THE POPULATION GENETICS OF GREEN MUSSEL (*Perna viridis*) FROM SABAH, MALAYSIA AND IMPLICATION TO ITS AQUACULTURE

LAU JEN SHI

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LAU JEN SHI

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Tarikh: 25 February 2016

(Assoc. Prof. Dr. JULIAN RANSANGAN)
Penyelia

(Dr. KENNETH FRANCIS RODRIGUES)
Penyelia bersama

CERTIFICATION

NAME : **LAU JEN SHI**

MATRIK NO. : **MY1221003T**

TITLE : **THE POPULATION GENETICS OF GREEN MUSSEL (*Perna viridis*) FROM SABAH, MALAYSIA AND IMPLICATION TO ITS AQUACULTURE**

DEGREE : **MASTER OF SCIENCE (MARINE BIOTECHNOLOGY)**

VIVA-VOCE DATE : **25 JANUARY 2016**

CERTIFIED BY

1. SUPERVISOR **Signature**

Assoc. Prof. Dr. JULIAN RANSANGAN

_____

2. CO-SUPERVISOR

Assoc. Prof. Dr. KENNETH FRANCIS RODRIGUES

_____

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I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

4 March 2016

Lau Jen Shi
MY1221003

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ABSTRACT

The extraordinary growth performance and simple culture method of green mussel (*Perna viridis*) make it a potentially profitable aquaculture species. However, green mussel aquaculture in Sabah still relatively undeveloped. As green mussel aquaculture in Sabah is completely relying on wild green mussel population for both spats and broodstock, it is imperative to understand various population parameters of green mussel including the population genetic information before green mussel aquaculture improvement initiatives commence. In this study, 200 green mussel specimens were collected from five locations in Sabah which included Kota Kinabalu, Kota Marudu, Kuala Penyu, Tuaran and Tawau. The population genetic of green mussel in Sabah was examined using mitochondrial DNA (D-loop) and microsatellite loci. Fifteen microsatellite loci were examined using polyacrylamide gel electrophoresis whereas mitochondrial DNA (d-loop) was examined using DNA sequencing method. Standard genetic diversity indices for mitochondrial DNA were calculated in DnaSP5.10.1 whereas microsatellite was calculated in Arlequin 3.5.1.2. Pairwise F-statistic, AMOVA and Mantel's test were calculated for both D-loop and microsatellite using the Arlequin 3.5.1.2. Nested clade analysis, UPGMA dendrogram and STUCTURE 2.3.4 were used to further elucidate the population structure of the green mussel. Besides, genetic bottleneck signature in the green mussel population was examined using BOTTLENECK 1.2.02 and neutral tests in Arlequin 3.5.1.2. Green mussel population showed high haplotype diversity and low nucleotide diversity (H_d=0.916; π=0.00915) in mitochondrial D-loop region whereas the microsatellite genetic diversity of green mussel in Sabah (A=3.08; H_e=0.43) was lower than other location such as Thailand. The low average allele number indicated the adaptability of the green mussel population to sudden change in environment and disease outbreak is weak. Introduction of green mussel stocks from Thailand would probably increase the genetic diversity of the green mussel population in Sabah and may manifest the genetic differences in commercially significant phenotypic traits. AMOVA and pairwise F-statistic for both microsatellite and D-loop showed low but significant population structuring. Nested clade analysis based on mitochondrial DNA was unable to identify the population structure clearly. However, STRUCTURE and UPGMA dendrogram based on microsatellite data showed that the individuals from Kota Marudu and Tawau constituted a cluster whereas individuals from Kota Kinabalu, Kuala Penyu and Tuaran formed the another cluster. The population structure pattern was probably affected by local marine current, larvae behaviour or even anthropogenic activities. From fisheries management perspective, it is desirable to manage the two groups separately. Significant genetic bottleneck signature was not detected in either microsatellite loci or D-loop of mitochondrial DNA albeit green mussel population in Sabah passed through a severe mass mortality which lasted for almost three years. In conclusion, the outcomes of this study have created a sound foundation for green mussel aquaculture improvement program in Sabah.

