PHYLOGENY OF PENAEID SHRIMPS AND POPULATION GENETIC STRUCTURE OF THE GREEN TIGER PRAWN (*Penaeus semisulcatus*) IN MALAYSIAN WATERS

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by

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November 2nd 2015, the day that I entered Molecular Systematics Laboratory (Lab 308), School of Biological Sciences, Universiti Sains Malaysia which I will never forget throughout my life. This date when I started working and furthered my study to the next level, a Masters Degree at this university, my very dream university to enter. Three years plus, and I have been through a lot during my study and Alhamdulillah, in the end, I able to finish my study in early 2019. Of course there are many people involved during these years, and on this page, I would like to convey my sincere thanks to all of these people who have been with me through thick and thin.

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LIST OF ABBREVIATIONS

AMOVA	Analysis of molecular variance
BI	Bayesian inference
BP	Bootstrap percentage
COI	Cytochrome c oxidase subunit 1
CR	Control region
CVA	Canonical Variate Analysis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
FDR	False discovery rate
G	Gamma
MCMC	Markov chain monte carlo
ML	Maximum likelihood
MSN	MSN
mtDNA	Mitochondrial DNA
NJ	Neighbor joining
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
SAMOVA	Spatial analysis of molecular variance
T92	Tamura 3-parameter
t	Tonnes

FILOGENI UDANG PENAEID DAN STRUKTUR GENETIK POPULASI UDANG HARIMAU (*Penaeus semisulcatus*) DI PERAIRAN MALAYSIA

ABSTRAK

Kajian ini melaporkan tentang filogeni udang penaied yang terpilih dan struktur genetik populasi udang harimau (*Penaeus semisulcatus*) yang mempunyai kepentingan komersial di perairan Malaysia. Pada bahagian pertama kajian ini, variasi bentuk morfologi di kalangan 12 spesies famili Penaeidae, terutamanya dari pantai barat laut Semenanjung Malaysia telah dikaji. Ini dicapai berdasarkan Morfometrik Geometri (GM) dari 18 penanda homolog, dianalisis dengan Analisis Komponen Prinsip (PCA) dan Analisis Variat Berkanun (CVA) dalam perisian Morpho J. Variasi bentuk di kalangan individu spesies udang dijelaskan oleh bentuk badan, kepala dan ekor telson. Empat komponen pertama menyumbang 76.24% dan 78.47% untuk PCA dan CVA. Terdapat kecenderungan untuk spesies yang berkait rapat untuk berkelompok bersama, walaupun tidak sepenuhnya konsisten. Untuk menilai isyarat filogenetik, data morfometrik telah dianalisis dengan memasukkan ketiga-tiga pohon filogenetik (Neighbor Joining -NJ, Maksimum Likelihood-ML dan Bayesian Inference-BI) yang dihasilkan daripada mitokondria sitokrom oksidase I (COI) pada 12 spesies udang. Hasil analisis menunjukkan tiada kesignifikanan (tiada isyarat filogenetik) untuk NJ tetapi isyarat filogenetik signifikan (kepentingan evolusi) bagi ML dan BI yang menunjukkan perbezaan bentuk di antara semua 12 spesies udang penaeid berkaitan dengan sejarah evolusi mereka. Ketidakseragaman ini boleh dijelaskan kerana pohon NJ cenderung kepada kesilapan melibatkan analisis perbezaan tempoh waktu yang lebih tinggi, sedangkan ML dan BI sangat sesuai untuk pembinaan semula pokok filogeni dan menggunakan model evolusi jujukan ke atas

data. Pada bahagian kedua, kajian genetik populasi dilakukan pada *P. semisulcatus* dari Selat Melaka (SOM), Laut China Selatan (SCS), Laut Sulu (SS) dan Laut Celebes (CS). Lima ratus empat puluh lapan pasang bes gen mitokondria COI dan 571 pasang bes gen kawasan kawalan (CR) dianalisis ke atas 263 spesimen dari 15 lokasi. Perbezaan genetik (Tamura 3-parameter) untuk COI adalah 0.1% hingga 7.2% dan CR 2.3% hingga 21.7% di kalangan populasi. Bagan Pasir (BGP) yang berlokasi di tengah SOM paling berbeza dari populasi lain (COI: 3.3-4.2% ; CR: 7.1-16.5%). Data genetik telah membezakan populasi kepada dua kumpulan; Keturunan 1 (pantai timur Semenanjung Malaysia, Sarawak dan Sabah) dan Keturunan II (pantai barat Semenanjung Malaysia dengan pengecualian BGP dan Batu Pahat (BPT)) seperti yang ditunjukkan dalam semua analisis statistik yang berkaitan. Populasi pantai barat (SOM) Bagan Pasir (BGP) dan Batu Pahat (BPT) berkelompok di Keturunan 1 di luar jangkaan. Populasi Bagan Pasir dipercayai merupakan kolam campuran gen di antara kedua-dua keturunan tersebut. Analisis kepelbagaian genetik menunjukkan kepelbagaian nukleotida adalah tinggi, π (Keturunan I: COI: 3.4%, CR: 7.4%) (Keturunan II: COI: 3.8%; CR: 12.6%) dan kepelbagaian haplotip yang tinggi, *h* (Keturunan I: COI: 0.81; CR: 1.0) (Keturunan II: COI: 0.57; CR: 0.99). Analisis ketidaksepadanan menghasilkan taburan unimodal bagi perbezaan pasangan di antara haplotip di dalam kedua-dua keturunan yang mengesahkan bahawa kedua-dua keturunan telah mengalami perkembangan populasi yang mendadak. Penemuan kajian ini bukan sahaja penting untuk pelan pengurusan strategik populasi liar dan kultur *P. semisulcatus* tetapi juga untuk pengurusan dan pemuliharaan keluarga Penaidae spesies di perairan Malaysia.

