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Evaluation of Genetic Variation in the Clown Knifefish, *Chitala chitala*, Using Allozymes, RAPD, and Microsatellites

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Abstract Twenty-seven enzyme systems, six random amplified polymorphic DNA (RAPD) primers, and two microsatellite loci were tested to determine intraspecific divergence in the natural population of the endangered Indian featherback fish, *Chitala chitala*, for the first time. The 262 samples of *C. chitala* were collected from six riverine locations in India: the Satluj, Ganga (Ghagra, Bhagirathi, and Brahmaputra), Mahanadi, and Narmada river systems. The analysis revealed population subdivisions, with an F_{ST} value from 0.1235 (95% confidence 0.0868–0.1621) for RAPD and a combined F_{ST} of 0.0344 (95% confidence 0.0340–0.0350) for microsatellite loci. An analysis of 38 allozyme loci did not reveal any polymorphism in the samples from any of the riverine localities; a possible explanation for this could be that the ancestors of *Chitala* could have faced a population reduction in prehistoric periods, as low allozyme variation is also reported for other species of *Chitala* from south Asia.

Keywords *Chitala chitala* · Genetic diversity · Allozymes · Microsatellites · RAPD

Introduction

Chitala chitala (Hamilton-Buchanan 1822), commonly known as the Indian featherback, belongs to one of the oldest groups of extant teleost freshwater fishes (subdivision Osteoglossomorpha, order Osteoglossiformes, family Notopteridae).

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It is widely distributed in freshwater bodies of the Indian subcontinent, including Bangladesh, Myanmar, Nepal, and Pakistan (Froese and Pauly 2003). Reported to grow up to 150 cm, it fetches high market value as a food fish (Lilabati and Vishwanath 1998) as well as for the ornamental trade (Hardwick 2005). Exploitation for those uses has caused *C. chitala* to decline in the wild, and it has been categorized as endangered (CAMP 1997; Goswami 2000). This fish is of considerable significance, not only as a resource that is under threat but also as a potential candidate species for aquaculture. Research on artificial propagation of the species for in situ conservation is being pursued actively (Sarkar et al. 2006).

Significant differences in biological parameters, such as specific rate of linear growth and specific rate of weight increase, have been reported recently for *C. chitala* collected from different rivers (Sarkar et al. 2008), but there are few stock management and restocking programs for such riverine fishes. Commensurate with the growing global concern for loss of biodiversity and efforts to protect biodiversity, steps have been taken in India to regularize the utilization of biodiversity and to plan conservation strategies for declining species (BDA 2002). Policies to protect the species will need scientific inputs on the genetic structure of the species in its native distribution range. Molecular markers have proven to be superior to the conventional ecological methods for determining gene flow of stocks of freshwater fishes (Wilson et al. 2004). Data on genetic variation can provide crucial input to plan effective strategies for conservation and rehabilitation of natural populations.

Population structure analyses of wild fish resources are primarily supported by allozyme, microsatellite, and random amplified polymorphic DNA (RAPD) techniques (Williams et al. 1990; DeWoody and Avise 2000). Within the past few years, microsatellite analysis has proved useful in detecting intrapopulation genetic variation (Brown et al. 2000; Robertson and Gemmell 2005; Chistiakov et al. 2006). Successful cross-species amplification of homologous microsatellite loci has been demonstrated in many fishes (Zardoya et al. 1996; Scribner and Pearce 2000; Mohindra et al. 2005). Recently, the combined use of different markers to determine genetic variability in fish species has received much more emphasis (Miller and Senanan 2003; Elliott and Reilly 2003; Alarcon et al. 2004).

Information on genetic markers and distribution of genetic variation in natural populations of *C. chitala* has been limited. Genetic research on the species is limited and restricted to karyotyping ($2n = 48$; Nayyar 1966) and genetic relatedness with another notopterid fish, *Notopterus notopterus* (Lal et al. 2006). The present study was designed to identify polymorphic genetic markers using different approaches, to describe the genetic variation of *C. chitala* populations across different rivers, and to determine its population structure.

Materials and Methods

Fish Samples

Chitala chitala specimens ($n = 262$) were obtained during May 2000 to May 2004 through commercial riverine catches from eight rivers located in various areas in

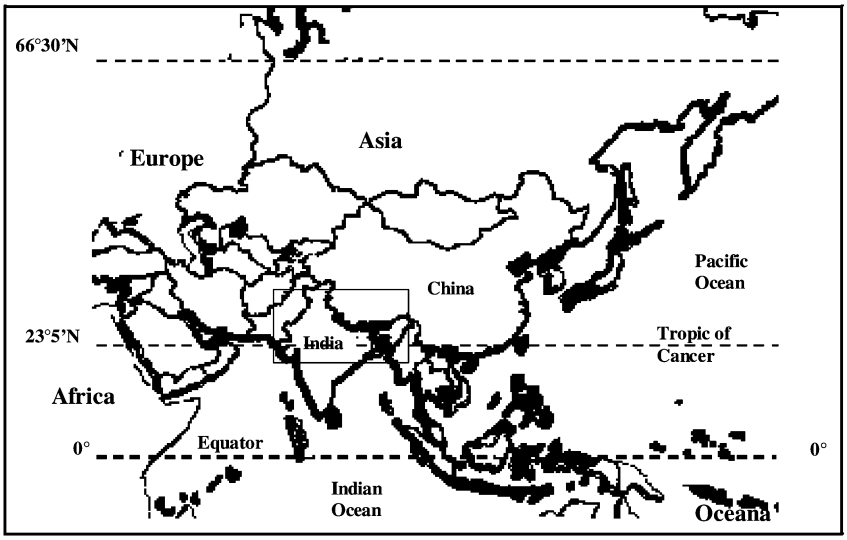
India (Fig. 1, Table 1). The riverine locations were carefully selected to cover geographically distant populations of *C. chitala*. The river Satluj is part of the Indus river system, whereas the Ghagra, Bhagirathi, and Brahmaputra are associated with the Ganga river system (ECAFE 1966). The rivers Narmada and Mahanadi originate from the Amarkantak mountain ranges in Central India, flowing west and southeast, respectively. The total length/body weight of *C. chitala* specimens ranged from 25 cm/250 g to 108 cm/10.5 kg. Liver and muscle tissues dissected for allozyme analysis were kept in liquid nitrogen (−196°C) immediately in the field and stored at −80°C till use. Blood from individual fish was collected through a puncture of the caudal vein at the fishing site, fixed in 95% ethanol in the ratio 1:5, and stored at 4°C till use.

