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Genetic Diversity in Three River Populations of the Giant Freshwater Prawn (*Macrobrachium rosenbergii*) in Bangladesh Assessed by Microsatellite DNA Markers

Sadequr Rahman Khan[1], Habiba Akter[2], Nargis Sultana[2], Mohd. Golam Quader Khan[2], Md. Abdul Wahab[1] and Md. Samsul Alam[2*]

[1]*Department of Fisheries Management, Bangladesh Agricultural University, Mymensingh, Bangladesh*
[2]*Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh*
*For correspondence: E-mail: samsul_bau@yahoo.com

Abstract

Information on intra- and inter-population genetic diversity of cultured species may help develop efficient management and stock improvement programs. The study was undertaken to assess the genetic diversity in three wild populations namely the Paira, the Naaf and the Pashur rivers of the giant freshwater prawn (*Macrobrachium rosenbergii* de Man). Varied number of alleles, 5-14 was detected at the seven polymorphic microsatellite loci with an average of 9.833, 9.167 and 10.167 in the Paira, Naaf and Pashur populations, respectively. The average heterozygosity observed (*Ho*: 0.634) and expected (*He*: 0.827) were highest in the Pashur population. The *Ho* values of all the loci were found to be lower than the respective *He* values and thus fixation index (Fis) values were positive in all the populations ranging from 0.203 to 0.268, reflecting a high level of inbreeding. Ten of the 21 tests comprising seven loci in three populations for deviations from Hardy-Weinberg expectation were significant. The population differentiation (F_{ST}) value ranged from 0.012 to 0.021 indicating a low level of differentiation among the populations. Results revealed a moderate level of genetic variability though there was deficiency in observed heterozygosity in the three river populations of *M. rosenbergii*. The detection of the reducing trend in the effective population size in the rivers indicates that a pragmatic management plan is needed to establish equilibrium between the catch and the maintenance of genetic diversity of the populations of *M. rosenbergii*. © 2014 Friends Science Publishers

Keywords: *Macrobrachium rosenbergii*; Microsatellite; Heterozygosity; Genetic diversity

Introduction

The giant freshwater prawn (*Macrobrachium rosenbergii* De Man) is naturally distributed in whole of South and South-East Asia, Northern Australia and the Western Pacific Islands (New, 2002). Rearing of freshwater prawn by collecting or trapping juveniles in tidal ponds had been an age-old practice in the Indian sub-continent and Malaysia (Wickins, 1976). However, with the technologies for captive breeding and postlarvae (PLs) production established, the freshwater prawn has appeared as one of the major commercial aquaculture species in many countries such as China, India, Thailand, Vietnam and Bangladesh. Bangladesh entered into commercial prawn farming in 1990s and has acquired a position in the top five prawn producing nations of the world (FAO, 2009).

Naturally, after mating the gravid females of *M. rosenbergii* migrate towards brackish water where the eggs hatch as free-swimming larvae (Aquacop, 1977). After passing through 8 (Ling, 1969) to 11 (Uno and Soo, 1969) developmental stages, PLs move upstream to freshwater area where they live up to their adult life. Though started with the wild-caught PL and PL collection has not been completely stopped, prawn farming in Bangladesh at present mostly depends on hatchery produced seed. However, for producing PL in the hatcheries, egg-bearing female prawns are collected from different rivers mostly from the south-west, south and south-east part of the country across the coastal districts. Therefore, the river populations are serving two important purposes: maintaining their own populations and providing mother prawn for the hatcheries. As the rivers are important reservoirs of this valuable prawn species, it is essential to conserve their genetic diversity. The genetic diversity has a positive correlation with the evolutionary potential and fitness of a population (Reed and Frankham, 2003; Vandewoestijne *et al.,* 2008). Estimation of the distribution of subpopulations based on population genetic structure may help protect weaker populations through regulating harvest based on their genetic properties (Utter, 1991). Genetic characterization also serves as a useful tool for evaluating the direction and magnitude of changes in genetic structure of species over time. Populations with a high level of genetic variation have greater prospects for

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improvements in production traits such as viability, growth rate, fecundity and resistance to environmental stress and disease. Genetic variation is the basic resource that is exploited in a successful breeding program. Therefore, conservation of genetic variation is considered as an essential component of efficient fisheries management programs.