PHYLOGENY OF PENAEID SHRIMPS AND POPULATION GENETIC STRUCTURE OF THE GREEN TIGER PRAWN (*Penaeus semisulcatus*) IN MALAYSIAN WATERS

ABSTRACT

This study reports on the phylogeny of selected penaieds and population genetics of the commercially important Green Tiger Prawn (*Penaeus semisulcatus*) in the Malaysian waters. In the first part of this study, morphological shape variations among 12 species of family Penaeidae, mainly from northwest coast of Peninsular Malaysia were investigated. This was achieved based on the Geometric Morphometrics (GM) of 18 homologous landmarks, analysed with Principal Component Analysis (PCA) and Canonical Variate Analysis (CVA) in Morpho J software. The shape variations was attributed to body shape, carapace head and telson tail among individuals. The first four components accounted for 76.24% and 78.47% for PCA and CVA, respectively. There is a tendency for closely related species to cluster together, although not absolutely consistent. To assess the phylogenetic signal, the morphometric data was mapped onto three phylogenetic trees (Neighbour Joining -NJ, Maximum Likelihood- ML and Bayesian Inference- BI) generated from the partial mitochondrial Cytochrome oxidase Subunit 1 (COI) on the same 12 species. Results revealed non significance (no phylogenetic signal) for NJ but significant phylogenetic signal (evolutionary significance) for ML and BI which suggest shape difference among all 12 penaeid prawn species was related to their evolutionary history. This discrepancy could be explained due to NJ tree are prone to errors when dealing with deeper divergence times, whereas ML and BI tree are ideal for phylogeny tree reconstruction which apply a model of sequence evolution on the data. In the second part of the study, a population genetics study was conducted on *P. semisulcatus*

from Strait of Malacca (SOM), South China Sea (SCS), Sulu Sea (SS) and Celebes Sea (CS). A 548 base pair region of mitochondrial COI and 571 base pair of control region (CR) were analysed among 263 specimens from 15 locations. Genetic divergences (Tamura 3-parameter) for COI was 0.1% to 7.2% and CR 2.3% to 21.7% among the populations, with Bagan Pasir (BGP) in central SOM being most genetically different from other populations (COI: 3.3-4.2%; CR: 7.1-16.5%). Genetic data differentiated the populations into two groups; Lineage 1 (East coast of Peninsular Malaysia, Sarawak and Sabah) and Lineage II (west Coast of Peninsular populations with the exception of BGP and Batu Pahat (BPT)) as revealed by all related statistical analyses. The west coast populations of Bagan Pasir (BGP) and Batu Pahat (BPT) unexpectedly clustered in Lineage 1. The Bagan Pasir population is believed to be a mixed gene pool between the two Lineages. The genetic diversity analysis showed high nucleotide diversity, π (Lineage I: COI: 3.4%; CR: 7.4%) (Lineage II: COI: 3.8%; CR: 12.6%) and high haplotype diversity, h (Lineage I: COI: 0.81; CR: 1.0) (Lineage II: COI: 0.57; CR: 0.99). A mismatch analysis produced a unimodal distribution of pairwise differences between haplotypes in both Lineages, supporting evidence from other demographic statistics that both Lineages had undergone sudden expansion. The findings of this study are not only important for the strategic management plan of wild and cultured stocks of *P. semisulcatus* but also for the management and conservation of species family Penaidae in Malaysian water.

CHAPTER 1
GENERAL INTRODUCTION

1.1 Introduction

Prawns of the family Penaeidae are an ecologically diverse group of species that live in estuarine and marine environments throughout the world's oceans (Gulland and Rothschild, 1984, Williams, 1984, Perez Farfante and Kensley, 1997). They widely contribute to the global crustacean fisheries and provide the basis for large aquaculture industries worldwide (Cai and Leung, 2006, Fast and Lester, 2013). According to De Grave et al. (2009), the classification of decapod crustacean consists of 233 families with 2,725 genera and an estimated 14,756 species. Penaeid prawns are the most economically important among all crustaceans (Holthuis, 1980, Dall et al., 1990, Perez Farfante and Kensley, 1997, Chan, 1998). Due to their ecological and economic importance, this family has been the subject of considerable biological and genetic research.

Mitochondrial DNA (mtDNA) is an effective molecular marker and widely used for evolutionary, genetic diversity and population genetic studies in fisheries management (Kyle and Boulding, 2000, Wilke and Davis, 2000, Collin, 2001, Marko, 2004) due to its high sequence variability compared to most single copy nuclear genes (Brown et al., 1979). The whole genome performs as a single, non-recombining locus where all sites share a single genealogical history since mtDNA genes are maternally inherited (Mcmillen-Jackson and Bert, 2003). It is also useful for interspecific and intraspecific phylogeographic analyses involving population history, demography and spatial patterns of genetic diversity (Wilson et al., 1985, Avise, 2000) as well as reconstructing phylogenetic relationships (Palumbi, 1997). Innumerable researches on phylogenetic relationships among selected penaeoids have been examined based on

mtDNA COI and 16S rRNA (Tong et al., 2000, Chan et al., 2008, Voloch et al., 2009, Chowdhury et al., 2018). Due to their rapid evolution, lack of introns and recombination, both genes have been widely applied in phylogenetic studies (Avise, 2012) and has also proven its efficiency to resolve species ambiguity (Chowdhury et al., 2018). Despite being widely studied, detailed study on the phylogenetic relationships of Penaeid prawn species between species and genera are limited worldwide and also in Malaysia.

This study also investigated the geometric morphometrics discrimination of 12 species of family Penaeidae employing mitochondrial DNA analyses. Geometric morphometrics (GM) is a low cost and reliable method for identification and comparing organisms based on shape variation (Corti, 1993, Rohlf and Marcus, 1993) and is a useful complement to molecular data particularly in situations when the facilities for the latter technique is not available. This method creates a set of shape variables that can be applied for statistical analysis which describes shape difference patterns from the data (Adams et al., 2013, Klingenberg, 2013). Thus, it provides insights to changes in body shapes or anatomical characters in response to environmental or genetic factors. Morphometric studies have been effectively used for describing variation in shapes of diverse populations and species over variable geographical distances and regions in crustaceans (Rufino et al., 2006, Konan et al., 2010, Silva et al., 2010, Srijaya et al., 2010) and freshwater decapods (Giri and Collins, 2004, Giri and Loy, 2008, Barría et al., 2011, Idaszkin et al., 2013). Most studies of GM analysis on prawns have focused on freshwater species (Zimmermann et al., 2012, Torres et al., 2014). However, geometric morphometric studies in marine prawn species is still limited. To date, there is a lack of a comprehensive study on morphometric analysis of Malaysian marine prawns. A few investigations have been

reported in Malaysia, mostly applying traditional morphometric studies. These include studies in the giant tiger prawn, *P. monodon* (Aziz, 2011), banana prawn, *P. merguensis* (Aziz, 2011), green tiger prawn, *P. semisulcatus* (Jahromi et al., 2018) and freshwater prawn, *Macrobrachium rosenbergii* (Ar et al., 2016).

