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Single Locus Microsatellite Development for the Malaysian Giant Freshwater Prawns, *Macrobrachium rosenbergii*

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ABSTRAK

Udang genus M. rosenbergii, *Bate 1868 (Palaemonidae) adalah kumpulan pelbagai jenis krustasea decopod yang didapati di kawasan tropika, laut, estuari dan air tawar. Pada masa ini, stok liar telah digunakan untuk ternakan pengeluaran benih di kebanyakan tempat di Malaysia. Walau bagaimanapun, pengambilan stok liar secara berterusan akan mengakibatkan kepupusan dan kehilangan kepelbagaian genetik. Oleh itu adalah penting bagi seseorang mempunyai kepekaan terhadap maklumat genetik untuk tujuan pemuliharaan dan pembiakan. Kajian ini menggunakan teknik RAMS untuk menghasilkan penanda lokus mikrosatelit tunggal. Sebanyak 13 kawasan mikrosatelit telah dikesan. Hasil kajian menujukkan kejayaan menggunakan teknik ini untuk memperoleh mikrosatelit dalam* M. rosenbergii. *Kajian ini akan diteruskan untuk mencari klon yang mengandungi mikrosatelit dan mereka bentuk primer bagi setiap kawasan mikrosatelit. Jujukannya adalah sama dengan dua spesies lain iaitu ikan zebra dan* Perna viridis. *Kewujudan persamaan pada kedua-dua spesies (ikan Zebra dan* Perna viridis*) akan digunakan sebagai 'model' bagi* M. rosenbergii *untuk masa hadapan.*

ABSTRACT

Prawns of the genus Macrobrachium Bate, 1868 (Crustacea: Palaemonidae) are a highly diverse group of decopod crustaceans found in circumtropical marine-, estuarine- and freshwaters. At present, wild stocks have been used to cultivate commercial cultures in many areas in Malaysia. Such repeated harvesting from wild stocks, however, will eventually lead to extinction and loss of genetic diversity. Therefore, it is important to know the genetic make up of these wild stocks in order to manage the populations for conservation and breeding purposes. In the present study, a novel method known as Random Amplified Microsatellites (RAMS) was used to develop single locus microsatellite markers. A total of 13 microsatellite regions were detected. The findings of this study showed a successful detection of the microsatellite sequences in M. rosenbergii *using RAMS. This study will be continued to identify more clones that contain microsatellite sequences and to design the forward and reverse primers for each microsatelite region.* M. rosenbergii *sequences were homologous with Danio rerio (Zebra fish) and* Perna viridis *(green lipped mussel).*

INTRODUCTION

Macrobrachium rosenbergii, the giant freshwater prawn (Family: Palaemonoidea, subfamily: Palaemoninae) is a component of the biodiversity in Malaysia's diverse water ecosystems. It inhabits tropical and subtropical zones of the world, in lakes, rivers, swamps, irrigation ditches, canals and ponds, as well as in estuarine areas. Most species require brackish water in the initial stages of their life cycle, and a minority complete their life cycle in inland saline and freshwater lakes. Their numbers in these natural habitats has been declining due to overexploitation and habitat

alterations. At present, wild stocks have been used to cultivate commercial cultures in many areas in Malaysia. Repeated harvesting from the wild stocks will lead to extinction and eventually loss of genetic diversity. To save this important bio-resource, effective conservation and propagation-assisted rehabilitation strategies are necessary. However, this may not be feasible unless data is available for *M. rosenbergii* on stock structure and genetic variation throughout its distribution range. The identification of polymorphic markers with consistent scorable alleles is a crucial step to generate population genetics data. A study conducted by Yuzine *et al.* (1996 using isozymes as genetic markers) revealed that *M. rosenbergii* from Rompin River, Linggi River and the Hatchery Unit at Universiti Putra Malaysia have low genetic variability when compared to *P. merguiensis*. The low genetic variability showed that these populations constitute species that need to be further investigated by using DNA markers. A recent study using the mitochondrial 16S ribosomal RNA gene placed Malaysian *M. rosenbergii* into the western 'form' along with those from Thailand, Java and Vietnam. (Mather and de Bruyn 2003). This information has implications for both the aquaculture industry and the conservation of wild stocks.

