) according to manufacturers instructions. All microsatellite sequences isolated from *L. dussumieri* have been submitted to the NCBI GenBank (Fig. 3).

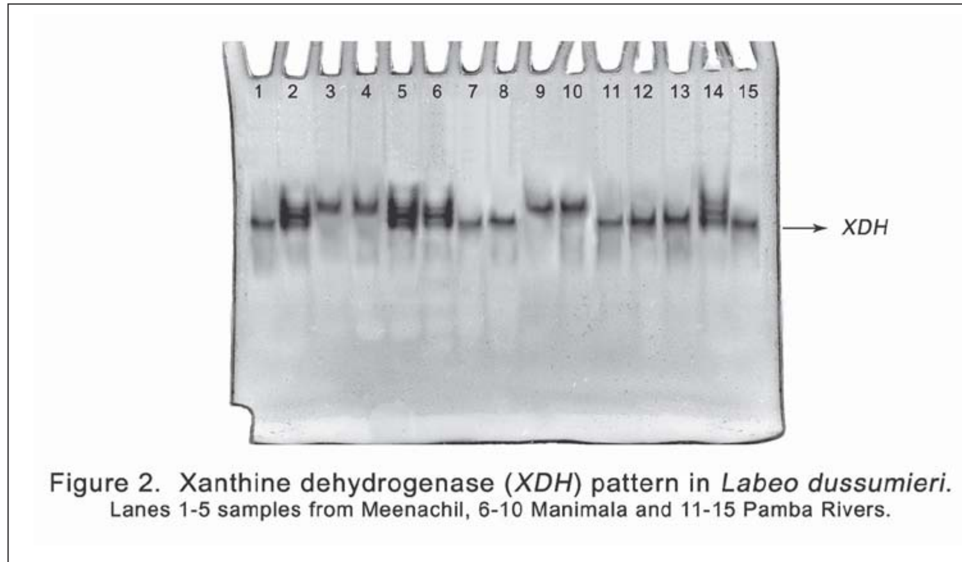

Figure 2. Xanthine dehydrogenase (*XDH*) pattern in *Labeo dussumieri*.
Lanes 1-5 samples from Meenachil, 6-10 Manimala and 11-15 Pamba Rivers.

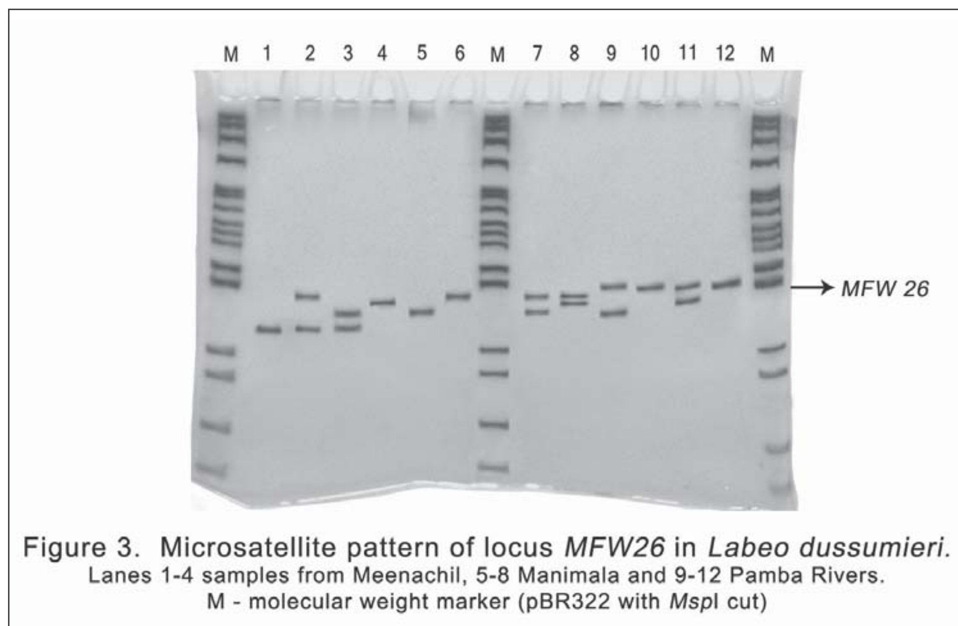

Figure 3. Microsatellite pattern of locus *MFW26* in *Labeo dussumieri*.
Lanes 1-4 samples from Meenachil, 5-8 Manimala and 9-12 Pamba Rivers.
M - molecular weight marker (pBR322 with *Msp*I cut)

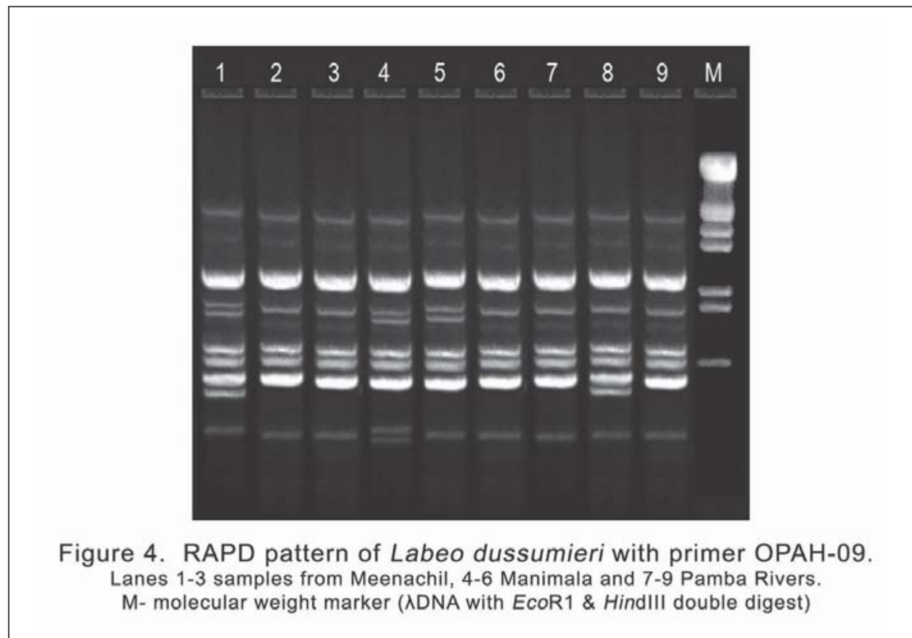

Figure 4. RAPD pattern of *Labeo dussumieri* with primer OPAH-09.
Lanes 1-3 samples from Meenachil, 4-6 Manimala and 7-9 Pamba Rivers.
M- molecular weight marker (λDNA with *Eco*R1 & *Hin*dIII double digest)

RAPD analysis

Eighty decamer primers from Operon Technologies, Alameda, USA (OPA1-20, OPAA1-20, OPAC1-20, OPAH1-10 and OPB1-10) were used for RAPD analysis. From these, 12 primers were selected for population genetic analysis taking into consideration of the repeatability, sharpness, intensity and polymorphic nature of the bands (Table 10). PCR amplifications were performed in a PTC 200 thermal cycler (M. J. Research, Inc., Watertown, USA) in 25µL reactions containing 1X reaction buffer [100mM Tris, 500mM KCl, 0.1% gelatin, pH9.0) with 1.5mM $MgCl_2$ (Genei, Bangalore, India), 6-8 pmoles of primer, 200 mM dNTPs, 2U *Taq* DNA polymerase (Genei, Bangalore, India] and 25ng of template DNA. To check DNA contamination, a negative control was made omitting template DNA from the reaction mixture. The reaction mixture was pre-heated at 95°C for 3minutes followed by 40 cycles (94°C for 3minutes, 40°C for 1.30minutes and 72°C for 2minutes). The reaction was then subjected to a final extension at 72^{0}C for 10 minutes. The resulting products were electrophoretically analysed through 1.5% agarose gels stained with ethidium bromide (5 µg/ml) in TBE buffer (90 mM Tris–borate and 2 mM EDTA, pH 8.0) and photographed using Image master VDS gel documentation system (Amersham Biosciences, USA). A known DNA size marker was run with every gel (ëDNA with *Eco*RI/*Hin*dIII double digest). The bands were designated according to the PCR product size in relation to the marker with Image master ID Elite v 3.01(Amersham Biosciences, USA).

