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Allozyme Variation in a Threatened Freshwater Fish, Spotted Murrel (*Channa punctatus*) in a South Indian River System

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Samples of the spotted murrel (Channa punctatus) *were collected from three rivers of Tamil Nadu and Kerala. The allozyme variation of* C. punctatus *was investigated by polyacrylamide gel electrophoresis. Eighteen enzymes were detected, but only 10 (EST, PGM, G3PDH, G6PDH, SOD, GPI, ODH, GDH, XDH, and CK) showed consistent phenotypic variations. Allele frequencies were estimated at the 18 polymorphic loci representing 10 enzymes. Two rare alleles, EST-4*C and G6PDH-2*C, were noted in the Tamirabarani and Kallada populations but were absent in the Siruvani population. The allele frequencies of the Tamirabarani and Kallada populations were similar, except for a few loci. Among the three populations, the maximum genetic distance (0.026) and* F_{ST} *(0.203) were found between the geographically distant Siruvani and Kallada populations. Overall the study showed that among the three populations, the Tamirabarani and Kallada have similar genetic structures.*

KEY WORDS: *Channa punctatus*; allozyme; population; heterozygosity.

INTRODUCTION

The spotted murrel, *Channa punctatus* (Bloch, 1793), commonly called the snakehead, is an important freshwater, food fish of Southeast Asia. It is found in rivers,

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ponds and lakes of India, Sri Lanka, Pakistan, Nepal, Bangladesh, Myanmar, Malaysia, China, Tahiti, and Polynesia (Jayaram, 1981). It has been identified as having potential for aquaculture in derelict and swampy water, since it is an air-breathing fish. It commands good consumer preference due to taste, high protein content and very few intramuscular spines (Haniffa *et al*., 2002). It breeds naturally during southwest and northeast monsoons in India. Parameswaran and Murugesan (1976) reported that induced-bred murrels never exhibited parental care, but we found breeding behavior and parental care under induced breeding conditions using different ovulating agents (Haniffa *et al*., 2004). The fecundity of this species is very low (3000 to 4000) compared with the major Indian carps, but it varies with the size of the fish.

Over the last 10 years, its wild population has declined steadily, mainly because of overexploitation, loss of habitat, introduction of alien species, disease, pollution, siltation, poisoning, dynamite, and other destructive fishing (CAMP, 1998). As a result, *C. punctatus* is listed among 66 low-risk, near-threatened fish species of India, according to IUCN status (CAMP, 1998). Information on the genetic structure of cultivable fish is necessary for optimizing identification of potential broodstock, stock enhancement, selective breeding programs, management for sustainable yield, and conservation of biodiversity. Previous studies have provided detailed knowledge of the length-to-weight relationship (Haniffa *et al*., 2006), embryology and development (Haniffa *et al*., 2002), biochemical composition (Singh and Singh, 2002; Sehgal and Goswami, 2001), hematology (Pandey *et al*., 1981), courtship behavior (Haniffa *et al*., 2004), and breeding (Haniffa and Sridhar, 2002) for this species. Basic knowledge of the levels of genetic variation within and among the populations is poorly represented, although a very few studies have been made on *C. punctatus*. Rishi *et al*. (2001) studied the LDH polymorphism of *C. punctatus* populations sampled from three natural bodies of water at Haryana (India), and Nabi *et al*. (2003) studied the genetic structure of *C. punctatus* populations collected from locations on the Rohilkhand plains of India using transferrin as a marker. In our previous study we analyzed the genetic variability of three *C. punctatus* populations using RAPD markers (Nagarajan *et al*., 2006). In this study, we used allozymes to investigate the genetic variability of three *C. punctatus* populations collected from three south Indian rivers.

