RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) FINGERPRINTING OF SIX INDONESIAN POPULATIONS OF GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii*

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ABSTRACT

Indonesia is rich of giant fresh water prawn (GFP) germ plasms. Best utilization of these resources for the purpose of either aquaculture development or conservation of genetic resources requires some information on the structure and levels of their genetic diversity. This study was aimed to characterize those GFP genetic resources by applying RAPD genetic markers. Six Indonesian populations of GFP from Asahan, Barito, Ciasem, Ogan, GImacro and Papua were collected and analyzed for their genetic variation using five RAPD primers. The results showed the diversity within the populations, as revealed by the level of polymorphism, ranged from 29% to 76% while genetic divergence between populations as shown by genetic distance ranged from 0.04 to 0.50. In terms of genetic divergence, two genetically distinct groups of GFP, namely the Papua GFP in one group and the remaining five GFP populations in the other, were identified. The results also showed the presence of specific population markers that are useful for genetic identification of GFP populations. Implication of these finding with regard to breed development is discussed.

KEYWORDS: randomly amplified polymorphic DNA (RAPD), giant freshwater prawn, molecular markers, *Macrobrachium rosenbergii*

INTRODUCTION

Giant Freshwater prawn, *Macrobrachium rosenbergii,* has been one of the important commodities of aquaculture industry in Indonesia. Development of commercial breeding units and growing out activities to reach marketable size of this species has been initiated since the decade of 70s and continuously expanding in order to cope with the increased demand of the market. The need to increase aquaculture production to meet market demand along with growing fishing pressure and deterioration of GFP natural habitats have attracted related stakeholders to study the species for the purpose of either aquaculture development or conservation of its natural resources.

Previous studies with regard to genetic variation within this species had been carried out an various scales using variety of approaches. At the global scale, in which sampling site covered most of the species natural distribution, study conducted by Mather & De Bruyn Mather (2003) and De Bruyn *et al.* (2004) using16S RNA mitochondrial gene found that there were two forms of GFP separated by Huxley's line. These forms were different by 6.2%, while differences between groups within the respective form were only about 0.9%. These two forms, based on

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allozymic and morphometric studies were classified as subspecies (Lindenfelser, 1984), while based on morphological characters, Wowor & Ng (2007) further classified the two forms as two different species namely *M. rosenbergii* for the eastern form and *M. dauqeti* for the western form. At the local scale, surveys of GFP genetic variation have also been conducted by several authors such as Sodsuk *et al.* (2007) and Charoentawee *et al.* (2006) in Thailand using allozyme and microsatellite markers, respectively and Chand *et al.* (2005) in Queensland using microsatellite markers.

In Indonesia, GFP germ plasms are widely distributed from the western to the eastern part of Indonesia. Several natural populations such as populations of Musi (South Sumatera) Kalipucang, Tanjung air (West Java) (Hadie *et al.*, 2004), Barito (Hadie *et al.*, 2005) and Papua have been identified. However, only a few of these genetic resources have been genetically surveyed. These included GFP genetic resources of Makassar (Sulawesi), Banjarmasin (Kalimantan), Jambi (Sumatera), Sukabumi (Java), and GIMacro, that have been characterized by Nugroho *et al.* (2008) using mitochondrial cytochrome oxidase I (mtCOI) marker. In addition to the abovementioned populations, there are numerous other geographic populations of GFP that remain to be characterized either morphologically or using molecular markers.

Among several molecular genetic markers available, particularly those targeting on DNA level, *Randomly Amplified Polymorphic DNA* (RAPD) markers have been known as one of the simplest method and posses several advantages over other markers (Williams *et al.*, 1990). The primer set is universal and commercially available, no preliminary information is required to perform RAPD analysis, and the number of loci that can be examined is unlimited. However, there are also caveats with this technique, particularly with regard to reproducibility issue (reviewed by Ali *et al.*, 2004). The technique has been successfully used to characterize the genetics of crustacean such as *Macrobrachium borelli* (D'Amato & Corach, 1996) and *Penaeus monodon* (Garcia & Benzie, 1995).

This study was aimed to elaborate the structures and levels of genetic variation both within and between populations of several Indonesian populations of GFP by applying RAPD genetic markers. The result obtained from this study is expected to serve as the baseline of genetic data that can be used either for conservation measures or aquaculture development.