Microsatellite DNA markers are widely used for genetic characterization of wild and cultured populations for management and breeding purposes. For example, establishing pedigrees and estimating heritabilities are very effective for a breeding program that can be conducted by using microsatellite DNA markers (Norris *et al.,* 2000; Vandeputte *et al.*, 2004). In freshwater prawn and marine shrimp, microsatellite markers have been used for studying genetic diversity (Chand *et al*., 2005; Chareontawee *et al*., 2007; Divu *et al*., 2008; Schneider *et al.,* 2012), for monitoring genetic variability in a breeding program (Cruz *et al*., 2004), for assigning parentage to progeny (Jerry *et al.*, 2006) and for identifying strain as an alternative to physical tagging (Karaket *et al*., 2011).

Detailed information on genetic structure of wild populations of *M. rosenbergii* generated by microsatellite DNA markers could play valuable roles in conserving genetic resources and managing capture fisheries as well as identifying potential source population for the development of broodstock. By applying microsatellite DNA markers, we report here for the first time the genetic population structure of three important river populations of *M. rosenbergii* from the South-West, South and South-Eastern part of Bangladesh that are used for collection of brooders and PL.

Materials and Methods

Pond and Hapa Preparation

Nine experimental ponds each with an area of 100 m^2 (0.01 hectare) were prepared by draining out of water and removing weeds and predators. Agricultural lime ($CaCO_3$) was applied at the rate of 250 kg ha^{-1} on the bottom of the pond and the pond was filled up with underground water using a deep tube well. For promoting algal growth, the ponds were fertilized with urea, Triple Super Phosphate (TSP) and cow dung at the rates of 25 kg ha^{-1}, 25 kg ha^{-1} and 750 kg ha^{-1} respectively. Nine *hapa*s, made of fine meshed nylon net, each having 10 m^2 in area (5 m × 2 m) with 1 m height were set up by using bamboo poles maintaining the top of the net 1ft above the water level.

Collection, Stocking and Management of Post-larvae (PLs) of *M. rosenbergii*

PLs were collected from three rivers namely the Pashur, the Paira and the Naaf under Bagerhat, Barguna and Cox's Bazar district, respectively (Fig. 1) and transported to the Fisheries Field Laboratory Complex, Faculty of Fisheries, Bangladesh Agricultural University, Mymensingh, Bangladesh. After conditioning for three days, the PLs with an average weight of 0.23 g were stocked at a rate of 3 PL m^{-2} (30 PL/*hapa*) with three replications. Supplementary feed having 32% protein was applied at 10% of total body weight up to 40 days and then the feeding ration was adjusted to 5% of the body weight for the next 30 days. The feed was applied twice a day, half in the morning and the other half in the afternoon.

Collection of Tissue Sample and Extraction of Genomic DNA

After 70 days of rearing, the juvenile prawns from the three *hapas* under each river source were mixed. The mean individual harvest weights (g) of the juvenile prawn were 1.51, 0.90 and 1.30 g in the Pashur, Paira and Naaf population, respectively. The pleopods of 36 randomly collected samples from each of the three river sources were clipped by using scissors and then preserved in 95% ethanol and stored at -20°C. The genomic DNA was extracted from approximately 30 mg of the tissue following standard phenol-chlorofrom-isoamyl alcohol extraction and ethanol precipitation method (Ayub *et al*., 2008). The quality of the DNA samples was checked by electrophoresis on 1% agarose gel and the quantity was measured using a spectrophotometer (Biophotometer Plus, Eppendorf, Germany).

PCR Amplification

Initially, ten pairs of microsatellite primers (*Mbr1, Mbr2, Mbr3, Mbr4, Mbr5, Mbr7, Mbr8, Mbr9, Mbr10, Mbr11*) developed by Charoentawee *et al.* (2006) from *M. rosenbergii* were screened but finally seven pairs were used for genetic diversity assessment of three river populations of the freshwater prawn. The PCR was conducted using 50 ng template DNA, 2.0 µM of each primer, 0.25 mM of each dNTPs, 1X PCR buffer and 1 unit of Taq DNA polymerase (GENEI, India) in a total volume of 12 µL. A gradient thermocycler (Eppendorf, Germany) was used to conduct the PCR under the following temperature profiles: an initial denaturation step at 94ºC followed by 35 cycles each consisting of denaturation at 94ºC for 30 sec, annealing at 56ºC for 30sec and extension at 72ºC for 1 min. A final step of elongation at 72ºC for 5 min followed the last cycle. For confirmation of PCR amplification, the products were mixed with 3 µL of loading dye (0.25% Bromophenol blue, 0.25% Xylene cyanol, 30% glycerol) and one half was run on 2% agarose gel.

