UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION AND APPLICATION OF DNA MICROSATELLITE MARKERS FOR THE GENETIC CHARACTERISATION OF THE GREEN-LIPPED MUSSEL, *Perna viridis*

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By

LILY ONG CHIN CHIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

IDENTIFICATION AND APPLICATION OF DNA MICROSATELLITE MARKERS FOR THE GENETIC CHARACTERISATION OF THE GREEN-LIPPED MUSSEL, *Perna viridis*

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May 2007

Chairman : Professor Tan Soon Guan, PhD

Faculty : Science

A total of 264 microsatellite sequences were successfully isolated from *P. viridis* by using 5' anchored PCR technique and 109 primer pairs were designed to amplify these repeat regions. Of these, 19 were found to be polymorphic and were used to analyse levels of genetic variation for 10 populations of *P. viridis* collected all over the Peninsular Malaysia. The populations involved in this study included Pulau Aman in Penang, Tanjung Rhu in Kedah, Bagan Tiang in Perak, Pulau Ketam in Selangor, Muar, Parit Jawa, Pantai Lido and Kampung Pasir Puteh in Johore, and Kuala Pontian and Nenasi in Pahang.

The number of alleles per locus ranged from 2 to 7 with an average of 3.1. The highest value of observed heterozygosity was 0.208 (Pulau Ketam) and the lowest value was 0.144 (Pulau Aman). Heterozygote deficiencies were observed across all the ten

populations. The Wahlund effect, inbreeding and null alleles are believed to be the most likely factors for the occurrence of heterozygote deficiencies in this study. Characterisation of the populations revealed that local populations of *P. viridis* in Peninsular Malaysia were genetically similar enough to be used as a biomonitoring agent for heavy metal contamination in the Straits of Malacca. Cluster analysis grouped the *P. viridis* populations according to their geographical distribution with the exception of Parit Jawa. The analysis also revealed that *P. viridis* from the northern parts of Peninsular Malaysia (Tanjung Rhu and Pulau Aman) were found to be the most distant populations among the populations of mussels investigated and *P. viridis* from the eastern part of Peninsular Malaysia (Kuala Pontian and Nenasi) were closer to the central and southern populations than to the northern populations.

Abstrak tesis yang dikemukakan untuk Senat Universiti Putra Malaysia sebagai keperluan ijazah Master Sains

IDENTIFIKASI DAN APPLIKASI PENANDA MIKROSATELIT DNA UNTUK PENCIRIAN GENETIK BAGI KUPANG HIJAU, *Perna viridis*

Oleh

LILY ONG CHIN CHIN

Mei 2007

Pengerusi : Professor Tan Soon Guan, PhD

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Sejumlah 264 jujukan mikrosatelit telah berjaya dipencilkan daripada *Perna viridis* dengan menggunakan teknik "5' anchored PCR" dan 109 pasangan primer telah direka. Daripada jumlah tersebut, 19 adalah polimorfik dan digunakan untuk menganalisis paras variasi genetik bagi *P. viridis* dari 10 lokasi yang berlainan di Semenanjung Malaysia. Populasi- populasi yang terlibat dalam kajian ini merangkumi Pulau Aman di Pulau Pinang, Tanjung Rhu di Kedah, Bagan Tiang di Perak, Pulau Ketam di Selangor, Muar, Parit Jawa, Pantai Lido dan Kampung Pasir Puteh di Johor, dan Kuala Pontian dan Nenasi di Pahang.

Bilangan alel per lokus berjulat daripada 2 hingga 7 dengan purata 3.1. Nilai tertinggi bagi heterozigositi cerapan ialah 0.208 (Pulau Ketam) dan nilai terendah ialah 0.144 (Pulau Aman). Kesemua populasi yang dikaji menunjukkan kekurangan heterozigositi.