MATERIALS AND METHODS

Fifty five individuals representing populations of Barito (Kalimantan), Ciasem (West Java), Asahan (North Sumatera), Ogan (South Sumatera), Papua and Genetically improved *macrobrachium* (GImacro) were sampled. The first five were newly collected GFP populations which were obtained in 2006 for Barito and in 2007 for Asahan, Ciasem, Ogan, and Papua. Due to this recency, they might resemble to their natural population counterparts. In contrast to the first five populations, the GImacro was a domesticated GFP strain maintained by the Research Institute for Freshwater Fish Breeding and Aquaculture (RIFFBA), Sukamandi for more than seven generations starting from its launching in 2001 (Imron *et al.*, 2008). The processes of domestication and breed development through selective breeding program had been started some five years earlier. A map illustrating the sampling locations is shown in Figure 1.

DNA Extraction

Genomic DNA (gDNA) was extracted from individual samples using Sigma DNA extraction kit. Brief description of the procedures is as follow. Some 25 mg of GFP muscle tissue were minced and placed in 1.5 mL tube. One hundred and eighty uL of lysis solution and 20 uL of protein K solution were added and the mixture was incubated at 55°C for 2-4 hours until the tissue was completely digested. Following this step, the mixture was added with 200 µL of 95%-100% ethanol, vortexed for 5-10 seconds to make it homogenous and put in a pre-treated binding column, which was prepared by adding 500 µL of column preparation solution and spun at 12.000 g for 1 minute. Following spinning for 1 minute at 6.500 g, the binding column was then transferred to a collection tube for twice consecutive washing, which was carried out by adding 500 µL of washing solution followed by spinning at 6.500 g for 1 minute. The procedure was terminated by elution step which was performed by adding some 200 µL of elution solution followed by 5 minute incubation at room temperature and terminated with spinning at 6.500 g for 1 minute.

Figure 1. Indonesian map showing the locations of sample collection of GFP, *M. rosenbergii*, populations except GIMacro; (1) Barito, (2) Ciasem, (3) Asahan, (4) Ogan, and (5) Papua

PCR Amplification

RAPD technique is notoriously known for its reproducibility issue (Ali *et al*., 2004). Hence, optimization procedures were carried out prior to the actual RAPD PCR. The optimization procedures were particularly focused to obtain the best quality genomic DNA template for PCR. These included the determination of concentration, purity, and the ratio of high and low sized of genomic DNA. The best result obtained from the optimization procedures were applied to this study. A complete report on the results of these optimization procedures will be reported elsewhere.

PCR reaction was conducted in 15 µL volume containing 6 µL of 2x mastermix *microsat-type it* (*Qiagen*), 1 µL of decamer primer, around 50-ng of DNA template, and 7 uL H_2O. The primers used are listed in Table 1. Amplification of the genomic DNA fragments was carried out using Mycycler thermal cycler (Biorad) for 35 cycles. The thermal profiles were 3 minutes at 94°C for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 37°C for 1 minute and extension at 72°C for 2 minutes. The whole cycles were terminated at 72°C for 10 minutes. The PCR product was run on horizontal agarose electrophoresis system (1.5% agarose in 0.5x Tris Boric EDTA (TBE) buffer), that was set at 10 volt and run for 6 hours. The product was then viewed using ultraviolet (UV transilluminator) following staining with ethidium bromide.

Data Analyses

RAPD bands resulting from the electrophoresis were scored as one (1) or zero (0) for the presence or absence of the bands, respectively. Sizes of RAPD bands were not precisely determined using individual sequences, instead they were arbitrarily determined by comparing with a known DNA size marker. Likewise, the differences in band intensity were not taken into account, namely the bands were scored the same regardless the intensity as long as they show the same size. The scores were then used to calculate several population genetic diversity parameters, particularly the level of polymorphism and genetic distance. The genetic distance was estimated using distance matrix and displayed in a dendrogram. Further, to asses whether there was correlation between genetic and geographic distances, a Mantel test was performed. For this purpose, only five populations excluding GImacro, were analyzed. The GImacro was excluded as it is not considered as geographic population, as previously described. Geographic distances among locations were determined as a straight and shortest distance on the map using Microsoft Encarta software. The analyses were carried out using Tools for Population Genetic Analysis program (TFPGA) (Miller, 1997).