ABSTRAK

Kepelbagain genetik kupang hijau (Perna viridis) Dari Sabah, Malaysia dan Implikasi Kepada Akuakultur

Kadar tumbesaran yang cepat dan kaedah penternakan yang mudah, menjadikan kupang hijau (Perna viridis) spesies akuakultur yang berpotensi menguntungkan. Tetapi perternakan kupang hijau di Sabah masih tidak maju. Perternakan kupang hijau di Sabah bergantung secara keseluruhan pada populasi kupang hijau liar untuk benih dan induk, jadi adalah penting untuk memahami parameter populasi termasuk populasi genetik sebelum memulakan program memajukan akuakultur kupang hijau. Dua ratus spesimen kupang hijau dikumpul dari lima tempat termasuk Kota Kinabalu, Kota Marudu, Kuala Penyu, Tuaran dan Tawau. Kupang hijau genetik populasi dikaji dengan DNA mitokondria (D-loop) dan mikrosatelit loci. Lima belas mikrosatelit lokus telah dianalisa dengan gel polyacrylamide manakala D-loop dikaji dengan kaedah penjujukan DNA. Indeks kepelbagaian genetik untuk mikrosatelit lokus telah dikira menggunakan Arlequin 3.5.1.2 manakala D-loop menggunakan DnaSP5.10.1. AMOVA, population pairwise F-statistik dan Mantel's test juga dikira untuk mikrosatelit loci dan D-loop dengan Arlequin 3.5.1, Nested clade analysis, UPGMA dendrogram and STUCTURE 2.3.4 diguna untuk menjelaskan struktur populasi kupang hijau. Cerutan genetik dalam populasi kupang hijau diperiksa dengan BOTTLENECK 1.2.02 dan neutral tests dalam Arlequin 3.5.1.2. Populasi menunjukkan kepelbagaian haplotaip tinggi namun kepelbagaian nukleotida yang rendah (H_d=0.916;π=0.00915) di D-loop jujukan manakala mikrosatelit loci menunjukkan kepelbagaian genetik (A=3.08;H_e=0.43) yang jauh lebih rendah berbanding tempat lain seperti Thailand. Memperkenalkan stok kupang hijau dari Thailand mungkin akan meningkatkan kepelbagaian genetik populasi kupang hijau di Sabah dan menunjukkan perbezaan genetik dalam ciri-ciri fenotip komersial yang penting. AMOVA dan population pairwise F-statistik menunjukkan penstrukturan populasi yang rendah tetapi signifikan. Nested clade analysis berdasarkan D-loop tidak dapat mengenal pasti populasi struktur dengan jelas. Namun, UPGMA dendrogram dan STUCTURE 2.3.4. menunjukkan populasi kupang hijau di Sabah dibahagi kepada dua kumpulan di mana Kota Marudu dan Tawau membentuk kumpulan satu manakala kupang hijau di Kuala Penyu, Kota Kinabalu dan Tuaran membentuk kumpulan kedua. Corak struktur penduduk itu mungkin terjejas oleh arus laut tempatan, kelakuan larva atau aktiviti antropogenik. Dari perspektif pengurusan perikanan, adalah wajar untuk menguruskan dua kumpulan berasingan. Cerutan genetik yang signifikan tidak dikesan dalam mikrosatelit lokus atau D-loop walaupun populasi kupang hijau di Sabah telah melalui kematian besar-besaran untuk hampir tiga tahun. Kesimpulannya, hasil kajian ini telah mewujudkan asas yang kukuh untuk program peningkatan akuakultur kupang hijau di Sabah.

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LIST OF SYMBOLS & ABBREVIATIONS

mtDNA	-	mitochondrial DNA
nDNA	-	nuclear DNA
DUI	-	doubly uniparental inheritance
M-type DNA	-	paternal DNA
F-type DNA	-	maternal DNA
PCR	-	polymerase chain reaction
PAGE	-	polyacrylamide gel electrophoresis
HWE	-	Hardy-Weinberg equilibrium
AMOVA	-	analysis of molecular variance
IAM	-	infinite allele model
TPM	-	two phase model
SMM	-	stepwise mutation model
UPGMA	-	unweighted pair group method with arithmetic mean
°C	-	degrees Celsius
cm	-	centimetre
g	-	gram
kb	-	kilo base
bp	-	base pair
%	-	percentage
ng	-	nanogram
µl	-	microlitre
dH_2O	-	distilled water
TBE	-	tris-borate-edta
H_d	-	haplotype diversity
π	-	nucleotide diversity
F_{st}	-	F-statistic
R_{st}	-	analog of F_{st} (assuming a stepwise mutation model)
Φ_{ST}	-	phi-st (analog of F_{st})
A	-	allele number
H_o	-	observed heterozygosity
H_e	-	expected heterozygosity
F_{IS}	-	inbreeding coefficient
ΔK	-	delta K
D_N	-	unbiased genetic distance
D_c	-	clade distance
D_n	-	nested clade distance
I-T	-	interior-tip distances
ppt	-	part per thousand
mg l^{-1}	-	milligram per litre

