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ORIGINAL ARTICLE

Genetic variation in wild and hatchery populations of giant freshwater prawn (*Macrobrachium rosenbergii*) revealed by randomly amplified polymorphic DNA markers

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KEYWORDS

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Abstract Sustainable improvement and conservation of any genetic resource depend on the assessment of its intra- and inter-population genetic variation. In order to estimate genetic variation in both wild and hatchery populations of *Macrobrachium rosenbergii*, randomly amplified polymorphic DNA (RAPD) analysis was performed. Analyses of 51 polymorphic loci amplified from genomic DNA by three decamer random primers revealed different degrees of genetic variation in two wild (Bhairab and Rupsha rivers) and hatchery-derived *gher* (*Gher*-1 and *Gher*-2) populations. The proportion of polymorphic loci was found to be higher in wild populations (0.90 and 0.65 for the Bhairab and Rupsha populations, respectively) than the hatchery-derived *gher* populations (0.29 and 0.16 for *Gher*-1 and *Gher*-2, respectively). Likewise, the river populations contained higher levels of gene diversity (0.221 and 0.179 for Bhairab and Rupsha populations, respectively) than the *gher* populations (0.114 and 0.045 for *Gher*-1 and *Gher*-2, respectively). These results suggest reduction of genetic variation and heterozygosity in the hatchery-derived *gher* populations. Inter-population similarity indices and pairwise genetic distance values showed that variation between the wild or between the *gher* populations were lower than those between the wild and hatchery populations. On average, 14 loci exhibited significant deviation from homogeneity in wild vs hatchery population pairs, whereas 2 and 3 loci showed heterogeneity in *Gher*-1 vs *Gher*-2 and Bhairab vs

Abbreviations PLs, post larvae; RAPD, randomly amplified polymorphic DNA; $MgCl_2$, magnesium chloride; mM, milli-molar; µM, micro-molar; µl, microliter; ng, nanogram; C, celsius; V, volt; h, hour; bp, base pair; kb, kilobase; DNA, deoxyribonucleic acid; dNTPs, deoxyribonucleotide triphosphates; TAE, tris-acetate-EDTA; h, gene diversity; H_t, total genetic diversity; H_s, average genetic diversity within populations; n_a, observed number of alleles; n_e, effective number of alleles; N_m, gene flow; S_i, intra-population similarity index; S_{ij}, inter-population similarity index

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Rupsha population pairs, respectively. A genetic distance-based UPGMA dendrogram segregated river populations from the *gher* populations. Our study, therefore, revealed substantial levels of genetic variation between wild and hatchery populations of *M. rosenbergii*.