MATERIALS AND METHODS

Fish Sampling

The majority of the rivers in the states of Tamil Nadu and Kerala originate from the Western Ghats, a mountain range in India that starts south of the Tapti River near the border of Gujarat and Maharashtra and goes approximately 1600 km through the states of Maharashtra, Goa, Karnataka, Kerala, and Tamil Nadu to

Kanyakumari at the southern end of the Indian peninsula. Recognized as one of the 21 biodiversity hotspots of the world, it harbors a rich and diversified fish fauna characterized by many rare and endemic species. In the present study we collected *C. punctatus* samples from three rivers of the Western Ghats, the Siruvani, Tamirabarani, and Kallada. The Tamirabarani River originates from the peak of the Periya Pothigai hills of the Western Ghats at Thirunelveli District (8.4°N, 77.44°E, Thirunelveli, Tamil Nadu). The Kallada River enters Kerala and runs into the Quilon and nearby districts (8.54°N, 76.38°E, Quilon, Kerala; Fig. 1). The Siruvani River originates about 500 km away from the Tamirabarani and Kallada rivers in the Western Ghats (11.00°N, 77.00°E, Coimbatore, Tamil Nadu). Of the three rivers, the Tamirabarani and Kallada have a rich fish biodiversity (Martin *et al*., 2000; Kurup *et al*., 2004). From each population, 60 fish samples were used for the present study. Liver tissues were dissected from the fish and were immediately stored at −80°C prior to analysis.

Extract Preparation

Adequate portions (250 mg) of liver tissue were first minced and homogenized using a glass homogenizer under cold condition. A buffer solution containing sucrose (50%), 0.2 M Tris HCl (pH 7.2), EDTA (64 mg/100 mL) and double-distilled water was used as homogenizing medium in selected proportions to the sample weight (250 mg/mL). The homogenates were then centrifuged at 12,000 rpm for 1 h at 4°C. The supernatant was again spun at 12,000 rpm for 40 min. After the second centrifugation, the supernatant was collected and used for further analyses.

Electrophoresis and Staining

Vertical polyacrylamide gel electrophoresis was used for the separation of allozymes at different enzyme loci. Gels consisted of 3.9% acrylamide and 3.36% bis-acrylamide, and electrophoresis was run at 30 mA and 150 V at 4°C with Tris-Boric acid-EDTA buffer (pH 8.0). The bands of each enzyme were revealed by incubating the gels in the dark at 37°C in the presence of specific histochemical staining solution until sharp bands were visualized. The locus and allele designations were followed according to the standardized genetic nomenclature for protein-coding loci (Shaklee *et al*., 1990).

Statistical Analysis

Genetic variation between the populations was assessed by the following standard measures; number of polymorphic loci (P), allele frequency, observed heterozygosity per locus (H_o), expected heterozygosity per locus (H_e), fixation index (F_{IS}),