Polyacrylamide Gel Electrophoresis and Silver Nitrate Staining

For separation of the microsatellite alleles, the PCR products were electrophoresed on 6% denatured polyacrylamide gel (19:1:polyacrylamide:bisacrylamide) containing 7 M urea using Sequigen GT (Bio-Rad, Hercules, USA) vertical gel electrophoresis system.

Fig. 1: Map of Bangladesh showing *M. rosengergii* PL collection sites from three rivers

The silver nitrate staining protocol of Promega (Madison) was followed for staininig the polyacrylamide gel fixed on the glass plate.

Statistical Analysis of Microsatellite Data

The length of the alleles against the molecular weight markers were estimated using the software Alpha Ease FC (Version 4.0). The genotype data on all loci of all samples of the three populations were compiled in a matrix using the Microsoft Excel program. Analysis of various population genetic parameters such as the number of alleles (*Na*), effective number of alleles (*Ne*), frequency of alleles, observed (*Ho*) and expected heterozygosity (*He*), Nei's (1972) genetic distance and gene flow (N_m) between the population pairs and chi-square test for fit to Hardy-Weinberg Expectation (HWE) and Analysis of Molecular Variance (AMOVA) were performed using the software GenAlEx version 6.4 (Peakall and Smouse, 2006). The software ARLEQUIN version 3.0 (Excoffier *et al.*, 2005) was used to test the statistical significance between the F_{ST} values of different population pairs applying 1000 permutations of the genotypes. The software MEGA version 4 of Tamura *et al.* (2007) was used to build a UPGMA (unweighted pair-group method of averages) dendrogram.

Results

Allele Size and Frequency

A total of 75 alleles ranging from 220 to 338 bp were detected at the seven microsatellite loci (*Mbr-1, Mbr-2, Mbr-3, Mbr-4, Mbr-5, Mbr-8* and *Mbr-9*). The number of alleles varied between 5 (*Mbr-8*) and 14 (*Mbr-3*) at the seven loci with an average of 10.21 alleles per locus.

Table 1: Allele frequencies at three microsatellite loci in three populations of *Macrobrachium rosenbergii*

Locus	Allele	Paira	Naaf	Pashur
Mbr1	260	0.000	0.100	0.067
	268	0.200	0.133	0.067
	272	0.000	0.033*	0.000
	284	0.067	0.067	0.300
	290	0.033	0.200	0.133
	294	0.067	0.033	0.100
	298	0.100	0.033	0.133
	302	0.233	0.233	0.033
	306	0.167	0.100	0.167
	314	0.133	0.067	0.000
Mbr2	296	0.000	0.014	0.014
	300	0.042	0.014	0.042
	304	0.056	0.153	0.069
	308	0.111	0.056	0.208
	312	0.194	0.083	0.111
	316	0.125	0.208	0.111
	320	0.250	0.222	0.139
	324	0.097	0.153	0.194
	328	0.083	0.097	0.069
	334	0.042	0.000	0.028
	338	0.000	0.000	0.014*
Mbr3	234	0.083	0.097	0.111
	238	0.042	0.111	0.153
	240	0.250	0.208	0.167
	244	0.000	0.222*	0.000
	248	0.250	0.000	0.236
	252	0.000	0.083*	0.000
	256	0.125	0.000	0.125
	258	0.125	0.194	0.097
	262	0.042	0.014	0.028
	268	0.014	0.014	0.014
	272	0.014	0.014	0.028
	276	0.028	0.028	0.028
	280	0.014	0.014	0.000
	284	0.014	0.000	0.014
Mbr4	280	0.000	0.028	0.042
	284	0.139	0.222	0.125
	288	0.014	0.028	0.056
	292	0.056	0.056	0.028
	296	0.069	0.028	0.028
	300	0.056	0.083	0.111
	304	0.056	0.069	0.111
	309	0.139	0.069	0.097
	312	0.194	0.222	0.264
	318	0.097	0.069	0.028
	322	0.097	0.083	0.042
	326	0.083	0.042	0.069
Mbr5	288	0.056	0.056	0.097
	292	0.181	0.319	0.097
	296	0.083	0.153	0.167
	300	0.306	0.125	0.278
	304	0.139	0.236	0.139
	308	0.069	0.028	0.056
	312	0.097	0.042	0.069
	318	0.014	0.000	0.028
	322	0.028	0.028	0.028
	328	0.028	0.014	0.042
Mbr8	252	0.014	0.083	0.222
	256	0.181	0.306	0.250
	258	0.431	0.403	0.389
	262	0.278	0.153	0.111
	266	0.097	0.056	0.028
Mbr9	238	0.014	0.000	0.069
	240	0.056	0.125	0.125
	244	0.167	0.278	0.236
	248	0.306	0.347	0.306
	252	0.292	0.083	0.139
	256	0.042	0.028	0.014
	259	0.028	0.042	0.000
	262	0.028	0.056	0.014
	268	0.028	0.028	0.014
	272	0.014	0.014	0.028
	276	0.014	0.000	0.028
	280	0.014	0.000	0.014
	284	0.000	0.000	0.014*