1. Introduction

Giant freshwater prawn *Macrobrachium rosenbergii*, locally known as 'Golda', contributes substantially to shrimp aquaculture in Bangladesh. Sub-tropical freshwater bodies influenced by huge adjacent brackish water favors commercial farming of the species in Bangladesh. The species also is found in natural water bodies in southern and southeastern Asia, parts of Oceania, and some Pacific islands, and has been recorded to be introduced in more than 40 countries [1]. As a commercial culture species, it possesses many biological advantages, including attaining maturation in captivity, relatively large size, and rapid growth rate. Due to higher market price in local and world markets, many farmers of Bangladesh, particularly those from the coastal region of southwest part of Bangladesh (the Khulna division) have converted their paddy fields to '*ghers*' to accommodate profitable prawn culture. The Bengali word *gher* means perimeter, and in this context, refers to ponds for shrimp or prawn cultivation made by modifying rice fields through building higher dikes around the field [2]. Farmers usually stock post-larvae (PLs) collected from either natural water bodies or a hatchery in *ghers* for grow-out. Due to overfishing, use of destructive gears, environmental degradation and pollution, collection of PLs from wild sources has declined in recent years [3]. Therefore, farmers depend on the hatchery-produced PLs for cultivations in *ghers*. The brood prawns required for the hatcheries are harvested directly from the river or from *ghers*, and no attention is paid to the genetic quality of the broodstock [4]. As the hatchery owners buy females carrying only gravid fertilized eggs for seed production, farmers tend to keep higher quantities of females in their brood banks. They often maintain 1♂: 4♀ or 1♂: 5♀, even 1♂: 7♀ sex ratios in the brood banks [5]. Hence hatchery-produced seedstock come mainly from but a limited pair of parents and poor quality seedstock often are produced. Recently, the prawn culturists have been facing a severe problem of slower growth performance in culture condition. Farmers claim that hatchery-produced PLs do not grow as fast as wild PLs (personal communication). That is why farmers are still interested in the natural seedstock, which could have a negative impact on biodiversity. For sustainable management and improvement of the species, study of its population structure is necessary. To address the issue, Khan et al. [6] characterized genetic diversity in three river populations of *M. rosenbergii* using microsatellite DNA markers. While they used wild populations, in our research we used both wild and hatchery populations of *M. rosenbergii* to compare the genetic variation between populations by randomly amplified polymorphic DNA (RAPD) analysis. Comparison of genetic diversity between wild and hatchery populations will enable us to understand population structure of the species. Among different DNA markers, RAPD is easy and simple to work. It functions based on amplification of discrete regions of the genome by polymerase chain reaction with short random primers. Changes of sequence at primer annealing sites or deletion of primer annealing site in the template DNA or large insertion or deletion between two primer annealing sites cause loss of amplification and detection of genetic variation [7,8]. This marker has extensive use in estimation of genetic variability and relatedness in many organisms [9]. The present study, therefore, used RAPD analysis in estimation of genetic variation in both wild and hatchery-derived *gher* populations of *M. rosenbergii* and to compare the level of genetic variation between the two stocks.

2. Material and methods

2.1. Sample collection

A total of eighty samples were collected from four different populations (20 from each) located in Khulna district of Bangladesh (Fig. 1). Among the four populations, two were wild and two were hatchery-derived *gher* populations. Wild samples were collected from the Bhairab and Rupsha rivers. Bhairab (approximately 160 km in length) is a tributary of the Ganges that passes through Khulna district. The Rupsha River on the other hand, is formed from the Bhairab. It connects to the Bay of Bengal through the Pashur River at Mongla Channel and its entire length is affected by tides. Hatchery-origin samples were collected from *Gher*-1 and *Gher*-2 of Rupsha and Paikgasa *upazilla* (sub-district) of Khulna district, respectively. *Gher* owners stocked PLs in *ghers* collected from nearby hatcheries (personal communication). Prawns from each location were placed on ice and taken to the laboratory.

2.2. Extraction of genomic DNA

Genomic DNA was extracted from tissue samples collected from pleopods. Approximately 25-mg tissues were cut into small pieces, homogenized by micro-tissue-grinder in a 1.5 ml microfuge tube, and total genomic DNA was extracted following the method described by Islam and Alam [10]. DNA quality was checked by electrophoresis on 0.8% agarose gel, and concentration was measured by a spectrophotometer (Spectro nic®Genesis™, Spectronic Instruments Inc., USA).

2.3. Primer selection

Initially, 30 decamer primers of random sequence (Sigma–Aldrich) were screened on two randomly chosen *M. rosenbergii* to test their suitability for amplifying RAPDs that could be accurately scored. Primers were evaluated on the basis of intensity of resolution of bands, repeatability of markers, consistency within individuals and potential to differentiate populations (polymorphisms). A final subset of three primers