CHAPTER 1

INTRODUCTION

1.1 Seafood Security and Aquaculture

Seafood is an important food source for human and the demand for seafood is increasing worldwide. According to FAO (2014), the global seafood consumption has increased from 117.3 million tonnes in 2007 to 136.2 million tonnes in 2012. However, global supply of marine seafood has changed very little over the past few decades and even undergoing alarming decrease in some of the traditional fisheries such as bluefin tuna (*Thunnus thynnus*) (Heinisch et al., 2008). Global fisheries have harvested near or beyond the maximum sustainable limits of the natural marine ecosystem (Garrison, 2010). Marine capture fishery is under great pressure from overfishing, ocean pollution and climate change (Garcia and Rosenberg, 2010). Capture fisheries alone is no longer sustainable and unable to support the demand from growing human population.

Aquaculture can provide an alternative food source to fulfill the increasing demand from human and lessen the fishing pressure on wild fish stocks. Many species of bivalves represent a source of inexpensive animal protein with high nutritional value. *Perna viridis* (Linnaeus, 1758) is one of the potential aquaculture species that is able to provide affordable protein source. *Perna viridis* is a type of mussel, which is an important aquaculture species in Asian countries. A typical total life span of a green mussel can last for 2 to 3 years (Asokan, 2011). However, it has been reported as an invasive species in some regions, which harm local ecosystem and economic in recent years (Gilg et al., 2013).

Extraordinary growth performance, natural abundance, high tolerance to wide range of environment conditions and relatively simple culture method make green mussel an excellent candidate for shellfish aquaculture (Vakily, 1989). Due to high growth rate of green mussel, it can reach marketable size in shorter culturing period as compared to many other species of bivalves. *Mytilus edulis* normally reaches marketable size (about 40mm) after 12-15 months of culture period (Goulletquer, 2014) whereas *Perna viridis* can reach marketable size (about 50-70mm) within a much shorter culture period, which is around 6-7 months (Vakily, 1989; Rajagopal et al., 2006).

1.2 Green Mussel Aquaculture

The FAO (2013) has reported that several countries including Cambodia, India, Malaysia, Philippines, Singapore and Thailand are actively involved in green mussel aquaculture activities (Figure 2.1). The culture activity of green mussel in Malaysia is confined mainly to southern Johor in the early 1980s (Al-Barwani et al., 2007). The experimental culture of green mussel in Sabah started in the late nineties. The green mussel broodstock from Peninsular Malaysia were collected on polypropylene and transplanted to Sabah. The major green mussel culture areas in Sabah were Marudu Bay (Kota Marudu), Mengkabong (Tuaran), Tasik Setompok (Kuala Penyu) and Tawau. As the green mussel aquaculture developed, the escaped green mussel spats established the natural green mussel population in the coastal waters of Sabah.