Allozyme Analysis

A total of 23 enzyme systems were analyzed using vertical polyacrylamide gel electrophoresis (Amersham Biosciences Ltd.). Gels of 10 × 8 cm were used for all the enzymes, except esterase, which was resolved on 10 × 12 cm gels. Electrophoretic enzyme profiles did not reveal any polymorphism in *C. chitala*. Therefore, another notopterid fish, *N. notopterus*, was used to evaluate different electrophoresis conditions with respect to clear scorability of bands, resolution of species-discriminating alleles, and polymorphism in *N. notopterus* (Lal et al. 2006). Allozyme profiles of seven samples for each species, collected from rivers Satluj and Brahmaputra, were simultaneously examined on the same gel for electrophoresis parameters such as buffer, time, tissue, and extract application volume. Both liver and muscle tissues were subjected to allozyme analysis, but muscle samples did not provide any additional loci (except *GPI**) or better resolution compared with that observed in liver. Therefore, liver was selected as the optimum tissue for all the enzyme systems, and muscle was used to resolve *GPI**. Tris-borate EDTA (TBE, pH 8.0) provided better resolution than Tris-citrate EDTA (TCE, pH 7.4) and Tris-glycine EDTA (TGE, pH 8.3); therefore, it was used as the running buffer for all the enzyme systems. Extract application volume (1–3 μl) and electrophoresis running time (40–130 min) were optimized for each enzyme system.

Frozen liver samples (60–80 mg) were crushed mildly and homogenized at 500 mg/ml in extraction buffer (0.17 m sucrose, 0.2 m EDTA, 0.2 m Tris–HCl, pH 7.0). Homogenates were centrifuged (Sigma Laborzentrifugen, Osterode) at 10,000 rpm for 1 h at 4°C. The supernatant was subjected to recentrifugation for 20 min. Sample extracts were electrophoresed on 8% polyacrylamide gel at a constant voltage of 150 V at 4°C in a cold chamber. Allozyme patterns were visualized by histochemical staining following the procedure outlined by Whitmore (1990). The nomenclature of loci and alleles recommended by Shaklee et al. (1990) was followed. At all the loci, the most common allele in the Satluj population was assigned 100. Alternate alleles were designated according to their mobility relative to the most common allele.

A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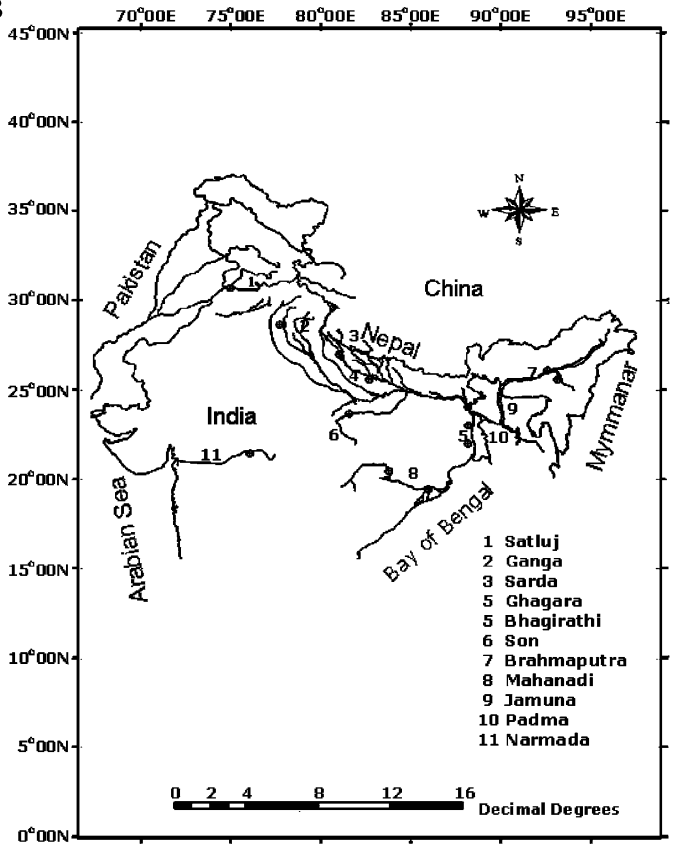
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5 Bhagirathi
6 Son
7 Brahmaputra
8 Mahanadi
9 Jamuna
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11 Narmada
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◀ **Fig. 1** **a** Location of study area in relation to Asia. The study area of and surrounding northern India is within the box. **b** Locations of sampling stations in various river basins for population structure study of *Chitala chitala*. Of the 12 rivers listed on the map, 8 were sampled, with multiple locations within some rivers

DNA Extraction

Genomic DNA was extracted from ethanol-fixed blood samples, using the phenol-chloroform extraction protocol (Ruzzante et al. 1996), resuspended in TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0), and the concentration was determined through serial dilutions on 0.7% agarose gel in 0.5 × TAE (1×= 40 mM Tris acetate, 1 mM EDTA).

RAPD Analysis

Screening of Primers

In the primary analysis, 80 random primers of 10 mer from Operon Technologies (arbitrary primers from OPA, OPB, OPP, and OPH kits) were tested on 32 individuals ($n = 4$, from each of eight rivers).