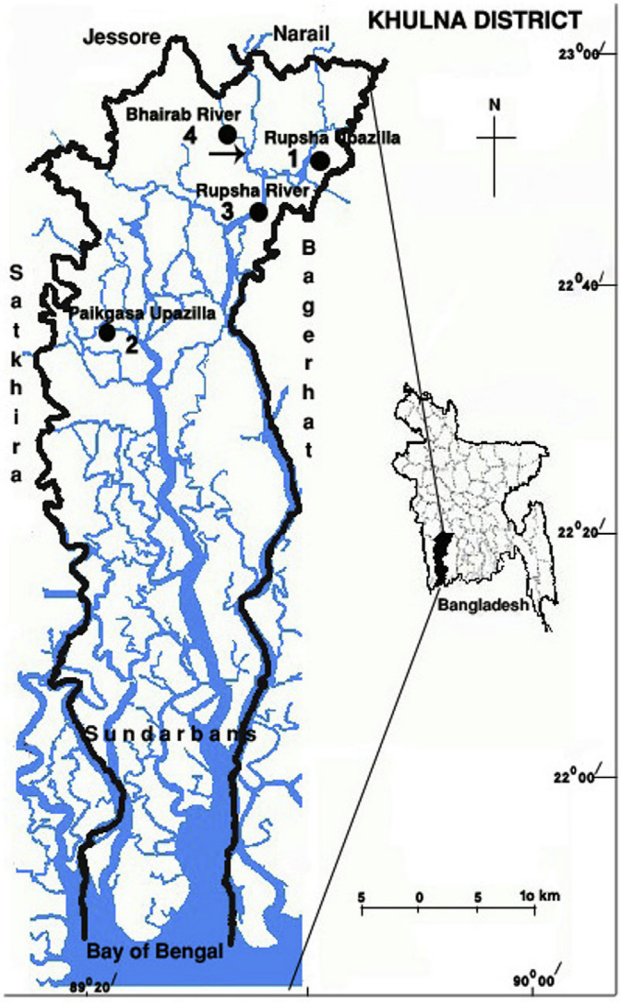

Figure 1 Map of Khulna district of Bangladesh showing sampling sites for *M. rosenbergii*. 1, 2, 3 and 4 refer to the locations of *Gher*-1, *Gher*-2, Rupsha and Bhairb populations of *M. rosenbergii*.

exhibiting good-quality, reproducible banding patterns was used for RAPD analysis of entire sample set. In order to ensure that RAPD bands were not merely artifact which is a common problem in RAPD analysis, RAPD profiles of whole sample set using the three primers were repeated and obtained consistent banding patterns.

2.4. PCR amplification

The amplification was performed following the method described by Williams et al. [8] with some modifications. The PCR reaction volume for each DNA sample was 10 µl, containing 1 µl of 10× Ampli *Taq* polymerase buffer, 2 µl of 10 µM primers, 1 µl of 250 µM dNTPs (Takara, Japan), 1 unit of Ampli *Taq* DNA polymerase (Takara, Japan), 100 ng of genomic DNA, and a necessary amount of sterile water. DNA amplification was performed in an oil-free thermal cycler (TProfessional Standard Gradient, Biometra, Germany). The thermal profile for PCR amplification was set as follows: pre-heating at 94 °C for 3 min, 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 36 °C and 2 min extension at 72°. To ensure complete extension of all amplified fragments, a final elongation step of 7 min at 72 °C was followed.

2.5. Electrophoretic separation of PCR-amplified products

The amplified products were separated electrophoretically through a 1.4% agarose gel (Invitrogen) in 1× TAE buffer at 120 V for 1.5 h. Molecular weight markers (1-kb and 100-bp DNA ladders) were run alongside the RAPD reactions. DNA bands were stained with ethidium bromide (10 mg/ml), observed on a UV-transilluminator, and photographed with a gel documentation system (Biodoc-It™ Gel Imaging System, Cambridge, UK).

2.6. Preparation of a data matrix and RAPD analyses

The size of each RAPD band was estimated by AlphaEaseFC™ Version 4.0 (Alpha Innotech Corporation). The presence (1) or absence (0) of bands having same molecular weight was scored separately for each prawn and each primer. The scores in respect of all primers used in the RAPD analysis were then pooled for constructing a single data matrix. This matrix was used for estimating allele frequency, proportion of polymorphic loci, Nei's [11] gene diversity (h), total genetic diversity (H_t), average genetic diversity within populations (H_s), observed (n_a) and effective number of alleles (n_e) [12], Nei's genetic distances [13] and gene flow (N_m) using POPEGENE (version 1.31) [14] and 'Tools for Population Genetic Analyses (TFPGA)' [15] computer programs. Allele frequencies for RAPD loci were estimated on the basis of an assumption of a two-allele system. One allele is capable of amplification of a RAPD-band by primer annealing at an unknown locus. The other is the 'null' allele incapable of amplification due to mutation at the primer-annealing site. The two-allele assumption is in most cases acceptable, because co-dominant loci showing band shifts are few [7]. In this system only a null-homozygote is detectable as negative for the RAPD-band of interest. Under the assumption of Hardy-Weinberg equilibrium, the null allele frequency (q) may be $(N/n)^{1/2}$, where N and n are the number of band-negative individuals observed and the sample size, respectively. The frequency of the amplifying allele (p) is $1 - q$. The assumption of the two-allele system enables us to calculate the Nei's genetic distance [16] from the RAPD pattern.

