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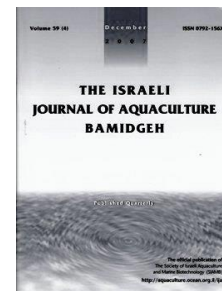
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Microsatellite DNA Analysis of Giant Freshwater Prawn (*Macrobrachium rosenbergii*) from India

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Abstract

Giant freshwater prawn (*Macrobrachium rosenbergii*), a commercially important crustacean species, is widely distributed across the Indo-Pacific region. Genetic diversity of this species from five different rivers (Krishna, Mahanadi, Hooghly, Narmada and Kalu) of India was investigated using 5 polymorphic microsatellite loci. The number of alleles across loci varied from 4 to 9. The mean expected and observed heterozygosity at all loci was 0.8359 and 0.5747 respectively. Most of the loci deviated significantly from Hardy-Weinberg expectations across all the populations. Pairwise F_{ST} estimates (0.0420 to 0.0841) revealed a significant genetic structure among *M. rosenbergii* populations of Indian rivers. The highest (0.5140) genetic distance was observed between Krishna and Kalu populations. All five wild populations exhibited significant variation across all five microsatellite loci. The results revealed in the study will be useful for breeding programs and conservation management of this species.

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Introduction

The giant freshwater prawn, *Macrobrachium rosenbergii* has been one of the most desirable candidate species for freshwater aquaculture in different parts of the India-Pacific region (Ranjeet and Kurup, 2002). Annual aquaculture production of *M. rosenbergii* dramatically increased from 178 tons in 1996 to 42,820 tons in 2005 in India (FAO, 2005) and then declined to 6,600 tons in 2010-11 (www.fis.com). The reason for declining production is inbreeding over several generations for seed production in commercial farms where productivity declined due to early sexual maturity, low fecundity and larval viability, and susceptibility to diseases (Mohanakumaran Nair and Salin, 2006). In spite of these problems, it has vast potential for global financial profits due to its rapid growth, disease resistance and high demand in both domestic and export markets. It is financially competitive with other cultured prawn provided the species is domesticated and genetically improved for better performance (Jahageerdar, 2003).

Genetic diversity in wild populations is declining as a result of over-exploitation. In order to develop sound conservation strategies it is important to understand the distribution of genetic diversity in wildstock. Recognition of unique genetic diversity will also improve choices in breeding programs, help genetic diversity of broodstock, and maintain genetic diversity in cultured stock (Chand et al., 2005). Genetic diversity is the fundamental resource on which stock improvements rely, therefore, populations can be selected on the basis of genetic criteria (Petit, et al., 1998; Vandeputte and Launey, 2004).

Microsatellite markers are a good choice for the characterization of genetic diversity in both wild and cultivated *M. rosenbergii* due to its reliable, informative, co-dominant nature and ease of exchange of data among different studies (Avisé, 1994).

Currently, little is known about the levels and patterns of genetic diversity in Indian populations of *M. rosenbergii*. In India, one study based on microsatellite markers was conducted on genetic variation of two wild populations of *M. rosenbergii* (Divu et al., 2008) and another study was conducted based on 24 unrelated individuals collected from the wild (Bhat et al., 2009). The present study was carried out to evaluate the levels of genetic diversity of five different populations of *M. rosenbergii* in India using five microsatellite markers. This study aims to assist the selection of individuals for prawn-breeding programs, in order to facilitate domestication of the species.

Materials and Methods

Sample collection. *M. rosenbergii* samples were collected from five different rivers in India: Krishna, Andhra Pradesh (16°20'35.51N, 80°45'16.91E), Mahanadi, Odisha (20°31'37.66"N, 85°07'56.99"E), Hooghly, West Bengal (22°19'46.13"N, 88°88'12.46"E), Narmada, Gujarat (22°21'50.50"N, 76°15'50.47"E) from the east coast, and Kalu, Maharashtra (19°19'18.09"N, 73°18'13.71"E) from the west coast. A total of 250 samples (50 from each location) were examined in the present study. Pleopods were removed from each individual, preserved in 95 % ethanol and kept at -20°C until DNA extraction.

DNA extraction and polymerase chain reaction (PCR) amplification. Total genomic DNA was extracted from the pleopod tissue using the standard phenol-chloroform extraction method described in (Sambrook et al., 2001) with minor modifications. DNA quality and quantity were determined by agarose gel electrophoresis and biophotometer (Eppendorf, Germany).