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Data Analysis

Individual fish genotypes at each allozyme and microsatellite locus were determined. These data were then analyzed for homogeneity between data sets for collections at different times within each river. Data sets within each river or neighboring tributaries that were not heterogenous (P>0.05) were later combined for further analysis for estimating 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 ver. 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, 1998). Fixation indices based on an infinite allele model (IAM, Kimura and Crow, 1964) and a stepwise mutation model (SMM, Kimura and Ohta, 1978) were estimated to determine the extent of population subdivision among samples in a quantitative way. Estimation of average F_{ST} and determining whether the values are significantly different from zero; and calculation of pair-wise population F_{ST} values (θ) of Weir and Cockerham (1984) and their significance levels, were carried out using Genetix ver. 4.05 software (Belkhir et al., 1997). Probability of è significantly deviating from zero was calculated using 1000 bootstraps. Under a SMM, estimates of R_{ST} (Slatkin, 1995) were made (only for microsatellite data - based on allele sizes) 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 of the loci with known inbreeding coefficient or fixation indices ($+F_{IS}$) were tested for the expected frequency of null alleles according to Van Oosterhout et al. (2004, 2006) using MICRO-CHECKER (http:// www.microchecker.hull.ac.uk/) and thereafter analyzed for population differentiation. Genetic similarity/identity and distance between pairs of populations of *L. dussumieri* were estimated using POPGENE Version 1.31 (Yeh et al., 1999). Nei and Li's (1979) pair-wise genetic similarity (SI) among these specimens were computed and converted by POPGENE into genetic distance (GD) according to Hillis and Moritz's (1990) formula, GD = 1- SI. The partitioning of genetic variation among and within populations of *L. dussumieri* was calculated by hierarchical analysis of molecular variance (AMOVA) (Excoffier et al., 1992) at 1000 permutations. The hierarchical components of genetic variation include (1) variance due to differences between individuals within a river; and (2) variance due to differences among populations. The AMOVA calculations were performed using ARLEQUIN v2.0 (Schneider et al., 2000; http://lgb.unige.ch/arlequin/). The Cornuet and Luikart (1996) programme BOTTLENECK ver. 1.2.02 was used to detect recent effective population size reduction (to assess the impact of population decline) using data from the allozymes under IAM and microsatellites under the more suitable two-phased model (TPM), in addition to IAM. BOTTLENECK detects past population reductions by testing for a transient

excess in measured heterozygosity compared with the heterozygosity expected at mutation-drift equilibrium (He>Heq). Excess in heterozygosity is generated because rare alleles are quickly lost due to drift during a bottleneck, but they contribute little to the expected heterozygosity (Luikart and Cornuet, 1998). To determine whether the 3 riverine populations of *L. dussumieri* exhibited a significant number of loci with gene diversity excess, "Wilcoxon Sign-Rank Tests" were employed in BOTTLENECK. In addition, a qualitative descriptor of the allele frequency distribution ("mode-shift" indicator) was also employed to discriminate bottlenecked populations from stable populations.

For the RAPD data, the images of gels were used to analyze the banding patterns. A binary matrix was produced whereby the presence or absence of each DNA fragment for each sample was recorded 1 or 0, respectively. Faint or poorly amplified fragments were excluded from the analysis as were fragments with very high (above 6500bp) or low (below 800bp) molecular weight. The analysis was based on few assumptions. First, all RAPD fragments scored represented 2-allele system, *i.e.,* presence (dominant) and absence (recessive) of bands. Second, fragments that migrated at the same position, had the same molecular weight, and stained with the same intensity were homologous bands from the same allele, and the alleles from different loci did not co-migrate. A third assumption was that the populations fit the Hardy-Weinberg equilibrium (Clark and Lanigan, 1993; Lynch and Milligan, 1994). From the binary matrix, the total number of RAPD fragments and polymorphic ones were calculated for each primer and for all primers. Genetic variability in three populations of *L. dussumieri* was estimated from the gene (allele) frequencies and percentage of polymorphic loci (%P) using POPGENE version 1.31 (Yeh et al., 1999). Tests for population differentiation were performed by calculation of G_{ST} and Nei's genetic distance between pairs of populations and for overall population using POPGENE version 1.31(Yeh et al., 1999).

Table 4. Distribution of dimeric G_6PDH genotypes in male and female *Labeo dussumieri* from different river systems.

Locus	Genotypes (Alleles & Rf value)	No. of individuals					
		Meenachil River		Manimala River		Pamba River	
		Male	Female	Male	Female	Male	Female
G_6PDH	*AA* (100/100)	14	09	18	08	10	14
	AB (095/100)	08	06	02	06	08	02
	AC (100/120)	06	03	04	03	03	01
	BB (095/095)	05	01	04	05	04	07
	BC (095/120)	04	03	01	02	02	02
	CC (120/120)	04	03	06	07	10	03

Table 5. Allozyme alleles and allele frequencies in *Labeo dussumieri* from three riverine populations and among populations.

Locus	Alleles	Meenachil River	Manimala River	Pamba River	Overall Populations
AAT-2	*100*	0.7270	0.7046	0.6800	0.7039
	108	0.1782	0.1811	0.1971	0.1855
	117	0.0948	0.1143	0.1229	0.1106
EST-1	*086*	0.3200	0.3214	0.4257	0.3557
	100	0.6800	0.6786	0.5743	0.6443
EST-4	*096*	0.4143	0.4495	0.4714	0.4451
	100	0.5857	0.5505	0.5286	0.5549
GLDH	*086*	0.4571	0.4143	0.3714	0.4143
	100	0.5429	0.5857	0.6286	0.5857
GPI-2	*088*	0.4071	0.3857	0.3753	0.3894
	100	0.5929	0.6143	0.6247	0.6106
G_6PDH	*095*	0.1400	0.0740	0.1000	0.1047
	100	0.7571	0.8143	0.8286	0.8000
	120	0.1029	0.1117	0.0714	0.0953
GAPDH	*090*	0.4071	0.4357	0.3714	0.4047
	100	0.5929	0.5643	0.6286	0.5953
αG_3PDH-2	*080*	0.2429	0.2643	0.2922	0.2665
	100	0.7571	0.7357	0.7078	0.7335
LDH-2	*077*	0.2000	0.2000	0.2714	0.2238
	100	0.5857	0.5143	0.5000	0.5333
	150	0.2143	0.2857	0.2286	0.2429
ODH	*085*	0.1571	0.1857	0.1714	0.1714
	100	0.5429	0.5143	0.6286	0.5619
	115	0.3000	0.3000	0.2000	0.2667
PGM-1	*075*	0.3714	0.4086	0.4143	0.3981
	100	0.6286	0.5914	0.5857	0.6019
PGM-2	*078*	0.4286	0.4445	0.4379	0.4370
	100	0.5714	0.5555	0.5621	0.5630
SOD-2	*100*	0.6357	0.6500	0.5857	0.6238
	125	0.3643	0.3500	0.4143	0.3762
XDH	*100*	0.5925	0.5643	0.6086	0.5885
	108	0.4075	0.4357	0.3914	0.4115

Number of specimens (n) = 66 from each river.