Band sharing based similarity indices referring to the fraction of shared bands of same molecular weight between the RAPD profiles of any two individuals on the same gel were calculated according the following formula, $SI = 2N_{AB}/(N_A + N_B)$, where N_{AB} is the total number of RAPD bands shared by individuals A and B, respectively [17]. Intra-population similarity (S_i) was calculated as the average of SI across all possible comparisons between individuals within a population. On the other hand, inter-population similarity (S_{ij}) was calculated as the average similarity between randomly paired individuals from populations of i and j [18].

3. Results

RAPD profiles of *M. rosenbergii* are given in Fig. 2. Three decamer random primers (i.e., S1189, S1265 and S1358) amplified 51 loci. Hence, the average number of loci generated per pri-

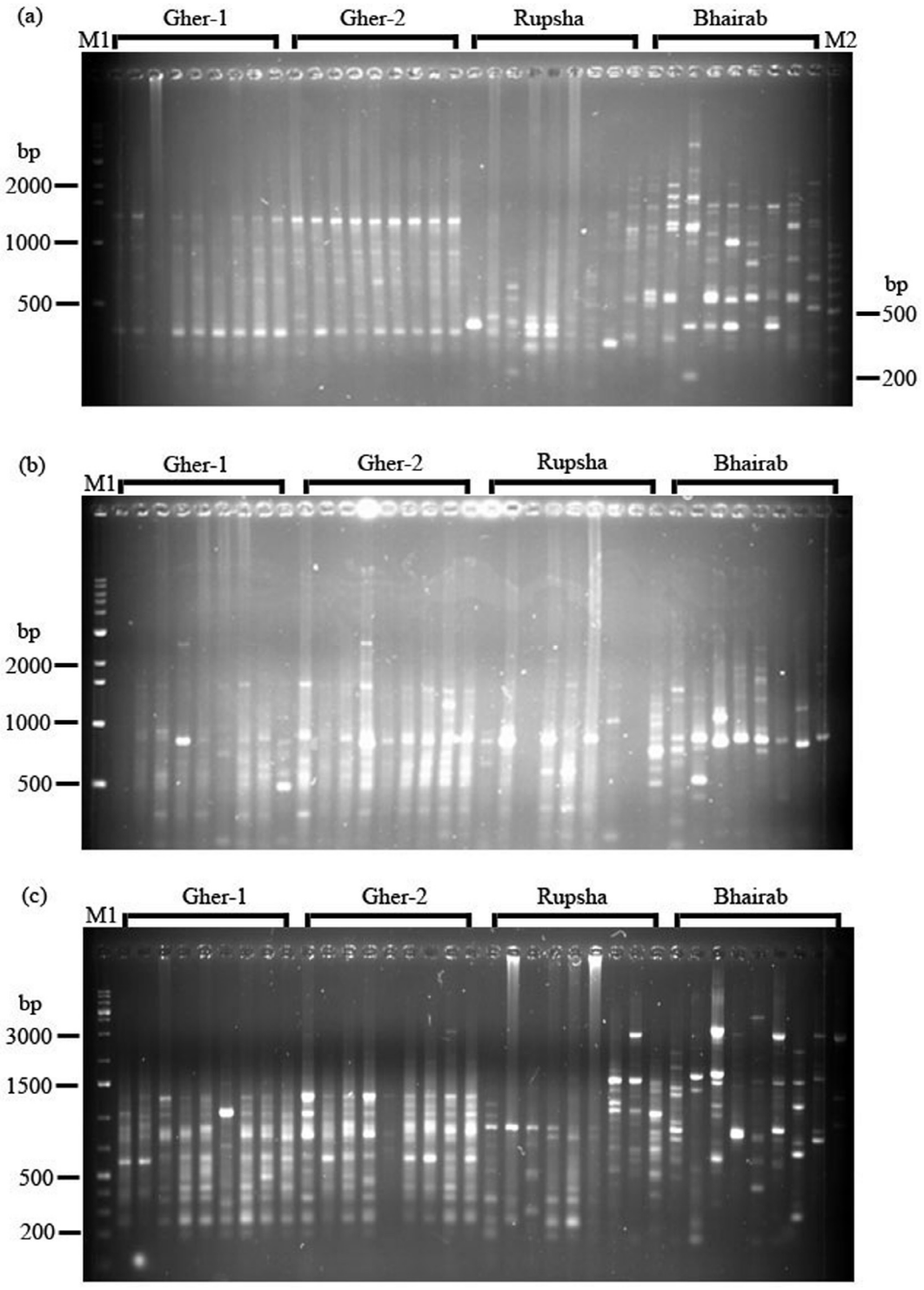

Figure 2 RAPD profiles of different populations of *M. rosenbergii* generated using primers S1189 (a), S1265 (b) and S1358 (c). M1 and M2 refer to 1 kb and 100 bp DNA ladder, respectively.

mer was 17 and sizes of the amplicons varied from 249 to 3789 bp. The S1358 primer produced the highest number of loci (20), followed by S1189 (19) and S1265 (12). All loci were found to be polymorphic in nature across the full sample set of the four populations. The S1358 primer revealed a higher level of gene diversity compared to other two primers in the four populations (Table 1). The total genetic diversity (H_t) considering all loci and populations and average genetic diversity within populations (H_s) were 0.243 and 0.140, respectively

3.1. Intra-population genetic variation and relatedness

Genetic variation between individuals within a population or intra-population genetic variations are shown in Table 2. The proportion of polymorphic loci, observed and expected number of alleles (n_a & n_e) and gene diversity (h) values were higher in the river populations compared to the hatchery-derived *gher* populations. The polymorphism estimated in Bhairab and Rupsha populations was 0.90 and 0.65, respectively, whereas the values for *Gher*-1 and Gher-2 were 0.29 and 0.16, respectively. Fourteen alleles ($S1189_{2942}$, $S1189_{2231}$, $S1189_{1897}$, $S1189_{1657}$, $S1189_{1409}$, $S1189_{1105}$, $S1189_{998}$, $S1358_{3789}$, $S1358_{2436}$, $S1358_{1995}$, $S1358_{1777}$, $S1358_{935}$, $S1265_{2093}$ and $S1265_{1817}$), which were found in the Bhairab population at a frequency range of 0.057–0.333, were absent in the other populations. Likewise, the Rupsha population contained a specific allele ($S1358_{477}$) at a frequency of 0.184. River populations contained higher levels of gene diversity (0.221 and 0.179 for Bhirab and Rupsha populations, respectively) than did *gher* populations (0.114 and 0.045 for *Gher*-1 and *Gher*-2, respectively). Band-sharing-based similarity indices are an important parameter to estimate both intra- and inter-population genetic relatedness. The intra-population similarity indices of the two hatchery-derived *gher* populations were higher (86.0% and 94.3% for *Gher*-1 and *Gher*-2, respectively) than those of two river populations (36.7% and 40.3% for Bhairab and Rupsha, respectively) (Table 3). The overall band-sharing-based similarity index was recorded to 64.32%.

3.2. Inter-population genetic variation and relatedness

There were 39 polymorphic loci which exhibited significant departure from homogeneity among different population pairs (Table 4). Among them, two loci (e.g., $S1265_{1603}$ and $S1265_{373}$) played a significant role in separation of the *Gher*-1 from the *Gher*-2 populations and four loci caused significant deviation from homogeneity in the Rupsha vs Bhairab population pair. The number of polymorphic loci that caused significant departure from homogeneity in Rupsha vs *Gher*-1, Rupsha vs *Gher*-2, Bhairab vs *Gher*-1 and Bhairab vs *Gher*-2 population pairs was 12, 16, 12, and 19, respectively (Table 4).