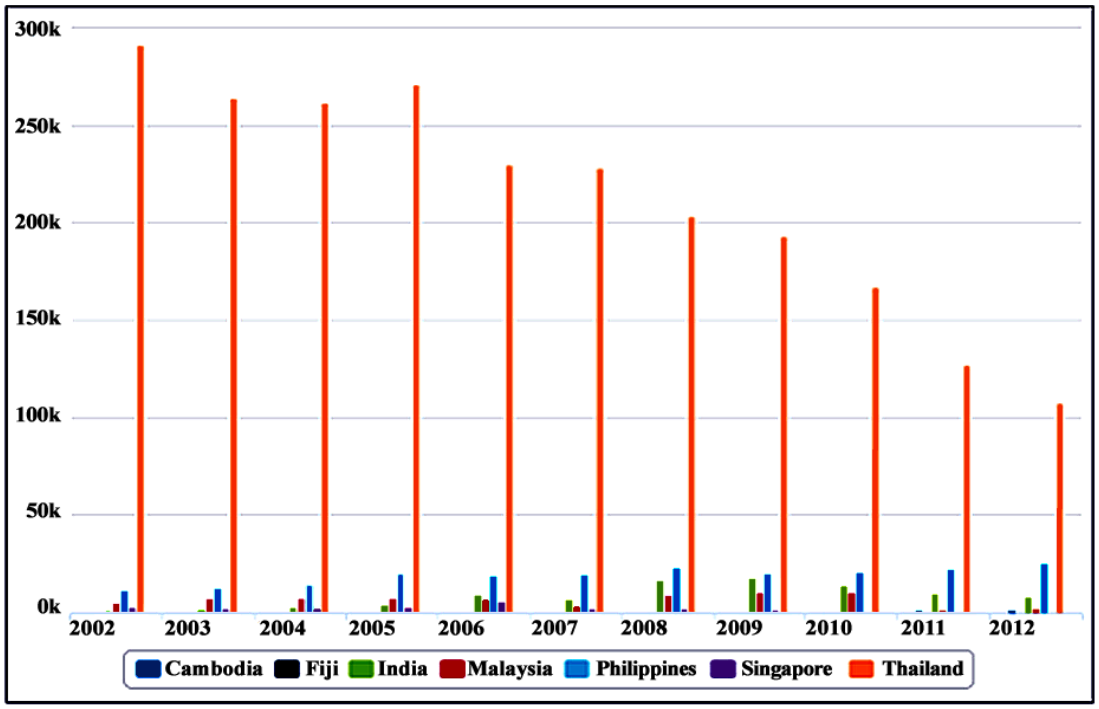

Figure 1.1: Global aquaculture production (tonnes) of green mussel (*Perna viridis*).

Source : FAO, 2013

The green mussel aquaculture in Sabah utilizes the off-bottom culture method that uses ropes as substrate for wild mussels spat to settle and grow. This method relies completely on spat from wild populations. However, the source of wild spats is often unreliable and unpredictable. Therefore, an initiative to produce seeds of green mussel in hatchery has been proposed by the Department of Fisheries Sabah. Many countries such as India and Malaysia have attempted to culture green mussel in hatchery (Laxmilatha et al., 2011; Mohd Salleh, 2011). However, currently the mass scale production of mussel spats in hatchery is still in its infancy stage. In 2010, there was a mass mortality of green mussel in Sabah of which cause serious economic loss to green mussel farmers and undermined the development of green mussel aquaculture in Sabah. Studies carried out by researchers from several institutions of higher learning and fisheries department in Malaysia have not been able to determine the cause of the mass mortality.

1.3 Significance of Study

The global demand for seafood is increasing worldwide and has surpassed the supply of traditional fisheries. Thus, aquaculture is important to provide alternative food source to fulfill the demand from human and lessen the fishing pressure on wild stocks. *Perna viridis* is one of the potential aquaculture species to provide affordable protein source. However, green mussel aquaculture in Sabah is still relatively undeveloped compared to that in Philippines and Thailand. Therefore, there is a necessity to develop management strategies for improving green mussel aquaculture. Nevertheless, lack of knowledge concerning various population parameters including the genetic information will hinder the management efforts. As wild populations provide both spat and broodstocks for aquaculture, it is imperative to understand the genetic properties of wild green mussel populations. Hence, this study is expected to provide the basic information regarding the population genetic of green mussel in Sabah. Such information is expected to contribute to better understanding of green mussel resource in Sabah leading to a more sustainable aquaculture of green mussel in Sabah in future.

1.4 Objectives

The general aim of this study is to provide basic information on population genetic of green mussel population in Sabah so that management strategies for improving green mussel aquaculture can be developed. Experiments were conducted with the following objectives to achieve the aim:

1. To estimate the genetic diversity of green mussel (*Perna viridis*) populations in Sabah using microsatellite and mitochondrial DNA analyses.
2. To determine the population structure of green mussel (*Perna viridis*) populations in Sabah using microsatellite and mitochondrial DNA analyses.
3. To examine the demographic history of green mussel (*Perna viridis*) populations in Sabah using microsatellite and mitochondrial DNA analyses.