Table 1 Collection of *Chitala chitala* samples from eight rivers in India

River system	River	Area/Location (Latitude; Longitude)	Period of sampling	Sample/ Collection	Total
Indus	Satluj	Hari Ke Pattan, Punjab (31°09′ N; 74°56′ E)	May 2000	58	70
			June 2001	12	
Ganga	Ganga	Bijnor, Uttar Pradesh (29°19′ N; 78°04′ E)	June 2002	04	04
	Bhagirathi	Farakka, West Bengal (24°05′ N; 88°06′ E)	January 2001	15	41
			June 2001	05	
			July 2002	05	
			July 2003	16	
	Sarda	Sarda Barrage, UP (28°21′ N; 88°11′ E)	December 2000	05	15
			June 2001	03	
			November 2001	07	
	Ghagra	Ajaypur, Uttar Pradesh (26°01′ N; 83°22′ E)	December 2000	24	34
			March 2002	10	
	Bramhaputra	Kalang, Assam (26°16′ N; 91°46′ E)	December 2000	09	63
			January 2001	54	
Narmada	Narmada	Bhopal, Madhya Pradesh (23°14′ N; 77°23′ E)	April 2003	11	11
Mahanadi	Mahanadi	Hirakund, Orissa (20°27′ N; 85°52′ E)	May 2004	24	24
Total					262

Amplification Conditions, Electrophoresis, and Genetic Diversity Analysis

RAPD-PCR reactions were performed in a total reaction volume of 25 µl, containing 100 ng template DNA, 1×buffer (500 mM KCl, 100 mM Tris–HCl; pH 9.0), 0.01% gelatin, 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 5 pmoles primer, and 1.5 U *Taq* DNA polymerase (Genei, India), using a MJ PTC-200 Research thermal cycler. The reaction conditions were denaturation at 94°C for 5 min, followed by 45 cycles of 94°C for 30 s and 36°C for 30 s, elongation at 72°C for 1 min, with a final elongation at 72°C for 4 min. One negative control (absence of template DNA) was included for each set of amplifications. Approximately 8 μl of amplified product was separated on 2% agarose gel (Sigma) by submarine gel electrophoresis of 12 × 10 cm in 1× TBE buffer (Tris-borate EDTA; 89.0 mM Tris, 2.0 mM EDTA, 89.0 mM boric acid), pH 8.0, for 2 h at constant voltage of 70 V. Allele sizes were estimated by interpolation from DNA standards (λ DNA double digest) run in every gel. Based on screening of RAPD profiles, for clear resolution, six primers (OPA-19, OPB-08, OPB-14, OPB-20, OPH-05, and OPH-18) were selected for assessment of genetic variation. Separate amplifications were performed to assess repeatability of results. Only the loci that exhibited absolute repeatability were considered markers.

RAPD fragments were interpreted under the following assumptions (Black 1993) during data analysis: (a) all fragments show complete dominance (Williams et al. 1990), (b) all loci have two alleles, (c) the population is in Hardy–Weinberg equilibrium (Lynch and Milligan 1994), and (d) each fragment, regardless of primer, was treated as an independent locus. Some of these assumptions may not be valid, in particular, that fragments with the same electrophoretic mobility are genetically identical and that absent fragments represent the same DNA fragment. Clearly defined and intensely stained fragments were scored, following Black (1993), to genotype the individual sample.

Microsatellite Analysis

Amplification Conditions and Electrophoresis

Amplification was carried out in a 25 µl reaction mixture, which included 1×PCR buffer (10 mM Tris–HCl, pH 9.0), 50 mM KCl, 0.01% gelatin, 0.2 mM each $dNTP_s$, 2.0 mM $MgCl_2$, 5 pmoles each primer, 1.5 U *Taq* DNA polymerase, and 25–50 ng template DNA, using an MJ PTC-200 thermal cycler, with initial denaturation at 94°C for 5 min, followed by 25 cycles of 94°C for 30 s and 52/55°C annealing temperature for 30 s, elongation at 72°C for 1 min, with a final elongation at 72°C for 4 min and final soak at 4°C. PCR-amplified products were screened by electrophoresis, on 10% nondenaturing polyacrylamide (19:1 acrylamide:bisacrylamide) gel of 12 × 10 cm and run with 1×TBE buffer for 4–5 h at constant voltage of 150 V at 4–6°C. The amplified microsatellite loci were visualized through a silver staining kit (Amersham Biosciences, USA). The alleles were designated according to PCR product size relative to a molecular marker (pBR322 DNA/*Msp*I digest) with Image Master 1D Elite version 3.01 (Amersham Biosciences). Wang et al. (2003) reported that the non-denaturing electrophoresis system provides

resolution comparable to that obtained with denaturing acrylamide gels and silver staining, with the additional advantage of ease of use in analysis of large sample size. Moreover, Bovo et al. (1999) demonstrated that non-denaturing electrophoresis is not responsible for spurious or multiple bands in microsatellite analysis.

Screening of Primers and Genetic Diversity Analysis

PCR primers for 21 microsatellite loci from *Cyprinus carpio* (Croojimans et al. 1997), 18 from *Scleropages formosus*, 14 from *Arapaima gigas* (Farias et al. 2003), 13 from *Catla catla* (Naish and Skibinski 1998), 5 from *Barbus barbus* (Chenuil et al. 1999), 5 from *Barbodes gonionotus*, 4 from *Pangasius hypophthalmus*, and one each from *N. notopterus* (microsatellite sequence within the RAG1 gene, referred to here as the NN90 locus; Venkatesh et al. 1999) and *Tropheus moorii* (Zardoya et al. 1996) were tested to amplify homologous microsatellite loci in *C. chitala*. Twelve specimens of *C. chitala* were used in this cross-species amplification experiment. The optimum annealing temperature was determined through experimental standardization for each primer pair. The primers yielding scorable, polymorphic amplified products were again evaluated with 262 *C. chitala* samples. Each individual band pattern was analyzed and alleles were identified according to their molecular weight. Different individuals were genotyped as homozygote or heterozygote at each of the microsatellite loci.