Population genetic markers including allozymes, mitochondrial DNA and microsatellites can be the markers of choice for addressing issues such as genetic variability, inbreeding, parentage assignments, species and strain identification and for the construction of high resolution genetic linkage maps for aquaculture species. Thus for this study, microsatellites was the marker of choice. The great popularity of microsatellite markers is mainly due to their presence in all prokaryotic and eukaryotic genomes, and their codominant, highly polymorphic and easy to score characteristics. These characteristics make them more informative than dominant markers such as RAPD, AFLP, and others. Microsatellites were used to estimate relative levels of genetic divergence within and among populations. Microsatellites can also identify genetically distinct populations, which contribute to conservation efforts. To date, only six microsatellite loci have been developed for the eastern form of *M. rosenbergii* and they failed to show any polymorphism in the western form of *M. rosenbergii*. These six loci exhibited null alleles in the western form. (Mather, pers. comm.). Thus it will be a breakthrough in the molecular genetics of *M. rosenbergii*, if steps are taken to develop microsatellite markers by isolating microsatellite regions and developing single locus microsatellite markers for this species. At present, a well developed protocol for single locus microsatellite identification has been developed for *Perna viridis* (Teh *et al.* 2002). Single locus microsatellites are highly repeated DNA sequences that have been markers of choice in resolving genetic variability in low variability populations, genome mapping, parentage, kinship assignments and stock structures. In this study, we have attempted to develop microsatellite loci for *M. rosenbergii*.

MATERIALS AND METHODS

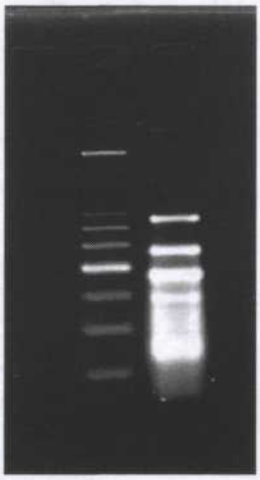
Prawns were collected from the wild stocks from Muda River (Kedah), Perak River (Perak) and Rompin River (Pahang). The samples were kept on ice for transportation and stored at -80°C for the study. Identification of the prawns collected was based on the taxonomic identification keys provided by New (2002). DNA was extracted from the muscle tissues by the protocol of Winnepenninckx *et al.* (1993). The microsatellite isolation technique followed the procedure of Teh *et al.* (2003). This involved the creation of an enriched genomic library based on the 5' anchored PCR technique. Degenerate primers were used for PCR and the DNA bands generated from each of the primers were ligated into the TOPO TA cloning vector (Invitrogen, USA). Vectors with inserts were then transformed into competent'*E. coli* cells. The transformed cells were then plated onto LB agar, and subsequently selection for white colonies were made after incubation at 37°C. Plasmid DNAs were extracted from positive clones, using the protocol of Sambrook *et al.* (1989) before

sequencing on an ABI PRISM 377 DNA sequencer.

Analysis of nucleotide sequences was carried out with BLAST-N program. This program was used to identify similarities of nucleotide sequences obtained with those of published sequences of other closely related species and to determine the significance of these sequences.

RESULTS AND DISCUSSION

A total of 16 degenerate RAMS primers were screened to check for amplification. All showed amplification. However, PCR optimization was done for some of these primers. A single degenerate primer containing $(CAG)_5$ microsatellite repeat motifs was used to detect the presence of microsatellite sequences. The primer sequence of Bp 11 (5' to 3') is $(K)_3(YH)_2Y(CAG)_5$, K= G/T, V= G/C/A, Y=T, H=A,C or T. The size of the amplified bands ranged from 200bp to 1000bp (Figure 1).



M 1

Figure 1: Banding pattern observed in one of the individuals in M. rosenbergii *using RAMS primer, Bp11. Lane M: 100 bp marker, Lane 1: Bp11 primer bands*

The whole PCR product of primer Bp-11 was cloned and almost 200 clones with an average insert size of 200 bp were isolated. A total of 12 positive clones were sequenced. Eight of the clones contained perfect CAG repeats and cryptic simple (GG)n(GGG)n repeats while the rest of the clones had no inserts (Table 1). These results suggest that the (CAG)n repeats are abundant in the genome of *M. rosenbergii.*

Nineteen cryptic simple regions were detected in the eight clones. Cryptic simple regions were found preferentially in the non-coding regions of the genomes but they are also found in the coding regions (Goldstein 1999). According to Tautz *et al.* (1986), microsatellites probably arose from cryptic simple regions. On the other hand, microsatellites may have undergone mutations to become regions of cryptic simplicity.

Random Amplified Microsatellites (RAMS) is proven to be an efficient starting point for developing single locus microsatellite markers in *M. rosenbergii.* This technique has been used to characterize species such as *Gremmeniella abietina* (Hantula *et al.* 1996); *Pinus radiata* (Fisher *et al.* 1998); *Vigna radiata* (Kumar *et al.* 2002) and blue mussel, *Mytilus edulis* (Presa *et al.* 2002). RAMS markers have provided useful information about population structures of these species. The findings of our study showed the success of using this method as a starting point for detecting microsatellite sequences in *M. rosenbergii.* The microsatellite sequences that we have obtained will be used to design primers based on the unique sequences flanking each microsatellite region. These primer pairs will then be tested for their abilities to amplify single locus microsatellite markers in *M. rosenbergii.* The development of polymorphic microsatellite loci will be useful in rebuilding wild stock populations, contributing significantly to environmental conservation and it will serve to provide valuable information for broodstock management of *M. rosenbergii.*