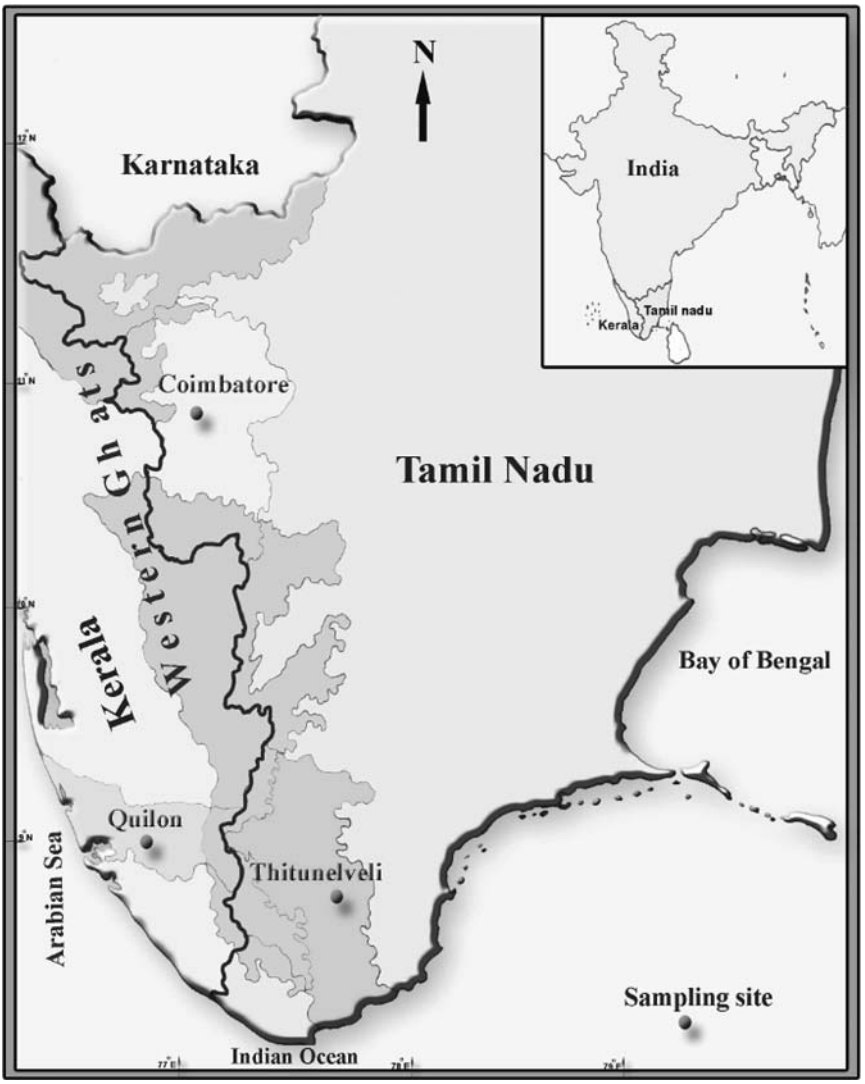

Fig. 1. Sampling sites of *C. punctatus* populations used in this study.

genetic diversity (F_{ST}) for the overall populations, and Nei's genetic distance (D_N) using the program PopGene 1.31 (Yeh *et al.*, 1999). Deviations from Hardy–Weinberg equilibrium of each locus for each population were tested by the Markov chain method of exact probability test using the program GenePop 3.3 (Raymond and Rousset, 1995). The *P* values were corrected using the Bonferroni correction

(Rice, 1989). AMOVA (analysis of molecular variance) and population pairwise F_{ST} were computed using the program Arlequin (Schneider *et al*., 2002). Isolation by distance (IBD) between populations was confirmed by Mantel's tests (Mantel, 1967) using the program IBD 2.1 (Jensen *et al*., 2005).

RESULTS

In this study, we checked for 18 enzymes in *C. punctatus*, but only 16 showed their presence in liver samples. The enzymes that could not be detected were aspartate amino transferase (AAT) and hexokinase (HK). Of the 16 enzymes detected, 12 showed phenotypic variations and 4 were monomorphic: glutamate dehydrogenase (GLUDH), adenylate kinase (AK), malic enzyme (MEP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Of the 12 that showed variation, lactate dehydrogenase (LDH) and isocitrate dehydrogenase (ICDH) exhibited inconsistent phenotypic patterns. The remaining 10 showed consistent phenotypic variation and were therefore useful for genetic analysis: EST, PGM, G3PDH, G6PDH, SOD, GPI, ODH, GDH, XDH, and CK. They are coded by 27 putative loci (Table I). A total of 38 alleles were detected from the 18 polymorphic loci of 10 enzyme systems, and their frequencies are presented in Table II. Of the 18 polymorphic loci, *EST-4** and *G6PDH-2** contained three alleles; the others had two alleles. The *EST-4** and *G6PDH-2** loci showed the allele C in very low frequencies, *EST-4** in the Tamirabarani population and *G6PDH-2** in Kallada, but they were absent in the Siruvani population. The allele frequencies were very similar in the Tamirabarani and Kallada populations. The frequency of the *ODH-1*, GDH-2*, EST-4*,* and *G6PDH-2** loci alleles in the Siruvani population was significantly different from that of the Tamirabarani and Kallada populations.