Kesan Wahlund, pembiakbakaan dalam dan “null alleles” dipercayai adalah antara faktor utama yang menyumbang kepada kekurangan heterozigositi dalam kajian ini. Pencirian populasi menunjukkan populasi tempatan *P. viridis* di Semenanjung Malaysia adalah serupa secara genetik untuk digunakan sebagai agen biomonitor bagi pencemaran logam berat di Selat Melaka. Analisis kelompok menunjukkan pengkelompokan yang selaras dengan kawasan geografi kecuali populasi *P. viridis* dari Parit Jawa. Analisis tersebut juga menunjukkan bahawa *P. viridis* dari bahagian utara Semenanjung Malaysia (Tanjung Rhu dan Pulau Aman) adalah populasi yang paling jauh dikalangan semua populasi kupang yang dikaji dan *P. viridis* dari bahagian timur Semenanjung Malaysia (Kuala Pontian dan Nenasi) adalah lebih dekat dengan populasi *P. viridis* dari bahagian tengah dan selatan Semenanjung Malaysia berbanding dengan populasi *P. viridis* dari bahagian utara Semenanjung Malaysia.

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I certify that an Examination Committee met on data of viva to conduct the final examination of Lily Ong Chin Chin on her Master of Science thesis entitled " Development and Application of DNA Microsatellite Markers for the Biodiversity Characterisation of the Green-lipped Mussel, *Perna viridis* " in accordance with Universiti Pertanian Malaysia (Higher degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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LILY ONG CHIN CHIN

Date: 5th JUNE 2007

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%	percent
°C	degree Celcius
µl	microliter
µg/ml	microgram per mililter
µM	micromolar
1X	one times
10X	ten times
A	adenosine
bp	base pair
C	cytosine
cDNA	complementary deoxyribonucleic acid
CTAB	cetyltrimetylammonium bromide
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
ddH_2O	double distilled water
dNTPs	deoxynucleotide triphosphate
EDTA	ethylenediamine tetraacetic acid
EMBL	European Molecular Biology Laboratory
EST	expressed sequence tags
G	guanosine
g	relative centrifugal force

HCl	hydrochloric acid
kb	kilobase
LB	Luria-Bertani
M	molar
$MgCl_2$	magnesium chloride
mg/ml	miligram per mililiter
min	minute
ml	milimeter
mM	milimolar
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
ng/µl	nanogram per microliter
PCR	polymerase chain reaction
pmol	picomole
RFLP	restriction fragment length polymorphism
RNase A	ribonuclease A
rpm	revolution per minute
s	second
SDS	sodium dodecyl sulphate
T	thymine
TBE	tris-borate-EDTA
TEMED	*N,N,N',N'*-tetramethylethylene diamine

U	unit
UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

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 but 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 biomonitoring 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 (Yap *et al.*, 2003b). It is a local seafood delicacy and is one of the few local species that is successfully cultured in the Straits of Malacca (Annual Fish Statistics, 1999). Furthermore, its sessile lifestyle and widespread distribution along the west coast of Peninsular Malaysia has prompted its use as a biomonitoring agent for heavy metal contamination in the Straits of Malacca (Ismail *et al.*, 2000).

Before this species can be used as a biomonitoring agent for heavy metal contamination in the Straits of Malacca, it needs to fulfil several recommended criteria. Among the criteria are that *P. viridis* collected from different geographical populations along the straits should have similar morphological characteristics for easy and correct species identification, and low degree of genetic differentiation as they may genetically adapt to heavy metals stresses (Gyllensten and Ryman, 1985; Rainbow, 1995). Therefore, studies on the population genetic structure of *P. viridis* in Malaysia should be done to validate its effectiveness for biomonitoring purposes as well as for proper mussel farming management.

So far, several molecular markers such as allozymes, Random Amplified Polymorphic DNA (RAPD) and Random Amplified Microsatellites (RAM) have been used to investigate the genetic relationships among populations of *P. viridis* collected along the west coast of Peninsular Malaysia (Yap *et al.*, 2002; Chua *et al.*, 2003). However, the results based on dominant DNA based RAPD and RAM markers (Chua *et al.*, 2003) showed clustering of populations that differed from those derived from the use of allozyme marker data (Yap *et al.*, 2002). In an attempt to clarify this situation, a more powerful codominant DNA microsatellite marker was employed for more detailed studies of the population genetic structure of *P. viridis* in Malaysia.

