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Genetic divergence in two featherback fishes, *Chitala chitala* and *Notopterus notopterus*

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Summary

Allozyme and RAPD profiles reveal markers that discriminate *Chitala chitala* and *Notopterus notopterus*. Thirty-five allozyme loci were scored from 23 allozyme systems. Species-specific differences were found at 16 loci. Fifteen RAPD markers with 77 size fragments, 244–2902 bp, were identified. The number of fragments specific to *C. chitala* and *N. notopterus* was found to be 20 and 31, respectively. Theta estimates of 0.9798 (allozymes) and 0.9471 (RAPD) indicated a large genetic divergence between *C. chitala* and *N. notopterus*. The observed genetic heterogeneity clearly demonstrated that the two genera, *Chitala* and *Notopterus*, are distinct from each other.

Introduction

Molecular markers are used extensively for detection of interspecific genetic divergence to establish species-specific markers, phylogenetic relationships and resolve taxonomic ambiguities (Rocha-Olivares et al., 2000; Asensio et al., 2002; Baker et al., 2002; Barman et al., 2003; Rasmussen et al., 2003). This provides precise identification of evolutionary significant units important for conservation and management of natural fish populations. The order Osteoglossiformes has at least 19 fossil genera found in upper Jurassic to lower Cretaceous deposits (Nelson, 1994; Otero and Gayet, 2001) considered as primitive (Taverne, 1975). Genetic variation in the fishes of this order can be of considerable interest to validate their evolutionary lineages and taxonomic status. Studies on the application of molecular markers to this group of fish species are limited (Farias et al., 2003; Yue et al., 2004). The ambiguity regarding the systematics of the family Notopteridae under the order Osteoglossiformes was reviewed several times (Lim and Furtado, 1986; Roberts, 1992; Guo-Qing et al., 1997). Roberts (1992) placed *Chitala chitala*, *C. ornata*, *C. blanci* and *C. lopis* under a new genus *Chitala*, while *Notopterus notopterus* was retained within the original genus *Notopterus*. Sodsuk and Sodsuk (2000) studied the allozyme variation in featherback fish in Thailand and found *C. ornata*, *C. blanci* and *C. lopis* to be genetically closer to each other than to *N. notopterus*.

Two species of the family Notopteridae, *C. chitala* and *N. notopterus*, are widely distributed in freshwater bodies of the Indian subcontinent including India, Bangladesh, Myanamar, Nepal and Pakistan (Froese and Pauly, 2003). In peninsular India and Sri Lanka only *N. notopterus* is reported, not *C. chitala* (Jayaram, 1999). The two species are important as food fishes as well as for the ornamental trade. Due to the decline in natural abundance, the *C. chitala* is categorized as endangered and *N. notopterus* as threatened and at lower risk, following IUCN criteria (CAMP, 1998). Therefore, research on artificial propagation for developing culture and *in situ* conservation is being actively pursued (Sarkar et al., 2004). Data on genetic variations can provide crucial input for evolving conservation plans. The present study analyses variation at allozyme and RAPD loci in the two species, *C. chitala* and *N. notopterus*. The objective is to determine species-specific markers and determine genetic relatedness of the two species.

Materials and methods

Sample collection

Specimens of *C. chitala* and *N. notopterus* were collected by commercial catch from two rivers, the Brahmaputra (lat. 26° 16′N; long.91°46′E) and the Satluj (lat. 31°09′N; long. 74°56′E). Blood from individual fish was collected from the caudal vein and fixed in 95% ethanol at 1 : 5 ratio and stored at 4°C until used. The specimens were dissected at site to excise liver and muscle tissue samples. The samples were immediately immersed in liquid nitrogen (−196°C). Frozen tissue samples were transported to the laboratory and stored at −80°C, until used for analysis. Sample size was 10 each for both species. For interspecific discrimination, the species is considered as a unit and genotype data from two locations of a species is pooled for analysis. Collections from the two distant rivers were intended to include a wide spectrum of putative genetic variation present within the species.