Penaeus semisulcatus De Haan, 1844 also called the green tiger prawn or grooved tiger prawn is one of the most commercially important species of prawns in the genus *Penaeus* and is widely distributed across the tropical Indo-West Pacific region (Chan, 1998). They are mainly caught offshore by trawl nets or drift nets (Holthuis, 1980, Chan, 1998, Annual Fisheries Statistics, 2015). In Malaysia, the prawn fishery is one of the most important components of the marine capture fishery. Based on the Annual Fisheries Statistics (2015), the total marine fish landing for tiger prawn is 1,309 tonnes comprising of landings from Sabah (713 t -54%), West Coast of Peninsular Malaysia (393 t), East Coast of Peninsular Malaysia (119 t), Federal Territory Labuan (56 t) and Sarawak (29 t). In terms of state contribution for the tiger prawn landing in 2015 (tonnes), Sabah ranked first with 54% followed by Selangor, 15% and Perak, 10%.

Several molecular studies on *Penaeus semisulcatus* have been reported based on mitochondrial DNA and nuclear markers (Munasinghe and Senevirathna, 2015, Alam et al., 2016, Jahromi et al., 2018). Still, there is lack of studies focusing on this species worldwide and particularly in Malaysia. Most of the Penaeid*s* studies conducted in Malaysia were focused on *P. monodon* and *P. merguiensis* (Daud, 1995, Aziz, 2011, Aziz et al., 2011, Nahavandi et al., 2011a, Nahavandi et al., 2011b). In general, Daud (1995) suggested that both species showed genetic distinction between the west (Strait of Malacca) and the east coasts (South China Sea), including southern populations of Peninsular Malaysia. She suggested that there is a major barrier to gene flow between

them, which may be related to the prevailing current patterns, variation in spawning periods and past biogeographical barriers in the region based on allozyme and mtDNA RFLP analyses.

1.2 Problem statement

To facilitate fishery stock assessment, stock identification of a species helps to discriminate among taxa of family Penaeidae through body size and shape differences by using morphological differentiations analyses. This study is essential for the fisheries management to achieve sustainable yield, avoid recruitment failures and rebuild overfished stocks and to conserve threatened and endangered species (Rawat et al., 2017). Studies on morphological differences among penaeid prawn species by Aktas et al. (2006) revealed uncertainty of the taxonomic status of the species under investigation; *Melicertus kerathurus*, *Metapenaeus monoceros* and *Penaeus semisulcatus.* They recommended further investigation of this penaeid prawn species with more intensive study of morphological differences. The current study is considered the first record of geometric morphometric information in family Penaeidae inhabiting Malaysian waters and would be invaluable in addressing the current dearth of information. By combining with molecular DNA analysis, the current study can contribute additional information on the relationship between molecular and morphological evolution through the assessment of phylogenetic signal of the twelve Penaeid prawn species.

One of the important aspects for fisheries and aquaculture organisms is the knowledge on population genetics. It is important to identify reproductively isolated and genetically differentiated population within a species for fisheries management

(Klinbunga et al., 1998) in order to conserve and sustain the existing resources of exploited species. Brooker et al. (2000) had observed potential stock differentiation in *Penaeus monodon* in northeastern Australia. However, documentation on the population genetics of this species within Malaysian waters is limited although there have been several recent studies of the green tiger prawn, *P. semisulcatus* in other regions (Munasinghe and Senevirathna, 2015, Alam et al., 2017, Jahromi et al., 2018). A recent study conducted by Jahromi et al. (2018) in the Persian Gulf and Penang waters reported two morphotypes of *P. semisulcatus* described hitherto in the Persian Gulf, namely the banded and non-banded antennae morphotypes, but the non-banded specimens were more abundant in Iran waters. Two subclusters distinctly grouped the Iranian from the Malaysian samples in the banded antennae lineage suggesting that a wide scale geographic variation between them.

This study aimed to quantify between molecular and morphological evolution through the assessment of phylogenetic signal relationships. This study will also assess intraspecific genetic structure of the commercially important green tiger prawn, *P. semisulcatus* within the Indo-Pacific region, with focus on Malaysian waters; Straits of Malacca (connects the Pacific Ocean in the east with the Indian Ocean in the west), South China Sea, Sulu Sea and Celebes Sea. Morphometric variability among selected species will also be conducted to complement existing morphological data. The information obtained from this study will address the wide gaps in the molecular and morphometric data records for this species and family, particularly in this country. Furthermore, this study will provide a better understanding of the genetic variations and morphological differences of the species, which is very valuable for long-term management and conservation strategies for the species.

1.3 Objectives

Thus, to address the issues described above, this study is aimed to achieve the following objectives:

1. To investigate morphometric variation among selected taxa of family Penaeidae in Malaysian waters based on Geometric Morphometrics
2. To analyse population genetic structure of the green tiger prawn, *Penaeus semisulcatus* in Malaysian waters based on mitochondrial cytochrome oxidase subunt 1 (COI) and control region.

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction to marine prawns (Malacostraca; Penaeoidea; Penaeidae)

2.1.1 Prawns from family Penaeidae

Shrimps or prawns constitute a large group of crustaceans, varying in sizes from microscopic to about 35 cm of body length (Chaitiamvong and Supongpan, 1992, Chan, 1998). Both shrimps and prawns taxonomically belong to the swimming group of decapoda, class Malacostraca. Generally, they have laterally compressed body and the pleopods are well formed. Their first abdominal segment called pleuron is well developed and usually has pointed telson (Chan, 1998). The terms use between "shrimp" and "prawn" have no specific reference to any studied taxonomic groups. According to (Chan, 1998), the term "prawn" is frequently used for larger species and "shrimp" used to smaller species, but there are no clear differences between both terms.