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, however, is that it needs to be isolated *de novo* from species that are being examined for the first time (Zane *et al.*, 2002). Although the conventional way of isolating microsatellites by screening of genomic libraries is a tedious, expensive and laborious procedure, this does not seem to be a strong deterrent factor. Over the last few years, numerous new protocols that overcome these limitations have appeared in the literature, and microsatellites now can be sourced through many ways, which include derivation from enriched genomic libraries, screening of BAC (bacterial artificial chromosome) or YAC (yeast artificial chromosome) and cDNA libraries, from public databases such as GenBank, from related species and from EST databases (Scott, 2001).

Until now, microsatellites have yet to be isolated for *P. viridis*. Hence, the main objective of this study is to isolate microsatellites from this species. To date, three protocols namely Direct Amplification of Length Polymorphism (DALP), Random Amplified Hybridisation Microsatellites (RAHM) and 5' anchored PCR have been applied in the Genetics Laboratory of the Department of Biology, Faculty of Science, Universiti Putra Malaysia to isolate microsatellites for various species such as *Mystus nemurus* (Usmani, 2002; Chan, 2003; Hoh, 2005) and *Vigna radiata* (Kumar, 2003). Of these, 5' anchored PCR was found to be the most efficient, fastest and cost effective way to obtain large numbers of microsatellites in the shortest period of time (Usmani, 2002; Chan, 2003; Kumar, 2003; Hoh, 2005). For these reasons, this protocol was chosen in this study to isolate microsatellites from *P. viridis*.

Thus, the objectives of this study are:

1. To identify and characterise microsatellite markers for the green-lipped mussel, *Perna viridis*, and
2. To characterise 10 *Perna viridis* populations using the newly identified microsatellite markers.

CHAPTER 2

LITERATURE REVIEW

2.1 Mussels of the Genus *Perna*

Mussels of the genus *Perna* are widespread, predominantly in the Southern Hemisphere and are replaced in temperate latitudes by *Mytilus* species (Nicholson and Lam, 2005). In Asia, they have become one of the most valuable mariculture organisms produced in the largest quantity, mainly because of their value as a cheap source of animal protein for human consumption (Tanabe, 2000). Besides being rich in protein, they are also an important food source for supplying essential trace metals and vitamins (Yap *et al.*, 2004a)

Currently, there are three species placed in the genus *Perna*: *P. perna* (the brown mussel), *P. viridis* (the green-lipped mussel) and *P. canaliculus* (the New Zealand greenshell mussel) (Siddall, 1980). Siddall (1980) conceded that the three species of *Perna* could hardly be differentiated without knowing from which locality the material was collected because of the great variation in characters of taxonomic importance within the genus.

P. viridis is native to the Indo-Pacific region, where it ranges longitudinally from the Persian Gulf to the Southwest Pacific and latitudinally from Southern Japan to Papua

New Guinea. *P. perna*, on the other hand, is indigenous to Africa and the western coast of South America (Vakily, 1989). Until recently, *P. viridis* and *P. perna* were geographically isolated and thus easily distinguished. However, the recent expansions of both mussels outside their native range, most likely by international shipping either as adults byssally attached to ships hulls or as larvae in ballast seawater, have made proper identification more reliant on detailed morphological features (Hicks *et al.*, 2001). Unlike *P. perna* and *P. viridis*, *P. canaliculus* was and is still only found in New Zealand. They are the only cultivated mussel species in New Zealand and are widely distributed throughout the three main islands, namely the North, South and Stewart Islands (Apte and Gardner, 2002; Apte *et al.*, 2003). Figure 2.1 shows the global geographical distribution of the three species of *Perna*.

P. perna adults are typically brown to red-maroon with irregular areas of light brown and green, while young *P. canaliculus* have light coloured zigzag markings on the outer shell. Brilliant green and blue-green predominate in *P. viridis* juveniles while the adult shells are less brilliant and have a greater proportion of brown (Siddall, 1980). Aside from the minor differences in shell colouration and patterns noted above, the presence of enlarged sensory papillae along the mantle margins in *P. perna* and variations in the number of diploid chromosomes (*P. perna* has 28 diploid chromosomes and *P. viridis* has 30) aid in species identification (Ahmed, 1974; Siddall, 1980).