Five microsatellite loci (MRMA27, MRMB7, MRMB10, MRMA8 and Mr5-26 (Divu et al. 2008 and Bhat et al. 2009) developed for *M. rosenbergii* were used to amplify DNA samples (Table 1). PCR was performed in 25 µl volume containing 1 X PCR Buffer (Bangalore Genei, India), 200 µM dNTPs, 10 pmol each primer, 50 ng DNA and 0.25 U *Taq* polymerase (Bangalore Genei). PCR cycles for each locus were as follows: initial denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, annealing temperature for 30 s, and 2 min at 72°C with the final extension at 72°C for 10 min. Holding temperature was set at 4°C. PCR products were subjected to electrophoresis on 7% non-denatured polyacrylamide gel at 80 V for 4 hrs after amplification. Gel was

stained using silver stain for further analysis. Allele sizes were determined with a gene runner DNA ladder.

Table 1. Details of primer and microsatellite in *M. rosenbergii* in the present study

Sl. No	Locus	NCBI GenBank Accession Number	Repeat motif	Primer sequence (5' to 3')	T _A (°C)	Allele size range (bp)	
						source reference	present study
1	MRMA27	DQ793616	(GT) ₁₃	F: TTAGGGTGTGGAGTAACAGG R: TTCGCTGAATACGCGCATGAC	44	384 - 422	380 - 420
2	MRMB7	EF515169	(GA) ₂₆	F: ACTTCGGAACAAGGGATTAT R: GAATCGAAAGCAGTCTCCTT	46	270 - 300	270 - 300
3	MRMB10	EF515168	(GA) ₃₅	F: AGAGGCACTACAGAAGACCAA R: ATCCTCAGGTCTCCCTTCGT	44	125 - 200	128 - 202
4	MRMA8	DQ793615	(GA) ₆₈	F: TTGACTAGGCTTCGAACCC R: AAACCGATTTCTGTCTTACGC	48	100 - 175	102 - 176
5	Mr5-26	EU847618	(GA) ₃₃	F: GGCTCAAGAACGCTATGAGG R: TCAAAGACCCAATTACTGCTCA	57	246	236 - 282

F: Forward primer; R: Reverse primer; bp: base pair; T_A: Annealing temperature

Data Analysis. The genetic variation within each of five populations including alleles per locus (A), observed (H_o) and expected (H_e) heterozygosity, and departures from Hardy-Weinberg equilibrium (HWE) were calculated using the software Genepop, version 3.3d (Raymond and Rousset, 1998). The Arlequin 3.11 software was used to calculate genotypic linkage disequilibrium between these loci (Schneider *et al.*, 2000).

Genetic differences between populations were evaluated by calculating pairwise F_{ST} values and testing their significance by bootstrapping analysis (1000 replicates) using Genepop, version 3.3d (Raymond and Rousset, 1998).

Expected frequency of null alleles were calculated across all populations according to Van Oosterhout *et al.* (2004, 2006) using Micro-Checker. Nei's (1978) genetic distances were calculated between all pairs of populations using Popgene, version 1.31 (Yeh *et al.*, 1999). A dendrogram was drawn based on the genetic distance between the populations following Unweighted Pair Group Method of Averages (UPGMA) using the software eMega4 (Tamura *et al.*, 2007).

Results

Overall genetic variability. Among the five populations 126 alleles with the allele numbers ranging from 4 to 9 were observed in 5 loci. Mean number of alleles per locus ranged from 4.40 to 6.20 across the 5 microsatellite loci. Mean values of expected heterozygosity for each population ranged from 0.7574 to 0.8049 and observed heterozygosity ranged from 0.5333 (Narmada population) to 0.6000 (Krishna population). All populations deviated significantly from HWE at all five microsatellite loci (Table 2). There was no significant association indicative of linkage disequilibrium between any pair of microsatellite loci for any population (P>0.05), indicating independence of the five genetic markers. Wright's (1978) fixation index (F_{IS}) a measure of heterozygote deficiency or excess (inbreeding co-efficient), and significance values (ranged from 0.0916 to 0.4781) for each locus in five populations are given in Table 2. F_{IS} values greater than zero (+ ve) indicating a deficiency of heterozygotes was evident in these cases. Microsatellite loci exhibiting + F_{IS} values were tested for presence of null alleles. Estimated null allele frequencies assessed with Microchecker were not significant (P<0.05) indicating the absence of null alleles and false homozygotes at any locus. Therefore information from all five microsatellite loci was considered for the population genetic analysis.

