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# Isolation of DNA Microsatellite Markers in the Green-lipped Mussel, *Perna viridis*

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## ABSTRAK

*Sejumlah 21 mikrosatelit DNA telah dipencilkan daripada* Perna viridis *dengan menggunakan teknik rantaian tindak balas '5' anchored polymerase '. Primer-primer telah direka untuk tujuh lokus mikrosatelit dan amplifikasi PCR bagi ketujuh-tujuh lokus mikrosatelit ini menunjukkan bahawa lima adalah polimorfik dengan jumlah alel per lokus menjulat daripada 2 hingga 4. Ini merupakan penanda set mikrosatelit pertama yang telah dibangunkan untuk spesies ini. Penanda-penanda ini amat berguna sebagai alat untuk kajian terperinci latar belakang genetik* P. viridis *di negara kita memandangkan spesies ini dikultur sebagai sumber protein murah bagi manusia dan telah dikenal pasti sebagai agen biomonitor yang berpotensi untuk pencemaran logam berat di Pantai Barat Semenanjung Malaysia.*

## ABSTRACT

*A total of 21 DNA microsatellites were isolated from* Perna viridis *by using a 5' anchored polymerase chain reaction technique. Primers were designed for seven microsatellite loci and the PCR amplifications of these seven microsatellite loci showed that five were polymorphic with the number of alleles per locus ranging from 2 to 4. These are the first set of microsatellite markers developed for this species. These markers are useful as tools for more detailed studies of the genetic backgrounds of the green-lipped mussel,* P. viridis, *in our country as this species is being cultured as a cheap source of protein for human consumption and has been identified as a potential biomonitoring agent for heavy metal pollution in the west coast of Peninsular Malaysia.*

## INTRODUCTION

The green-lipped mussel, *Perna viridis*, is widely distributed in the Indo-Pacific region (Siddall 1980). This mussel is important ecologically because of its widespread distribution and biological filtration activity, and also economically because of its value as a cheap source of animal protein for human consumption. It was once regarded as a nuisance by oyster farmers in the Philippines and today it is being extensively cultured in many Asian countries including Malaysia (Rosell 1991; Monirith *et al.* 2003). Recently, this species has been used as a biomonitor for a wide range of contaminants such as metals, organochlorines, polycyclic aromatic hydrocarbons and organotins throughout the Indo-Pacific region and has considerable potential as a pollution monitoring agent throughout its geographical range (Nicholson and Lam 2005).

In Malaysia, *P. viridis* is widely distributed along the west coast of Peninsular Malaysia and to a lesser extent, in certain parts of Sabah and the east coast of Peninsular Malaysia. It is a local seafood delicacy and is one of the few local species that is successfully cultured in the Straits of Malacca. Furthermore, its sessile lifestyle and widespread distribution in the Straits has prompted its use as a bioindicator of heavy metal contamination in the Straits of Malacca; which is one of the busiest shipping lanes in the world (Ismail *et al.* 2002). Therefore, information

on the population genetic structure of *P. viridis* along the Straits of Malacca is undoubtedly very important since different species may accumulate metals at different rates (Gyllensten and Ryman 1985). Besides, this information can be applied to improve the broodstocks of this commercially important seafood delicacy and hence increase the productivity of mussel farms.

In the last few years, microsatellites have become one of the most popular molecular markers used in various fields of study. Being codominant, PCR-based, highly polymorphic and easy to score contribute to the popularity of microsatellites as a marker of choice. One of the major drawbacks of this DNA marker is that it needs to be isolated *de novo* from species that are being examined for the first time (Zane *et al.* 2002). Although microsatellite isolation is a tedious, expensive and laborious procedure, this does not seem to be a strong deterrent factor. The conventional method of isolating microsatellites is like searching for a needle in a haystack. It involves the creation of a size-fragmented genomic library, cloning of bands and subsequently screening thousands of colonies for the presence of microsatellite repeats through hybridization. The success rate usually ranges from 12% to less than 0.04% (Zane *et al.* 2002). Here we report on the development of microsatellites from *P. viridis* using a modification technique of the 5' anchored PCR technique of Fisher *et al.* (1996).

