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**Molecular Ecology of Two Commercially Important  
Crustacean Species, *Nephrops norvegicus* and  
*Macrobrachium rosenbergii*: Implications for the  
Management of Fisheries and Aquaculture**

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This thesis is submitted in fulfilment of the requirement of the degree of Doctor  
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Medical, Veterinary & Life Sciences

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## Abstract

Molecular ecology is one of the most important branches of evolutionary biology, and it uses the advantages of molecular techniques such as PCR-RFLP, sequencing, microsatellite analysis, and most recently the introduction of next generation sequencing, to address outstanding issues in the fields of population genetics and phylogeny. The genomic approach has been influential in providing new information relevant to traditional questions in ecology, such as genetic differentiation, speciation, species adaptation and others. The rationale of the present thesis was to incorporate the advantages of both the PCR-RFLP and sequencing techniques to gain information on the genetic variability of two commercially-important crustacean species, namely the Norway lobster *Nephrops norvegicus* and the giant freshwater prawn *Macrobrachium rosenbergii*.

*Nephrops norvegicus* exhibits large morphological variability even between adjacent populations. The first objective of this thesis was to investigate the genetic variability of *N. norvegicus* from three localities, the Clyde Sea area and North Minch (North Sea) as well as Icelandic waters. The phylogeographic study found no significant differentiation between populations from the studied areas ( $F_{ST}$ : 0.01819). This finding is consistent with outcomes from previous studies that *N. norvegicus* populations were not geographically structured. Outcomes from the present study strongly suggest that environmental factors, rather than genetic factors, are more likely to play a more significant role in the high morphological differentiation observed in this species.

A study of the most important freshwater crustacean species, *Macrobrachium rosenbergii* was then undertaken as a contribution to understanding the most complex biogeography in the world, the Indo Australian Archipelago (IAA). The IAA has incredible species richness and endemism and is the location of 4 out of 25 world's biodiversity hotspots, namely the Sundaland, the Philippines, Indonesia and Wallacea. Within the IAA is the location of Wallace's line and Huxley's line, the most abrupt faunal transition in the world that lies between the Sunda and Sahul shelves. The studied species used in the present thesis, *M.*

*rosenbergii* is an ideal model species as it has a wide geographical distribution across the IAA.

The present phylogeographic study screened the COI segment using the sequencing technique to study *M. rosenbergii* populations collected from eight locations in Malaysia in the peninsular and east of Malaysia. These populations exhibited high genetic differentiation ( $F_{ST}$ : 0.62503) mainly due to the sample from Sabah. However, the adjacent population (Sarawak) was similar to that in Peninsular Malaysia, even though Northern Sarawak showed sub-population differentiation from the main cluster (cluster I) indicating that the genetic diversity of Northern Sarawak was more restricted. Beside, cluster II observed in the study indicated and confirmed the recent aquaculture activities of restocking the Kedah, Perak and Sarawak populations.

Knowledge of the levels of genetic differentiation in *N. norvegicus* and *M. rosenbergii* could assist in the management of the species. *N. norvegicus* could be managed as one stock and conservation and recovery programme could be carried out based on the knowledge that all studied populations exhibited lack genetic differentiation within and between populations. In contrast, *M. rosenbergii* that possessed high level of genetic differentiation have to be managed separately, especially for a unique population such as the one in Sabah. The outcomes of this study could also be useful for future research in the conservation of wild population, as well as aquaculture management and product improvement purposes.

The finding of Sabah as a unique population could potentially be useful for aquaculture improvement programmes. One of the most important aspects was to examine whether the Sabah population possessed high resistance to disease infection. A preliminary study was conducted to investigate the susceptibility of a Malaysian wild population of *M. rosenbergii* to infection by the human food-poisoning bacterium *V. parahaemolyticus*. Nonetheless, the virulence stage of the bacterial strain, the status of the immune system of the host, the size and age of the experimental animals as well as the dose of injected bacteria might all have contributed to the inconclusiveness of the results. However, the principle of screening wild populations for disease resistance is sound, and may

lead to improvements in the quality of the broodstock used in the Malaysian aquaculture industry.

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## Preface

The rationale of the present thesis is that it has incorporated the advantages of two molecular methods, which are PCR-RFLP and the sequencing technique, for ecological studies to gain information on the genetic differentiation of two commercially-important crustacean species, *Nephrops norvegicus* (Section I) and *Macrobrachium rosenbergii* (Section II). Such applications of molecular methods for ecological studies constitute 'Molecular Ecology'. Molecular ecology is useful not only to explain the demographic history of the studied species but also could benefit the management of commercial species, improvement of aquaculture product and its sustainability as well as for biodiversity conservation purposes.

Phylogeographic comparison between the North Atlantic and Icelandic waters populations of *N. norvegicus* has been made to provide information on the genetic differentiation between the two studied populations. On the other hand *M. rosenbergii*, which has wide geographic distribution across one of the world's biodiversity hotspots, Sundaland located in Southeast Asia, is a model species to understand the complex biogeography of the Indo-Australian Archipelago (IAA). The IAA region is the location of the so-called Wallace's line and Huxley's line, which is a biogeographic barrier that has facilitated the distribution of species, creating a region that has extreme species richness and endemism on the two sides of the lines. The information of genetic differentiation of Malaysian populations of *M. rosenbergii* obtained has also been used to study the response of immune system of the prawn to the bacteria infection from *V. parahaemolyticus*.

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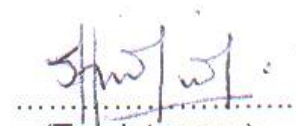
a blessing in my life, for their forbearance of my long hours at work that always continue at home which at times caused me to neglect them and sometimes ignore the 'mommy's duties'. To my precious gift, Umaira, thanks for always 'kept mommy accompanied'. To my parents, family and family in-law, thanks for the love and dhua' through the challenge, achievement and disappointment that has been part of this journey.

## **Author's declaration**

I declare that the work recorded in this thesis is entirely my own, with the following exception, and has not been submitted as part of a degree elsewhere.

### **Chapter 6: Isolation and confirmation of the bacteria strain**

Carried out by Fazirah Abdullah, Microbiology Department, University of Malaya.

A handwritten signature in black ink, appearing to read 'Hasnita Binti Che Harun', written over a horizontal dotted line.

Hasnita Binti Che Harun

March 2013

# Chapter 1

## 1.1 General introduction

The application of molecular genetic tools in the field of ecology provides the knowledge to understand genetic variation and population differentiation (Lindblom and Ekman, 2006). Genetic variation that is attributable to three importance events; DNA mutation, gene flow and sex reproduction, leads to the creation of population differentiation. Information on the genetics of natural populations is importance to the management of the commercial species as it is the only source to maintain the genetic variation of transplantation and aquaculture populations. The advantage of having high genetic variation in a population is that the genetically variable population will adapt to the changing environment better than those populations that not have genetic variation (Chauhan and Rajiv, 2010). Genetic variation and population differentiation are also among the important criteria which are needed to facilitate the aquaculture improvement programmes by identifying and manipulating the economically important traits such as reproduction, sex, growth and disease resistance.

Molecular genetic tools and techniques are useful to resolve genetic variation at different levels; species, population and individual (Chauhan and Rajiv, 2010). The application of these tools and techniques could benefit studies on the genetic of natural populations, genomics, breeding and production of novel traits (Kapusinski and Miller, 2007).

Genetic markers that have been intensively used in many studies are proteins (allozymes), mitochondrial (mtDNA) and nuclear DNA (nDNA) markers. Allozyme studies detect genetic variation as polymorphic proteins. Differences in protein sequence result from the differences in the amino acid composition that have different structures. The most common proteins used in the electrophoretic studies are enzymes (Kapusinski and Miller, 2007). Nevertheless, studies using allozymes markers have been reduced due to its limitations including low

repeatable rates and produce less sensitive and less reliable results (Belaj et al., 2003).

mtDNA markers have been successfully utilised in many research areas such as maternity testing, forensic, medical, phylogenetic and population genetic studies. Different regions of mitochondria could be used as genetic markers, which could be either a single gene or could also consist of a combination of several genes. The most popular single mitochondrial markers are the control region and cytochrome oxidase I, while genes such as *cyt b*, NADH1 and 16S rRNA or 12S rRNA can be combined as one marker to provide more information on the polymorphism site, if it exists. Choosing the most appropriate region for detection of polymorphism is very important in order to obtain a significant number of polymorphism sites to determine the level of polymorphism in the studied population. mtDNA is inherited from the maternal side (Chat et al., 1999, Zhao et al., 2004, Schwartz and Vissing, 2002). However, there are also some suggestions that the inheritance of mtDNA can involve the paternal side (Nakamura, 2010). The advantage of the maternal inheritance is the ability to use this marker to trace the phylogenetic position of the studied animal. Moreover, animal mtDNA is a rapidly evolving marker, which evolves 5-10 times faster than nuclear DNA, making it an excellent marker for phylogenetic and evolutionary studies (Brown et al., 1979). Factors that contribute to this rapid evolution include the fact that mitochondria lack both excision and recombination repair systems, but rely only on enzymes such as uracil-DNA glycosylase and AP endonuclease as well as 3' to 5' exonuclease activity of mitochondrial DNA polymerase for DNA repair system (Clayton et al., 1974). Moritz et al. (1987) reported that the three mechanisms that have led to the evolution of mitochondrial mtDNA were base substitution, length differences (duplication, deletion or replication slippage) and rearrangement of the genes. They agreed that recombination is rare or may even be absent, although in recent years this fact has been questioned in many studies (Ladoukakis and Zouros, 2001, Rokas et al., 2003, Hoarau et al., 2002).

On the other hand, genetic variation of nDNA markers is characterized either at single loci or simultaneously at multiple loci. Single loci nDNA markers are microsatellites (a 1-6 base-pair repetitive sequence) and single nucleotide polymorphisms (SNPs) (single nucleotide changes occur in DNA sequence).

Multilocus nDNA detects genetic differences that occur at multiple loci. Variation of DNA sequences at multiple loci produce different sizes of DNA fragment that can be detected with electrophoresis. Examples of multilocus nDNA markers are RADP and AFLP. The advantage of multilocus nDNA markers is that they do not require background information of the sequence, although these markers are less powerful compared to single locus nDNA markers.

Molecular genetic techniques commonly used to characterize the genetic markers as stated above are electrophoresis, polymerase chain reaction, restriction enzymes (PCR-RFLP) and microarray. Electrophoresis is applied to understand genetic variability at protein and DNA levels. Single locus DNA electrophoresis is determined from the pattern of the fragments that are labelled with fluorescent before undergoing gel electrophoresis. For the multilocus DNA electrophoresis technique, restriction enzymes are used to cut at specific regions and these are then amplified with specific PCR primers. Detection of variation is based on whether or not the DNA was cut and amplified, resulting in different banding patterns. The size of the fragments and the presence or absence of the bands provides information on the genetic variation of the studied species.

PCR is a technique that requires only very small or even degraded samples. Amplification of a specific region using specific primer produces millions of copies of DNA fragments. The DNA copies have to be processed by gel electrophoresis or subjected to further analyses such as DNA sequencing, cloning and etc.

A combination of polymerase chain reaction and restriction length polymorphism (PCR-RFLP) is a molecular technique commonly used to study genetic variability and phylogenetics. This method utilises restriction enzymes on the amplified sequence (PCR product) in order to detect DNA polymorphism at a specific site if any. PCR-RFLP is a reliable method that is efficient at detecting any variation that occurs at the recognition site of each of the restriction enzyme applied (Stamatis et al., 2004, Triantafyllidis et al., 2005, Vekemans et al., 1998). The basic principle of the PCR-RFLP method is that different restriction enzymes are applied, each of which will cleave at a specific site so that different fragment sizes will be produced when the alleles differ. The lengths of the fragments



produced depend on the availability of an adjacent recognition site, which in turn determines the distance between each of the cutting sites (Silva and Russo, 2000). The fragments with different sizes are then separated using electrophoresis, with the smaller fragments running faster than the larger fragments. Each variation found in the populations is used to generate the haplotype data. The different sizes of fragment produced reflect the pattern of restriction sites which will eventually reveal any polymorphism that exists in the studied populations. The haplotype profile can then be used as input data in downstream analyses.

DNA microarray or DNA chips is a method in which nucleic acid fragments are hybridised to produce a large set of oligonucleotide probes. Only nucleic acid fragment that are similar to the sequence of the probe will successfully bind to the DNA microarray, and can be used to determine gene sequence, genetic variation, expression and gene mapping.

Genetic tools and techniques are very important for identifying hybrids or for parentage studies using species-specific markers. Meanwhile, stock identification, particularly in commercial species, could benefit the management of the species based on their degree of homogeneity within stocks, the importance of reproductive isolation and their relevance for exploitation. Management of commercial species is important due to the unsustainable use of fish and fish stocks, pollution, habitat loss and various human activities that have reduced the genetics resources and variability.

The advancement of genetic tools such as gene mapping, quantitative trait loci (QTL), genome sequencing and microarray expression analysis have had a great impact on genomic studies. Gene mapping is defined as mapping of genes to specific locations on the chromosome (Brown, 2002). Genetic mapping is performed using DNA markers (RFLP, AFLP, RADP, microsatellite and SNPs). The ultimate goal of gene mapping is to clone the genes of interest for disease resistance, growth, reproduction, sex etc. and manipulate them for various purposes, especially to improve the quality of hatchery populations.

QTLs analysis is a statistical method that relates two components which are phenotypes and genetic data, in order to gain information of certain complex

phenotypes to specific locations on chromosome (Miles and Wayne, 2008). Many studies have used QTLs to map the genes of interest, for instance rapid growth of embryonic, disease tolerance and etc. QTLs analysis is carried out by crossing the selected parents to get the heterozygous (F1) individuals which are crossed again to obtain the F2 generation. The phenotypes and genotypes of the derived F2 generation are then scored. Theoretically, markers that are genetically linked with the traits of interest will segregate together with the trait value, whereas markers that are not linked with trait will not associate with phenotypes.

Genome sequencing reveals the complete genome sequences of that particular species. This technique provides the identification of all genes in organism and is a link between gene mapping and QTLs mapping.

Gene expression microarray provides a snapshot of all transcriptional activity that occurs in biological samples. The striking benefits of this advanced technique are the ability to reveal novel genes useful for understanding the aetiology of new diseases and the possibility of designing new diagnostic tools and to study the responses of patients to treatments. However, such microarray methods require both high technology machines, which are costly, and highly skilled operators, which could constrain their use.

Phenotypic variation could be arise due to environmental and/or genetic factors. Genetic factors existing in a population can be divided into additive and non-additive genetic variation. Non-additive genetic variation can be further divided into dominance and epistatic variation. Genetic tools could improve captive breeding programmes by manipulating the genetic variation exhibited in a population. Several breeding techniques such as mass selection, family selection and multiple trait selection are examples of using genetic tools for calculating the effect of additive genetic variation. Meanwhile hybridization is used to test for non-additive genetic variation. Other methods such as crossing, outcrossing and rotational line are useful to minimise the occurrences of inbreeding, whereas indirect selection, sib selection and progeny selection are crucial to calculate genetic variation in cases where the phenotype is difficult to measure. Genetic tools also being incorporated into another type of indirect selection process, termed the marker-assisted selection (MAS) technique, where DNA

based markers are used instead of other types of marker (e.g: biological, morphological, biochemical and cytological).

Production of novel traits is one of the most important factors in enhancing the genetics of aquaculture. Several techniques such as gene transfer and the creation of monosex populations have been practised. Gene transfer technology is the production of transgenic organisms by inserting the heterologous DNA into the nucleus of a target cell. A variety of techniques has been applied to facilitate the gene transfer processes such as microinjection, electroporation, sperm-mediated gene transfer, lipofection and retroviral infection. Among these, electroporation is the preferred technique in many laboratories because of its efficiency and simplicity (Sarmasik, 2003). The advancement of gene transfer technology provides many benefits, particularly in the aquaculture field, via the production of genetically-modified species. Gene transfer technology could promote growth rates by inserting an extra growth hormone (GH) gene, and could also produce disease-resistant lines. In addition, gene transfer can also be used for freeze resistance and feral pest control (Galli, 2002). The insertion of an antifreeze protein gene to produce animals that have the ability to tolerate the cold water environment could broaden the aquaculture options. On the other hand, many studies have been devoted to the creation of monosex populations, such as in giant freshwater prawn. The genetically-modified organism will have traits of commercial interest. For instance, the production of male-only populations of freshwater prawns creates large sized animal, for which a high price can be demanded. Meanwhile, the production of female-only freshwater prawns could reduce the size variability in the population.

The present thesis has incorporated various molecular genetic tools to study two commercially-important crustacean species. The PCR-RFLP method and sequencing techniques were used to characterize the genetics of a commercial fisheries species, namely the Norway lobster *Nephrops norvegicus*. Meanwhile, a sequencing technique was used to characterise the genetics of an aquacultured species, namely *Machrobrachium rosenbergii*. The studies were carried out to investigate the level of the genetic differentiation of these species collected from several localities. In addition, the present thesis also had the aim to understand the demographic history that shapes the genetics of these highly

exploited species. Lastly, based on the outcomes from the *M. rosenbergii* study, a disease challenge study was performed in order to gain knowledge on the susceptibility of *M. rosenbergii* to develop disease caused by *Vibrio parahaemolyticus* infection.

## 1.2 Chapter objectives

A general introduction to the importance of studying the genetic variability and population differentiation and its benefits to the management of commercial species marker is presented in **Chapter 1**.

An introduction to the studied species in Section I, *N. norvegicus* as well as information on the differentiation observed in this animal is provided in **Chapter 2**.

In **Chapter 3**, three geographically isolated locations (Clyde Sea area, North Minch and Icelandic waters) were chosen to characterize the genetic structure of *N. norvegicus* within and among populations. A comparison between the two methods (PCR-RFLP and sequencing) is made, based on the outcomes from this study as well as other general factors involved in the analyses.

**Chapter 4** provides a general introduction of the second studied region, the Indo Australian Archipelago (IAA), and to the studied animal presented in Section II, *M. rosenbergii* populations from Malaysia, and its importance in the aquaculture industry, as well as a bacteria pathogen, *V. parahaemolyticus*, to be used in a challenge on *M. rosenbergii* in Chapter 5.

**Chapter 5** aims to investigate if there is any significant difference between Peninsular, Northern Sarawak, Southern Sarawak and Sabah population based on the recent transplantation history of *M. rosenbergii* by screening the cytochrome oxidase I (COI) segment using a sequencing technique. The findings are then compared with published data on other populations of *M. rosenbergii*, as well as another form of *M. rosenbergii* currently known as *M. spinipes* Schenkel, 1902. A literature review is presented to correlate the findings from the present thesis with other published data on the genetics of *M. rosenbergii*, as well as *M. spinipes*.

The objective of the study described in **Chapter 6** was to challenge *M. rosenbergii* with *V. parahaemolyticus* and to investigate the response of the affected prawns to the bacterial infection, using various measures: total haemocytes counts (THC), measures of bacteria spread and the activities of the enzymes phenoloxidase (PO) and superoxide dismutase (SOD).

Lastly, **Chapter 7** provides a general discussion of the thesis. This chapter also suggests future research plans that can be extended from the *M. rosenbergii* research project presented in this thesis.

# **SECTION I: *Nephrops norvegicus***

## **Chapter 2**

### **General introduction**

#### **2.1 The Norway lobster *Nephrops norvegicus***

The Norway lobster *Nephrops norvegicus* (Linnaeus, 1758), also known as scampi or Dublin Bay prawn, is one of the most commercially-important marine decapods in Europe. *N. norvegicus* is distributed along the Eastern Atlantic coast from Icelandic waters in the north and Morocco to the south, and including North-Western Norway and the Faeroes (although it is reportedly absent from several isolated areas such as the Baltic sea, the Levantine sea and the Black sea (Bell et al., 2006)). Because of its high economical value, *N. norvegicus* has been exploited throughout its geographic distribution. According to the Food and Agriculture Organization (FAO), global landings of the animal in 2010 were 66,544 tonnes. The largest catches were in the United Kingdom where the total landings by UK vessel were 38,200 tonnes worth about £95.3 million (Ellis et al., 2010). The majority of the catches are in Scotland (28,900 tonnes, worth about £79.7 million) where most of the fishing activities occur in the North Sea area (Fladen ground: FU VI) followed by Ireland where the fisheries area is in the Irish Sea (Ellis et al., 2010). In terms of animal numbers, the landed catches recorded are higher in the summer compared to the winter. This can be explained by the seasonal emergence pattern of this species which have separate sexes, whereby the females are mainly resident in burrows within the sediment through the winter, but emerge in the late winter and through the summer to search for food, and to moult and mate. The proportion of males to females being caught during summer is 1:1 whereas females are almost absent from the catches during winter (Sarda, 1991).

*N. norvegicus* can be found at depth of 20-800 meters in the burrows of a sandy mud sediment type. Adults normally occupy a 20-30 mm deep burrow along with juvenile *N. norvegicus* and other species such as the echiuran worm

(*Maxmuelleria lankesteri*), the goby (*Lesueurigobius friesii*) and the thalassinidean (*Jaxea nocturna*) (Tuck et al., 2000). The density of the burrows varies across populations and shows a non-linear relationship with the type of sediment. The highest burrow densities have been observed in populations that occupy seabed sediments with intermediate particle sizes (Campbell et al., 2009). The main fishing method for this burrowing benthic species is bottom trawling, but this is limited to offshore areas. On inshore fishing grounds such as along Scottish West coast, creels are used. The body length of this species ranges from 8 to 24 cm with the males being larger than the females. However most of the catch lengths are between 10 and 20 cm. Juvenile males and females show similar moulting patterns, but once matured the females grow slower than males. Female *N. norvegicus* moult on average between 0 and 1 times a year, while males moult 1-2 times a year (Sarda, 1991). However, Castro et al. (2003) reported no significant differences in the size increments at moult between males and females. Female *N. norvegicus* mature at different body lengths depending on their habitat. In general, females occupying shallow water mature at about 23-30 mm carapace length (CL) but at around 30-36 mm CL in deep-water habitats (Bell et al., 2006). At this size the animals are around three to five years of age, and they will reproduce every year or alternate year depending on the latitude. Generally, after moulting and when still soft the animals that reproduce every year will mate and spawn in the summer (around July). They incubate their eggs through the winter for about nine months, and then hatching and release of larvae occur from April through June the following year. The larvae pass through three stages before reaching the post-larval stage; this process takes about 6 months (Engelhard and Pinnegar, 2008). The first larval stage has a body length of about 6.5 mm, while the second stage is 8 mm and the third stage is 10 mm; the first post-larval stage is 11 mm in length (Jorgensen, 1925). Dickey-Collas et al. (2000b) reported that the duration of larval development is temperature-dependant, and thus it is likely that there is differentiation between populations living in different water temperatures. Besides, the effect of climate change may play a role, as warmer water could cause a reduction in the duration of larval development. However, unlike temperature-dependence, no significant correlation has been found between larval development and salinity (between 30-40 PSU) (de Figueiredo and Vilela, 1972). Mente et al. (2009) showed a seasonal timing of ovarian maturation and

brooding period, which also varies slightly depending on the latitude and depth. They also reported that these two factors also contributed to the differences observed on the size at onset of maturation.

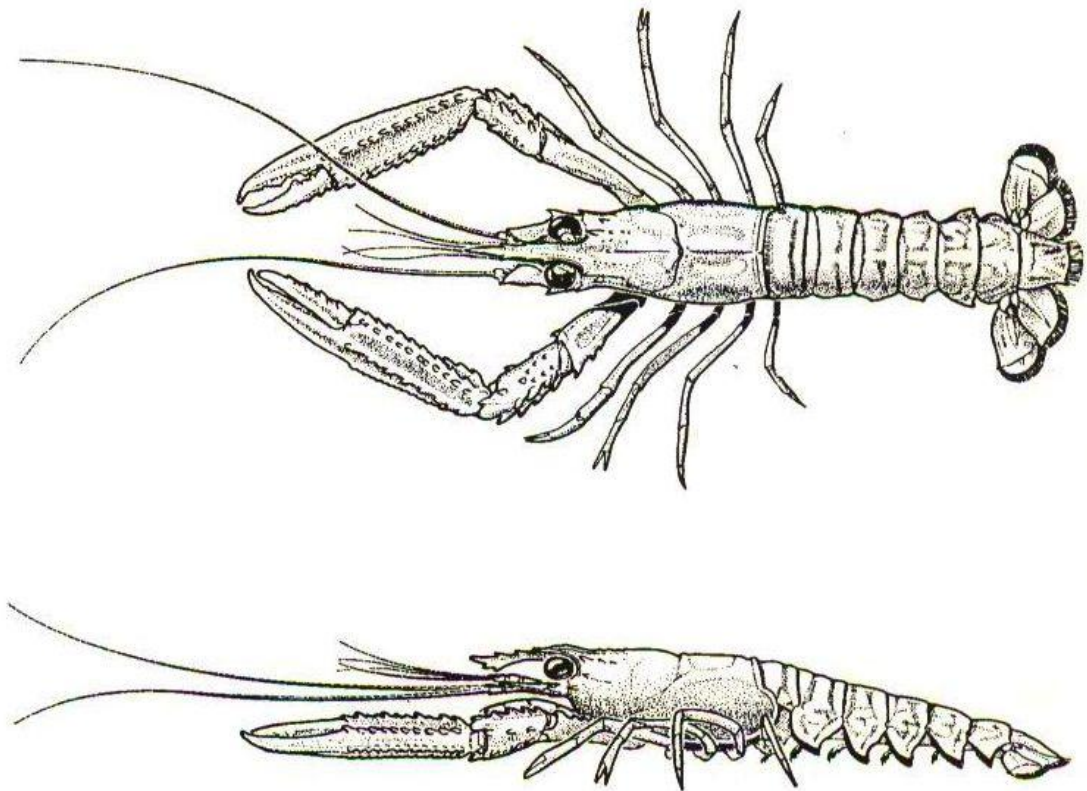


Figure 2-1: Dorsal and lateral view of *N. norvegicus* (L). Source: Howard, F.G. 1989.



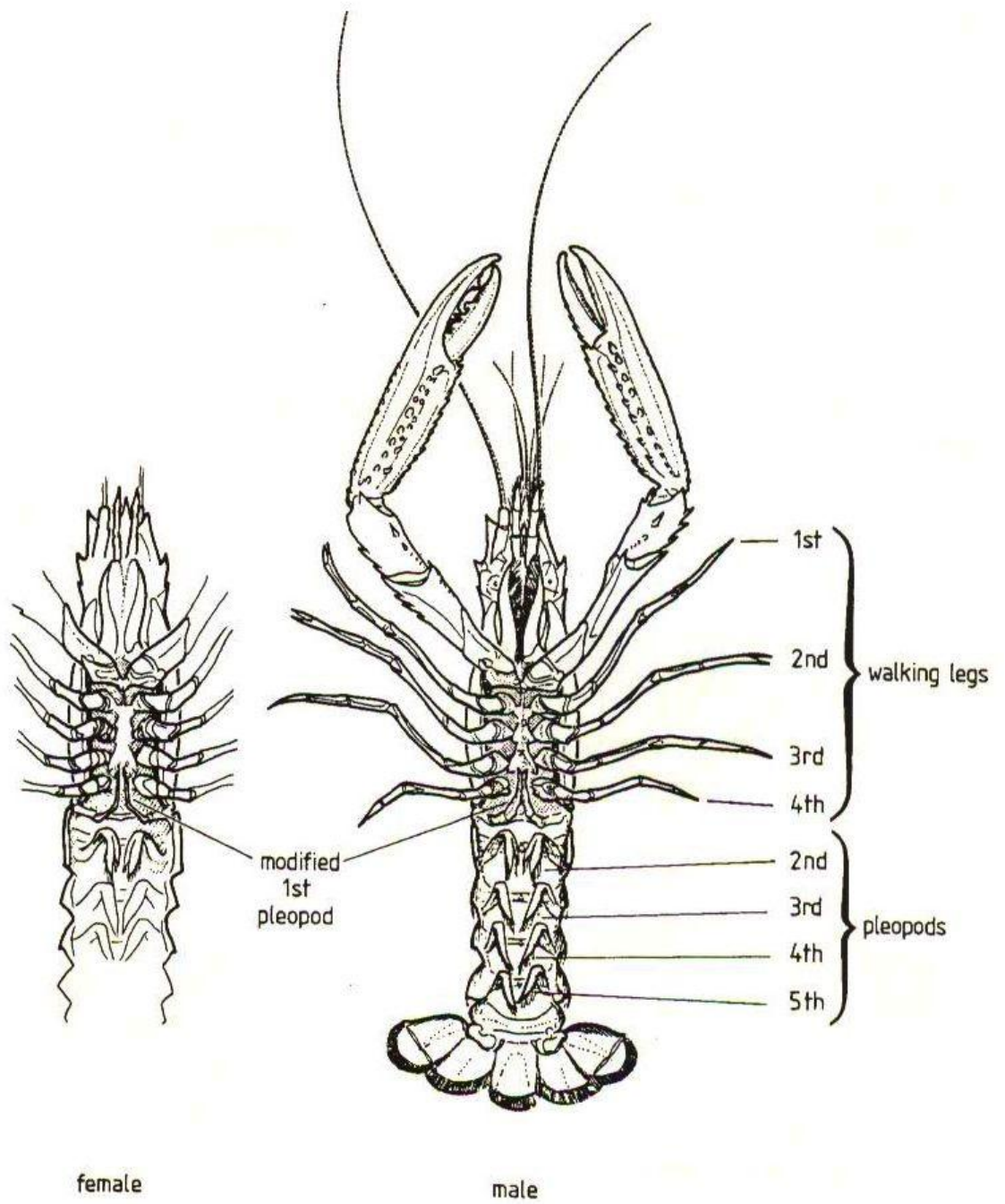


Figure 2-2: Ventral view of male and female of *N. norvegicus*. Source: Howard, F.G. 1989.

## 2.2 Differentiation in *N. norvegicus*

*N. norvegicus* is known to have markedly distinct morphological characteristics between populations. High morphological differentiation was found within populations from the Adriatic sea (Frogliola and Gramitto, 1981), the northern Irish Sea (Tully and Hillis, 1995), northwest Irish Sea populations (Briggs, 1995), Scottish coasts (Tuck et al., 1997) and the Mediterranean coast (Abello et al., 2002, Maynou and Sarda, 1997). Besides, about 30 different *N. norvegicus* populations, which differ in phenotype, were identified in European waters (Bell et al., 2006). This animal differs in size, colour, density, natural mortality, growth rate and also the size at maturity even between adjacent trawling sites. The fact that *N. norvegicus* favour walking over swimming and only migrate around a few hundreds metres, which is also limited to the availability of the suitable sediment (Chapman and Rice, 1971), raises the question of whether morphological differentiation in *N. norvegicus* is due to genetic and/or environmental factors.

Information on the genetics of *N. norvegicus* has important implications for the conservation of their populations and for future stock management. For a unique or markedly distinct population, a good management practice should be applied to sustain the existence of the animal.

To date, molecular markers and approaches such as mtDNA, PCR-RFLP, microsatellite, allozyme and sequencing have been applied to *N. norvegicus* using samples from different populations. Until now, a low to moderate level of differentiation was found among and between the studied areas. Low yet significant levels of differentiation in mtDNA were reported by Stamatis et al. (2004) on populations from the North Sea, Irish Sea, and Mediterranean populations. However, no evidence of glaciations was discovered. Another study by Stamatis et al. (2006) on allozymes, with additional samples from Portugal and the Irish Sea, also showed a similar result. In addition, an allozyme study on eight Mediterranean populations and one Atlantic population by Maltagliati et al. (1998) showed moderate genetic differentiation, but an unclear geographical pattern of genetic differentiation was detected. A biometric study on the same populations was conducted and compared with genetic data from Maltagliati et al. (1998) study. Castro et al. (1998) found significant morphological differences

between populations but suggested that environmental factors are more likely to play a role in the differentiation observed in the morphology of *N. norvegicus*, compared to the genetic factors. Passamonti et al. (1997) compared a Scottish population (from south Cumbrae) and two Aegean populations with published data of two Adriatic populations using an allozyme marker (Mantovani and Scali, 1992) showed low levels of genetic differentiation. A fine scale population study using microsatellite analysis on Scottish populations from North Sea, Firth of Forth and Moray Firth found extensive larval dispersal in these populations, though low levels of migration when compared with other populations such as those from the Irish Sea and Portugal (Murphy, 1999). This phenomenon might be attributed to hydrology effects (e.g. ocean current flow and gyre). The most recent microsatellite study on *N. norvegicus* using Icelandic populations showed low genetic differentiation (Pampoulie et al., 2011) despite a high genetic polymorphism detected in the microsatellite markers used in the study (Skirnisdottir et al., 2010). Another microsatellite study found a high level of differentiation in five different markers, but also showed no significant differentiation among and between loci were found when tested on two populations from the Portuguese coast (Streiff et al., 2001). These findings have been confirmed by further investigations carried out by the same authors on the same populations that revealed that multiple paternity occurs in *N. norvegicus* (Streiff et al., 2004). High genetic diversity was found at marker loci, although no significant differentiation was revealed among these populations, which supports the previous finding. Multiple paternity increases genetic diversity and will thus increase the reproductive rate, which may have a strong effect on increasing or at least maintaining the effective size of the population which will assure its stability (Pearse and Anderson, 2009, Yue et al., 2010, Theissinger et al., 2009). Multiple paternity was also reported in the American lobster (Jones et al., 2003), even though an earlier finding (Tam and Kornfield, 1996), is inconsistent with this result. This inconsistency might be due to the sensitivity of the genetic markers used in the later studies. Gosselin et al. (2005) revealed a strong correlation between multiple paternity and higher fishing pressure, as well as its positive effect on the mean adult size in American lobster, *Homarus americanus*.

## 2.3 Factors contributing to the differentiation in *N. norvegicus*

The morphological differentiation observed in *N. norvegicus* species does not depend on only one factor, but on a combination of many genetic and/or environmental factors, which can be difficult to define. Beside genetic factors, there are many possible environment factors that might also play an important role in the differentiation in *N. norvegicus*: sediment composition, population density, food availability, hydrology effects, water temperature, water salinity and fishing effort.

*N. norvegicus* is known to be a very sedimentary benthic species. This selective pressure may have caused differences in the biological features such as size (length and weight), growth rate, sex composition and size at first maturity (Tuck et al., 1997 and Bell et al., 2006). Besides, it is also linked with the variation in the densities (Farina et al., 1994) as well as mortality rate. The most frequent associated macro-fauna species in this habitat are polychaete worms (Hensley, 1996, Briggs et al., 1997). Campbell et al. (2009) studied the sediment types in Scottish waters and described two slightly different relationships between densities and the percentages of silt and clay, which were a linear relationship; and a non-linear or dome-shape relationship between burrows density and percentage of silt and clay (Tuck et al., 1997) indicating population-specific relationships. Meanwhile, Tully and Hillis (1995) reported that in Irish sea area, *N. norvegicus* was found to have the characteristics of small size which reflected a slow growth rate but high abundance in a finer sediment area. In general, the characteristics of the sediment should be muddy sand composed of more than 40% silt and clay (Bell et al., 2006).

While population density of *N. norvegicus* is closely related to the sediment type of the habitat, it is also influenced by the characteristics of the sea floor, e.g. whether it is a shelf or slope. For instance, in Catalan sea area, the density of *N. norvegicus* populations is related to both depth and sediment composition of the sea floor where they inhabit (Maynou and Sarda, 1997). However, Farina et al. (1994) obtained data that was inconsistent with this finding but suggested that sediment may play a more important role than depth or characteristics of the sea floor in determining the abundance and distribution of *N. norvegicus*.

Parslow-Williams et al. (2001) carried out a starvation study on two populations from Clyde sea area with different densities in order to understand the correlation between food availability with morphological differentiation in *N. norvegicus*. They concluded that food availability, if low, would lead to slow growth of the animal. This will eventually produce a population with a smaller mean size.

Fishing pressure could also be a factor for morphological differentiation in *N. norvegicus*. Sarda (1998) reported significant reduction in the mean size of *N. norvegicus* over a period of 20 years that could be the sign of overexploitation of the stock. Increased mortality of the mature *N. norvegicus* as consequence of intensive fishing activities has caused a reduction in the number of adult animal available to maintain their reproduction rate. This phenomenon will increase the possibility of a genetic bottleneck, which will then raise the chances of heterozygote deficiency or elimination of the rare alleles in the population and genetic drift and/or selection.

There are many other factors that might cause morphological variation, such as adverse environmental conditions e.g. a too high or too low water temperature. Temperature has been shown to play more important role in survival of the larvae, compared to water salinity (de Figueiredo and Vilela, 1972, Dickey-Collas et al., 2000b). The study of Dickey-Collas et al. (2000b) showed low levels of larvae survival, attributed to low temperature. Moreover, Tully and Hillis (1995) reported that low temperature attributable to stratification events is correlated with a fine type of sediment.

A factor such as the hydrological condition is also one of the contributors (Triantafyllidis et al., 2005). Ocean current flows as well as seasonal gyre have a great impact on the settlement of the larvae. It is well known that adult *N. norvegicus* prefer to walk rather than swimming, which supports the fact that they do not migrate over a distance longer than a few hundred metres (Chapman and Rice, 1971). However, the possibility of exchange between populations might occur during planktonic larval stages, which would increase the possibilities of genetic differentiation in this animal. Extensive studies have been carried out on Irish Sea fishing grounds to understand the effect of the gyre in Irish Sea water. White et al. (1988) revealed that most of *N. norvegicus* larvae in

western Irish Sea were retained within the centre of the gyre throughout their larval phases. This has raised the question concerning the genetics of *N. norvegicus* of whether they have spatial differentiation in genetic information between populations. Studies on other species, *Calanus* spp. and *Meganyctiphanes* spp. supported this fact (Emsley et al., 2005, Dickey-Collas et al., 1997). Dickey-Collas et al. (1997) demonstrated that pelagic juvenile fish such as haddock and cod (but not sprat) were also associated with the gyre and were retained in the system.

However, genetic factors could possibly interact with environmental factors such as water salinity and temperature to produce differentiation in the phenotype of the animal, as is the case for European hake (*Merluccius merluccius*) (Cimmaruta et al., 2005) and Atlantic herring (Bekkevold et al., 2005). Specific environmental conditions (variation in the water salinity and temperature) has confined only for certain alleles that managed to adapt in this condition and able to survive in this population. This has created a distinct population that differs in both their genetic and their environmental features from other populations. Besides, evolutionary events such as Pleistocene glaciations are most likely to shape the genetic structure of many marines invertebrate (Kenchington et al., 2009) though could not be detected in *N. norvegicus* populations by (Stamatis et al., 2004). Others, like bathymetric barriers could also restrict the gene flow between two locations (Hemmer-Hansen et al., 2007).

## **2.4 The differentiation of *N. norvegicus* populations from Scottish and Icelandic waters**

The present study used *N. norvegicus* samples from two locations in Scotland, which are the Clyde Sea and North Minch, as well as from the Icelandic waters. Literature review from previous works on these populations was carried out in order to understand and gain information on the morphological differentiation that exists in these populations. However, there were limited information available on the Icelandic population (most of them were ICES documents published by Eiriksson research group as reported by Pampoulie et al., 2011) compared to the other *N. norvegicus* populations for which many studies exist, mainly on the Clyde Sea area (Tuck et al., 1997, Tuck et al., 2000, Parslow-Williams et al., 2001, Campbell et al., 2009), the North Sea (Stamatis et al.,

2006, Stamatidis et al., 2004), the Irish Sea (Briggs, 1995, McQuaid et al., 2009), (Dickey-Collas et al., 2000a, Herraiz et al., 2009) as well as Mediterranean Sea (Sarda, 1998, Maynou et al., 1996, Maynou, Sarda, 1997). This might be due to the interest of its high catches, whereby Scotland contributes about 1/3 of total *N. norvegicus* global landing (14,139 tonnes in 2007 from the west coast area only) followed by the Irish Sea. In contrast, Icelandic vessels have landed only around 2,500 tonnes annually since the 1950s, except for increases in 1960s and 1970s when landings were around 4,000 tonnes a year. However, it declined again in the mid-1990s when only 1,500 tonnes were landed annually. In recent years, the number has recovered to around 2,500 tonnes in 2008 and 2009 (Pampoulie et al., 2011).

Highly morphological differentiation was observed in *N. norvegicus* from Scottish and Icelandic waters. There are large differences in the size of landed animal, with the mean size in Scottish population being 36.4 mm compared to 49.6 mm in the Icelandic population (Pampoulie et al., 2011). Despite being geographically isolated, Scottish and Icelandic waters share the same ocean current. In Icelandic waters, the North Atlantic current branches at the south of Iceland to give two main currents that flow to the southeast and southwest of the island. These two main currents could restrict the possibility of mixture between *N. norvegicus* populations in Iceland. Another striking feature is the coastal current that flows clock-wise around Iceland, providing the possibility of larval dispersal. On the other hand, the Clyde Sea area on the west coast of Scotland is a partially enclosed basin which only exchanges water with the adjacent shelf via a shallow sill (45 m depth) (Kasai et al., 1999, Simpson and Rippeth, 1993), through the entrance of about 42 km. The shallow sill with only 45 m depths confines the mixture of the water between the North Channel (depth >150 m) and Clyde Sea (depth >150 m). Moreover, it receives large amount of freshwater that flows from the Clyde river and other rivers (Kasai et al., 1999, Simpson and Rippeth, 1993). However, the other sampling location in the present study, the North Minch in the north west of Scotland, is an open sea that receives current from the Gulf stream that flows to the Norwegian sea. The average depth of the Clyde Sea is between 40-60 m, while *N. norvegicus* inhabits different depths in Icelandic water, especially along the south coast where troughs are at a depth of 150-250 m between banks at depth between 100-120

m. In Iceland, the warm north Atlantic current (Irminger current) is around 6-10°C when it branches at the south of Iceland, while the cold east Greenland and east Iceland currents are <0°C, and the average annual temperature does not exceed 6°C. The waters of the Clyde Sea and North Minch have higher temperatures, between 4°C in winter and 14°C during summer with fjordic characteristics (Kasai et al., 1999, Simpson and Rippeth, 1993, Hughes et al., 2010).

## 2.5 Objectives of Section I

The main objective for the Section I were:

- To gain information regarding the genetic differentiation of *N. norvegicus* from the Clyde Sea area, North Minch and Icelandic waters in order to assist in the management of *N. norvegicus* fisheries.
- To understand the demographic history that shapes the genetic of *N. norvegicus* of Atlantic and Iceland populations.

In addition, this thesis was also focused on the development of different molecular techniques useful for the detection of genetic differentiation. The objective for the purposed was therefore:

- To compare the sensitivity of PCR-RFLP and sequencing techniques for detecting polymorphism.