To date, De Grave et al. (2009) has classified decapod crustacean into 233 families with 2,725 genera and an estimate of 14,756 species based on compilation of the scientific literature. Among these are the superfamily Penaeoidea, which is composed of five families, 1) Aristeidae 2) Benthesicymidae 3) Penaeidae 4) Sicyoniidae and 5) Solenoceridae (De Grave and Fransen, 2011). Most commercial prawns belong to the superfamily Penaeoidea. Penaeidae is the largest family of marine crustacean in the suborder Dendrobranchiata within the superfamily Penaeoidea. Species of this family are typically of moderate to large size and frequently occur abundantly in shallow waters along the continental shelf. They are widely fished by trawls, seines, set nets, traps, and artisanal gear (Chan, 1998). They are locally consumed or exported and are normally marketed fresh or frozen or

sometimes live, except *Acetes* which is often processed into prawn paste (Chan, 1998). The members of this family contribute the largest number of commercial prawn and valuable species throughout the warm waters of the world (Grey et al., 1983). They grow rapidly and live up until about one year. The taxonomic hierarchy of Penaeidae according to ITIS (Integrated Taxonomic Information System) is as below:

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Superclass: Multicrustacea
Class: Malacostraca
Subclass: Eumalacostraca
Superorder: Eucarida
Order: Decapoda
Suborder: Dendrobranchiata
Superfamily: Penaeoidea
Family: Penaeidae

Presently, 32 genera have been recorded in the family Penaeidae (De Grave and Fransen, 2011). The most prolific among them, genus *Penaeus* Fabricius, 1798 is composed of various groups of marine prawns that distribute mainly in the tropical and subtropical areas but also expand into temperate coastal waters in certain areas (Lavery et al., 2004). They are found in abundance along the shallow coastal waters but some species are found in open waters. This genus is the most economically important group among the shrimps and prawns (Holthuis, 1980, Dall et al., 1990, Chan, 1998, Perez Farfante and Kensley, 1997). Within this genus, *P. merguiensis* and *P. monodon* are the two most important species caught. The green tiger prawn, *P. semisulcatus* is also commercially important. *Penaeus* shrimps are important in the aquaculture industry of many nations, especially *P. monodon* (Chan, 1998). The second most important prawn genus is *Metapenaeus*. This genus is also widely used for culture, normally in ponds. The genus *Parapenaeopsis* is the third most

commercially important genus. They are often caught as bycatch in prawn fisheries and are common in the western part of the Western Central Pacific. Other genera appear not to be plentiful as these three genera although species of *Metapenaeopsis* and *Trachypenaeus* are often caught along with the main catches in the prawn fisheries (Chan, 1998). Both these genera have some commercial importance. Studies of deep-water trawling operations have observed the presence of two other genera, *Parapenaeus* and *Penaeopsis* although they are not widely caught. The genus *Parapenaeus* is sometimes caught in substantial amount and probably have some limited importance in fisheries. Members of the other genera such as *Atypopenaeus* are usually too small and not consumed. *Heteropenaeus* and *Trachypenaeopsis* are small and live in coral reefs, while *Funchalia* is a deep-sea pelagic shrimp genus- none of these are of economic importance (Chan, 1998).

2.1.2 The Biology of the Green Tiger Prawn, *Penaeus semisulcatus* (De Haan 1844)

2.1.2(a) *Penaeus semisulcatus :* Synonymy and vernacular name

Penaeus semisulcatus De Haan, 1844 also called the green tiger prawn or grooved tiger prawn is a commercially important species of prawn in the genus *Penaeus* (Figure 2.1). Previously, this species has been misidentified as *P. ashiaka* Kishinouye, 1900, *P. monodon manillensis* Villaluz and Ariola, 1938, and *P. semisulcatus paucidentatus* Parisi, 1919 (Chan, 1998).

According to Holthuis (1980), this prawn is known by numerous local names namely green prawn (South Africa), Kamba (Kenya), Rebian (Arabian), Kalri (Pakistan), Kuma-ebi (Japan), Fa ha or flower prawn (Hong Kong), Kung kula lai (Thailand), northern tiger prawn (Australia) and Kastanie (Israel). In Malaysia, it is called 'udang harimau' (Ahmad and Mohd Isa, 1986).

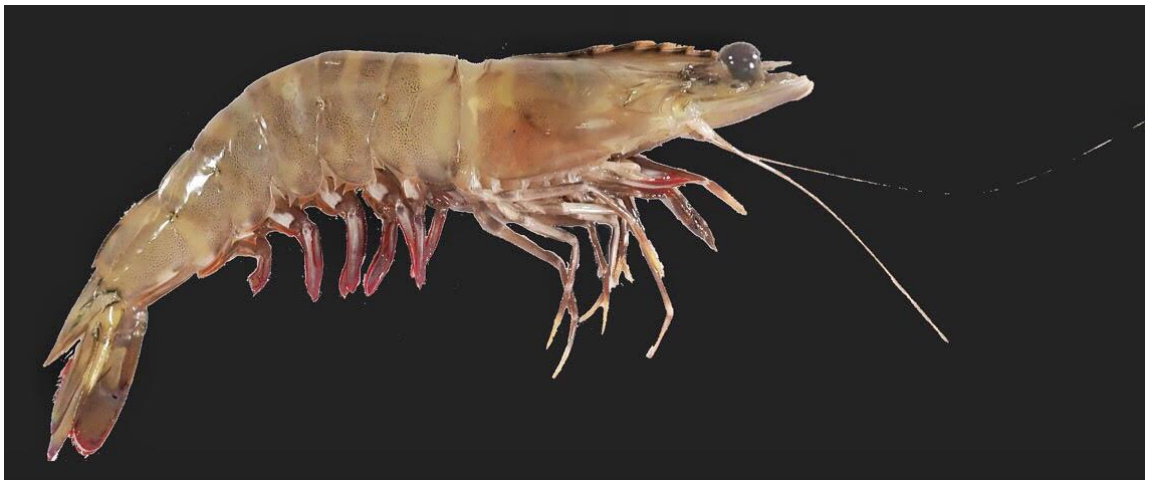

Figure 2.1: Species *Penaeus semisulcatus* used in this study.

2.1.2(b) Physical features, body colouration and size of the prawn

The green tiger prawn has well-defined grooves and crest at the carapace. It has a rostrum that is armed with 6 to 8 upper teeth and three lower teeth. Its postrostral crest is well developed and extend to the posterior margin of the carapace along with a distinct median groove. The adrostral crest reaches the postrostral tooth. The gastrofrontal crest is absent but it has a long hepatic crest extending behind the antennal crest. In addition, this species has an exopod on the fifth leg. It has a short distomedian projection of petasma in males and the thelycum of females form two sub-oval lateral plates with tumid lips. In addition, the thelycum has a sub-triangular anterior process with raised edges. The telson of this prawn is developed without lateral spines (Chan, 1998).