The observed heterozygosity ranged from 0.194 in the Siruvani population to 0.236 in the Tamirabarani population. The expected heterozygosity was 0.262 in Siruvani and 0.327 in Kallada (Table III). Of the 38 polymorphic loci from the three populations, 31 showed Hardy-Weinberg equilibrium, and 7 deviated significantly after the Bonferroni corrections (Rice, 1989). These statistically significant values were produced at *PGM-3** and *GDH-3** in the Siruvani population, *ODH-2*, GDH-2*,* and *XDH-2** in the Tamirabarani population, and *GDH-1** and *GDH-3** in the Kallada population. In each of the statistically significant cases, the fixation index (F_{IS}) was very high, indicating significant heterozygote deficiency at the population level. The mean F_{IS} per population was 0.262, ranging from 0.226 in the Tamirabarani population to 0.301 in the Kallada population (Table IV). The F_{ST} for the overall population ranged from 0.007 in *PGM-2** to 0.068 in *ODH-1**, with a mean of 0.028, indicating that about 2.8% of the total genetic variation exists between populations due to population differentiation (Table IV). AMOVA analysis revealed 12.46% variation among the populations (Table V). We obtained 13.55% variation when we combined the Tamirabarani and Kallada populations

Table I. Allozymes Screened in *Channa punctatus*

S. no.	Enzyme	Abbreviation and enzyme code	Subunit structure	Locus	Monomorphic or Polymorphic
1	Esterase	EST 3.1.1	Monomeric	*EST-1**	Monomorphic
				*EST-2**	Monomorphic
				*EST-3**	Monomorphic
				*EST-4**	Polymorphic
				*EST-5**	Polymorphic
2	Phosphoglucomutase	PGM 5.4.2.2	Monomeric	*PGM-1**	Polymorphic
				*PGM-2**	Polymorphic
				*PGM-3**	Polymorphic
3	Glycerol-3-phosphate dehydrogenase	G3PDH 1.1.1.8	Dimeric	*G3PDH**	Polymorphic
4	Glucose-6-phosphate dehydrogenase	G6PDH 1.1.1.49	Dimeric	*G6PDH-1**	Monomorphic
				*G6PDH-2**	Polymorphic
5	Superoxide dismutase	SOD 1.15.1.1	Dimeric	SOD-1*	Polymorphic
				*SOD-2**	Polymorphic
6	Glucose-6-phosphate isomerase	GPI 5.3.1.9	Dimeric	*GPI**	Polymorphic
7	Octanol dehydrogenase	ODH 1.1.1.73	Dimeric	*ODH-1**	Polymorphic
				*ODH-2**	Polymorphic
				*ODH-3**	Monomorphic
8	Glucose dehydrogenase	GDH 1.1.1.47	Monomeric	*GDH-1**	Polymorphic
				*GDH-2**	Polymorphic
				*GDH-3**	Polymorphic
9	Xanthine dehydrogenase	XDH 1.1.1.204	Dimeric	*XDH-1**	Monomorphic
				*XDH-2**	Polymorphic
				*XDH-3**	Polymorphic
10	Creatine kinase	CK 2.7.3.2	Dimeric	*CK-1**	Polymorphic
				*CK-2**	Monomorphic
				*CK-3**	Monomorphic
				*CK-4**	Monomorphic

as one group to compare with the Siruvani population. There was only 2.87% variation between the Tamirabarani and Kallada populations (interpopulational variation). Among the three populations, the maximum genetic distance and F_{ST} were found between the Siruvani and Kallada populations (Table VI). We checked for a correlation between the geographic distance and corresponding F_{ST} value for the three populations using the IBD program. A significant positive correlation was obtained ($r = 0.59$, $P < 0.02$), confirming that these three populations are in isolation by distance. The overall analysis indicated that the Tamirabarani population is genetically closer to the Kallada population, whereas the Siruvani population is closer to the Tamirabarani population.

Table II. Estimated Allele Frequency at 18 Polymorphic Loci in Three Populations of *C. punctatus*

