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Morpho-genetic characterization of morphotypes of the giant freshwater prawn, *Macrobrachium rosenbergii*

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Abstract. Different morphotypes of *Macrobrachium rosenbergii* show variations in growth. The objective of the study is to characterize the morphological and genetic variations in *M. rosenbergii* male and female morphotypes. Juveniles of *M. rosenbergii* (average body wt. 5.33 ± 0.72 g) were stocked in an earthen pond at a stocking density of 20,000 per hectare and reared for five months using shrimp quality feed. After harvesting, males were characterized into three morphotypes such as blue- and orange-clawed males (BC and OC, respectively) and small males (SM) based on color and claw length. Here, morphometric characteristics of the BC morphotype demonstrated significantly higher ($p < 0.05$) values followed by OC, SM, and females. Likely, the proportion of CL and BL were found to be highest in the OC morphotype (1.25 ± 0.01) followed by BC (1.16 ± 0.10), SM (0.84 ± 0.10), and female morphotypes (0.64 ± 0.16). For the genetic variation study, four allozyme enzymes were screened that were encoded by six loci (*Ldh-1**, *Mdh-1**, *Mdh-1**, *Pgm**, *Gpi-2**, and *Gpi-2**). Two loci, *Gpi-1** and *Pgm** showed polymorphism ($p < 0.05$) in all morphotypes. The observed (H_o) and expected (H_e) heterozygosities were higher in the BC morphotype followed by OC, female and SM. In the UPGMA dendrogram, the BC morphotype made a one cluster and was differentiated from other morphotypes by the genetic distance $D = 0.0047$. The results suggest that considerable morphogenetic variations existed in BC, OC and SM morphotypes even though they were siblings.

Keyword: *M. rosenbergii,* Allozyme, Genetic variation, Body and claw length ratio

Introduction

The freshwater prawn, *Macrobrachium rosenbergii* is considered a prime candidate species for aquaculture world-wide for its distinctive taste, rapid growth rate, larger size, and greater disease resistance compared to other prawn (Jasmine *et al.* 2011). This species is native to Bangladesh, Brunei Darussalam, Cambodia, China, India, Indonesia, Malaysia, Myanmar, Pakistan, Philippines, Singapore, Sri Lanka, and Thailand (De Grave *et al.* 2013). In Bangladesh, the freshwater prawn has been introduced to aquaculture in the coastal areas and in few other areas depending on naturally collected post larvae because prawn hatcheries are not well established. Numerous rice fields and ponds are used in prawn culture. The farming system of prawn is well-established and increasing day by day (New 2002). Bangladesh is an important exporter of farmed freshwater prawns. Frozen shrimp and prawn (mainly *Penaeus monodon* and *M. rosenbergii*) contributed 92% of the total export value among fish and fishery products of which freshwater prawn contributes less than a quarter of the total export by quantity and value (FRSS, 2017). However, the production of prawn from natural sources is decreasing day by day due to various man-made and natural causes such as chemical water pollution, over harvesting of the post larvae (PL), destruction of prawn habitats, and unavailability foods.

The breeding behavior of *M. rosenbergii* was observed with regard to mating behaviour and reproductive probabilities. The dominant males actively court and protect the females prior to mating. The intermediate male demonstrates less reproductive activities in the presence of the dominant male. The smallest male performs a form of sneak mating consistent with their small size and high mobility. New (1988) reported that males of giant freshwater prawns appeared in three distinct size categories and the average growth of males was higher than female. The differential growth pattern of *M. rosenbergii* exhibited by the male morphotype is termed as "Heterogeneous Individual Growth" (HIG) and is one of the major bottlenecks for increasing profitability of its farming (Ranjeet and Kurup 2001). Size composition of the harvested morphotype is crucial for prawn culture since prawn prices are size-dependent. Sexually mature morphotype of *M. rosenbergii* is composed of three male morphotypes such as small males (SM), orange-clawed (OC) and blue-clawed (BC) males which differ in their size, morphologies, and behaviours (Kuris *et al*. 1987, Barki *et al.* 1991). Several studies on freshwater prawns have been undertaken to reveal size variations in different region of the world based on morphology (Lindenfelser 1984), allozymes (Hedgecock *et al.* 1979), mitochondrial DNA (De Bruyn *et al.* 2004), and microsatellite markers (Khan *et al.* 2014, Banu *et al.* 2015).