The body colour of this prawn is reddish brown to pale brown or dark green. The carapace is covered with mud yellow transverse bands and the abdomen including tail fan is greyish brown with mud yellow cross bands. This species has a pair of light brown eyes with numerous black dots. The antennal flagella are alternated with white and brown bands. Both legs or pereiopods and pleopods are reddish in colour coated with a few white marks. The uropod is dark reddish brown (Chan, 1998).

The maximum body length of this prawn for females is 25 cm and males is 18 cm. Normally the size is between 13 and 18 cm, respectively (Grey et al., 1983, Chan, 1998) with the female prawns usually larger than males. A study conducted in the Pilar and Capiz Bays reported the minimum sizes at sexual maturity for male and female green tiger prawn at 12.3 cm and 14.5 cm, respectively (Villarta et al., 2007). Both male and female penaeids exhibit the highest growth rate during the first three months of life but with increase in age the growth lengths slowly decline (Villarta et al., 2007).

2.1.2(c) Habitat and distribution

The green tiger prawn inhabits the continental shelf from the coastline to depths from 2 to 130 m but normally less than 60 m over the sandy and muddy substrates (Holthuis, 1980, Chan, 1998). The juveniles of this prawn are often found in shallow waters associated with seagrass beds (Grey et al., 1983, Chan, 1998) and also found on the coral reef platforms (Chan, 1998) while the adults live in the deeper waters (Grey et al., 1983).

This species is widely distributed in Indo-West Pacific from Red Sea, East and South East Africa to Japan, Korea, the Malay Archipelago and northern Australia (Holthuis, 1980, Dall et al., 1990, Chan, 1998) (Figure 2.2). In eastern Atlantic, this species has reached the eastern Mediterranean through the Suez Canal (Holthuis, 1980, Dall et al., 1990, Chan, 1998) and can be found in Egypt, Israel, Lebanon, Syria and Southern Turkey along the coasts (Holthuis, 1980). In Australia, the green tiger prawn dominates northern Australia from Bowen (Queensland) through the Gulf of Carpentaria, Northern Territory to northern Western Australia (Grey et al., 1983). In Malaysia, this species can be found along the West Coast and East Coast of Peninsular

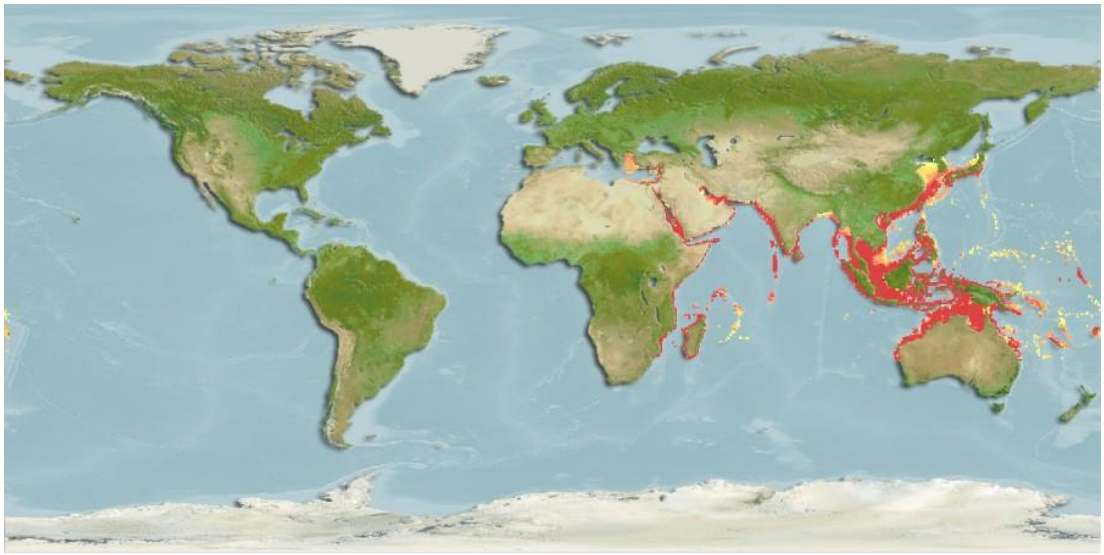

Figure 2.2: Distribution of *Penaeus semisulcatus* throughout the Indo-West Pacific. Image retrieved from http://www.sealifebase.org.

2.2 Morphological studies using geometric morphometrics

Morphometrics is the study of variation of size and shape and its covariation among the studied variables (Bookstein, 1997, Dryden and Mardia, 1998). Through the years, morphometric stock identification studies have highlighted the historical development of morphometric analysis, which appears as a complex discipline with applications in many biological fields of study (Cadrin, 2000). Thus, the analysis of shape and size, upon which morphometric analysis are dependent on, play an important role in various areas of biological research (Adams et al., 2004, Zelditch et al., 2012a).

In the late 1960's and 1970's, biometricians introduced multivariate statistical tools to describe patterns of shape variation within and among groups in traditional morphometrics (Marcus, 1990, Reyment, 1991), also known as multivariate morphometrics (Blackith and Reyment, 1971). This technique was and still is based on employing multivariate statistical analyses (e.g., principal components analysis, canonical variate analysis, discriminant function analysis, or multivariate analysis of variance) to sets of morphological variables measured (Parsons et al., 2003). In the 1980's, fundamental changes were introduced in terms of data collection and analyses. The changes were more focused on the coordinates of landmarks and geometric information compared to traditional morphometrics where measurements were made from random "convenient" anatomical parts with no consideration for shape variation. Based on this approach, the multivariate analysis results can be displayed as a configuration of landmarks representative of the original spatial shape of the organisms rather than only statistical scatter plots. This new approach introduced by Rohlf and Marcus (1993) is called "geometric morphometrics". Geometric morphometrics is a landmark-based geometric morphometric methods of two or three-

dimensional coordinates of biologically definable landmarks or anatomical structures of the organism (Adams et al., 2004). Its objective is to understand factors such as ecology and environment, underlying evolutionary changes that are precursors of variability of shapes among species (Adams and Otárola-Castillo, 2013).