Locus	Allele	Siruvani	Tamirabarani	Kallada
EST-4	A	0.833	0.589	0.544
	B	0.167	0.394	0.428
	C	0.000[a]	0.017[b]	0.028[b]
EST-5	A	0.739	0.544	0.522
	B	0.261	0.456	0.478
PGM-1	A	0.650	0.556	0.511
	B	0.350	0.444	0.489
PGM-2	A	0.639	0.556	0.544
	B	0.361	0.444	0.456
PGM-3	A	0.650	0.600	0.578
	B	0.350	0.400	0.422
G3PDH-1	A	0.689	0.611	0.544
	B	0.311	0.389	0.456
G6PDH-2	A	0.672	0.533	0.567
	B	0.328	0.439	0.411
	C	0.000[a]	0.028[b]	0.022[b]
SOD-1	A	0.678	0.600	0.522
	B	0.322	0.400	0.478
SOD-2	A	0.7000	0.861	0.678
	B	0.3000	0.139	0.322
GPI-1	A	0.717	0.583	0.544
	B	0.283	0.417	0.456
ODH-1	A	0.833[a]	0.556[b]	0.589[b]
	B	0.167	0.444	0.411
ODH-2	A	0.833	0.806	0.611
	B	0.167	0.194	0.389
GDH-1	A	0.800	0.639	0.589
	B	0.200	0.361	0.411
GDH-2	A	0.822[a]	0.733[a,b]	0.567[b]
	B	0.178	0.267	0.433
GDH-3	A	0.783	0.639	0.567
	B	0.217	0.361	0.433
XDH-2	A	0.672	0.828	0.689
	B	0.328	0.172	0.311
XDH-3	A	0.750	0.589	0.511
	B	0.250	0.411	0.489
CK-1	A	0.667	0.533	0.533
	B	0.333	0.467	0.467

Note. Values with different superscripts (a,b) in the same row are significantly different ($P < 0.05$).

DISCUSSION

The genetic variability in the three natural populations of *C. punctatus* was evident in this study using allozyme markers. The allozyme allele frequency of the Siruvani population was significantly different from that of the Tamirabarani and Kallada populations at *ODH-1*, GDH-2*, EST-4*,* and *G6PDH-2** loci, but the Tamirabarani and Kallada populations were closely similar. The variation in allele frequency in the populations can be due to environmental factors such as

Table III. Genetic Variability Estimates of Three *C. punctatus* Populations

S. No	Parameter	Siruvani	Tamirabarani	Kallada
1	Sample size	60	60	60
2	Number of loci found	27	27	27
3	Number of polymorphic loci	18	18	18
4	Observed heterozygosity	0.194 ± 0.152	0.236 ± 0.195	0.227 ± 0.176
5	Expected heterozygosity	0.262 ± 0.196	0.300 ± 0.226	0.327 ± 0.237
6	Observed number of alleles	1.667 ± 0.480	1.741 ± 0.594	1.741 ± 0.594
7	Effective number of alleles	1.439 ± 0.342	1.561 ± 0.444	1.6388 ± 0.465

Table IV. Fixation Index for Polymorphic Loci in Three *C. punctatus* Populations

	Fixation Index (F_{IS})			
Locus	Siruvani	Tamirabarani	Kallada	Overall F_{ST}
EST-4	0.300	0.196	0.316	0.0086
EST-5	0.107	0.194	0.286	0.0395
PGM-1	0.292	0.280	0.378	0.0137
PGM-2	0.254	0.280	0.373	0.0073
PGM-3	0.603*	0.398	0.180	0.0204
G3PDH-1	0.119	0.018	0.462	0.0147
G6PDH-2	0.269	0.234	0.258	0.0118
SOD-1	0.289	0.028	0.198	0.0168
SOD-2	0.101	0.303	0.135	0.0352
GPI-1	0.261	0.017	0.194	0.0230
ODH-1	0.120	0.190	0.449	0.0683
ODH-2	0.120	0.539*	0.252	0.0521
GDH-1	0.236	0.061	0.633*	0.0370
GDH-2	0.316	0.546*	0.186	0.0542
GDH-3	0.575*	0.013	0.548*	0.0363
XDH-2	0.118	0.493*	0.119	0.0247
XDH-3	0.378	0.082	0.244	0.0419
CK1	0.200	0.196	0.196	0.0162
Mean	0.259	0.226	0.301	0.0281

*Locus deviates significantly from Hardy-Weinberg equilibrium after Bonferroni correction.