## Chapter 3

# Molecular characterization of mtDNA of *Nephrops norvegicus* using the PCR-RFLP and sequencing technique.

### 3.1 Introduction

As described in Chapter 2.1, *Nephrops norvegicus* (Linnaeus, 1758) is a marine decapod species that inhabits the North-eastern Atlantic Ocean and parts of Mediterranean Sea. This species is one of the most commercially important crustacean species in Europe particularly the UK. *N. norvegicus* possess high morphological differentiation even between adjacent populations (Bell et al., 2006). In contrast, low to moderate significant genetic differentiation was reported in the previous studies (Maltagliati et al., 1998, Streiff et al., 2001, Stamatis et al., 2004, Stamatis et al., 2006, Pampoulie et al., 2011). It has been known that adult *N. norvegicus* do not migrate, and rarely venture over one hundred metres from their home burrows (Chapman and Rice, 1971). However, the chances of mixing between populations at the larval stage cannot be ruled out. High morphological differentiation observed within and between the Atlantic and Icelandic populations (see Chapter 1.4) has raised the question of whether this differentiation is attributable to genetic and/or environmental factors. As far as it is known, neither mtDNA markers nor the PCR-RFLP technique has ever been applied to study *N. norvegicus* from the Atlantic area or Icelandic populations, except for a few populations from the southeast of Scotland (Moray firth) (Stamatis et al., 2004). However, Pampoulie et al. (2011) have used microsatellite markers to study the population genetic structure of Icelandic populations, and in fact used one Clyde Sea area population as an outgroup.

The polymerase chain reaction, followed by DNA sequencing is a method that determines the sequence of the nucleotide bases (adenine, guanine, thymine

and cytosine) in a particular DNA segment. This technique has been known for its reliability and is informative in phylogeography studies, as well as in other fields such as disease diagnostics (Cameron et al., 2008, Nuryanto et al., 2009, van der Kuyl et al., 2010). On the other hand, PCR-RFLP analysis is less reliable and informative compared to sequencing, but nevertheless can be used to answer questions in phylogeographic studies, particularly when the cost is limited. Both of these techniques have been widely used and become conventional and practical for the detection of genetic differentiation between populations (Launey et al., 2002, Evans et al., 1999, Smith, 2005, Chow et al., 2006).

The objective of the present study was to gain information regarding genetic differentiation between *N. norvegicus* from North Atlantic and Icelandic localities and to understand the demographic history that shapes the genetic of these populations. In addition, comparison between the sensitivity of PCR-RFLP and sequencing techniques for detecting polymorphism was also carried out.

## **3.2 Materials and methods**

### ***3.2.1 Sampling***

A total of 49 samples were collected from two different sites in the Clyde Sea area in May 2008 using a research vessel from USBSM Millport. Twenty samples were obtained for each of these two sites. An additional 9 samples obtained from the same area (but without exact locations) were used for the initial optimisation of the PCR cycling conditions and restriction analysis only. In addition, 19 samples were obtained from an unspecified location in the North Minch, landed to Stornoway by commercial trawlers (collected in August 2006). A further 19 samples were obtained from a location off the South coast of Iceland in 2008 from commercial vessels, provided by a research group at the University of Reykjavik in Iceland (Figure 3-1).

A portion of the deep abdominal flexor muscle was taken in the field from each individual sampled from the Clyde Sea area, and then quickly frozen in liquid nitrogen and stored at -80°C until used. Portions of muscle samples from the

North Minch were only kept on ice following capture and during transportation back to University of Glasgow and then stored at  $-80^{\circ}\text{C}$  until used. Pre-treatment of the Icelandic samples of abdominal muscle following capture is unknown, but they were transported to the University of Glasgow via express delivery in an ice pack.

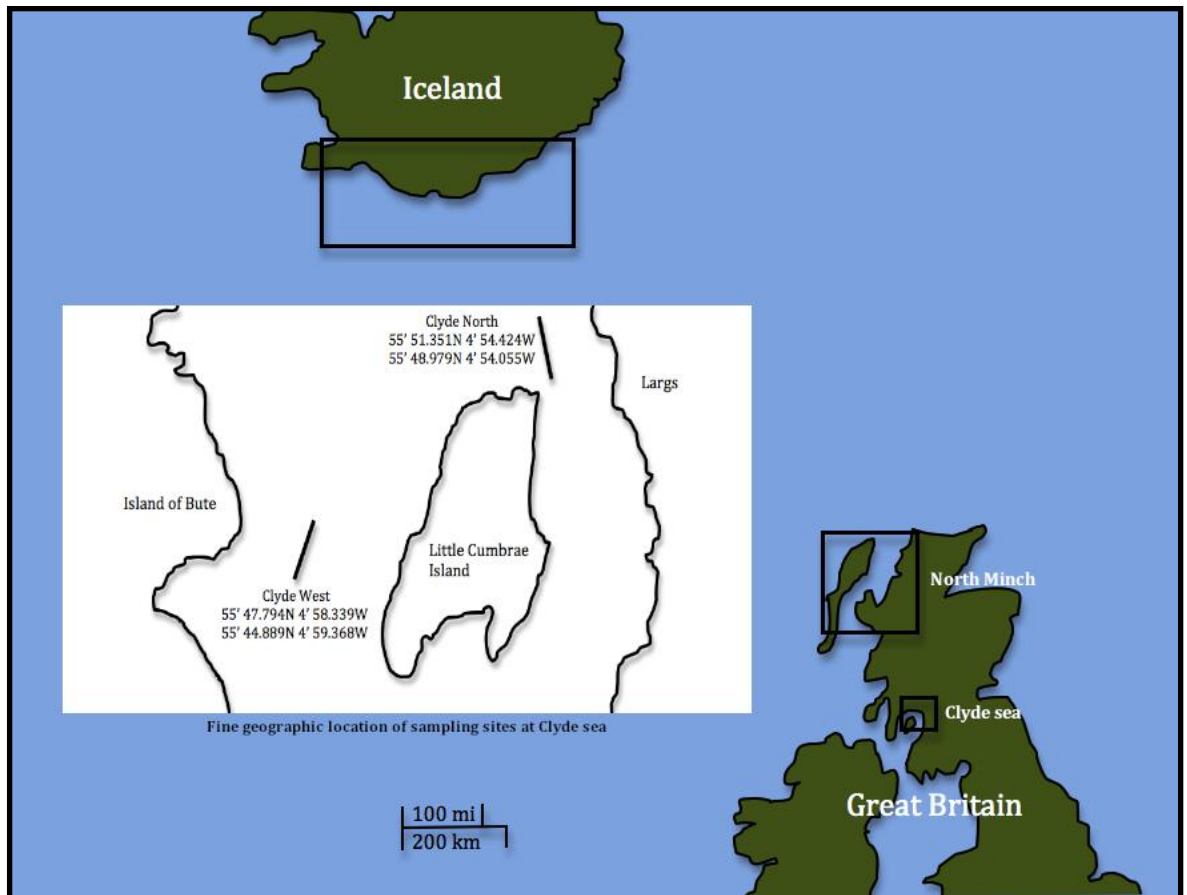


Figure 3-1: Geographic location of sampling sites: Icelandic, Clyde Sea area and North Minch samples.

### 3.2.2 DNA purification

Approximately 25 mg of tissue was used to isolate the genomic DNA from each muscle sample using a commercial DNA extraction kit (QIAquick DNA purification kit, QIAGEN Inc.) with slight modifications. Longer incubation (5 minutes) was applied during the elution of the DNA to increase the efficiency of recovering the DNA from the spin column filter. DNA was eluted in a smaller than recommended volume of AE buffer, 50  $\mu\text{l}$ , and this was repeated twice to give a final volume of 100  $\mu\text{l}$ . Extracted genomic DNA was kept at  $-20^{\circ}\text{C}$  until used.

Genomic DNA purified from muscle tissues was checked for the quality as well as for the quantity of the product as described in the following section.

### **3.2.3 Gel electrophoresis and visualization**

Purified genomic DNA was loaded onto a 1.5% agarose gel after 5 µl of 6X Orange loading dye (New England Biolabs (UK) Ltd.) and run in 1.5% Tris borate EDTA (TBE) buffer, with 2 µl of 10 ng/ml ethidium bromide added. 5 µl of 100 bp DNA marker (New England BioLabs Inc.) was used as a size standard. The PCR products were visualized and photographed using an ultraviolet (UV) transilluminator system with an attached camera (Gel Doc System, Bio-Rad Laboratories (UK) Ltd.), driven by the Quantity One software (Bio-Rad Inc.). All the samples from the Clyde Sea area and North Minch were extracted with satisfactory results where high concentration of genomic DNA was obtained and the DNA was not degraded. However, most of the Icelandic samples gave low quantity and quality of the genomic DNA, with DNA degradation being indicated by smeared DNA in the gel images.

### **3.2.4 Nucleic acid measurement using spectrophotometer**

The quality and quantity of the purified DNA were also checked using a spectrophotometer. The nanodrop method can check for the purity of the DNA and RNA in the samples from the ratio of the absorbance at 260 nm and 280 nm indicates the presence of contaminants that absorb at 230 nm.

### **3.2.5 PCR amplification**

Two mtDNA regions of *N. norvegicus* which were the 1.4 kb segment of the cytochrome oxidase subunit I gene (COI) (Normark et al., 1991) and the 2.2 kb fragment that includes part of the cytochrome b gene, ND-1 gene, and part of the 16S rRNA gene, amplified using primers designed by (Stamatis et al., 2004) based on alignment of the 16S rRNA gene from European lobster (*Homarus gammarus*) (Kornfield et al., 1995) and a complete mitochondrial sequence of the crustacean *Artemia franciscana* (Valverde et al., 1994) were used in the present study (Table 3-1).

The amplification of the COI and of the 2.2 kb region of mtDNA was performed according to Stamatis et al. (2004) with minor modification. Several parameters were taken into account during optimization of the PCR reaction, such as the amount of the DNA template, the concentration of MgCl<sub>2</sub>, as well as annealing

Table 3-1: Nucleotide sequences of forward and reverse primers used for amplification of COI and 2.2 kb region of mtDNA segment, as well as the internal primer that was designed in the present study.

Primer	Size (kb)	mtDNA segment	Sequences	Sources
L5950	1.4	COI	5'-ACAATCACAAAGAYATYGG-3'	Normark et al., 1991
H7196	1.4		5'-AGAAAATGTTGWGGGAARAA-3'	
Internal primer	0.9	COI	5'-GAAAAGGAATAACAATAGACCG-3'	Designed in the present study
Forward	2.2	cyt <i>b</i> , ND-1 and 16S rRNA	5'-GCTATTTTGCGGTCTATTCCTAA-3'	Stamatis et al., 2004
Reverse	2.2		5'-CAAACAAAGGGCCCGGTAT-3'	

temperature. Gradient PCR using a UNO TGradient thermal cycler (Eppendorf (UK) Ltd.) was run to find the optimum annealing temperature for each primer. Also different concentrations of MgCl<sub>2</sub> and template DNA were applied to find the optimum concentration using the UNO II thermal cycler (Eppendorf (UK) Ltd.). Annealing temperatures at 48°C and 50°C were selected for COI and 2.2 kb region, respectively. 2 µl of MgCl<sub>2</sub> (25 mM, Promega (UK) Ltd.) and template DNA (100 ng) were used in the amplification of both regions.

Using the optimised parameters, double stranded DNA amplifications were performed using the UNO II thermal cycler (Eppendorf (UK) Ltd.) in 50 µl volumes, containing 1.25 U of Taq polymerase (New England Biolabs (UK) Ltd.), 10 µl of 5X reaction buffer (green GoTaq<sup>®</sup> reaction buffer, Promega (UK) Ltd.), 0.2 mM each of dNTPs (New England Biolabs (UK) Ltd.), 0.08 µM (COI) and 0.25 µM (2.2 kb region) of each of primer, 2.5 mM MgCl<sub>2</sub> (Promega (UK) Ltd.) and approximately 100 ng of DNA. PCR amplification cycles were as follows: 95°C for 5 minutes to denature double-stranded DNA, followed by strand denaturation at 95°C for 1 minute and annealing at 48°C for 50 seconds (COI) or at 50°C for 1 minute (2.2 kb region). Primer extension then proceeded at 72°C for 1 minute and 30 seconds (COI) and 2 minutes and 20 seconds (2.2 kb region). The denaturation, annealing and extension steps were repeated for 33 cycles. Lastly,

the reactions underwent final primer extension for 5 minutes at 72°C and the product was then held at 10°C.

The amplified PCR products were visualized and photographed as described in Chapter 3.2.3. These steps are crucial to determine whether the expected fragment is amplified and also to confirm the specificity of the primer to obtain only the desired fragment. Beside, it is also useful to decide which extraction methods need to be used (precipitation or gel purification). It proved difficult to amplify the 2.2 kb region from the Iceland population, which might be due to the poor quality of the DNA (due most probably to the treatment after capture, or the conditions during the transportation). However all samples were successfully amplified for the COI region.

### **3.2.6 PCR product purification**

The purification method is important to remove any residuals such as dNTPs and excess primers that might interfere in the RFLP or sequencing analyses. PCR products were first purified using one of two methods as described below.

Where the quantity of the amplification products was low (e.g. Icelandic samples), DNA was precipitated to simultaneously purify and increase the concentration. The DNA precipitation method was carried out on the amplified product (Chapter 3.2.5) of the 2.2 kb segment of Icelandic samples (only on the sample with low quantity of PCR product). The principle of DNA precipitation is that interaction between the different electric charges of a salt solution (NaCl) (+) and nucleic acid (-) makes the nucleic acid become less hydrophilic, and thus precipitates from the elution buffer. First, 3M Sodium Acetate, pH 5.2 was added in a ratio of 1:10 to the PCR product, followed by 3X volumes of cold absolute ethanol. The mixture was allowed to stand at -80°C for an hour. After that, the mixture was centrifuged for 15 minutes at 18 000 x g in a microcentrifuge at 4°C. The supernatant was discarded and 200 µl of cold 70% ethanol was added. The sample was centrifuged for 5 minutes in a microcentrifuge at 4°C. The supernatant was discarded and the pellet was air dried at 37°C. The pellet was then re-suspended in 30 µl of sterile distilled water.

Where good amplification of PCR products was achieved, purifications of the PCR products were carried out using the gel purifications method. Gel purification was optimized using two different brands of gel purification kit, which were from Qiagen (QIAquick<sup>®</sup> gel extraction kit) and Promega (Wizard<sup>®</sup> SV gel and PCR system). This comparison was done to exclude the possibility of salt carried over during purification of the DNA that has been suggested to interfere with downstream analyses such as RFLP and sequencing (Fangan et al., 1999 and Pikor et al., 2011). Before the PCR product was purified using the PCR purification kit, the remaining PCR product (40 µl) was loaded onto another 1.5% gel and the procedures as described in the Chapter 3.2.3 were repeated, except the 6X orange dye was not applied since the 5X green GoTaq<sup>®</sup> reaction buffer used in to amplify the DNA already contained two dyes (a yellow and a blue dye). To excise the gel for extracting the bands, the gel was then put on the ultra-violet (UV) light box attached to a video camera (Sony Corporation) and connected to a black and white monitor (Sony Corporation) and video graphic printer (Sony Corporation). The desired band at 1.4 kb size was then excised from the gel using a clean scalpel and transferred into a weighed 1.5 ml microcentrifuge tube carried out under UV light. Each of the microcentrifuge tubes containing a gel slice was weighed again and the reading was recorded. The weight of each of the band slice is important to estimate the volume of membrane binding solution provided with the Wizard<sup>®</sup> SV gel and PCR system or QIAquick<sup>®</sup> gel extraction kit that is needed to bind the DNA to the membrane, as well as to help dissolve the agarose gel. The remaining steps were followed as per the instructions provided by the manufacturer, as follows.

Gel purification steps can be divided into four parts: (1) dissolving the gel slice, (2) binding of the DNA onto the filter, (3) washing out the DNA and evaporate any ethanol residues, and finally (4) elution of the purified DNA. Promega purification kit steps were much simpler than those of the Qiagen purification kit but the cost for the Promega kits was higher. Minor modification was done on both of the purification kit steps. During the steps to elute the DNA, the Minicolumn (filter) placed in 1.5 ml centrifuge tube containing nuclease free water was incubated at room temperatures for 5 minutes and only 30 µl of nuclease water was used. However, no significant differences were found between these purification kits that can improve the quality of the sequencing.

### **3.2.7 Sequencing analysis**

A few 2.2 kb samples (6 Clyde Sea area and 3 North Minch samples) were sent for sequencing the 2.2 kb region and a total of 14 samples were sent for sequencing the COI region. None of the sequencing of the 2.2 kb were successful. The sequencing results showed a very short sequence while from a few samples no good sequences could be read (giving NNNN readings). In contrast, a full sequence of the COI region of the sequenced samples was obtained by applying any one of the three methods describe below.

Analyses of the 2.2 kb sequences detected heterozygous peaks, and since heterozygosity can only be found in nuclear DNA, although mtDNA was being amplified, it indicates that there was contamination with nuclear DNA that was also amplified during the PCR reaction. This was due to the contamination of nuclear insertions of mitochondrial sequences (NuMts) also known as mitochondrial pseudogenes in the nuclear genome. Several studies of mitochondria DNA have shown that contamination with nuclear DNA can occur if the primer sequence is complementary to the nuclear DNA sequence, leading to co-amplification of mtDNA and nuclear DNA, especially in a case where mtDNA is degraded or in low amounts or the NuMts copy number is high (Ramos et al., 2009, Goios et al., 2008). The problem of NuMts was also reported in crustacea (Buhay, 2009). Unlike the primers used to amplify the 2.2 kb region in the present study, the primers used to amplify the COI gene (IH7196 and L5950) have been widely used as mtDNA marker in phylogeographic studies (Mamuris et al., 2001, Mamuris et al., 2005, Apostolidis et al., 2009) with significant genetic differentiation detected. As far as is known, no other study has utilised the 2.2 kb region except that of Stamatis et al. (2004).

#### **Sequencing from direct PCR products**

All samples that were sent for sequencing were purified using a purification kit (Promega (UK) Ltd.). Dilution of each sample was based on the concentration of the PCR product visualised by agarose gel electrophoresis. The final volume of each sample was 30  $\mu\text{l}$  (15  $\mu\text{l}$  for each sequencing reaction). Meanwhile each of the primers was also prepared separately. The concentration of the primer was 3.2  $\mu\text{M}$   $\mu\text{l}^{-1}$  for each reaction, which was diluted from a 100  $\mu\text{M}$  stock. For



instance, a batch of 10 samples sent for sequencing needed at least 15 µl of each primer. The larger amount of the primer (15 µl for 10 samples where only 1 µl is needed to sequence one sample) was provided to allow for any repetitions of sequencing if required. The optimum concentration of both the PCR product and primer are critical as they are one of the main keys to success in sequencing analyses. Sequencing was carried out on the both strands of the PCR product (L5950 used to sequence one stand and H7196 to sequence the other strand) using the Sanger method for cycle sequencing, which labels the four dideoxynucleotides with different fluorescent dyes so that the peak intensities for each position in the sequence can be detected by a laser in an automated sequencer. Sequencing both strands provided two copies of DNA strand for each samples to increase the confidence to identify the nucleotide when dealing with an ambiguous peak in the case of poor quality sequencing results. Sequencing was performed on an ABI 3730 sequencer by the Sequencing Service, School of Life Sciences, University of Dundee, Scotland, United Kingdom. However, only three samples (from Clyde Sea area) were successfully sequenced using L5950 and IH7196 primers.

### **Sequencing using an internal primer**

The internal primer was designed based on the full sequence of the 1.4 kb segment of COI region from animals sampled in the Clyde Sea area (Clyde 47), using Primer3 (Rozen and Skaletsky, 2000) available from the Geneious software package (Drummond et al., 2011). This internal primer was designed due to the size of the COI region being too long to be sequenced all the way through using both forward (L5950) and reverse primers (IH7196). For the forward primer, a readable sequence around 500 to 700 bp was obtained, while the reverse primer gave a shorter length of readable sequence, around 300 - 400 bp. The internal primer was designed about 400 bp away from the first base pair of the amplified segment of the 1.4 kb segment of the Clyde 47 sample. The internal primer was designed and chosen based on the percentage of Guanine (G) and Cytocine (C) in the primer (36.4%), and the length was 22 bp which produced an annealing temperature of 60°C, which is optimum for sequencing analysis. Beside, three base pairs at the 3' end of the primer is not a stop codon.

Sequencing using the internal primer produced sequences of about 900 bp. The PCR product to be sequenced was prepared as in Chapter 3.2.5 and 3.2.6. Sequencing was carried out as in as Chapter 3.2.7 (sequencing from direct primer) using the newly designed internal primer (5'-GAAAAGGAATAACAATAGACCG-'3). In this analysis, only one strand of the PCR product was sequenced since only one primer was used.

By using the combination of the L5950, IH7196 and internal primers, a total of eight samples (2 Clyde Sea area, 3 North Minch and 2 Icelandic) were successfully sequenced. Preliminary analysis on the sequences obtained from sequencing of the original primer used in the PCR amplification of the COI segment and internal primer revealed that this set of primers was highly polymorphic to detect variation in the studied population. Thus, only the COI segment was sequenced in this study. The other region, the 2.2 kb segment, was excluded from the sequencing analysis.

## **Cloning and Sequencing of PCR products**

### ***Ligation and Transformation***

Another three samples (from Clyde Sea area) that could not be sequenced using the above mentioned method were first cloned and then sequenced using another set of primers, the forward and reverse M13 primers. The procedures were as follows.

Firstly, the COI gene segment was amplified using the primers designed to amplify the COI segment (Chapter 3.2.5) and then purified using a gel purification kit (Promega (UK) Ltd.) prior to cloning (Chapter 3.2.6). Purified DNA was then cloned using a TOPO TA cloning kit (Invitrogen Corp., vector pCR TOPO® II). 1.5 µl DNA was pipetted into a 1.5 ml microcentrifuge tube and then 0.5 µl salt solution and 0.5 µl vector were added. The mixture was swirled using the tip to mix it. It is very crucial to treat all the components involved in cloning procedure very gently, otherwise the efficiency of the cloning process will be greatly reduced. The ligation was left at room temperature for 15 minutes. Meanwhile, the competent cells that had had been stored at -80°C until a few minutes before they were ready to be used, were then immediately placed on

ice. 2 µl of ligation was added into a tube containing competent cells placed on ice, just before the cells had completely thawed. The mixture was incubated on ice for another 15 minutes. The mixture was heat shocked at 42°C in a water bath for exactly 30 seconds and then placed on ice for 2 minutes, after which 250 µl of SOC solution was added. The heat shock step is crucial for the plasmid to be able to enter the cell. Lastly, the mixture was incubated at 37°C in a shaking water bath for 1.5 hours.

In the meantime, 100 ml of Luria Bertani Agar (LB) (Sigma-Aldrich, Inc. USA) was prepared and left to cool to around 50°C, to avoid the destruction of Kanamycin. Then, 200 µl of Kanamycin was added to produce Kanamycin selective plates. Bacteria cells without the plasmid will be lost in a few generations in the present of Kanamycin. This is because plasmids inserted into the bacteria cell contain the Kanamycin resistance gene. The agar was poured into 4 Petri dishes (90 mm diameter) and after a few minutes 40 µl of X-gal was pipetted onto the agar surface and spread with a plastic spreader. X-gal (bromo-chloro-indolyl-galactopyranoside) is a substrate used in the cloning for blue-white screening. Cells that contain the desired DNA (insert) will produce a blue colour but cells that do not pick up this DNA are white. When the insert incorporated into the open reading frame of LacZ gene, galactose and 5-bromo-4-chloro-3-hydroxyindole will be expressed and then oxidized into 5,5'-dibromo-4, 4'-dichloro-indigo, an insoluble blue product. Oxidation of 5-bromo-4-chloro-3-hydroxyindole is useful to distinguish between the empty cells (white colonies) and cells that contain insert (blue colonies).

After incubation for 1.5 h, 100 µl of the competent cells were then plated onto the agar surface and spread using a sterilised glass rod. The plates were then incubated overnight at 37°C.

### ***Screening for positive clones***

A total of 12 colonies per sample were picked and then directly amplified using the COI primers using the same PCR protocol as in Chapter 3.2.5, except that the colonies were added into the PCR master mix directly using toothpick instead of liquid form of DNA. Only half of the colony size was used for the amplification process. After that, the amplified products were visualized using ethidium

bromide dye and photographed using the UV transilluminator (Bio-Rad Laboratories (UK) Ltd.) (for details see Chapter 3.2.3). Each of the amplified products was checked to see whether they were at the correct size of 1.4 kb. Only three DNA fragments at the right size per sample were chosen to be cultured. The other half of the colony (remaining from the plated cells) was cultured in 3 ml of Luria Bertani (L-Broth) (Sigma-Aldrich Inc. USA) culture media. The culture media was left overnight at 37°C in a shaking water bath to allow replication of the bacterial cells.

### ***Pre-sequencing preparation***

A QIAprep<sup>®</sup> spin miniprep kit (Qiagen Inc. Valencia, CA) was used to purify plasmid DNA. The basic principle of plasmid DNA purification is to first lyse the bacteria cells followed by absorption of plasmid DNA onto the QIAprep<sup>®</sup> miniprep membrane in the presence of high salt buffer. Finally the DNA was washed and incubated in 30 µl of EB buffer for 5 minutes. The purified plasmid DNA was then run in 1.5% agarose gel (for details see Chapter 3.2.3). 5 µl of 1 kb DNA marker was used as a standard size (New England BioLabs Inc.) Amplified plasmid DNA was sent to the Dundee Sequencing Service for sequencing (for details see Chapter 3.2.7 (sequencing from direct primer)). Each of the samples was diluted with ddH<sub>2</sub>O to make the final concentration of 200-300 ng of DNA in 15 µl elution buffer, which was required for one sequencing reaction. The concentration of the DNA obtained from plasmid DNA is usually consistent. Thus, 4 µl of each of the samples was diluted with 26 µl ddH<sub>2</sub>O and sent for sequencing of both strands using the forward and reverse M13 primer.

### ***3.2.8 PCR-RFLP analysis***

#### **Restriction digestion**

A larger sample size (78 samples) was used in the PCR-RFLP analysis. Genomic DNA that was amplified (Chapter 3.2.5) and then purified (Chapter 3.2.6) was subjected to RFLP analyses using the restriction enzymes used by Stamatis et al. (2004) in their study of the same species. For both regions (13 enzymes used in each COI and 2.2 kb region), a total of sixteen restriction enzymes were used to digest the amplified products in order to screen for genetic differentiation: *Acil*, *Alul*, *AseI*, *DdeI*, *HhaI*, *HincII*, *HinfI*, *MboI*, *MspI* and *TaqI* were used for both

regions. In addition, *EcoRI*, *PstI* and *MseI*, were applied to the COI region only, while *BanI*, *HaeIII* and *AvaI* were applied to the 2.2 kb region only. Only one restriction enzyme at a time was applied to the PCR product to avoid confusion and misinterpretation. All restriction digestions followed the standard procedure provided by the manufacturer. The master mix containing 1 µl of 10X compatible buffer, 0.1 µl of bovine serum albumin (BSA) provided with the restriction enzymes, 0.25 µl of restriction enzyme and ddH<sub>2</sub>O to make a final solution of 10 µl for each samples. BSA is useful to prevent adhesion of the enzyme to the reaction tube or pipette surface as well as to stabilize some proteins during incubation. The master mix was mixed using a pipette and 6 µl was added into a 1.5 ml microcentrifuge tube containing 4 µl of purified PCR products. The mixture in this tube was then incubated in a water bath set at 37°C for 1 hour. After that, the sample was transferred into another water bath set at 72°C for 20 minutes, in order to inactivate the enzyme. Different concentrations of agarose gel were used (1%, 1.5%, 2%, 2.5% and 3%) in order to find an optimum gel concentration to visualise the digested fragments. The concentration of 2.5% was chosen to obtain a good separation of the bands. The bands were stained with 2.4 µl ethidium bromide dye followed by imaging using a UV transilluminator, as described in Chapter 3.2.3. The digested fragments were compared to a 100 bp DNA marker (5 µl) run on the same gel to establish the sizes of the bands cut for each sample.

### **3.2.9 Data analyses**

#### **Sequencing analysis**

Sequences were analyzed and edited manually using Sequencher v4.7 (Gene Codes Inc.). Consensus sequences were aligned using the Geneious alignment option (using default settings) available in the Geneious Pro 4.8.4 software package (Drummond et al., 2011). Optimisation of the alignment through manual editing was achieved using the same software package. Geneious Pro 4.8.4 was also used to map the restriction sites within sequenced segments from a total of 13 restriction enzymes applied in the PCR-RFLP analysis. Aligned sequences were then used to calculate the number of conserved and variable sites using *MEGA* v5.03 (Tamura et al., 2011). Beside, the nucleotide composition was also calculated using *MEGA* v5.03. The relationships between populations were

visualised by a maximum likelihood tree (Felsenstein, 1981) generated also by *MEGA* v5.03 using the best fitting model suggested by Model selection and calculated with 1000 bootstrap replicates (Felsenstein, 1985). The same software was also utilized to calculate the genetic divergence rate using the best test model, Kimura 2-parameter (Kimura, 1980) and gamma distribution model with 5 categories. This model was chosen based on the lowest number of Bayesian Informative Criterion (BIC) scores. The molecular clock available from *MEGA* v5.03 was applied to test the evolutionary rate using the maximum-likelihood method based on the Kimura-2 model with 5 gamma discrete categories. The final tree was visualised and manipulated using Figtree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/> accessed on 10<sup>th</sup> April 2012). Gene diversities (haplotype and nucleotide diversity) were calculated using *DNA<sub>SP</sub>* v5.10.01 using the aligned nucleotide sequences created using Geneious. A neutrality test was conducted to postulate whether the sequences are evolving naturally or under selection pressure using the Tajima'D (Tajima, 1989) implemented in *DNA<sub>SP</sub>* 5.10.01. Aligned DNA sequences were assigned into the coding region of mitochondrial DNA, and the reading frame was started at the second base pair to carry out the synonymous and non synonymous test. Haplotype data were generated by *DNA<sub>SP</sub>* v5.10.01 (Librado and Rozas, 2009) using an aligned nucleotide sequences data in a fasta formatted file created using Geneious. Based on the haplotype data created using *DNA<sub>SP</sub>* v5.10.01, the sequence for each haplotype was manually copied from one of the sequences of the sample that belonged to that haplotype for further analysis using ARLEQUIN v3.1 (Excoffier et al., 2005). Assessment of relative genetic diversity within and between populations was computed using Analyses of Molecular Variances (AMOVA), as implemented in ARLEQUIN v3.1 using the Kimura-2 parameter model. Fisher's exact P-value was calculated using Arlequin v3.1 with 1000 number of Markov chain length. The exact P-value is to test the differentiation between all pairs of populations. TCS (Clement et al., 2000) was employed to construct the network between haplotypes. All these software packages have been demonstrated to be useful in analysing data for phylogenetic studies (Stamatis et al., 2004, Triantafyllidis et al., 2005, Mamuris et al., 2001). Comparison between the total number of restriction sites detected in PCR-RFLP and sequencing results was also carried out at the end of the study. The

sequencing results were also used to map the size of digested fragments reported in PCR-RFLP analysis.

### **PCR-RFLP analysis**

A specific letter was used for each restriction site detected in the sample, which represents a locus. For example, if sample 1 of COI segments has two cutting sites (three fragments of around 300 bp, 400 bp and 700 bp were detected in the agarose gel), then the cutting sites were labelled with A and B. If the following sample (sample 2) appeared to also have two cutting sites then these were labelled with the same letter if they produced a fragment with the same size. In such cases if a third example had three cutting sites, (four fragments of around 300 bp, 400 bp, 500 bp and 200 bp detected in the agarose gel) then the third site, which differed from the previously identified sites A and B, was labelled with C.

The TCS network (Clement et al., 2000) was used to create the network among studied populations using the aligned sequences from Geneious Pro 4.8.4. Because TCS is not specifically designed for RFLP data, input data were created that were compatible with the TCS software. The input data were created based on the haplotype sequence (letter) (Table 3-5) and each variation in the letters represents the variation in the fragment size produced by each restriction enzyme applied to different samples. Variation in the fragment size was due to the nucleotide substitution that occurred in the restriction sites of certain samples.

## **3.3 Results**

Evaluation of the different pre-treatments of the muscle tissue revealed that samples kept in liquid nitrogen and on ice gave excellent quality of DNA without any problem for downstream analyses. The purified genomic DNA treated with treatments (1) and (2) showed a high concentration of DNA that was not degraded as in the samples treated without ice during transportation. However, samples that were kept without ice for several hours and then transferred to -20°C gave a poor quality of DNA. Meanwhile, gel electrophoresis and nucleic acid measurement of the purified DNA using a spectrophotometer showed that the

Clyde Sea area and North Minch samples gave a high quantity of DNA and the purity of the samples was within the acceptable range, while most of Icelandic samples showed a low quantity of DNA although no contamination was observed.

### **3.3.1 mtDNA sequence information**

A total of 14 samples were sequenced using the combination of the three methods described in Chapter 3.2.7. After analysing and editing the sequences, ambiguous nucleotides were excluded to give a total of 1155 bp sites for tracing any polymorphisms that occurred within the fragment sequenced. This segment contained 36 variable sites, with a total of 37 nucleotide substitutions, and 1119 conserved sites. Of the variable sites, 10 were parsimony informative and 26 were singleton variable sites, which were observed only once. 30 out of 36 variable sites revealed in the sequencing analysis were not detected through the PCR-RFLP analyses (Table 3-2). Haplotype diversity observed in this study was 0.9890 and nucleotide diversity was 0.00609. No significant deviation from neutrality was found for Tajima's  $D$  ( $-1.68127$ ;  $0.10 > P > 0.05$ ). 35 of the variable sites were synonymous and only 1 was a non-synonymous site. From a total of 7 nucleotide substitutions detected in PCR-RFLP analysis, only 4 nucleotide substitutions occurred at the restriction sites, which altered the sequence of the restriction sites. Consequently, the altered restriction sites are no longer restriction sites. Meanwhile, 3 nucleotide substitutions have created restriction sites for *MspI*, *MboI* and *HinfI* (Table 3-3). In addition, sequence analyses revealed that 3 nucleotide substitutions occurred inside the restriction sites of unapplied restriction enzymes (*RsaI*, *NdeI* and *BglI*) in the PCR-RFLP analysis (Table 3-4). A total of 33 restriction sites were detected in this analysis.

### **3.3.2 PCR-RFLP analyses**

A total of 78 samples from four *N. norvegicus* populations of Clyde North, Clyde West, North Minch and Icelandic water were successfully amplified and resolved with all the restriction enzymes except *AccI* and *HpaI*. However, amplification of the 2.2 kb segment of the Icelandic samples was unsuccessful, and thus excluded from the study. The low quantity of PCR products from amplification of 2.2 kb region was still obtained after this DNA precipitation process, so no further analysis was done on these samples. The low quantity of these PCR products was



attributed to the quality of the template DNA or PCR amplification that may not have been fully optimised.

All the restriction enzymes were successfully optimized, except for *Acil* and *HpaI*, which did not work with the standard procedures. For COI, the *Acil* restriction enzyme showed some signs of partial digestion in that the original band (1.4 kb) still appeared in the gel (Figure 3-2). Partial digestion by the restriction enzyme could be due to several factors such as suboptimum incubation temperature and/or time, as well as a suboptimum concentration of the restriction enzyme in the master mix. However, these might not be the only factors in this case. Another restriction enzyme, *HpaI*, did not cut the 1.4 kb region at all (Figure 3-3). Preliminary analyses carried out on several sequences of COI regions obtained from the sequencing analysis indicated that no recognition sites exist for both of the enzymes (*Acil* and *HpaI*). These enzymes were thus excluded from the experiment.

The results from restriction digestions are shown in Figure 3-4 where no variation in the cutting sites were detected in all samples, while samples 1, 4 and 5 of Cldye Sea area in Figures 3-5 show variation, where the *MboI* restriction enzyme cut the 1.4 kb region into 3 fragments. The remaining samples that did not show variation in Figure 3-5 were cut into 2 fragments. In total, 29 restriction sites were found in the 1.4 kb segment from digested with 11 restriction enzymes. From PCR-RFLP analysis, two polymorphisms led to the alteration of the restriction sites (1 in North West and 1 in Icelandic population); consequently, they were no longer recognised as a restriction site while 12 polymorphisms created restriction sites (Table 3-5).

**Table 3-2: Thirteen haplotypes (letters A-M) from the sequencing analysis. Sequencing revealed 30 polymorphic sites within the restriction sites in each haplotype that were not detected by PCR-RFLP analysis. The number 0 indicates the absence and 1 the presence of the polymorphic sites. The location and nucleotide that was substituted (e.g. C2T) are also shown.**

Haplotype	A	B	C	D	E	F	G	H	I	J	K	L	M
Polymorphic sites													
C2T	0	0	0	0	0	0	0	0	0	0	1	0	0
G26A	0	0	0	0	0	0	0	0	0	0	0	1	0
T35G	0	0	1	0	0	1	0	0	0	0	0	0	0
T59C	0	0	0	0	0	0	0	0	0	0	1	0	0
G95A	0	0	1	0	0	1	0	0	0	0	0	0	0
C122T	0	0	0	0	0	1	0	0	0	0	0	0	0
T146C	0	0	0	0	0	0	0	0	0	0	0	1	0
T152G	0	0	0	1	0	0	0	0	0	0	0	0	0
C233T	0	0	1	0	0	1	0	0	0	0	0	0	0
A254G	0	0	0	0	0	0	0	0	0	0	0	1	0
A269G	0	0	1	0	0	1	0	0	1	0	0	0	0
G311A	0	0	1	0	0	1	0	0	1	0	1	0	1
T491C	0	0	0	0	1	0	0	0	0	0	0	0	0
G485A	0	0	0	0	0	0	0	0	0	1	0	0	0
T518C	0	0	0	0	1	0	0	0	0	0	0	0	0
C521T	0	0	0	0	0	0	0	0	0	1	0	0	0
C614T	0	0	1	0	0	1	0	0	1	0	0	0	0
A638G	0	0	0	0	0	0	0	0	1	0	0	0	0
T662C	0	0	0	0	0	0	0	0	0	0	0	0	1
C728T	0	0	1	0	0	1	0	0	1	0	0	0	0
T749C	0	0	0	0	0	0	0	0	0	0	1	0	0
T791C	0	0	0	0	0	0	0	0	0	0	1	0	0
G797A	0	0	0	0	0	0	1	0	0	0	0	0	0
G869A	0	0	0	0	0	0	0	0	1	0	0	0	0
A890G	0	0	0	0	0	0	0	0	0	0	0	1	0
C926T	0	0	0	0	0	0	1	0	0	0	0	0	1
C959T	0	1	0	0	0	0	0	0	0	0	0	0	0
C1028T	1	0	0	0	0	0	0	0	0	0	0	0	0
T1052C	0	0	0	0	0	0	0	0	0	1	0	0	0
T1061C	0	1	0	0	0	0	0	0	0	0	0	0	0

Table 3-3: Seven polymorphic sites reported in the PCR-RFLP analysis that were confirmed by the sequencing analysis. Haplotypes obtained from PCR-RFLP are indicated by the combination of letter and number. Haplotypes obtained by sequencing analysis are indicated by letter. Location and nucleotide that were substituted are also shown. A number 1 in the table indicates the presence of the restriction cut and 0 indicates the absence of the

polymorphic sites. Respective restriction enzymes that have nucleotide substitutions are also shown.

		Haplotype							
PCR-RFLP		H8	H3	H2	H4	H12	H10	H14	
Sequencing		D	E	G	H	J	K	L	
Polymorphic sites									Restriction enzyme
T18C		1	0	0	0	0	0	0	MseI
C338A		0	0	0	0	1	0	0	MspI
A323T		0	0	0	0	0	1	0	PstI, AluI
A479G		0	0	0	0	0	0	1	MboI
C521G		0	0	0	1	0	0	0	MspI
T854C		1	0	1	0	0	0	0	MboI
C908G		0	1	0	0	0	0	0	HinfI

Table 3-4: Polymorphic sites that occurred at the restriction sites of several restriction enzymes that were not applied in this study. Frequency of the haplotype (F), nucleotide substitution and the polymorphic site (P), haplotype (letter A-M) and the respective restriction enzymes (R).

Population	F	P	Haplotype												R	
			A	B	C	D	E	F	G	H	I	J	K	L		M
Icelandic	1	T59C	0	0	0	0	0	0	0	0	0	0	1	0	0	BgII
Clyde North	1	C728T	0	0	1	0	0	1	0	0	1	0	0	0	0	RsaI
Clyde West	2															
Clyde North	1	G797A	0	0	0	0	0	0	1	0	0	0	0	0	0	NdeI

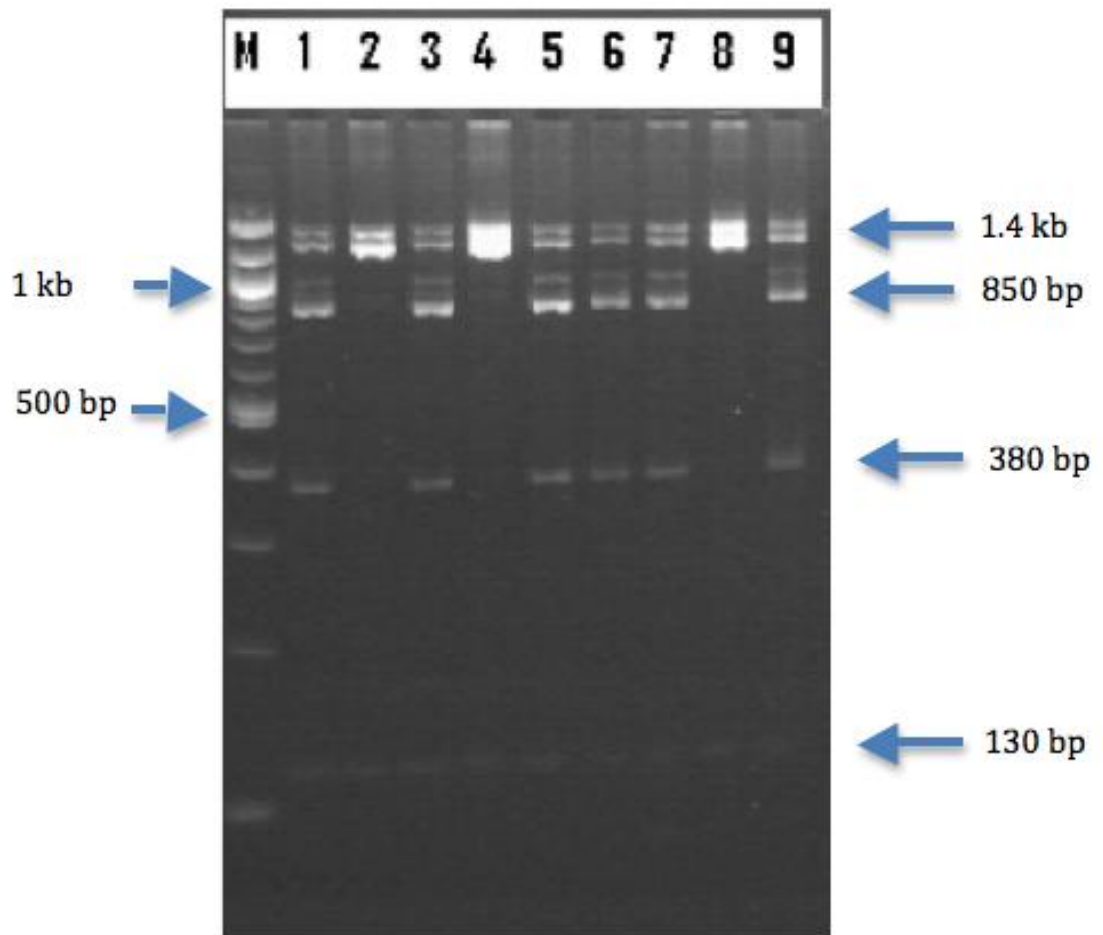


Figure 3-2: Restriction digestion on the 1.4 kb COI gene region for Clyde Sea area samples (lanes 1-9) using the *Acil* restriction enzyme. Samples 2, 4 and 8 were not cut with *Acil* restriction enzyme while sample 1, 3, 5, 6, 7 and 9 produced three fragments. Lane M: 100 bp DNA size standard.