Table 2. Summary statistics of 5 populations of *M. rosenbergii* using 5 microsatellite loci

Locus name	Parameters	Populations				
		Krishna	Mahanadi	Hooghly	Narmada	Kalu
MRMA27	n_a	4	5	6	4	4
	H_{obs}	0.6667	0.6667	0.6000	0.6333	0.7333
	H_{exp}	0.7328	0.8006	0.8158	0.7580	0.7544
	F_{is}	0.0916	0.1696	0.2679	0.1677	0.0297
	P_{HW}	0.0601	0.0001**	0.0001**	0.0006**	0.1785**
MRMB10	n_a	7	6	5	4	7
	H_{obs}	0.6000	0.6000	0.6333	0.6000	0.6000
	H_{exp}	0.7842	0.8226	0.7910	0.7156	0.8514
	F_{is}	0.2380	0.2740	0.2020	0.1641	0.2989
	P_{HW}	0.0001*	0.0003**	0.0684**	0.0341	0.0007**
MRMB7	n_a	9	9	8	5	5
	H_{obs}	0.6333	0.4667	0.5000	0.5667	0.5667
	H_{exp}	0.8870	0.8870	0.8633	0.8062	0.8062
	F_{is}	0.2895	0.4781	0.4250	0.3007	0.3007
	P_{HW}	0.0001*	0.0114**	0.0001**	0.0003**	0.0001**
Mr5-26	n_a	6	4	6	4	4
	H_{obs}	0.6000	0.6333	0.5667	0.4667	0.4333
	H_{exp}	0.8023	0.7136	0.7847	0.7508	0.7305
	F_{is}	0.2553	0.1141	0.2813	0.3825	0.4109
	P_{HW}	0.0023**	0.1993	0.0019*	0.0015**	0.0001**
MRMA8	n_a	5	5	5	5	6
	H_{obs}	0.5000	0.5667	0.4667	0.5000	0.5000
	H_{exp}	0.7972	0.8006	0.7497	0.7554	0.8229
	F_{is}	0.3768	0.2957	0.2921	0.3418	0.4017
	P_{HW}	0.0071**	0.0001*	0.0029**	0.0001*	0.0001*
Mean overall loci	H_{obs}	0.6000	0.5867	0.5667	0.5333	0.5667
	H_{exp}	0.8007	0.8049	0.8009	0.7574	0.7947
	A_n	6.20	5.80	6.00	4.40	5.20

n_a , Number of alleles; H_{obs} , Observed Heterozygosity; H_{exp} , Expected Heterozygosity; F_{is} , Inbreeding Coefficient; P_{HW} , Probability value of significant deviation from Hardy-Weinberg equilibrium; A_n , Mean number of alleles per locus

* Is significant at $P < 0.05$

** Is significant at $P < 0.05$ after Bonferroni adjustment

Population differentiation. Overall F_{ST} value was estimated to be 0.0666. Pair-wise F_{ST} estimates between population pairs differed significantly ($P < 0.01$) from 0.0420 to 0.0911 for all the pairs of populations (Table 3).

Table 3. Pairwise F_{ST} among five populations

	Krishna	Mahanadi	Hooghly	Narmada	Kalu
Krishna	***				
Mahanadi	0.0911	***			
Hooghly	0.0420	0.0484	***		
Narmada	0.0553	0.0428	0.0795	***	
Kalu	0.0841	0.0808	0.0715	0.0778	***

All pairwise F_{ST} values were significant at $P < 0.05$

Among the five populations, Krishna and Mahanadi population pairs were the most divergent (highest F_{ST} value=0.0911), followed by the Krishna and Kalu populations (F_{ST} value=0.0841). The highest genetic distance (0.5140) was observed between Krishna and Kalu populations while the lowest genetic distance (0.2190) was observed between Krishna and Narmada populations (Table 4).