Table V. AMOVA of Three *C. punctatus* Populations

	Percentage of variation*			
Source of variation	No grouping	Two groups[a]	Two groups[b]	Two groups[c]
Among groups	12.46	13.55	−1.76	−12.98
Among populations within groups	—	2.87	13.71	21.66
Within populations	87.54	83.58	88.05	91.32

[a]Tamirabarani + Kallada versus Siruvani.
[b]Tamirabarani + Siruvani versus Kallada.
[c]Tamirabarani versus Kallada + Siruvani.
*$P < 0.01$.

Table VI. Genetic Distance and Genetic Diversity Between *C. punctatus* Populations

Population	Siruvani	Tamirabarani	Kallada
Siruvani	—	0.152	0.203
Tamirabarani	0.017	—	0.027
Kallada	0.026	0.007	—

Note. Below diagonal, Nei's unbiased genetic distance. Above diagonal, F_{ST} genetic diversity value.

temperature, alkalinity, and pollution (Ponniah, 1989). The role of temperature in maintaining alleles at different frequencies has been proved in natural populations (Nyman and Shaw, 1971) and experimentally (Mitton and Koeh, 1975). Two rare alleles, *G6PDH-1*C* and *EST-4*C*, were found in the Tamirabarani and Kallada populations with low frequencies, though sample size was sufficient. This represents an inherent genetic stock difference between the Siruvani and the Tamirabarani/Kallada populations (Levy and Neal, 1999). The rare alleles can be utilized as genetic markers for selection of a candidate stock for controlled breeding programs (Lester and Pante, 1992). Significant deviations from Hardy-Weinberg expectations were found at *XDH-2**, *G3PDH-1**, and *GDH-3** in Siruvani; *XDH-1**, *ODH-1**, and *PGM-1** in Tamirabarani; and *SOD-1* in Kallada because of an excess of homozygotes. The excess of homozygotes is also confirmed by positive fixation index values (Table IV). Homozygote excess for allozyme has been reported quite commonly in many fish species (Engelbrecht and Mulder, 2000; Steenkamp *et al*., 2001). Several hypotheses have been mentioned to explain homozygote excess in fish species, including inbreeding, population admixture (Wahlund effect), or the presence of a nonexpressed allele (Appleyard *et al*., 2001; Ward *et al*., 2003).

The population structure of freshwater organisms is primarily dependent on the distribution of the river systems, as has been reported by several authors (Ikeda *et al*., 1993; Hara *et al*., 1998). The present study also showed a significant correlation between genetic distance and geographic distance, confirmed by the Mantel test. The three populations used in the present study were collected from three different rivers, all of which originate in the Western Ghats. The Tamirabarani and Kallada rivers originate at the south end of the Western Ghats and are geographically closer to each other than to the Siruvani, which originates more than 500 km away from the other rivers. The results of this study consistently showed that the Tamirabarani and Kallada populations were genetically closer to each other, compared with the Siruvani population. It supports the concept that genetic differentiation is primarily dependent on geographic isolation. Theoretically, the result of the present study shows a certain level of gene flow between the Tamirabarani and Kallada populations when compared with the Siruvani population. In fact,

natural interbreeding between the Tamirabarani and Kallada populations was impossible, and there has been no record of transplantation of *C. punctatus* between the two rivers. It is likely that these two populations have come from a single stock introduced into these two rivers in the past, or migration or transportation between the two rivers could be a possibility.

In conclusion, allozyme analysis revealed, as did the RAPD markers used by Nagarajan *et al*. (2006), that of the three *C. punctatus* populations, the Tamirabarani and Kallada populations have similar genetic structures. The estimated values of average number of alleles, percentage of polymorphic loci, and heterozygosity for populations are considered indicators of the actual level of genetic variability of the species. Genetic variability data may also be used as a tool by an aquaculturist for stock selection for selective breeding programs. The present study provides basic information about the genetic structure of these three populations that can be used to improve the quality of the populations by selective breeding or out-breeding programs, and it would also help to conserve the population. Microsatellite markers, however, would be better suited than isozyme and RAPD analysis to detect population bottlenecks and losses of variation due to inbreeding, with allele richness being a more sensitive variability measure than mean heterozygosity (Bentzen *et al*., 1996; Wright and Bentzen, 1994). Hence, further research on microsatellite markers for these populations is in progress.

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