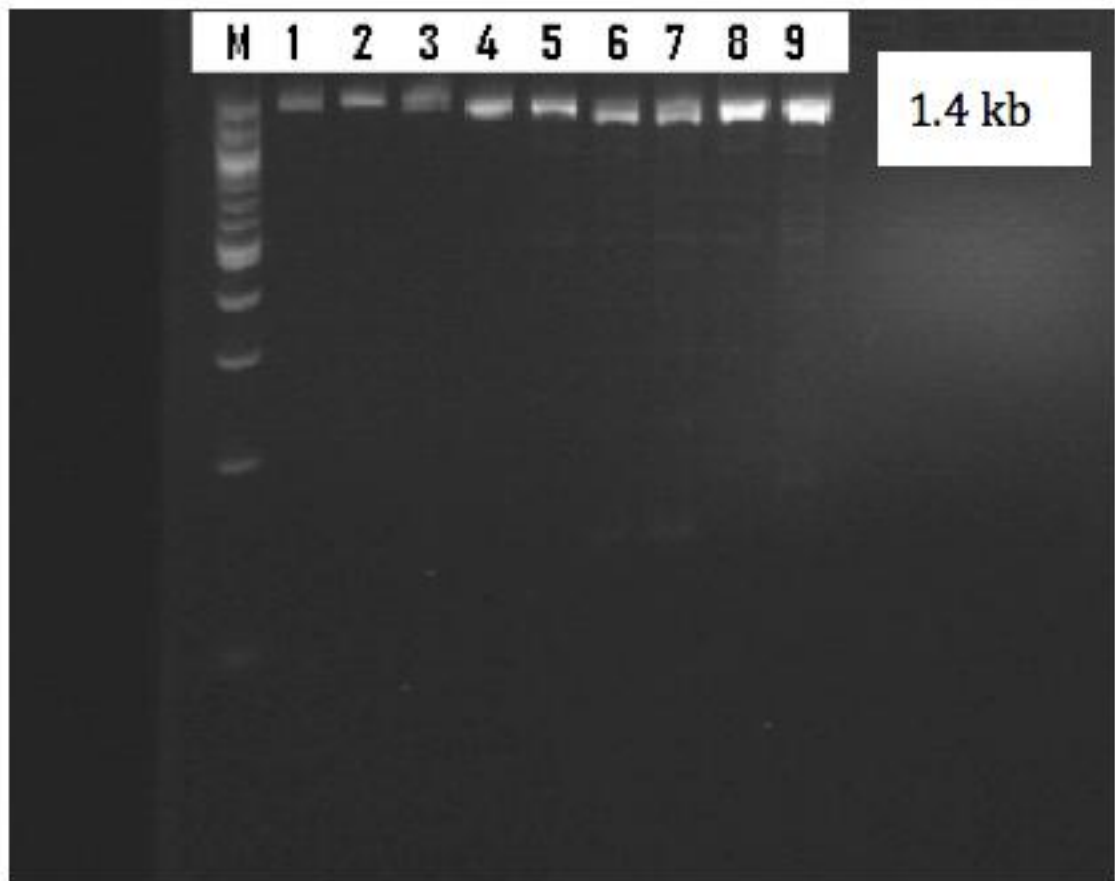


Figure 3-3: Restriction digestion of the 1.4 kb region of COI for the Clyde Sea area samples (lanes 1-9) using the *HpaI* restriction enzyme. The amplified COI segments are not cut by *HpaI*.

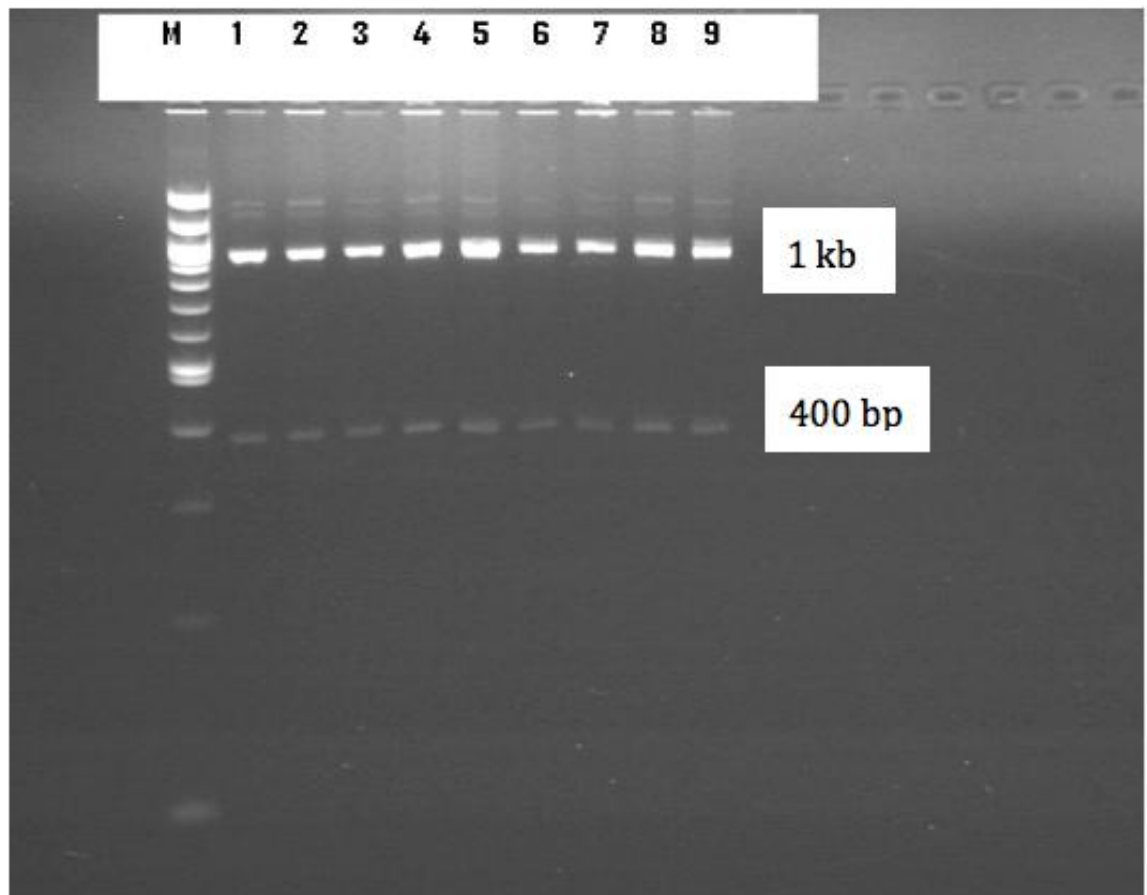


Figure 3-4: *EcoRI* digestion on Clyde Sea area samples of amplified COI segment (lanes 1-9). Samples were run on a 2.5% agarose gel. Lane M: 100 bp DNA ladder marker. Two fragments (400 bp and 1000 bp) were observed for each sample, indicating that no variation occurs at the restriction site.

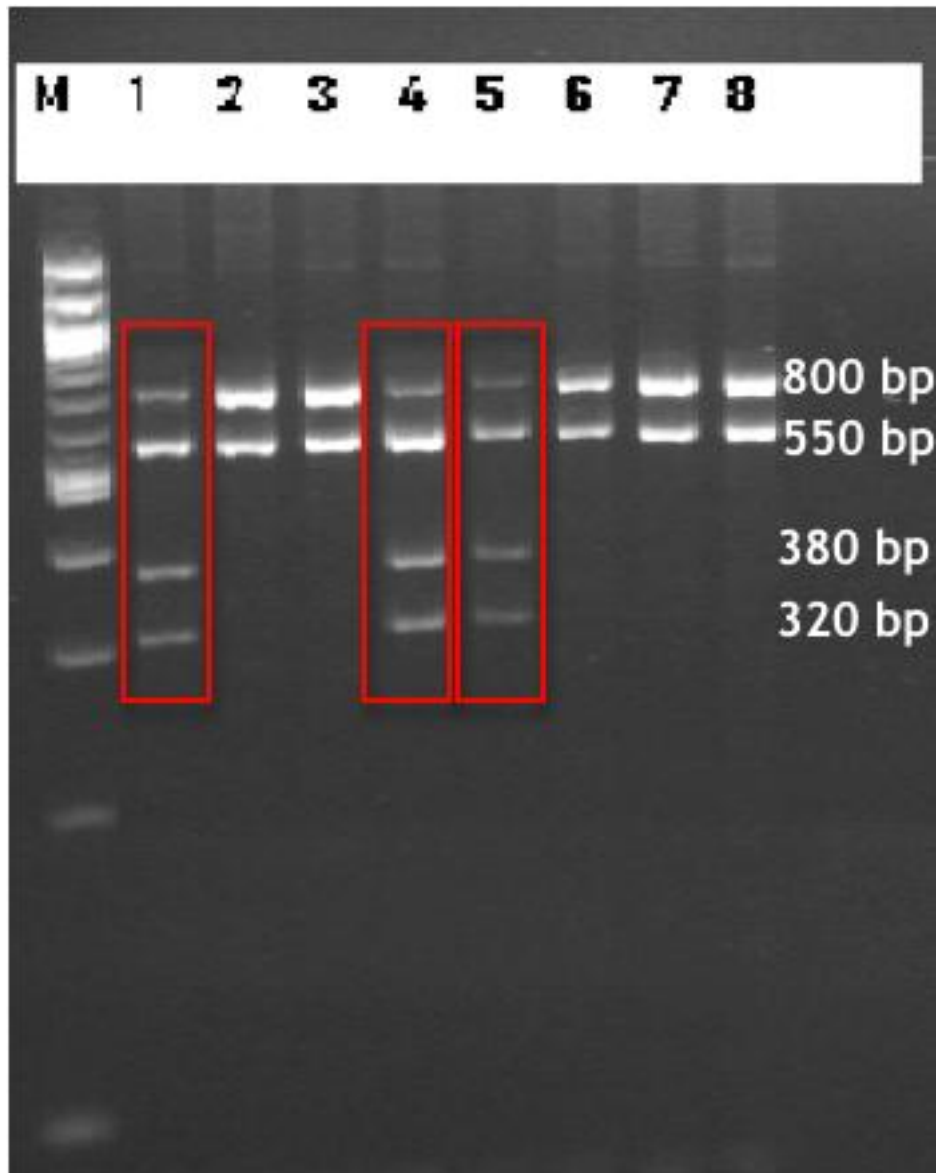


Figure 3-5: *Mbol* digestion of Clyde Sea area samples (lanes 1-8) on a 2.5% agarose gel. Sample numbers 1, 4 and 5 show variation compared to the other lanes. The 1.4 kb of COI segments are resolved into 3 bands (around 320 bp, 380 bp and 550 bp). The bands at size around 800 bp are still not fully resolved in the samples that show variation. Lane M: 100 bp DNA ladder marker.



Table 3-5: Composite haplotype created from the restriction enzyme banding patterns of the COI segment before cross checking with sequencing analysis. The absolute frequency of samples in every population for each haplotype are provided. Haplotype frequency is the total number of samples with each haplotype and TOTAL is the sample size for each population. A total of 11 restriction enzymes were applied. The order of the restriction enzymes in the composite haplotype are *AluI*, *DdeI*, *EcoRI*, *HincII*, *HinfI*, *MboI*, *MspI*, *PstI* and *TaqI*.

Haplotype	Composite haplotype	Clyde North	Clyde West	Icelandic	North Minch	Haplotype frequency
H1	AAAAAAAAAAAA	17	14	10	14	55
H2	AAAAAABAAAA	2	-	3	-	5
H3	AAAAABAAAAA	1	-	1	3	5
H4	AAAAAAAABAA	-	1	1	-	2
H5	BAAAAAAAAAAAA	-	1	-	-	1
H6	AAAAACAAAAA	-	1	-	-	1
H7	AAAAAACDAAA	-	-	-	1	1
H8	AAAAAABCAAA	-	-	-	1	1
H9	AAAAABABAAA	-	1	-	-	1
H10	AAAAAAAABAA	-	-	2	-	2
H11	AAAAAAAADAA	-	-	1	-	1
H12	AAAAAAAACAA	-	1	-	-	1
H13	CAAAAAAAAAAAA	-	1	-	-	1
H14	AAAAAACAAAA	-	-	1	-	1
<b>TOTAL</b>		<b>20</b>	<b>20</b>	<b>19</b>	<b>19</b>	<b>78</b>

### 3.3.3 Cross checking of PCR-RFLP results

The PCR-RFLP results were cross-checked with information on the sequences obtained from sequencing analysis. This was carried out to increase the reliability of each of the variations found in PCR-RFLP analysis.

After cross-checking the PCR-RFLP with the sequencing results, 6 haplotypes were excluded from a total of 14 haplotypes reported in the PCR-RFLP analyses, as the haplotypes showed similarity to each other in the restriction pattern. For instance, the PCR-RFLP analysis of Clyde 34 and Icelandic 9 has created haplotype 6 and 4, respectively. This was determined from the number of digested fragments obtained from the PCR-RFLP analysis. Clyde 34 produced three fragments when digested with *HinfI* at size of 700 bp, 400 bp and 250 bp compared to the other samples that produced three bands but at different sizes, which were 890 bp, 400 bp and 100 bp. This sample also showed variation in the fragment size when digested with *MspI* restriction enzyme, producing three fragments of size 700 bp, 400 bp and 300 bp but other samples produced only two fragments which were at 1000 bp and 400 bp. The other samples, Icelandic 9 showed variation that similar to Clyde 34 only when digested with *MspI* restriction enzyme. On the other hand, results from sequencing analysis show no variation in Clyde 34 at the *HinfI* restriction sites compared to other samples. However, the sequencing analysis has confirmed the variation at *MspI* restriction site. Thus, haplotypes 6 and 4 (Clyde 34 and Icelandic 9) were combined together as they contained the same variation only at *MspI* restriction sites. Consequently, the Clyde 34 sample appeared to have a similar restriction pattern to Icelandic 9. In addition, haplotype 7 was also excluded as it showed similarity to haplotype 14 since no polymorphisms were detected in the *MseI* recognition site but only within the *MboI* recognition site. Another 4 haplotypes were also excluded since sequencing analyses detected no polymorphisms in their sequences. These haplotypes were combined with haplotype 1 (main haplotype) (Table 3-6). No significant genetic differentiation was detected among studied groups ( $F_{ST}$  value: 0.0058) (Tables 3-7).

Table 3-6: Haplotype frequencies after cross-checking with sequencing analysis. Haplotypes (*H*), Composite haplotype, sample size, total haplotypes in each population and total numbers of unique haplotype. The frequency of haplotypes reported in the PCR-RFLP analysis (A) and modified haplotypes after cross-checking with the sequence analysis (B). *F* is total number of samples with each haplotype. *T* is the type of haplotype: S indicates the shared haplotypes and U indicates a unique haplotype. The grey shaded rows are the haplotypes that were excluded from the study after cross-checking with the sequencing technique. The red numbers represent the changes made to the PCR-RFLP data after cross checking with sequencing analysis.

		Haplotype frequency									
<i>H</i>	Composite haplotype	Clyde North		Clyde West		Icelandic		North Minch		<i>F</i>	<i>T</i>
		A	B	A	B	A	B	A	B		
H1	AAAAAAAAAAAA	17	17	14	18	10	11	14	14	60	S
H2	AAAAAABAAAA	2	2			3	3			5	S
H3	AAAAABAAAAA	1	1			1	1	3	3	5	S
H4	AAAAAAAABAA			1	1	1	1			2	S
H5	BAAAAAAAAAAAA			1						1	-
H6	AAAAACAAAAA			1						1	-
H7	AAAAAACDAAA							1		1	-
H8	AAAAAABCAAA							1	1	1	U
H9	AAAAABABAAA			1						1	-
H10	AAAAAAAABA					2	2			2	U
H11	AAAAAAAADAA					1				1	-
H12	AAAAAAAACAA			1	1					1	U
H13	CAAAAAAAAAA			1						1	-
H14	AAAAAACAAAA					1	1		1	2	S
Sample size		20	20	20	20	19	19	19	19	78	
Total haplotype		3		3		6		4		8	
Total unique haplotype		0		1		1		1		3	

Table 3-7: Values of  $F_{ST}$  obtained from PCR-RFLP technique. The haplotypes data were divided into North Atlantic and Icelandic. Genetic differentiation is explained among groups, among populations within groups and within populations.

Hierarchy	Source of variation	Percentage of variation	P-value
Two structures: 1) North Atlantic 2) Icelandic	Among groups	FCT: 0.582	0.245±0.015
	Among populations within groups	FSC: -0.390	0.328±0.014
	Among populations	FST: 94.57	0.101±0.008

### **3.3.4 Phylogenetic analyses**

The Kimura 2-parameter was chosen as the best model for nucleotide substitution pattern and used to build the maximum likelihood tree. A star like shape of the maximum likelihood tree depicts homogenous genetic composition between the studied populations, confirmed by the high percentage of bootstrap replicates (1000 replicates was used) (Figure 3-6). However, haplotypes 9, 3 and 6 were differentiated from other samples. Only haplotype 8 was shared between Clyde Sea area (Clyde West) and Icelandic samples. The TCS network to show the relationship of the haplotypes derived from PCR-RFLP analysis before cross checked with sequencing analysis was obtained (Figure 3-7). Furthermore, the TCS network of composite haplotypes derived from both PCR-RFLP (after cross-checking with sequencing analysis) and sequencing analyses were also obtained. The networks between these two sources were not identical as they contain different information (Figure 3-8 and 3-9). TCS analysis depends on 36 polymorphisms detected by the sequencing technique, but only 11 restriction sites in PCR-RFLP from 11 restriction enzymes. Because of this, sequencing analysis is more informative than PCR-RFLP. The TCS network created from PCR-RFLP results after cross-checking with sequencing results showed that the haplotypes were not structured by populations. The main haplotype (H1) from PCR-RFLP analysis was shared among all populations. Only three unique haplotypes were observed in the studied populations where the frequency was similar in Clyde West, North Minch and Icelandic populations (1 unique haplotype/population) but none were observed in Clyde North. The frequency of the haplotypes was highest in the Icelandic population (6 haplotypes/19 samples) and lowest in Clyde North (3 haplotypes/20 samples) and Clyde West (3 haplotypes/20 samples). Meanwhile the North Minch has 4 haplotypes/19 samples (Table 3-6).

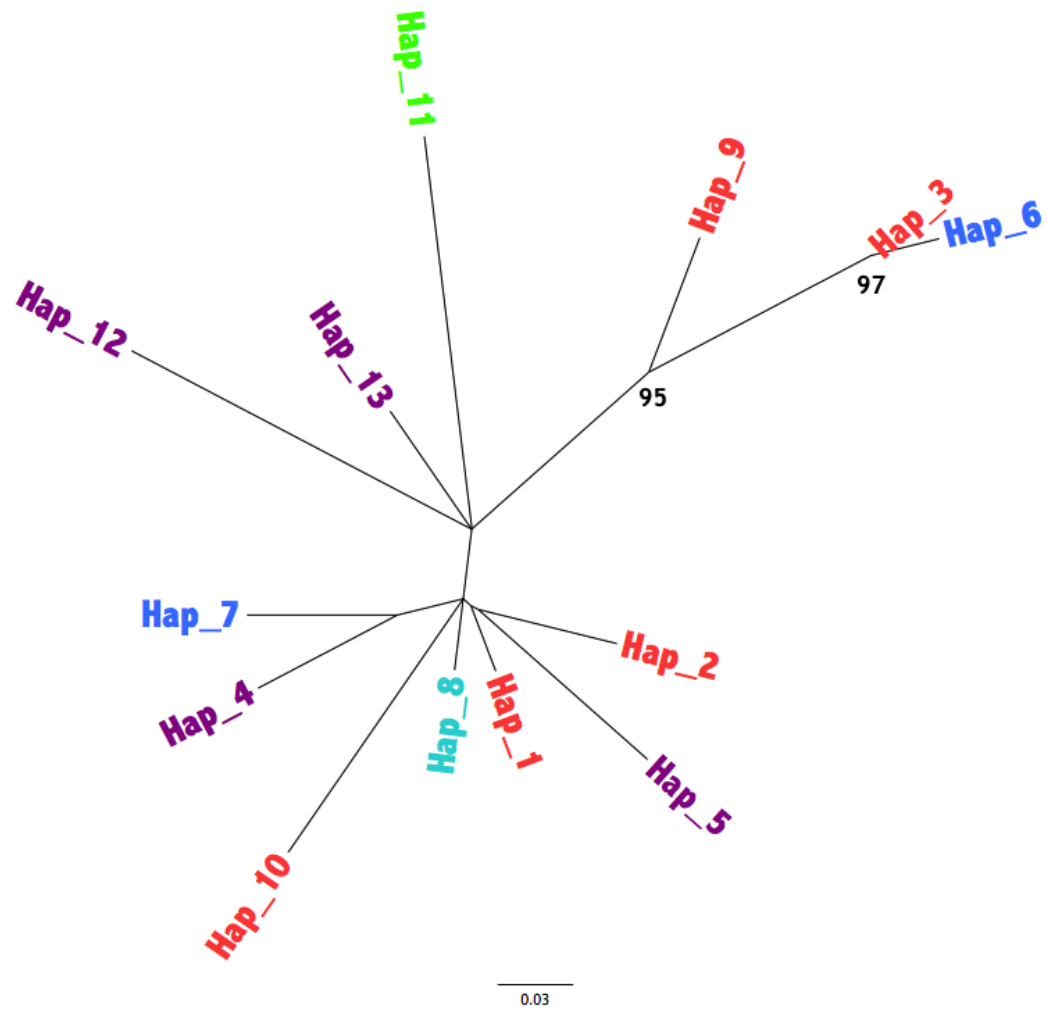


Figure 3-6: Maximum likelihood tree depicting that no haplotypes were structured by populations. However haplotypes 3, 6 and 9 are differentiated from the other samples. Haplotype colours; blue: Clyde North, red: Clyde West, purple: North Minch, green: Icelandic samples, turquoise: combination of Clyde West and Icelandic. Numbers indicate the percentage of bootstrap value/1000 replicates (only value above 70% are shown).

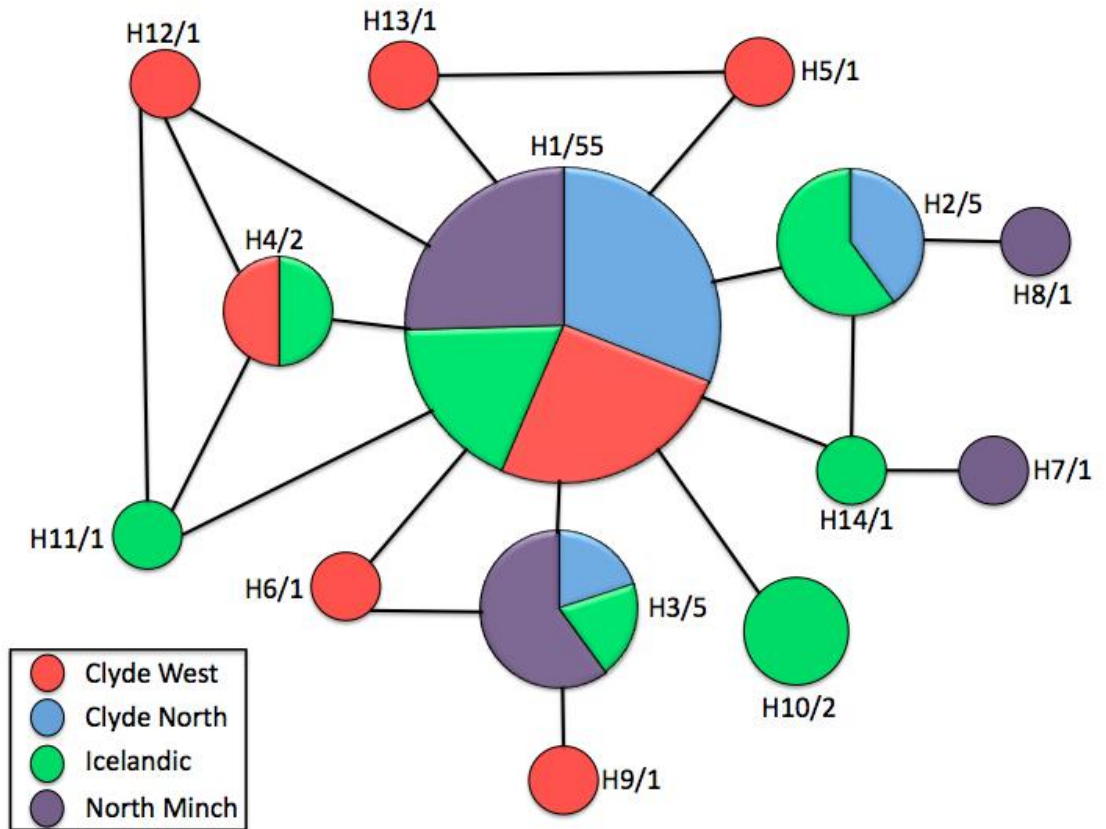


Figure 3-7: Haplotype network based on composite haplotypes constructed from the banding pattern of the PCR-RFLP analysis. Haplotypes were not structured by populations. Each population is colour coded as indicated in the key. The name and frequency of the haplotypes are indicated (in order).

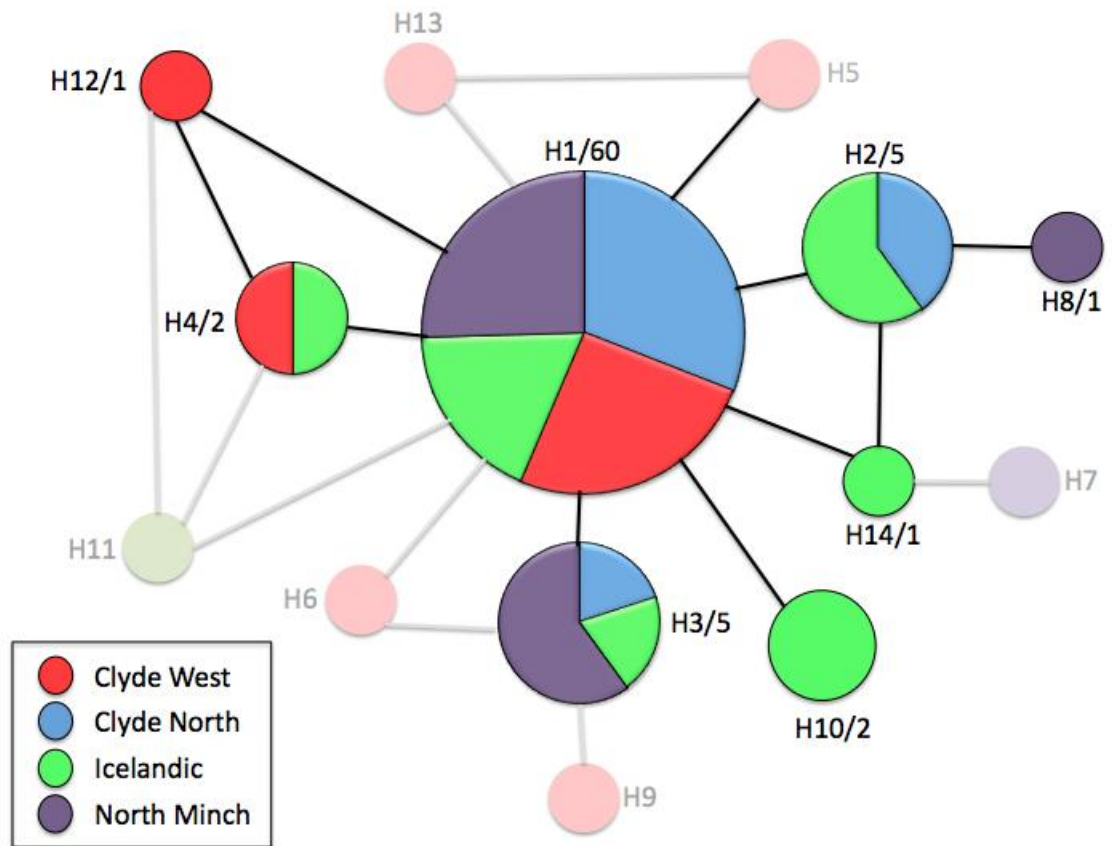


Figure 3-8: TSC network between composite haplotype sequences of the PCR-RFLP technique after it was cross-checked with the sequencing analysis. A star-like shape indicates a homogenous genetic structure in the studied populations. The haplotype names and frequency are indicated (in order). Each population is colour coded as indicated in the subset. The faint circles are the haplotypes that were excluded after cross-checking with the sequencing technique.

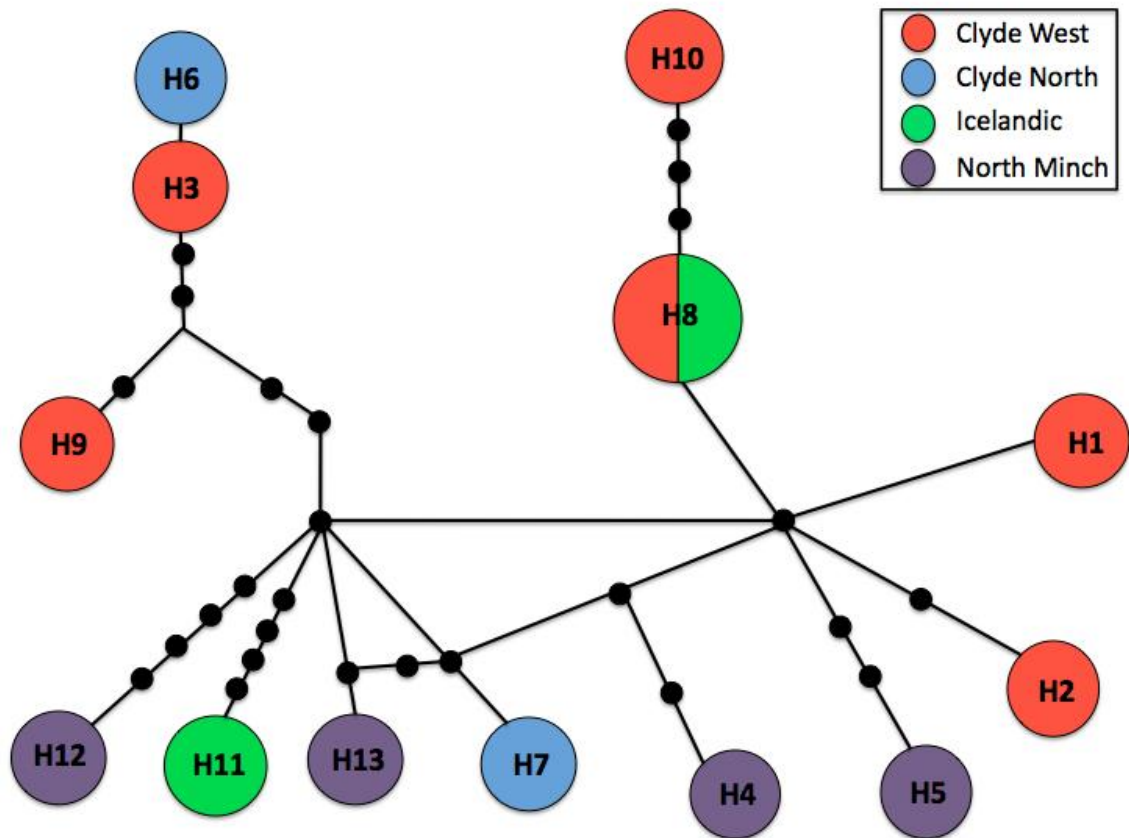


Figure 3-9: TCS network between 13 haplotypes (H1-H13) created from the sequencing of 14 polymorphic samples reported in the PCR-RFLP analysis show that the haplotypes were not structured by populations. Small black solid circles indicate the individual mutations separating haplotypes. Each population is colour coded as indicated in the subset.

### 3.3.5 Comparison between sequencing and PCR-RFLP data

The 33 restriction sites found in the sequencing analyses were inconsistent with the number reported from PCR-RFLP (29 restriction sites). From the sequencing analyses, 13 haplotypes were constructed based on the polymorphic sites derived from 14 sequences (each sequence represented the sequence of each haplotype reported by PCR-RFLP). However only 8 haplotypes from 78 samples were created in the PCR-RFLP analysis. Compared to PCR-RFLP, the sequencing technique was able to detect 81% (30) more sites with nucleotide substitutions. Analyses of the data produced from both methods using the TCS network supported the fact that all haplotypes were not structured by populations. The number of restriction sites revealed by RFLP is not equal to the restriction sites found in the sequencing results might be due to the small fragment size that was beyond agarose gel resolution. However, previous studies have reported that 2% and 2.5% agarose gels were successfully utilised for RFLP (Jones et al., 2003,



Chow et al., 2006), while other gel types with higher resolution such as 6% polyacrylamide (Stamatis et al., 2004) and 1.5% Metaphor agarose (Hiraishi et al., 1995) could also be used to avoid this problem.

### 3.4 Discussion

Both sequencing and PCR-RFLP analyses carried out in the present study suggested that haplotypes derived from the studied populations were not structured by populations. Mitochondrial markers only provide information of the genetic from maternal side only. The genetic information obtained from this study explained that there is no differentiation among and within population of *N. norvegicus* from maternal side from the studies area. Nevertheless the genetic information at paternal side is uncertain. The morphological differentiation observed in *N. norvegicus* could be due to the differentiation inherited from paternal side. However outcomes from Streiff et al. (2001) study that used microsatellite markers has proved that their studied populations (south and west of Portuguese coast) showed no significant genetic differentiation. In addition, the recently published work that also used microsatellite markers has supported the outcomes from the present study (Pampoulie et al., 2011). These authors used the same samples as in the present study (Clyde population) and concluded that *N. norvegicus* populations were unlikely to be structured on a geographic scale between the Icelandic and Scottish samples. The evidence from both the deep phylogeographic pattern (from mitochondrial markers) and population genetic analyses and resolution of population structure based on recent allele frequency distribution and changes (from microsatellite markers) were consistent in both the present study and that of Pampoulie et al. (2011), regarding the genetic differentiation between North Atlantic and Icelandic populations. Thus, the combination from the present and Pampoulie et al. (2011) studies has provided the genetic information from both sides of maternal and paternal.

After comparison between the outcomes from the present and other studies, it was decided not to increase the sample size of the studied populations and to terminate the study at that stage. In addition, all the above mentioned studies

used either microsatellite marker, PCR-RFLP or allozyme marker, hence a direct comparison between the sequences of the haplotypes to see the relationship between the haplotypes derived from the present study and those studies are not possible due to the differences of the data type. However larger sample size will provide a better relationship between studied populations.

Based the evidence from the  $F_{ST}$  value obtained from the present study, it can be concluded that the high morphological differentiation in *N. norvegicus* may not be associated with the genetic factors, but rather with environment factors. Sediment type of the habitat may play a particularly significant role. The strong relationship between sediment type and *N. norvegicus* density that leads to the variation in the size was reported in a previous study (Tuck et al., 1997). Nevertheless other factors such as temperature, location, depth, hydrology condition and fishing pressure (as explained in Chapter 1.3) cannot be ruled out.

The current genetic pattern observed could be explained by two inconclusive theories, supported also by Pampoulie et al. (2011) and Stamatis et al. (2004), which are the high gene flow among populations and the recent population expansion. It is well known that *N. norvegicus* is a sedimentary, burrowing species, that they favour walking over swimming and rarely migrate over a distance greater than 100 m from their burrow (Chapman and Rice, 1971). The only possibility of genetic mixing is at the planktonic larval stages where there is the possibility of larval dispersal by ocean current flows. Pampoulie et al. (2007 and 2011) have explained the possibility of ocean current flows in shaping the genetics of both Icelandic cod (*Gadus morhua*) and *N. norvegicus* from Icelandic waters. Icelandic currents can be divided into two main currents which are the North Atlantic current and the coastal current. These two currents have promoted the larval dispersal between populations. The North Atlantic currents branch at the mid-south of Icelandic coast, with the main branch flowing to the southwest and the other branch flowing to the southeast. Meanwhile, the coastal currents flow clockwise around the country. However for long distance populations such as between Icelandic and North Atlantic populations, this theory is very unlikely even though it cannot be totally excluded. Meanwhile, Stamatis et al. (2004) invoked the theory that a recent population expansion occurred after the last glacial maximum (LGM) during Pleistocene age and proposed that this might play a more important role in shaping the current

genetics of *N. norvegicus*. A population that possessed a high level of haplotype diversity but a low to moderate level of nucleotide diversity as also detected in the present study (haplotype diversity, Hd: 0.9890 and nucleotide diversity, Pi: 0.00609) is a characteristic of population that expands after a period of low effective population size. Consequently, the rapid population growth has promoted the retention of new mutations. Beside, Stamatis et al. (2004) also showed the evidence of mutation-drift disequilibrium which is attributable to a rapid population growth. Nonetheless, other *Nepropiadae*, from the same studied area show a different level of genetic differentiation. A low level but not significant of genetic differentiation has been reported in the American lobster, *Homarus americanus* (Kenchington et al., 2009, Crivello et al., 2005). Surprisingly, the result of the mtDNA study on the Norway populations of the European lobster (*Homarus gammarus*), indicating high diversity, was found to be opposite to that in *N. norvegicus* and *H. americanus* (Jorstad et al., 2005). Another study that supported the previous finding has revealed a positive correlation between geographic and genetic distances in European lobster populations throughout their geographical distribution, mainly due to the samples from northern Norway, the Netherlands and the Mediterranean Sea populations (Triantafyllidis et al., 2005). Meanwhile, studies on other marine invertebrates and vertebrates with similar distributions showed different levels of genetic differentiation. High genetic differentiation was found in the prawn *Palaemon elegans* (Reuschel et al., 2010), European hake, *Merluccius merluccius* (Cimmaruta et al., 2005) and white seabream, *Diplodus sargus sargus* (Gonzalez-Wangueemert et al., 2010).

Sequencing more of or all the samples will provide a better picture of the relationships among sequences. However, applying more restriction enzymes that have restriction sites in the COI region will not produce more informative data as only 3 nucleotide substitutions were detected inside the restriction sites that occur at a very low frequency rate in the sequences.

The PCR-RFLP method required information on the availability of restriction sites in the targeted region is needed in order to select the most appropriate restriction enzyme. Besides, scoring the banding can be difficult, especially with low quality of banding, as well as for enzymes that have multiple cutting sites on the PCR product. The experiments need to be repeated at least two or three

time to avoid uncertainty. Confirmation with other methods such as sequencing is sometimes required for confirmation. Despite the less informative marker over sequencing analysis, PCR-RFLP analysis is able to produce the same level of genetic differentiation among the studied species as evidenced by the comparison between of the  $F_{ST}$  value obtained in the present study with other *N. norvegicus* studies. This method is considered less reliable and informative than sequencing, but preferable when cost is a constraint.

On the other hand, the sequencing technique is more informative and reliable to detect variation compared to PCR-RFLP technique. Moreover, no information of the polymorphism sites is needed prior to the experiment. This method is considered to be cheap in cases where no repetition of sequencing analysis is required for good quality DNA samples, which normally produce a good quality of sequencing result (Table 3-8).

**Table 3-8: Comparison between the PCR-RFLP and sequencing techniques, based on the outcomes of the present study and general comparison.**

	PCR-RFLP	Sequencing
<b>Outcomes from the present study</b>		
Cutting sites	29	33
Polymorphic sites	7	37
Total haplotypes	8 (78 samples)	13 (14 samples)
Total unique haplotype	3	1
<b>General comparison</b>		
Time	Time consuming	Less time
Reliability	Less reliable data	More reliable data
Informative	Less informative	More informative

## **SECTION II: *Macrobrachium rosenbergii***

### **Chapter 4**

#### **General introduction to *Macrobrachium rosenbergii***

##### **4.1 Biogeography of the Indo-Australian Archipelago (IAA)**

The Indo-Australian Archipelago (IAA), also known as Malesia or the Malay Archipelago, has the most complex biogeography found in the tropical region (Lohman et al., 2011). The IAA is the largest archipelago in area and the fourth in the number of islands in the world (more than 20,000 islands straddling the equator in Southeast Asia), with extraordinary species richness and endemism even though most are under anthropogenic threat. The IAA comprises two continents: the Thai-Malay peninsular, Borneo, Sumatra, Java and Bali on the Sunda shelf and New Guinea and Australia on the Sahul shelf. Between the two continents is Wallacea, a biogeographical region comprising islands that separates Asia from Australasia (Michaux, 2010). The name Wallacea was coined by Dickerson in 1928 for an area already delimited by Wallace in 1863. Wallacea includes the Philippines, Sulawesi, the Lesser Sunda Islands, and the Moluccas (Figure 4-1). Although the area of the IAA is only 4% of the land area in the world, it is home to almost 25% of all terrestrial species, and has the most species-rich coral reefs in the world (Corlett, 2009). Wallacea, Indo-Burma, the Sundaland (terrestrial extension of mainland Southeast Asia formed during periods when low sea levels exposed sea beds between the Malay Peninsula, Java, Sumatra, and Borneo) and the Philippines are parts of the IAA recognized as internationally important biodiversity hotspots by Conservation International (<http://www.biodiversityhotspots.org> accessed on 10<sup>th</sup> April 2012). Hotspots are defined on the basis of biodiversity, degree of endemism and level of threat (ICBP, 1992; Cardillo *et al.*, 2006). The provenance of IAA's biota particularly in the Wallacea area has been debated.