*Indicates private alleles in the respective population

A total of five alleles were detected as private alleles: three in the Naaf population and two in the Pashur population. No private allele was detected in the Paira population (Table 1).

Intra-population Genetic Variation

A 100% of the loci were polymorphic (P_{95}) in the three studied populations. The mean number of alleles (*Na*), mean effective number of alleles (*Ne*) and the observed (*Ho*) and expected (*He*) heterozygosityy values of the Pashur population were higher than those of the Paira and the Naaf population (Table 2). The *Fis* (inbreeding coefficient) values were positive at all the loci in all the populations. The *Fis* value of the Pashur population was the lowest and that of the Paira population was the highest. χ^2 tests revealed deviations from the Hardy-Weinberg expectation (HWE) at varied number of loci in the three populations due to deficiencies in heterozygosity: five in the Naaf, three in the Paira and two in the Pashur population (Table 2).

Inter-population Genetic Diversity

The population differentiation value (F_{ST}) between the Paira and the Naaf population was the highest (0.021) and that between the Paira and Pashur population was the lowest (0.012) (Table 3). However, the F_{ST} values between the population-pairs were low and non-significant (P>0.05). Highest and lowest gene flow (N_m) values were observed between the Paira-Pashur (20.910) and the Naaf-Pashur population-pairs (11.887), respectively (Table 3). AMOVA revealed that of the total molecular variation, 6% existed among the stocks while 94% existed within stocks (data not shown).

Genetic Distance and Dendrogram

The genetic distance between the population-pairs ranged from 0.110 to 0.200. The highest and lowest genetic distances were estimated between the Pashur and Naaf population and between the Pashur and Paira population respectively (Table 3). The UPGMA dendrogram built on the basis of Nei's (1972) genetic distance included the Pashur and the Paira populations in one cluster and the Naaf population another cluster (Fig. 2).

Discussion

The abundance of alleles and heterozygosity are extensively used as tools for assessing the intra- and inter-population genetic diversity. Genetic variability is fundamental to ensuring the evolutionary potential and fitness of populations (Koljonen *et al*., 2002; Reed and Frankham, 2003). The present study revealed a moderate level of genetic variability in terms of average number of alleles and expected heterozygosity at seven microsatellite loci in the three river populations of *M. rosenbergii*. We detected 75 alleles at seven loci (*Mbr1, Mbr2, Mbr3, Mbr4, Mbr5, Mbr8* and *Mbr9)* averaging 10.71 alleles per locus and their sizes were comparable to the findings of Chareontawee *et al.* (2006). Variations in the number of alleles have been observed in *M. rosenbergii* by different authors; e.g., 12-18 (Chand *et al*., 2005), 5-17 (Chareontawee *et al*., 2006),