Differences were found in inter-population identities, ranging from 0.73 to 0.97 for different population pairs (Table 3). A higher level of similarities was observed in the *Gher*-1 vs *Gher*-2 and Rupsha vs Bhairab population pairs (0.97 and 0.96, respectively) and a lower level of genetic identity in the Bhairab vs *Gher*-2, Rupsha vs *Gher*-2, Bhairab vs *Gher*-1, and Rupsha vs *Gher*-1 population pairs. Likewise, the Rupsha vs Bhairab population pair had the highest level of estimated gene flow (4.62), followed by 2.24 between *Gher*-1 and *Gher*-2 populations, whereas the lowest level of gene flow was found in the *Gher*-2 v Rupsha and *Gher*-2 vs Bhairab population pairs. The two river populations or hatchery-derived popula-

Table 1 Genetic variation in *M. rosenbergii* detected by three different RAPD primers.

Primer codes	Sequence (5′-3′)	% GC	No. of loci scored	Size of loci (bp)	No. of polymorphic loci	Gene diversity (h)
S1189	5′gagctaccgt3′	60	19	323–2942	19	0.207
S1265	5′agtccccctc3′	70	12	373–2648	12	0.243
S1358	5′accccaacca3′	60	20	249–3789	20	0.276

Table 2 Estimation of genetic variation in different populations of *M. rosenbergii*.

Populations	Number of polymorphic loci	Number of private loci	Proportion of polymorphic loci	Observed number of alleles (n_a)	Effective number of alleles (n_e)	Gene diversity (h)
Gher-1	15	0	0.29	1.294	1.202	0.114
Gher-2	8	0	0.16	1.157	1.075	0.045
Rupsha	33	1	0.65	1.647	1.283	0.179
Bhairab	46	14	0.90	1.902	1.335	0.221
Overall	51	15	1.00	2.00	1.416	0.243

Table 3 Summary of pair-wise inter-population similarity indices (S_{ij}, above diagonal), gene flow (N_m, above diagonal in parentheses), intra-population similarity indices (S_i, underlined) and Nei's [13] genetic distances (below diagonal) in *M. rosenbergii*

Populations	*Gher*-1	*Gher*-2	Rupsha	Bhairab
Gher-1	86.0	0.97 (2.24)	0.86 (1.14)	0.81 (1.01)
Gher-2	0.034	94.3	0.78 (0.55)	0.73 (0.55)
Rupsha	0.152	0.253	36.7	0.96 (4.62)
Bhairab	0.208	0.320	0.040	40.3

Table 4 Chi-square values for the loci showing significant departure from homogeneity in different population pairs of *M. rosenbergii*.

Loci		*Gher*-1	*Gher*-2	Rupsha
Gher-2				
	$S1265_{1603}$	9.00**		
	$S1265_{373}$	5.55*		
Rupsha				
	$S1189_{1198}$	7.24**	16.05****	
	$S1189_{838}$	18.00****	18.00****	
	$S1189_{622}$	16.05****	7.24**	
	$S1189_{353}$	7.30**	7.30**	
	$S1358_{1352}$	9.00**	18.00****	
	$S1358_{1191}$	5.68*	14.20***	
	$S1358_{1050}$	10.69**	10.69**	
	$S1358_{851}$		9.00**	
	$S1358_{758}$		10.69**	
	$S1358_{577}$	4.30*	12.42***	
	$S1358_{403}$	5.68*	5.68*	
	$S1358_{344}$	9.00**		
	$S1265_{1603}$		16.05****	
	$S1265_{884}$	5.5*	5.55*	
	$S1265_{621}$		7.30**	
	$S1265_{535}$	5.68*	14.20***	
	$S1265_{373}$		12.42***	
Bhairab				
	$S1189_{1497}$		6.47*	4.83*
	$S1189_{1198}$	7.24**	9.00**	
	$S1189_{838}$	18.00****	10.69**	
	$S1189_{622}$	16.05****	4.30*	
	$S1189_{551}$		9.00**	5.68*
	$S1189_{353}$	7.30**	12.42***	
	$S1358_{1352}$	9.00**	14.20***	
	$S1358_{1191}$	5.68*	14.20***	
	$S1358_{1050}$	10.69**	16.05****	
	$S1358_{851}$		12.42***	
	$S1358_{758}$		7.30**	
	$S1358_{577}$	4.30*	10.69**	
	$S1358_{403}$	5.68*	7.24**	
	$S1358_{344}$	9.00**	9.00**	
	$S1358_{249}$		7.24**	
	$S1265_{1603}$		16.05****	
	$S1265_{884}$	5.55*		5.55*
	$S1265_{621}$		18.00****	4.82*
	$S1265_{535}$	5.68*	16.05****	
	$S1265_{373}$		18.00****	