## MATERIALS AND METHODS

### *Sample Collection*

Mussels were collected from five different locations along the west coast of Peninsular Malaysia. The five collection sites were Tanjung Rhu (Pulau Langkawi, Kedah), Bagan Tiang (Perak), Pulau Ketam (Selangor), Muar (Johor) and Kampung Pasir Puteh (Johor) with a sample size of 20 individuals per location. The adductor muscle was excised from the mussel and kept at -80ºC prior to DNA analysis.

### *DNA Extraction*

DNA was extracted from approximately 20-30 mg of the adductor muscle by using a CTAB based protocol described by Winnepennincks *et al.* (1993) with minor modifications.

### *5' Anchored PCR Amplification*

Two degenerate RAMs primers, VJ1 and BP2, were used to produce a genomic library enriched for microsatellites (Table 1).

A polymerase chain reaction (PCR) was carried out in a total volume of 10 $\mu$L containing 25 ng of genomic DNA, 1X PCR buffer, 0.2 mM each of dATP, dGTP, dCTP and dTTP, 15 pmol of either primer VJ1 or BP2, 2mM $MgCl_2$, and 1.5 U of *Taq* DNA polymerase (Promega, USA). Amplifications were performed in a Peltier Thermal Cycler PTC-220 (MJ Research, USA) with an initial 3 min of predenaturation at 96°C, followed by 40 cycles of denaturation at 96°C for 15 seconds, an appropriate annealing temperature for 15 seconds (as shown in Table 1) and extension at 72°C for 30 seconds. A final extension step at 72ºC for 7 min was included for the attachment of a dATP at the 3' terminal for the cloning reaction. The PCR product was run on a 2% agarose gel and visualised by ethidium bromide staining to confirm the presence of bands. After performing the PCR reaction, the resultant products were then ready for cloning.

TABLE 1
Degenerate RAMs primers used to produce a genomic library enriched for microsatellites and the annealing temperatures for the PCR amplifications

| Primer | Sequence 5' to 3' | Annealing temperature (°C) |
|---|---|---|
| VJ1 | NNN NNN NKK VRV RV $(CT)_{10}$ | 56 |
| BP2 | NNN NNK KYW $(BD)_3B(CA)_{10}$ | 55 |

*Note:* K = G/T, N = A/C/G/T, V = G/C/A, R = G/A, Y = T/C, B = C/G/T, D =A/G/T (IUB code)

*Cloning of the 5' Anchored PCR Products into pCR 2.1-TOPO Vector*

The PCR products were then cloned into the TOPO TA cloning vector according to the manufacturer's instructions (TOPO TA Kit, Invitrogen). Five recombinant clones from each primer were randomly selected for plasmid extraction.

*Plasmid Extraction*

The plasmid extractions were performed according to the protocol of Sambrook *et al.* (1989) and the clones were sequenced by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied biosystems, USA) on the ABI PRISM 377 DNA sequencer at Institute of Biosciences, Universiti Putra Malaysia.

*Primer Design*

Primer pairs flanking each of the microsatellite regions were designed by using a free online primer designing software, PRIMER 3 (Rozen and Skaletsky 1997) provided at http://www.genome.wi.mit.edu/genome_software/other/ primer3.html.

*Amplifying Microsatellites*

The primer pairs so designed were then used to screen for polymorphisms in *P. viridis.* PCR amplifications were performed in a 10 $\mu$L volume containing 25 ng of genomic DNA, 1X PCR buffer, 0.2 mM each of dNTPs, 0.15 $\mu$M of each reverse and forward primers, 1 – 3 mM $MgCl_2$, and 0.5 – 1 U of *Taq* DNA polymerase (Promega, USA). The PCR amplifications were performed in a Peltier Thermal Cycler PTC-220 (MJ Research, USA) with the following temperatures: a predenaturation for 3 min at 95°C, followed by 35 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at appropriate temperatures (as shown in Table 2), 30 seconds extension at 68°C and concluded with a 5 min final extension at 68°C. The PCR products were run on a 4% (w/v) horizontal MetaPhor gel (BMA, USA) and visualized over UV after ethidium bromide staining. A 20 bp DNA ladder (BioWhittaker Molecular Applications) was used as the molecular weight standard. Twenty samples from each of the following *P. viridis* populations: Tanjung Rhu, Bagan Tiang, Pulau Ketam, Muar and Kampung Pasir Puteh were typed for each of the five microsatellite loci. The population data were analysed by using the POPGENE (version 1.32) computer software (Yeh & Boyle, 1997). An Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) dendrogram was constructed based on Nei's (1978) genetic distance estimates by using the NTSYS software (Rohf 1990).