Blast analysis was performed to identify homology sequences between *M. rosenbergii* with its closely related species. The list of sequences between *M. rosenbergii* and other species are shown in Table 2. The analysis showed that the majority of the homologous

TABLE 1
Microsatellite loci isolated from *Macrobrachium rosenbergii* using Bp-11 RAMS clone and their GenBank accession numbers

Clone	Type of repeat units			
	Perfect	Imperfect	Compound	GenBank Accession
SUGbp11-1	$(CAG)_5$, $(TC)_4$, $(GCT)_5$, $(TAG)_3$	$(GG)_4(GGG)_2$	–	AY841999
SUGbp11-2	$(CAG)_5$	$(AG)_5$,$(GG)_5(GGG)_2$	–	AY842000
SUGbp11-3	$(CAG)_6$,$(TGC)_4$	$(GG)_5(GGG)_2(GG)_2$	–	AY842001
SUGbp11-4	$(CAG)_5$	$(CA)_2(CA)_3$	–	AY842002
SUGbp11-5	$(CAG)_5$, $(TGC)_4$	$(GG)_5(GGG)_2$	–	AY842003
SUGbp11-6	$(CAG)_7$, $(TGC)_4$	$(GG)_5$ $(GGG)_2(GG)_2$	–	AY842004
SUGbp11-7	$(CAG)_5$	$(GG)_5(GGG)_2$	–	AY842005
SUGbp11-34	$(CAG)_6$	$(GG)_5(GGG)_2$	–	AY842006

TABLE 2
Nucelotide sequence comparisons of the five clones of *M. rosenbergii* with other species

Organism		Sequences		No. of nucleotides	Homology (%)
Clone SUGbp11-1	8	cagcagcagcagcaggaagagcaggagcagtagtagtag	46		
Cepaea nemoralis microsatellite Cne1 sequence (AF139019)	343	cagcagcagcagcagcagcagcagcagtagtagtag	30	39	89
Clone SUBbp11-1	9	agcagcagcagcaggaagagcaggagcagtagtagtag	46		
Fonscochlea accepta clone Sn49 microsatellite sequence. (AY805231)	130	agcagcagcagcagcagcagcagcagtagtagtag	167	39	87
SUGbp11-2 Zebrafish DNA sequence from clone CH211-11P18 in linkage group 22,complete sequence. (AL845526)	2	tttcagcagcagcagcagaa	21	20	100
	16074	tttcagcagcagcagcagaa	160934		
SUGbp11-2 Zebrafish DNA sequence from clone DKEY-4P13 in linkage group 2, (BX255907)	4	tcagcagcagcagcagaagnagtagct	30	27	97
	183686	tcagcagcagcagcagaagcagcagct	183712		

Table 2 Cont'd

Organism		Sequences	No. of nucleotides	Homology (%)
SUGbp11-2 Danio rerio zgc:56721, mRNA (cDNA clone MGC:56721 IMAGE: 2601288), complete cds. (BC044205	2 1694	tttcagcagcagcagcagaa 21 ||||||||||||||||||| tttcagcagcagcagcagaa 16752	20	100
SUGbp11-2 Danio rerio zgc:56721 (zgc:56721), mRNA. (NM_213521)	2 1694	tttcagcagcagcagcagaa 21 ||||||||||||||||||| tttcagcagcagcagcagaa 167	20	100
SUGbp11-2 Argopecten irradians clone ScaE_6721 microsatellite sequence. (AY485259)	2 398	tttcagcagcagcagcagaa 21 |||||||||||||||||| tttcagcagcagcagcagaa 417	20	100
SUGbp11-2 Argopecten irradians clone ScaE_1153 microsatellite sequence. (AY496639)	2 361	tttcagcagcagcagcagaa 21 ||||||||||||||||||| tttcagcagcagcagcagaa 380	20	100
SUGbp11-2 Zebrafish DNA sequence from clone BUSM1-101L20 in linkage group 2AL591481)	5 70639	cagcagcagcagcagaagnagtagct 30 |||||||||||||| || ||||||| cagcagcagcagcagcagcagtagct 70664	26	92
SUGbp11-5 Perna viridis microsatellite PV1 sequence.(AY190018)	143 24	gcctgctgctgctgctgatata 164 |||||||||||||||||||| cctgctgctgctgctgatata 3	22	100
SUGbp11-5 Zebrafish DNA sequence from clone DKEY-4P13 in linkage group 2, complete sequence.(BX255907)	8 183686	tcagcagcagcagcagaagcagtagct 34 |||||||||||||||||||| |||| tcagcagcagcagcagaagcagcagct 183712	27	96
SUGbp11-5 Zebrafish DNA sequence from clone BUSM1-101L20 in linkage group 2 (AL591481)	9 70639	cagcagcagcagcagaagcagtagct 34 ||||||||||||||| |||||||||| cagcagcagcagcagcagcagtagct 70664	26	96