Data Analysis

RAPD Data

The allele frequency of every fragment was calculated on the basis of the inferred homozygous recessive genotype using the TFPGA program (Miller 1997). Because of the dominant nature of the alleles, and in order to correct the bias originated by calculating the recessive allelic frequencies, the Taylor expansion (Kendall and Stuart 1977) was implemented through the TFPGA program. This reduction in the bias is based on the equation resulting from the second-order expansion of Taylor (Lynch and Milligan 1994).

An exact probability test was used to test the homogeneity of RAPD allele frequencies (Raymond and Rousset 1995b), consisting of a contingency analysis for every polymorphic locus and an estimation of probability values by the combined probability of Fisher (Sokal and Rohlf 1995), as implemented in the software TFPGA (Miller 1997). Weir and Cockerham's (1984) methods of calculating Wright's F-statistics were applied to the data. Pairwise comparisons were conducted to determine allele frequency differences among samples in order to define sources of variation. Standard error and confidence intervals (95%) were obtained through jackknife and bootstrapping (1,000 replicates) procedures. Genetic relatedness between sampling localities was also assessed through the unweighted pair group method (UPGMA) based on Nei's (1978) unbiased minimum genetic distance. The significance of node was computed through 1,000 bootstraps.

Allozyme and Microsatellite Data

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). Parameters of genetic variation, such as allele frequency, heterozygosity (observed and expected), proportion of polymorphic loci ($P_{0.95}$ and $P_{0.99}$) in each population, and mean heterozygosity over all loci for each population, were determined using Genetix version 4.05 software (Belkhir et al. 1997). Tests for conformity to Hardy–Weinberg expectations (probability and score test) and linkage disequilibrium were undertaken in Genepop version 3.3d software (Raymond and Rousset 1995a). The genetic heterogeneity of all population and pairwise localities was determined using an exact test based on log-likelihood (G) that assumes random samples of genotypes (Genepop version 3.3d, Genotype differentiation test, Raymond and Rousset 1995a). This test is performed on genotype tables, and possible nonindependence of alleles within genotypes does not affect test validity (Raymond and Rousset 1995b; Goudet et al. 1996). The null hypothesis (H_o) tested was that the genotype distribution was identical across all populations. Fixation indices were estimated to determine the extent of population subdivision among samples. For the former, Genetix version 4.05 software (Belkhir et al. 1997) was used to estimate F-statistics (Wright 1951) computed as estimators θ, F, and f of Weir and Cockerham (1984). The probability of θ significantly deviating from zero was calculated using 1,000 bootstraps. To correct for multiple simultaneous comparisons, sequential Bonferroni corrections were applied using a global significance level of 0.05 (Lessios 1992). Nei's (1978) unbiased minimum genetic distance was computed to construct UPGMA using the software TFPGA (Miller 1997). The significance of each node was computed through 1,000 bootstraps.

Results

Genotype proportions from multiple data sets (collection in different years) in the Satluj, Bhagirathi, Sarda-Ghagra, and Brahmaputra rivers were tested for genotype homogeneity, for both RAPD and microsatellite data. The test did not indicate any significant divergence among the multiple sets within each of these rivers ($P > 0.05$). The genotype data sets within a river were pooled; six combined genotype data sets (Satluj, Bhagirathi, Ghagra, Brahmaputra, Mahanadi, and Narmada) were available for investigation of genetic variation and differentiation.

Profiling and Genetic Variation

Allozyme

An analysis of 27 enzyme systems (list not given) yielded 38 loci in *C. chitala*, but none of the allozyme loci exhibited alternate alleles in any of the individual samples from riverine localities, and therefore none of the statistical tests was feasible.

Table 2 Allele frequencies of RAPD loci in *Chitala chitala* populations of six rivers in India

Locus	Population					
	Satluj	Bhagirathi	Ghagra	Brahmaputra	Narmada	Mahanadi
OPB-14-2711	0.4539	0.7959	0.5830	0.5224	1.0000	0.7915
OPB-14-1314	0.7351	0.7113	0.6703	0.6811	0.3876	0.7915
OPB-14-852	0.6254	0.6181	0.8526	0.7745	0.6464	0.5830
OPB-14-775	0.5412	0.5918	0.7915	0.6556	0.6464	0.7915
OPH-05-2499	0.7038	0.8509	1.0000	0.6864	1.0000	1.0000
OPH-05-912	0.4380	0.5528	0.6773	0.6379	0.3333	1.0000
OPA-19-3054	0.6697	0.3316	0.4947	0.3567	0.1056	1.0000
OPA-19-1733	0.7303	0.3158	0.4947	0.2808	0.1056	0.7113
OPA-19-819	0.8652	1.0000	0.7937	1.0000	1.0000	1.0000
OPA-19-695	0.7303	0.2562	0.3642	0.1800	0.1056	0.7113
OPH-18-2166	0.7160	0.7143	0.7113	0.6349	1.0000	1.0000
OPH-18-1363	0.7800	0.8571	0.8557	1.0000	1.0000	1.0000
OPH-18-1035	0.5421	0.7526	0.5918	0.7113	0.5286	0.5918
OPH-18-662	0.6408	0.4110	0.5213	0.4373	0.4226	0.4226
OPH-18-449	0.5421	0.7980	0.6181	0.6349	0.5286	0.7113
OPB-08-2223	0.8698	0.7143	1.0000	0.7706	1.0000	1.0000
OPB-08-2027	0.8698	0.7143	1.0000	0.7706	1.0000	1.0000
OPB-08-1617	1.0000	1.0000	1.000	1.0000	1.0000	1.0000
OPB-08-1122	0.2081	0.3939	0.233	0.2745	0.4523	1.0000
OPB-08-716	0.5490	0.6220	0.6703	0.6756	0.4523	1.0000
OPB-08-516	0.5306	0.6220	0.6099	0.7351	0.4523	1.0000
OPB-20-1709	1.0000	0.7389	1.0000	0.8652	0.5528	1.0000
OPB-20-1352	1.0000	0.8492	1.000	1.0000	1.0000	1.0000
OPB-20-1069	1.0000	1.0000	1.0000	1.0000	0.6838	1.0000
OPB-20-748	0.6307	1.0000	0.6056	0.8652	1.0000	1.0000
OPB-20-722	0.8492	0.4778	1.0000	0.8093	0.2254	1.0000
Avg. sample size	55.6154	47.2692	46.5000	57.8846	9.4231	23.8462
Avg. exp. heterozygosity	0.3463	0.3461	0.2956	0.3229	0.2703	0.1446
% Polymorphic loci	84.6154	84.6154	69.2308	80.7692	61.5384	34.6154