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
**** $P < 0.0001$.

tions exhibited lower genetic distances (GDs for *Gher*-1 vs *Gher*-2 and Rupsha vs Bhairab were 0.034 and 0.040, respectively), whereas GD values increased to a range of 0.152 to 0.253 between river and *gher* populations (Table 3). A UPGMA dendrogram (Fig. 3) was constructed using genetic distances between different population pairs. In the dendrogram, the *Gher*-1 and *Gher*-2 populations grouped together to make one cluster, whereas the Rupsha and Bhairab populations with their genetic distance value of 0.040 made another cluster.

4. Discussion

One of the major objectives of our study was to determine intra-population genetic variation in *M. rosenbergii*. Populations having higher within-population similarity and lower proportion of polymorphic loci are likely to be less genetically diverse [10]. The *Gher*-1 and *Gher*-2 populations showed higher intra-population similarity indices and lower percentage of polymorphism indicating that hatchery-derived *gher* populations are less diverse than wild populations. Moreover, wild

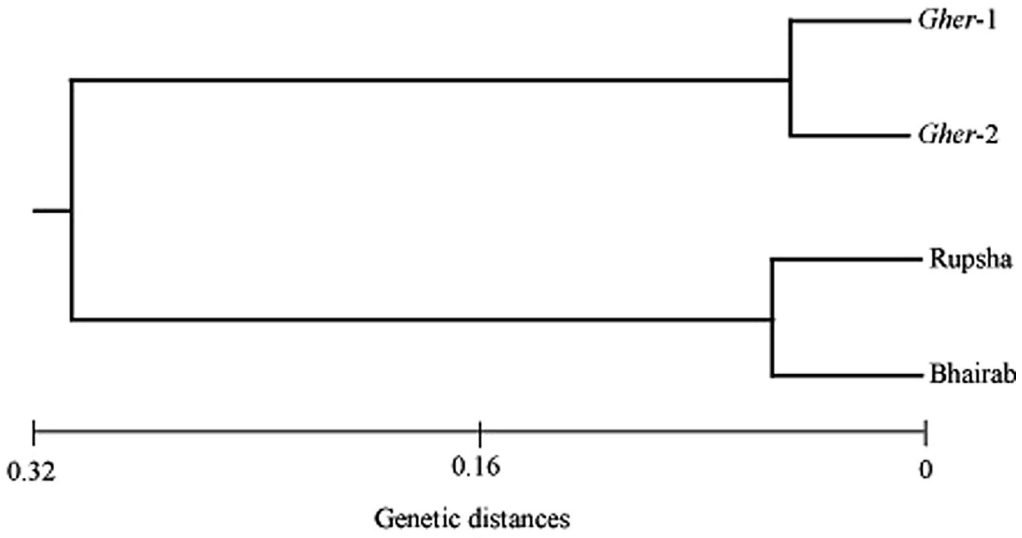

Figure 3 Clustering of different populations of *M. rosenbergii*, using the Unweighted Pair Group Method of Arithmetic Mean (UPGMA) algorithm, which was constructed on the basis of Nei's [13] genetic distances summarizing differentiation according to RAPD profiles.