Results

Genetic variability

Of the 23 allozymes examined, 15 enzymes (30 loci) yielded scorable activity and of these 14 loci – *AAT-2*, EST-1*, EST-4*, GLDH*, GPI-2**, G_6PDH*, aG_3PDH-2*, *GAPDH*, LDH-2*, ODH*, PGM-1*, PGM-2*, SOD-2** and *XDH** were polymorphic (46.7%) in *L. dussumieri* (Table 2; Fig. 2). Unlike in humans G_6PDH did not exhibit sex-linked inheritance in *L. dussumieri*. Both male and female specimens from all three rivers exhibited both homozygotes and heterozygotes. A sex-wise break up of G_6PDH is

given in Table 4. Seven out of the 36 microsatellite loci screened (Table 3; Fig. 3) gave clear scorable products with 4-7 alleles per locus. These seven polymorphic loci – *LdussG1* (AF517937), *MFW19* (DQ780025), *MFW26* (EU272894), *Bgon22* (EU272893), *Barb37* (DQ780024), *R3-1* (DQ780026), *R6* (DQ780027) - were confirmed to contain repeats and further considered for the population genetic analysis of *L. dussumieri.* Among these, two loci contained perfect repeats (*LdussG1* and *R3-1*) and sequence information of all the loci is presented in Fig. 5. The tandem repeats of five loci were same as that of the resource species while repeat motif of the locus *MFW19* and *Barb37* differed from that of the resource species. No significant genotype heterogeneity was observed between the multiple data sets (collections at different time intervals) within the rivers (Table 1). After combining the genotypic data from multiple data sets within each river, three data sets for the rivers - Meenachil, Manimala and Pamba were available for analysis of genetic variation and differentiation among *L. dussumieri* populations. Allele frequencies at polymorphic allozyme and microsatellite loci in *L. dussumieri* samples from the three riverine locations are presented in Tables 5 and 6 respectively. No population-specific alleles were observed for any allozyme or microsatellite locus. All the seven microsatellite loci exhibited considerable variation in all the sampled populations. In RAPD analysis, the PCR reagents were from the same source (GENEI Ltd., Bangalore, India) and the template DNA quantity and concentration were kept uniform across the samples (1ml and circa 25 ng respectively per single reaction). This resulted in high level of reproducibility and sharpness of RAPD profiles in *L. dussumieri* even from the DNA sample that was stored at -20°C for more than six months, demonstrating the robustness of the technique (Fig. 4). A total of 117 random amplified DNA fragment from specimens of *L. dussumieri* were detected consistently with the 12 decamer primers in three populations. The size of the fragment ranged from 200 to 3000bp. The number of fragments generated per primer varied from 06 to 14 and altogether 65 bands (55.56%) were found to be polymorphic (Table 10). The average gene diversity (H) for each primer in each population ranged from 0.000 (OPA-07) to 0.2380 (OPAA-08) and the mean value for overall populations was 0.1848 (Table 14). No test for linkage disequilibrium was statistically significant ($P>0.05$) for any pair of allozyme or microsatellite or RAPD loci within each of the sample sites and when all samples were considered together.

Summary statistics for parameters of genetic variation at each allozyme and microsatellite locus and across all loci are given in Tables 7 and 8 respectively. Mean number of alleles per locus ranged from 1.326 to 1.342 for allozyme loci and 3.000 to 3.750 for microsatellite loci. Mean values of observed heterozygosity ranged from 0.1445 to 0.1898 for allozyme loci and from 0.4948 to 0.5360 for microsatellite loci respectively. Six allozyme loci (*AAT-2**, *EST-4**, *GLDH**, *GPI-2**, *G_6PDH** and *LDH-2**) and three microsatellite loci (*LdussG1*, *MFW19* and *Bgon22* in all samples from three rivers) (Tables 7 and 8) exhibited consistent significant deviation from Hardy-Weinberg

Equilibrium expectations in populations after probability level (P<0.05) was adjusted for sequential Bonferroni correction. Significant deviation at the *AAT-2** and *GLDH* loci was found in all samples from three rivers; at locus *EST-4** and *LDH-2** only in the Meenachil and Manimala samples; and for the remaining two loci (*GPI-2** and G_6PDH) only in the Manimala and Pamba samples. F_{IS} values greater than zero (+ve) indicating deficiency of heterozygotes was evident in these cases (Tables 7 and 8). The three microsatellite loci (*LdussG1*, *MFW19* and *Bgon22*) exhibiting $+F_{IS}$ values were tested

Table 6. Microsatellite alleles and allele frequencies in *Labeo dussumieri* from three riverine populations and overall populations.

Locus	Allele size (bp)	Meenachil	Manimala	Pamba	Overall Populations
LdussG1	079	0.2323	0.2986	0.2753	0.2687
	087	0.4357	0.3925	0.4250	0.4177
	099	0.1028	0.1106	0.1201	0.1112
	115	0.2292	0.1983	0.1796	0.2024
MFW19	160	0.1818	0.1397	0.1714	0.1643
	172	0.2528	0.2750	0.2365	0.2548
	187	0.3275	0.3249	0.3369	0.3298
	196	0.1454	0.1479	0.1297	0.1410
	205	0.0925	0.1125	0.1255	0.1101
MFW26	145	0.2375	0.2222	0.2426	0.2341
	149	0.0425	0.0532	0.0698	0.0552
	155	0.0125	0.1103	0.0700	0.0643
	163	0.2053	0.2217	0.2021	0.2097
	171	0.2592	0.1263	0.1915	0.1923
	185	0.1063	0.1542	0.1253	0.1286
	193	0.1367	0.1121	0.0987	0.1158
Barb37	146	0.0963	0.1382	0.1179	0.1175
	162	0.2626	0.3250	0.3123	0.2999
	182	0.3613	0.2635	0.2845	0.3031
	206	0.2798	0.2733	0.2853	0.2795
Bgon22	118	0.1964	0.1673	0.1855	0.1831
	127	0.0625	0.0526	0.0723	0.0624
	136	0.2432	0.2516	0.2614	0.2521
	145	0.3743	0.4099	0.3476	0.3773
	157	0.1236	0.1186	0.1332	0.1251
R3-1	109	0.2549	0.2613	0.2984	0.2715
	117	0.3152	0.3048	0.2902	0.3034
	133	0.2637	0.1994	0.2349	0.2327
	145	0.1662	0.2345	0.1765	0.1924
R6	166	0.0889	0.0867	0.0728	0.0828
	174	0.0497	0.0381	0.0129	0.0336
	182	0.2163	0.3250	0.2843	0.2752
	194	0.3653	0.2647	0.3347	0.3215
	198	0.2798	0.2855	0.2953	0.2869

Number of specimens (n) = 66 from each river.

for the occurrence of null alleles. The estimated null allele frequency using MICRO-CHECKER was not significant (P<0.05) at all the three tested loci in all populations (Table 9). There was also the absence of general excess of homozygotes over most of the allele size classes in all the three loci in different rivers and no instance of non-amplification of samples in repeated trials - all indicating the absence of null alleles and false homozygotes. Therefore, for population genetic analysis, information from all the seven microsatellite loci was considered.