CHAPTER 2

LITERATURE REVIEW

2.1 Green Mussel (*Perna viridis*)

The systematic classification of green mussel (*Perna viridis*) (Figure 2.1) is as the following (Rajagopal et al., 2006):

Kingdom: Animalia
Phylum: Mollusca
Class: Bivalvia
Subclass: Ptriomorphia
Order: Mytiloida
Family: Mytilidae Rafinesque, 1815
Genus: *Perna* Philipsson, 1788
Species: *Perna viridis*

The genus *Perna* is a marine bivalve mollusc under the family Mytilidae. The genus *Perna* consists of three extant species, *Perna perna* (Linnaeus, 1758), *Perna viridis* (Linnaeus, 1758) and *Perna canaliculus* (Gmelin, 1791) (Siddall, 1980). Anterior adductor muscle is absent in adult *Perna* species (Siddall, 1980; Asokan, 2011). Some researchers included three other species, under genus *Perna*, which are *Perna picta*, *Perna indica* and *Perna magellanica*. Notwithstanding, *Perna indica* has been synonymized with *Perna perna* (Siddall, 1980; Vakily, 1989). *Perna magellanica* also has been synonymized with *Perna perna* (Huber, 2015). In addition, Wood et al. (2007) had confirmed *Perna picta* clustered with *Perna perna* clade based on nuclear (ITS1 and ITS2) and mitochondrial (COI) DNA sequence analyses. The appearance and colour of external shell can be used to differentiate species in genus *Perna* albeit rather inaccurate taxonomically (Vakily, 1989). The external shell of *Perna perna* normally is brown to red-maroon colour surrounded with irregular light brown and green. On the other hands, the external shell of *Perna viridis* typically is bright green or blue-green in juvenile but turn to greater proportion of brown colour internally during adult stage. Juveniles of *Perna*

canaliculus have light coloured zigzag markings. *Perna viridis* can also be differentiated from *Perna perna* and *Perna canaliculus* with the presence of enlarged sensory papillae along the edge of the mantle (Siddall, 1980). The most accurate diagnostic method is based on chromosome number, *Perna viridis* has 30 diploid chromosomes whereas *Perna perna* and *Perna canaliculus* have 28 diploid chromosomes (Ahmed, 1974; Muhammed Zafar Iqbal et al., 2008).

2.1.1 Morphology

Adult green mussels generally range from 80 millimeter to 100 millimeter in length but in rare cases it can reach up to 150 millimeter (FAO, 2013). Green mussel has two hinged shells, which connected with posterior adductor muscle. The shells have smooth surface with concentric growth rings. The shells are important as skeleton for the attachment of muscles and protect mussel against predators (Gosling, 2015). The periostracum is the proteinaceous outer layer of the shell, which gives its colour. The periostracum of green mussel is green and blue green in juveniles but turns brown with irregular areas of light brown and green in adult (Siddall, 1980). The foot in green mussel secretes byssus which is a bundle of tough threads of tanned protein that enables adult mussels to attach to the substrate or to other mussels (Vakily, 1989; Gosling, 2015). Figure 2.1 shows the general morphology of a green mussel. In bivalves, the mantle forms two separate siphons, which are the inhalant opening and the exhalant opening. Inhalant opening is the entry point to allow water to enter the mantle cavity whereas exhalant opening is to allow water to exit the mantle cavity (Karleskint et al., 2012; Gosling, 2015). The water enters via inhalant opening then passes through gill with the help of beating cilia located on the lining of mantle cavity and gill structure. Green mussel has 4 rows of gills which have both feeding and respiratory roles (Vakily, 1989; Gosling, 2004). Green mussel obtains oxygen and filters food particles including phytoplankton, zooplankton and suspended fine organic materials from the incoming waters via the gills. The gills of *Perna viridis* have the ability to retain particles down to at least 0.46μm in diameter (Vakily, 1989). The labial palp then secretes mucus to entangle food particles and move it to the mouth for digestion (Karleskint et al., 2012). The rejected materials are then discharged through exhalant opening