Isozymes of the components of different alleles at the same locus are named as allozymes. Allozymes are co-dominant markers in which both alleles are individually expressed in heterozygous individuals (Hallerman 2003). Allozymes were also found to be supportive for generating species-specific profiles and resolving taxonomic ambiguities in several species (Gopalakrishnan *et al.* 1997; Pouyaud *et al.* 2000). Chauhan and Rajiv (2010) reported that allozyme electrophoresis can be an effective tool for fish population studies and fishery management. Khan *et al.* (2014) reported a moderate level of genetic variability in three river stocks of *M. rosenbergii* by microsatellite DNA marker analysis. However, in Bangladesh not much work on molecular characterization of *M. rosenbergii* specially on morphotypes was done. Therefore, the current research was aimed at understanding the morpho-genetic variations of different morphotypes of *M. rosenbergii*.

Materials and Methods

Collection of samples: The PL of *M. rosenbergii* were collected from a hatchery and reared in a pond at the Field Laboratory Complex of the Fisheries Faculty, BAU for minimizing the heterogeneous individual growth (HIG). The average body weight of the prawn at stocking was 5.33 ± 0.72 g and the stocking density was maintained at 20,000 per hectare. After five months' of culture period, the prawns were harvested and BC, OC, and SM males were identified according to the color and size of claws as described by Karplus *et al.* (1986). Claw lengths ([CL] from the basis to the tip of dactylus) and body lengths ([BL] from the eye socket to the tip of the telson) of the prawns were measured and compared the claw to body length ratio of different male morphotypes. A total of 120 specimens, 30 of each morphotype were collected for the study.

***Morphometric analysis*:** Morphometric characters such as total length (TL) in cm, BL in cm, CL in cm, body weight (BW) in g, and ratio between CL and BL of 30 samples from each morphotype were measured following the conventional method described by Hubbs and Lagler (1958). Non-parametric statistical analyses were used in all the comparisons due to limited numbers of prawn in each group. Differences in these characters of prawn were analyzed using

the Kruskal-Wallis non-parametric analysis of variance (ANOVA). In instances significant differences between groups were detected, a non-parametric post hoc test (Zar 1996) was conducted. All of the data were analyzed using SPSS program (Version 21).

***Allozyme electrophoresis*:** Allozyme markers were analyzed by horizontal starch gel electrophoresis following Shaw and Prasad (1970). Allozyme electrophoresis was performed by using amine-citrate buffers (CA 6.1) (Clayton and Terataik 1972). Gel slices (1 mm) were stained histochemically for different enzymes following the protocol described by Aebersold *et al*. (1987).

***Genetic variation data analysis*:** Observed genotypes were used to calculate the allelic frequencies. When the most common allele existed in a frequency of <0.95 at a given locus, it was regarded as polymorphic. The mean proportion of heterozygous loci per individual, mean proportion of polymorphic loci per morphotype, and average number of alleles per locus were calculated so as to show the extent of genetic variability for each morphotype. Expected heterozygosity (H_e) and observed heterozygosity (H_o) were examined according to Nei (1978). Nei's formula was used to calculate the genetic distance (D). Based on the D-values, dendrogram was made by the Unweighted Pair Group Method using Arithmetic Average (UPGMA) method (Nei 1972).