Table 2: Allelic variation and deviation from Hardy-Weinberg expectations at seven microsatellite loci in a sample of 36 *M. rosenbergii* of the three populations: N- Number of sample, *Na*- number of alleles, *Ne*- effective number of alleles, *Ho*-observed heterozygosity, *He*- expected heterozygosity, *Fis*- inbreeding coefficient; H-W test- X^2 values followed by the degrees of freedom in parenthesis

Locus	Parameters	Population		
		Paira	Naaf	Pashur
	Na	8	10	8
	N_e	6.250	6.923	5.769
	H_o	0.733	0.600	0.800
	H_e	0.840	0.856	0.827
	Fis	0.127	0.299	0.032
	H-W Test	35.77(28)^{ns}	82.28(45)***	21.74 (28)^{ns}
Mbr2	Na	9	9	11
	Ne	6.612	6.276	7.261
	Ho	0.583	0.611	0.611
	He	0.849	0.841	0.862
	Fis	0.313	0.273	0.291
	H-W Test	62.40 (36)**	57.74 (36)*	75.66 (55)*
Mbr3	Na	12	11	11
	Ne	5.945	6.216	6.803
	Ho	0.611	0.556	0.583
	He	0.832	0.839	0.853
	Fis	0.265	0.338	0.316
	H-W Test	61.78 (66) ^{ns}	72.94 (55) ^{ns}	68.75 (55) ^{ns}
Mbr4	Na	11	12	12
	Ne	8.583	7.448	7.513
	Ho	0.611	0.667	0.611
	He	0.883	0.866	0.867
	Fis	0.308	0.230	0.295
	H-W Test	129.00 (55)***	76.96 (66) ^{ns}	133.44 (66)***
Mbr5	Na	10	9	10
	Ne	5.838	4.918	6.480
	Ho	0.556	0.500	0.722
	He	0.829	0.797	0.846
	Fis	0.330	0.372	0.146
	H-W Test	66.36 (45)*	79.04 (36)***	48.74 (45) ^{ns}
Mbr8	Na	5	5	5
	Ne	3.281	3.461	3.620
	Ho	0.528	0.639	0.556
	He	0.695	0.711	0.724
	Fis	0.241	0.101	0.232
	H-W Test	15.775 (10)^{ns}	42.540 (10)***	16.103 (10) ^{ns}
Mbr9	Na	12	9	12
	Ne	4.670	4.408	5.226
	Ho	0.556	0.583	0.722
	He	0.786	0.773	0.809
	Fis	0.293	0.246	0.107
	H-W Test	76.48 (66)^{ns}	57.61 (36)*	32.69 (66) ^{ns}
Average no. of alleles (*Na*)		9.57	9.28	9.85
Average no. of effective alleles (*Ne*)		5.883	5.664	6.096
Average H_o over loci		0.574	0.592	0.634
Average H_e over loci		0.812	0.804	0.827
Average *Fis*		0.268	0.265	0.203
Polymorphism (P_{95})		1.00	1.00	1.00

Key: ns=not significant, * P<0.05, ** P<0.0[1, *** P<0.001

4-20 (Charoentawee *et al*., 2007), 3-12 (Bhassu *et al*., 2008), 3-16 (Divu *et al*., 2008), 10-20 (Karaket *et al*., 2011) and 3-25 (Schneider *et al*., 2012). The number of alleles observed in our study ranged from 5 to 14 were consistent with those of Chareontawee *et al.* (2006) and Divu *et al.* (2008) but higher than reported in a wild population in India (Schneider *et al*., 2012). The Pashur population showed better variation than the other two populations in terms of the number of alleles and average effective number of alleles.