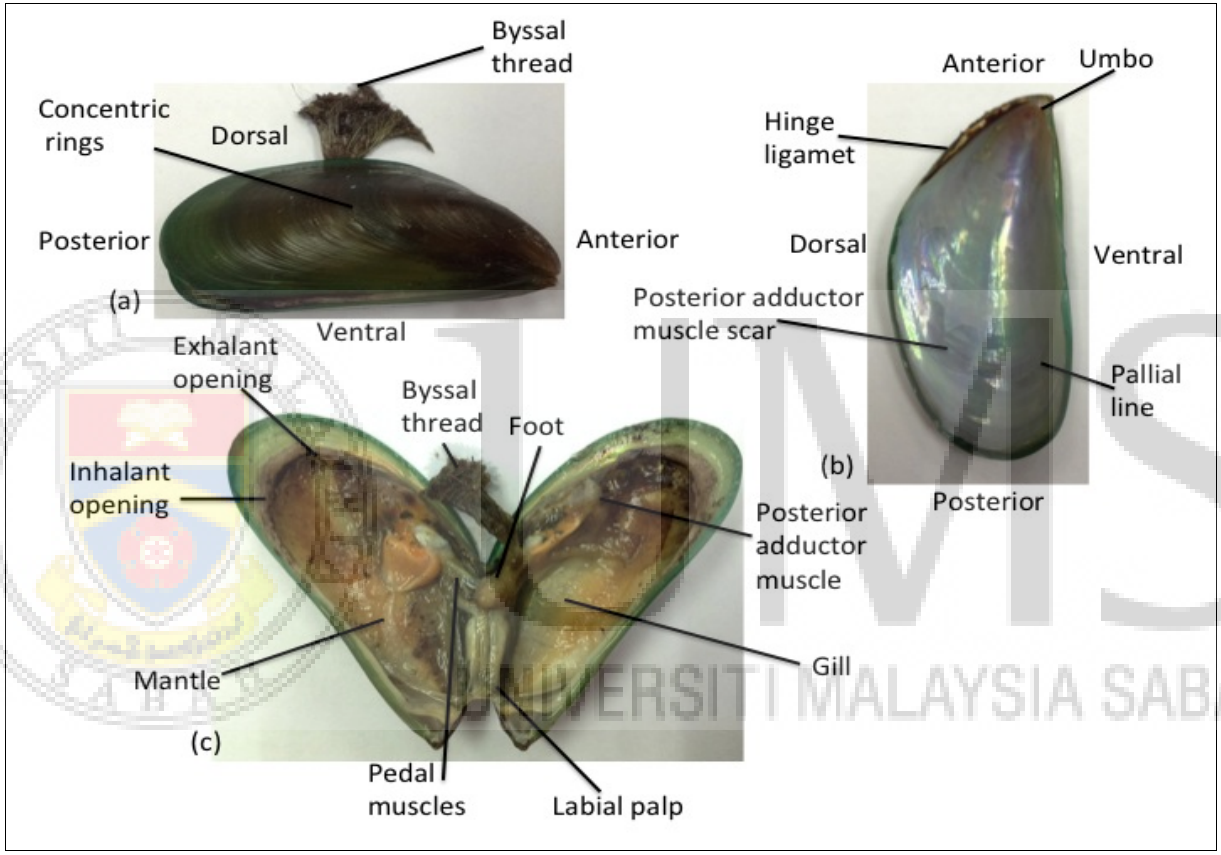

Figure 2.1: The general features of green mussel (a) external shell (b) internal shell (c) internal features.

Source : Modified from Gosling, 2015

2.1.2 Habitat and Physical Tolerance

Green mussel thrives in coastal waters less than 10m depth (Vakily, 1989). They generally inhabit marine intertidal, subtidal and estuarine environments, which have high salinity and receive more nutrients from terrestrial run-off. Green mussel can attach on various hard structures including vessels and mariculture equipment, and even muddy sea bottoms such as seagrass beds (Vakily, 1989; Rajagopal et al., 2006). Green mussel tolerates a wide range of salinities and temperatures. Optimum salinity for green mussel is around 27 to 33ppt and optimum temperature is around 26°C to 32°C (Power et al., 2004). However, green mussel has been shown to survive in extremely low salinity (24ppt) to extremely high salinity (80ppt) and extremely low temperature (10°C) to extremely high temperature conditions (35°C) (Sivalingam, 1977). They also exhibit high tolerance toward turbidity and pollution caused by high suspended particulate matter. Shin et al. (2002) reported that green mussel could tolerate up to 1200mg l^{-1} suspended particulate matter.