LdussG1 (NCBI GenBank Accession # AF517937)

AAAGAATATT TGGGAAAAAG AACAGAGTTG CACCACAGGA AAGGAACA**GA**
TAGATAGATA **GATAGATAGA** **TA**CAATTCA

Barb37 (NCBI GenBank Accession # DQ780024)

AAATACGCTC TCCTCATTAC TGTGG**AAATA** **AATAAAT**GTG G**AAATAAATA**
AATGTCT**AAA** **TAAATAAATA** **AATAAATAAA** **T**CTGGAAA**AA** **ATAAATAAAT**
AAGG**AAAT**AA **AATAAATAAA** **TAAAT**AATTT ATTTTTGCTT TTGTAC

MFW19 (NCBI GenBank Accession # DQ780025)

AGCCTAGGCT CGAGAAGCTT GTCGACGAAT TCAGATTACA GATGGGGAAA
GAGAGCATCC GAGCGTGACT GTACAATGAG TCTGCTAATT ACTTCATTCC
GGAGGAGGAG **GAGGAGGAGG** **AGGAGGAGGA** GCTCCGTGTG ATCAACAAGA
AATCACGAAT

R3-1 (lower locus) (NCBI GenBank Accession # DQ780026)

TATTCACCCC AAATCCATTA TCATTA**GTGT** **GTGTGTGTGT** **GTGTGTGTGT**
GTGTGTGAGA GCTGTCCTGG CCTGGGTGTT TGGCTGTGGT GGTCTTATGC
ACAAGGGTC

R6 (NCBI GenBank Accession # DQ780027)

TATCCTGGCT GAAAACTTTG CTGAAAACTT TGGAGGGAAA TG**GTGTGTGT**
CTC**GTGTGTG** **TGTGTGTGT**G ACGCTCAATG ATTTTCGGAA TCAGTAAAGT
AAAATTTTGT GTCTTTCTCA CAGCCTTTGT GGCTGTCTCC AGTGCAGGTG
ATGGTTGTTC CTGTAG

Bgon22 (NCBI GenBank Accession # EU272893)

TCTTGTTGAT CACACGGACG TCC**CCTCCT**T **CCTCCTCCTC** **CTCCTCCTCC**
TCCTCCTCCT **CCT**GGAATGG AATAATATTG ATACAGTGCT CGGATGCTCT
CTTTCCCCAT CTGTGTAA

MFW26 (NCBI GenBank Accession # EU272894)

CCCTGAGATA GAAACCACTG GACATTAATT AACAACCAGA TTAATTGTCT
TTTTAAA**CAC** **ACACACA**G**CA** **CACACACACA** A**CACACACAC** **ACACACACA**C
TGTGGAAATT TCCACGTTTC TTTTCTTTTG CATCCAAGCA TGGTG

Figure 5. Nucleotide sequences of seven microsatellite loci in *Labeo dussumieri*. Microsatellite regions are depicted in bold letters.

Population structure

The coefficient genetic differentiation (F_{ST}) under IAM model was consistent for both allozyme and microsatellite markers. The mean F_{ST} value across all the populations and all loci was 0.0034 (allozymes) and 0.0041 (microsatellites). Pair-wise comparisons of F_{ST} with probabilities of significance are given in Tables 11 and 12. Fixation indices under SMM Model (R_{ST}) for microsatellite loci were found to be comparable with F_{ST} values in pair-wise comparisons of samples (Table 12). The G_{ST} values were low for each RAPD primer across all populations (ranging from 0.0000 to 0.0115) and for overall populations (0.0081) (Table 13). Nei's (1978) unbiased genetic distance values estimated between pairs of three populations of *L. dussumieri* with allozymes, microsatellites and RAPD markers were also very low (Tables 14 and 15). AMOVA analysis revealed that 99.12% (allozymes) and 98.60% (microsatellites) variance was explained within populations/within rivers (Table 16). There was evidence of only very weak genetic differentiation among different populations of *L. dussumieri* sampled from three river basins.

Bottleneck analysis

The bottleneck results based on allozymes (IAM) and microsatellites (TPM) exhibited the expected L-shaped distribution indicating that all the populations followed mutation drift equilibrium (Fig.6). The probability values (Sign and Wilcoxon Tests) also indicated absence of genetic bottleneck in *L. dussumieri* populations (Table 17).

Table 7. Parameters of genetic variation for the fourteen polymorphic allozyme loci in *Labeo dussumieri* from three different riverine locations.

Locus	Populations (n = 66 each)		
	Meenachil River	Manimala River	Pamba River
AAT-2			
H obs.	0.2429	0.3429	0.1857
H exp	0.3907	0.5710	0.2535
F_{IS}	+0.385	+0.406	+0.268
P_{HW}	<0.0001***	<0.0001***	<0.0001***
EST-1			
H obs.	0.4771	0.4272	0.5214
H exp	0.4800	0.4362	0.4996
F_{IS}	+0.005	+0.002	-0.139
P_{HW}	1.0000	1.0000	1.0000
EST-4			
H obs	0.0797	0.1856	0.4562
H exp	0.1868	0.3150	0.4984
F_{IS}	+0.284	+0.310	+0.090
P_{HW}	<0.0001***	<0.0001***	0.4799
GLDH			
H obs.	0.2143	0.1948	0.1429
H exp	0.3367	0.3082	0.3024
F_{IS}	+0.265	+0.323	+.0664
P_{HW}	<0.0001***	<0.0001***	<0.0001***
GPI-2			
H obs.	0.3976	0.2855	0.1143
H exp	0.4193	0.3976	0.2971
F_{IS}	+0.072	+0.227	+0.311
P_{HW}	0.4228	<0.0001***	<0.0001***

G₆PDH			
H obs	0.2585	0.2414	0.1714
H exp	0.3678	0.3253	0.2212
F_{IS}	+0.230	+0.451	+0.316
P_{HW}	0.0970	<0.0001***	<0.0001***
αG₃PDH-2			
H obs	0.2612	0.3000	0.3714
H exp	0.2678	0.3374	0.3770
F_{IS}	+0.009	+0.068	+0.002
P_{HW}	1.0000	0.6997	1.0000
GAPDH			
H obs	0.1811	0.2000	0.1823
H exp	0.1909	0.2323	0.1877
F_{IS}	+0.009	+0.032	+0.002
P_{HW}	1.0000	0.5099	1.0000
LDH-2			
H obs	0.2857	0.2571	0.3618
H exp	0.3298	0.2996	0.4853
F_{IS}	+0.151	+0.091	+0.241
P_{HW}	<0.0001***	<0.0001***	0.0513
ODH			
H obs	0.4755	0.3857	0.4213
H exp	0.4963	0.3696	0.4669
F_{IS}	+0.086	-0.134	+0.150
P_{HW}	0.0926	0.2209	0.1036
PGM-1			
H obs	0.2400	0.3429	0.1925
H exp	0.2669	0.3898	0.2053
F_{IS}	+0.077	+0.142	+0.099
P_{HW}	0.0121*	0.0143*	0.0201*
PGM-2			
H obs.	0.1857	0.4000	0.1400
H exp.	0.1898	0.4291	0.1705
F_{IS}	+0.001	+0.005	+0.090
P_{HW}	1.0000	1.0000	0.3118
SOD-2			
H obs.	0.4286	0.3000	0.3143
H exp.	0.4632	0.4415	0.4553
F_{IS}	+0.297	+0.226	+0.329
P_{HW}	0.0192*	0.0014*	0.0033*
XDH			
H obs	0.2909	0.4515	0.2432
H exp	0.3228	0.4917	0.2955
F_{IS}	+0.010	+0.028	+0.024
P_{HW}	0.3211	0.5278	0.4646
Mean overall loci			
H obs	0.1660	0.1898	0.1445
H exp	0.2128	0.2355	0.2135
Fis	---	---	---
$P_{(0.95)}$	0.2486	0.3142	0.3482
$P_{(0.99)}$	0.2468	0.3142	0.3482
A_n	1.3261	1.3354	1.3421

H obs. = Observed heterozygosity; H exp. = Expected heterozygosity; F_{IS} = Inbreeding coefficient; P_{HW} = Probability value of significant deviation from HWE; $P_{(0.95)}$ = Polymorphism at 0.95 criteria; $P_{(0.99)}$ = Polymorphism at 0.99 criteria; A_n = Mean number of alleles per locus; * = Significant at P<0.05; ***= Significant after Bonferroni adjustment.