Table 2 Cont'd

Organism		Sequences	No. of nucleotides	Homology (%)
SUGbp11-6 Zebrafish DNA sequence from clone CH211-245M19 in linkage group 5, complete sequence. (CR385090)	144 124120	gtttcgcctgctgctgctgctgaaataa 171 |||| |||||||||||||||||||| gtttggcctgctgctgctgctgaaataa 124147		
SUGbp11-6 Zebrafish DNA sequence from clone CH211-137C4 in linkage group 8, complete sequence. (BX248231)	151 50048	ctgctgctgctgctgaaataacca 174 |||||||||||||||| ||||| ctgctgctgctgctgaaacaacca 50071	24	96
SUGbp11-6 Zebrafish DNA sequence from clone DKEY-32D10 in linkage group 5, complete sequence.(BX469907)	149 128335	gcctgctgctgctgctgaa 167 |||||||||||||||||| gcctgctgctgctgctgaa 128353	19	100
SUGbp11-6 Perna viridis microsatellite PV3 sequence.(AY190020)	151 24	ctgctgctgctgctgaaataac 172 |||||||||||||||||||||| ctgctgctgctgctgaaataac 3	22	100
SUGbp11-6 Perna viridis microsatellite PV1 sequence. ACCESSION AY190018	149 24	gcctgctgctgctgctgaaataac 172 |||||||||||||||||| ||||| gcctgctgctgctgctgatataac 1	24	96
SUGbp11-34 Blast analysis is the same with SUGbp11-6 to the similar organism and same homologous sequences				

sequences were identified with *Danio rerio* or commonly known as zebra fish and *Perna viridis*, or green lipped muscle.

The presence of homologous sequence with zebra fish is a a very important focal point in understanding *M. rosenbergii* genome. Zebra fish is an important model system for cell fate decisions and pattern formation and for assembling molecular components of responsible pathways (Fishman 1999). By identifying more sequences from *M. rosenbergii* and with the understanding on the possible relationship that might exist between zebra fish and *M. rosenbergii*, the role of *M. rosenbergii* genes from zebra fish mutations and identification of zebra fish models for candidate genes could be established. The synthetic relationship between zebra fish and human genome is well documented by Barbazuk *et al.* (2000), and shows that similar

work can be carried out with *M. rosenbergii*. However, there is a possibility that the expected relationship might not exist between these two species as Zebra fish is a vertebrate and *M. rosenbergii* is an invertebrate. However, Ohno (1970) proposed that without duplicated genes the creation of metazoans, vertebrates and mammals from unicellular organisms would have been impossible. Ohno (1970) was not the first to suggest that genome wide redundancy could lead to new evolutionary opportunities.

Intragenome similarities through phylogeny and synteny data suggest that the common ancestor of zebra fish and puffer fish, a fish that gave rise to 22,000 species experienced a large scale gene duplication event and puffer fish has lost many duplicates that the zebra fish has retained (Taylor *et al.* 2003). Similar possible relationship could exist between Zebra fish and *M. rosenbergii* because ancestor of these two species (zebra fish and puffer fish) experienced a genome duplication event lends support to the idea that genome duplication and speciation might be casually linked (Amores *et al.* 1998). Sequence level studies on *M. rosenbergii* will help to answer possible evolutionary lineages in the development of an organism.

The homologous sequences that existed between two families, *Palaemonidae* and *Mytiloidea*, was very interesting due to the fact that similar studies on marine turtles indicated conservation of flanking sequences spanning approximately 300 million years of divergent evolution (FitzSimmons *et al.* 1995). Few primers that were obtained from Teh *et al.* (unpublished) showed positive amplification in *M. rosenbergii*. Some of the primers showed polymorphic banding patterns (Bhassu *et al.* unpublished data).

CONCLUSION

This study will be continued to test the hypothesis that microsatellite loci have great potential for broader applications such as comparative gene mapping and assessing genetic population structures within species. The use of microsatellite loci across species depends on the conservation of priming sites within flanking sequences, which enables amplification and maintenance of repeat arrays long enough to promote polymorphism (Weber 1990). In this study, the use of RAMS primer Bp11 was designed to amplify (CAG)n repeats which are trinucleotide regions that are more conserved than the dinucleotide regions because they are found in the coding region (Young *et al.* 2000) This could explain the possible existence of conservation of these sequences between species such as Zebra fish and *Perna viridis* and possibly other species.

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