## RESULTS AND DISCCUSION

5' anchored PCR is a technique that involves the use of arbitrary primers, which are specifically designed to incorporate an anchor and a microsatellite repeat motif to produce a library, which is highly enriched for microsatellites. This technique offers a number of advantages. One of the advantages is that the amplification of genomic DNA yields PCR products that contain a microsatellite region at both the 5' and the 3' terminal ends. This means every band sequenced would theoretically contain at least two microsatellites. Another advantage of this technique is its ability to target a specific repeat motif that is required, which means we can produce a library that is highly enriched for the specific repeat motif that we want (Fisher *et al.* 1996).

In this study, a total of ten clones (five from each of the degenerate RAMs primer, VJ1 and BP2) were randomly selected and sent for sequencing. The ten clones sequenced revealed a total of 21 microsatellite regions. Of the 21 microsatellite regions, ten were perfect microsatellites while eight were imperfect or interrupted microsatellite and the rest were compound microsatellites. Five of the clones had one or more additional microsatellites in the internal sequence. The presence of additional internal microsatellites was an unexpected bonus, implying that there may be clustering of microsatellites in some genomic regions (Fisher *et al.* 1996).

Primer pairs were designed for seven microsatellite loci. These are the first set of microsatellite markers that have been identified and characterized in *P. viridis.* Out of these seven loci, five were polymorphic with the number of alleles per locus ranging from 2 to 4 with an average of 3.2. The observed heterozygosity ranged from 0.0104-0.3776 (Table 2) and was similar with those reported using allozyme studies (Yap *et al.* 2002).

Cluster analysis revealed two major groups. Tanjung Rhu, Pulau Ketam, Bagan Tiang and

TABLE 2
Microsatellite variation in five populations of *Perna viridis*

| Locus | Repeat motif | Primer sequence (5'-3') | $T_a$ (°C) | Allele size range (bp) | No. of alleles | $H_O$ | $H_E$ | GenBank Accession no. |
|---|---|---|---|---|---|---|---|---|
| VJ1-18-1 | $(CT)_n$ | F: GTAGCGGCTCTCTCTCTCT<br>R: GCGTGACACTCTTTTTCTTT | 55 | 260-290 | 4 | 0.3776 | 0.7105 | AY850126 |
| VJ1-12-2 | $(AG)_n(GACA)_N$, $(AG)_N$ | F: ATAGGATAGAGTCACGTTAG<br>R: TAAGACCTCTCTCTCTCTC | 40 | 200-210 | 2 | 0.1579 | 0.1462 | AY850124 |
| VJ1-23-2 | $(GAAA)_N$, $(AG)_n$ | F: CAGGACTCCCGCTGGGTAAA<br>R: TCCACTGGCCGGCTCTCT | 44 | 205-220 | 2 | 0.0104 | 0.0104 | AY850125 |
| BP2-49-1 | $(CA)_n$, $(CAAC)_N$ | F: GGTACTTTTCTCACTTCACA<br>R: GGAGTGAACCTCTTCGAC | 44 | 165-230 | 4 | 0.2418 | 0.2541 | AY850129 |
| BP2-49-2 | $(TG)_N$ | F: GTTAAACAACCAACCAACG<br>R: GTCTTTTTGTCATTGCACAC | 44 | 180-260 | 4 | 0.2366 | 0.2471 | AY850129 |

Repeat motif: N, pure; n, interrupted. $T_a$ = annealing temperature, $H_O$ = observed heterozygosity, $H_E$ = expected heterozygosity

Muar were clustered together while Kampung Pasir Puteh was clustered by itself (*Fig. 3*). The Kampung Pasir Puteh population was collected from a highly polluted environment and this finding is in line with the results obtained by Yap *et al.* (2004) based on codominant allozyme markers but contradicted those of Chua *et al.* (2003) which were based on the dominant RAPD and RAMs markers. No diagnostic alleles that could differentiate between samples from polluted and non-polluted environments were found in this study.

This is the first report that microsatellite markers were isolated and characterized for *P. viridis*. Seven microsatellite primer pairs were designed based on the *P. viridis* DNA sequences obtained from a modified 5' anchored PCR technique (Fisher *et al.* 1996). This study is currently ongoing and the primers described will be used for more detailed studies on the population genetic structure of the green-lipped mussel, *P. viridis* from along the west coast of Peninsular Malaysia.