Allozyme analysis

Twenty seven enzyme systems were analysed using the vertical polyacrylamide gel electrophoresis (Amersham Biosciences Ltd). Gels of 10 × 8 cm were used for all enzymes, except esterases that were resolved on 10 × 12 cm size gel. Both liver and muscle tissue were subjected to allozyme analysis, but muscle samples did not provide any additional loci except *GPI** or better resolution in comparison to that observed in the liver. Therefore, the liver was selected as an optimum tissue for all enzyme systems, and muscle was used to resolve *GPI**. Tris–borate EDTA (TBE, pH 8.0) provided better resolution than TCE or TGE, and was therefore used as the running buffer. Extract application volume (1–3 μl) and electrophoresis running time (40–130 min) were optimized for different enzyme systems.

Frozen liver samples (60–80 mg) were lightly crushed and homogenized at 500 mg ml^{-1} in extraction buffer (0.17 M

sucrose, 0.2 M EDTA, 0.2 M Tris–HCl, pH 7.0). Homogenates were centrifuged (Sigma Laborzentrifugen GmbH, Osterode, Germany) at 10 000 rpm for 1 h at 4°C. The supernatant was subjected to recentrifuge for 20 min. Sample extracts were electrophoreses on 8% polyacrylamide gel at a constant voltage of 150 V at 4°C in a cooling chamber. Allozyme patterns were visualized by histochemical staining following the procedure outlined by Whitmore (1990). Nomenclature of loci and alleles was followed as recommended by Shaklee and Bentzen (1998).

At all loci, the most common allele in *C. chitala* was assigned as 100. Alternate alleles for the two species were designated as per their mobility, relative to the most common allele. Parameters of genetic variation, such as the proportion of the polymorphic loci ($P_{0.95}$ and $P_{0.99}$) group and mean heterozygosity over all loci for the two species, were calculated with Genetix ver. 4.05 (Belkhir et al., 1997). Theta (θ, Weir and Cockerham, 1984) was estimated and significance of the probability calculated through 1000 bootstraps (Genetix ver. 4.05, Belkhir et al., 1997).

RAPD analysis

Total DNA was extracted from ethanol fixed blood samples stored at 4°C, using the method of Ruzzante et al. (1996). To ensure good quality DNA and stable template concentrations, all DNA samples were tested qualitatively as well as quantitatively on agar gels (0.7%), before introducing them to the PCR-RAPD protocol.

Eighty primers from Operon (20 each from the kit OPA, OPB, OPP and OPH) were used in *C. chitala* and *N. notopterus* to find random primers giving repeatable and polymorphic bands. Thirty-seven primers, out of eighty, amplified bands in both species. Sixteen primers (OPA8, OPA17, OPB1, OPB2, OPB3, OPB7, OPB11, OPB13, OPB17, OPP3, OPP5, OPP13, OPP20, OPH1, OPH3 and OPH6) were used to differentiate the two species. Amplification reactions of RAPD-PCR were performed in a total reaction volume of 25 μl, 100 ng template DNA in 500 mM KCl, 100 mM Tris–HCl (pH 9.0), 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 (Genei, India), using PTC-200 MJ-Research thermal cycler. The reaction conditions

Table 1
Enzyme systems, tissues (M = Muscle, L = Liver) examined, enzyme commission (EC) number, loci, genotypes with number of individuals and parameters of genetic variation in *Chitala chitala* and *Notopterus notopterus*