Discussion

The present study reports the distribution and patterns of genetic variation in the natural population of *L. dussumieri* estimated from allozyme, microsatellite and RAPD markers. Ruzzante (1998) demonstrated that sample sizes larger than 50 individuals are adequate to minimize bias due to large number of alleles in microsatellite data and Silva and Russo (2000) inferred that sample size should be more than 30; in the present study sample size was 66 individuals per site in three localities analyzed. Therefore, estimates of population differentiation obtained, are unlikely to be confounded by small sample sizes.

Table 8. Parameters of genetic variation for the seven microsatellite loci in *Labeo dussumieri* from three different rivers.

Locus	Populations (n= 66 each)		
	Meenachil	Manimala	Pamba
LdussG1			
H obs.	0.6571	0.6310	0.5501
H exp.	0.7976	0.7832	0.6308
Fis	+0.3292	+0.2741	+0.3703
P_{HW}	<0. 0001***	<0. 0001***	<0. 0001***
MFW19			
H obs	0.6943	0.6927	0.5571
H exp	0.7652	0.7479	0.6604
Fis	+0.3344	+0.0470	+0.1631
P_{HW}	<0. 0001***	<0. 0001***	<0. 0001***
MFW 26			
H obs	0.3336	0.2743	0.2571
H exp	0.3261	0.2767	0.2743
Fis	-0. 0123	+0.004	+0.0031
P_{HW}	0.9867	1.0000	0.8842
Barb37			
H obs	0.6098	0.5857	0.5671
H exp	0.5894	0.5539	0.6037
Fis	-0.0193	-0.0225	+0.0676
P_{HW}	0.9862	1.0000	0.0741
Bgon22			
H obs	0.3857	0.3180	0.3286
H exp	0.5291	0.4168	0.4321
Fis	+0.3748	+0.2143	+0.2827
P_{HW}	0.0111*	0.0122*	<0.0001***

R3-1			
H obs	0.7429	0.7387	0.6014
H exp	0.6871	0.7236	0.5644
Fis	-0.0743	-0.0254	-0.0834
P_{HW}	0.8072	1.0000	0.7828
R6			
H obs	0.6143	0.5802	0.6071
H exp	0.5949	0.5674	0.6098
Fis	-0.0318	-0.0022	+0.004
P_{HW}	0.9517	0.9687	1.0000
Mean Overall Loci			
H obs	0.4948	0.5360	0.5239
H exp	0.5067	0.5996	0.5619
Fis	---	---	---
$P_{(0.95)}$	0.8684	0.7896	0.7143
$P_{(0.99)}$	0.8684	0.7896	0.7143
A_n	3.7500	3.0000	3.3750

H obs = Observed heterozygosity; H exp = Expected heterozygosity; F_{IS} = Inbreeding coefficient; P_{HW} = Probability value of significant deviation from HWE; $P_{(0.95)}$ = Polymorphism at 0.95 criteria; $P_{(0.99)}$ = Polymorphism at 0.99 criteria; A_n = Mean number of alleles per locus; * = Significant at P<0.05; *** = Significant after Bonferroni adjustment.

Table 9. Summary statistics of null allele frequencies in microsatellite loci of *Labeo dussumieri.*

Microsatellite Locus	Populations showing positive F_{IS} values	Null allele frequency * (from MICRO-CHECKER)			
		Van Oosterhout	Chakraborty	Brooksfield 1	Brooksfield 2
LdussG1	Meenachil	0.0313	0.0402	0.0411	0.0411
	Manimala	0.0293	0.0278	0.0281	0.0281
	Pamba	0.0253	0.0189	0.0218	0.0218
MFW19	Meenachil	0.0043	0.0038	0.0048	0.0048
	Manimala	0.0116	0.0095	0.0108	0.0108
	Pamba	0.0163	0.0171	0.0188	0.0188
Bgon22	Meenachil	0.0380	0.0414	0.0373	0.0373
	Manimala	0.0013	0.0014	0.0017	0.0017
	Pamba	0.0025	0.0027	0.0032	0.0032

(* P< 0.05)

For allozyme loci, genetic variability in *L. dussumieri* was relatively high (mean H_o for overall loci 0.1445 - 0.1898), when compared with the values described for many freshwater teleosts (0.043 - Gyllensten, 1985; 0.046 - Ward et al., 1994). As reported for several vertebrates (Nevo et al., 1984) and plants (Frankham, 1996), populations of widespread species often show significantly higher heterozygosity

estimates than for population of species with more restricted distribution; as observed in the widespread and opportunistic European roach *Rutilus rutilus* (H_e = 0.0972 - 0.124; Bouvet et al., 1991) and mrigal, *Cirrhinus mrigala* in the Indian sub-continent (H_e = 0.1082 - 0.1224; Chauhan et al., 2007) in contrast to the endemic and rare species such as *Leuciscus* species (H_e = 0.0000 - 0.057). However, higher heterozygosity rates are also reported using allozyme markers in endemic teleosts with restricted distribution. Muneer et al. (2007) and Gopalakrishnan et al. (2006) in the endemic bagrid fish, *Horabagrus brachysoma* (H_o = 0.170 - 0.191); Musammilu (2008) in an endangered cyprinid, *Gonoproktopterus curmuca* (H_o = 0.145 - 0.156) and Tessier et al. (1995) in landlocked Atlantic salmon (H_e = 0.18 - 0.42) have recorded high heterozygosity rates as reported in the present study. Genetic variability estimates for *L. dussumieri* using microsatellite loci (H_o = 0.4948 - 0.5360; 4 - 7 alleles per locus) closely approximate the values reported for many freshwater fishes (H_o = 0.38 - 0.42; 2 - 7 alleles per locus; Chauhan et al., 2007). Estimate of gene diversity (H) is a measure of genetic variation with RAPD markers (Silas et al., 2005). In *L. dussumieri*, the H ranged from 0.0000 to 0.1998 with 12 decamers for overall populations and the values are in proximity to the gene diversity figures reported in other freshwater teleosts (0.132 - 0.215 in *G. curmuca*; Musammilu, 2008; 0.0558 - 0.1640 in *Tor malabaricus*; Silas et al., 2005). The percentage of polymorphism (%P) estimates (21.5 to 25.0%) with RAPD primers in *L. dussumieri* are also congruent with the values reported for many freshwater fishes (Musammilu, 2008; Silas et al., 2005; Cagigas et al., 1999).