Results

Morphometric analysis: All of the observed morphometric characteristics of the BC morphotype demonstrated significantly higher ($p<0.05$) values followed by OC, SM, and females (Table I). On the other hand, the proportion of CL and BL were found to be highest in the OC morphotype (1.25 ± 0.01) followed by BC (1.16 ± 0.10), SM (0.84 ± 0.10), and female morphotypes (0.64 ± 0.16). In addition, the mean of CL to BL of all of the morphotypes were significantly different ($p<0.05$) from each other. In case of size, BC males had larger size compared to OC, SM, and females (Fig. 1)

Table I. Morphometric characters (mean ±SE) as recorded from M*. rosenbergii* sample of four morphotypes (n=120)

Morphometric characters	Morphotype			
	BC	OC	SM	Female
Total length (TL)	12.50 ± 0.33^a	11.50 ± 0.42^b	8.57 ± 0.47^c	8.05 ± 0.44^c
Body length (BL)	9.60 ± 0.25^a	8.00 ± 0.39^b	7.40 ± 0.26^c	7.00 ± 0.37^c
Claw length (CL)	11.19 ± 0.59^a	10.03 ± 0.39^b	6.22 ± 0.41^c	4.5 ± 0.11^d
Body weight (BW)	23.5 ± 2.14^a	19.4 ± 1.11^b	12.35 ± 2.16^c	11.25 ± 1.85^c
CL : BL	1.16 ± 0.01^b	1.25 ± 0.10^a	0.84 ± 0.10^c	0.64 ± 0.16^d

Values in the same row having different superscripts are significantly different ($p<0.05$).

Fig. 1. Size variation of *M. rosenbergii* male and female morphotypes where blue-clawed males (BC) showed significantly ($p < 0.05$) higher average values followed by OC (orange-clawed males), SM (small males), and females, respectively (BC > OC > SM > Female).

Genetic analysis: The enzymes were controlled and regulated by the genes at six presumptive loci as indicated by the electrophoretic patterns of muscle tissue. Two of the 6 loci, *Gpi-1** and *Pgm**, formed three genotypes (**aa*, **ab*, and **bb*) by two alleles (**a and *b*) (Table II). Four loci such as *Gpi-2**, *Ldh-1*, Mdh-1*,* and *Mdh-2** formed only homozygous genotypes (**aa*) with fixed allele **a*. On the average, 1.5 genotypes were produced by 1.3 alleles at the six loci (Table II). Allele frequencies were measured from observed genotypes at six loci in 120 samples of four morphotypes (Table III). The dimeric enzyme, glucose-6- phosphate isomerase (GPI), was presumably controlled by two loci, *Gpi-1** and *Gpi-2** (Fig. 2). The *Gpi-1** locus was heterozygous with two alleles (**a* and **b*) while allele **b* was dominant in all morphotypes and its frequency ranged from 0.550 to 0.866. In addition, *Gpi-2** was monomorphic with a frequency of **a* = 1.00. (Table III).

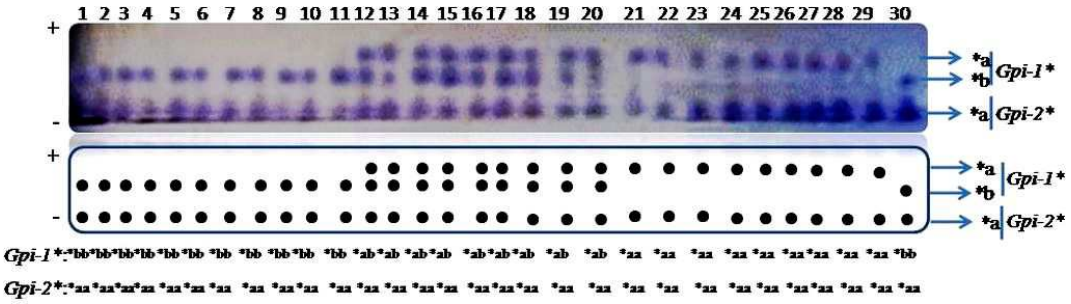

Fig. 2. Electropherogram of glucose-6-phosphate isomerase (GPI) and the schematic representation of electrophoretic patterns of *Gpi-1** and *Gpi-2** loci in the BC morphotype.