According to Adams et al. (2013), the first step to be carried out in the geometric morphometric technique is to define a set of two or three dimensional landmark coordinates from each specimen. Next, a generalized Procrustes analysis (GPA) (Gower, 1975, Rohlf and Slice, 1990, Klingenberg and McIntyre, 1998, Rohlf, 1999) is applied to all specimens to superimpose the configurations of landmarks to produce a new set of shape variables. This procedure translates the specimens to the origin, scales to a unit of centroid size and rotates the specimens to minimize the total sums of squares deviations of the landmark coordinates from all specimens to the average configurations. The aligned coordinates, now called Procrustes shape coordinates define the location of each specimen in a curved space which is related to Kendall's shape space (Rohlf, 1999, Slice, 2001). They are orthogonally projected into a linear tangent space producing Kendall's tangent space coordinates (Dryden and Mardia, 1993, Dryden and Mardia, 1998, Kent and Mardia, 2001). Finally, to test biological hypotheses of geometric morphometrics, statistical analysis is performed using the Procrustes shape coordinates. For instance, principal component analysis (PCA) can be performed to determine the features of shape variation in a specimen and as an ordination analysis for analyzing the arrangement of specimens in morphospace. A canonical variate analysis (CVA) can contribute a divergent type of ordination analysis, which can maximize the separation of specified groups, i.e. species or/and ecotypes (Klingenberg, 2011). According to Klingenberg et al. (2002), the shape variation patterns can be differentiated between groups by a matrix

correlation and statistically estimated with permutation test matrix. Another important aspect of morphometric studies is to test the covariation of shape with other variables. For example, multivariate regression analysis can be applied to estimate relationships between variables or allometry, for example shape changes over time could be determined by regressing shape on size or on time (Monteiro, 1999, Drake and Klingenberg, 2008).

2.2.1 Applications of geometric morphometrics in marine prawns

The morphological characteristics in decapod crustaceans have been widely studied for various applications. These include in the setting up of suitable management planning for threatened species protection or to identify and distinguish fish stocks (Cadrin, 2000). Numerous researches have previously used traditional morphometrics based on distances, angles and proportions to determine allometric growth relationships (Strauss and Bookstein, 1982, Rohlf and Bookstein, 1990, Adams et al., 2004, Natarajan et al., 2011), taxonomic relationships, morphological divergence (Wahidah et al., 2015, Teodoro et al., 2016, Barros-Alves et al., 2018) and stock identification (Tzeng, 2004, Freire et al., 2017). For example, traditional morphometric measurements in penaeid prawn was widely used in the past (Lester et al., 1990) (Figure 2.3). However, in the last two decades more advanced approaches like geometric morphometrics based on homologous landmarks have been applied for morphological analysis in crustaceans (Cadrin and Friedland, 1999, Rosenberg, 2002, Zimmermann et al., 2012, Torres et al., 2014). Geometric morphometrics (GM) is based on landmarks of anatomical points, which act as origins of projections forming a coordinate system. These are incorporated with superimpositions methods of homologous points into mathematical spaces to remove the main sources of bias that

are present in traditional analyses such as translation, scaling and rotational effects. Hence, the comparisons are united only in the geometrical shape of the objects (Rohlf and Marcus, 1993, Adams et al., 2004). Only a few past documentations have successfully used geometric morphometrics in penaeid prawns. These include studies on the marine prawns such as the pink prawn, *Farfantepenaeus brasiliensis* and *F. paulensis* (Teodoro et al., 2016), sea-bob prawn, *Xiphopenaeus kroyeri* (Gusmão et al., 2013, Bissaro et al., 2013) and white prawn, *Litopenaeus vannamei* (Accioly et al., 2013) as well as the freshwater prawns, *Macrobrachium australe* (Zimmermann et al., 2012) and *Macrobrachium borellii* (Torres et al., 2014). Furthermore, to determine if the shape data has any phylogenetic signal i.e if the shape has phylogenetically evolved across the genus (Ferdous, 2013), the dendogram generated from the landmark data could be mapped onto a phylogenetic tree of the molecular data of the species in common.

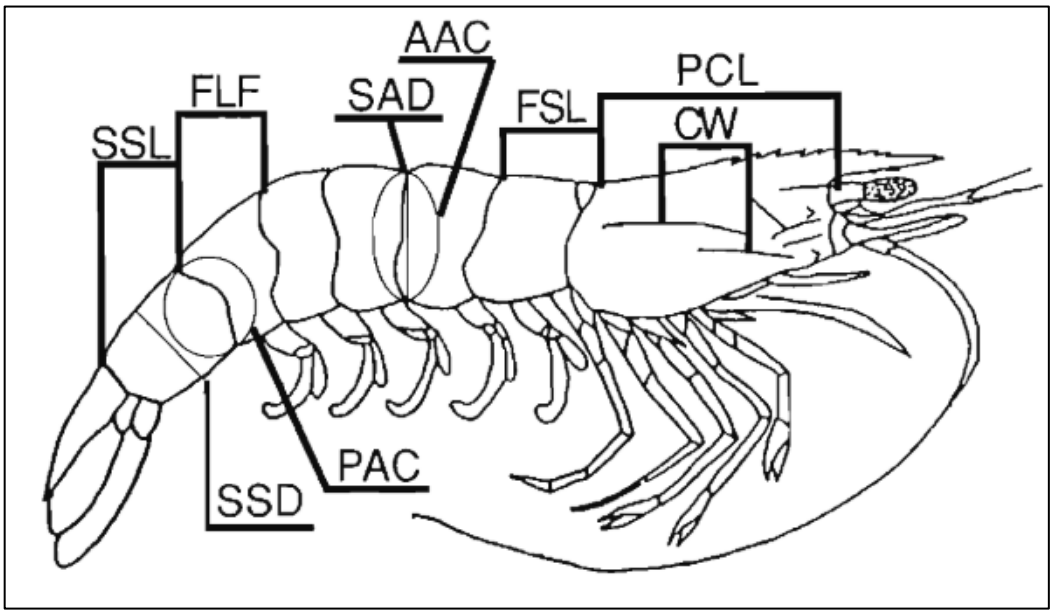

Figure 2.3: Traditional morphometric measurements in penaeid shrimp based on Lester et al. (1990) with no consideration of shape