In the IAA, a famous zoological boundary based on the biogeographical work of Wallace is found (i.e. Wallace's Line). Wallace defined Wallace's line in 1860,

starting from Selat Lombok, between Bali and Lombok in the south to the Celebes Sea in the north. This line is based on studies of birds, especially parrot species (Simpson, 1977). Wallace was uncertain with regard to the position of Sulawesi; placing the line west of Sulawesi in 1859 and 1863-1876, and east of Sulawesi in 1860 and 1910. The position of Sulawesi was discussed in Wallace's famous book, 'Island life' published in 1880 in which he regarded Sulawesi as an 'anomalous island' with no continental connections because Sulawesi lacks Sundaic groups and contains (old) endemic and Australasian species. Later on Huxley (1868) extended the line in the north in between Kalimantan and the Sulu Archipelago and across the Sulu Sea along the Mindoro Strait between the islands of Mindoro and Palawan. The new modified Wallace line was termed the Huxley modification line. According to this modified line, the Philippines (except the Calamian, Palawan and a few adjacent islands categorised to be faunally Asiatic) is designated into either the Australian Region or Wallacea (Simpson, 1977) (Figure 4-1). In 1895, Richard Lydekker proposed a line that runs in between Wallacea in the west and Australia and New Guinea in the east. This line is known as Lydekker's line (Figure 4-1). A more complete historical overview is presented in Simpson (1977), who describes how a number of variants of Wallace's Line have been proposed based on the study of different groups of organisms.

Borneo and Sulawesi are separated by a deep sea known as the Makassar strait (Figure 4-1). The northern part of the Makassar strait is deeper (about 2.5 km depth) while the southern region is shallower (less than 2 km depth) (Hall et al., 2009). This strait is also known to play an important role in the global climate system, whereby it functions as the main passage to transfer the water and heat from Pacific to India Ocean (Hall et al., 2009). These features (depth and temperature) have shaped a complex biogeographical break of flora and fauna, and have led to many researches, mainly in the mammals (Heaney, 1986).

The complex history of the IAA has contributed to an incredible diversity, and to an endemic biota. Contemporary patterns of species distribution across the IAA have been shaped largely by pre-Pleistocene dispersal and vicariance events (i.e. the separation or division of a formerly continuously distributed population or species due to the formation of geographic barrier, such as a mountain or a body

of water, resulting in differentiation of the original group into new varieties or species) including plate tectonics. Plate tectonic concepts (Lohman et al., 2011) have great potential to explain the distribution of a species on different islands or the dispersal of some organisms within a region on collision and fragmentation of different terranes (Michaux, 1996).

The islands of the IAA are of more varied origin, comprising pieces of continental crust (terranes), oceanic islands, and landmasses of composite origin, such as Sulawesi and New Guinea. These two islands attained their present configuration after separate terranes collided, potentially with any resident biota. Oceanic islands such as the islands in the Wallacea area and Philippine have never had a connection to the adjacent lands since their emergence approximately 23 Mya ago due to the collision between Australian plate and submerged Sundaland. Thus, a dispersal mechanism is more likely to explain the provenance of the biota of these islands. At the beginning of Cenozoic (about 65 My ago), the geography of this region was not as it is at present (Figure 4-2). The ancient vicariance events (e.g. plate tectonic) have given rise to remarkable patterns in the distribution of higher taxa. For instance, the island of Sulawesi was formed from collision of different terranes and fragmentations were suggested to occur about 15 Mya ago (Figure 4-2d,e). Consequently, its biota could originate from either Sundaland in the west or Australian/New Guinea in the east, even though most of its faunas are reported to be Asian origin (van Oosterzee, 1997). The collision between the Australian plate and submerged Sundaland also caused the emergence of New Guinea and also the rise of mountains in the centre of Borneo to Palawan. Changes in Sundaland's geography, particularly in Borneo, have gradually increased the area of shallow seas surrounding it through the massive amount of sediment brought from these mountains through the rivers. The elevation of the mountains and changes in the ocean circulation have affected the climate in Sundaland, causing it to become wetter. The extension of the vicariance event that contributes to the climate changes has also affected the changes in the sea levels. Changes in the connectivity of islands within the Archipelago due to series of changes in the sea level during the Pleistocene, which repeatedly sunder and fuse the islands on the Sunda shelf with each other or the mainland, have influenced the partitioning of intraspecific variation. When the sea level was low, the sea floor was exposed. This allowed the

connection between the islands and/or mainland and dispersal of the species to occur. However when the sea level rose, the sea floor was submerged and the distance between the emergent terrains was increased.

Up until now, the application of molecular genetic approaches which have immediate impact on the understanding of the complexity of biogeography of IAA have stimulated much recent biogeographic work in the IAA. The giant freshwater prawn *Machrobrachium rosenbergii* is one of the model species that could explain the complexity of the IAA biogeography, based on evidence of the existence of two forms of *M. rosenbergii*: the western form (*M. rosenbergii* de Man 1879) and the eastern form (*M. spinipes* Schenkel 1902) that segregate to either side of Huxley's line.

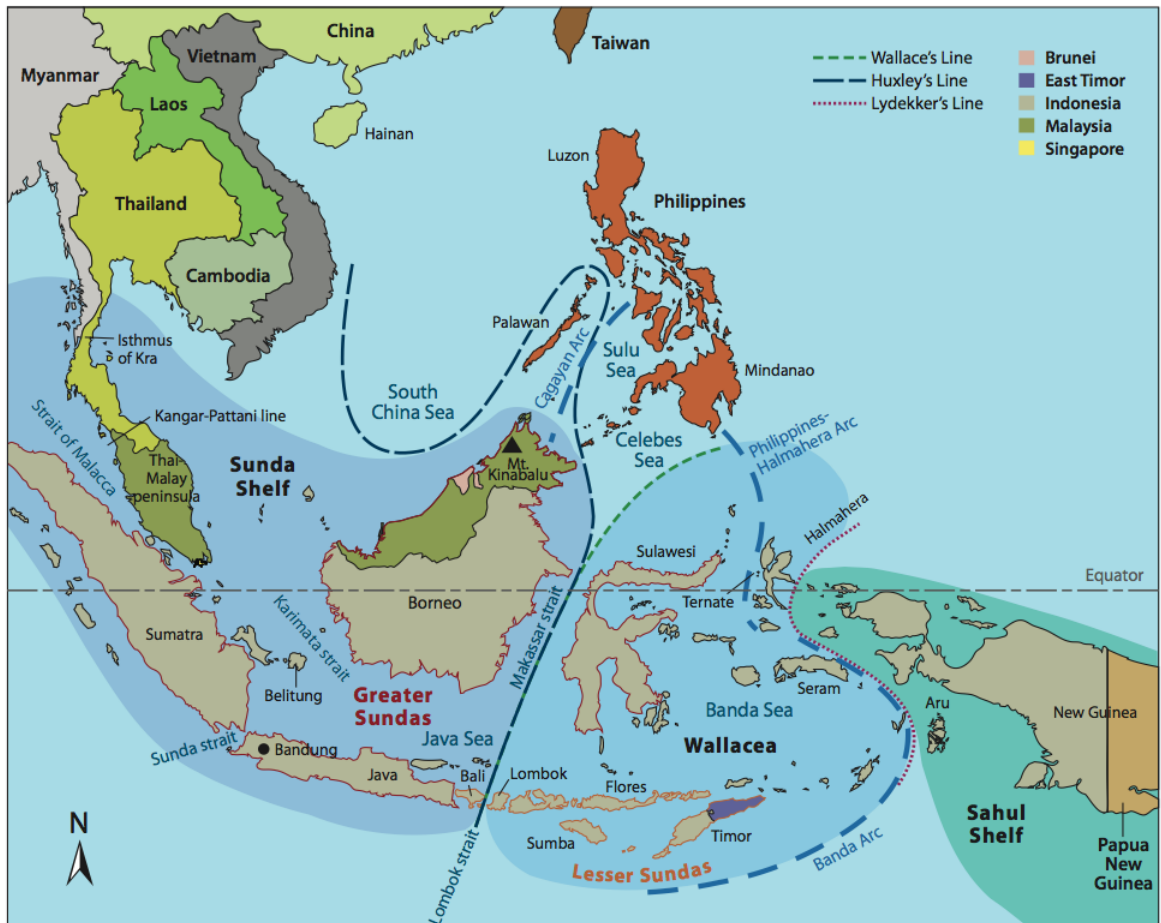


Figure 4-1: Map showing the location of Sunda shelf, Sahul shelf and Wallacea. The map also shows the location of Huxley's line, Wallace's Line, Lydekker's line and the Makassar Strait.



Each country is colour coded as indicated in the key. Source: Lohman et al., 2011.

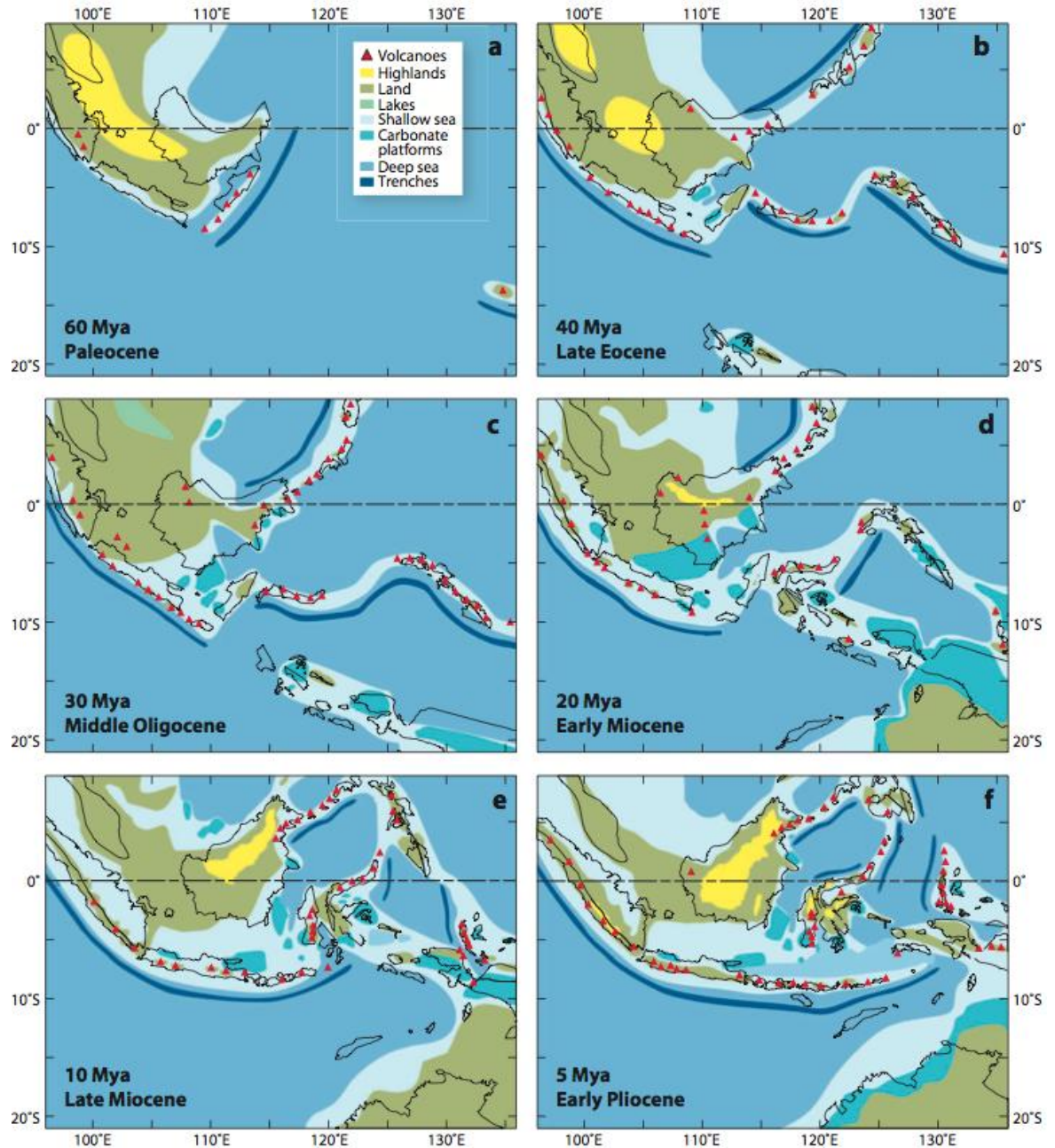


Figure 4-2: Six Cenozoic reconstructions of land and sea in the Indo-Australian Archipelago. Source: Lohman et al., 2011.

## 4.2 *M. rosenbergii* (de Man, 1879)

*Macrobrachium rosenbergii*, de Man (1879), also known as the giant freshwater prawn or Malaysian prawn, is a cultured freshwater species that inhabits tropical freshwater environments that have access to an adjacent low salinity area. This accommodates the need of the animal for different environments at different times throughout its life cycle (Figure 4-3). The requirement of *M. rosenbergii* for brackish water to survive at the early stages of life was first discovered by

Shao-Wen Ling (Food and Agriculture Organisation (FAO)), from research carried out at the Marine Fisheries Research Institute in Penang, Malaysia. From egg hatching to the post larval stage, *M. rosenbergii* prawns live in low salinity water (Figure 4-4). Only after metamorphosis to the post larval stage do they migrate to an adjacent freshwater area. Ling (1969) showed the ability of post larval *M. rosenbergii* to migrate against strong currents to as far as 200 km away from the brackish water area.

Known as the largest prawn species in its genus (Wowor and Ng, 2007), *M. rosenbergii* is one of the most commercially-important freshwater crustacean species across its natural habitat, and as an introduced species in other countries such as North, South and Central America, Africa, Europe, Asia and Oceania (New and Kutty, 2010). Until recently, other *Macrobrachium* species have been produced on a large scale in China (*M. niponese*) and India (*M. malcomsanii*), the two major aquaculture producers in South East Asia (New and Kutty, 2010). According to the Food and Agriculture Organization (FAO 2009), the global catches of this species were 221,174 tonnes in 2007 with the major producer being China, which produced about 56.3% of global production (124,520 tonnes worth about US\$ 452 million), followed by Thailand (12.5%) and India (12.3%) (New, 2010).

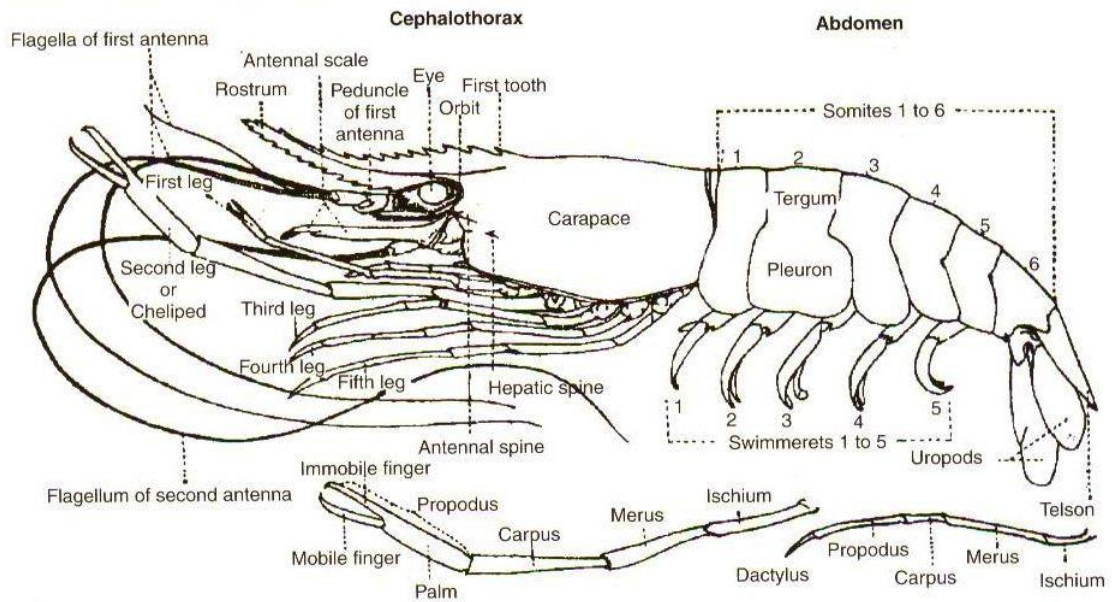


Figure 4-3: Morphology of *M. rosenbergii* (De Man). Source: Cowles, 1914.

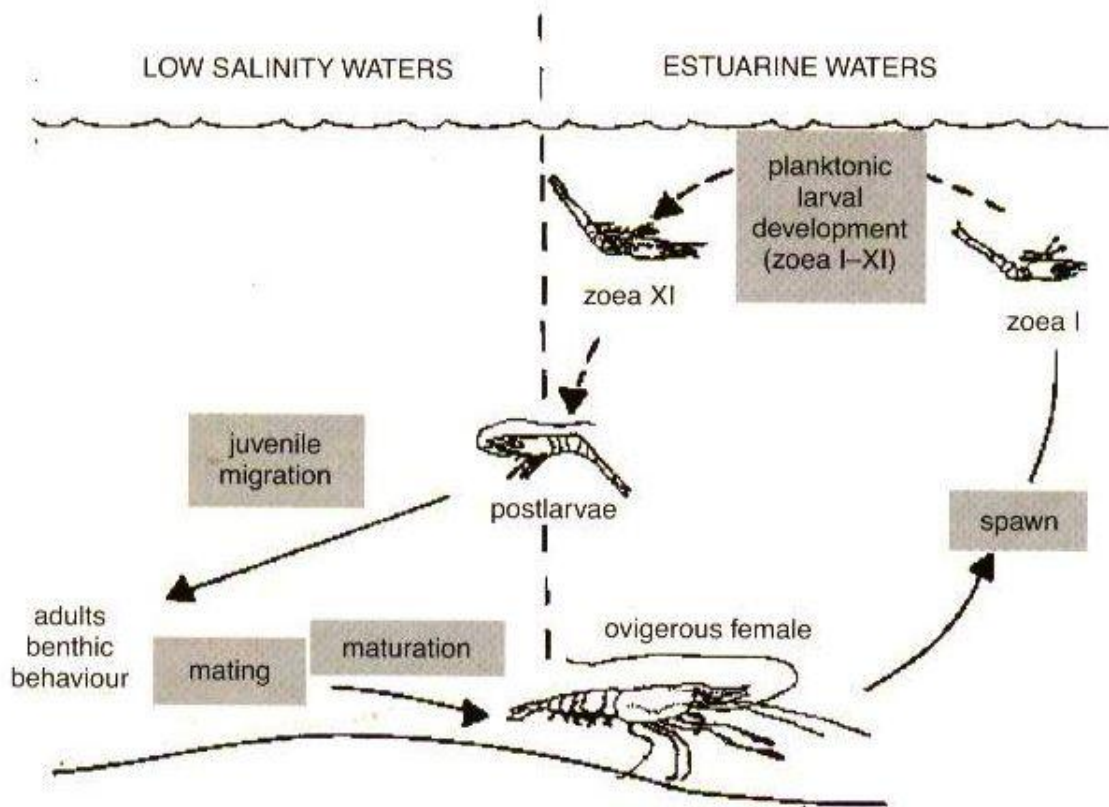


Figure 4-4: Life cycle of *M. rosenbergii*. Source Brown et al., 2010.

### 4.3 The nomenclature of *M. rosenbergii*

*M. rosenbergii* has long been recognised to exist in two forms that can be found either side of Huxley's line. Recently, however, Wowor and Ng (2007) pointed out the significant requirement to give both forms of *M. rosenbergii*, the western and eastern, unique taxonomic names based on their distinct morphological and morphometric characteristics which are also congruent with the studies on the genetics of the species (Hedgecock, 1979, Chand et al., 2005, de Bruyn et al., 2005, de Bruyn et al., 2004a, De Bruyn et al., 2004b). Urgent attention is needed to solve the problem regarding the nomenclature of this species since it is so widely used in freshwater aquaculture world-wide, and so many research studies have been conducted on it. It is only the western form of *M. rosenbergii* that has been primarily used in aquaculture and generated high economic value to the producer countries. The problem arose when Wowor and Ng (2007) reported that *M. rosenbergii*, the name that has been used by aquaculturist and researchers is actually *M. rosenbergii dacqueti* (the western form). However, the form belonging to the holotypes of *M. rosenbergii* is actually the eastern form, which is currently the less important species for aquaculture.

In this chapter, the early studies of how this species was first recognised and recorded to possess different morphological characteristics, as well as the studies that added the weight to give a new taxonomic classification, will be reviewed. The guidance of Wowor and Ng (2008) is followed, who advise that the name *M. rosenbergii* is reserved for the western form, whereas many existing publications have used the name *M. rosenbergii* to refer to the eastern form. If the name *M. rosenbergii* is reserved for the western form then a new taxonomic name will have to be given to the eastern form.

The early studies on the identification and differentiation in *M. rosenbergii* were based solely on the morphological characteristics of the animal, even though a large number of specimens has been analysed and collected throughout its natural geographic range. The morphological characteristics were regarded as relatively weak and thus not strong enough to treat the identified forms of *M. rosenbergii* as a distinct species (Wowor and Ng, 2007).

De Man (1879) was the first to identify what he quoted in his paper as a new form of *Palaemon carcinus* Fabr., *P. rosenbergii*, based on a female specimen collected by Mr von Rosenberg from Andai, New Guinea. This new form, *P. rosenbergii* showed high similarity with *P. carcinus* but can be clearly distinguished by the structure of the rostrum and of the terminal joint of the abdomen. Later on, Cowles (1914) noticed the consistent differences between the specimens collected from the Philippines (eastern form) and India (western form). Nevertheless he continued to use the name *P. carcinus* for both forms of this species. It was only Sunier (1925) (article in Dutch cited by Wowor and Ng 2008) who challenged the identification of the species of *P. carcinus* (based on a specimen from Java). According to Sunier, *P. carcinus* actually refers to a closely related species of *M. rosenbergii* that originated from East America (currently known as *Macrobrachium carcinus*) (Wowor and Ng, 2008). He then gave *P. carcinus* a new name, *P. dacqueti* (Sunier, 1925) and kept the eastern form as *P. rosenbergii*. Holthuis (1950) has agreed with this new identification but he commented that it is more appropriate to give the name *P. rosenbergii* to the *P. carcinus* since it is the oldest available name for the western subspecies of *M. rosenbergii*. This is to follow the principles of priority stated in the ICZN code as well as the Art.11 (e) as one of the main criteria for naming a species. The studies on *M. rosenbergii* carried out after Holthuis (1950) have recognised and used only one name for the giant freshwater prawn, namely *M. rosenbergii*, de Man (1879).

However, Johnson (1960) acknowledges the presence of two subspecies that could be clearly distinguished through the shapes of the rostrum and the body (see Chapter 6.2 for details). He proposed the name of *M. rosenbergii sckenkeli* for the western form and *M. rosenbergii rosenbergii* for the eastern form. The existence of these subspecies was then supported by early molecular work carried out on *M. rosenbergii* using electrophoresis (Hedgecock et al., 1979). The electrophoretic study detected a significant genetic divergence between the western and eastern forms, as well as another genetic break within each form. More recent studies that applied mtDNA molecular markers (de Bruyn et al., 2005, de Bruyn et al., 2004a, De Bruyn et al., 2004b) and microsatellite markers (Chand et al., 2005, Charoentawee et al., 2006, Divu et al., 2008, Bhat et al., 2009, Bhassu et al., 2008, See et al., 2009, See et al., 2011) also supported the

previous molecular findings. Holthuis (1995), while supporting the existence of the two subspecies also commented that if two subspecies are to be recognised, the eastern form should be *M. rosenbergii rosenbergii* (de Man, 1879) and the western form *M. rosenbergii dacqueti* (Sunier, 1925) which is the oldest available name for the western form.

In 2007, Wowor and Ng requested a unique name for each of the two forms based on their accumulated morphological and morphometric data, colour changes and different environment requirement for both forms. However this has created confusion in the usage of *M. rosenbergii* since it has long been reserved for the form that has been used extensively in freshwater aquaculture. Such changes also have a large impact on scientific knowledge, as well as in the aquaculture industry itself since this species has been widely cultured not only in its natural habitat but also beyond.

To satisfy this important requirement of having the correct name for both forms, as well as not to alter a name that has been used in many studies, Wowor and Ng (2008) requested the International Commission for Zoological Nomenclature (ICZN) to use its plenary power (Article 78.1 under conditions stated in Article 81) to set aside the previous assigned name *Palaemon rosenbergii* de Man 1879 (a holotype female from Andai, New Guinea, in the Nationaal Natuurhistorisch Museum, Leiden, catalogue number RMNH D 1097) and to designate the lectotype specimen of *Palaemon dacqueti* (Sunier, 1925) (a male specimen from Java, Indonesia, in the Nationaal Natuurhistorisch Museum, Leiden, catalogue number RMNH D 1065), as its neotype. This has conserved the name *M. rosenbergii* for *P. rosenbergii* de Man 1879, the species that has been used by researchers and aquaculturists. In 2010, the ICZN accepted the request to retain the name *M. rosenbergii* for the western form and to replace *P. rosenbergii* de Man 1879 and any other previously given neotype (ICZN 2010, Opinion 2253). However, this has also created another problem for the eastern form found on the east side of Huxley's line, which was previously known as *P. rosenbergii*. Wowor and Ng (2008) requested to use *Macrobrachium wallacei* sp. nov. to replace the name *M. rosenbergii* de Man, 1879 (the eastern form) since the only synonym, *Palaemon spinipes* Schenkel, 1902 is a junior homonym of *Palaemon spinipes* Desmarest, 1817, is unavailable. However, the name depended on a fresh specimen collected from Papua New Guinea as the holotype. Thus, the



name is not appropriate to be used as a replacement name for the previous *M. rosenbergii* de Man, 1879. Later on, Ng and Wowor (2011) mention that the most appropriate name to replace *M. rosenbergii* de Man, 1879 from Sulawesi, Bali, Sumba, Moluccas, Irian Jaya, Papua New Guinea, Australia and Phillipines is *Macrobrachium spinipes* (Schenkel, 1902) since the name *Palaemon spinipes* Schenkel, 1902, still valid and available.

By following the most updated nomenclature for this species, the name *M. rosenbergii*, used in this study refers to what was previously known as the western form of *M. rosenbergii* from Southeast Asia starting from India, Thailand, Sri Lanka and Malaysia.

#### **4.4 *M. rosenbergii* farming in Malaysia: aquaculture trends, improvements and sustainability**

*M. rosenbergii* is one of the most popular freshwater species and is marketable at a high price because it is the largest freshwater prawn in its genus (Wowor and Ng, 2007). This has created great interest in the farming of this species even beyond its natural distribution. Up until now, a considerable number of research studies have been carried out on the biology, genetics, diseases and nutrition of *M. rosenbergii*, as well as the development and enhancement of the farming industry for this species. The country of Malaysia, where *M. rosenbergii* is indigenous, has made a very significant contribution to the methodology for culturing *M. rosenbergii*. The discovery of the requirement of brackish water for larval development by Shao-Wen Ling in 1961, and the introduction of a Malaysian strain into Hawaii, which was successfully developed by Takuji Fujimura in 1965 for intensive culture, are particularly notable.

Despite all the pioneer works carried out on the Malaysian strain, the development of the *M. rosenbergii* industry in Malaysia is modest and highly variable. The production is left far behind by that of its neighbours, Thailand and Indonesia (New and Kutty, 2010). In Thailand, the farming of *M. rosenbergii* began in the mid-1970's and the production was consistently increased from 1998 (4,764 tonnes) to 2004 (32,583 t) with a slight decrease in 2005 (28,740 t) and 2006 (25,353 t) which recovered again in 2007 (27,650 t) (New and Kutty, 2010). Inbreeding depression was suspected to be the cause of this problem but analysis

at the molecular level showed no correlation between the two factors. Genetic comparison between two hatchery populations and wild stock populations showed high genetic differentiation in both wild populations and hatchery products (Chareontawee et al., 2007). In Indonesia the farming of this species was started around the same time as in Thailand, and the first production reported was 400 t in 2002, rising to 989 t in 2007 (higher than that in Malaysia). In Malaysia, the production never exceeded 800 tonnes per year except for a dramatic increase in 2000 to 1338 t. The *M. rosenbergii* farming industry suffered a great reduction in production in 2006 to only 194 t but the figures improved in 2007 to 246 t and in 2009 to 552 t (New, 2010).

Both polyculture and monoculture systems are practiced in prawn farming in Malaysia, carried out only in freshwater ponds, producing about 619 t in 2010 worth about MYR 18, 848.95 (Aquaculture statistic, Department of Fisheries, Malaysia, 2010, ([http://www.dof.gov.my/html/themes/moa\\_dof/documents/jadual\\_pendaratan\\_marin\\_%20aquaculture.pdf](http://www.dof.gov.my/html/themes/moa_dof/documents/jadual_pendaratan_marin_%20aquaculture.pdf) accessed on 10<sup>th</sup> April 2012). *M. rosenbergii* production was discontinued in Kelantan state in 2010 even though it was about 0.77 t from both freshwater pond and pen culture in 2009. However no production was ever reported from Perlis, Pulau Pinang, Melaka, Kelantan and Sabah states. Attempts to establish integrated culture in Malaysia were also initiated and the potential for the commercial success of these is promising (Idris et al., 2011). Most of the hatcheries operate a green water system with minimal water exchange (New and Kutty, 2010). However, mass mortality was reported in the hatchery that used a static green water technique (Anderson et al., 1990b). The green water system is a system that polycultures phytoplankton with zooplankton in larval rearing tank, thus simulating a natural situation (Fujinaga, 1969). A common scenario in Malaysia *M. rosenbergii* farming is, in many hatcheries, for production to be high initially (range between 3.0 t ha<sup>-1</sup> to 6.5 t ha<sup>-1</sup> per cycle) but to decrease after a few generations (New and Kutty et al., 2010). Financial problems resulting from low production have constrained the farmers to maintain the operation and some have rotated cultures with fish species such as catfish or tilapia. The harvested prawns are supplied to local markets but most of the production is marketed directly to hotels and seafood restaurants. Sometimes, live berried females or superior males and females are



also sold to other *M. rosenbergii* hatcheries. Live prawns are also supplied to Singapore where wild stock of *M. rosenbergii* was reported to be extinct (New and Kutty, 2010). The majority of the farming activities occur in peninsular Malaysia, especially in Perak and Kedah (production of 157 and 308 t respectively (Aquaculture statistic, Department of Fisheries, Malaysia, 2010, [http://www.dof.gov.my/html/themes/moa\\_dof/documents/jadual\\_pendaratan\\_marin\\_%20aquaculture.pdf](http://www.dof.gov.my/html/themes/moa_dof/documents/jadual_pendaratan_marin_%20aquaculture.pdf) accessed on 10<sup>th</sup> April 2012). Until 2010, Kelantan, Perlis, Penang, Malacca and Sabah, had no *M. rosenbergii* production recorded, with the supply of prawns to the local markets being solely from local wild stocks or imported from other places in Malaysia. Sarawak and Sabah were reported as actively exporting their wild stocks to peninsular Malaysia to compensate for the higher demand of the prawn industry. In Malaysia, farmers use wild population of *M. rosenbergii* as the broodstock for grow-out but some used broodstock from hatcheries that exhibit superior characteristics (New and Kutty, 2010).

The prognosis for the *M. rosenbergii* industry in Malaysia is difficult to predict. Despite high prices being achievable (ranging between MYR 16.00 in Sabah and to MYR 47.90 in Penang), there is high demand for the product at local markets, potential for exportation and support from government (for funding, consultation and training) and academic institutions (for research and consultation), while the industry has remained relatively undeveloped. This can be attributed mainly to the shortage of high quality juvenile supply. The availability of such high quality post larvae at lower prices from Thailand, Indonesia and Myanmar has constrained the production of high quality juveniles in Malaysia itself. Most of the farms are family oriented, but there is a need to use foreign labour because of the mentality of Malaysian people, which does not consider farming as a career that can promise a better life and secure finances. Lack of knowledge and technology are among the factors that have limited the development of the industry despite the strong support from both government and academic institutions. There is no specialist nursery for broodstock, and improper management of the grow-out hatcheries (very high density up to 1000 post larval m<sup>-2</sup> with lack of management and technology) was reported (New and Kutty, 2010).

The difficulties encountered in farming *M. rosenbergii*, as well as the urgent actions needed to improve the industry, particularly in Malaysia, have been discussed in the 'Giant Malaysia Prawn 2008' conference organised by the Malaysia Fisheries Department and other organisations which take consultation and advice from experts around the world (New and Kutti, 2010). The current priorities in Malaysia are to overcome the shortage of supply of high quality post larvae as well as to initiate genetic selection for disease resistance and improve the growth rate and sex ratio (New and Kutti, 2010). However, the above-mentioned objectives can only be achieved by manipulating the genetic differentiation of the wild stock. This is where genetics studies could play an important role in the development and sustainability of aquaculture industry. Increasing demand for broodstock supply mainly from wild stocks, as well as increasing loss of natural habitat due to human activities (logging, pollution, overfishing and illegal fishing) could threaten the existence of wild stocks (Bhassu et al., 2007). The importance of the availability of the wild stocks has to be stressed, as it is the only source for a genetic selection programme to be possible. Genetic information on the wild stock is crucial to initiate conservation and to facilitate the management of wild stock as a source of genetic differentiation. Genetic selection which will produce better broodstock quality, and genetic improvement programmes for disease-free and better quality cultured product (size), can be achieved by manipulating the genetic differentiation in the wild stocks. This is also useful for the development of quantitative trait loci (QTL) markers for genetic selection. Beside, inbreeding depression as experienced in some *M. rosenbergii* farms such as those in Vietnam could be avoided if proper broodstock management is followed and a genetic selection programme is established (Thanh et al., 2009, 2010). All the earlier discussed factors would facilitate the development and sustainability of the freshwater prawn industry in Malaysia.

Currently, the level of genetic differentiation of *M. rosenbergii* wild stocks in Malaysia is still unclear. As far as is known, only two studies have been carried out extensively in Malaysia. Bhassu et al. (2007) reported no genetic structure retrieved from 18 populations in Malaysia using the RAMs marker. The second study on the same populations using another molecular marker, termed LP-RADP, also failed to detect any significant genetic variation in the populations

(See et al., 2008). Moreover, a research group in University of Malaya is now developing microsatellite markers (Bhassu et al., 2008, See et al., 2009, See et al., 2011). However the level of the genetic differentiation of Malaysia populations at the nuclear level is yet to be determined. Genetic information is very useful for the management and conservation of the local stock, and is the only resources to resolve the current problems in farming *M. rosenbergii*. Beside, the improvement and sustainability of the farming industry is only possible by manipulating the genetics of the local stock. Following the outcomes from the 'Giant Malaysia Prawn' conference in 2008, the present study initiated a phylogeographic investigation (reported in Chapter 4) in parallel with a microsatellite study carried out by a research group in Malaysia based at University of Malaya. The mitochondrial study utilized the cytochrome oxidase I (COI) gene of mtDNA using a sequencing technique. The aim of the study was to carry out a phylogeographic study of wild populations of *M. rosenbergii* using a mtDNA segment.

#### **4.5 Diseases associated with *M. rosenbergii* and implications for the aquaculture industry**

*M. rosenbergii* is known to possess moderate disease-resistance to viral, bacterial or fungal infections compared to penaeid shrimps (Peng et al., 1998, Sahul Hameed et al., 2000, Ramalingam and Ramarani, 2006, Ravi et al., 2010). Many aquaculturists choose to culture *M. rosenbergii* over *Penaeus monodon* because of the high susceptibility of the latter species to many infections, resulting in mass mortality and severe economic losses (Karunasagar et al., 1994, Thaithongnum et al., 2006, Bhaskar et al., 1998, Lavilla-Pitogo et al., 1998).

Until recently, white tail disease (WTD), also known as white muscle disease (WMD), a uniquely important disease in *M. rosenbergii* farming, has been the only major concern that caused high mortality in the hatchery-rearing *M. rosenbergii* post-larvae, inflicting severe economic losses (Ravi et al., 2010, Bonami and Widada, 2011, Pillai et al., 2010). WTD is caused by a nodavirus known as MrNV, which is always associated with a non-autonomous satellite-like virus (XSV). This disease was first reported in Guadeloupe (Arcier et al., 1999). In fact the disease symptoms expressed in WTD are similar to those previously reported for the disease in *M. rosenbergii* known as *Macrobrachium* muscle virus

(MMV) in Taiwan (Tung et al., 1999). Since then, WTD was widely reported especially in major producer countries: China (Qian et al., 2003), India (Hameed et al., 2004b, Vijayan et al., 2005), Thailand (Yoganandhan et al., 2006) and more recently in Taiwan (Wang et al., 2008) and Australia (Owens et al., 2009). Another important viral agent in *M. rosenbergii* culture is a parvo-like virus from the Parvoviridae family which is a *Macrobrachium* hepatopancreatic parvo-like virus (MHPV). MHPV was first isolated from a single hatchery rearing *M. rosenbergii* post larvae in Peninsular Malaysia. Nonetheless, MHPV was not reported to be associated with mass mortality (Anderson et al., 1990a).

Another causative agent for disease infection in *M. rosenbergii* is bacteria. Compared to viral diseases, bacterial diseases have less serious effects on the economics of *M. rosenbergii*. However, high intensive culture systems together with poor management of nursery and grow-out systems have caused serious disease problems in *M. rosenbergii*, including bacteria necrosis, black spot disease and vibriosis. Many studies have been conducted to identify bacteria in *M. rosenbergii* hatchery systems (Dass et al., 2007, Kennedy et al., 2006, Phatarpekar et al., 2002). The most abundant bacteria isolated either from hatchery water, larvae or *Artemia* used as live feed were gram-negative bacteria of the genera *Pseudomonas*, *Vibrio* and *Aeromonas* but some gram-positive bacteria such as *Bacillus* and non-spore formers (NSF) were also isolated at high percentages (Phatarpekar et al., 2002, Kennedy et al., 2006, Dass et al., 2007). The potential routes of contamination are via feeds, broodstock and rearing water. Live feed *Artemia* could potentially become a vector that transmitted bacteria into the larvae when consumed (Kennedy et al., 2006, Sudhakaran et al., 2006b). Beside, introduction of infected broodstock or other infected animals will contaminate the existing broodstock in the hatchery. Hence, consistent screening of the broodstock to be introduced will reduce the possibility of infecting the local stock. The pH, salinity and temperature of the water are among the factors that have a significant role in the success of larval rearing. However, optimum conditions of salinity, pH and temperature provide an optimum environment not only for the species of interest but also favour the multiplication of certain viruses, bacteria and parasites in the rearing water (Hameed, 1993). Daily water exchange and tank cleaning would reduce the establishment of unnecessary species (Kennedy et al., 2006). However, most of

the commonly-found bacteria species in hatchery systems are non pathogenic since no major mortalities or abnormalities were reported in the above discussed studies, and Sriwongpuk et al. (2006) reported that the high prevalence of normal bacteria alone is unlikely to directly cause mass mortality. Nevertheless environmental stressors (high density, water quality) could be secondary factors. For instance, non-pathogenic strains of the *Vibrionaceae* family and *Pseudomonase* group, as well as *Bacillus* and gram-positive non-spores formers such as *Lactobacillus* spp., have the ability to protect the prawn by suppressing the growth of other pathogenic strains using their antagonistic effect (Kennedy et al., 2006, Rengpipat et al., 2000, Moriarty, 1998).

A symptom of black spot disease or shell disease is the presence of one to several focal melanised lesions on the general body surface (Pillai et al., 2010). Although black spot disease infects prawns at any life stages, this disease has been reported more frequently in juveniles and adults than in larval stages. This has been related to the moult frequency at different stages of life. If the infection is confined to the exoskeleton, the infected prawn recovers after moulting. However, in the case of larval infection, the higher frequency of moulting prevents the infection progressing far within any instar. In contrast, the lower frequency of moulting in juvenile and especially adults provides ample time for the infection to progress to an advanced stage and cause a more severe effect, especially if it contaminates the haemolymph and spreads to the whole body. Poor hatchery management, high intensive culture and poor sanitation are the major contributors to the development of black spot disease.

Another important bacterial disease is bacterial necrosis. Le Bitoux (1988) defined necrosis as similar to black spot disease in its gross signs, but bacterial necrosis only infected prawns at larval stages, especially at stages IV and V. Gross signs of this disease are necrosis of appendages, especially the antennae and the abdominal appendages (Le Bitoux, 1988). The disease progresses rapidly within just a few days and is very likely to cause mass mortality if not treated (Le Bitoux, 1988). Necrosis infection can be minimised by controlling environmental stressors such as temperature and physical stress, by daily examination of the prawn and by application of antibiotics only when necessary (Le Bitoux, 1988). Sharshar and Azab (2008) studied necrosis disease in *M. rosenbergii* infected with *Vibrio vulnificus*.

Vibriosis, a disease associated with luminescent *Vibrio* spp. has been reported in Brazil, and has been widely reported in the penaeid shrimp *Penaeus monodon*. The unique clinical sign of this disease is a glowing appearance of the moribund and dead larvae that can be seen at night (Saurabh and Sahoo, 2008 and Pillai et al., 2010). *Vibrio* spp. associated with vibriosis in *M. rosenbergii* larval are *V. parahaemolyticus cholerae*, *V. alginolyticus*, *V. carchariae* and *V. mimicus* (Oanh et al., 2001). (Cheng and Chen, 1998b) reported a mass mortality and significant economic loss in a *M. rosenbergii* hatchery in Taiwan due to the *Enterococcus*-like bacterium which later was identified as *Lactococcus garvieae*, a gram-positive bacteria (Chen and Wang, 2001).

## 4.6 Prawn immune system

It is well known that the haemolymph has an important role in the crustacean defence system. In general, haemolymph consists of plasma and circulating haemocytes of various types: hyaline cells, granular cells and semi-granular cells (Pillai et al., 2010, Saurabh and Sahoo, 2008). However, Vazquez et al. (1997) classified the haemocytes of *M. rosenbergii* into a large fraction of hyaline cells, followed by granule cells which can be divided into Type I and Type II depending on the characteristic of the granules. Another type of haemocyte, which he called undifferentiated cells, is the least abundant haemocyte cell type and shows no phagocytic activity. In another study by Sierra et al. (2001), circulating haemocytes of *M. rosenbergii* were divided into fusiform, rounded and large ovoid cells. The granule cells, rounded and large ovoid cells showed phagocytic activities, similar to the function of granule and semi-granule cells (Sierra et al., 2001, Vazquez et al., 1997).

The interaction between circulating haemocytes and plasma is important in the coagulation mechanism, wound repair, restoration of damaged tissue and the defence system (Pillai et al., 2010). The crustacean defence system, known as the innate immune system, differs from the adaptive immune system in vertebrates because of its lack of immunological memory, which means no production of immunoglobulins. In the vertebrate defence system, specific interactions between the antigen and antibody facilitate the elimination of foreign organisms. In contrast, the crustacean innate immune system involves direct interaction between haemocyte and non-self microorganisms, which

efficiently removes non-self microorganisms by both a phagocytosis mechanism and the activation of a prophenoloxidase (proPO) defence system.