Table II. List of the alleles and genotypes examined in *M. rosenbergii* morphotypes

Locus	Allele		Genotype	
	No	Type	No	Type
*Gpi-1**	2	**a*,**b*	3	***aa, * ab, *bb***
*Gpi-2**	1	**a*	1	***aa***
*Pgm**	2	**a*,**b*	2	***ab *bb***
*Ldh-1**	1	**a*	1	**aa*
*Mdh-1**	1	**a*	1	**aa*
*Mdh-2**	1	**a*	1	**aa*
Average	1.3	-	1.5	-

The single *Pgm** locus controls the monomeric enzyme phosphoglucomutase (PGM) (Fig. 3). The *Pgm** was polymorphic in all the four morphotypes with the allelic frequency of **a* ranged from 0.133 to 0.284 and allele **b* with frequencies ranged from 0.716 to 0.867 (Table III). The tetrameric enzyme, lactate dehydrogenase (LDH), was presumably controlled by the *Ldh-1** locus and exhibited a five-banding pattern. The results indicated that the *Ldh-1** locus was monomorphic in all of the four morphotypes (BC, OC, SM, and female), and the allelic frequency of **a* was found to be 1.00 (Table III). The dimeric enzyme, malate dehydrogenase (MDH) was presumably controlled by two different loci, *Mdh-1** and *Mdh-2**, and exhibited three banding patterns consisting of two heterodimers and one homodimer. The results also indicated that both the *Mdh-1** and *Mdh-2** were monomorphic in all of the four morphotypes (Table III). Significant variations in allele frequency of *Gpi-1** locus were observed in only BC morphotype, and in case of the *Pgm** locus, significant variations were observed in all of the morphotypes. The genetic variability at six presumptive loci of *M. rosenbergii* morphotypes is shown in Table IV. The mean fractions of polymorphic loci in all four morphotypes were 40%. The number of alleles per locus (N_a) ranged from 0.092 (SM) to 1.280 (BC) with a mean value of 0.907 for all morphotypes. The average fraction of heterozygous loci per individual was found to be 16.21% for all of the morphotypes. H_o ranged from 0.106 (SM) to 0.186 (BC) with a mean value of 0.131. H_e ranged from 0.094 (SM) to 0.144 (BC) with a mean value of 0.104 for all morphotype.

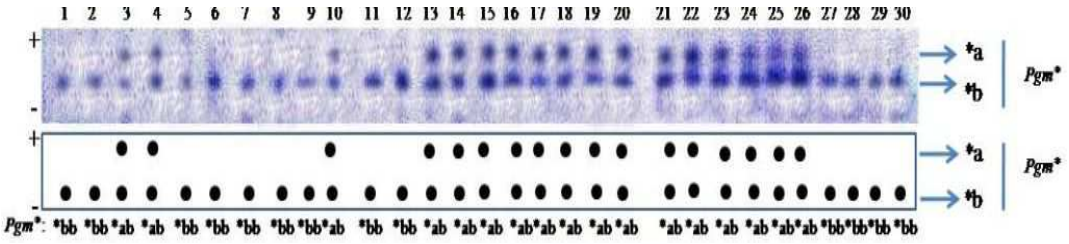

Fig. 3. Electropherogram of phosphoglucomutase (PGM) and the schematic representation of electrophoretic patterns of *Pgm** locus in the BC morphotype.

Table III. Allele Frequency of six presumptive loci of all *M. rosenbergii* morphotypes

Locus	Allele	Morphotype			
		BC	OC	SM	Female
*Gpi-1**	**a*	0.450	0.167	0.133	0.133
	**b*	0.550	0.833	0.867	0.867
	P	0.044*	0.447 NS	0.571 NS	0.732 NS
*Gpi-2**	**a*	1.000	1.000	1.000	1.000
*Pgm**	**a*	0.284	0.150	0.133	0.133
	**b*	0.716	0.850	0.867	0.867
	P	0.048*	0.047*	0.001*	0.001*
Ldh-1,*	**a*	1.000	1.000	1.000	1.000
Mdh-1,*	**a*	1.000	1.000	1.000	1.000
*Mdh-2**	**a*	1.000	1.000	1.000	1.000

P: Probability Value; * Significant Level: $p < 0.05$; NS: Non-Significant.