RESULTS AND DISCUSSION

The illustration of RAPD finger printing resulting from the analysis are presented in

Tabel 1. RAPD primer used to amplify genomic DNA fragments on six Indonesian populations of *M. rosenbergii*

Primer	Sequence (5' – 3')	Nucleotide length	G+C (%)
OPA-01	CAG GCC CTT C	10-mer	70
OPA-02	TGC CGA GCT G	10-mer	70
OPA-03	AGT CAG CCA C	10-mer	60
OPA-05	AAT CGG GCT G	10-mer	60
OPA-09	AGG GGT CTT G	10-mer	60

Figure 2 and 3, while the summary of polymorphism levels within the six populations of Indonesian GFP and genetic distances between the combinations of all 6 populations are presented in Table 2. Dendrogram showing relatedness among populations and constructed using these genetic distances is shown in Figure 4. The correlation between genetic and geographic distances is presented in Figure 5.

General RAPD Performance

Five decamer primers employed within this study produced 55 DNA fragments, or in average 11 fragments per primer, that vary in size from 400 to 3500 bp. Some fragments were specific population, meaning that they existed in particular population but were absent in other populations. DNA fragments sized 250-500 amplified by primer OPA2 in GImacro population for instance, were population-specific fragments, since they appeared only within this population, and did not exist in the other populations. Discovery of these specific markers is highly desirable in most genetic surveys, particularly those aiming at finding genetic markers for population identification. However, more primers are required to run if more specific markers are needed.

Levels of Polymorphism

Genetic polymorphism, along with other population genetic parameter such as gene/ allele diversity, allele richness and heterozygosity may indicate the level of genetic stability of populations. Those with a higher level of polymorphism will have a better ability to adapt to changing environment (Frankham *et al*., 2002). Information on population polymorphism can be used to evaluate populations relatedness, domestication history, and estimation of

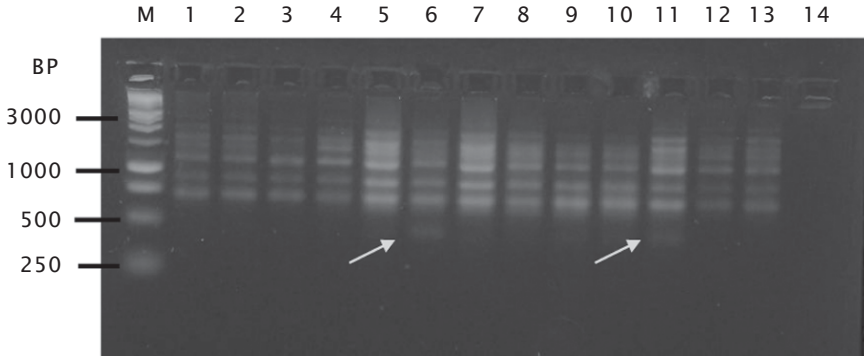

Figure 2. Patterns of RAPD fragment of *M. rosenbergii* of Ogan and Barito populations amplified using primer OPA2. M represents DNA size marker, (GeneRuler 1kb DNA ladder), lane 1-4 and lane 5-15 indicate individual samples representing Ogan and Barito populations. Arrow heads indicate polymorphic status of the bands

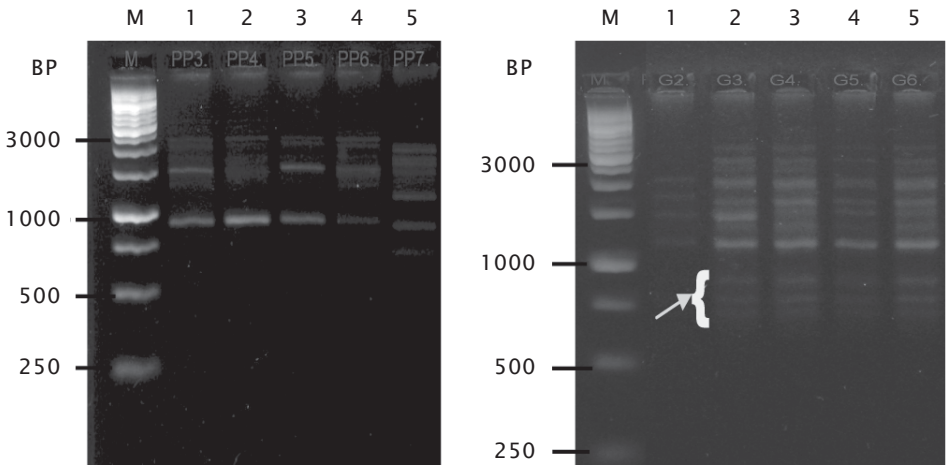

Figure 3. Patterns of RAPD fragments of *M. rosenbergii* of Papua (left picture) and GImacro (right picture) populations amplified using primer OPA2. M represents DNA size marker, (GeneRuler 1kb DNA ladder). Lane 1-5 indicate individual samples representing Ogan and Barito populations. Arrow head indicate both polymorphic status and specificity of the bands

population inbreeding level (Bowditch *et al.*, 1993).