2.3 Genetic diversity in species and populations

2.3.1 The uses of mitochondrial DNA (mtDNA)

Mitochondria are cytoplasmic organelles in the eukaryotic cells and have their own DNA. They contain several genes that are important for cell respiration and other functions (Park and Moran, 1994). These mitochondrial genes or mitochondrial DNA (mtDNA) is widely used in various molecular research such as for population and evolutionary studies (Wilson et al., 1985, Avise, 1986). The mtDNA has several characteristics that confers it several advantages over single nuclear genes. It is haploid and have a compact size and is maternally inherited (Gyllensten et al., 1991, Magoulas and Zouros, 1993). Since mtDNA is haploid and transmitted maternally, these factors merge to reduce the effective population size of mtDNA to 1/4 of the nuclear genes of the same organism (Nei and Tajima, 1981, Mcmillen-Jackson and Bert, 2003). This smaller effective population size allows genetic drift in isolated gene pools to be more easily detected in mtDNA than in nuclear DNA. Furthermore, mutations in mtDNA have been observed to be more rapidly accumulated than in nuclear genes of numerous organisms (Brown et al., 1979, Lynch and Jarrell, 1993). Due to its large variability and sensitivity to genetic drift, and ease of detecting population distinction of a species, the mtDNA is appealing for population genetics (Avise, 1986, Zwanenburg et al., 1992, Geller et al., 1993), phylogeography and to deduce underlying historical demographic processes (Avise, 2000).

2.3.2 Applications of mtDNA genes in population study

MtDNA has been successfully applied to resolve population genetic structure and genetic variability in various marine taxa (Kyle and Boulding, 2000, Wilke and Davis, 2000, Collin, 2001, Marko, 2004) including crustaceans (Chu et al., 2003) due to its rapid rate of evolution, lack of recombination and maternal inheritance (Hoelzel et al., 1991). Mcmillen-Jackson and Bert (2003) stated that mtDNA has also been useful for both intraspecific population differentiation and phylogenetic relationships studies of penaeid prawn for applications in preserving fisheries production and sustainable harvesting of populations (Park and Moran, 1994). The mtDNA Cytochrome C Oxidase Subunit I (COI) gene is a coding gene, widely used in many studies due to its robustness, universal primer pair and has been successfully applied for many invertebrate species (Folmer et al., 1994). The mtDNA control region or D-loop is a non-coding protein and is widely utilised for population studies due to its high variability and rapid evolution (Upholt and Dawid, 1977, Walberg and Clayton, 1981, Chu et al., 2003). The gene segment size differs among animal species ranging from -200 to 4,100 base pair (Brown, 1985). Simon (1991) stated that this region is an ideal marker to determine the geographical patterns of genetic variation within and between prawn populations since it has been successfully proven to be the most variable region for both vertebrates and invertebrates. Both COI and D-loop genes have been successfully utilised to resolve the genetic diversity and population structure in numerous penaeid species (Benzie et al., 2002, Chu et al., 2003, Hualkasin et al., 2003, Tsoi et al., 2007, You et al., 2008, Li et al., 2009, De Croos and Pálsson, 2010).

2.4 Genetic diversity of marine prawns in the Southeast Asia region (Indo-Pacific)

Strong genetic divergences in various prawn species have been reported between Indian and Pacific Ocean or Indo-Pacific regions. Several studies have also been conducted on population and genetic diversity of penaied prawn species in the region such as in the giant tiger prawn, *Penaeus monodon* (Duda and Palumbi, 1999, Benzie et al., 2002, You et al., 2008, Waqairatu et al., 2012, Abdul-Aziz et al., 2015), green tiger prawn, *P. semisulcatus* (Alam et al., 2017), Kuruma prawn, *P. japonicus* (Tzong et al., 2004, Tsoi et al., 2007), brown tiger prawn, *P. esculentus* (Ward et al., 2006), banana prawn, *P. merguiensis* (Hualkasin et al., 2003, Wanna et al., 2004) and Indian white prawn, *P. indicus* (De Croos and Pálsson, 2010, Alam et al., 2015). Most of the studies indicated very strong genetic differentiation between populations from both oceans. In contrast, the Chinese white prawn, *P. chinensis* displayed lack of genetic differentiation in Northwestern Pacific (Zhaoxia et al., 2007, Li et al., 2009, Xiao et al., 2010, Hui et al., 2012, Wang et al., 2014). Such low genetic differentiation was also discovered in the brown prawn, *Farfantepenaeus aztecus* (Mcmillen-Jackson and Bert, 2003) and pink prawn *F. duorarum* (McMillen-Jackson and Bert, 2004b) in southeastern United States.

CHAPTER 3

MORPHOLOGICAL AND MOLECULAR PHYLOGENY OF PENAEID PRAWNS (PENAEIDAE) INFERRED BY GEOMETRIC MORPHOMETRICS AND MITOCHONDRIAL CYTOCHROME C OXIDASE SUBUNIT I

3.1 Introduction

Morphological variation studies within and among species of penaeid prawns became popular in the 1970s, mainly for aquaculture purpose (Aktas et al., 2006). Morphological characteristics among species of penaeid prawns may differ due to genetic differences. Within a species, variability in growth, development, and maturation create variations in body shape. Thus, body shape constitutes one important set of features to differentiate the species or populations (Cadrin, 2000). However, data on morphological variation among crustacean species in regards to size, shape or other commercial characters are still limited (Aktas et al., 2006).

Quantitative morphological differentiation have been investigated through traditional methods of measuring from one anatomical landmark to another, where most often the same points of origin are repeatedly used (Bookstein, 1990, Marcus, 1990, Rohlf and Bookstein, 1990, Rohlf and Marcus, 1993, Adams et al., 2004) resulting in biasness as a particular region is over emphasized. These methods have since advanced to geometric morphometrics (GM) which is now widely applied as it contributes a robust technique for the study of shape variation and identification (Corti, 1993, Rohlf and Marcus, 1993) and this has now been further developed into more efficient mathematical design through sophisticated statistical software, like MorphoJ (Klingenberg, 2011). Several investigations have successfully applied geometric morphometrics in marine penaeid prawns, i.e. pink prawns, *Farfantepenaeus brasiliensis* and *F. paulensis* (Teodoro et al., 2016), sea-bob prawn, *Xiphopenaeus*

kroyeri (Gusmão et al., 2013, Bissaro et al., 2013) and white prawn, *Litopenaeus vannamei* (Accioly et al., 2013) as well as in freshwater prawns, *Macrobrachium australe* (Zimmermann et al., 2012) and *M. borellii* (Torres et al., 2014). Geometric morphometrics are efficient in detecting shape variation and may provide insights into the causes of variation within and between species such as sex, age, geographic region, disease, or phylogenetic relationships (Lawing and Polly, 2010).