populations appear to contain some unique genetic variants. For instance, population specific alleles were found in the Bhairab and Rupsha populations. Reduction of genetic diversity in cultured populations has been reported in many aquatic species including freshwater prawn [19–21] and giant tiger prawn (*Penaeus monodon*) [22]. Bhairab and Rupsha are two inter-connected rivers. These two natural water bodies are important for the life cycle of *M. rosenbergii*. Naturally, after mating, females move toward brackish water for hatching. As soon as larvae grow to PLs, they migrate upstream to freshwater rivers such as the Rupsha and Bhairab, where they grow to maturation [6]. The higher level of genetic polymorphism in both Rupsha and Bhairab populations is presumably due to the large effective population size (N_e) and random mating within populations. The individuals collected from both Rupsha and Bhairab might come from a large number of different parental pairs. The *Gher*-1 and *Gher*-2 on the other hand, are two small populations. In both *Gher*-1 and *Gher*-2 populations, hatchery-produced PLs were stocked for grow-out. A major concern is that it is a common practice in hatcheries to keep effective population size (N_e) small for running hatcheries at a minimum cost (personal communication). Therefore, there is a great possibility that individuals (in hatcheries such as the *gher* populations) might come from a limited number of matings, causing substantial reduction of genetic variation.

Another major focus of our study was to estimate inter-population genetic variation in *M. rosenbergii*. Estimation of pair-wise genetic identity, genetic distance and gene flow values showed that there is a lower level of genetic variation between the two *gher* or two river populations, and they are closer to each other in the dendrogram. Chi-square values for loci that caused significant departure from homogeneity in different population pairs support the lower level of genetic differentiation between the two *gher* or river populations. On the other hand, all the parameters mentioned above showed a high genetic variation between *Gher*-1 vs Rupsha, *Gher*-1 vs Bhairab, *Gher*-2 vs Rupsha and *Gher*-2 vs Bhairab population pairs. Smaller genetic distance between the Rupsha and Bhairab populations could be explained by the connection between these two rivers (Fig. 1). This might be due to random mating and hence, a higher level of gene flows between the two. The low genetic distance and high gene flow between the *Gher*-1 and *Gher*-2 populations may indeed be due to common origin, but also due to random genetic drift having caused fixation of the same common alleles.

5. Conclusions

We found a significant genetic difference between river and *gher* populations of the species. Reduction of genetic variation in *gher* populations of the species compared to river populations reflects improper management practices in the shrimp hatcheries where effective population size is apparently small due to a limited number of matings. The reduction of genetic variation in hatchery populations of *M. rosenbergii* could pose negative consequences, including lowered viability, survival, growth, fecundity, increased percentage of abnormalities, and pronounced depression of productivity. While collecting samples from the sites, farmers expressed concern that growth of hatchery-origin PLs is much lower and that they are more prone to diseases compared to PLs from wild sources. Based on the present study, we conclude that the possible reason for the lowered productivity in the hatchery populations of the species is the loss of their genetic variation. Since PLs from the hatchery are derived from a limited number of gravid females, they are more homogenous than those collected from wild sources. Moreover, hatchery owners often collect gravid females from nearby *ghers* where PLs of that hatchery were stocked. This practices could cause inbreeding, resulting in increased homozygosity. Our study therefore, showed the poor genetic condition of hatchery-origin PLs of *M. rosenbergii*. Based on our preliminary data, breeding strategies in the hatcheries of the species could be improved by formulating and implementing effective breeding strategies for this important crustacean species. Moreover, to conserve a diversified

gene pool for river populations, indiscriminate capture of PLs from rivers should be stopped and effective management strategies should be formulated by management agencies in Bangladesh. In future, large numbers of both wild and hatchery populations and additional primers could be included in the RAPD analysis of the species. Moreover, other molecular tools, such as microsatellite DNA markers, amplified fragment length polymorphisms, or single nucleotide polymorphisms, could be incorporated in the study to reveal the genetic structure of the species more precisely.

Contribution of authors

BB, SS, MM, and MSI designed, performed and analyzed data. MSI supervised research activities of the three authors. BB and MSI wrote the paper. All authors went through and approved final version of the manuscript.

Competing interests

The authors have declared that no competing interests exist.

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