Table IV. Genetic variability at 5 loci of *M. rosenbergii* morphotypes

Morphotype	Mean proportion of polymorphic loci* (%)	Mean proportion of heterozygous loci per individual (%)	Mean No. of alleles per locus (N_a)	Heterozygosity		
				H_o	H_e	H_o/H_e
BC	40.000	23.330	1.280	0.186	0.114	1.630
OC	40.000	14.990	1.137	0.120	0.103	1.165
SM	40.000	13.000	0.092	0.106	0.094	1.127
Female	40.000	13.500	1.122	0.113	0.098	1.153
Average	40.000	16.205	0.907	0.131	0.104	1.268

The genetic distance (D) values ranged from 0.0001 to 0.0047 among the four *M. rosenbergii* morphotypes. The lowest genetic distance ($D=0.0001$) was detected between the female and the SM morphotypes, while the highest value ($D=0.0047$) was found between the female and the BC morphotypes (Fig. 4). The UPGMA dendrogram constructed based on genetic distance displayed two main clusters among the four different morphotypes (Fig. 4). The BC morphotype formed one cluster while the SM, female, and OC morphotypes formed another cluster. The dendrogram showed that the BC morphotype was separated from the other three morphotypes by a genetic distance of 0.0047. While the OC morphotype was separated from the SM morphotype by a genetic distance of 0.0002, and the SM morphotype was separated from the female by a genetic distance of 0.0001 (Fig. 4). Therefore, based on genetic analysis, OC showed higher genetic variability then BC, SM, and female (OC > BC > SM > Female).

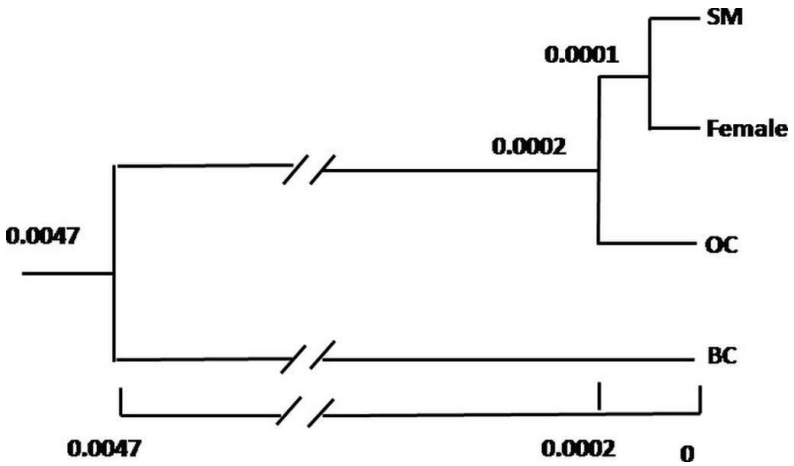

Fig. 4. The UPGMA dendrogram of three male morphotypes including the female morphotype. The dendogram showed three clusters, one for BC morphotype, and another for OC, female, and SM morphotypes.

Discussion

In the present study, the morphometric characteristics and allozyme variations in different groups of *M. rosenbergii* were determined. Each group represented a specific variation in morphometric characters (BL and CL) as demonstrated by ANOVA. Similar morphometric variations of *M. rosenbergii* were reported by Kuris *et al.* (1987), who revealed distinct morphotypes on the basis of measurement of ratios between the CL and BL. Ra'Anan and Cohen (1985) reported that SM of *M. rosenbergii* are small animals with fine translucent or rose-colored claw, BC males are large and have very long blue claws, which was similar to the present study. The findings of the present study also revealed that the morphotypes of *M. rosenbergii* were composed of four distinct groups differing in claw morphologies and sizes, which are consistent to the findings of Banu *et al.* (2015) who characterized BC, OC, and SM morphotypes of *M. rosenbergii* in Malaysia.