The front line of such an innate defence system is the exoskeleton, known as the external defence system, which functions as a physical barrier and may contain antimicrobial factors (Pillai et al., 2010, Vazquez et al., 2009, Saudabh and Sahoo, 2008 and Braak, 2002). This provides protection to all exposed body surfaces against pathogen infection. However, damage to the exoskeleton such as a lesion would expose the tissue and haemocoel cavities to the pathogen infection. The crustacean defence system involves a combination of cellular and humoral mechanisms. Cellular defence includes the reactions performed directly by haemocytes such as phagocytosis, encapsulation, cell-mediated cytotoxicity and clotting (Braak, 2002 and Sahoo et al., 2007). On the other hand, humoral defence involves the activation and release of molecules stored in defence cells as inactive forms, such as coagulation protein, agglutinins, hydrolytic enzymes and antimicrobial peptides. Once the non-self microorganism crosses the external defence, some sort of recognition process is carried out by several types of pattern recognition proteins (PRPs) such as lectin, peptidoglycan recognition/binding protein (PGBPs) and LPS-binding protein (LGBP) which will recognise the microbial component (exogenous elicitor), associate it with the non-self molecules of the microorganism such as lipopolysaccharides or LPS (gram-negative bacteria), peptidoglycan (gram-positive bacteria) and B-1,3-glucan carbohydrate (fungi and algae) and activate the haemocytes, which then migrate to the site of infection (Aspan et al., 1990, Vazquez et al., 1997, Vazquez et al., 2009, Jones and Khan, 2010 and Braak, 2002). The recognition of exogenous elicitor by PRPs will promote the release of the inactive form of proPO from granular and semi-granular cells through exocytosis (Johanson and Soderhall, 1985 and 1988). Activation of proPO to form PO is mediated by the active form of the proPO-activating enzyme (ppA) which involves a series of cascade enzymatic reactions (Aspan et al., 1990). Exocytosis in semi granular cells is induced by the same molecules that induces the activation of proPO system, namely LPS or B-1,3-glucan (Johansson and Soderhall, 1985). Another molecule, the adhesion factor, a 76kDa protein, induces the release of proPO from granular cells (Kobayashi et al., 1990). The 76 kDa protein has multi-function properties that mediate the cell adhesion, agglutination and

encapsulation processes, resulting in the immobilisation and/or isolation of non-self particles, as well as opsonization (Aspan et al., 1990 and Pillai et al., 2010). The PO will mediate the oxidative conversion of the phenols into quinones which will accumulate as the product melanin, which can be seen as a dark spot. Moreover, the opsonic action of lectins in the plasma also enhances recognition between haemocytes and invader microorganism which intensify phagocytosis activities (Sahoo et al., 2007). Hence, the PO defence system is a central to the immobilisation, encapsulation and melanisation of large invader microorganism, which cannot be phagocytised. At the same time, when the external defences are breached this will also stimulate the coagulation process mediated by the release of TGase from haemocyte, most probably hyaline cells. Coagulation is a crucial process that prevents loss of haemolymph and entraps the invading microorganisms.

Direct interaction of circulating haemocytes with small non-self particles is called phagocytosis. During the activation of PO, antimicrobial factors, known as peroxinectins are formed. Peroxinectins play an important role in cell-adhesion and in peroxidase activity, which helps to eliminate non-self microorganism. The function of peroxidase in cell-adhesion includes cell attachment, spreading, phagocytosis, encapsulation, nodule formation and agglutination. At the same time, antimicrobial peroxidase activities also assist in eliminating the invading pathogens. However, the specific cell type that is responsible for the phagocytosis mechanism is unclear. Sung et al. (2000) suggested the hyaline cell as the primary cell type responsible for phagocytosis. Vazquez et al. (1997) reported the large granular haemocytes to be the only cell type that possessed phagocytic activity. Meanwhile, Sierra et al. (2001) and Sahoo et al. (2007) observed phagocytosis activity in ovoid and small round haemocytes.

Phagocytosis can be initiated by a receptor-mediated endocytosis interaction between circulating haemocytes and fixed phagocytes with the non-self invaders. Phagocytosis starts with the attachment of the haemocyte to the non-self microorganism, followed by its ingestion into endocytic vesicles known as phagosomes and then the destruction is carried out by variety of degradation enzymes and antimicrobial peptides released into the phagosome (Saurabh and Sahoo, 2008). During phagocytosis, activation of the host's NADPH-oxidase by the non-self microorganism will reduce the oxygen level but increase the



production of reactive oxygen intermediates (ROIs) such as the superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), the hydroxyl radical ( $\cdot OH$ ) and singlet oxygen ( $^1O_2$ ) which is toxic to the invading microorganism. The release of ROIs is known as respiratory burst activity, which is important in eliminating invader microorganisms. Unfortunately, increasing the amount of ROIs has an adverse effect on the host itself but this is balanced by antioxidant defence mechanisms such as NADH oxidase, superoxide dismutase (SOD), peroxidase and catalase (Saurabh and Sahoo, 2008). These antioxidant enzymes have the ability to scavenge the superoxide anion and convert it to molecular oxygen and hydrogen peroxide. Besides phagocytosis, haemocytes are also involved in the processes called encapsulation and nodule formation. Encapsulation is a process of eliminating large invader microorganism which cannot be phagocytosed, while nodule formation is a process to eliminate a high number of invading microorganisms (Pillai et al., 2010 and Saurabh and Sahoo, 2008).

#### **4.7 *V. parahaemolyticus***

*Vibrio parahaemolyticus* was first discovered in 1950 from a food poisoning outbreak in Japan that killed 20 people (Broberg et al., 2011, Yeung and Boor, 2004). Since then *V. parahaemolyticus* has been known as one of the most important causative agents for foodborne disease (Daniels et al., 2000, Yeung and Boor, 2004, Su and Liu, 2007, Hlady and Klontz, 1996). *V. parahaemolyticus* is a curved rod shape and motile gram-negative halophilic bacterium. It is widely distributed in warm marine and estuarine environments as a free swimmer or as attached to surfaces. The optimum environmental conditions for survival and multiplication of *V. parahaemolyticus* are marine water with temperatures between 35°C to 39°C (Barrow and Miller, 1974) and salinity ranges from 14.9-29.3 ppt (DePaola et al., 2000). Temperature has a stronger effect on the survival and multiplication of *V. parahaemolyticus* compared to salinity (DePaola et al., 2000, Cook et al., 2002). *V. parahaemolyticus* has the ability to survive and multiply even in hostile environments such as those containing high concentrations of the metal iron. This natural adaptation is essential particularly during infection of a mammalian (Yeung and Boor, 2004).

*V. parahaemolyticus* is well known to be associated with food-borne disease in human, most commonly gastroenteritis, which manifests as diarrhea, abdominal

pain, vomiting, headache, fever and chills (Yeung and Boor, 2004). *V. parahaemolyticus* infection in individuals with pre-existing medical problems such as liver disease, cancer, diabetes or recent gastric surgery have a high potential to cause morbidity and mortality due to septicemia (Yeung and Boor, 2004, Hlady and Klontz, 1996, Daniels et al., 2000). Septicemia is attributed to inflammation and increased vascular permeability, and leads to hypovolemic shock, multisystem organ failure and ultimately death (Broberg et al., 2011 and Su and Liu, 2007). In addition, this bacterium may also cause wound infection following exposure (e.g. in injured fisherman). Transmission of the disease is from the consumption of raw or undercooked fish or shellfish (including crustaceans). The occurrence of foodborne disease related to *V. parahaemolyticus* is high, particularly in a country such as Japan, where the consumption of under-cooked food is not uncommon. The first outbreak in Japan was related to the consumption of Shirasu, a partially dried sardine (Yeung and Boor, 2004) while the first outbreak in the USA was caused by the consumption of contaminated crab meat (Molenda et al., 1972). *Vibrio* species have been known to be one of the major human pathogens and have attracted great public concern due to the severe foodborne disease outbreaks (Su and Liu, 2007). Many studies have been conducted to gain understanding and information on the aetiology and pathogenicity as well as detection and prevention of the disease (Yeung and Boor, 2004, Broberg et al., 2011, Molenda et al., 1972, Nishibuchi and Kaper, 1990, Nishibuchi et al., 1985, Makino et al., 2003, Nishibuchi and Kaper, 1995).

Even though *V. parahaemolyticus* can be found in almost all aquatic environments, it does not cause serious mortality to all infected aquatic animals. The affected animals may serve only as transmission agents of the pathogen to the human body. This fact is supported by the finding that most of the virulent *V. parahaemolyticus* strains were found in clinical samples (Miyamoto et al., 1969). Hatcheries that operate with sea water are highly exposed to the *V. parahaemolyticus* infection, especially at the larval stage. Studies have reported that *V. parahaemolyticus*, *V. harveyi*, *V. alginolyticus* and *V. mimicus* were the most abundant *Vibrio* species isolated from *M. rosenbergii* hatcheries. Nonetheless, the pathogenicity of *V. parahaemolyticus* in *M. rosenbergii* is considered mild, even though some strains from environmental

samples also possess virulence factors. Miyamoto et al. (1969) reported only about 5% occurrence of virulent strains from environment isolates. AlapideTendencia and Dureza (1997) reported that the most abundant bacteria isolated from *P. monodon* showing symptoms of red disease syndrome were *V. harveyi* and *V. parahaemolyticus*. However, pathogenicity tests conducted in the same study showed higher mortalities in juvenile *P. monodon* infected with *V. parahaemolyticus* compared to *V. harveyi*. Recently, a study carried out by Khuntia et al. (2008) on *M. rosenbergii* larvae of strains from India experimentally infected with *V. parahaemolyticus* showed 80% mortality due to the infection. Within 6 days of infection, affected prawns exhibited some signs of black colouration of the carapace, red discolouration of the exoskeleton and loss of appendages.

It is highly important to understand the pathogenicity of this species as well as to establish efficient screening and treatment methods. One of the objectives of this thesis was therefore to investigate the pathogenicity of *V. parahaemolyticus* in a Malaysian wild population of *M. rosenbergii*, collected from the Siput River, Negeri Sembilan. This is exceptionally important because of the trend in *M. rosenbergii* farming in Malaysia to use wild populations as the main source of broodstock for the hatcheries. Understanding the pathogenicity of the bacterial diseases in *M. rosenbergii* will be useful for the development of effective treatments and for screening purposes.

## 4.8 Objectives of Section II

The main objective of Section II was to gain information on the level of genetic differentiation of wild population of *M. rosenbergii* in Malaysia populations in order to assist in the management of the wild populations as well as improvement and sustainability of aquaculture industry and identify the potential population that can be subjected for genetic improvement programmes.

The objectives of *Chapter 5* were:

- I. To investigate if there is any significant difference between Peninsular and Borneo Malaysia.
- II. To test if there is any significant difference between Peninsular, Northern Sarawak, Southern Sarawak and Sabah population based on the recent transplantation history.

and the objective of *Chapter 6* was:

- III. To investigate if *M. rosenbergii* is susceptible to developing diseases caused by bacterial infection from *V. parahaemolyticus*.

## Chapter 5

# Molecular characterization of wild populations of the Malaysian giant freshwater prawn (*Macrobrachium rosenbergii*) using the cytochrome oxidase I (COI) gene.

### 5.1 Introduction

As described in Chapter 4.1, the Indo-Australian Archipelago (IAA) is a geographically complex tropical region, across which there exists the most abrupt biogeographical faunal transition in the world. This complex break of flora and fauna is delimited by the so called Wallace's line and Huxley's line, the latter being an extension of Wallace's line in the north in between Kalimantan and the Sulu Archipelago which crosses the Sulu Sea along the Mindoro Strait between the islands of Mindoro and Palawan.

*Macrobrachium rosenbergii* is known to have high genetic diversity throughout its geographic range, which spans the whole of the IAA. Nominated as the largest species of the genus *Macrobrachium* (Wowor and Ng, 2007), this is one of the most popular species for freshwater aquaculture and has made a significant contribution to the industry regardless of whether it is an indigenous or an introduced species (New, 2010, New and Kutty, 2010). Based on evidence from both morphological and genetic data, two forms of *M. rosenbergii* can be recognised that are distributed to the east and west of the biogeographical barrier, namely *Macrobrachium rosenbergii* de Man 1879 (the eastern form) and *Macrobrachium spinipes* Schenkel 1902 (the western form) (Ng and Wowor, 2011). Based on the analysis of the 16S rRNA gene, de Bruyn et al. (2004) concluded that it is more appropriate to categorize the species *Macrobrachium rosenbergii* and *Macrobrachium spinipes* according to Huxley's line rather than Wallace's line, so that the Philippine species is included as an eastern form.

Another study by de Bruyn et al. (2005) that screened the COI gene found significant differences between northern and southern populations of the western form that were divided by the Isthmus of Kra Seaway. Surprisingly the Kraburi River population, which is located in Thailand, just north of the seaway showed a combination of haplotypes from northern (9 haplotypes) and southern (1 haplotype) populations. Meanwhile, another study by de Bruyn et al. (2004), which used the COI gene on the eastern form of *M. rosenbergii* (samples from the Lake Carpentaria region, located in Australia) also showed moderate genetic differentiation between the studied populations. However, little is known about the diversity of *M. rosenbergii* in Malaysia.

As far as is known, only two studies have been carried out extensively on *M. rosenbergii* samples (i.e. the western form) from Malaysia. Bhasu et al. (2007) reported the development of a set of DNA markers of long random amplified polymorphic DNA (LP-RADP) and random amplified microsatellite (RAMs) markers, and have tested these on 11 populations in Malaysia. In this study no cluster was retrieved from the studied populations, even though high polymorphic markers were used. Meanwhile, a second study developed another type of DNA marker, the random amplification of polymorphic DNA (RAPDs), and has applied this to the same populations as in Bhasu et al. (2007). The developed markers have each proved informative in indicating the genetic differences in *M. rosenbergii*. See et al. (2008) found no significant similarity between their data and that of Bhasu et al. (2007). Nevertheless this study did not confirm the existence of a highly differentiated population in Sabah, as reported in the preliminary study by de Bruyn et al. (2003) that used 16S rRNA gene.

The present study utilised the advantages of the COI gene as a widely used region for phylogenetic studies (da Silva et al., 2011). Moreover, this region has already been tested on both the western form of *M. rosenbergii* collected from 11 different localities from peninsular Malaysia, Bangladesh, Thailand, Vietnam and Indonesia (de Bruyn et al., 2005) as well as on the eastern form (de Bruyn et al., 2004), so that global data are available for comparison.

The purpose of the present study was to investigate patterns of genetic differentiation of wild populations of *M. rosenbergii* from different parts of

Malaysia; Peninsular, Northern Sarawak, Southern Sarawak and Sabah), based on a phylogenetic analysis of sequence variation in the mitochondrial gene, cytochrome oxidase I (COI).

## **5.2 Materials and methods**

### ***5.2.1 Sample collection and pre-treatment***

A total of 232 prawns were used in this study, which were collected from 9 different locations in Peninsular Malaysia and East Malaysia (Figure 5-1). The collection of Sarawak samples was subdivided into northern and southern populations. A portion of fresh muscle samples was immediately preserved in 70% ethanol and kept at -20°C prior to molecular analysis. The samples were transported to the University of Glasgow via next day special delivery (World courier (M) Sdn. Bhd.) in 1.5 ml microcentrifuge tubes containing 1 ml of 70% ethanol at ambient temperature. The importation license to bring the samples from Malaysia was obtained from the Scottish Government (POAO(S)/2009/238 and POAO(S)/2011/27).

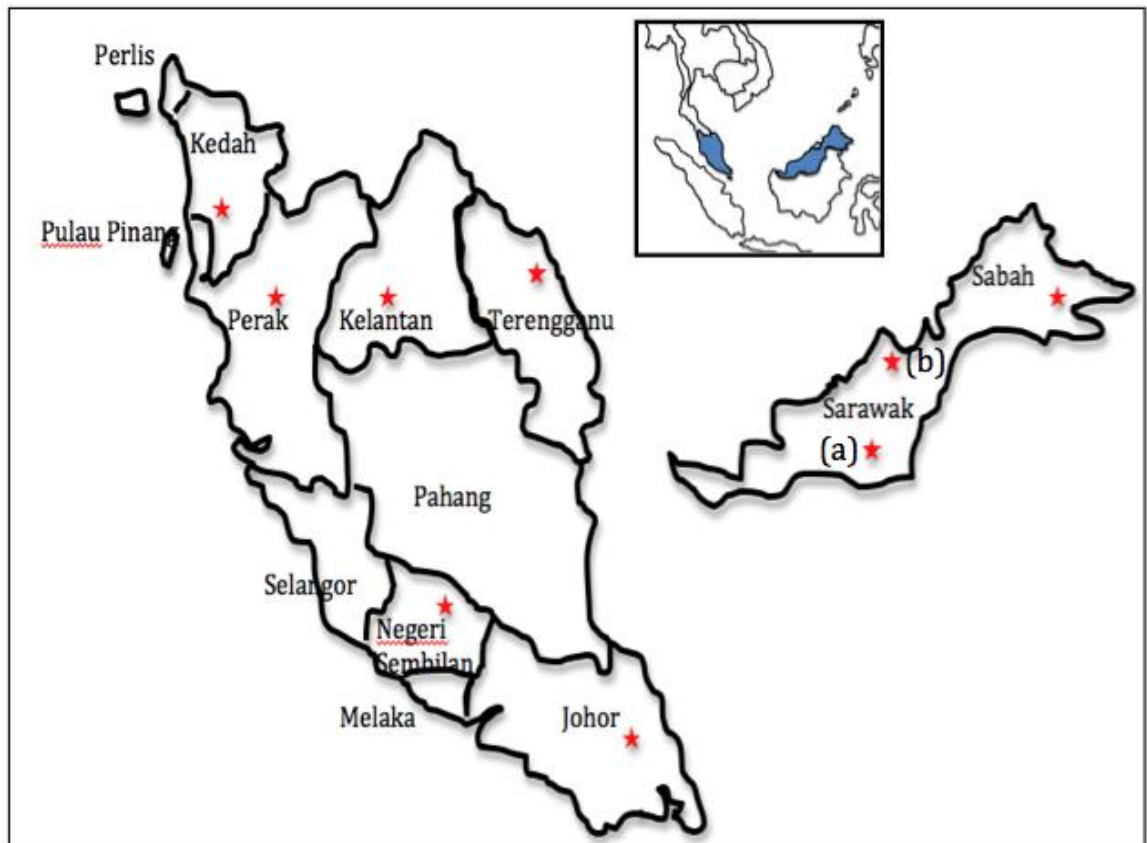


Figure 5-1: Map showing the sampling sites of 9 localities in Malaysia. Two locations were from the (b) Northern and (a) Southern regions of Sarawak, Malaysia. Two locations were from the (b) Northern and (a) Southern regions of Sarawak. The inset picture shows the location of Malaysia in the Asia Pacific region.

## 5.2.2 Molecular analyses

### DNA purification

About 25 mg of muscle tissue was used for genomic DNA extractions, using the standard protocol for the Qiaquick DNA extraction kit (Qiagen, (UK) Inc.), with minor modifications (refer to Chapter 3.2.2). A negative control of ddH<sub>2</sub>O was also included in every extraction. The genomic DNA was checked for the quality and quantity using a 1.5% agarose gel (for details see Chapter 3.2.3). Beside, the quality and quantity of the genomic DNA was also checked using a spectrophotometer (Nanodrop, ND 1000). However, the genomic DNA showed smears DNA in all samples. To overcome this problem, 11 samples from Perak were treated with RNAase to exclude the possibility of contamination with RNA in the samples. 2  $\mu$ l of RNase (1  $\mu$ g  $\mu$ l<sup>-1</sup>) was added to each 90  $\mu$ l genomic DNA.



## PCR amplification

Despite the poor quality and quantity of the samples, it was decided to proceed with the PCR amplification using a published method (de Bruyn et al., 2005), using a set of universal primers (LCO1490 and HCO2198) that was adapted from Folmer et al. (1994), with a design based on the conserved sequence of the COI gene from several invertebrate species (Folmer et al., 1994). Double stranded DNA amplifications were performed in 50  $\mu\text{l}$  volumes, containing 1.25 U of Taq polymerase (New England Biolabs (UK) Ltd.), 10  $\mu\text{l}$  of 5X reaction buffer containing  $\text{MgCl}_2$  (Promega (UK) Ltd.), 2  $\mu\text{l}$  of 25 mM  $\text{MgCl}_2$  (Promega (UK) Ltd.), 0.2 mM dNTPs (New England Biolabs (UK) Ltd.), 8  $\mu\text{M}$  of each primer, and approximately 400 ng of genomic DNA. Thermal cycling was performed on a UNO II thermal cycler (Eppendorf (UK) Ltd.) using PCR conditions used in the de Bruyn et al. (2005) study, except for the annealing temperature being set at 50°C. Negative controls were run in all PCR amplifications. PCR amplification was started with pre-denaturation of the DNA strand at 94°C for 3 min, followed by 30 cycles of strand denaturation at 94°C for 30 s and annealing at 50°C for 30 s. The reaction then proceeded with primer extension at 72°C for 30 s. Lastly, the reactions underwent final primer extension at 72°C for 10 min and cooling at 10°C. Amplified fragments were visualized on a 1.5% agarose gel under an ultra-violet (UV) transilluminator (BIO-Rad Laboratories (UK) Ltd.) (for details see Chapter 3.2.3).

However, the PCR amplification failed. The success of PCR amplification depends on many factors but the most crucial ones are annealing temperature, the amount of  $\text{MgCl}_2$ , and the quantity and quality of the templates. PCR optimization was carried out for annealing temperatures ranging between 45°C to 65°C. Beside the amount of  $\text{MgCl}_2$  was also optimised using the concentration of 1  $\mu\text{l}$ , 1.5  $\mu\text{l}$ , 2  $\mu\text{l}$  and 2.5  $\mu\text{l}$ . Finally the DNA templates were optimised using two different concentrations (25  $\text{ng } \mu\text{l}^{-1}$  and 77  $\text{ng } \mu\text{l}^{-1}$ ) with three different amounts of 5  $\mu\text{l}$ , 10  $\mu\text{l}$ , 15  $\mu\text{l}$  for each concentration. All the PCR amplification cycles were carried out using a UNO TGradient thermal cycler (Eppendorf (UK) Ltd.). PCR amplification using the optimised condition produced a fragment at the expected size of 1.4 kb. Nevertheless, the PCR amplification also produced smear DNA. To overcome this problem, a serial dilution of 1  $\mu\text{l}$  DNA templates/5  $\mu\text{l}$  ddH<sub>2</sub>O, 1  $\mu\text{l}$  DNA templates/10  $\mu\text{l}$  ddH<sub>2</sub>O, 1  $\mu\text{l}$  DNA templates/30  $\mu\text{l}$  ddH<sub>2</sub>O and

1 µl 30/50 µl was carried out on the DNA templates prior for PCR amplification. With this modification, a clean and strong band at the correct size was ultimately obtained. Diluting the DNA template will dilute the contaminants that had been carried over with the DNA template. In the case of a low quantity of DNA template, contaminants that have been carried over with the template will have a more significant effect on the PCR amplification process.

## DNA sequencing

Sequencing was conducted on both strands of the PCR products using the same primers used for PCR amplification (LCO1490 and HCO2198) and performed on an ABI 3730 automated sequencer carried out by the Sequence Service, School of Life Sciences, University of Dundee, Scotland, United Kingdom. The volume of each of the samples needed for sequencing was determined, based on the concentration of the fragment of PCR product visualised in agarose gel electrophoresis. Dilution of samples and the volume of the primers were as explained in Chapter 3.2.7. The first sequencing failed, which might have been due to several factors related to the quality and quantity of the PCR products. First, the concentration of PCR products might have been either too high or too low. The amount of PCR products sent to the sequencing service was estimated based on the suggested amount provided by the sequencing service and the gel image of the PCR products. A few samples at different concentrations were sent for sequencing. Secondly, the salt and/or ethanol concentration that was carried over during DNA purification of PCR products might have caused the low signal peaks that drop after a few hundred base pairs. To exclude the possibility of salt or ethanol being carried over, 2 samples that were prepared in different ways, either by purification using commercially available kit (Wizard<sup>®</sup> SV gel and PCR clean-up system, Promega (UK)) or SAP-EXO treatment ([http://www.nucleics.com/DNA\\_sequencing\\_support/exonuclease1-SAP-PCR-protocol.html](http://www.nucleics.com/DNA_sequencing_support/exonuclease1-SAP-PCR-protocol.html) accessed on 10<sup>th</sup> April 2012), were sent for sequencing. The SAP-EXO treatment is useful to digest nucleotides and any single-stranded DNA left over after PCR amplification. However, this purification method is only possible if a clean and strong band from PCR amplification is obtained. A 1X master mix was prepared by combining the exonuclease enzyme (0.04 µl) with Shrimp Alkaline Phosphate (SAP) (0.4 µl) in a proportion of 1:10. The mixture was then made up to 10 µl by adding sterile dH<sub>2</sub>O. For each amplified PCR sample, 10 µl of

the SAP-EXO master mix was added to 40 µl of the remaining PCR product (10 µl was initially used in gel electrophoresis for confirmation). Strong and long reading was obtained from the sample treated with SAP-EXO, suggesting that it was the salt or ethanol being carried over that had interfered with sequencing results. The PCR products amplified in the present study were suitable for SAP-EXO treatment since they produced a distinct and clean band. Thirdly, the concentration of the primer itself might have also contributed to this problem even though not in the case of the present study. Finally, there is the probability of inhibitors being present that could inhibit the sequencing reaction. All these parameters were taken into consideration during optimizing of the sample for sequencing.

### **5.2.3 Phylogenetic analyses**

Sequences were analyzed and edited manually using Sequencher v4.7 (Gene Codes Inc.). Consensus sequences were aligned using the Geneious alignment option (using default settings) available in the Geneious Pro 4.8.4 software package (Drummond, 2010). Optimisation of the alignment through manual editing was achieved using the same software package. Aligned sequences were then used to calculate the number of conserved and variable sites using *MEGA* v5.03 (Tamura et al., 2011). Beside, the nucleotide composition was also calculated using *Mega* v5.03. The transition and transversion bias (R) was calculated using Kimura (1980) 2-parameter with gamma distribution model of 5 categories was also carried out using *MEGA* V5.03 to estimate the pattern and rates of nucleotide substitution. Maximum likelihood of the substitution matrix of transition and transversion was also calculated using Kimura (1980) 2-parameter with gamma distribution model of 5 categories using *MEGA* v5.03. The model which best described the nucleotide substitution was evaluated using the Model selection option available in the *MEGA* v5.03 software package. The relationships between populations were visualised by a maximum likelihood tree (Felsenstein, 1981) generated also by *MEGA* v5.03 using the best fitting model suggested by Model selection and calculated with 1000 bootstrap replicates (Felsenstein, 1985). The maximum likelihood approach, which is based on probability, chooses the best tree that has highest probability. Beside, maximum likelihood will produce a reliable tree even if the evolving rates between the lineages is different and it use all sequence information to construct the tree

(Felsenstein, 1981). The same software was also utilized to calculate the genetic divergence rate using the best test model, Kimura 2-parameter (Kimura, 1980) and gamma distribution model with 5 categories. This model was chosen based on the lowest number of Bayesian Informative Criterion (BIC) scores. The molecular clock available from *MEGA* v5.03 was applied to test the evolutionary rate using the Maximum-likelihood method based on the Kimura-2 model with 5 gamma discrete categories. The final tree was visualised and manipulated using Figtree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/> accessed on 10<sup>th</sup> April 2012). Gene diversities (haplotype and nucleotide diversity) were calculated using *DNA<sub>SP</sub>* v5.10.01 using the aligned nucleotide sequences created using Geneious. Neutrality tests to postulate whether the sequences are evolving naturally or under selection pressure were conducted using the Tajima'D (Tajima, 1989) and Fu and Li's tests (1000 replicates) (Fu and Li, 1993) implemented in *DNA<sub>SP</sub>* V5.10.01. Synonymous and non-synonymous tests were carried out using *DNA<sub>SP</sub>* V5.10.01 to investigate whether the polymorphism occurred in the sequences (codon) has altered the protein encoded by that particular codon. To do this, the aligned sequences were assigned into mitochondrial genetic code and the reading frame was started at the second base pair. Haplotype data were generated by *DNA<sub>SP</sub>* v5.10.01 (Librado and Rozas, 2009) using aligned nucleotide sequences data in a fasta formatted file created using Geneious. Based on the haplotype data created using *DNA<sub>SP</sub>* v5.10.01, the sequence for each haplotype was manually copied from one of the sequence of the sample that belong to that haplotype for further analysis using ARLEQUIN v3.1 (Excoffier et al., 2005). Assessment of relative genetic diversity within and between populations was computed using Analyses of Molecular Variances (AMOVA), as implemented in ARLEQUIN v3.1 using the Kimura-2 parameter model. The pairwise  $F_{ST}$  values calculated using Arlequin v3.1 (Excoffier et al., 2005) were used to conduct Multidimensional Scaling analysis (MDS) using R software package (<http://cran.r-project.org/mirrors.html> accessed on 10<sup>th</sup> December 2012 and Beckerman and Petchey, 2012). TCS (Clement et al., 2000) was employed to construct the network between haplotypes in Malaysia populations.

The data were further aligned with other published COI sequences of both the eastern form (GeneBank accession number: AY614545 until AY614587 (43

haplotypes) published by de Bruyn et al., 2004) and the western form (GeneBank accession number: AY554293 until AY554327 (35 haplotypes) published by de Bruyn et al., 2005) of *M. rosenbergii* available from the National Centre for Biotechnology Information (NCBI) website. These sequences were amplified using the same set of primers used in the present study (LCO1490 and HCO2198). A maximum likelihood tree was built using T92+G model (gamma: 0.25) suggested by model selection implemented in *MEGA* v5.03. Two COI sequences of *M. nipponense* (GeneBank accession number: FJ958198 and FJ958199) available from the NCBI website also amplified using the same set of primers (LCO1490 and HCO2198) and were used as an outgroup (Zhang et al., 2009) (Table 5-1).

**Table 5-1: Haplotypes obtained from the present study and other published data in GenBank. *P* is the sampling locations in the study, *n* is the sample size and *h* is the total number of the haplotypes. Studied populations, their forms and the paper that published the haplotypes are indicated.**

Form	Studied population	<i>p</i>	<i>n</i>	Accession number	<i>h</i>	Sources
Western	Malaysia	Setiu, Semenyih, Bahand	404	AY554293 until AY554327	35	de Bruyn et al., 2005
	Bangladesh	Raimangal, Meghna				
	Thailand	Kraburi, Tapi				
	Vietnam	Dongnai, Mekong				
	Sumatra	Musi				
	Kalimantan	Barito				
	Malaysia	Kedah, Kelantan, Terengganu, Perak, Negeri Sembilan, Endau, Southern Sarawak, Northern Sarawak, Sabah	232		45	Present study
Eastern	Australia	Katherine, Keep, Roper, McArthur, Wenlock, Norman, Archer, Limmen, Bight, Lennard	378	AY614545 until AY614587	37	de Bruyn et al., 2004
	Irian Jaya	Ajkwa				
	Papua New Guinea	Hann				
Out-group ( <i>M. nipponense</i> )				FJ958198 and FJ958199	2	Zhang et al., 2009

### 5.2.4 Demographic analysis

The combined nucleotide sequence data from the present study and Bruyn et al. (2005) were also subjected for Bayesian Skyline Plot (BSP) method using BEAST v1.6.1 software that contains BEAUti, BEAST programme and TreeAnnotator (Drummond et al. in press). The nucleotide sequence data saved in Nexus format was imported into the BEAUti programme and used to estimate effective population size through time, using an MCMC sampling procedure. All taxa were set at date of zero by default (samples were sampled at the same time). The substitution model was set at default but gamma and invariant sites was chosen

for site heterogeneity model. The clock model was set based on the nucleotide substitution rates of snapping shrimp (0.014/million year). The option coalescent: Bayesian Skyline was chosen for the tree prior and the length of chain in Markov Chain Monte Carlo (MCMC) was set at 70 millions. BEAST XML file was generated and run with BEAST programme with parameters set at default. Meanwhile the tree file, which is the output file from BEAST, was summarized using TreeAnnotator and viewed using Figtree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/> accessed on 10<sup>th</sup> December 2012) to estimate the time since divergence of the populations based on the nucleotide sequences information. The Tracer v1-1.5 was used to explore the output of BEAST and estimated the demographic history of the studied populations. Bayesian Skyline reconstruction was carried out by selecting the tree log file that corresponded with the log file, run in Tracer.

### 5.3 Results

Genomic DNA of extracted samples gave a very poor quality and quantity, which might be due to the treatment of the tissue samples either after being caught, or during transportation and preservation. The genomic DNA of purified tissue viewed using gel electrophoresis showed smears of DNA, and nanodrop readings were around only 10-50 ng  $\mu\text{l}^{-1}$ , indicating that the concentration of DNA was very low. Moreover, the gel pictures also show multiple bands at a very low size between 400-100 bp. Besides, the gel images obtained after RNase treatment showed no differences between before and after treatment. The methods to preserve samples for later DNA study depend on the storage duration, the purpose of the analyses at later stage and the types of sample (Michaud and Foran, 2011). For instance, DMSO-EDTA solution was found to be better for long-term storage as the salt concentration in the solution could precipitate the DNA, making it less susceptible to DNA degradation. However, differences in the concentration of the ethanol 40%, 70% and 100% did not show significant differences. Preservation of tissue samples in liquid nitrogen described in Chapter 3.2.1 proved to be the best method, but might be costly and quite difficult for field use.

Despite the low quality and quantity of the genomic DNA, the purified DNA was used to optimise the PCR condition. Annealing temperatures at 50°C, 2 µl of MgCl<sub>2</sub> at concentration of 25 mM MgCl<sub>2</sub> (Promega (UK) Ltd.) and 10 µl of each template DNA were used. Furthermore, the template DNA was diluted depending on the starting amount of the stock DNA. Meanwhile, the SAP-EXO treatment method was proved to be a better treatment of the PCR products prior to sequencing, given a good quality of PCR products was obtained.

### **5.3.1 COI sequence information**

A fragment of 582 bp of the COI gene was successfully sequenced from a total of 232 samples of *M. rosenbergii*. The sequenced fragments consisted of 532 conserved and 50 variable sites, of which 25 were polymorphic and the remainder included substitutions in only a single sequence (i.e. singletons) (Figure 5-2). A total of 45 haplotypes was identified. The average nucleotide composition was T: 27.5 A: 26.2 C: 27.3 G: 19.0. The nucleotide substitution rate calculated using maximum composite likelihood estimation indicated that the rates of transition and transversion substitutions were 24.24 and 0.38, respectively. The overall transition/transversion bias (R) was 31.87. The haplotype diversity (Hd) was 0.8 and nucleotide diversity (Pi) was 0.00801. From a total of 193 codons detected in the sequences, the synonymous test value was 161.37 from 44 mutations and the nonsynonymous value was 417.63 from 6 detected mutations. A total of 8 shared haplotypes were identified. Among the 8 shared haplotypes, two were the main haplotypes which are H2 (92/232 samples) and H8 (40/232 samples) that have a wide geographical distribution, shared among all populations except Sabah. Each of the remaining haplotypes was shared among at least 2 populations at low frequency (Table 5-2). The genetic diversity is lowest in Northern Sarawak (6 haplotypes/30 samples) but highest in Kedah (7 haplotypes/13 samples) population. Most of haplotype 8 consists of Northern Sarawak samples (19/40 samples) but it is also shared with other populations, except Sabah, with lower frequency. Meanwhile the unique population, Sabah, only has 1 shared haplotype with other samples from Sabah (H33) but a high number of unique haplotypes (8/28 samples).

Haplotype	T	A	T	G	G	C	A	A	C	A	C	A	A	C	A	T	C	A	G	T	T	T	A	G	T	C	G	G	T	C	G	A	G	A	G	A	A	T	C	A	T		
H1	.	.	.	.	.	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H2	.	.	.	.	.	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H3	.	.	.	.	.	.	G	C	A	.	.	.	G	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	
H4	C	.	.	.	.	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	
H5	.	.	.	.	.	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	
H6	.	.	.	.	.	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	
H7	.	.	.	.	.	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	
H8	.	.	.	.	.	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	
H9	.	.	.	.	.	G	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	G	.	.	.	G	
H10	.	.	.	.	.	.	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	G	
H11	.	.	.	.	.	.	.	G	C	A	.	.	.	.	.	T	.	G	T	.	.	.	.	A	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	G	
H12	.	.	.	.	.	.	.	.	G	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
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H17	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H18	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H19	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H20	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
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H22	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
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H24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H26	G	.	.	.	.	.	T	G	C	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H27	.	.	.	.	.	.	G	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
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H36	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
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H38	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H39	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H40	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H41	.	.	.	.	.	A	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H42	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H43	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H44	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G
H45	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G

Consensus identity\* sequence:  
 CAGGCATGGTAGGTACGTCACTAAGACTCTTAATTCGAGCAGAATTAGGGCAGCCGGGCAGACTGATCGGAAATGACCAAACTACAACGTAATTGTCACTGCCACGCATTTCGTA  
 ATAAATTTTTCATGGTTATACCGATCATAATTGGTGGTTTCGGTAATTGACTAGTACCCCTAATATTAGGGGCCAGACATAGCATTCCACGCATAAAACAACATAAGATTCTG  
 ACTCCTACCCCATCTCTAACACTTCTTCTCTCCAGAGGAATAGTAGAAAGAGGGGTTGGCACAGGATGAACGTGTTATCCACCCTAGCGGCTGGTACCGCCACGCCGGGGCATC  
 GGTAGATCTAGGTATTTTCCCTCCACCTAGCAGGAGTTCTTCAATCTTAGGGGCTGCAACTTTATTACCACAGTGATTAAACATACGAGCCCGAGGAATAACTATAGATCGAC  
 TGCCCTATTGATAGCGGTATTTCTAACAGCCATCTGCTTCTTCTCACTACCAGTTTAGCCGGAGCCATTACCATACTCTTAAGTATGAAACCTAAATACATCTTTT  
 Variable sites: 14, 26, 45, 53, 56, 59, 71, 80, 104, 116, 125, 131, 140, 164, 176, 182, 200, 203, 206, 212, 215, 239, 242, 254, 260, 263, 275, 287, 305, 308, 311, 314,  
 323, 326, 338, 344, 350, 365, 371, 404, 425, 428, 464, 467, 482, 518, 528, 539, 548 and 552.

Figure 5-2: Polymorphic sites and nucleotide changes in each haplotype. Full consensus identity sequence of 582 bp is provided. Variable sites are the base pair numbers of each of the polymorphic sites.



Table 5-2: The absolute frequency of the haplotypes. Population abbreviation; PRK: Perak, EDU: Endau, NSE: Negeri Sembilan, TRG: Terengganu, KDH: Kedah, KEL: Kelantan, SS: Southern Sarawak, NS: Northern Sarawak and SBH: Sabah.  $n$  is the sample size for each population.  $I$  is the total samples of each haplotypes and  $T$  is the total haplotype in each population.

Population	PRK	EDU	NSE	TRG	KDH	KEL	SS	NS	SBH	
$n$	25	28	28	30	13	22	28	30	28	
$H$	HAPLOTYPES FREQUENCY									$I$
H1	6	0	0	0	1	0	3	0	0	10
H2	9	19	16	17	6	13	12	0	0	92
H3	1	0	0	0	0	0	0	0	0	1
H4	1	0	0	0	0	0	0	0	0	1
H5	3	0	0	0	0	0	0	0	0	3
H6	1	0	0	0	0	0	0	0	0	1
H7	1	0	0	0	0	0	0	0	0	1
H8	2	2	4	5	2	3	5	19	0	42
H9	1	0	0	0	1	0	0	0	0	2
H10	0	0	0	1	1	1	1	0	0	4
H11	0	0	0	0	0	0	1	0	0	1
H12	0	1	2	2	0	0	1	0	0	6
H13	0	0	0	0	0	0	1	0	0	1
H14	0	0	0	0	0	1	3	5	0	9
H15	0	1	1	0	0	2	1	0	0	5
H16	0	0	1	0	0	0	0	0	0	1
H17	0	0	1	0	0	0	0	0	0	1
H18	0	0	1	0	0	0	0	0	0	1
H19	0	0	1	0	0	0	0	0	0	1
H20	0	0	1	0	0	0	0	0	0	1
H21	0	0	0	0	0	2	0	0	0	2
H22	0	1	0	0	0	0	0	0	0	1
H23	0	2	0	0	0	0	0	0	0	2
H24	0	1	0	0	0	0	0	0	0	1
H25	0	1	0	0	0	0	0	0	0	1
H26	0	0	0	0	1	0	0	0	0	1
H27	0	0	0	0	1	0	0	0	0	1
H28	0	0	0	1	0	0	0	0	0	1
H29	0	0	0	1	0	0	0	0	0	1
H30	0	0	0	1	0	0	0	0	0	1
H31	0	0	0	1	0	0	0	0	0	1
H32	0	0	0	1	0	0	0	0	0	1
H33	0	0	0	0	0	0	0	0	20	20
H34	0	0	0	0	0	0	0	0	1	1
H35	0	0	0	0	0	0	0	0	1	1
H36	0	0	0	0	0	0	0	0	1	1
H37	0	0	0	0	0	0	0	0	1	1
H38	0	0	0	0	0	0	0	0	1	1
H39	0	0	0	0	0	0	0	0	1	1
H40	0	0	0	0	0	0	0	0	1	1
H41	0	0	0	0	0	0	0	0	1	1
H42	0	0	0	0	0	0	0	1	0	1
H43	0	0	0	0	0	0	0	1	0	1
H44	0	0	0	0	0	0	0	3	0	3
H45	0	0	0	0	0	0	0	1	0	1
$T$	9	8	9	9	7	6	9	6	9	232

### 5.3.2 Phylogenetic analyses

The best-fit model for nucleotide substitution was K2+G (Gamma shape parameter: 0.1097). The TCS network clearly supported the existence of three differentiated clusters in the Malaysian populations (Figure 5-3). Cluster I has a wide distribution that covered all studied populations except Sabah. Moreover, a sub-cluster (marked as I-I in Figure 5-3) was also identified in cluster I where majority of the samples were from Northern Sarawak (30/42 of Northern Sarawak samples were found in sub-cluster) and Northern Sarawak haplotypes can only be found in sub-cluster I-I. This sub-cluster also consisted of other populations in Malaysia except Sabah, but at lower frequency. Besides, cluster II which was derived from the main haplotype in cluster I, included populations from Perak, Kedah and Southern Sarawak but these populations were also identified in cluster I. Sabah was identified as an isolated population (cluster III). Beside, the maximum likelihood tree with a high percentage of bootstrap replicates (>70%) strongly supports the existence of three clearly different clusters in Malaysian populations. Significantly high genetic differentiation was observed in Malaysia populations with 60% of the variation occurring among groups ( $F_{CT}$ : 0.57600) and 40% of the variation occurred among population within groups and within populations, (Table 5-3) which was also evident with the value from the pairwise  $F_{ST}$  (Figure 5-5). The genetic differentiation of the studied populations was also tested at a higher hierarchy level by separating the Sabah haplotypes into another group. However high genetic differentiation was still observed among populations. No significant deviation from the neutrality was found for Tajima's D (-1.29124;  $P > 0.10$ ) but was significant for Fu and Li's tests ( $D = -3.56516$ ;  $P < 0.02$ ;  $F = -3.07404$ ;  $P < 0.02$ ). The maximum likelihood test implemented in *MEGA* could not reject the hypothesis that the lineages were evolving according to a molecular clock null hypothesis, indicated by the higher value obtained without the molecular clock enforced. This indicates that each of the Malaysia populations, including the highly differentiated population of Sabah, is evolving at the same rate ( $-\ln = 1852.210$  when the molecular clock was applied versus  $-\ln = 1239.978$  without the molecular clock enforced,  $P < 3.188$ ).