Deviations from Hardy-Weinberg genotypic expectations were observed at some allozyme and microsatellite loci in the present study. One possible explanation for these observations, especially with the microsatellite loci is the presence of null alleles that do not amplify producing heterozygotes which cannot be distinguished (Van Oosterhout et al., 2004, 2006). But, the analysis of data using MICRO-CHECKER did not give any evidence for null allele homozygotes in *L. dussumieri* populations. Moreover, in *L. dussumieri*, significant departures from the Hardy-Weinberg equilibrium were found within samples across loci rather than within loci across most of the samples. Such a situation is not consistent with null alleles at these loci (Van Oosterhout et al., 2004; Musammilu, 2008) or null alleles were not present in significant frequency to be a major cause of observed heterozygotes deficit. Where homozygote excesses were detected, generally such deviations indicate that factors such as non-random mating, reduction in effective breeding populations or specific locus could be under selection pressure were the causes for the observed violations (Ferguson, 1995; Chauhan et al., 2007). Heterozygote deficits can also result from mixing of undetected genetically divergent stocks within the samples, referred to as Wahlund effect (Hartl and Clark, 1997). With respect to *L. dussumieri* fish escaping to rivers in large numbers from farms or hatcheries to create such a situation does not arise, as aquaculture and hatchery seed production of this species is yet to become a commercial activity in Kerala. Hence, the possible causes for the excess homozygosity levels can be speculated as over-

Table 10. The total number of RAPD fragments; number and percentage of polymorphic bands and average gene diversity for each and overall populations of *Labeo dussumieri*.

Primer Code	Meenachil River				Manimala River				Pamba River				Overall populations			
	Total no. of bands	No. of poly-morphic band s	% of poly-morphic bands (%p)	Average Gene diversity (H)	Total no. of bands	No. of poly-morphic bands	% of poly-morphic bands (%p)	Average Gene diversity (H)	Total no. of bands	No. of poly-morphic bands	% of poly-morphic bands (%p)	Average Gene diversity (H)	Total no. of bands	No. of poly-morphic bands	% of poly-morphic bands (%p)	Average Gene diversity (H)
OPA-07	08	0	00.00	0.0000	08	0	00.00	0.0000	08	0	00.00	0.0000	08	0	00.00	0.0000
OPA-08	12	3	25.00	0.0778	12	3	25.00	0.0778	12	3	25.00	0.0778	12	3	25.00	0.0778
OPA-09	08	2	20.00	0.0378	10	3	30.00	0.0424	10	3	30.00	0.0424	12	5	41.67	0.1584
OPA-10	06	1	16.67	0.0315	06	0	00.00	0.0000	06	1	16.67	0.0315	06	1	16.67	0.1370
OPAA-08	10	3	23.08	0.1051	10	2	20.00	0.2380	10	2	20.00	0.2380	10	5	50.00	0.1540
OPAA-12	07	2	28.57	0.0892	08	1	12.50	0.1241	06	0	00.00	0.0000	10	5	50.00	0.1540
OPAC-06	07	2	28.57	0.0892	07	2	28.57	0.0892	07	2	28.57	0.0567	07	2	28.57	0.0892
OPAC-14	07	2	28.57	0.0540	07	3	42.86	0.1889	07	3	42.86	0.1889	14	9	64.29	0.1998
OPAC-17	10	4	40.00	0.1047	10	4	40.00	0.1047	10	4	40.00	0.1047	10	6	60.00	0.1901
OPAH-09	10	3	30.00	0.0860	10	3	30.00	0.0860	10	3	30.00	0.0860	10	3	30.00	0.0860
OPB-05	06	2	33.33	0.0603	06	2	33.33	0.0603	06	2	33.33	0.0603	06	2	33.33	0.0603
OPB-08	07	2	28.57	0.0892	08	0	00.00	0.000	06	0	00.00	0.0000	10	2	20.00	0.0941
Total	98	26	--	--	102	23	--	--	98	23	--	--	115	43	--	--
Mean Primers	--	--	25.20	0.0687	--	--	21.86	0.0843	--	--	22.20	0.0739	--	--	34.96	0.1167

exploitation of the species over the years and habitat alteration leading to reduction in catches, ending with inbreeding, as reported by Kurup (1990) and CAMP (1998) and revealed from our constant interactions with the fishermen and local people during the study period.

Table 11. Pair-wise F_{ST} (θ) (above diagonal) and their significance levels (below diagonal) between riverine populations of *Labeo dussumieri* using allozyme markers.

Populations	Meenachil	Manimala	Pamba
Meenachil	—	0.0028	0.0064
Manimala	NS	—	0.0009
Pamba	NS	NS	—

NS = Not significant.

Comparable F_{ST} (θ) estimates from allozymes (0.0034), F_{ST} and R_{ST} values (0.0041 and 0.0048 respectively) from microsatellite loci and G_{ST} estimates 0.0081 from RAPD loci clearly indicate that the wild *L. dussumieri* populations are only weakly sub-structured and that only 0.34 to 0.81% of the total observed genetic variation is resulted from population differentiation. Wright (1978) and Hartl and Clark (1997) suggested that F_{ST} estimates in the range of 0.00 to 0.05 indicate only little genetic differentiation among populations. Ward et al. (1994) reviewed 49 freshwater species and observed F_{ST} estimates ranging from 0 to 74% with a mean of 22.2%. In their survey, 23 fish species out of 49 exhibited genetic differentiation (F_{ST}) ranging from 0 to 10% (Ward et al., 1994; Chauhan et al., 2007). Nei's (1978) genetic distance estimates between pair-wise populations of *L. dussumieri* were also low with all three classes of markers in the present study. AMOVA analysis of the data also did not indicate any significant genetic differentiation among the sampled populations of *L. dussumieri*. Comparing the selected morpho-meristic characters and life history parameters of *L. dussumieri* from Meenachil, Manimala, Pamba and Achankovil Rivers, Kurup (1990) also concluded that these populations constituted a homogeneous stock.

Table 12. Pair-wise Fisher's F_{ST} (θ) (above diagonal) and Rho-statistics R_{ST} (below diagonal) between riverine samples of *Labeo dussumieri* using microsatellite markers.

Populations	Meenachil	Manimala	Pamba
Meenachil	—	0.0047(NS)	0.0063(NS)
Manimala	0.0052(NS)	—	0.0012(NS)
Pamba	0.0069(NS)	0.0022(NS)	—

NS = Not significant.

It is crucial to identify populations that have undergone ancient or recent bottlenecks, because they may have been affected by the small population size through

demographic stochasticity, inbreeding or fixation of deleterious alleles, possibly leading to a reduced evolutionary potential and increased probability of extinction (So et al., 2006). Bottlenecked populations may exhibit gametic disequilibrium; reduced genetic diversity – particularly reduced allelic diversity and loss of rare or unique alleles; and increased heterozygosity relative to that expected at mutation drift equilibrium (Cornuet and Luikart, 1996). No evidence for a recent genetic bottleneck was observed in *L. dussumieri* populations based on allozyme and microsatellite data set analysis. Avise (2004) and So et al. (2006) opined that the ancient or recent bottlenecks will have little or no effect on the distribution of microsatellite genetic diversity and hence mitochondrial DNA markers may be a better choice for detecting genetic bottlenecks. However, allozyme and microsatellite markers were useful in identifying recent genetic bottlenecks in many freshwater teleosts, such as *G. curmuca* (Musammilu, 2008) and *H. brachysoma* (Muneer et al., 2009) and marine fishes like *Sardinella aurita* (Chikhi et al., 1998). While the present analysis based on allozyme and microsatellite loci did not pinpoint the evidence for a recent genetic bottleneck in *L. dussumieri* populations, it can be ascertained only through further studies employing mitochondrial DNA markers.

Table 13. Co-efficient of genetic differentiation (G_{ST}) using RAPD primers for overall populations of *Labeo dussumieri*.