In the present study, all the samples were collected at the same time from the same source, that's why the age difference among the prawn was very low. Therefore, the characteristic CL to BL ratio of the OC morphotype compared to other morphotypes might be due to some of species' feature because variations in male morphology have been reported for other crustaceans. This variation might be correlated with enlargement of the androgenic gland in males (Carpenter and de Roos 1970). One likely explanation is that the size of a particular species could be influenced by food, space, different environmental factors, and interaction with different genes, which in turn, reflect the reproductive activity of that species (Mookerjee and Majumder 2006, Aziz *et al.* 2017). The proportions of CL and BL of *M. rosenbergii* as obtained in the present study (1.25–0.64) was higher than in the previous study (0.16–0.10) as reported by Soundarapandian *et al.* (2013). The present findings are close to the study of Cohen and Ra'Anan (1983) who reported that the ratios of CL and BL were 1.6 $\pm$ 0.1, 1.0 $\pm$ 0.05, and 0.5 $\pm$ 0.1, respectively for BC, OC, and SM. The present findings agreed with those of a previous study by Soundarapandian *et al.* (2013) who reported that the morphometric characters of shellfish are considered to be affected by environmental factors such as water temperature and pH in freshwater.

Four enzymes *viz*. lactate dehydrogenase (LDH), malate dehydrogenase (MDH), phosphoglucomutase (PGM), and glucose-6-phosphate isomerase (GPI) produced clear resolution in the muscle tissue of the four morphotypes of *M. rosenbergii*. The fraction of polymorphic loci (*P*) can be commonly used to estimate electrophoretically noticeable variations in a morphotype. Other commonly used measurements of genetic variations are allele frequency in loci, mean number of alleles per locus, average frequency of heterozygous loci per individual (*H*), H_o, and H_e. In the present study, all morphotypes showed two common alleles, **a* and **b*. The average fraction of polymorphic loci per morphotype of the three male morphotypes and the female was the same (40%). This finding indicates that the gene pool diversity among the male morphotypes and female was the same. It may be occurred due to the same source of all the morphotypes. In the present study, the mean number of heterozygous loci per individual (16.205%) indicated that the status of heterozygous loci remarkably remained through all of the male and female morphotypes. The H_o obtained in the present study (1.268) was higher than that 0.5747 reported by Suresh *et al*. (2015). In case of male *M. rosenbergii* in Malaysia, Banu *et al*. (2015) reported an average heterozygosity of 0.5067, which was also lower than the present study. The H_e values (0.094–0.114) obtained in the present study did not exceed the range of values (H_e = 0.062 to 0.118) which are generally considered as the higher margins of genetic variability described by Nevo *et al*. (1984).

The UPGMA dendrogram showed that the three male morphotypes and female can be congregated into two groups. The BC morphotype formed the first group was separated by a $D=0.0047$ from the second group consisted of the OC, female, and SM morphotypes. Then OC morphotype was separated from SM and female by $D=0.0002$. It was found that in a variety of animals, *D* is approximately 1.0 for inter species comparisons, around 0.1 for subspecies, and 0.01 for local races. The *D*-value between subspecies is approximately 0.20 (Ayala 1975). Considering the above-mentioned criteria, the studied *M. rosenbergii* may be categorized as morphotype or local race, which is agreed with the findings of Hurwood *et al*. (2014) who reported three clades of *M. rosenbergii* populations such as Eastern, Central, and Western morphotypes. These morphotypes corresponded geographically with the wild populations of *M. rosenbergii* in India. The present study represents different morphotypes of *M. rosenbergii* which is supported by the findings of Pillai *et al*. (2017) who stated that genetic variations existed in the different morphotypes of giant freshwater prawn, *M. rosenbergii*, in India. This genetic variation might be correlated with several candidate genes such as those associated with visual, olfactory, and inter-male aggressive behaviors that are differentially expressed in eyestalk, testis, and hepatopancreas, respectively, and can potentially provide cues to directly influence the formation of male social dominance hierarchy (Aziz *et al*. 2018). Therefore, formation of the male morphotype is obviously a complex process, and subtle changes in allelic expression during their growth likely impact a cascade of signaling pathways that affect morphotype differentiation.

To best of our knowledge, this is the first research to report morphogenetic variations in *M. rosenbergii* morphotypes in Bangladesh. Although they maintained subtle allelic variations, little is known about the underlying size variations that might be caused by gene-environment interaction. Hence, we concluded that the optimum yield of giant freshwater prawn (GFP) can be achieved by partial harvesting of large sized BC morphotype from culture pond as SM gradually become BC males (SM to OC and then OC to BC).

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