					Observed genotypes	
					No. of individuals	
Enzyme	E. C. no.	Locus	Tissue	Genotype	*C. chitala*	*N. notopterus*
Glucose phosphate isomerase	5.3.1.9	*GPI-1**	M	075/088	0.00	2.00
				088/088	0.00	8.00
				100/100	10.00	0.00
Lactate dehydrogenase	1.1.1.27	*LDH-1**	L	100/100	0.00	10.00
				200/200	0.00	10.00
-do-	-do-	*LDH-2**	L	088/088	0.00	8.00
				088/118	0.00	2.00
				100/100	10.00	0.00
Esterase	3.1.1.1	*EST**	L	084/092	0.00	2.00
				092/092	0.00	8.00
				100/100	10.00	0.00
Malate dehydrogenase	1.1.1.37	*MDH**	L	089/089	0.00	8.00
				089/144	0.00	2.00
				100/100	10.00	0.00
Phosphogluco mutase	5.4.2.2	*PGM-1**	L	090/090	0.00	10.00
				100/100	10.00	0.00
-do-	-do-	*PGM-2**	L	090/090	0.00	10.00
				100/100	10.00	0.00
6-Phosphogluconate dehydrogenase	1.1.1.44	*6PGDH**	L	082/082	0.00	10.00
				100/100	10.00	0.00
Aldolase	4.1.2.13	*ALD**	L	085/085	0.00	10.00
				100/100	10.00	0.00
Alcohol dehydrogenase	1.1.1.1	*ADH-1**	L	017/017	0.00	10.00
				100/100	10.00	0.00
-do-	-do-	*ADH-2**	L	035/035	0.00	10.00
				100/100	10.00	0.00
Fumerase	4.2.1.2	*FUM**	L	100/100	10.00	0.00
				163/163	0.00	10.00
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	*G3PDH**	L	075/075	0.00	10.00
				100/100	10.00	0.00
Glucose dehydrogenase	1.1.1.47	*GDH**	L	074/095	0.00	2.00
				095/095	0.00	8.00
				100/100	10.00	0.00
α-Glycerophosphate dehydrogenase	1.1.1.8	*GPDH-1**	L	100/100	10.00	0.00
				125/125	0.00	10.00
-do-	-do-	GPDH-2*	L	100/100	10.00	0.00
				136/136	0.00	10.00
Heterozygosity expected					0.000	0.056
Heterozygosity observed					0.000	0.063
% of polymorphic loci (P = 0.95)					0.000	0.313
Mean no. of alleles per locus					1.000	1.313

Table 2
Comparison of *Chitala chitala* and *Notopterus notopterus* based on RAPD analysis. Presence of bands in individuals (proportion) studied, probability of genetic homogeneity at each locus (* not significant, P > 0.05) and theta (θ) value between the two species is given