RAPD

Amplification with six RAPD primers yielded 26 loci in *C. chitala*. The 26 loci exhibited scorable and consistent patterns across the amplification trials. The proportion of polymorphic loci ranged from 34.6% to 84.6% (Table 2) in *C. chitala* samples from six rivers. Mean heterozygosity (exp) value ranged from 0.145 (Mahanadi) to 0.346 (Satluj). None of the 26 loci was found to be restricted to any specific population; however, frequencies were different (Table 2).

Microsatellites

Out of 82 primer pairs tested, 13 (16%) primers pairs yielded amplified PCR products in *C. chitala*. Five out of 19 (22%) primer pairs of *Scleropages formosus* (order Osteoglossiformes), one out of four (25%) of *Pangasius hypothalamus* (order Siluriformes), and one *N. notopterus* (order Osteoglossiformes) primer provided amplification of homologous microsatellite loci in *C. chitala*. The primers of the order Cypriniformes did not amplify any locus. The primer sequences, repeat motif, and specific annealing temperature in *C. chitala* for these primer sets are given in Table 3. Two microsatellite loci, D16 and NN90, were polymorphic and characterized by a symmetrical two-banded pattern in heterozygotes. Allele size and frequencies for the samples from the six localities are given in Table 4.

Table 3 Characteristics of amplified microsatellite loci in *Chitala chitala*

Resource species	Locus	Primer sequence (5′→ 3′)	Repeat motif	T_a (°C)	Alleles
Notopterus notopterus	NN90	CATGCATCCATTGTACAGCAA CTCCCAATGCTCTCCCAATA	CA	55	5
Scleropagus formosus	D13	GCTCTCAGCTGCTGTGTCTG GTGCATGCCCATGGAGAG	$(GT)_{12}$ $(CT)_{23}$	55	1
	D16	CCTGTGTTGCGGGTTAGG GGCCTTTTCTGCTGGTAAAA	$(GT)_{20}$	52	4
	D27	GAATCTGTAGAACTTGTTGATATGGG TGAAAATGACAATGGCAGCA	$(CA)_{17}$	50	1
	D33	CACATGCATGGAATTATGGC GAGCCAGAAGCAGGACTGAC	$(CA)_{12}AA$ $C(CA)_4$	53	1
	D94	GAAGCAAGGCTGGCTGTTAC CGCAGGCTGATTAAAGGTGT	$(CA)_{16}$	50	1
Pangasius hypothalamus	Phy07	AGTCACTTCAGCACCTGCCT ATCTTGTGATGGTGAGCCA	CA	57	Multiple bands

Table 4 Allele frequencies at two microsatellite loci in *Chitala chitala* of six riverine locations in India

Locus	Allele (bp)	Satluj	Bhagirathi	Ghagra	Brahmaputra	Narmada	Mahanadi
NN90	134	0.1452	0.1750	0.2614	0.0902	0.3000	0.0000
	154	0.0887	0.0455	0.0455	0.0164	0.1500	0.1190
	160	0.6855	0.7045	0.6136	0.8279	0.4000	0.6905
	164	0.0806	0.0568	0.0795	0.0656	0.1500	0.1667
	180	0.0000	0.0227	0.0000	0.0000	0.0000	0.0238
D16	424	0.0106	0.0541	0.1026	0.0424	0.1667	0.2222
	448	0.6702	0.4865	0.4615	0.5763	0.3333	0.2778
	462	0.1915	0.3108	0.3333	0.2881	0.5000	0.5000
	480	0.1277	0.1486	0.1026	0.0932	0.0000	0.0000

Table 5 Genetic variation of two microsatellite loci in *Chitala chitala* from six riverine systems in India

Locus[a]	River (Number of samples)					
	Satluj (65)	Bhagirathi (49)	Ghagra (45)	Brahmaputra (63)	Narmada (11)	Mahanadi (24)
NN90						
H_{obs}	0.4839	0.5455	0.6136	0.3115	0.8000	0.5238
H_{exp}	0.4947	0.4688	0.5467	0.3019	0.7050	0.4807
P_{HW}	0.0969	0.5263	0.5446	0.5310	0.9002	0.5600
P_{score}	0.5688	0.9692	0.7667	0.2721	0.7178	0.7641
F_{IS}	+0.030	−0.152	−0.111	−0.023	−0.083	−0.065
D16						
H_{obs}	0.2340	0.6757	0.6154	0.4237	1.0000	1.0000
H_{exp}	0.4977	0.6417	0.6548	0.5744	0.6111	0.6235
P_{HW}	0.0000	0.3088	0.3180	0.0126	1.0000	0.0001
P_{score}	0.0020	0.7718	0.2813	0.0207	0.4000	0.2313
F_{IS}	+0.537	−0.039	+0.073	+0.270	−0.500	−0.585
Mean						
H_{obs}	0.3590	0.6106	0.6145	0.3676	0.9000	0.7619
H_{exp}	0.4962	0.5552	0.6008	0.4382	0.6581	0.5521
$P_{(0.95)}$	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
$P_{(0.99)}$	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
A_n	4.0000	4.5000	4.0000	4.0000	3.5000	3.5000