In order to determine the shape variation of the penaeid prawns, the truss network system was applied in this study to construct a network on the prawn body (Lester et al. (1990)Aktas et al. (2006). The truss network system is a landmark-based technique using geometric morphometrics proposed by Strauss and Bookstein (1982). Truss network system, incorporated with landmark points is more effective for capturing information of an organism's body shape and consequently for stock and species identification (Strauss and Bookstein, 1982, Cavalcanti et al., 1999, Sathianandan, 2003). Morphometry based on truss network data has been applied in previous studies of other penaeid prawns, to elucidate taxonomic relationships (Aktas et al., 2006), stock identification (Rebello et al., 2014, Marini et al., 2017) and phylogenetic relationships (Rajakumaran et al., 2014).

3.1.1 Geometrics morphometrics as a tool to elucidate phylogenies

Another important aspect for understanding the relationship between molecular and morphological evolution is the phylogenetic signal (Felsenstein, 1988, Zelditch et al., 1995, MacLeod, 2002, Klingenberg and Gidaszewski, 2010, Zelditch et al., 2012b). With a null hypothesis of not having any phylogenetic signal in the data set, the morphological variation of penaeid prawn body shape is tested by mapping the body shape onto an independently derived molecular phylogeny (Klingenberg and Gidaszewski, 2010). Significant phylogenetic signal is concluded when genetically

closely related taxa is phenotypically more similar to each other than distantly related taxa, whereas weak phylogenetic signal is when taxa is more similar to genetically distant relatives or similarity is randomly distributed across phylogeny (Blomberg et al., 2003, Klingenberg and Gidaszewski, 2010). In addition, phylogenetic signal is a measure of the statistical dependence of trait values among species due to phylogenetic relationships (Revell et al., 2008). Many studies have applied geometric morphometrics data to contribute in phylogenetic information. For example, Armbruster et al. (2016) examined the potential for convergence between *Enteromius aspilus* and *E. guirali* from family Cyprinidae in Dja river in Cameroon, Congo, using geometric morphometrics in combination with molecular phylogeny. In another study, Ferdous (2013), developed a database of Cyprinid specimens to test for presence of phylogenetic signal in the geometric morphometric data by mapping the morphometric data onto the molecular phylogeny of Cyprinid data sets.

3.1.2 Problem statement

The study of shape analysis using this method is still lacking in prawn specimens. The aims of this study are firstly, to explore the potential of geometric morphometrics to discriminate among taxa of family Penaeidae through body shape differences. The diversity pattern among members of this family were evaluated with reference to current taxonomic classification. Secondly, phylogenetic signal in the geometric morphometric data was assessed by mapping the morphometric data onto a molecular phylogeny generated from COI gene. This current study provides new information of morphological variation in family Penaeidae using GM analysis.

3.1.3 Objectives

1. To investigate morphometric variation among 12 selected taxa of family Penaeidae in Malaysian waters based on geometric morphometrics (GM).
2. To determine the phylogenetic signal in geometric morphometric data for the 12 taxa from family Penaeidae by comparison with molecular data.

3.2 Materials and Method

3.2.1 Taxonomic sampling

A total of 149 individuals from 12 species of family Penaeidae collected from four landing sites from northwest Peninsular Malaysia were analysed for the geometric morphometric study. The prawn specimens were identified using the following references; FAO Species Identification Guide for Fishery Purposes (Chan, 1998), A guide to the Australian penaeid prawns (Grey et al., 1983) and A guide to penaeid shrimps found in Thai waters (Chaitiamvong and Supongpan, 1992). Whole samples were then subjected to geometric morphometric analysis. For conducting a molecular phylogenetic analysis, a single specimen per species was immediately iced or frozen after collection and later stored at -20ºC or preserved in 96% ethanol. A detailed list of specimens and sampling locations are given in Figure 3.1, Figure 3.2 and Table 3.1.

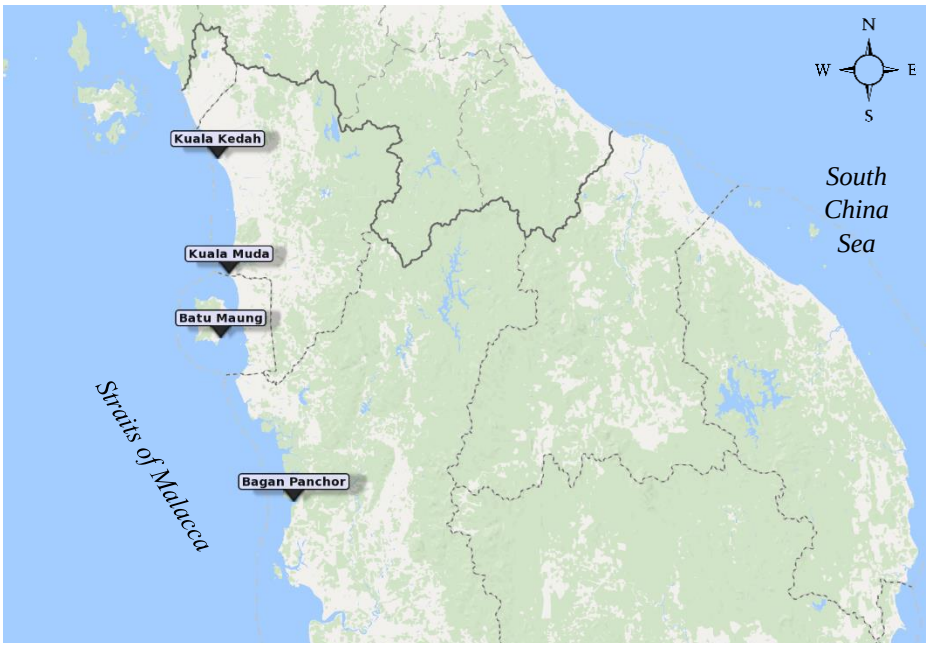

Figure 3.1: Sampling locations of 12 penaeid shrimp species.