Primer code	Sequence (5'- 3')	G_{ST}
OPA-07	GAAACGGGTG	0.0000
OPA-08	GTGACGTAGG	0.0075
OPA-09	GGGTAACGCC	0.0081
OPA-10	GTGATCGCAG	0.0096
OPAA-08	TCCGCAGTAG	0.0106
OPAA-12	GGACCTCTTG	0.0095
OPAC-06	CCAGAACGGA	0.0092
OPAC-14	GTCGGTTGTC	0.0115
OPAC-17	CCTGGAGCTT	0.0098
OPAH-09	AGAACCGAGG	0.0077
OPB-05	TGCGCCCTTC	0.0111
OPB-08	GTCCACACGG	0.0068
	Mean	0.0086
	Overall populations	0.0081

Genetic differentiation can be influenced by a number of evolutionary forces and their interaction that act on natural populations including; migration, random genetic drift, mutation etc (Hartl and Clark, 1997). Random genetic drift will tend to cause genetic differentiation, after subpopulations are fragmented and gene flow between them is either reduced or absent. *L. dussumieri* from different river basins sampled

here, are likely to have evolved from common ancestral gene pool. The genus *Labeo* is considered to have entered India during the Eocene (54-38 million years ago) following migration of Indo-Malayan fishes via the Indo-Brahma River, flowing westward from Assam in the north-east to the present-day Arabian Sea (Daniels, 2001). Migration of fishes that evolved during the Eocene (60 million years ago) continued until dismemberment of the Indo-Brahma River and formation of the modern river systems such as Indus, Ganga and Brahmaputra during the late Pleistocene. The Western Ghats were uplifted during the later half of the Eocene (circa 50 million years ago) even before India collided with mainland Asia. According to Radhakrishna (1993), before the uplift of the Western Ghats, there came into being the 67–68 million year old Deccan Traps and the 115-117 million year old Rajmahal Traps in peninsular India. These uplifted surfaces together with the Western Ghats and the Deccan Plateau were topographically ideal for a wide network of torrents, streams and rivers in peninsular India, providing conducive conditions for westward and southwestward migration of Malayan fishes (Radhakrishna, 1993). The still young and diversifying carps and catfishes found extensive habitats, as they were amongst the earliest colonizers invading peninsular India including Sri Lanka during the Eocene [Ceylon - which was a part Indian Peninsula up to the Miocene (24-5 million years ago) and again intermittently connected with the mainland upto the Holocene (10,000 years back)] (Silas, 1953). During the late Pleistocene (1.5-0.011million years ago), the Western Ghats came to be known more or less as they are today and the river capture, waterfalls and deep gorges gave rise to the present structure of watersheds in the region including Kerala (Daniels, 2001, Unnithan, 2001).

Table 14. Nei's pair-wise genetic distance using allozymes (above diagonal) and microsatellite markers (below diagonal) in *Labeo dussumieri*.

Populations	Meenachil	Manimala	Pamba
Meenachil River	****	0.0029	0.0032
Manimala River	0.0061	****	0.0014
Pamba River	0.0073	0.0041	****

The low genetic divergence among wild 'thooli' populations in spite of fragmentation may be as a result of the ongoing high gene flow among populations across the Meenachil, Manimala and Pamba Rivers. Originating from the Western Ghats, these rivers flow in close proximity, drain into the southern end of the Vembanad Lake and are connected to each other in the lower reaches through an extensive network of natural canals (Fig. 1). This could be the source of mixing of fish populations resulting in lack of significant allelic heterogeneity among the *L. dussumieri* populations. The southern end of the Vembanad Lake was converted into a freshwater lagoon following the construction of the Thanneermukkom Barrage in 1975 and Padmakumar et al. (2004) reported that this part of the lake as well as the interconnecting canals of the rivers act as a fish reserve, especially for the species such as *L. dussumieri*, *Puntius sarana*

subnasutus and *Horabagrus brachysoma*. Besides direct migration, a stepping stone model of migration that attributes effective gene flow to gene exchange among neighbors (Gaggiotti et al., 1999; Olivier et al., 2003) may also explain the lack of significant allelic heterogeneity, among thooli population in the river systems sampled here. The observed lack of private or locality specific allele at any of allozyme, microsatellite or RAPD loci argues in favour of effective ongoing gene flow. Therefore, common ancestry in the past and possible continuous exchange of individuals among rivers belonging to different river basins may explain the observed low levels of genetic differentiation among *L. dussumieri* populations. Comparable values for fixation indices based on the SMM (R_{ST}) and IAM (θ) estimates for microsatellite data indicate that the observed genetic structure of *L. dussumieri* population is likely to be of recent origin. In effect, there may have been insufficient time for isolation and mutational events to give rise to new alleles and unique genotypes as reported in *C. mrigala* (Chauhan et al., 2007).

Table 15. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) using RAPD markers in *Labeo dussumieri*.

Populations	Meenachil	Manimala	Pamba
Meenachil River	****	0.9923	0.9889
Manimala River	0.0077	****	0.9937
Pamba River	0.0111	0.0063	****

Allozyme, RAPD and microsatellite markers could be considered as random indicators to discriminate the three populations of *L. dussumieri*. Therefore, it would be of interest to compare the results obtained from the application of these three approaches to the same individuals. To date, only few studies have compared the results of allozymes with RAPD and microsatellites (Cagigas et al., 1999; Muneer, 2007, 2008, 2009; Musammilu, 2008). The three methods in the present study probably might have generated markers pertaining to different parts of thooli genome. But, all produced similar results and indicated only low genetic differentiation among the populations of *L. dussumieri*, indicating the robustness of the techniques applied. Although it was possible to gain a clear understanding of population structure using allozyme data alone, the use of more variable markers such as microsatellites and RAPDs could further confirm the analysis using allozymes which reinforced reliability of interpretations. These DNA techniques involved the examination of putative non-coding genes thought to be neutral, which permits high rates of mutation and lead not only to different alleles at each locus but also to an increase in the amount of genetic variation (Cagigas et al., 1999). Although the three techniques could clearly discriminate the populations, microsatellites as a basic genetic tool overcome some of the disadvantages displayed by the other two. First, because specific primer development for a particular species

can be both time-consuming and costly, primers developed in one species can be used to amplify homologous loci in closely related species (Scribner et al., 1996). Second, many microsatellite loci are thought to be neutral (Zardoya et al., 1996) but some allozyme loci may be influenced by selection pressure, allowing only a few alleles at each locus (Musammilu, 2008). Furthermore, because *L. dussumieri* populations are under endangered category, killing specimens to collect liver and muscle for allozyme analysis becomes a significant inconvenience (fin clips and body slime may not give satisfactory results for all allozymes), which makes it advisable to adopt other techniques. Transportation of tissue samples from remote areas in liquid nitrogen and their subsequent storage in -85^{0}C freezer until further analysis are other disadvantages associated with allozyme analysis. The sampling for microsatellites and RAPD is usually non-lethal or minimum invasive unlike for allozymes. The RAPD methodology also involves some disadvantages compared with microsatellites. The dominant character of RAPDs makes it impossible to distinguish between homozygote and heterozygote of a particular fragment, and the comparison of bands across different gels often makes data scoring more difficult. Although reproducibility both within and among laboratories has been proved for RAPD polymorphisms (Cagigas et al., 1999; Muneer, 2008, 2009; Musammilu, 2008; Nagarajan et al., 2006; also in the present study) some confusion still exists regarding its application in population genetics especially of endangered species (basic assumption in RAPD analysis is, the populations fit the Hardy-Weinberg equilibrium). The apparent disadvantages of the allozyme and RAPD techniques further enhance the utility of microsatellites for the analysis of population genetic problems.