[a] Parameters: H_{obs} Observed heterozygosity. H_{exp} Expected heterozygosity. P_{HW} Probability value of significant deviation from Hardy–Weinberg equilibrium. F_{IS} Inbreeding coefficient. $P_{(0.95)}$ Polymorphism at 0.95 criterion. $P_{(0.99)}$ Polymorphism at 0.99 criterion. A_n Mean number of alleles per locus

The mean number of alleles ranged from 3.5 (Narmada and Mahanadi samples) to 4.5 (Bhagirathi samples), and observed heterozygosity ranged from 0.359 in Satluj to 0.900 in Narmada (Table 5). The number of individuals assayed, heterozygosity value (H_{obs} and H_{exp}), probability of conformance to expected Hardy–Weinberg proportion per locus per population, and F_{ST} (inbreeding coefficient) are given in Table 5. Genotype proportion at two polymorphic loci was tested for departure from Hardy–Weinberg equilibrium expectation. Significant deviation was evident in the Satluj and Mahanadi samples at locus D16 ($P < 0.05$). The probability test indicated that deviation from the expectation was associated with deficiency of heterozygotes in the Satluj samples and excess of heterozygotes in the Mahanadi samples at the D16 locus (Table 5). A more powerful score test (Rousset and Raymond 1995) that assesses a specific alternate hypothesis of heterozygote deficiency, or excess, confirmed the deficit of heterozygotes in Satluj. The probability values through the score test were not sufficient to confirm the heterozygosity excess in Narmada and Mahanadi samples. The test for genotypic disequilibrium confirmed that no value was statistically significant ($P > 0.05$) for the pair of loci in any samples and across all the samples. This indicated that genotypes were independent and not linked at these microsatellite loci for *C. chitala*.

Interpopulation Diversity and Population Structure

RAPD

Pairwise comparisons were performed to assess allelic homogeneity (Table 6), which revealed that 9 out of a possible 15 tests had significant heterogeneity after sequential Bonferroni corrections ($P < 0.0033$). Satluj samples were significantly differentiated from Bhagirathi, Ghagra, Brahmaputra, Mahanadi, and Narmada samples. Bhagirathi samples were also divergent from Mahanadi and Ghagra samples. Other pairs that revealed heterogeneity included Brahmaputra with Mahanadi, and Narmada with Ghagra as well as Mahanadi samples ($P < 0.0033$). The UPGMA relationship among *C. chitala* samples from the six localities agrees with the conclusion drawn from Fisher's exact test for genetic heterogeneity (Fig. 2).

Table 6 Genetic distance of *Chitala chitala* collected from six riverine systems in India, using RAPD loci

	Satluj	Bhagirathi	Ghagra	Brahmaputra	Narmada	Mahanadi
Satluj	–	0.0461	0.0204	0.0347	0.1001	0.0800
Bhagirathi	<0.0001**	–	0.0385	0.0121	0.0337	0.0892
Ghagra	0.0030**	0.0002**	–	0.0190	0.0781	0.0674
Brahmaputra	<0.0001**	0.3033	0.1259	–	0.0569	0.0851
Narmada	<0.0001**	0.6700	0.0001**	0.0261*	–	0.1505
Mahanadi	0.0009**	<0.0001**	0.0078	<0.0001**	<0.0001**	–

Above the diagonal: Nei's (1978) minimum unbiased genetic distance. Below the diagonal: Probability; * significant before correction; ** significant after sequential Bonferroni correction ($P < 0.0033$)

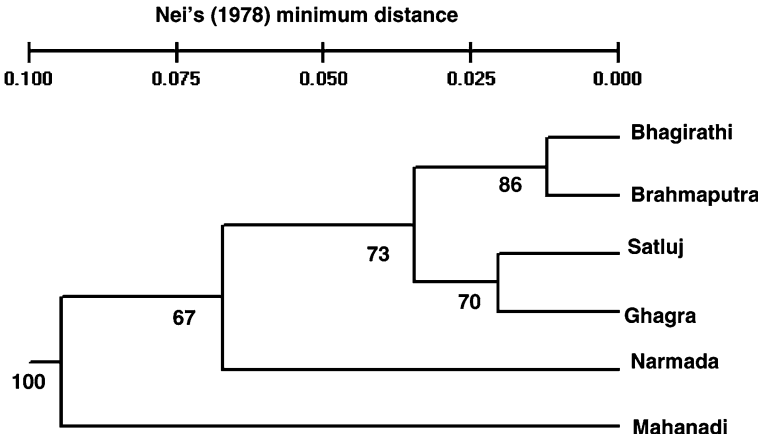

Fig. 2 UPGMA dendrogram from RAPD analysis of *Chitala chitala* from six rivers in India. Based on Nei's (1978) minimum genetic distance, with number of bootstraps (%) at each node

Microsatellite

Genetic differentiation was observed among *C. chitala* populations at both loci. Spatial homogeneity in genotype distribution at each microsatellite locus was tested over all populations. When considered for each locus, Mahanadi samples exhibited significant heterogeneity from the Satluj, Bhagirathi, and Brahmaputra samples at the D16 locus after sequential Bonferroni correction ($P < 0.0033$), and the Mahanadi samples had significant differences from Bhagirathi, Ghagra, Brahmaputra, and Narmada samples at the NN-90 locus (Table 7). The Brahmaputra samples were significantly heterogeneous in comparison with the Narmada samples at the NN-90 locus. Probability values for each population pair across all loci based on Fisher's exact test indicated that five population pairs had significant values after sequential Bonferroni correction ($P < 0.0033$). Mahanadi samples had significant heterogeneity among all populations except the Narmada samples (Table 8).