The highest level of polymorphism occurred in Papua (76%) followed by Ciasem (45%), Asahan (41%), GIMacro (39%), Ogan (33%), dan Barito (29%). Compared to the level of polymorphism obtained by other studies, these figures were comparable with or even slightly higher than that found in other populations. Polymorphism level in palaemonidae, *Macrobrachium borelli* for instance, ranged from 33,33% to 50,00%, (D'Amato & Corach, 1996), while Garcia & Banzie (1995) found that the level of polymorphisms in penaeidae, assessed with RAPD and allozyme markers ranged from 39% to 77%. The high levels of polymorphism in most investigated GFP populations suggest that the populations were relatively genetically stable. For natural populations, these polymorphism levels suggest that they have good evolutionary potentials to adapt to ever-changing environment as a response to natural selection (Frankham *et al.*, 2002). For cultivated populations or under domestication process, these may suggest that they have potential to respond positively to artificial selection, such as selective breeding. Therefore, it is highly desirable to see how good these populations would respond to artificial selection currently undertaken at the RIFFBA.

Genetic Distance

Genetic distance between populations as presented in Table 2 and Figure 3 suggests at least two interesting phenomena, namely relatively low levels of genetic differences between western populations, excluding GImacro, and relatively high levels of genetic differences between Papua and the remaining populations. Genetic distances between the members of western Indonesia populations, excluding GImacro, range from 0.04 between Ogan and Barito and 0.19 between Asahan and Barito. Other pairwise distances are within the range of these two. Genetic distances between GImacro and other western Indonesia populations ranged from 0.25 (GImacro against Ogan) to 0.34 (GImacro against Barito).

A relatively close genetic distances between the populations of Asahan, Ogan, Barito, and Ciasem may be explained by geological history of the regions, in which these western Indonesia islands (Java, Sumatera, and Kalimantan) were all connected during Pleistocene era (10.000-20.000) allowing gene flow to occur (Kottelat *et al.*, 1993). This situation is different from that of Papua and the remaining populations. It is widely recognized that there is geographical barrier dividing western and eastern of Indonesia, which is called Wallace line. This

Tabel 2. Levels of polymorphism and Nei's Genetic distances of six Indonesian populations of GFP, *M. rosenbergii*

Population	Polymorphism (%)	Genetic distance					
		Papua	GIMacro	Barito	Ciasem	Asahan	Ogan
Papua	76.47	*****					
GIMacro	39.21	0,49	*****				
Barito	29.41	0,41	0,34	*****			
Ciasem	45.00	0,37	0,31	0,09	*****		
Asahan	41.10	0,37	0,37	0,18	0,15	******	
Ogan	33.18	0,38	0,24	0,04	0,08	0,16	*****

hypothetic line is hypothesized to restrict the continuous distribution of flora and fauna from the western to the eastern part of Indonesia and vice versa. Due to this barrier, there was almost no natural gene flow between the western and eastern Indonesia populations causing both populations to evolve independently. Additionally, differences in ecological conditions may also promote local adaptation of the populations leading to accumulated genetic differences. It is not surprising when most genetic survey sampling flora and fauna covering both regions found a high level of genetic differences, which is also the case with GFP populations (Mather & de Bruyn, 2003; Nugroho *et al*., 2008).

Patterns of genetic distance observed within this study seemed to associate with geographic distance. As can be seen in Figure 4, the correlation between these two parameters is very tight as indicated by the high values of coefficient of correlation (r=0.9) and coefficient of determination (R=0.81). Overall, the patterns of genetic distance between populations seemed to follow isolation by distance (IBD) pattern, namely genetic distance increases with geographic distance (Bielawski & Pumo, 1997).