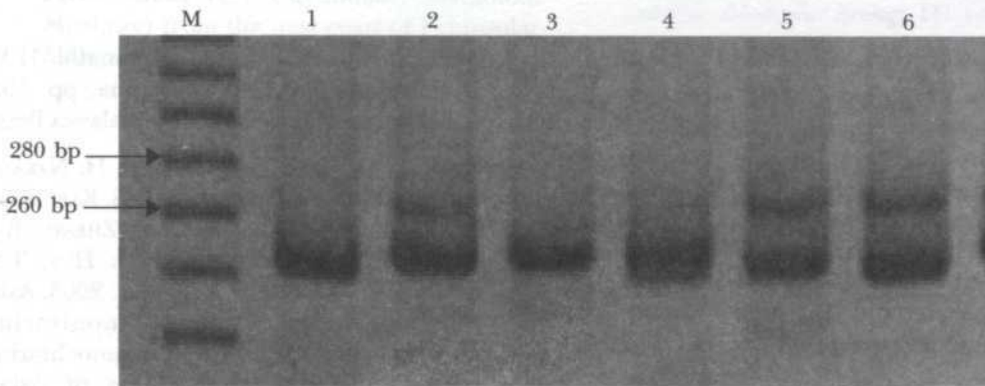


*Fig. 1: Microsatellite profile of samples from the Bagan Tiang population generated by primer pairs VJ1-18-1. Lane M: 20 bp marker; lanes 1-6: microsatellite profiles of samples from Bagan Tiang*

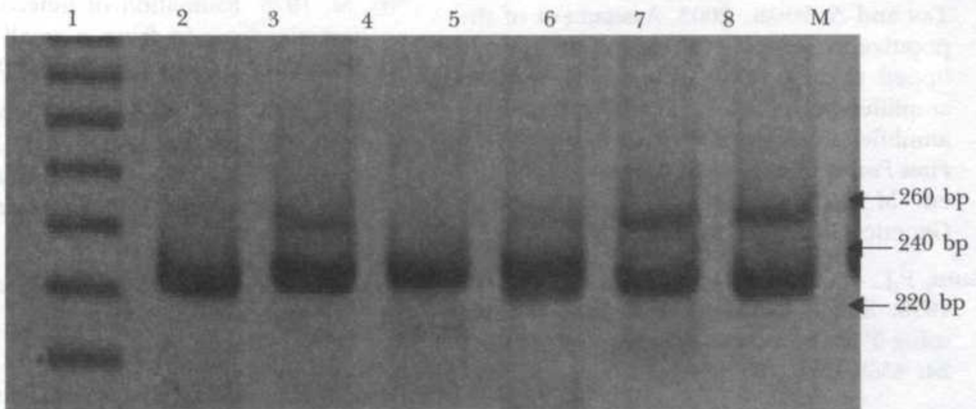


*Fig. 2: Microsatellite profile of samples from the Muar population generated by primer pairs BP2-49-1. Lane M: 20 bp marker; lanes 1-8: microsatellite profiles of samples from Muar*

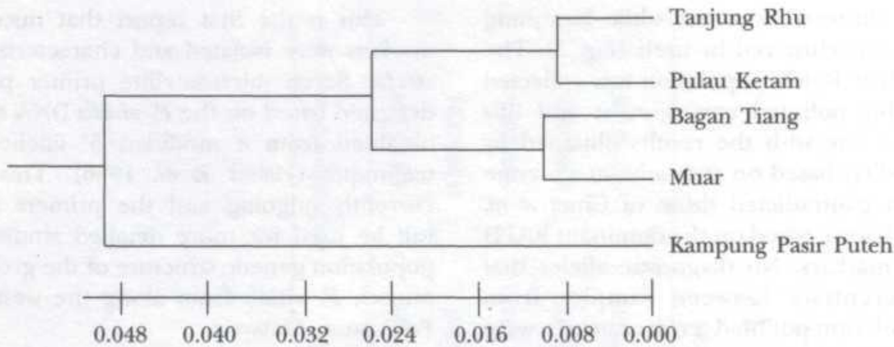


*Fig. 3: UPGMA dendrogram of genetic relationships among five populations of Perna viridis based on Nei's (1978) genetic distance*