Estimates of F_{ST} over all loci were found to be 0.0344 (95% confidence 0.0340–0.035). Pairwise comparison of F_{ST} values indicated that the results agreed with the test for genetic homogeneity. Satluj and Mahanadi exhibited the highest F_{ST} value (0.126, $P < 0.0001$). The high F_{ST} value of the Narmada sample compared with some of the other localities (0.035–0.109; Table 8) was, however, not supported by statistical significance. UPGMA analysis indicated that the Mahanadi and Narmada samples are close to each other and divergent from other localities (Fig. 3).

Table 7 Pairwise comparison of allele homogeneity in *Chitala chitala* microsatellite loci collected from six riverine systems in India

Population pair	Allele homogeneity		
	Locus NN90	Locus D16	Over all loci
Bhagirathi and Satluj	0.2639	0.1021	0.1244
Ghagra and Satluj	0.1430	0.0111*	0.0118*
Ghagra and Bhagirathi	0.2808	0.6048	0.4709
Brahmaputra and Satluj	0.0224*	0.2420	0.0337
Brahmaputra and Bhagirathi	0.0895	0.5801	0.2055
Brahmaputra and Ghagra	0.0042*	0.3356	0.0108*
Narmada and Satluj	0.1434	0.0638	0.0521
Narmada and Bhagirathi	0.0673	0.3064	0.1007
Narmada and Ghagra	0.2525	0.7772	0.5158
Narmada and Brahmaputra	0.0005**	0.3498	0.0017**
Mahanadi and Satluj	0.0047*	<0.0001**	<0.0001
Mahanadi and Bhagirathi	0.0011**	0.0002**	<0.0001**
Mahanadi and Ghagra	0.0001**	0.0066	0.0001**
Mahanadi and Brahmaputra	0.0003**	0.0001**	<0.0001**
Mahanadi and Narmada	0.0018**	1.0000	0.0133*

* Significant before correction; ** Significant after sequential Bonferroni correction ($P < 0.0033$)

Table 8 Pairwise F_{ST} in *Chitala chitala* collected from six riverine systems in India, based on microsatellites

	Satluj	Bhagirathi	Ghagra	Brahmap	Narmada	Mahanadi
Satluj	–	0.0131	0.0291	0.0146	0.0859	0.1260
Bhagirathi	0.0850	–	−0.00197	0.0065	0.0354	0.0589
Ghagra	0.2200	0.4970	–	0.0342	−0.0140	0.0534
Brahmap	0.0530	0.1590	0.0030**	–	0.1091	0.0911
Narmada	0.0350	0.0940	0.6860	0.0060*	–	0.0364
Mahanadi	<0.0001**	<0.0001**	<0.0001**	<0.0001**	0.0900	–

Above the diagonal: F_{ST}; Below the diagonal: Probability; * significant before correction; ** significant after sequential Bonferroni correction ($P < 0.0033$)

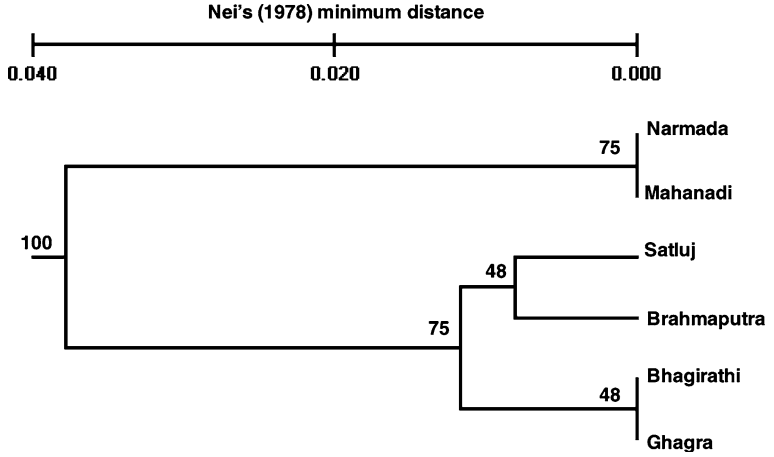

Fig. 3 UPGMA dendrogram from microsatellite loci in *Chitala chitala* natural populations in India. Based on Nei's (1978) minimum genetic distance, with number of bootstraps (%) at each node

Discussion

The present investigation is the first attempt to determine genetic variation and differentiation in the *C. chitala* population from various geographic locations in India. The results from RAPD and microsatellite markers indicated population substructuring in *C. chitala* from different rivers.

The observed deviation from Hardy–Weinberg equilibrium was associated with a deficiency of heterozygotes at one microsatellite locus (D16) only in Satluj populations. The null alleles could be one cause of concern that gives rise to a deficiency of heterozygotes in the computation for microsatellite data (Jones et al. 2001). In the genotype data, however, no individual was observed in the null homozygous state at locus D16, and a significant deficit of heterozygotes is found in only one locality sample. Therefore, the null alleles may not be the likely cause for the observed deficit of heterozygosity.