Table 16. Analysis of Molecular Variance (AMOVA) based on allozyme and microsatellite markers in three populations of *L. dussumieri*.

Sources of Variation	Degrees of freedom	Variance component	Percentage of Variation (%)	Fixation indices
		Allozymes		
Among populations (Among Rivers)	2	0.1423 (Va)	0.878	0.00878 (NS)
Within populations (Within River)	393	16.0610 (Vb)	99.122	---
Total	395	16.2033 (Vt)	---	---
		Microsatellites		
Among populations (Among Rivers)	2	0.2259 (Va)	1.40	0.014(NS)
Within populat ions (Within River)	393	15.9111(Vb)	98.60	
Total	395	16.137 (Vt)		

NS = Not significant.

However, microsatellites are not free from short comings. Non-specific amplification, presence of stutter bands and very high level of polymorphism demanding large sample sizes (to adequately characterize the genetic variation both within and among populations, to ensure that apparent differences among populations are not due to sampling error) are often encountered with microsatellites, complicating the genotyping and analysis. But in the present study, the number of alleles per locus was relatively less compared to other teleosts (Na-Nakorn et al., 1999). Also, the PCR conditions were optimized to overcome the problem of stutter bands and non-specific amplification in *L. dussumieri*. The non-denaturing PAGE coupled with silver staining could resolve the alleles up to 2 base pair difference in the present study.

Table 17. Bottleneck analysis in three populations of *Labeo dussumieri* with allozyme and microsatellite markers

	I A M (allozymes)			T P M (microsatellites)		
	Meenachil	Manimala	Pamba	Meenachil	Manimala	Pamba
Sign Test (P)	0.06923	0.07946	0.07300	0.62501	0.30974	0.57712
Wilcoxon Test (P)	0.07391	0.08391	0.07810	0.67712	0.56183	0.54688

The distribution of genetic variation evidenced from allozyme, microsatellite and RAPD data clearly indicate only low genetic differentiation among *Labeo dussumieri* populations in the rivers of the Western Ghats. Gene flow across river basins, after common ancestry, probably did not allow evolutionary forces to result in significant genetic differentiation. For management of wild thooli stocks, an important challenge will be to maintain high levels of genetic variation over time. Regulated water flows in the rivers will be crucial to maintain necessary large effective breeding population sizes that may be threatened due to habitat alteration. The stocks from the three rivers do not require different management strategies and for propagation assisted river ranching programme of this species, large effective breeding population can be developed by mixing individuals from three rivers.

Though the confirmed range of natural distribution of *L. dussumieri* is Kerala and Sri Lanka, Johal and Tandon (1979; quoted from Jayaram & Dhas, 2000) reported the species from East Punjab, which was afterwards treated as doubtful (Talwar and Jhingran, 1991). Another closely related species of *L. dussumieri* with dubious identity is *Labeo rajasthanicus* Datta and Majumdar 1970 from Jaisamand Lake, Rajasthan, India (Jayaram & Dhas, 2000). In an independent study in our laboratory, *L. rajasthanicus* specimens collected from its type locality exhibited overlapping morphological and meristic counts with that of *L. dussumieri* from Kerala (Raymond, 2006). Genetic

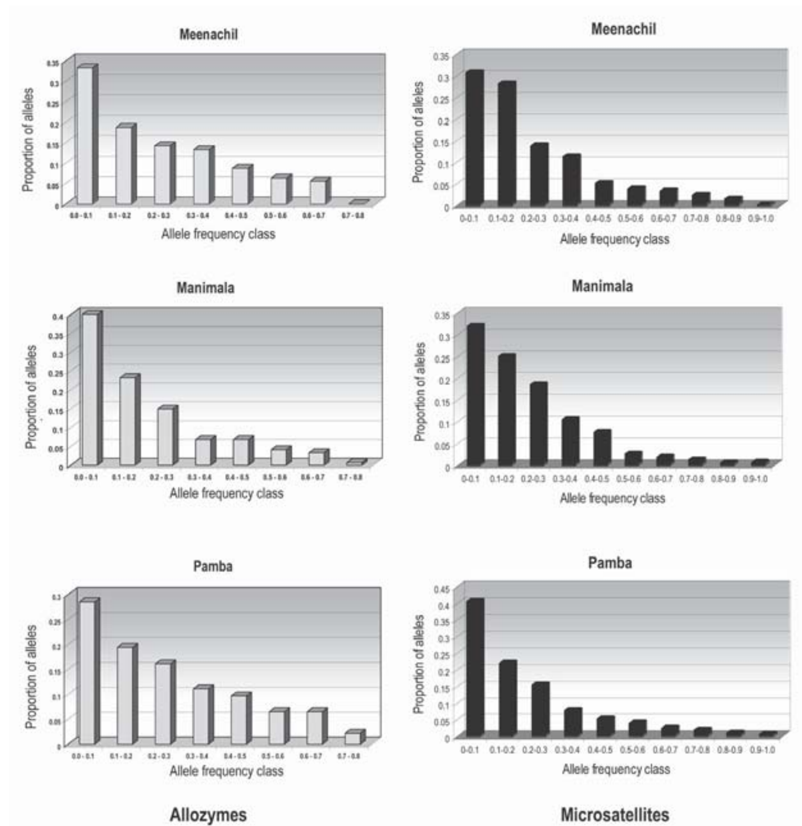

Figure 6. Qualitative "mode-shift" indicator test to discriminate bottlenecked populations of *Labeo dussumieri* from three rivers, based on allozyme and microsatellite allele frequency distribution.

divergence values between these two separate (?) species based on partial sequence information of mitochondrial 16SrRNA and Cyt*b* genes were very low (0.53% and 0.74% respectively; NCBI GenBank Accession #s DQ 520910 -13, DQ 520918 - 21; Raymond, 2006). Such low values of haplotype differences were rather unexpected especially since both species are separated by large geographical distances (~2500 km) and when there are no chances of migration and gene flow between Kerala and Rajasthan. Possibility of man-made introduction of *L. dussumieri,* even though the chances are remote, also needs to be explored. It is noted that the classic works like that of Hamilton-Buchanan (1822), McClelland (1839), Valenciennes (1847) and Day (1878) failed to make any mention about *L. rajasthanicus* or its closely related species *L. dussumieri* from Rajasthan (quoted from Jayaram & Dhas, 2000). In spite of the Indian and Sri Lankan land masses having been connected terrestrially from time to time upto the Holocene, the seperation of biotas of India and Sri Lanka in many cases is much more ancient (Silas, 1953; Bossuyt et al., 2004) and it would not be suprising if further research were to show the mainland and insular populations of *L. dussumieri* to be genetically distinct. The natural extension of the present study is to examine the finer scale of

phylogeography, population genetics and life history traits of these two species on a broad geographic range including Sri Lanka with intensive sampling not only to further investigate the evolutionary relationships, but also to assist in the management and recovery of rare and endangered populations.

Acknowledgements

The Indian Council of Agricultural Research (ICAR) and the National Agricultural Technology Project (NATP Sub-project MM-18), which supported the study financially, are gratefully acknowledged. The authors are grateful to Dr. S.P Singh (PI, NATP, NBFGR) for the encouragement and support. Technical assistance provided by Mr. Alfred Dourom is also duly acknowledged.

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Received: 05 February 2008; Accepted: 23 May 2009