2.1.3 Distribution

The native habitat of green mussel is in the Indo-Pacific region (Figure 2.2), which encompasses regions between Japan to New Guinea and from Persian Gulf to South Pacific Islands (FAO, 2013). However, in recent years, green mussel has expanded its geographical range through shipping activities, either as spat in ballast water or as adults attached to the hulls of ships (Rajagopal et al., 2006). Green mussel was first found in Trinidad in 1990 as an invasive species (Agard et al., 1992). Since then, green mussel have also been found in Venezuela, Jamaica, Florida and throughout the Caribbean coastal areas (Gilg et al., 2013; Gobin et al., 2013). Green mussel is considered an invasive species in those regions because green mussel is able to outcompete and displace many endemic bivalves, and modify the local natural ecosystems significantly (Rajagopal et al., 2006; Gilg et al., 2013). As green mussel thrives in warmer region, global warming is expected to further increase the geographical distribution of *Perna viridis* (Urian et al., 2011).

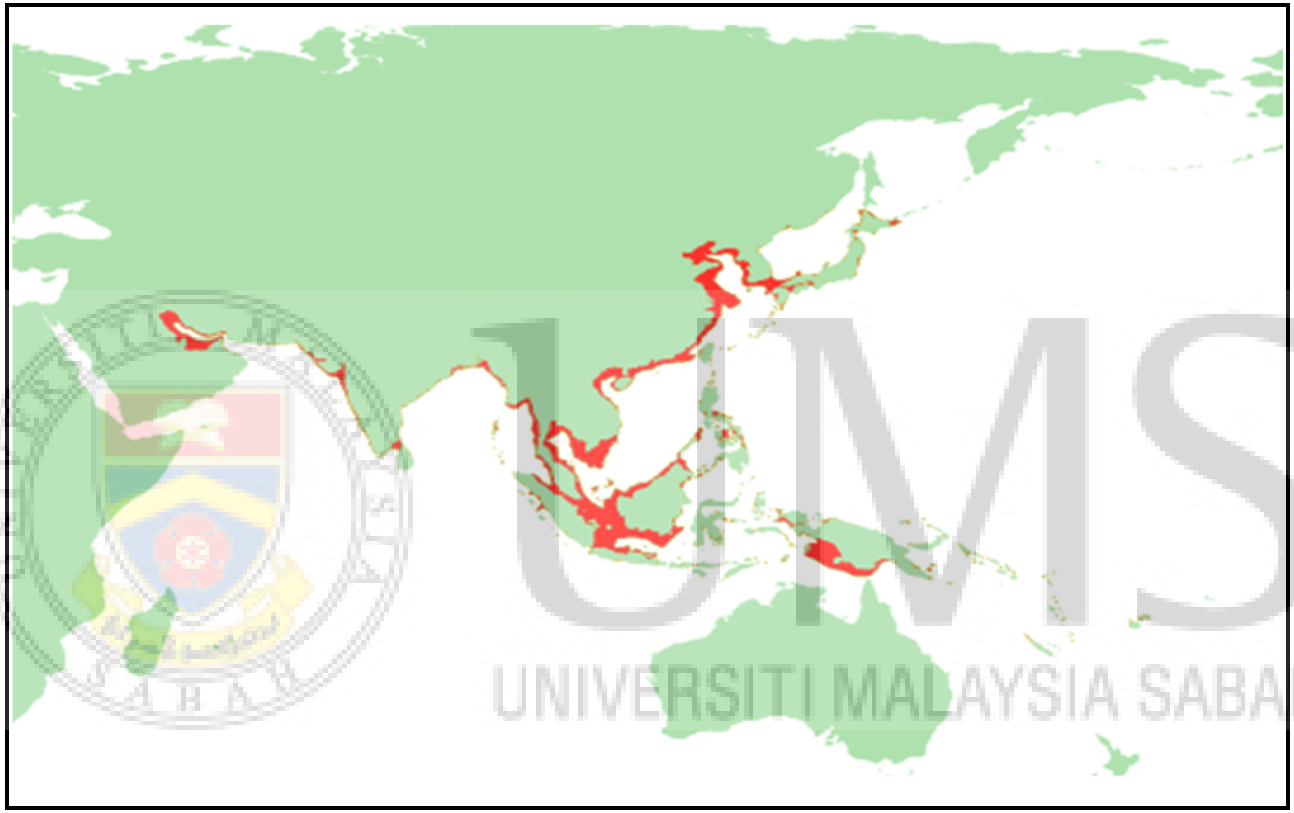

Figure 2.2: Native distributions of *Perna viridis* (red colour).

Source : FAO, 2013