RAPD locus	Bands present		Genetic homogeneity (p)	Theta (θ)
	C. chitala	*N. notopterus*		
OPP03-2055	0.0000	1.0000	0.0080	1.0000
OPP03-1149	1.0000	0.0000	0.0065	1.0000
OPP03-0737	0.0000	1.0000	0.0075	1.0000
OPP03-0557	1.0000	0.0000	0.0094	1.0000
OPB13-1584	0.0000	1.0000	0.0096	1.0000
OPB13-0864	0.0000	1.0000	0.0069	1.0000
OPB13-0715	1.0000	0.0000	0.0084	1.0000
OPP05-1193	0.0000	1.0000	0.0093	1.0000
OPP05-0909	0.0000	1.0000	0.0087	1.0000
OPP05-0825	1.0000	0.0000	0.0087	1.0000
OPP05-0634	0.0000	0.8000	0.0483	0.4109
OPB11-1919	0.0000	1.0000	0.0095	1.0000
OPB11-1476	0.2000	0.0000	1.0000*	-.0001
OPB11-1050	0.0000	1.0000	0.0091	1.0000
OPB03-1159	1.0000	0.0000	0.0081	1.0000
OPB03-0932	0.0000	0.8000	0.0491	0.4479
OPB03-0486	0.0000	0.8000	0.0438	0.4479
OPB02-1111	1.0000	0.0000	0.0080	1.0000
OPB02-0788	0.0000	1.0000	0.0102	1.0000
OPB02-0615	1.0000	0.0000	0.0078	1.0000
OPB17-0341	0.0000	1.0000	0.0085	1.0000
OPB17-0244	1.0000	0.0000	0.0065	1.0000
OPA17-1579	0.0000	1.0000	0.0077	1.0000
OPA17-1494	0.0000	1.0000	0.0098	1.0000
OPH03-1403	0.0000	1.0000	0.0069	1.0000
OPH03-0803	0.0000	1.0000	0.0086	1.0000
OPH03-0643	1.0000	0.0000	0.0093	1.0000
OPP20-1406	1.0000	0.0000	0.0085	1.0000
OPP20-1085	0.0000	1.0000	0.0078	1.0000
OPH06-1341	0.0000	1.0000	0.0081	1.0000
OPH06-1127	1.0000	0.0000	0.0079	1.0000
OPH06-1028	0.0000	1.0000	0.0083	1.0000
OPH06-0983	1.0000	0.0000	0.0092	1.0000
OPH06-0762	0.0000	1.0000	0.0083	1.0000
OPH06-0610	1.0000	0.0000	0.0065	1.0000
OPH01-1945	0.0000	1.0000	0.0069	1.0000
OPH01-1650	1.0000	0.0000	0.0066	1.0000
OPH01-0992	0.0000	1.0000	0.0073	1.0000
OPH01-0887	1.0000	0.0000	0.0077	1.0000
OPH01-0741	0.0000	1.0000	0.0084	1.0000
OPH01-0564	0.0000	1.0000	0.0078	1.0000
OPB01-2902	0.0000	1.0000	0.0077	1.0000
OPB01-1963	1.0000	0.0000	0.0103	1.0000
OPB01-1807	0.0000	1.0000	0.0079	1.0000
OPB01-1388	1.0000	0.0000	0.0094	1.0000
OPB01-1339	0.0000	1.0000	0.0083	1.0000
OPB01-0756	0.0000	1.0000	0.0070	1.0000
OPB07-2235	1.0000	0.0000	0.0074	1.0000
OPB07-1650	1.0000	0.0000	0.0067	1.0000
OPB07-1130	0.0000	1.0000	0.0105	1.0000
OPB07-1017	0.0000	1.0000	0.0098	1.0000

Table 3
Theta (θ) values with 95% confidence interval (from bootstrapping over loci; replications performed: 5000) based on allozyme and RAPD analysis between *Chitala chitala* and *Notopterus notopterus*

Theta (θ) (95% confidence limits)	Nei (1978) distance	UPGMA analysis	
		Useable loci supporting node (%)	Bootstrap values supporting node (%)
Allozyme 0.979 (0.963–0.992)	0.969	100.00	100.00
RAPD 0.947 (0.910–0.978)	0.650	100.00	100.00

were: denaturation at 94°C for 5 min followed by 45 cycles of 94°C for 30 s, and at 36°C annealing temperature for 30 s, elongation at 72°C for 1 min, with a final elongation at 72°C for 4 min and finally soaking at 4°C. One negative control (absence of template DNA) was included for each set of amplifications. Approximately 8 μl of amplified products were separated on 2% Agarose gel (Sigma) by submarine gel electrophoresis of 12 cm × 10 cm dimensions 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 a constant 70 V. Gels

stained with ethidium bromide were visualized and documented using VDS (Amers). Allele sizes were estimated from interpolation from DNA standards (λ DNA double digest) run in each gel.

For all primers, the presence (1) and absence (2) of a fragment was visually scored and RAPD patterns of individuals were compared within and between the two species. Allele and genotype frequencies were analysed using the TFPGA program (v. 3.1; Miller, 1997). Percent of polymorphism, average number of alleles per locus, and average heterozygosity were calculated. Weir and Cockerham (1984) θ, corresponding to Wright (1978) F_{st}, was calculated and θ was bootstrapped over loci with 5000 interactions to generate 95% confidence levels. The θ was jackknifed (Felsenstein, 1985) over loci to obtain variance estimates, allowing judgment as to whether θ values were significantly different from one another. UPGMA analysis based on Nei's minimum unbiased genetic distance was conducted and the significance of the formed node was tested through 1000 bootstraps.