Highly significant divergence of Mahanadi samples from all the other localities is supported by both RAPD and microsatellite markers. The Narmada population did not have significant genetic heterogeneity (after Bonferroni correction) from the Brahmaputra samples; it could, however, be conclusively differentiated based on the microsatellite loci. The microsatellite loci did not indicate significant genotype heterogeneity between Satluj and the riverine localities of Bhagirathi, Brahmaputra, and Narmada. In contrast, RAPD loci not only revealed heterogeneity between Satluj and Bhagirathi, Ghagra, Brahmaputra, Narmada, and Mahanadi samples but also pointed out significant divergence between two samples within the Ganga river system, Bhagirathi and Ghagra. Based on RAPD analysis, it was possible to differentiate at least five genetic stocks, for Satluj, Ghagra, Mahanadi, Narmada, and the shared gene pool of Bhagirathi-Brahmaputra. The relatively lower level of genetic heterogeneity observed with microsatellite loci could be due to an inadequate number of available polymorphic loci. Nevertheless, the potential of microsatellite loci to determine divergence in the *C. chitala* population is demonstrated beyond ambiguity as indicated by the combined F_{ST} (0.034) and significant F_{ST} estimates (0.03–0.12) between Mahanadi samples and different localities. Therefore, to meet the needs of future genotyping programs, species-specific polymorphic microsatellite markers were identified through construction of a genomic library (Punia et al. 2006). The parameters of genetic variation ($H_{obs} = 0.475$ to 0.672) at eight loci with alleles 2–10 were comparable to these observed in the present study. With the limited sample size tested ($n = 25$ for three rivers), significant genetic heterogeneity ($P < 0.002$) was observed at two loci and over all loci. Due to the lack of statistical significance, high F_{ST} values (up to 0.109) observed between Narmada and other localities (except Ghagra) could not be considered conclusive evidence of genetic heterogeneity. The small sample size for the Narmada locality ($n = 11$) might have resulted in the observed lack of significant probabilities.

The migration of Indo-Malayan fishes via the Indo-Brahma River, flowing westward from Assam in the northeast to fall in the present-day Arabian Sea, probably helped to disperse fish species (Hora 1953). The migration of fishes initiated during the Eocene (60 million years ago) continued until dismemberment of the Indo-Brahma River and formation of the Indus, Ganga, and Brahmaputra river systems during the late Pleistocene. Modern rivers such as the Satluj, Beas, Yamuna, Ghagara, and other Himalayan rivers were formed as lateral rivers to the Ganges over the course of time. The Mahanadi river flowed westward as a part of the Narmada river started flowing southward through the Eastern Ghats during the Pleistocene. Therefore, low to moderate levels of genetic differentiation between the various river samples and paleogeographic reconstructions indicate the possibility that the *C. chitala* from different river basins sampled here are likely to have evolved from a common ancestral gene pool. A similar inference has been indicated by the low genetic variation found in the major Indian carp, *Cirrhinus mrigala*, a natural inhabitant of the Indo Gangetic rivers (Chauhan et al. 2007).

The lack of genetic variation in the survey of 38 allozyme loci for 262 *C. chitala* samples is interesting. Such a phenomenon, though rare, has been reported in some animal species, including three species of *Chitala* (*C. ornata*, *C. blanci*, and *C. lepis*)

in Thailand, where very little intraspecific variation was found within *C. ornata* and *C. blanci* and no variation in *C. lepis* (Sodsuk and Sodsuk 2000). One of the possibilities is suboptimal electrophoresis conditions, but that does not appear to be a cause here. In a previous study, the same electrophoresis conditions could resolve the alleles that discriminated *C. chitala* from another notopterid fish, *N. notopterus*, at 16 of the 35 loci analyzed (Lal et al. 2006). *Notopterus notopterus* also exhibited a high level of polymorphism, 31.3% (total 35 loci); the observed heterozygosity and mean number of alleles were 0.063 and 1.313, respectively. Zhang et al. (2002) did not detect any polymorphism at 44 allozyme loci in 32 individuals of the endangered snub-nosed monkey, *Rhinopithecus roxellana*. Dever and Densmore (2001) reported high variation at microsatellite loci in contrast to the low polymorphism at allozyme loci in Morelet's crocodile. In *C. chitala*, mean heterozygosity for two microsatellite loci ($H = 0.46$) is comparable to the value reported for teleostean fishes ($H = 0.49$, DeWoody and Avise 2000). Frentiu et al. (2001) reported low allozyme polymorphism (0.8%) in an ancient air-breathing dipnoan lineage Australian lungfish, *Neoceratodus fosteri*. In all such cases, the severe genetic bottleneck that species might have suffered during prehistoric periods, especially during Pleistocene aridity, has been considered a possible reason for the lack of genetic variation at slow-mutating loci like allozymes, compared with loci with high mutation rates such as microsatellites (Kemp 1991; Glenn et al. 1998; Zhang et al. 2002). Therefore, absence of genetic variation at allozyme loci observed in *C. chitala* and other *Chitala* species from Southeast Asia (Sodsuk and Sodsuk 2000) probably indicates that these species or their common ancestor might have suffered a reduction in population size in the historical past, parallel to the observations of other animals that evolved during the pre-Pleistocene period, such as crocodiles, lungfish, and snub-nosed monkeys. The observations highlight the need for studies to determine the time of population expansion after bottleneck and divergence between *Chitala* species using mitochondrial and microsatellite DNA markers.

The present study provided baseline information on the genetic variation parameters at 26 RAPD loci and two microsatellite loci. Based on the RAPD markers, a substructure is described, revealing at least five genetically distinct stocks. The RAPD markers proved useful in generating information on the population structure of *C. chitala* where allozymes did not exhibit polymorphism. A larger number of polymorphic microsatellite loci may be necessary to draw conclusions regarding fine-scale population structure. RAPD alleles have dominant expression, and therefore, analysis of RAPD data assumes that the population is in Hardy–Weinberg equilibrium (Ali et al. 2004). In the present study, however, the microsatellite data (on the same individuals) revealed nonconformity to the Hardy–Weinberg expectation at a locus in the Satluj population. Therefore, caution may be necessary in the conservation application of these results, due to the limitations associated with RAPD loci. The low success in cross-species amplification of primers from other fish species in *C. chitala* indicated that this approach is unlikely to yield adequate microsatellite loci. The study highlights the potential utility of microsatellite loci in genetic divergence. In view of this, development of species-specific microsatellite markers through genomic library construction could be a step

forward in the exploration of population structure and might ascertain if the wild *C. chitala* population in any locality faced a genetic bottleneck in recent times.

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