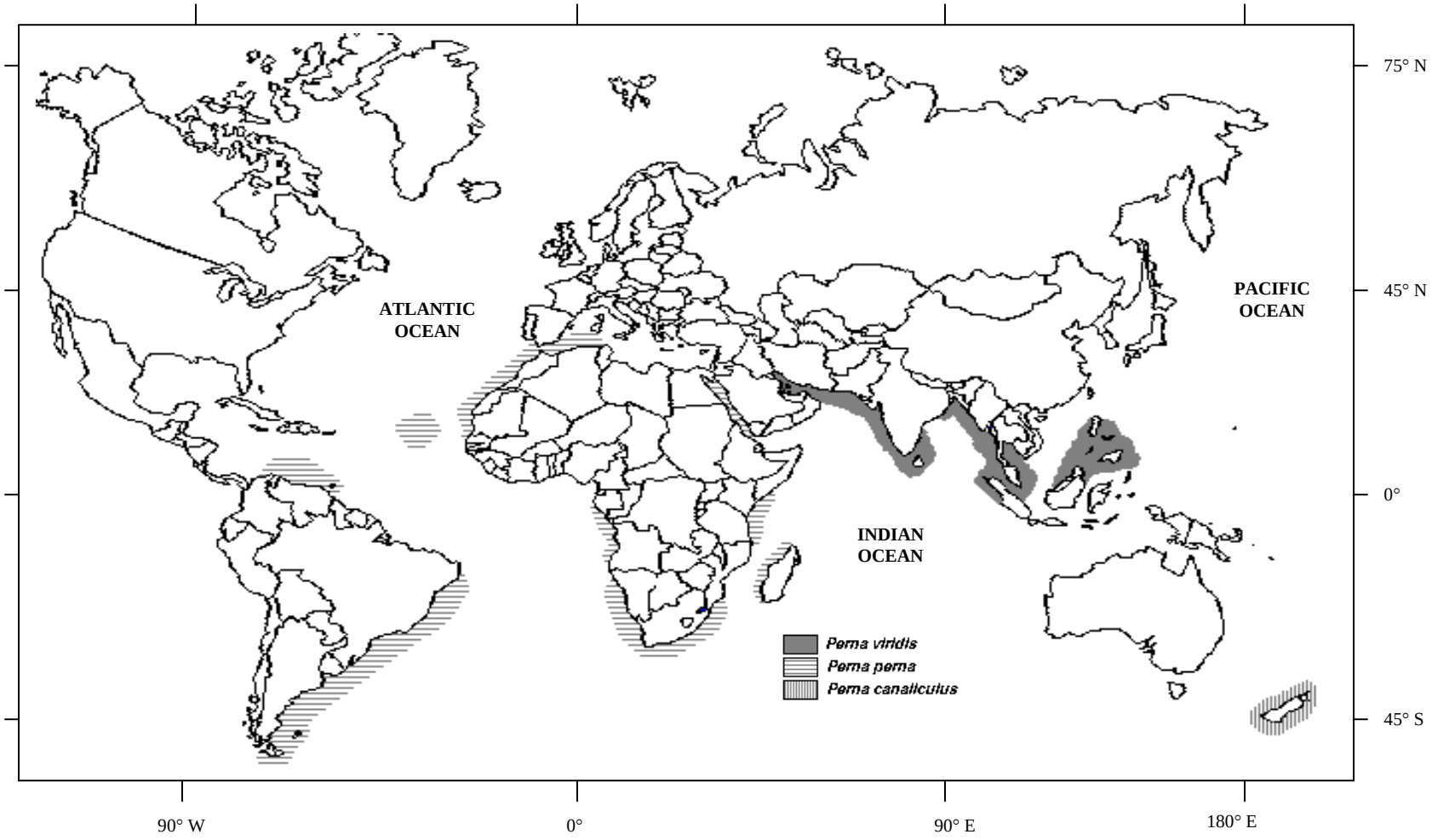

Figure 2.1: Global geographical distributions of the three living species of *Perna* (modified from Siddall, 1980).