The analysis between these data with the haplotypes from de Bruyn et al. (2005) (western form) and de Bruyn et al., 2004 (eastern form) carried out in the

present study separated the haplotypes of western form from de Bruyn et al. (2005) into two sub-clusters, termed as western forms 1 and 2 in Figure 5-4. According to Bruyn et al. (2005), the western form can be separated into northern and southern populations at the Isthmus Kra-seaway located between Thailand and Malaysia. In the present study, cluster I showed similarity with the southern population, while cluster II showed similarity with the northern population (Figure 5-4). However, the maximum likelihood tree depicted Sabah as a distinct population. Mismatch distribution analysis showed demographic expansion in each of the cluster identified in Malaysian population of *M. rosenbergii*. The Bayesian Skyline Plot reconstruction shows that the western form of *M. rosenbergii* underwent a strong population expansion around 50,000 years ago. However convergence problems were encountered with the analyses, thus, the estimation should be treated with caution.

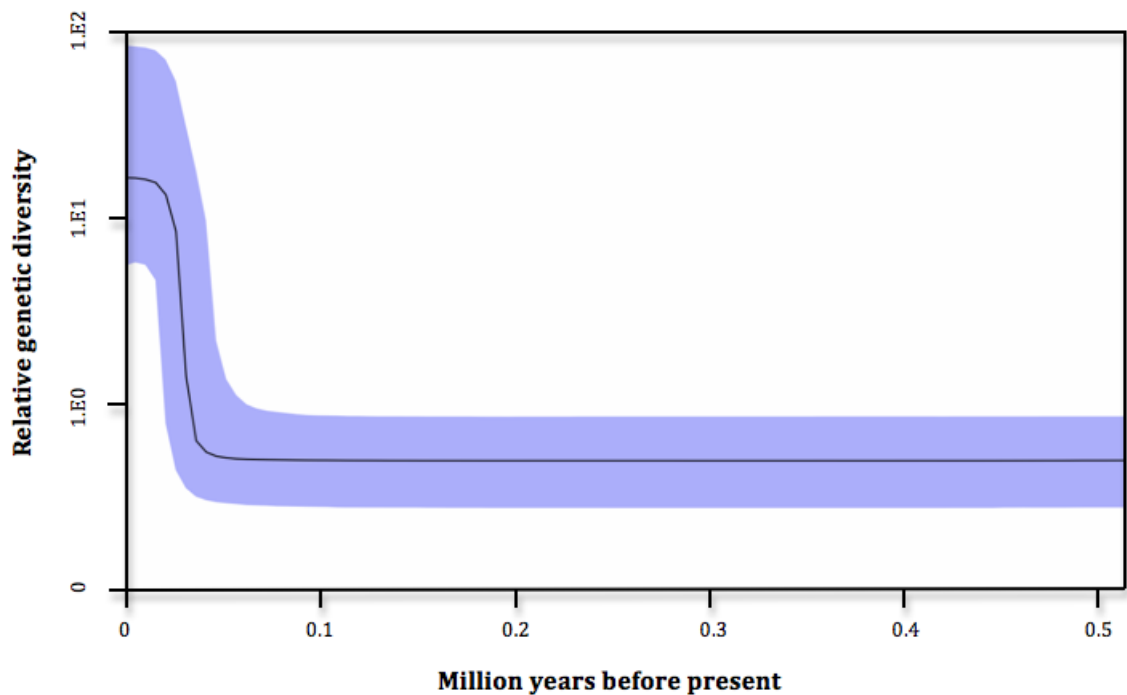


Figure 5-3: Bayesian Skyline Plot reconstruction from mtDNA of *M. rosenbergii* populations from the present study and Bruyn et al. (2005). Y Axis is the relative genetic diversity and X Axis is the time before present in units of million years. The thick solid line is the median estimate and the solid interval (purple colour) is 95% HPD interval.

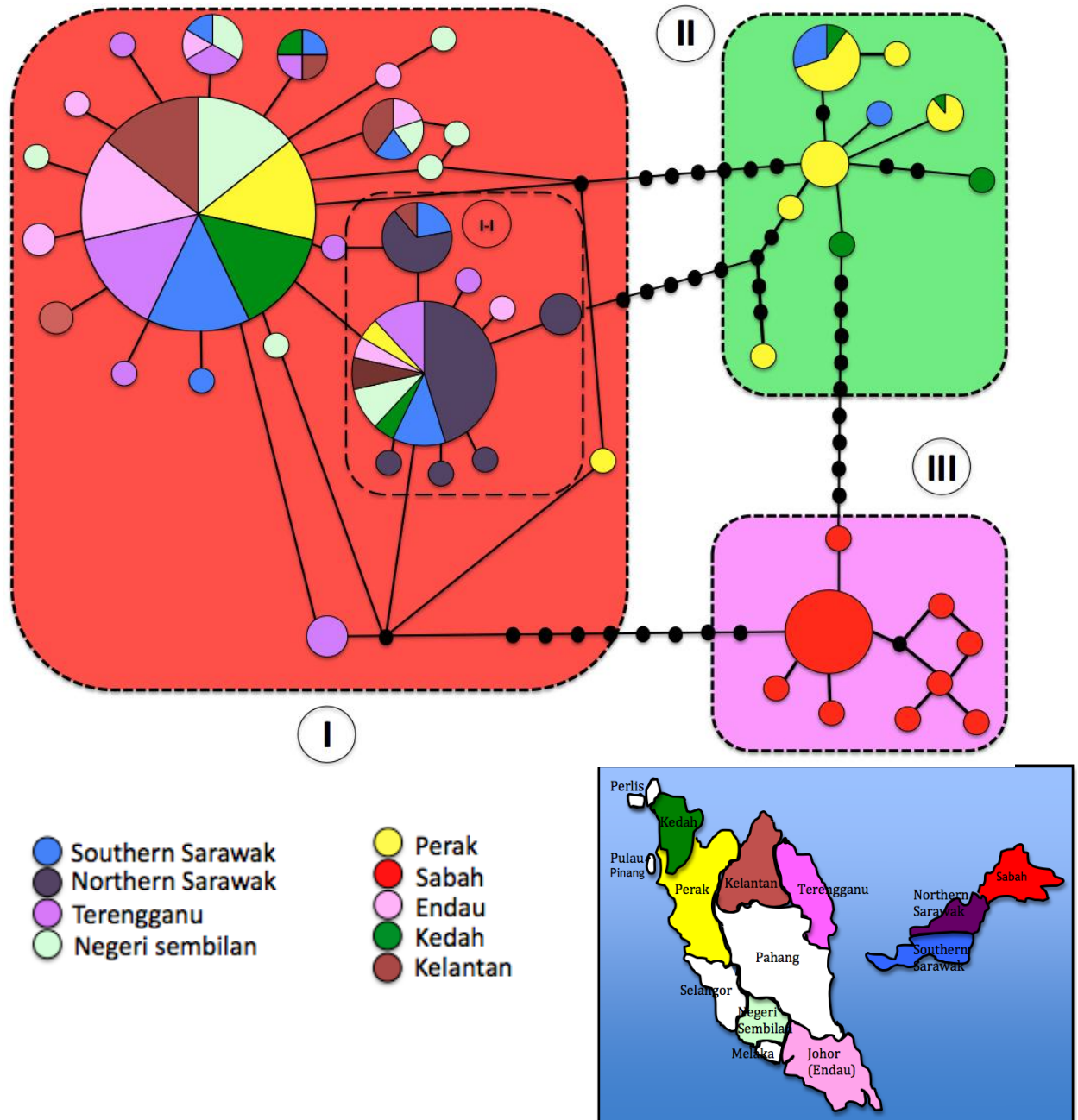


Figure 5-4: TCS network between haplotypes revealing that three clusters (cluster I, II and III) exist in Malaysian populations of *M. rosenbergii* species. I-I is a sub-population in cluster I. Each colour represents a population and the sizes of the circles indicate the frequencies of the haplotypes. The small black circles indicate inferred missing haplotypes not observed in the data set. The location of each of the populations is indicated on the map with colour coding, as in the TCS network.

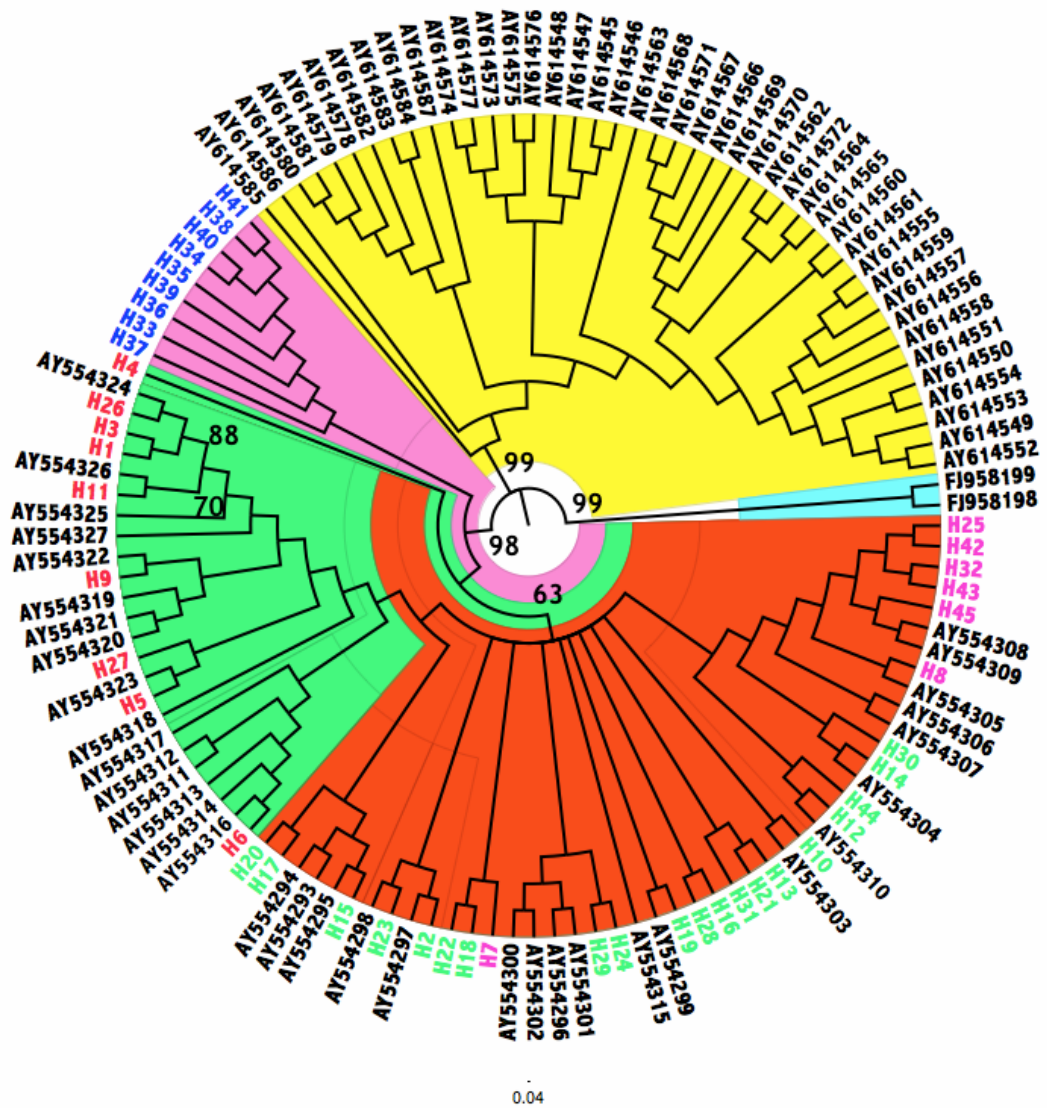


Figure 5-5: Maximum likelihood tree of Malaysian populations previously assigned to the Western form by Bruyn et al. (2005), populations assigned to the Western form from other locations (Bruyn et al., 2005) and populations assigned to the Eastern form (Bruyn et al., 2004). *M. nipponense* was used as an outgroup. The tree was rooted at the outgroup. The Malaysian populations (H1-H45) showed similarity with other western forms except for the Sabah population (branch highlighted with pink). Some of the Perak, Kedah and Sarawak haplotypes showed similarity with Western form 1 (branch highlighted with green) while haplotypes from the remaining populations (excluding Sabah) showed similarity with Western form 2 (branch highlighted with red). The haplotypes highlighted with turquoise are the outgroup. A high genetic differentiation observed between Eastern (branch highlighted with yellow) and Western forms of *M. rosenbergii*. Tip node labels of Malaysia haplotypes (H1-H45); Cluster 1: green, sub-cluster I: pink, cluster II: red and cluster III: blue. Numbers are the percentage of bootstrap replicates (1000 replicates) (only values >60% are shown).

Table 5-3:  $F_{ST}$  values of population genetic differentiation conducted using AMOVA. All populations were assigned into three groups. Percentages of variation are shown among groups, among populations within groups and within populations.

Hierarchy	Source of variation	Percentage of variation	P-value
Three structures: 1a) Cluster I 1b) Sub population of Cluster I 2) Cluster II 3) Cluster III	Among groups	FCT: 57.60	0.326 ±0.015
	Among populations within groups	FSC: 10.63	0.000±0.000
	Within populations	FST: 31.77	0.000±0.000

Table 5-4: Pairwise  $F_{ST}$  value of differentiation between populations of *M. rosenbergii* from Malaysian population. The values were used to conduct the Multidimensional Scaling analysis (MDS) using R software package.

Population	Perak	Endau	N. Sembilan	Terengganu	Kedah	Kelantan	S. Sarawak	N. Sarawak	Sabah
Perak	0	0.43013	0.40652	0.42149	0.02527	0.39269	0.18286	0.43262	0.72729
Endau	0.43013	0	-0.01103	-0.00059	0.27517	0.00065	0.10252	0.54529	0.92547
N. Sembilan	0.40652	-0.01103	0	-0.00664	0.24027	-0.00787	0.07819	0.48273	0.91437
Terengganu	0.42149	-0.00059	-0.00664	0	0.25555	-0.00888	0.07782	0.45584	0.91763
Kedah	0.02527	0.27517	0.24027	0.25555	0	0.22754	0.00903	0.32988	0.78884
Kelantan	0.39269	0.00065	-0.00787	-0.00888	0.22754	0	0.06852	0.4802	0.9178
S. Sarawak	0.18286	0.10252	0.07819	0.07782	0.00903	0.06852	0	0.18413	0.80933
N. Sarawak	0.43262	0.54529	0.48273	0.45584	0.32988	0.4802	0.18413	0	0.92165
Sabah	0.72729	0.92547	0.91437	0.91763	0.78884	0.9178	0.80933	0.92165	0



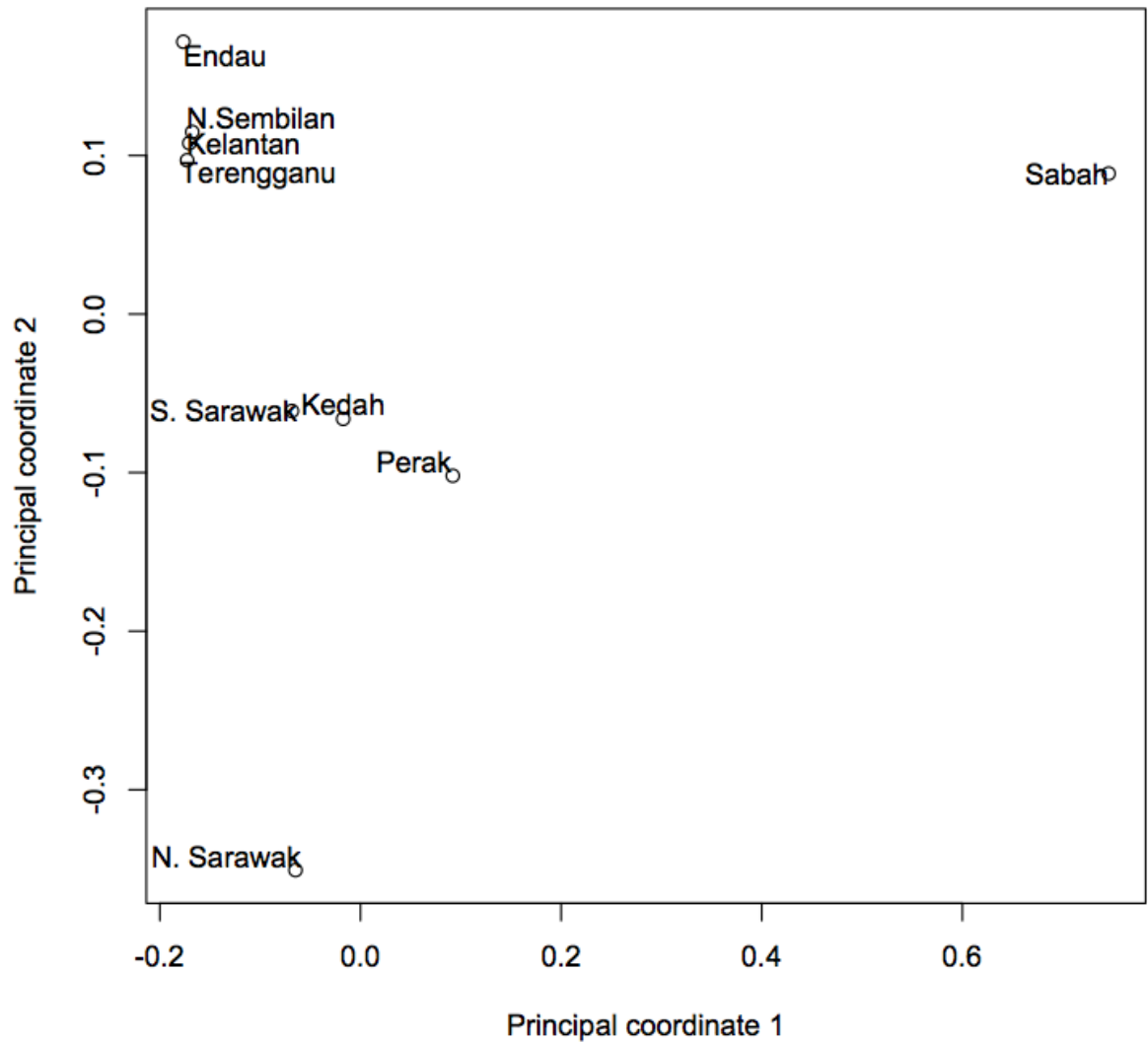


Figure 5-6: The pairwise  $F_{ST}$  value demonstrated the three clusters and a sub population of Cluster 1 in Malaysia population of *M. rosenbergii*. Cluster 1 contain Endau, N. Sembilan, Kelantan and Terengganu populations, while majority of the sub population of Cluster 1 contains N. Sarawak population. Cluster 2 contains S. Sarawak, Kedah and Perak populations and Cluster 3 contains only Sabah population.

## 5.4 Discussion

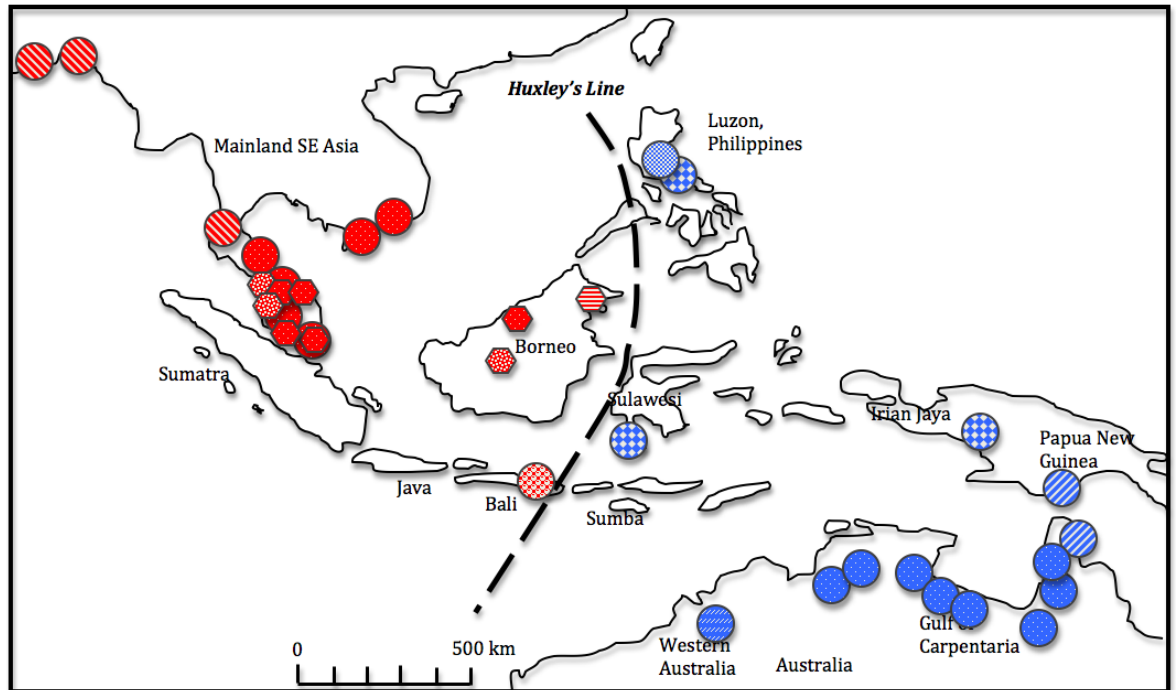
In the early studies of the genetic differentiation of *M. rosenbergii*, electrophoretic markers (allozyme markers), and morphological data have demonstrated the high differentiation of *M. rosenbergii*. Hedgecock (1979) carried out the first molecular study using allozyme markers on *M. rosenbergii* that detected genetic discontinuity which divided *M. rosenbergii* along the Wallace line. This finding was later supported by Lindenfelser (1984) using both morphological and electrophoretic markers. Malecha (1980) agreed with the division of the species into two forms but also reported that the eastern form can be further divided into the Australian type, found only in northern Australia

and a Philippine type found in southern Taiwan, the Philippines and Palau in the Micronesian Marshall Islands. He limited the distribution of the western form from India, Sri Lanka, Myanmar, Thailand, Malaysia and south to Indonesia. Further genetic researches on *M. rosenbergii* were carried out mainly by an Australian research group using both mtDNA and nuclear markers (de Bruyn et al., 2004a, 2004b, 2005, 2007, Chand et al., 2005). A few attempts have also been made to determine the genetic differentiation of Malaysian populations, but no striking findings were revealed (Bhassu et al., 2007, See et al., 2008).

The present study revealed the existence of three clusters in Malaysia populations. The first cluster included all populations from Malaysia except Sabah. A previous study carried out by de Bruyn et al. (2005) focused on the western form of *M. rosenbergii* using the same COI gene used in the present study. The Malaysian populations used their study were Setiu, Bahand and Semenyih. Among these populations, only Setiu was used both in the present and de Bruyn et al. (2005) studies. de Bruyn et al. (2005) revealed a sharp genetic break that separated the northern and southern population at the Isthmus of Kra Seaway, but all Malaysia populations tested in their study were homogenous and categorized into the southern population. The Isthmus of Kra Seaway has acted as a geographic barrier to restrict migration between the two clades. Interestingly, the Kraburi River population, which is located in Thailand adjacent to the seaway, showed a mixed genetic profile between the northern and southern clades. This characterization indicates a recent expansion of the southern clade towards the northern clade, confirmed by the detection of a low frequency of the northern haplotype in Kraburi River population (de Bruyn et al., 2005). The same COI region was used in another intraspecific study carried out by de Bruyn et al. (2004b) to extensively screen the eastern populations. They found moderate genetic differentiation in the eastern form. The COI region identified four discrete genealogical lineages in the eastern form: the Western Australia lineages (I), the Northern territory and Lake Cartenparia lineages (II), Irian Jaya lineages (III) and Papua New Guinean lineages (IV). The high genetic differentiation reported in this study was ascribed to a late Pleistocene event in which the Lake Cartenparia region was inundated by the marine environment (de Bruyn et al., 2004b). Analysis of COI gene in Malaysian populations in the present study showed higher haplotype diversity compared to other populations of the



western ( $h = 0.500$ ) (de Bruyn et al., 2005) or eastern ( $h = 0.376$ ) forms (de Bruyn et al., 2004), mostly due to the Sabah population. The distributions of the populations used in the studies of mtDNA using the COI region as outlined above were demonstrated in the Figure 5-6.



**Figure 5-7:** Map showing the distribution of lineages for mtDNA marker of COI haplotypes obtained from 26 locations on the two sides of Huxley's line from Bruyn et al., 2007 study (circle shape) and 8 locations (hexagon shape) from the present study. The red colour indicate the western population and the blue colour indicate the eastern population. Different shaded pattern indicates differences in the genetic of the studied species. The dash line indicate the location of Huxley's line.

Meanwhile, the Kedah, Perak and Northern Sarawak included haplotypes from two clusters (cluster I and II) where cluster II was connected from the main haplotype (H1) in cluster I. This can be explained by the recent restocking activities of *M. rosenbergii* from northern population of the western form into the Northern Sarawak, Kedah and Perak populations. Restocking activities of some populations that started about 25 years ago in order to recover the depleted wild population was reported in *M. rosenbergii* populations in Malaysia (New 2000). Meanwhile, the high frequency of Northern Sarawak haplotypes clustered in the sub-cluster of cluster I indicates the more restricted gene diversity in this population compared to the other populations. The genetic and morphological differentiation between northern and southern Sarawak has also

been noted in fish species. Ryan and Esa (2006) reported the existence of two types of *Hampala bimaculata*. The *H. bimaculata* from southern and central region of Sarawak was identified as Type A and samples from northern Sarawak were recognised as Type B. These two types possessed high genetic and morphological distinction. Following this the authors suggested that the Type A *H. bimaculata* should be recognised as new species.

The third cluster consisted of only a highly genetically differentiated population, namely Sabah located in the East Malaysia. Another study by de Bruyn et al. (2004a) also used the 16S rRNA region tested on populations collected from both western and eastern forms, including several populations in the Peninsular Malaysia (Semenyih, Bahand and Setiu) and Sabah. de Bruyn et al. (2004a) revealed the most significant discoveries in the studies of *M. rosenbergii* differentiation that divided *M. rosenbergii* into two forms which are the western and eastern forms. As other previous studies on delimiting of *M. rosenbergii* subspecies, de Bruyn et al. (2004a) also agreed to include Philippine populations in the eastern form despite its location close to the mainland (which is the location of the western form). However, because of the classification of the Philippine populations into the eastern form, they commented that this species is more appropriate to be categorised according to Huxley's line rather than Wallace's line. According to de Bruyn et al. (2004a), the eastern form of this species is distributed from the Philippines through Australia and Papua New Guinea, while the western form has a wider geographical distribution which occurs in South and Southeast Asia, including Indo-China. Besides the remarkable finding in the study of 16SrRNA region, both studies (de Bruyn et al., 2003 and 2004a) also revealed Sabah as a distinct population from other population in the western form except Java. Nevertheless, this study also supported the unstructured haplotypes by population in other Malaysia populations sampled from northwest (Bahand), northeast (Setiu) and southwest (Semenyih) Malaysia.

The interpretation of Sabah as a distinct population is still uncertain. Another western population that showed high genetic distinction is the Bali (Indonesia) population which is located close to the Makassar Strait. The Makassar Strait has the characteristic of a strong stratification, which might have restricted mixing between the two forms of *M. rosenbergii*. Johnson (1960) had noted that a specimen from Bali exhibited somewhat intermediate characteristics between

the western and eastern forms, while Short et al. (2004) noted that individuals from Bali showed a close identity with the eastern form compared to the other western form based on the morphological characteristic (rostrum, second cheliped and body) as well as second cheliped colour and life history, despite being categorized in the western form by de Bruyn et al. (2004).

Sabah drainage was still isolated during the Pleistocene period, while the East Sunda River system located in Java drained eastward to exit near Bali, which might explain the unique characteristic of the Bali population. Bali is adjacent to Java which has been known to have genetic similarity with the Sabah population, according to the 16S rRNA marker. Nevertheless there is no single marker, either 16S rRNA, COI or microsatellite, that has been tested on all populations discussed (Sabah, Java and Bali) to allow the genetic information to be compared between these three populations (the Bali population was tested with COI and microsatellite markers while the Java and Sabah populations were tested with 16S rRNA marker only). Similar to the Bali population, the Sabah population is also located close to the Makassar Strait, but the event that shaped the genetics of Sabah population could be more complex, and interpretations can only be preliminary at this stage. The most likely explanation is via the vicariance hypothesis (explained later). Whether these three populations, Sabah, Bali and Java, have genetic similarity is yet to be determined, but when this is done the outcomes will provide a better understanding of the differentiation of the species. Population divergence has also been reported in other freshwater and marine species from Sabah. Preliminary work on Malaysia populations of *Tor douronensis*, a freshwater species of Mahseer, identified a distinct population in Sabah (Esa et al., 2008). Mahseer is the common name for the family Cyprinidae (carps) (subfamily Cyprininae) from the genus *Tor*. Three crustacean decapods collected from the Kinabatangan River located in Sabah were also classified as new species (Ng, 1994). The three species were *Macrobrachium sabanus* sp. nov., *Parathelphusa ovum* sp. nov. and *Hymenicoides microrhynchus* sp. nov.. Besides, the study of Ryan and Esa (2006) on Hampala fishes from Malaysian Borneo discovered a close phylogenetic relationship of the species between northern Sarawak and Sabah, compared to Sabah from central or southern of Sarawak.

In contrast, the outcome from the present study is inconsistent with previous studies on Malaysian populations reported by Bhassu et al. (2007) using LP-RAPD and RAMs markers, and by See et al. (2008), using RAPDs. The LP-RADP and RAMs study showed genetic homogeneity between all Malaysia populations including Sabah while the RADPs maker detected the existence of three major clusters among Malaysia population. This inconsistency could be due to the less reliability of the markers to detect polymorphism. Several studies have reported the low reliability and reproducibility of bands as well as less informative results with the RADP marker than with other DNA marker such as amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) (Yu et al., 2005, Kafkas et al., 2006, Belaj et al., 2003). This is due to the difficulties in scoring the bands, and the fact that the amplification reaction needs to be repeated at least two or three times for confirmation. Consequently the bands were difficult to reproduce in other labs. However the RAPD marker is preferred over other markers such as microsatellites if the cost is constrained. However, preliminary analysis based on EST microsatellite markers carried out by Bhassu et al. (unpublished data) on the same population used in the present study showed that no genetic structure exists in *M. rosenbergii* population in Malaysia. Inconsistency of the outcomes derived from two different sources of genetic information has been reported in many studies and the possible factors leading to the differences between the two markers have been discussed (Bernatchez and Osinov, 1995, Ferguson et al., 1991, Sekino et al., 2011, Yannic et al., 2010, Nyakaana and Arctander, 1999, Moritz et al., 1992). mtDNA and microsatellites analyse different scales of sequences divergence. mtDNA is useful for resolving deep phylogeographic patterns but microsatellites are more appropriate for population genetic analyses and resolution of population structure based on the recent allele frequency distribution and changes under evolutionary processes such as natural selection, genetic drift, mutation and gene flow.

Other recent studies of the genetic differentiation in *M. rosenbergii* have been aimed towards the development of microsatellite markers, which are nuclear DNA markers, in order to investigate the population genetic structure at the nuclear level and to compare them with the information on the deep phylogeography pattern revealed at the mitochondrial level. The first

researchers to successfully isolate and develop microsatellite markers from *M. rosenbergii* were Chand et al. (2005). They developed a set of microsatellite markers based on the eastern form of *M. rosenbergii*. However, their attempts to apply the same markers in both the western and eastern forms were unsuccessful (Malecha et al., 2010). Nonetheless, this gives a positive indication of the possibility that the genetic information from nuclear DNA might be consistent with the differentiation found in mitochondrial DNA (Malecha et al., 2010). This finding initiated another microsatellite study by Charoentawee et al. (2006) that developed a set of microsatellite markers using a Thailand population (the western form). However, the ability of these markers to amplify in the eastern form population is yet to be demonstrated. These two studies have motivated more development of microsatellite markers, but only in the western form. Recent publications on the development of microsatellite markers are from India (Divu et al., 2008 and Bhat et al., 2009) and Malaysia (See et al., 2009, 2011). Nonetheless, until now no microsatellite markers have been tested on the wild populations, and so comparison of the data from two different markers is not yet possible either between the two forms of *M. rosenbergii* or within the forms.

*M. rosenbergii* is an ideal species for interpreting the biogeographic complexity of the Indo-Australian Archipelago (IAA). The centre of accumulation hypotheses (Wallace, 1869, Archbold et al., 1982, Audley-Charles, 1983) such as the plate tectonic evolution hypothesis could explain the dispersal of the *M. rosenbergii* ancestral line. According to this hypothesis, *M. rosenbergii* might have dispersed across the Makassar Strait located along Huxley's line facilitated by the meeting of the Sunda and Sahul shelves during the Miocene through a series of collisions of the shelves dated back to occur during 45, 25 and 5 million years ago (Ma) (Hall, 2002 and de Bruyn et al., 2007). Huxley's line is the extension from Wallace's line, the most discussed line that divides the fauna and flora between the Sunda and Sahul shelves (de Bruyn et al., 2007). Meanwhile, the Makassar Strait is known to play an important role in the global climate system where its function as the main passage to transfer the water and heat from Pacific to India Ocean (Hall et al., 2009). These features (depth and temperature) have shaped a complex biogeographical break of flora and fauna, leading to many researches mainly on the mammals (Heaney, 1986). In the case of *M. rosenbergii*, the

Makassar Strait has restricted the gene flow between the western and eastern forms. However, the outcomes from mtDNA analysis in de Bruyn et al. (2004b, 2005) were not strong enough to postulate the direction of *M. rosenbergii* dispersal (de Bruyn et al., 2007). Nevertheless, the close phylogenetic relationship between *M. rosenbergii* with western species from the genus *Macrobrachium*: *M. malcomsanii* and *M. gangeticum* indicated that the ancestral form of this species most probably is the western form. This also implies that the species has been dispersed southwards (de Bruyn et al., 2007, Johnson, 1973 and Short, 2004).

The vicariance hypothesis (Heaney 1986, Schmitt et al. 1995) invokes an event that shapes the contemporary species diversity across IAA through uplift of the plates and increase in the area of shallow seas. Borneo (location of Sabah, Sarawak, Brunei and Kalimantan) and the mainland of Thai-Malay Peninsular was an emergent terrestrial region separated by a shallow sea since the Paleocene (60 Mya) (Lohman et al., 2011). Through a series of vicariance events, approximately 20 Mya ago, the land in Borneo was uplifted to create mountains in Borneo and Palawan. This process caused the changes in the ocean circulation and gradually increased the area of shallow seas. Moray (2000) suggested that the uplift process in Borneo might also contribute to the wetter climate in Sundaland from the Miocene onward. In contrast, the land area of Sundaland has been gradually reduced, attributed to the uplift process of Borneo during the Neogene until Pliocene (approximately 5 Mya). About 7 million years ago, Mount Kinabalu (located in Sabah) has risen from the process of crustal melting in Borneo to its present height of more than 4 km. These processes might explain the distinct characteristic of *M. rosenbergii* population in Sabah compared to other Malaysia populations.

An extension of the vicariance hypothesis is the refugium hypothesis (Hewitt 2000, Gathorne-Hardy et al. 2002) that postulates the dispersal of animals due to the climate changes associated with the changes of the sea level. The close phylogeographic relationship between peninsular Malaysia and Sarawak haplotypes of *M. rosenbergii* reported in the present study, as well as between the two forms, could also be explained by glacial separation during the Pleistocene period (de Bruyn et al., 2007). Widespread marine dispersal occurred during the Pleistocene glacial maxima but then in the mid-Pleistocene

the sea levels were subsequently elevated, resulting in the wide spread of fresh watersheds that limit the ability of the freshwater species to disperse due its dependence on a freshwater environment. Freshwater species possess greater genetic and morphological differentiation, even on a small geographic scale, compared to marine or anadromous species, as discussed in many papers (Gyllensten, 1985, Ward et al., 1994 and De Woody and Avise, 2000). The high genetic and morphological differentiation found in freshwater species is facilitated by the existence of geographic barriers which restrict migration among lineages, as evidence by the Isthmus of Kra Seaway and the ancient Lake of Carpentaria. Similar to other freshwater species, the giant freshwater prawn *M. rosenbergii* shows significant genetic and morphological differentiation even on a small geographic scale (de Bruyn et al., 2004, Munasinghe and Thushari, 2010). After completing the eight stages of larval development, the post larvae of *M. rosenbergii*, a catadromous species, still remain for about one or two weeks in brackish water before starting to migrate to a lower salinity environment (Ling, 1969). Thus the possibility for dispersal is limited to the larval stages alone (that occupy about 30-45 days from hatching) which encounter fewer geographic barriers in the brackish water that could restrict the gene flow among the lineages (Ling, 1969). Wowor and Ng (2007) suggested that high water salinity and current flow pattern might make a more significant contribution in dividing the eastern and western form. The ability of larvae to tolerate marine conditions is unknown. According to Sandifer et al. (1975), based on a laboratory study, some postlarvae have the capability of surviving up to 20 days but adults normally do not survive more than a week in marine environment. However *M. rosenbergii* are found on some true oceanic islands (e.g. Christmas Island, Palau, Sulawesi, the Philippine Archipelago), which suggests that at least limited marine dispersal occurred in the past (de Bruyn et al., 2007).

Data from the present study should be useful in assisting management and conservation, as well as informing a recovery plan for wild populations. This is important particularly for a unique population such as that in Sabah, since the majority of the *M. rosenbergii* broodstocks for the hatcheries in Malaysia depend on wild populations (New and Kutty, 2010). Besides, the outcomes from this study are also useful for future research to improve the quality of hatchery

products, to obtain a high quality of broodstocks and also to solve the problems arising in the aquaculture industry of *M. rosenbergii* in Malaysia, such as disease infection and large variations of the animal (especially in the male morphotypes). Most of the hatcheries in Malaysia are operated using the green water system which was reported to be associated with many disease and mortality, and has limited the development of the industry in Malaysia (New, 2010 and New and Kutty, 2010). The knowledge of the genetic differentiation in Malaysia population could be potentially used in the future research to select desired populations prior to genetic improvement programmes.



## Chapter 6

# The immune response of *Macrobrachium rosenbergii* to a *Vibrio parahaemolyticus* challenge

### 6.1 Introduction

Previous studies have shown the active involvement of the haemocytes in prawn defence systems (Smith et al., 2003, Cheng and Chen, 2001, Vazquez et al., 2009, Smith and Soderhall, 1991). According to Vazquez et al. (1997), the *M. rosenbergii* haemocytes comprise hyaline cells, granular cells and undifferentiated cells. The hyaline cells are responsible for the phagocytosis mechanism which is a method of eliminating small foreign microorganisms by ingestion into a vacuole termed the phagosome. The trapped molecules in the phagosome are then destroyed by degradation enzymes, together with antimicrobial peptides released into the phagosome. During phagocytosis, NADPH-oxidase of the host is activated, producing the reactive oxygen intermediates (ROIs). ROIs such as superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical (OH) and singlet oxygen ( $^1O_2$ ) are toxic to the foreign microorganism. The release of ROIs is known as respiratory burst (RB) activity. On the other hand, excess activity of RB creates a phenomenon called the oxygen toxicity defence system which has an adverse effect on the host itself. However, the amount of ROIs produced is controlled by another mechanism termed superoxide dismutase (SOD). Lastly, phenoloxidase (PO) activity mediated by granular and semi-granular cells is regarded as the central defence system in prawns. It is responsible for immobilisation, encapsulation and melanisation of the larger foreign microorganism which cannot be phagocytosed. All these factors contribute to an efficient and rapid mechanism for the elimination of foreign microorganisms by innate defence systems (Van de Braak, 2002, Saurabh and Sahoo, 2008, Pillai et al., 2010 and Vazquez et al., 2009) (also refer Chapter 4.6 for further details of the prawn immune system).

The present study was designed based on the information obtained from Chapter 5 of the present thesis. Knowledge that the genetic of Sabah population is distinct from other populations required further explanation whether this population carrying desirable traits in aquaculture industry such as higher resistance to disease infection, big size and fast growth rates. In line with the focus of Malaysia's prawn industry to stabilise and develop the prawn industry, the knowledge of prevention, treatment and management of the disease are also greatly important. Therefore the aim of the present study was to investigate if populations with different genetic background could respond differently to the bacterial infection particularly *V. parahaemolyticus* that caused black spot disease. Black spot disease was chosen based on the finding that many of the experimental prawns obtained from wild populations, as well as a few samples obtained from a grow-out hatchery (Sri Sendayan hatchery, Negeri Sembilan), showed signs of black spot disease. They presented a focal melanisation that can be observed on the gill bailer base as well as on the tail (Saurabh and Sahoo, 2008). Black spot disease, also known as shell disease, affects *M. rosenbergii* at any life stage and is known to be associated with Gram-negative bacteria which are *Vibrio*, *Pseudomonas* and *Aeromonas* (Sharshar and Azab, 2008). Susceptibility of *M. rosenbergii* to infection from *Vibrios* that cause diseases such as black spot disease, bacterial necrosis and vibriosis has been reported in many studies (Ramalingam and Ramarani, 2006, Cheng and Chen, 1998 and Sindermann, 1976). Even though severe effect on the economic is rarely reported due to the black spot disease but could limit the expansion of prawn industry. In this study, *M. rosenbergii* was challenged with *V. parahaemolyticus* to investigate the susceptibility of this prawn species to developing diseases caused by bacterial infection. To achieve this, the experiment was divided into a virulence test and an immunity test. In the virulence test, a hatchery population of *M. rosenbergii* was infected with *V. parahaemolyticus* and the lethal dose ( $LD_{50}$ ) for the prawn was determined. In the immunity test, a wild population of *M. rosenbergii* was subjected to an immunity test based on the  $LD_{50}$  obtained in the first test. The immunity test was performed to investigate the responses of the *M. rosenbergii* defence system to the bacterial infection, measured at different time points. Comparison between the infected and uninfected prawns in terms of their responses to the infection was investigated using the main parameters of the immune system of *M. rosenbergii*, namely total haemocyte

counts (THC), phenoloxidase (PO) activity and superoxide dismutase (SOD) tests. In addition, the bacteria spread was also determined to show the process of eliminating the injected bacteria in the test prawns, compared to the control groups. Meanwhile, the THCs reflect the responses of haemocytes to the bacteria infection, whereby the number of circulating haemocytes is expected to fall rapidly because they migrate from the haemolymph to the infection sites. Finally, the steps that should be practised in prawn culture for the prevention and treatment of disease outbreaks, particularly from bacteria infection, are discussed.