Results

Analysis of 23 enzyme systems yielded 35 loci in *C. chitala* and *N. notopterus*. Out of the total 46 alleles expressed, 43.5% were shared between the two species. Genetic heterogeneity between the two species was detected at 16 loci (Table 1). The observed genotypes in the two species and other parameters of genetic variation at these sixteen loci are given in Table 1. It is evident at 11 loci that the two species were fixed for alternative alleles (diagnostic loci). Six loci (*PGM**, *LDH2**, *MDH**, *EST**, *GDH** and *GPI1*-M)* were polymorphic in *N. notopterus*. In contrast, *C. chitala* did not yield any polymorphic locus in the enzyme systems analysed. For the six polymorphic *N. notopterus* loci, no allele was shared with *C. chitala*. With the frequency of most common alleles < 0.95, *N. notopterus* had 31.25% polymorphic loci, with 0.0625 observed heterozygosity.

Out of 80, 37 RAPD primers amplified bands in both species. From these, 15 primers amplified species-specific patterns with a total of 77 bands. The number of fragments per primer ranged from 3 (OPA8, OPA17, OPP20, OPP13) to 9 (OPP-5) and fragments size from 244 to 2902 bp. The number of species-specific bands was 20 in *C. chitala*, 31 in *N. notopterus*, and 26 fragments were common in both species (Table 2). F_{st} values between *C. chitala* and *N. notopterus* were estimated to be 0.9798 for allozymes and 0.9471 for RAPD markers (Table 3). The value of Nei's minimum unbiased genetic distance between the two species was 0.969 (allozymes) and 0.650 (RAPD). The UPGMA analysis based on Nei's minimum unbiased genetic distance clearly indicated that the node formed was found to be significant and supported by all loci (Table 3).

Discussion

The analysis of allozyme and RAPD loci provided species-specific diagnostic markers that could discriminate *C. chitala* and *N. notopterus*. These loci might be of specific interest to discriminate early life history stages of the two species. Identification of morphologically close species, especially at early stages of life, has been acknowledged as a difficult task (Olivar et al., 1999). The need for screening early life history stages could arise from the facts: (i) the two species have a similar breeding ground and season; (ii) the artificial propagation research efforts and limited culture activity are dependent upon fish stocked from natural collections; and (iii) the market potential of *C. chitala* is approximate six to sevenfold more than *N. notopterus*. Observed values of heterozygosity and polymorphism obtained with allozyme loci agreed with the range reported for teleost fishes (Nevo, 1978). However, the lack of polymorphism found in *C. chitala* is interesting and appears to be characteristic of the *Chitala* sp. A poor level of genetic variation in three species of *Chitala* found in Thailand (*C. ornata*, *C. blanci* and *C. lopis*) has been reported (Sodsuk and Sodsuk, 2000). Nevertheless, assessment with larger sample sizes will give better insight as to whether allozyme loci will be able to resolve the intraspecific fine scale population level variation in *C. chitala*.

Individual fish genotype data analysed to estimate theta (θ) as a measure of genetic divergence revealed highly concordant results from allozyme and RAPD markers. Sodsuk and Sodsuk (2000) reported the θ value as ranging from 0.865 to 0.919 between *N. notopterus* and three species of *Chitala* with allozyme marker. This agrees well with the estimates and inference from the present study. The theta (or F_{st}) value ranges from 0 (no divergence) to 1 (total divergence). The estimated theta values of 0.94 and 0.97 are close to 1, indicating high level of divergence between *C. chitala* and *N. notopterus*. The UPGMA analysis and bootstrap estimates also supported the divergence between these two featherback fish species. Therefore, based on the various estimators of genetic heterogeneity, it can be concluded that the genus *Chitala* is different from the genus *Notopterus*.

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