The closeness in genetic distance between the GImacro and Ogan is similar to other studied species when the similar samples were analyzed morphologically using a set of morphometric characters (Imron *et al*., 2008). The closest genetic distance between the two populations, apparently, could be explained by their ancestral sharing. The GImacro is a domesticated population synthesized under selective breeding program from three GFP germ plasms namely Kalipucang, Citarum (West Java) and Musi (South Sumatera). The Ogan population that was analyzed genetically in the current study was obtained from Ogan River which is part of Musi river system. Therefore, it is interesting to note that despite a long history of domestication, GImacro population still shows some level of similarity to its ancestral root. This finding suggests that genetic traces can last for many generations. However, whether these long lasting genetic traces occur due to the dominant contribution of Musi germ plasm over the others could not be verified since samples representing the Kalipucang and Citarum populations were not available by the time the genetic analysis was carried out.

High genetic distance observed between several pairs of populations, particularly between Papua and GImacro, provides good opportunity to exploit it for genetic improvement purposes through the mean of hybridization or cross breeding. Crossing between genetically distant groups is expected to produce progeny possessing heterotic effect, namely a performance better than that of its pure lines. Although no guarantee that hybridization will produce heterosis, several studies (Dong & Zhou, 1998; Melchinger *et al*., 1990a; Melchinger *et al*., 1990b; Visscher *et al*., 2000) suggested that genetic distance measures could be indicative of success; the greater the genetic distance the more the chance to obtain heterosis. Viewed within this context, the chance to obtain heterosis from hybridization between the Papua and GImacro populations is quite prospective.

CONCLUSION

RAPD fingerprinting was able to show genetic diversity both within and between Indo-

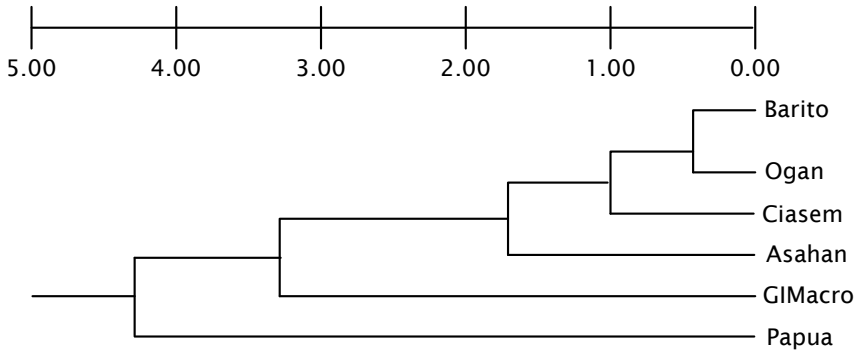

Figure 4. Dendrogram of six populations of Indonesian GFP, *M. rosenbergii* constructed using Nei's Genetic distance

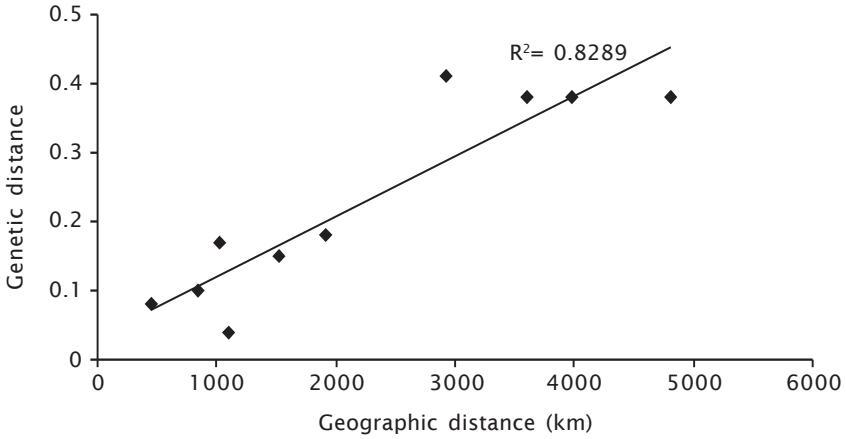

Figure 5. Graph produced from Mantel test showing correlation between geographic and genetic distances

nesian GFP populations. GFP population's genetic diversity as revealed by the level of polymorphism and genetic distance was quite high. Exploitation of these genetic variations through the means of either selective breeding or cross breeding is allowable, particularly using Papua population that showed high level of polymorphism and was genetically most distant from the other populations.

ACKNOWLEDGEMENTS

Authors would like to thank all team members of the genetic laboratory group at the RIFFBA for sharing time, materials and knowledge during the analysis. This research was funded by research fund of Agency for Research of Marine and Fisheries, fiscal year 2009.

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