## 6.2 Material and methods

Prawns weighing between 25-32 g fished from the Timun river (Negeri Sembilan) were purchased from a *M. rosenbergii* hatchery and reared in fibreglass tanks (60 cm x 30 cm x 35 cm) held at 25°C with aeration. The water used to rear the prawns was tap water that was treated using Nutrafin Aquaplus, Tap Water Conditioner (Swell UK Ltd.) to neutralise chlorine, chloramines and dissolved metals. Prawns were fed twice a day with artificial feed for at least three days. The prawns were kept at a density of 10 prawns/tank prior to the experiment.

### 6.2.1 Isolation of bacteria strain

A strain of the *V. parahaemolyticus* bacterium was isolated from the hatchery water used to rear *M. rosenbergii* larvae. Isolation of the bacteria was carried out by Microbiology Division, Institute of Biological Sciences, Faculty of Science, University of Malaya. Sequencing analysis of the DNA sample of the bacteria strain was carried out and confirmed that it was *V. parahaemolyticus*. Isolated *V. parahaemolyticus* was then cultured on plates containing nutrient agar with different salinities (sodium chloride was used) at 0%, 1%, 2% and 3%. All cultured bacteria were incubated overnight at 37°C. Bacteria cultured with 1% and 2% sodium chloride gave optimum bacterial cell growth. No bacterial cell growth was observed in 0% sodium chloride, while 3% sodium chloride produced very small cell sizes. Bacterial cells were then grown on nutrient agar slants prepared using 1% sodium chloride as a stock.

### **6.2.2 Preparation of bacteria suspension**

A total of 5 bacteria suspensions with different concentrations were prepared prior to the virulence test. In order to estimate the concentration of bacteria cells required to give the concentration of  $10^9 \text{ ml}^{-1}$  a graph of bacteria cell versus OD reading at 600 nm was plotted. Based on the estimated number of the OD reading, obtained from the graph, bacteria cells at a concentration of  $10^9 \text{ ml}^{-1}$  was prepared and confirmed by haemocytometer counts under a light microscope (Leica Microsystems (SEA) Pte. Ltd.). A number of 10x serial dilutions were then performed using bacteria cell suspension at concentration of  $10^8 \text{ ml}^{-1}$  to prepare the final concentrations of  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$  and  $10^2 \text{ ml}^{-1}$ .

### **6.2.3 Virulence test**

*M. rosenbergii* samples were divided into 6 test groups (A-F) and two control groups (one group injected with sterile saline solution and a second group of uninjected prawns). Prawns were placed in separate tanks at a density of 5 prawns/tank. The prawns in Group A were injected with 10  $\mu\text{l}$  of bacteria suspension at the dose of  $10^2$  bacteria/prawn, Group B with  $10^3$  and Group C with  $10^4$ , Group D with  $10^5$ , Group E with  $10^6$  and Group F with  $10^7$ . The injected prawns were then placed into separate new tanks held at 25°C with aeration. One of the controls was injected with 10  $\mu\text{l}$  of sterile saline water while the other control group did not receive any treatment. The number of dead prawns was monitored for 7 days.

The mortality percentage of the prawns from each group was calculated using the following formula:

$$\text{Percentage of mortality} = (B - A) / (C - A) * 100$$

- (i) A: number of prawn dead within 12 hours
- (ii) B: number of dead prawn after 12 hours
- (iii) C: total number of prawns

About 100  $\mu\text{l}$  of haemolymph from each of the prawns that still survived after day 7 was drawn using a 1 ml syringe containing shrimp anti-coagulant (1:1). The

mixture was then spread onto a CHROMagar™ Vibrio (Focus Biotech (M) Sdn. Bhd.) agar plate, which incubated overnight at 37°C. The colonies grown on the plate were counted based on the differences in the colour of the bacteria colonies. A mauve colour of the colony indicates *V. parahaemolyticus*, while blue to turquoise blue colonies are either *V. cholera* or *V. vulnificus* and colourless colonies are specific for *V. alginolyticus*. Other bacteria will not grow on this agar even though they may be present in the prawn. Hence contamination with other bacteria cannot be detected.

#### **6.2.4 Immunity test**

The experimental prawns were divided into 3 groups (A, B and C). Each group (A, B and C) contained 40 prawns, 8 of which were exposed for one of five different exposure times (3h, 12h, 24h, 48h and 72h). The ventral muscle of each of the experimental prawns in group A was injected with 30 µl of bacteria suspension at  $10^3$  dose of bacteria cell/prawn. Each of the injected prawns was placed in a new tank held at 25°C with aeration according to their group (Figure 5-1). Step 1 was repeated for other time points (12, 24, 48 and 72 hours). As for the control groups, group B was injected with 30 µl of sterile saline solution while group C did not receive any treatment. Their density in the tank was 8 prawns/tank.. A total of [8(3x5)] prawns were used in this experiment. Mortality percentage was calculated using the formula described in Chapter 5.2.3. The number of the dead prawns was monitored for 7 days.

#### **Haemolymph preparation**

About 250 µl of haemolymph was drawn from the ventral sinus of the first abdominal segment of each prawn using a 1 ml sterile syringe, and placed in a collection tube containing 2250 µl anti-coagulant solution (Figure 5-2). 100 µl of the mixture was dropped onto the CHROMagar™ Vibrio agar plate to confirm the existence of the bacteria in the haemolymph. The remaining mixture was then divided for the subsequent tests: 1000 µl (for PO activity test), 100 µl (for THC count) and 1000 µl (for SOD activity test).

## Total haemocyte counts

About 100 µl of haemolymph and anti-coagulant mixture was introduced into the haemocytometer counting chamber. The total haemocyte count was carried out under light microscope (Leica Microsystems (SEA) Pte. Ltd.) using standard procedures. The number of haemocytes was recorded for each prawn. The cell counting procedure could not be carried out on all haemolymph samples at time points 1 and 2. This is because the haemolymph was clotted just after the formalin-formaldehyde (37%) was added into the haemolymph-anti-coagulant mixture. To overcome this, cell counting on the remaining haemolymph samples (time point 3, 4, and 5) were carried out on fresh haemolymph containing anti-coagulant without any preservation steps.

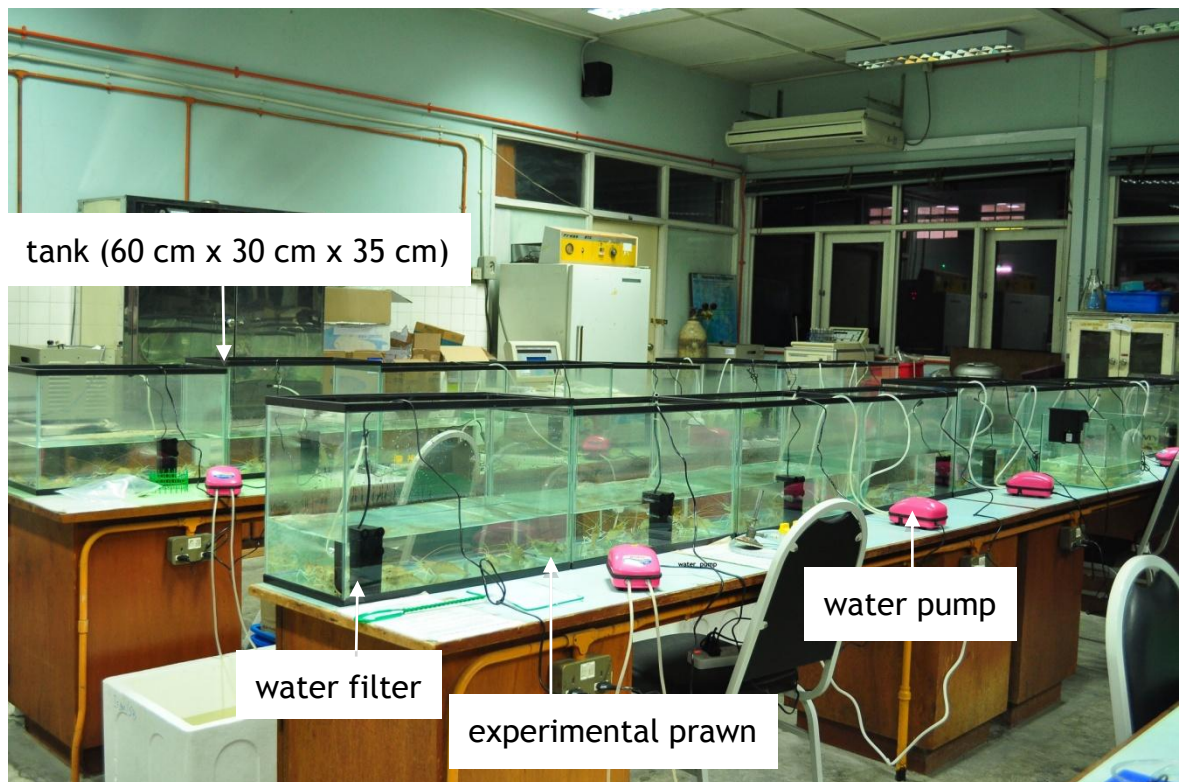


Figure 6-1: The aquarium setup for the immunity test.



Figure 6-2: Drawing haemolymph from the ventral sinus of the first abdominal segment for the PO and SOD tests, and for THC and bacteria spread.

### **Haemocyte Lysate Supernatant (HLS) preparation for PO activity**

The 1.5 ml eppendorf tube containing 1000  $\mu$ l of haemocyte-anticoagulant mixture was centrifuged at 800 g for 20 min at 4°C. The supernatant was discarded and the pellet was rinsed before being re-suspended in 1 ml cacodylate-citrate buffer. The 1.5 ml centrifuge tube was then centrifuged again and supernatant was discarded. The pellet was re-suspended in 200  $\mu$ l of cacodylate buffer. The cell suspension was homogenised using a Microson™ XL-2005 ultrasonic cell disruptor and then centrifuged at 40,000  $\times$  g for 30 minutes at 4°C. The resultant HLS was used for the PO activity test. Finally the HLS was divided into two tubes containing 100  $\mu$ l each (100  $\mu$ l for PO activity and another 100  $\mu$ l for the background of PO activity). The HLS was kept at -20°C until used.

### **PO activity**

About 50  $\mu$ l of HLS was incubated for 30 min at 25°C with 25  $\mu$ l of trypsin as an elicitor. 25  $\mu$ l of L-DOPA was added later and the optical density was measured after 5 s. Meanwhile, 50  $\mu$ l of the HLS sample mixed with 25  $\mu$ l of cacodylate

buffer and 25  $\mu$ l of L-DOPA was run for each of the samples to obtain the background of PO activity. Each of the samples was also run in parallel with a blank sample that contained 75  $\mu$ l of cacodylate buffer and 25  $\mu$ l L-DOPA. PO activity was measured as optical density at 490 nm on a multi-scan spectrophotometer (Thermo scientific (UK) Inc.). Beside, protein concentration of each of the samples was determined using a Bicinchoninic Acid Protein Assay kit (BCA1) (Sigma-Aldrich Co. LLC.). The method for protein determination followed the standard protocol for the BCA1 kit.

### **SOD activity**

A Ransod Kit (Randox Laboratories Ltd. UK) was used to determine SOD activity of the experimental prawns. About 1000  $\mu$ l of the haemolymph-anti-coagulant mixture was centrifuged at 10, 500 x g for 20 min at 4°C. Plasma was removed and the pellet was re-suspended with 1 ml of 0.85% sodium chloride (NaCl) before re-centrifugation. After that, the supernatant was discarded and re-suspended with 100  $\mu$ l of dH<sub>2</sub>O at 4°C for 15 min. A 30  $\mu$ l aliquot was placed in a well of a 96 well flat-bottom microplate (Greiner Bio-one Ltd. UK) containing 200  $\mu$ l of reaction mixture (available in the kit). 50  $\mu$ l of xanthine oxidase (available from the kit) was added. The microplate was placed in multi-scan spectrophotometer (Thermo fisher scientific (UK) Inc.) and the optical density was measured at 505 nm at 37°C. A reference standard was used, which was supplied with the kit. The Ransod standard was prepared according to the manufacturer's guideline. A standard curve was plotted using the percentage of inhibition against the log<sub>10</sub> of standard concentration. Beside, a diluted Ransod control was also run at least once a day and the activity was confirmed to be 199 +/- 52 U/ml.

### **6.2.5 Statistic analysis**

Graphs were plotted for each of the experiments (THC, SOD activity, PO activity and PO activity corrected with the total amount of protein in 1 ml HLS) to show the effect of each experiment with different groups (control, injected with saline and test) and time point (1-5). A General linear model (GLM) from analysis of variance (ANOVA) was applied within Minitab® 16.2.2 (Minitab Inc.), using a pairwise comparison Tukey test to compare the significant differences between



each group, time point and the interaction between group and time point. Furthermore the effect of the interaction, group or time point that showed a significant effect was then analysed by grouping using a pairwise comparison Tukey test to calculate the significant level. For statistically significant differences, it was required that  $p < 0.05$ .

## 6.3 Results

### 6.3.1 Virulence test

The virulence test was repeated twice but each time failed to determine the LD<sub>50</sub> for the experimental prawns, even though multiple injections with increasing doses were applied. In the first virulence test, the doses were in the ranges of  $10^2$  to  $10^7$  per prawn (6 test groups and two controls). No mortality was observed within the first 12 hours after injection. However, two prawns died on the second day. Physical examination showed that the exoskeleton of one of the dead prawns was soft and it had lost its appendages and tail. The other prawn that died lost both of the first and second maxillae (used to hold and position food). The experimental prawns showed low feeding rates from the first day until the fourth day, particularly the prawns injected with doses of  $10^6$  and  $10^7$  bacteria. The rearing water in all tanks was changed on the fourth day by replacing 10% of the rearing water with tap water that has been treated with water conditioner. On the 7<sup>th</sup> day, all the survived prawns looked healthy and were feeding readily.

In the second virulence test, the same prawns from the first test were used and the concentration of bacteria/prawn was increased ( $10^6$ ,  $10^7$  and  $10^{12}$  per prawn). Two prawns died within 12 hours after injection, one from the control group injected with saline and one from the test group injected with a dose  $10^6$ . After 12 hours and within 7 days of the experiment, only 1 further prawn died which was on the third day. This prawn was newly moulted, and likely succumbed to injury from dominant prawns (with blue or orange claws), rather than from the infection. On the first and second days of injection, the experimental prawns showed different eating behaviour; the uninjected and injected with saline water groups were eating very well, whereas the test prawns had a low eating rate.

Bacteria spread using CHROMagar™ was carried out on 5 test prawns (injected with bacteria) from the first virulence test after 14 day of injection. It showed two prawns with no bacteria cells and one prawn with only 5 bacteria cells, while the other two had many bacteria cells grown on the CHROMagar™ (750 and 483 cell each). The colonies were identified as *V. parahaemolyticus* (mauve colony) with also some from *V. alginolyticus* species (colourless colony).

Nonetheless the immunity test was continued without the information of the LD<sub>50</sub> of the prawns, but using a higher dose than in the virulence test and the volume of bacteria suspension was also increased (30 µl) from the volume used in the virulence test.

### **6.3.2 Immunity test**

All the prawns in the control group that received no treatment survived until the end of the experiment, an 8% mortality was observed in the control group injected with saline water while 13% mortality was observed in the test group. All the prawns were feeding very well but the prawns in the test group were inactive and sluggish compared to the prawns in the control groups.

No significant effect was found between the interaction of group and time point on the total haemocyte count ( $F_{8,66}=0.92$   $P=0.51$ ) (Table 6-1). However the effect of group was significant when run separately ( $F_{4,66}=3.15$ ,  $P=0.022$ ) (Table 6-2). The post hoc Tukey test comparing the means of total haemocyte count in animal irrespective of time point showed a significantly higher number of haemocytes in the control groups, with the average number of haemocytes in the untreated control group (mean±standard error) being  $0.093\pm0.011$  compared to the number in the test group,  $0.057\pm0.009$  (Figure 6-3).

**Table 6-1: Effect of different treatments on THC, PO, PO corrected with the amount of protein and SOD activity. F is F ratio=mean square (MS) factor/MS error, DF is degree of freedom and P is the probability (P<0.05).**

	THC			PO			PO II			SOD		
	F	DF	P	F	DF	P	F	DF	P	F	DF	P
Group	3.84	2,27	0.04	0.56	2,66	0.58	0.73	2,35	0.50	0.07	2,50	0.94
Time point	1.46	2,27	0.26	3.15	4,66	0.02	7.65	4,35	0.00	2.02	4,50	0.11
Group vs Time point	0.96	4,27	0.45	0.92	8,66	0.51	1.21	8,35	0.34	2.19	8,50	0.05

**Table 6-2: Means of THC in each group, PO activity and PO activity corrected with the amount of protein in 1 ml HLS in each time point. Data in the same column with same letter are not significantly different (P>0.05). Values are mean±SE.**

	THC		PO		POII	
control	0.0930±0.0114 <sup>A</sup>		0.0698±0.0028 <sup>AB</sup>		0.2041±0.073 <sup>A</sup>	
saline	0.0695±0.0068 <sup>AB</sup>		0.0736±0.0032 <sup>AB</sup>		0.2025±0.024 <sup>AB</sup>	
test	0.0572±0.0088 <sup>B</sup>		0.0838±0.0044 <sup>AB</sup>		0.0587±0.016 <sup>ABC</sup>	
			0.0879±0.0114 <sup>A</sup>		0.2436±0.034 <sup>BC</sup>	
			0.0590±0.0028 <sup>B</sup>		0.0675±0.008 <sup>C</sup>	

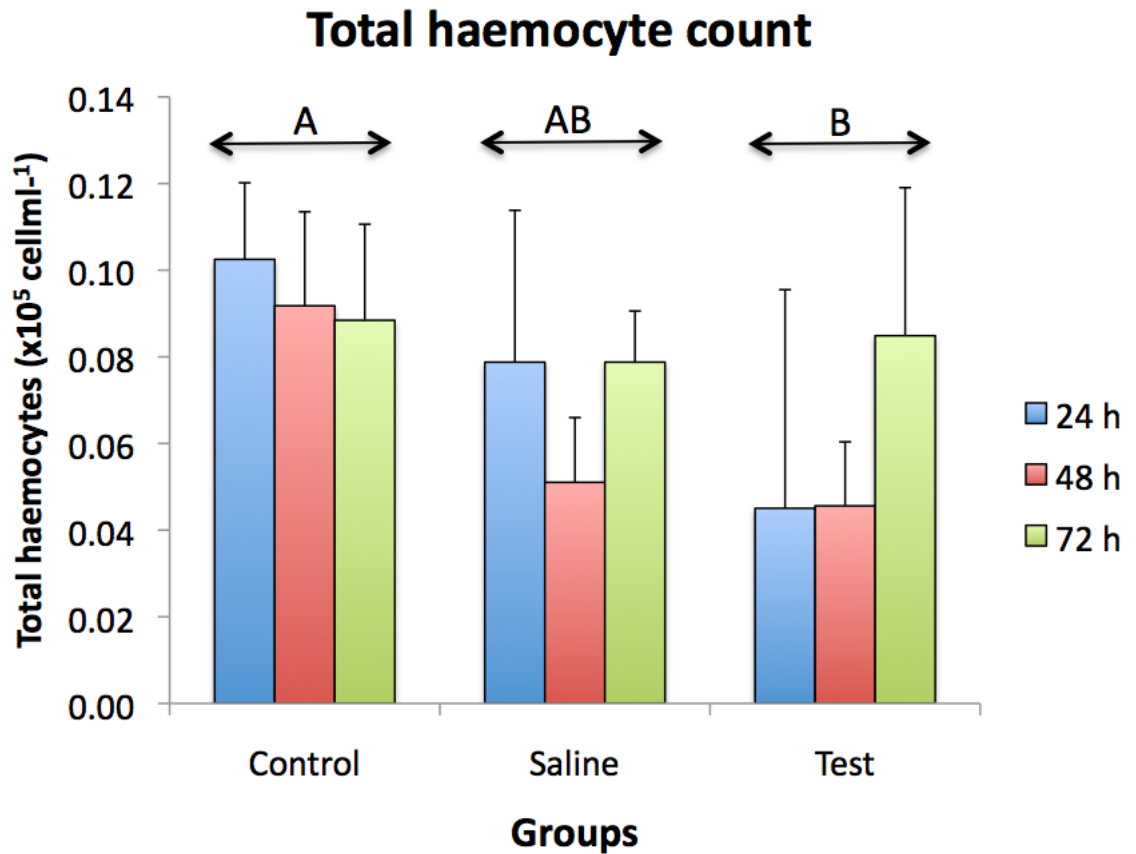


Figure 6-3: The mean (+StDev) value of total bacteria cell counts carried out on days 1 (24 h), 2 (48 h) and 3 (72 h). An increased number of haemocytes in the test group was detected after 48 h. The number of haemocytes in the control uninjected group is maintained from day 1 until day 3. Significant different detected between the groups but not between the timepoint ( $p < 0.05$ ). Refer to Table 6-2 for the value of the significance levels indicated by the letters.

Meanwhile, the bacteria spread carried out on the prawns from T5 (control: 4, saline: 2 and test: 2) showed the presence of *V. alginolyticus* (indicated by colourless colonies on the CHROMagar™) together with *V. parahaemolyticus* (mauve colonies) even in the control uninjected group (Figure 6-4).

There was no significant effect between the interaction of group and time point on the SOD activity ( $F_{8,50}=2.19$   $P=0.05$ ) (Table 6-1). The effect of both group and time point were not significant even when run separately. The SOD test showed increased activities in T4 (48 h) with average SOD activity of  $0.3522 \pm 0.0425$ , but then decreased in T5 (72 h) with average SOD activity of  $0.2255 \pm 0.0389$ . However this differentiation was not significant (Figure 6-5).

Another test, which measured the phenoloxidase (PO) activity showed a similar pattern to the SOD activity (Figure 6-6). No significant effect was found between the interaction of group and time point on the PO activities ( $F_{8,66}=0.92$   $P=0.51$ ) or after the PO activity was corrected for the total amount of protein present in 1 ml of HLS measured using the BCA1 method ( $F_{8,35}=1.21$ ,  $P=0.342$ ) (Table 6-1). However the effect of time point before and after correction for the amount of protein in 1 ml HLS was significant when run separately ( $F_{4,66}=3.15$ ,  $P=0.022$  and  $F_{4,35}=7.65$ ,  $P=0.01$ , respectively) (Table 6-2). The post hoc Tukey test comparing the mean of the PO activity showed increasing PO activity in the animal starting in T4 (48 h) with the average of PO activity in T4 being  $0.0879\pm 0.0114$  and decreasing in T5 (72 h) ( $0.0590\pm 0.0028$ ). The pattern of PO activity when corrected with the amount of protein in animal irrespective of group was significant at all time point, where time point 4 (48 h) was found to be highest, with average activity of  $0.244\pm 0.034$ , and T5 (72 h) lowest, with average activity of  $0.068\pm 0.008$  (Figure 6-7).

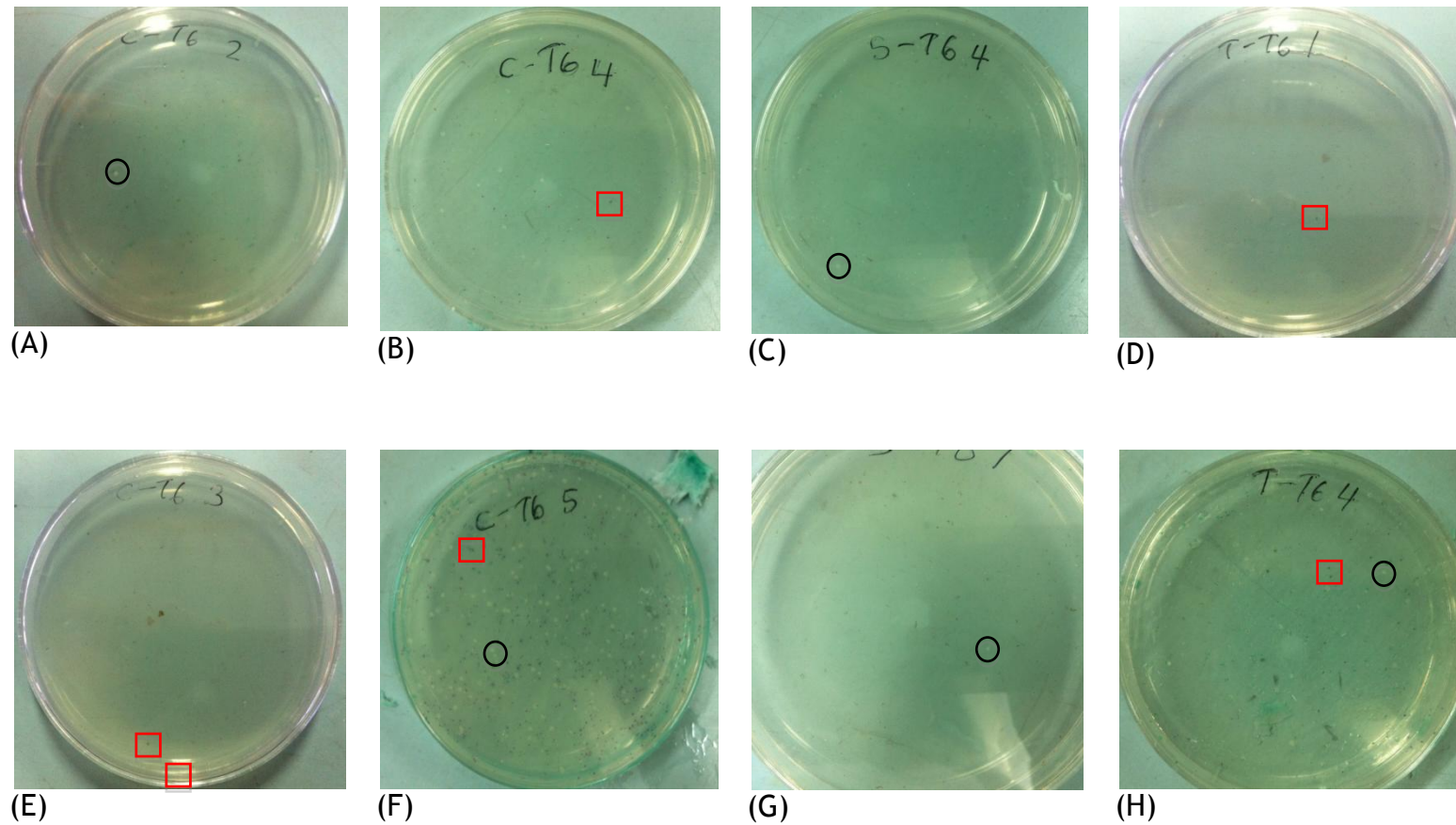


Figure 6-4: Bacteria spread from time point 5 (T5). (A)-(D) are bacteria spreads for control uninjected (individual 1,2,3,4), (E)-(F) are for control injected with saline water (individual 1 and 2) and (G) and (H) are test prawns (individuals 1 and 4). Circles indicate *V. alginolyticus* and square indicates *V. parahaemolyticus*.

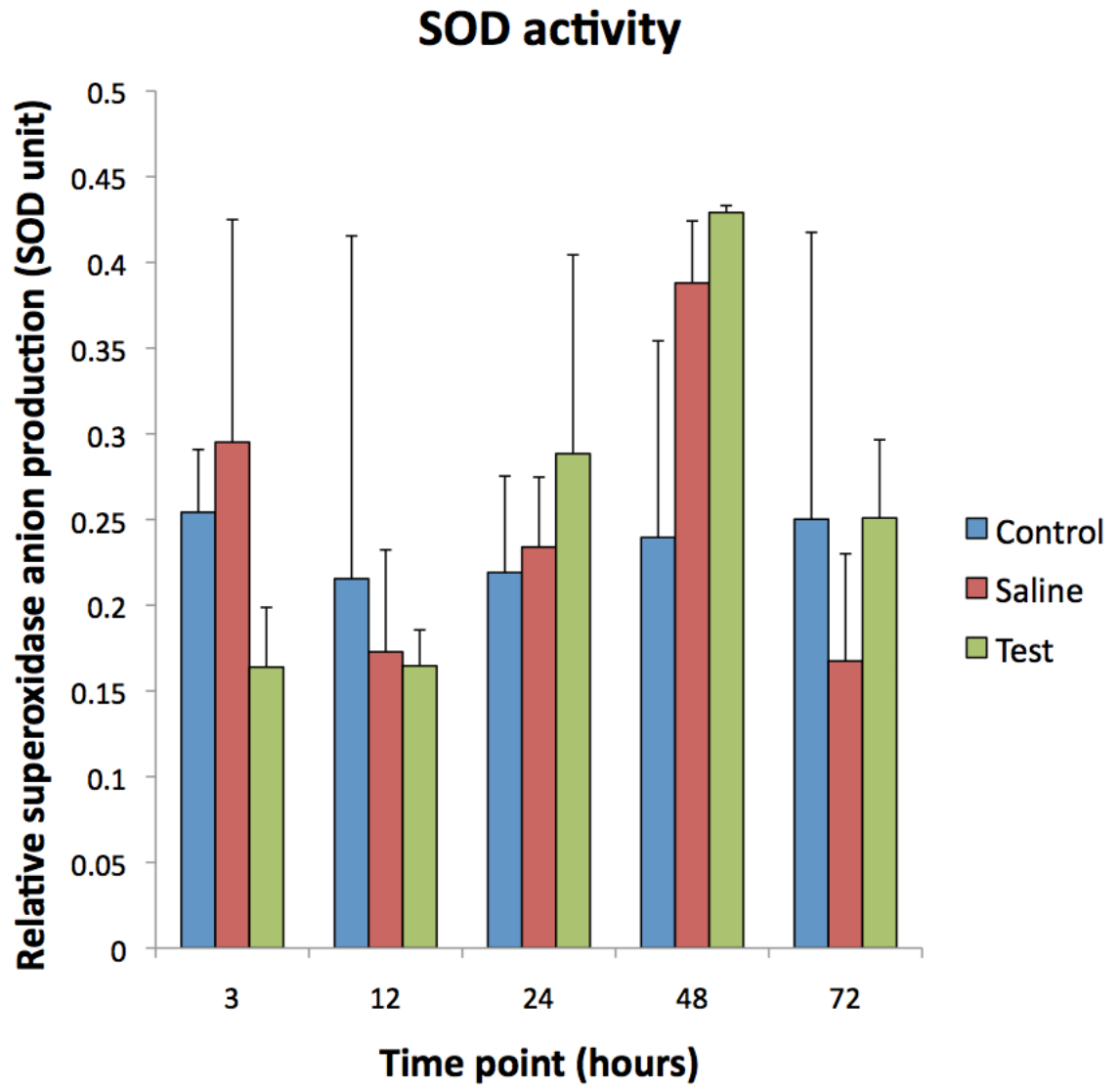
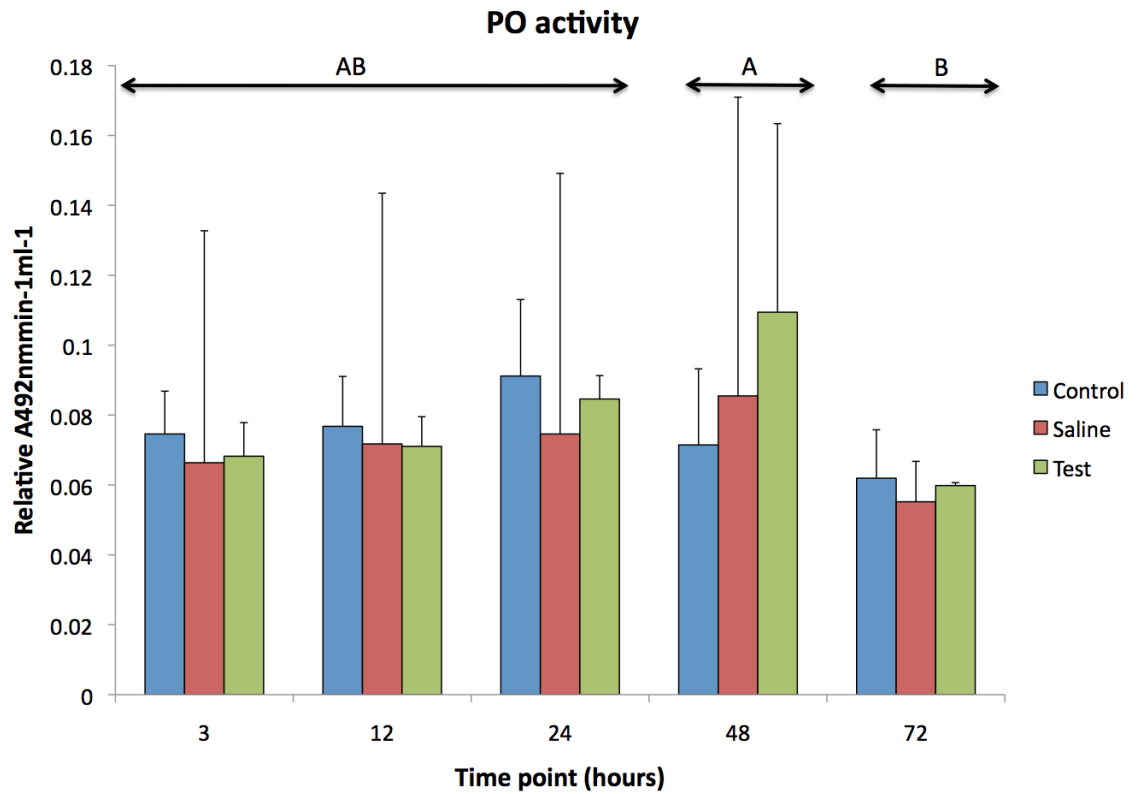


Figure 6-5: Graph shows the mean values (+StDev) of SOD activities of control groups and test group. No significant effect of the groups, time point or interaction of group and time point ( $p < 0.05$ ).



**Figure 6-6:** Graph shows the mean value (+StDev) of PO activity of three different groups of experimental prawn (control that did not received any treatment, control injected with saline water and test group). Significant differences detected between the time points but not between the groups ( $p < 0.05$ ). Refer to Table 6-2 for the value of the significance level indicated by the letters.



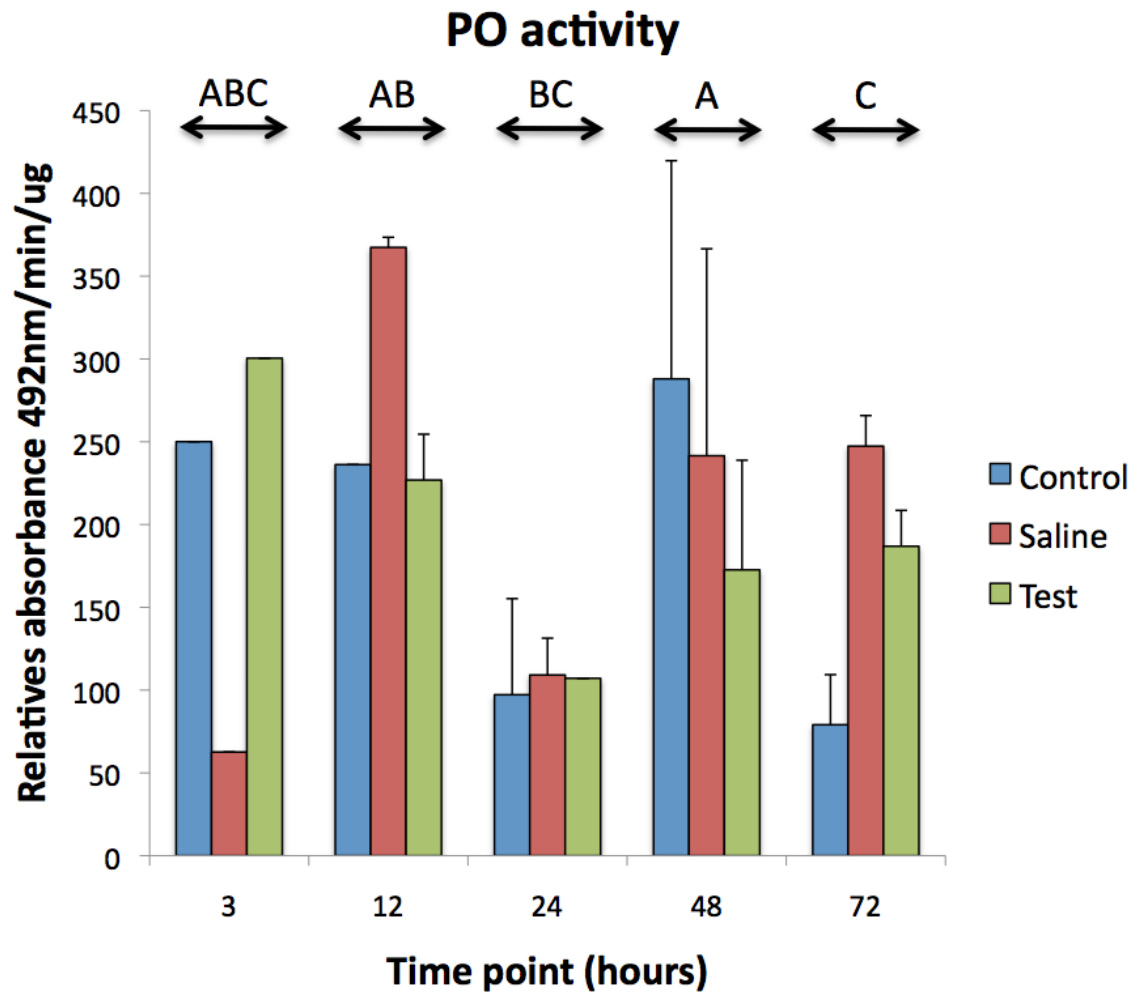


Figure 6-7: PO activities of each group (control that did not received any treatment, control injected with saline water and test group) show increased PO activities at T4 (48 h) (after correction for the actual amount of protein in 1 ml). Significant differences detected between the time points but not between the groups ( $p < 0.05$ ). Refer to Table 6-2 for the value of significance level indicated by the letters.

## 6.4 Discussion

The present study was carried out in Malaysia between November 2010 and January 2011 except for the downstream experiments on the haemolymph of the experimental prawns that were carried out at the University of Glasgow. Hence, even though the study was designed with great care and took into account all the possible factors that could affect the experiment, nonetheless, time limitation and technical difficulties encountered during the experiments were inevitable. Moreover, unforeseen difficulties also disrupted the experiments.

All results from the present study failed to determine the susceptibility of *M. rosenbergii* to *V. parahaemolyticus*, even when multiple injections with increasing doses were applied. The LD<sub>50</sub> of the prawns cannot be determined because no prawn died due to the infection, but could have been stressed from the injection, cannibalism or lost of their 1<sup>st</sup> and 2<sup>nd</sup> maxillae use for feeding. Meanwhile the different eating behaviour observed in the experiments is not due solely to the reaction to the infection but might also relate to the moulting process. Nevertheless the total haemocytes count showing a decreased number of haemocytes present in the blood of the test group reflects the process of elimination of the bacteria cells. This is a typical indication of a response by the affected prawns to remove the injected bacteria, carried out by the host defence system (haemocytes) through the phagocytosis and the PO mechanism (Vazquez et al., 2009 and Saurabh and Sahoo, 2008). Previous studies have demonstrated that infected prawns have the ability to eliminate the injected bacteria or even viruses from haemolymph (Cheng et al., 2003, Sarathi et al., 2008). However in the present study, the results from experiments carried out to measure the changes in the activity of PO and SOD were not reliable. Hence, no conclusive data could be produced from these experiments, and limitation on the supply of samples prevented repetition of this experiment. The increased activity of PO and SOD which was observed in the test group T4 (48 h) but also in the control groups could have been due to stress, or perhaps to changes in the osmolarity of the blood (in the case of control injected with saline and test groups) rather than a direct response of the host to the infection.

Physical examination of the prawns carried out before the injection showed signs of black spot disease in some of the samples (Figure 6-8). This was confirmed by the bacteria spread carried out on the prawns from all groups (control, injected with saline and test) in T5 (72 hours). Bacterial cells grew not only from the test group but also in both of the control groups (Figure 6-4). The use of only healthy animal prior for the experiments is very crucial to confirm the cause of the disease or mortality. However, bacteria infections can be secondary rather than primary causes of disease and continuous exposure of bacteria infection may lead to the development of primary vibriosis. Beside, identification of the disease symptoms cannot rely solely on the physical examination but it would be better to carry out molecular tests (e.g. PCR) to rapidly screen the prawns in

order to ensure that only disease-free individuals are used. Molecular tests that use specific disease markers have proved to be simple, reliable, rapid and accurate (Roque et al., 2009, Suthienkul et al., 1996, Fabbro et al., 2010). Recently, Khuntia et al., (2008) reported the susceptibility of a *M. rosenbergii* population from India to the *V. parahaemolyticus* infection. The gross signs of affected prawns were black colouration of the carapace, red discolouration of the exoskeleton and loss of appendages within 6 days. In comparison with the present study, Khuntia et al. (2008) used a high dose of  $2 \times 10^9$  CFUs ml<sup>-1</sup> and the prawns were monitored for 6 days. 80% mortality was observed in the group injected with  $2 \times 10^9$  CFUs ml<sup>-1</sup> but no mortality reported in the concentration of  $2 \times 10^7$  and  $2 \times 10^8$  CFUs ml<sup>-1</sup>. Beside, the size of the prawns used in the present study were larger (25-32 g) compared to the samples used in the Khuntia et al., (2008) study (10-15 g). The size of the prawn is important, since differences in prawn size require different numbers of bacterial cells to be injected to be able to cause disease. Hence, the size of the prawn should be minimised. Beside, the Khuntia et al. (2008) study used captive-reared animals while the present study used wild population. It is known that wild populations have a better immune system compared to captive-reared animal due to the potential loss of genetic differentiation that might affect the fitness of the animal.