The average number of alleles, *Ho* and *He* obtained in the present study were lower than those reported in three strains of *M. rosenbergii* in Thailand (Karaket *et al*., 2011). In all cases the He_s were higher than the respective Ho_s. *He* is considered as gene diversity as it is the expected probability that an individual will be heterozygous at a given locus. It depends on the number and relative frequencies of the alleles present in a locus (Hale *et al.,* 2012). The average observed and expected heterozygosities were found to be 0.574 to 0.634 and 0.804 to 0.827, respectively are in agreement with the range reported by other authors (Chareontawee *et al*., 2007; Schneider *et al*., 2012). Inbreeding causes decrease in observed heterozygosity but does not affect the allele frequencies and thus the expected heterozygosity of a population. Significant departures from HWE have been observed in all three populations- at three loci in the Paira population, at five loci in the Naaf and at two loci in the Pashur population due to reduction in heterozygosity through inbreeding. Similar to our observation, Chareontawee *et al*. (2006) also reported higher levels of *He* compared to respective *Ho* in a natural population of *M. rosenbergii* in Thailand. These may be attributed to the presence of null alleles in the loci examined. Deficiencies in heterozygosity relative to HWE may result from several factors such as differences in chances of mating among individuals, reduction in effective breeding number due to over exploitation (Schneider *et al.,* 2012), or selection pressure on certain locus (Ferguson, 1995; Garcia de Leon *et al*., 1997), Wahlund effect (Hartl and Clark, 1997) and preferences of the individuals to mate with geographically nearer individuals. Among these, reduction in effective population is the most likely factor causing nonconformity to HWE in the giant prawn populations in Bangladesh. The excess of homozygosity resulted in positive F_{is} values in all populations, ranging from 0.032 to 0.372 that were higher than those reported by Schneider *et al*. (2012). *Fis* is a measure of the frequencies of heterozygotes compared to that expected when the populations are under H-W equilibrium.

The F_{ST} values between the population-pairs ranged from 0.012 to 0.021 in the present study. This level of genetic differentiation between the pairs of populations is considered as low (Balloux and Lugon-Moulin, 2002). The lowest value of F_{ST} (0.012) between the Paira and the Pashur population indicated relatively closer relation between these rivers over the Naaf population. The hierarchy of molecular variance tested by AMOVA revealed that 94% of the variation existed within the population and 6% existed among the populations, which suggesting little differentiation among the studied populations. The higher level of gene flow was found between the Paira and the Pashur populations. The higher genetic distance between the Naaf and the Pashur and between the Naaf and the Paira populations may be explained by the geographical distance between the pairs as the distance of the Naaf river from the other two rivers are more than the distance between the Pashur and the Paira River (Fig. 1).

Table 3: Multilocus F_{ST} (above diagonal) and N_m (above diagonal in parentheses) and genetic distance (below diagonal) values between pairs of three stocks of *M. rosenbergii* across all loci

Population	Paira	Naaf	Pashur
Paira	***	0.021 (12.436)	0.012 (20.910)
Naaf	0.197	***	0.020 (11.887)
Pashur	0.110	0.200	***

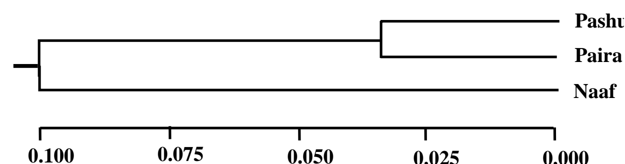

Fig. 2: Unweighted Pair Group Method of Averages (UPGMA) dendrogram based on Nei's (1972) genetic distances between the three population pairs of *M. rosenbergii*

The average number of allele was lowest in the Naaf population (9.28), while average number of allele of the Pashur population (9.85) was higher with highest average effective number of alleles (N_e= 6.096). The effective number of alleles is much less than the actual number of alleles indicating that the frequencies are not equal for all alleles.

The genetic structure of a population is not a stable phenomenon and subjected to change over time. The magnitude and trend of the change largely depend on the intensity of natural and human interventions. The parent prawn and PL are harvested from the rivers and the sizes of the existing populations are determined by the intensity of catch and recruitment. The present study detected a reducing trend in the effective population size of *M. rosenbergii* in the rivers as reflected by deficiencies in heterozygotes and increasing inbreeding (F_{IS} values). Genetic diversity of smaller populations declines at a faster rate than that of a larger population and loss of genetic variation is considered to be the loss of fitness of a population.

In conclusion, among seven microsatellite loci Pashur population showed highest average number of alleles, and observed and expected heterozygosity. Moreover, Hardy-Weinberg expectation found fewer deviations in Pashur

population compared to the other two populations. So, genetic characterization elucidated Pashur population's PLs are superior over the Paira and the Naaf population in terms of genetic variability. These findings and catch data of last few years could help develop a pragmatic management plan to establish equilibrium between the catch and the maintenance of genetic diversity of the populations.

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