Table 4. Nei's genetic identity values (above diagonal) and genetic distances (D_A) (below diagonal) among five populations

	Krishna	Mahanadi	Hooghly	Narmada	Kalu
Krishna	***	0.8721	0.7777	0.2577	0.5981
Mahanadi	0.1368	***	0.7462	0.8033	0.6074
Hooghly	0.2514	0.2928	***	0.6599	0.6529
Narmada	0.2775	0.2190	0.4156	***	0.6720
Kalu	0.5140	0.4985	0.4263	0.3974	***

Among the five populations, Krishna and Mahanadi population pairs were the most divergent (highest F_{ST} value=0.0911),

followed by the Krishna and Kalu populations (F_{ST} value=0.0841). The highest genetic distance (0.5140) was observed between Krishna and Kalu populations while the lowest genetic distance (0.2190) was observed between Krishna and Narmada populations (Table 4).

UPGMA dendrogram. The dendrogram based on genetic distance computed by Nei (1978) showed four major clusters: Krishna & Mahanadi populations formed in single cluster, and the remaining populations (Hooghly, Narmada and Kalu) formed separate clusters (Fig. 1).

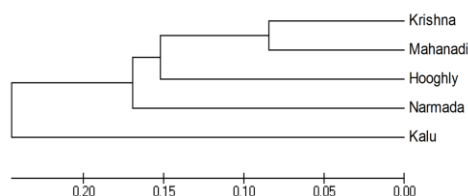


Fig.1 UPGMA clustering using Nei's unbiased genetic distance (1978) of *M. rosenbergii* population

Discussion

Five polymorphic microsatellite loci (Divu *et al.* 2008 and Bhat *et al.* 2009) developed for *M. rosenbergii* were used to evaluate genetic diversity and population differentiation in *M. rosenbergii* (N=250) collected from five different rivers of India. Ruzzante (1998) confirmed that sample sizes larger than 50 individuals are adequate for minimizing bias due to a large number of alleles in microsatellite data. Silva and Russo (2000) also reported that sample sizes should exceed 30. In the present study, collected sample sizes were 50 from each location. Therefore, estimates of population differentiation obtained, were unlikely to be confounded by small sample sizes.

Number of alleles varied from 4 to 9 and mean number of alleles per locus ranged from 4.4 to 6.2 across all microsatellite loci (Table 2). This finding is similar to the results reported by Divu *et al.* (2008) and Bhat *et al.* (2009) for *M. rosenbergii* sampled from two South Indian rivers. Much of the variation in polymorphism at microsatellite loci that exist between species can be attributed to population biology and life history and to a lesser extent to differences in natural selection acting at the loci directly or indirectly (Neff and Gross, 2001). Hence, lesser number of alleles at microsatellite loci in *M. rosenbergii* suggests lower mutations rates in the species.

Significant deviations from HWE, resulting from heterozygote deficiencies, were detected at most loci in the sampled populations. A similar finding was reported by Chareontawee *et al.* (2007) and Bhat *et al.* (2009) in *M. rosenbergii*. Low microsatellite heterozygosity values were observed for four species of penaeid prawns and *P. monodon* respectively (Benzie, 2000; Mandal, 2012).

Deviations from HWE with homozygote excesses are often attributed to either null alleles (Garcia DeLeon *et al.* 1995; Gopalakrishnan *et al.* 2009), selection (Garcia DeLeon *et al.* 1995), unrecognized sampling of divergent gene pools (Wahlund effect) (Gibbs *et al.* 1997), inbreeding, or non-random mating (Beaumont and Hoare, 2003). Micro-Checker analysis did not indicate the presence of any null alleles in all five populations sampled. This may be due to inbreeding caused by over-exploitation, which might result in deficiency of heterozygotes and deviation from HWE. The decline of *M. rosenbergii* due to over-exploitation was corroborated in another study by Bhat *et al.* (2009).

F_{ST} value (0.0666) and pairwise F_{ST} estimates (0.0420 to 0.0911) obtained in this study indicate a significant level of genetic differentiation among different riverine *M. rosenbergii* populations. This result suggests separation of breeding populations, restriction in movement of populations between different areas, and existence of distinct stock structure among populations. Nei's (1978) genetic distance estimates between population pairs among sampled *M. rosenbergii* populations were high with 5 microsatellite markers in the present study. The UPGMA also showed a distinct population structure related to geographical location.

In conclusion, this study provides baseline genetic data for freshwater prawn culture and useful for devising stock-specific conservation management plans and traceability analysis in mixed catch scenarios.

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