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## REFERENCES


CHUA, B.H., S.Y. YONG, C.H. TEH, C.K. YAP, S.G. TAN and A. ISMAIL. 2003. Assessment of the population genetic structure of the green-lipped mussel, *Perna viridis* using random amplified polymorphic (RAPD) and random amplified microsatellite (RAMs) markers. In *From Peas to Chips: The Globalisation of Genetics*, ed. M.K. Thong, p. 47-48. Bangi: The Genetics Society of Malaysia

FISHER, P.J., R.C. GARDNER and T.E. RICHARDSON. 1996. Single locus microsatellites isolated using 5' anchored PCR. *Nucleic Acids Research* **24:** 4369-4371.

GYLLENSTEN, U. and N. RYMAN. 1985. Pollution biomonitoring programs and the genetic structure of indicator species. *AMBIO* **14:** 29-31.

ISMAIL, A., C.K. YAP, P.M. ZAKARIA, S. TANABE, H. TAKADA and A.R. ISMAIL. 2000. Green-lipped mussel *Perna viridis* (L.) as a biomonitoring agent for heavy metals in the west coast of Peninsular Malaysia. In *Towards Sustainable Management of the Straits of Malacca*, ed. M. Shariff, F.M. Yusoff, N. Gopunath, H.M. Ibrahim and R.A. Nik Mustapha, pp. 553-559. Serdang: Universiti Putra Malaysia Press.

MONIRITH, I., D. UENO, S. TAKAHASHI, H. NAKATA, A. SUDARYANTO, A. SUBRAMANIAM, S. KARUPPIAH, A. ISMAIL, M. MUCHTAR, J.S. ZHENG, B.J. RICHARDSON, M. PRUDENTE, N.D. HUE, T.S. TANA, A.V. TKALIN and S. TANABE. 2003. Asia-Pacific mussel watch: monitoring contamination of persistent organochlorine compounds in coastal waters of Asian countries. *Marine Pollution Bulletin* **46:** 281-300.

NEI, M. 1978. Estimation of heterozygosity and genetic distance from a small number of individuals. *Genetics* **89:** 583-590.

NICHOLSON, S. and P.K.S. LAM. 2005. Pollution monitoring in Southeast Asia using biomarkers in the mytilid mussel *Perna viridis* (Mytilidae: Bivalvia). *Environment International* **30:** 121-132.

ROHF, F.J. 1993. NTSYS-pc. *Numerical Taxonomy and Multivariate Analysis System, version 1.60.* Exeter Software, New York.

ROSSEL, N.C. 1991. The green mussel (*Perna viridis*) in the Philippines. In *Estuarine and Marine Bivalve Mollusk Culture*, ed. W. Menzel, pp. 297-305. Boca Raton: CRC Press, Inc.

ROZEN, S. and H.J. SKALETSKY. 1997. Primer 3. Code available at http:// www.genome.mit.edu/genome_software/other/primer3.html.

SAMBROOK, J., E.F. FRITSCH and T. MANIATIS. 1989. *Molecular Cloning: A Laboratory Manual.* New

York: Cold Spring Harbor Laboratory, Cold Spring Harbor.

SIDDALL, S.E. 1980. A clarification of the genus *Perna* (Mytilidae). *Bulletin of Marine Science* **30:** 858-870.

WINNEPENNINCKS, B., T. BACKELJAU and R.D. WACHTER. 1993. Extraction of high molecular weight DNA from mollusks. *Trends in Genetics* **9:** 407.

YAP, C.K., S.G. TAN, A. ISMAIL and H. OMAR. 2002. Genetic variation of the green-lipped mussel *Perna viridis* (L.) (Mytilidaae: Mytiloida: Mytilicae) from the west coast of Peninsular Malaysia. *Zoological Studies* **41(4):** 376-387.

YAP, C.K., S.G. TAN, A. ISMAIL and H. OMAR. 2004. Allozyme polymorphisms and heavy metals level of the green-lipped mussel, *Perna viridis* (Linnaeus) collected from the contaminated and uncontaminated sites in Malaysia. *Environment International* **30:** 39-46.

YEH, F.C. and T.H.B. BOYLE. 1997. Population genetic analysis of codominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129:** 157.

ZANE, L., L. BARGELLONI and T. PATARNELLO. 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology* **11:** 1-6.

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