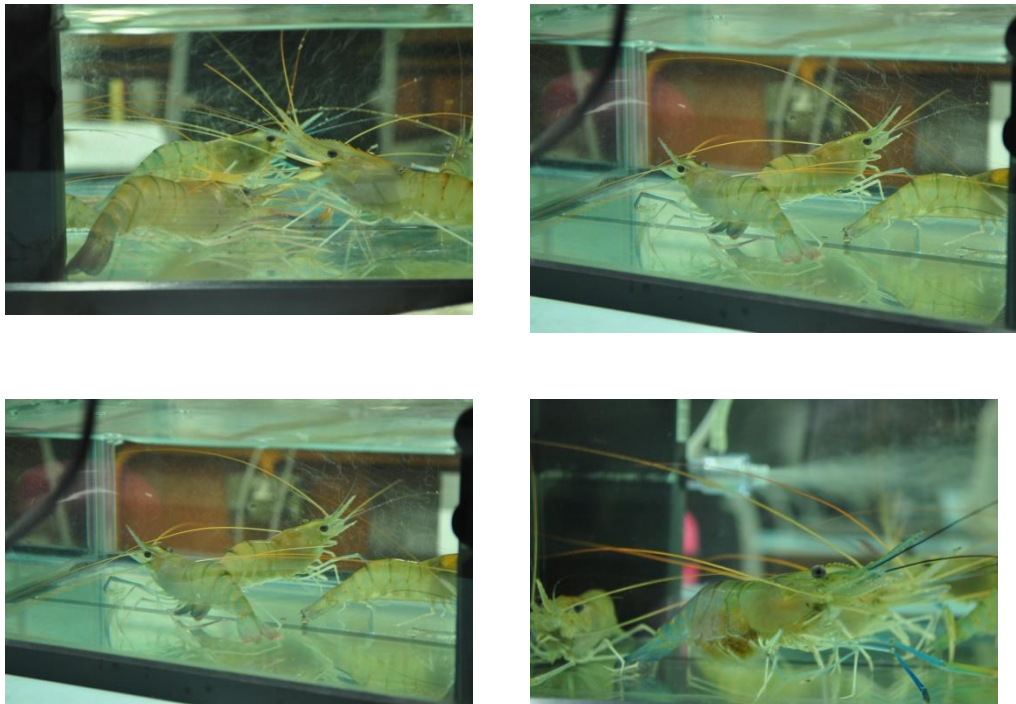


Figure 6-8: The test prawns at time point 5 (72 h) before being injected with 30 µl of bacteria suspension at  $10^3$  bacteria/prawn.

Even though both virulence and immunity tests failed, the prawns in the immunity test did show signs of black colouration on the carapace, appendages and tail fan (Figure 6-9). It is suggested that the duration of 3 day might not sufficient to allow the development of the disease into its later stages. Thus it is suggested to extend the experiment up to 30 days to provide ample time to monitor the progression of the disease if this happens, or to follow the process of elimination of the bacteria cell from the haemolymph (that can be carried out using the bacteria spread method) (Sung et al., 2000a, Sritunyalucksana et al., 2005). Finally, information from a histopathology study of the lesions from infected prawns (which was not carried out in the challenge study due to technical difficulties) would provide information on the pathological state of the tissues, possibly clarifying the cause of any mortalities as due to bacterial infection.

While failure in the virulence test might be attributed to an insufficient dose of the bacteria injected, other factors may have also have contributed to the lack of success in these experiments. These include the bacteria strain not being in a virulent state, or the two assays (SOD and PO) not being fully optimised. The changes of the activities in SOD and PO of the test group and control group injected with saline water did not differ significantly from the control prawns. In the present study there were some technical problems and difficulties in obtaining a virulent *V. parahaemolyticus* strain. The identity of the bacteria used as *V. parahaemolyticus* has been confirmed using a molecular test (carried out by microbiology group in University of Malaya). The virulence stage of the bacteria was, however, unknown and it did not prove possible to carry out a virulence test due to a technical problem. It is crucial to determine the virulence stage of the bacterial strain prior to a challenge experiment since only virulent strains can induce disease in the infected prawns. Certainly, the amount of bacteria injected to infect the prawn will not reflect the potential for disease development if it is a non-virulent strain.

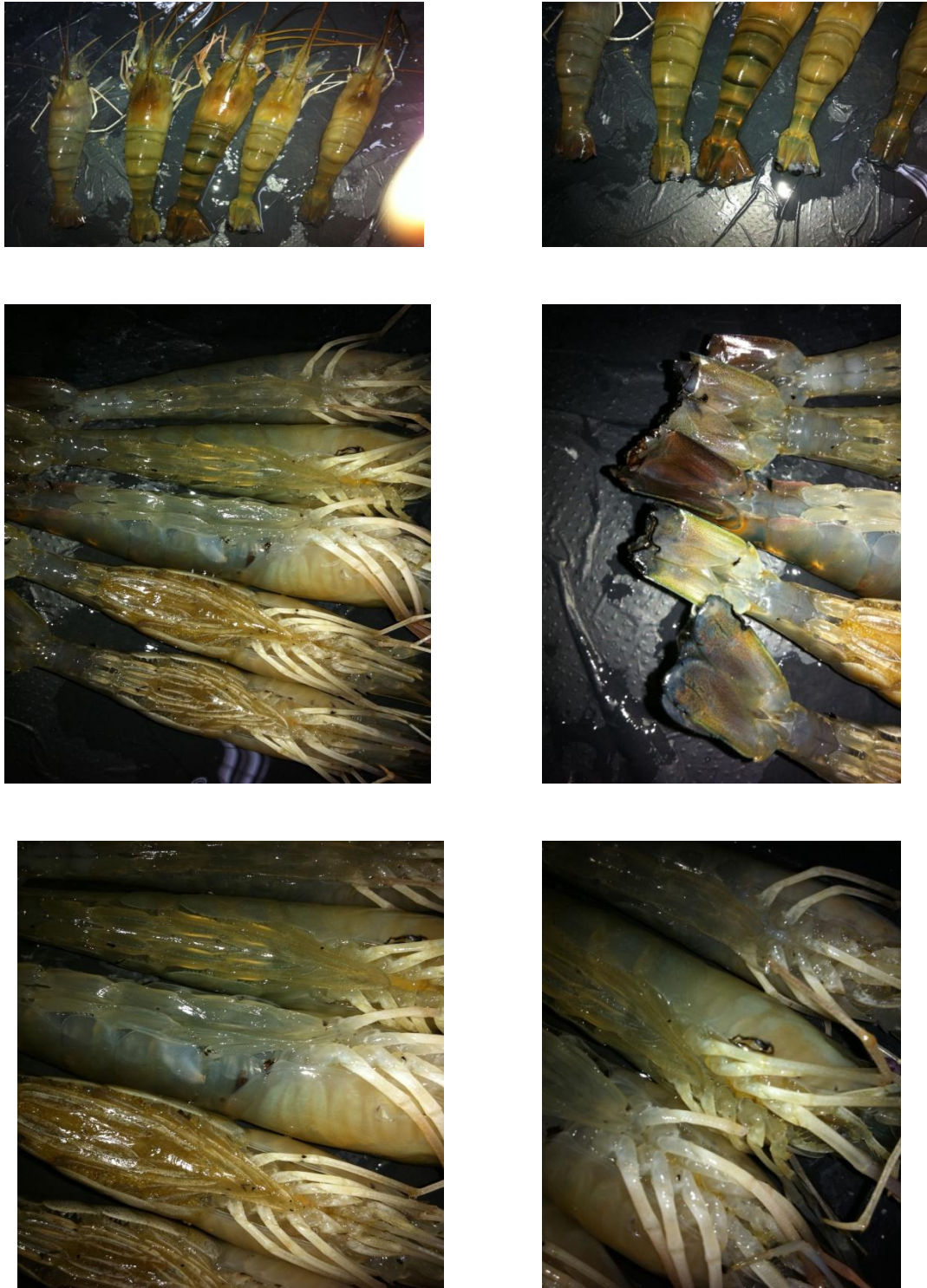


Figure 6-9: The test prawns at time point 5 (72 h) after injection. Note that the appearance of black spot is increased.

Knowledge of the susceptibility of *M. rosenbergii* to bacterial infection is important in order to establish the prevention and treatment steps required to sustain the prawn industry. Bacteria contamination, particularly from *V. parahaemolyticus*, an important human pathogen, is known to limit the sales of



aquaculture product. Thus, it is crucial to have knowledge about this bacteria for prevention, treatment and improvement of both the immune system of the prawn as well as the management of the pond. The preliminary works on *M. rosenbergii* challenge with *V. parahaemolyticus* carried out in this study were unsuccessful. Repetitions of the experiment with consideration of factors discussed above are required. Nonetheless the basic information of *M. rosenbergii* immune system produced in the present study is useful for future research on the susceptibility of different populations of *M. rosenbergii* in Malaysia. The experiment should target the distinct population from Sabah and compare this with other populations including those from Northern Sarawak and the recently restocked populations (Perak, Kedah and Southern Sarawak). Populations that show higher resistance to the bacteria infection will then be targeted for future research to produce broodstocks that are more resistance to the bacteria infection particularly black spot disease. Whether Sabah population will be of beneficial to the *M. rosenbergii* industry in Malaysia is still uncertain and beg explanations from future researches that should focus on the study of the genomic of this prawn. The outcomes from this preliminary study are important to provide basic knowledge on the studied animal which showed that the immune system of the host might be highly resistant to the disease infection as reported in other studies (Sarathi et al., 2008, Rajendran et al., 1999, Sahul Hameed et al., 2000 and Longyant et al., 2005) or might also due to the ability of *V. parahaemolyticus* to develop disease in prawns, particularly in *M. rosenbergii*. Even though the study by Khuntia et al. (2008) showed high susceptibility of Indian population of *M. rosenbergii* to *V. parahaemolyticus* infection, however, the differentiation in the susceptibility of different populations to the infection has to be concern. Indian population of *M. rosenbergii* has been reported to possess many diseases (Khuntia et al., 2008 and Saurabh and Sahoo, 2008) compared to Malaysian population. Many factors especially from environment may contribute to this differentiation such as the water quality, the density of the stocking animal and the management and sanitation of the hatcheries. Others such as inbreeding depression due to the pressure from high production of the prawn industry may reduce the ability of the animal to resist to the disease infection. India is the third major *M. rosenbergii* production beside China and Thailand. Compared to India, the *M. rosenbergii* production in Malaysia is considered as low and most of the

broodstock are applied directly from wild populations which could maintains the high genetic differentiation in the hatchery product.

# Chapter 7

## General discussion

### 7.1 The importance of molecular ecology in the study of commercial species

The advancement of molecular genetic approaches has had a profound impact on ecological studies and has overcome the limitations of traditional ecology approaches. It is known that genetic information is passed through generations and encoded at the DNA level. Molecular genetic tools have the ability to detect the genetic differences among and within populations using specific molecular markers. Development of many molecular markers such as microsatellite, mitochondrial and RFLPs marker were broadly utilised to understand species formation, and the diversification and ecological adaptation of a species to the environment.

Of many available markers, COI is one of the important mitochondrial markers that have been used in ecology studies. The ability of the COI region to differentiate species, even allied species, and to detect intra- and inter-specific variation has facilitated many studies that aim to understand the diversity of many species, particularly in mammals (Francis et al., 2010) and crustaceans (Costa et al., 2007, da Silva et al., 2011). The COI region has the characteristic of uniparental inheritance (in the majority of the animal phyla), high evolutionary rates, lack of introns, high copy number in every cell and almost absence of recombination (Radulovici et al., 2010). Many studies have utilised the COI region to understand phylogenetic relationships and enhance knowledge of phylogeographic patterns. More recently the use of a standardized size of 650 bp of the COI region has been used as a tool for DNA barcoding for animals (Hebert et al., 2003, Radulovici et al., 2010, Ros and Breeuwer, 2007). DNA barcoding is important to assist in the identification and assigning of unknown specimens to a species that has previously been described, identification of new species using a threshold of sequence divergence as well as aiding in



conservation plans, particularly for threatened species (da Silva et al., 2011). For regions of the world with incredible species diversity such as the Southeast Asia region (the IAA), which is part of the world's most important biodiversity hotspot based on both its flora and fauna, the advantages of COI barcoding could be used to enhance the knowledge of the high diversity and taxonomy of its many known species, as well as to uncover cryptic species. However the size of this task is indicated by the fact that decapod crustaceans, which comprise the majority of benthic species and include many that are commercially important, are known to comprise a total of 17635 species based on morphological characteristics, but only 5.4% of these are represented with DNA barcoding sequences (Francis et al., 2010).

Traditional ecology approaches based on direct observation of studied animal for a certain period of time provide less information than molecular ecology approaches. This is because the information is limited to the time frame of the study, unless it has been carried out for a long period and the data have been properly collected and archived. In contrast, molecular ecology provides information beyond the time frame of the study. Molecular ecology is very important to explain the demographic history of marine, estuarine and freshwater species, particularly commercial ones such as *N. norvegicus* and *M. rosenbergii*. Demographic history provides information of the population size and mortality rates. It is also useful to understand the mating behaviour of dispersing animal and detects recent immigration where the genetic differences will appear in the offspring. For instance, previous studies have postulated that *M. rosenbergii* were dispersed to the southward direction based on the evidence of the close phylogenetic relationship between *M. rosenbergii* with other genus of *Macrobrachium* (*M. malcomsanii* and *M. gangeticum*). Meanwhile, *M. rosenbergii* populations from Peninsular Malaysia share a common haplotype with Sarawak but not with Sabah, even though there is a close distance between Sarawak and Sabah. This indicates that *M. rosenbergii* populations found in Borneo are dispersed from Peninsular Malaysia, but that other vicariance events have probably shaped the genetics of the Sabah population.

Molecular approaches can also calculate the divergence time using a molecular clock, provided that it is associated with significant historical and environmental events. Generally, phylogeographic studies on marine species have revealed lack

of spatial genetic differentiation, as compared to that of catadromous or freshwater species (Ward et al., 1994, De Woody and Avise, 2000). This is due to the high potential for dispersal and lack of barriers in the marine environment that could restrict the gene flow between lineages. In contrast, dispersal of catadromous and freshwater species is limited by intervening terrestrial features and by the marine environment. Nevertheless, studies on some commercially important marine species such as the Icelandic cod (Pampoulie et al., 2008), swordfish *Xiphias gladius* (Kotoulas et al., 1995), and *Mytilus galloprovincialis* (Quesada et al., 1998) showed evidence of species divergence mostly due to the distance that limit the gene flow. They could also be attributed to the secondary contact between the diverted lineages, most probably after a period of separation (e.g. glaciations) (Perez-Losada et al., 2002). Both of the species studied in this thesis possessed high morphological differentiation even between adjacent populations. However, previous phylogeographic studies revealed that there was a different level of genetic differentiation in the same species. Previous studies on *N. norvegicus* carried out across a large geographical range from Icelandic and Scottish waters to the Mediterranean Sea reported either that the populations were not structured according to the genetic region investigated (COI region) or no population genetic structure (microsatellite and allozymic marker) was detected. In contrast to the results obtained from populations of *N. norvegicus*, studies on the genetic differentiation of *M. rosenbergii*, a commercially-important freshwater species in the aquaculture industry, not only in the countries of its natural distribution but also beyond, have revealed a high level of genetic differentiation. Perhaps a geological explanation such as plate tectonic evolution and vicariance events could possibly be invoked to understand the differences exhibited in the biology of these species (Lohman et al., 2011). In addition, the refugium hypothesis and climate changes, or the extension of the vicariance hypothesis, may also have played important roles in shaping the contemporary distribution of the species.

It is well documented that *N. norvegicus* is a sedimentary, burrowing species, that favours walking over swimming and rarely migrates over a distance greater than 100 m from their burrow systems (Chapman and Rice, 1971). In addition, female *N. norvegicus* carry the eggs under their tail and spend most of this brooding time in the burrow. Nonetheless the possibility of genetic mixing at the

planktonic larval stages, where there is the possibility of passive larval dispersal by local oceanic currents, cannot be ruled out. In a similar way, *M. rosenbergii*, a catadromous species, spend their early life stages in brackish water and there is thus also a possibility of their dispersal at these planktonic larval stages.

The contemporary genetic pattern of *N. norvegicus* could be explained by the recent expansion of *N. norvegicus* from the refuge area suggested to occur after the LGM. *N. norvegicus*, lives in a marine environment perceived to lack biogeography barriers to limit the dispersal of the animal. Meanwhile *M. rosenbergii* distributes across the IAA region which has restricted the gene flow between the forms at the biogeographic boundary delimited by Huxley's line. In Chapter 5, the role of Makassar Straits, located along Huxley's line to restrict the gene flow between the two forms was discussed. Moreover, the subsequently divergence of the populations within the form after the LGM was facilitated by the existence of the ancient biogeographic barrier such as the Isthmus of Kra-Seaway and the Lake of Carpentaria region. Lack of gene flow between lineages has contributed to the high genetic differentiation in *M. rosenbergii*. The dispersal of the species before the LGM could explain the homogeneous genetic differentiation between the Peninsular and East Malaysia. However, the biological explanation of the distinct population, Sabah is still unclear. Perhaps the vicariance event that formed Mount Kinabalu and the climate changes that caused repeated changes of the sea levels could explain the current genetics of the Sabah population. In addition, de Bruyn et al. (2004) stated that Sabah drainage was still isolated during the Pleistocene age.

Delimiting of *M. rosenbergii* subspecies is not straightforward. Based on the morphological observation of the variation in the rostrum length and dentition, Short et al. (2004) suggested that a step cline exists where the western form (longest rostrum length) is on one side and the eastern form from northwest Australia (shortest rostrum length) is on the other side (Appendix E). The Bali population, as quoted by Johnson (1973) to have an intermediate characterization between western and eastern forms, falls somewhere on the cline, suggesting that this is the area where the division of the two forms has taken place.

Traditional ecology cannot detect variation in the organism that does not influence the physical appearance. However molecular approaches are able to detect the differences at DNA level even though they may appear the same in physical appearance. For populations that possess close identity, such as the northern and southern population of the western form, it is proved that molecular ecology could differentiate these populations at DNA level.

## **7.2 Implications for the management of *N. norvegicus* and *M. rosenbergii***

Several important aspects of using molecular ecology techniques that are of benefit to the fisheries and aquaculture industry have been identified in this thesis. Knowledge about the high level of genetic differences (as explained earlier in this chapter) would be tremendously important in the management, conservation and recovery programme particularly for a highly exploited species.

Molecular ecology has a great impact in assisting the management of the fisheries of commercial species such as *N. norvegicus*. Even though report of *N. norvegicus* in 2011 showed that all *Nephrops* populations were fished at sustainable rates, however, identification of the pattern and extent of genetic diversity that are present in the wild populations is tremendously important to identify which population possess unique genetic composition. Such population has to be prioritized, as it could assist in the conservation efforts. For instant, ICES 2009 reported low migration activity between the west and east Irish Sea populations, resulting in two distinct populations. Thus, these populations are managed separately. The presence of a gyre in the western Irish Sea during spring and summer each year which coincides with the planktonic larval stage of *N. norvegicus* at this time. It is suggested that the gyre has retained the larvae within their population rather than them being swept away by current flow (Hill et al., 1997). A highly exploited population can be recovered by simply introduce animal from other population that have similar genetic composition.

Meanwhile, knowledge of the molecular ecology of *M. rosenbergii* also has important implications for the sustainable development of the aquaculture industry for this species, particularly in Malaysia where the giant freshwater prawn farming industry is unstable. The ability to produce good quality

brockstock is one of the initial steps required to overcome the problems encountered in the *M. rosenbergii* industry in Malaysia. Luckily, the high genetic variability that exists in the wild populations could facilitate attempts to produce high quality broodstock. However, regardless of whether the degree of the genetic variability obtained is significant or not, the information should be valuable in assisting the conservation, management and recovery of the wild population of the studied species, as well as the improvement and sustainability programmes for the aquaculture industry.

Firstly, genetic information would provide great benefits if incorporated into population genetic studies of wild population management. They could also be applied in the production of good quality broodstock, sex reversal studies for developing a better quality aquaculture product and assisting in the management of the broodstock.

Secondly, the conservation of wild populations is very important, as it is the only source of genetic variability available for selection. Low genetic variability soon eliminates the heterozygous alleles, resulting in the inbreeding depression phenomenon that would have adverse effects such as high mortality, vulnerability to disease infection, decreased individual fitness in the affected population, low reproduction rate and slower growth rate (which associates with smaller adult size). Moreover, populations that suffer from inbreeding depression will also find it difficult to evolve and adapt to new environmental conditions due to the loss of alleles that can fit with new conditions. On the other hand genetic selection programmes that manipulate genetic information by maintaining or increasing the level of genetic variability of the species could offer a better aquaculture product which would eventually be useful to further enhance and sustain the aquaculture industry of that species. Moreover, the information on the genetic variability could also be expanded to investigate the susceptibility of different populations to disease which is another important aspect in the aquaculture industry. In the scenario of the unstable industry of prawn farming in Malaysia, it has to be highlighted that the outcome from the present study, together with the advantage of the recent development of microsatellite markers when applied in wild populations, will assist the initiatives to produce a high quality broodstock to meet the increasing local market demand for grow-out ponds.

Thirdly, the genetic information will also be useful as a guideline for recovery programmes to avoid extinction of the population, attributable mainly to human activities such as overexploitation of the wild population, habitat loss due to modernisation as well as environmental pollution. In Malaysia a restocking programme was initiated about 20 years ago in peninsular Malaysia, particularly in Perak and Negeri Sembilan, the two major production areas of the *M. rosenbergii* industry where the main broodstock supply for the hatcheries is from the wild population. The restocking programme successfully recovered the treated populations and also maintained the genetic variability of the wild population, as described in the Chapter 5.

The success of the *M. rosenbergii* aquaculture industry, particularly in Malaysia, can only be guaranteed if there is serious actions, dedication and cooperation from all the stakeholders in the industry, including the government bodies, aquaculturists, researchers and funding agencies. *M. rosenbergii* farming in Malaysia has a great potential to develop because of the high demand from local markets, as well as a potential for exportation. As stressed at the 'Giant Malaysia Prawn' meeting held in 2008, the two main foci must be on the production of good quality broodstock and the initiation of genetic selection programmes. The objectives can only be achieved by manipulation of the high genetic differentiation known to exist in the wild population of *M. rosenbergii* in Malaysia. The sustainability of the *M. rosenbergii* industry relies on the availability of high quality post larvae for broodstock nurseries. The genetic selection programme would solve the problem of disease infection, slow growth rate and the production of a larger marketable size of prawn for a better market price. The genetic selection programme itself will open another window, with many research fields to be explored that could greatly benefit the Malaysian freshwater prawn industry, and hopefully make it more stable. A first step towards this is indicated by the fact that the 2008-2010 budget plan of the Malaysian Government clearly indicates strong support to improve the freshwater aquaculture industry by maximising the usage of inland water.

### **7.3 Future research plans for *M. rosenbergii***

The main focus in the *M. rosenbergii* farming industry in Malaysia is to find solutions to improve and sustain the industry. The present thesis has provided

important genetic knowledge that should initiate more efforts to explore the potential routes for the successful development of the industry. The benefit of manipulation of the genetic knowledge from the present study into studies that could have direct effect on the development of *M. rosenbergii* farming such as the establishment of the genetic selection programme and the production of good quality broodstock has to be initiated. Here I suggest the areas that are of most interest:

### **7.3.1 Confirmation of mitochondrial DNA information**

The reported genetic data at the mitochondrial level in this thesis would be more significant if it could be compared with genetic information from the nuclear DNA. Thus, screening of nuclear DNA of the populations used in the present study using microsatellite markers and comparison between these two important genetic markers should have a great impact in the development of *M. rosenbergii* farming industry. A research group based in University of Malaya which is currently collaborating in a *M. rosenbergii* study, is now at the final stage of developing the microsatellite markers to be available for the population genetic study using nuclear DNA, as well as to be applied in the quantitative trait loci (QTL) mapping in the genetic selection programme. Meanwhile support from the morphology data for each of the populations, particularly Sabah, should also make a significant contribution to our understanding of the complex genetic structure possessed by *M. rosenbergii* populations from Malaysia, as well as other *M. rosenbergii* populations, including *M. spinipes*.

### **7.3.2 Development of *M. rosenbergii* farming**

#### **To investigate the morphological variability of populations from different genetic backgrounds**

The study of the morphological data of the species will provide information on the populations that can be related to their different genetic backgrounds. The population found to possess superior traits such as larger adult size (that could demand high market price) or a high resistance to disease infection could be subjected to a disease challenge to confirm their susceptibility to particular diseases.

## **To determine the susceptibility of populations from different genetic backgrounds to disease infection**

Clades I, II and III reported in the present study showed different genetic backgrounds, and these could be tested for their susceptibility to the disease infection. The outcome could possibly reveal the different levels of susceptibility to the disease infection due to their different genetic backgrounds. The most highly resistant population to disease infection could then be subjected to a genetic selection programme.

## **Transcriptome of *M. rosenbergii***

Characterization of the transcriptome of *M. rosenbergii* is important in order to identify the potential genes that associate with desired traits for a higher quality of aquaculture product such as pathogen defence responses to the disease and sexual maturation traits. The populations identified in the disease challenge experiment will be targeted for pyrosequencing to sequence the ESTs coding sequence to produce the genome dataset of the species. This dataset will be useful to construct further researches to manipulate or select the genes responsible for prawn defence system.

## **The genetic selection programme**

Genetic selection is a method of crossing between individuals that have the traits of economic importance (physical traits) such as disease resistance, fast growth rate or high reproductive rate with wild types from the population (normal phenotype) to obtain a F<sub>2</sub> generation that carries the desired characteristics. This process is not straightforward, and the F<sub>2</sub> generation needs to be tested to check that it consistently inherits the desired trait. The physical traits are known to always associate with genes or combinations of genes that express it. To start the genetic selection, the link between that particular gene/genes and the trait of interest must be determined. A large number of DNA markers link to the QTL controlling the desired trait needs to be developed prior to the experiment. The most popular DNA markers to date are microsatellite markers, but single nucleotide polymorphisms (SNPs) have also been used in many studies. The QTL mapping is required to map the region that closely links with the desired gene by using the developed DNA markers. Identification of DNA



marker-assisted selection is promising to enhance the aquaculture industry of *M. rosenbergii*.

### **The production of good quality broodstock**

The availability of good quality broodstock at a low price would resolve the current problem of the shortage of supply of good quality broodstock encountered in *M. rosenbergii* farming in Malaysia. A limited supply of good quality broodstock forced the farmers to import broodstock from the neighbouring countries, particularly Thailand, that could provide good quality broodstock at low prices. However the production of good quality broodstock would only be possible if it was based on a successful genetic selection programme. Research focusing on maintaining the production of good quality broodstock and a high scale of production will solve the above mention problem of good quality broodstock supply.

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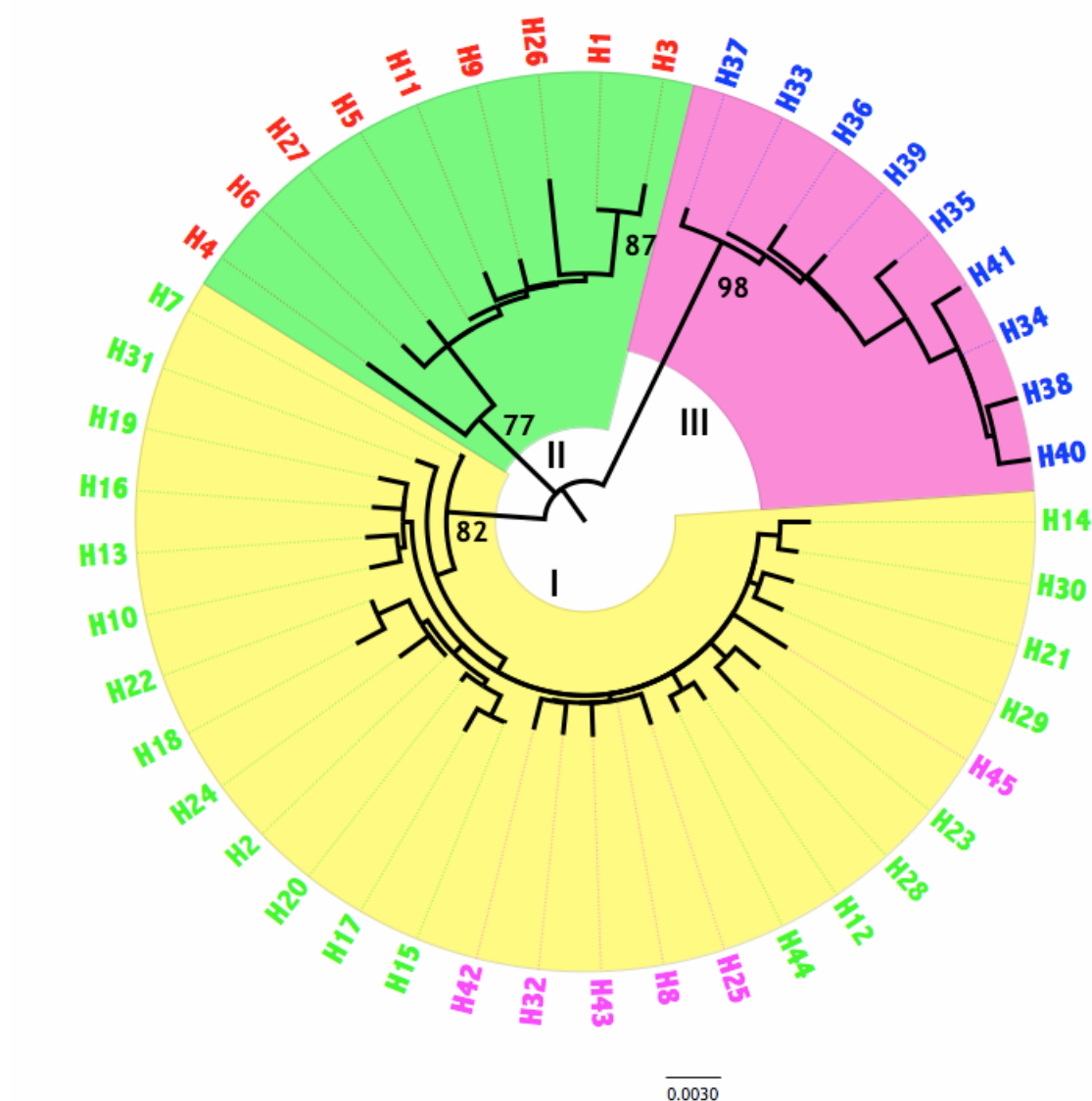
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## Appendices

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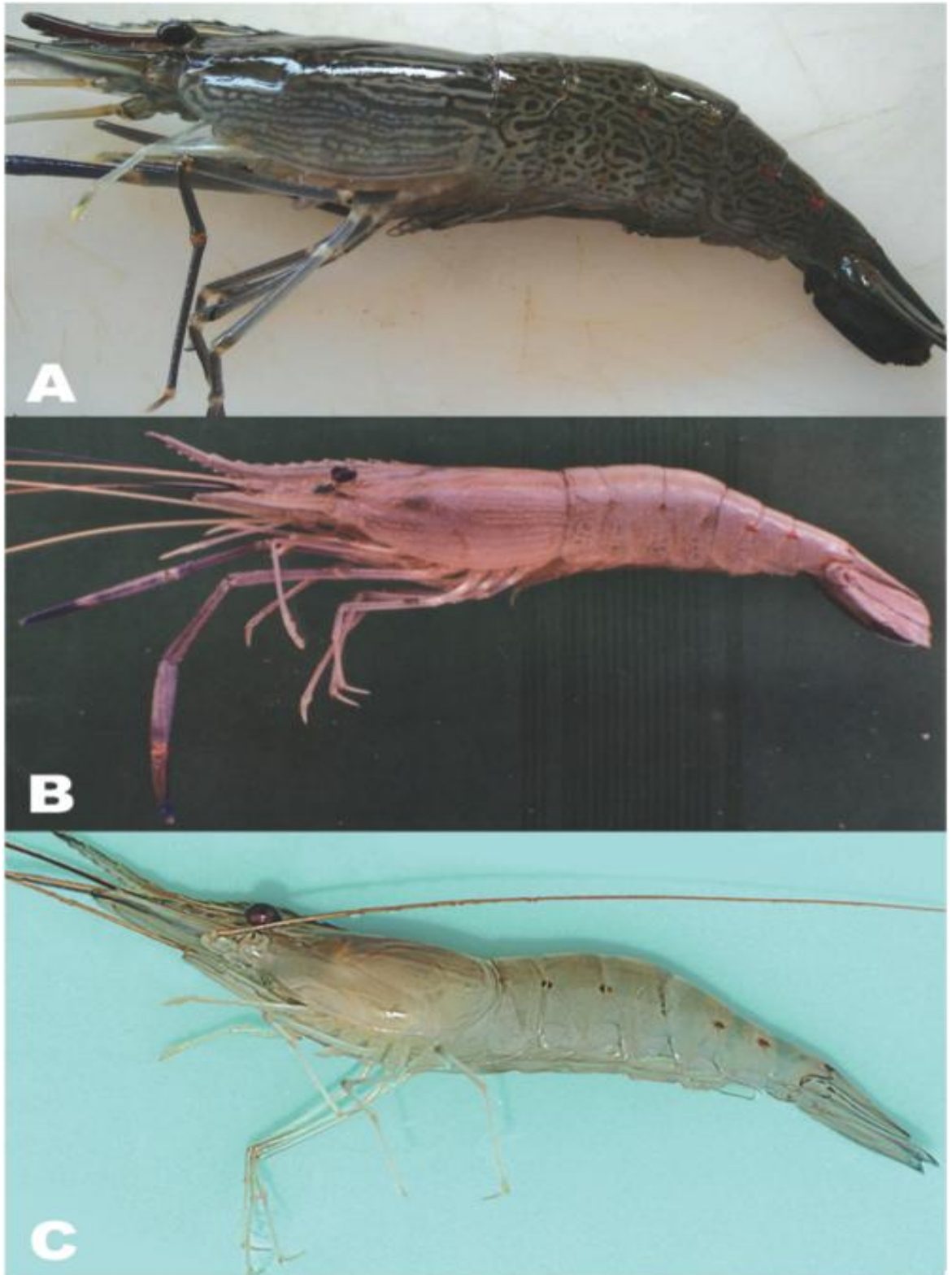


- Cluster I: Main haplotype consist of all population except Sabah.
- Cluster II: Perak, Kedah, Southern Sarawak
- Cluster III: Sabah

Appendix A: Maximum likelihood tree depicting genetic relationship among *M. rosenbergii* populations from Malaysia. Cluster I is the main haplotype which includes haplotypes found in all populations except Sabah. Cluster II includes of several haplotypes from Perak, Sarawak and Kedah and cluster III consists of only haplotypes from Sabah population. The bootstrap values are the percentages over 1000 replicates (only percentage of bootstrap value above 70% were shown). Tips branch; Green: Cluster I, Pink: sub-cluster I, Red: Cluster II and Blue: Cluster III.

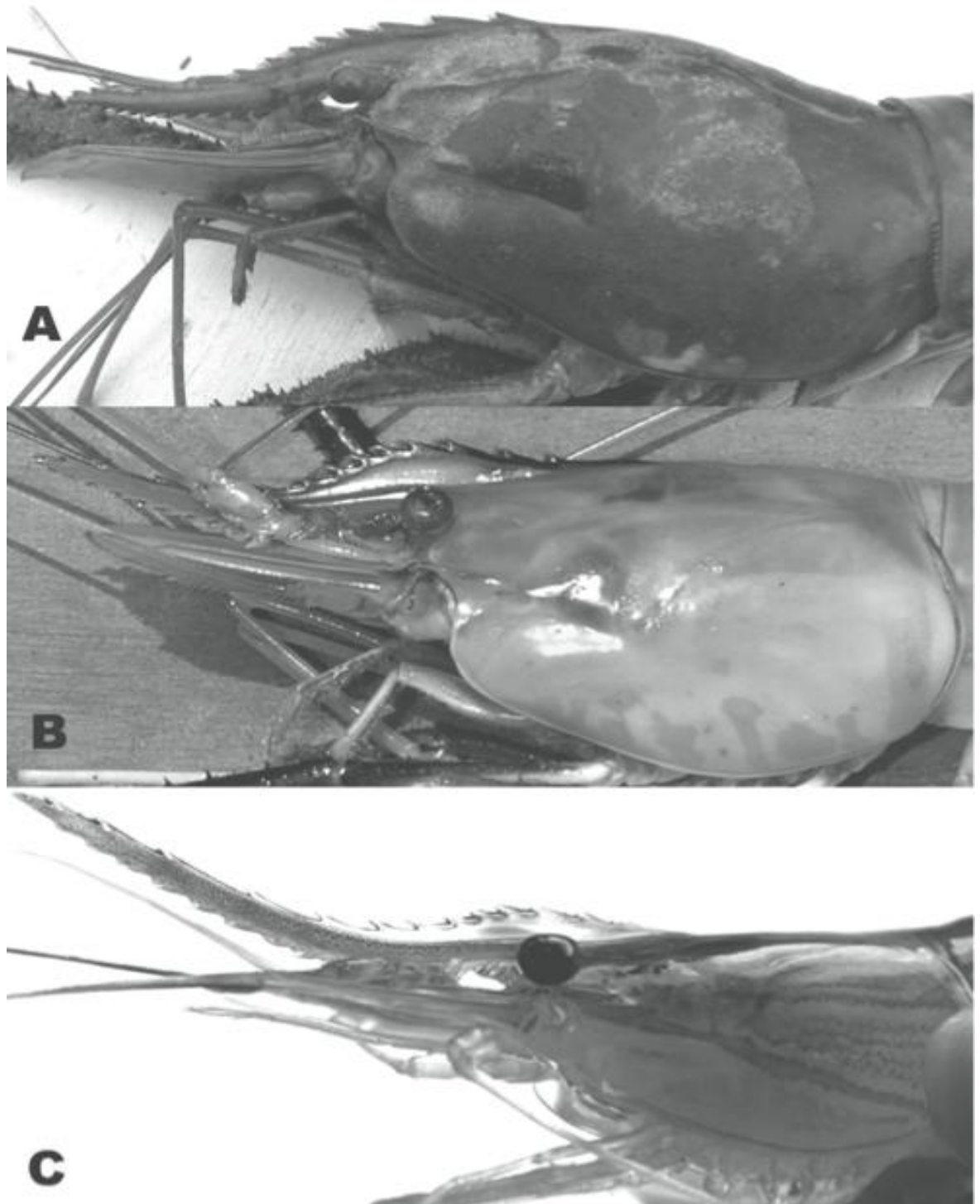
<i>Characters</i>	<i>Macrobrachium rosenbergii</i>	<i>Macrobrachium dacqueti</i>
Height of basal crest	Low to moderately low	High to moderately high
2 <sup>nd</sup> Pereiopods <sup>1,2</sup>	All segments covered with abundant spinules interspersed with widely-spaced, elevated large spines	All segments covered with widely-spaced, elevated large spines only
3 <sup>rd</sup> -5 <sup>th</sup> Pereiopods <sup>1,2</sup>	All segments covered with abundant spinules interspersed with widely-spaced, elevated medium-sized spines	All segments covered with widely-spaced, elevated medium-sized spines only
T4	With posterior submedian plate in form of low ridge	Without posterior submedian plate
Live colouration <sup>3</sup>	Carapace lightly brown with several brown bands; abdomen lightly brown with brown mottled pattern and bright red markings on each pleural condyle	Carapace with several black bands; several dark blue areas visible near margins of abdomen segments, telson and uropods, and reddish brown (=dark orange) markings present on each pleural condyle*
Live colouration	Carapace and abdomen uniformly olive brown to very dark brown; pleural condyle of 4 <sup>th</sup> -6 <sup>th</sup> segments with bright red markings <sup>4</sup>	Carapace and abdomen uniformly translucent olive grey to grayish blue; pleural condyle of 4 <sup>th</sup> -6 <sup>th</sup> segments with dark orange markings <sup>5</sup>
Live colouration <sup>6</sup>	Brown in undeveloped males, bright violet in developing males, dark violet brown or black in fully developed males**	Unpigmented or pink in undeveloped males, orange in developing males, dark blue in fully developed males***

**Appendix B: Informative characters for identification of *M. rosenbergii* species. Source: Wowor and Ng, 2007.**

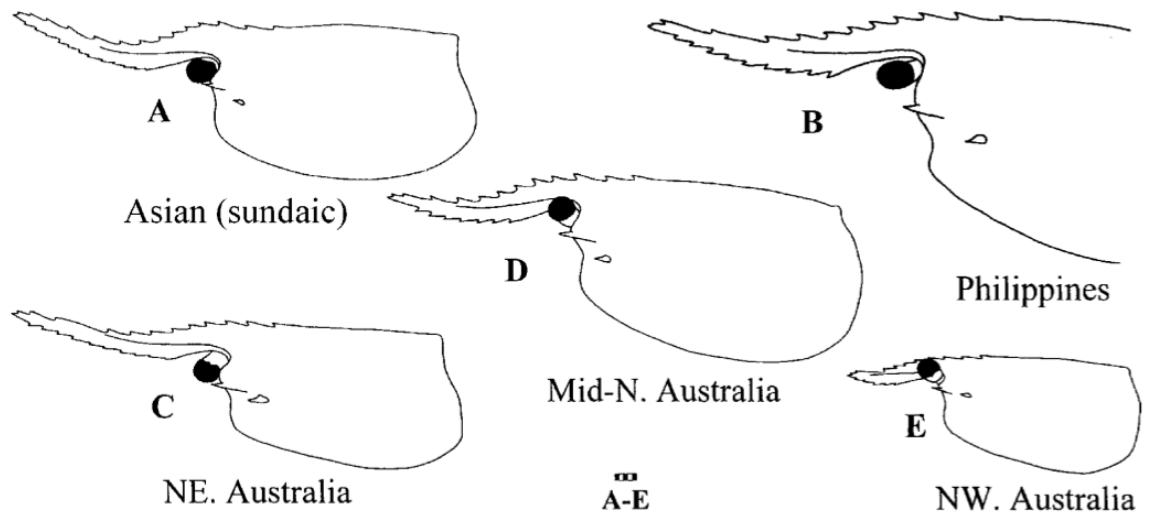


Appendix C: A and B are *M. spinipes*. Specimen A from Philippines and B from Papua, Indonesia. C is *M. rosenbergii* from Sarawak, Malaysia. Source: Wowor and Ng, 2007.





Appendix D: Variation in the rostrum shape. A is *M. spinipes* (specimen collected from Papua, Indonesia). B and C are *M. rosenbergii*. B adult and C young specimen. Both from Sarawak, Malaysia. Source: Wowor and Ng, 2007.



Appendix E: Geographical rostrum variation in mid-grown, sexually-mature males of *M. rosenbergii* (De Man, 1879). (A) QM W17187, 58.1 mm CL, Kelian R., Kalimantan, (B) 245-250 mm TL, Philippines (source: Cowles, 1914), (C) QM W22028, 45.0 mm CL, Mclvor R., northeast Australia, (D) NTM Cr005849, 58.5 mm, Coppermine Billabong, Daly R. catchment, mid-northern Australia, (E) WAM C21445, 43.2 mm, Prince Regent N.P., northwest Australia. Scale bar divisions 1 mm. Source: Short et al., 2004.