UTILIZATION OF CYTOCHROME OXIDASE SUBUNIT 1 GENE TO INVESTIGATE THE GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii* RESOURCES IN MALAYSIA WITH A SPECIAL FOCUS ON THE SOUTHEAST ASIAN POPULATION PHYLOGENY

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2018

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: AYU CERA LUTAS

Matric No: SGR120091

Name of Degree: Master of Science

Title of Project Paper/Research Report/Dissertation/Thesis:

UTILIZATION OF CYTOCHROME OXIDASE SUBUNIT 1 GENE TO INVESTIGATE THE GIANT FRESHWATER PRAWN, *Macrobrachium rosenbergii* RESOURCES IN MALAYSIA WITH A SPECIAL FOCUS ON THE SOUTHEAST ASIAN POPULATION PHYLOGENY.

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ABSTRACT

Productivity of Giant Malaysian Prawn (GMP) in 2013 to present has demonstrated a decrease in yield and survival that caused farmers to move to an easier crop, for example, tilapia and catfish, despite the fact that the demand of Giant Malaysian Prawn (GMP) is still high. Translocations of broodstocks for breeding purposes and restocking program from hatchery population to riverine systems could lead to loss of genetic diversity. By utilising mitochondrial DNA cytochrome oxidase subunit-1 (*COI*), we believe it can be a quick system, to assess genetic diversity in domesticated culture in farms from nine locations dispersed all through Peninsular Malaysia. AMOVA between wild and domesticated GMP showed that the genetic variation between these two groups was not significantly different, however, based on maximum-likelihood tree and Tajima's D value Johor (wild stock) is unique and different from other populations. Domesticated populations, KKL, BLG and LGG populations have high genetic diversity, thus, these populations are suitable to become base population, instead of wild stocks, for genetic improvement program. The temporal analysis between two different time cohorts, 2004 and 2013, revealed that there was reduction of haplotype diversity and nucleotide diversity. This is supported with high F_{ST} value of Sg. Johor and Sg. Kedah between 2004 and 2013. Analysis between GMP from Malaysia with Southeast Asian countries revealed that the geographic origin location could be from Malaysia as haplotype 5, 14 and 31 were ancestral haplotype. These findings could show that mtDNA *COI* genetic marker, is useful to evaluate population genetic structure of the freshwater prawn in Malaysia, very quickly and helps to manage the stocks in a more sustainable manner.

Keywords: *Macrobrachium rosernbergii,* genetic diversity*, COI*, aquaculture

PENGGUNAAN GEN "CYTOCHROME OXIDASE SUBUNIT 1" UNTUK MENYIASAT SUMBER UDANG GALAH, *Macrobrachium rosenbergii* DI MALAYSIA DENGAN FOKUS ISTIMEWA DALAM FILOGENI POPULASI ASIA TENGGARA

ABSTRAK

Produktiviti "udang galah" (GMP) pada tahun 2013 telah menunjukkan bahawa pengurangan hasil ternakan dan kadar hidup udang galah menyebabkan penternak beralih ke akiviti ternakan yang lebih mudah seperti tilapia dan ikan keli, walaupun permintaan udang galah masih tinggi. Translokasi stok liar untuk tujuan pembiakan dan program pengembalian stok semula dari populasi penetasan ke kawasan sungai boleh menyebabkan kehilangan kepelbagaian genetik. Dengan menggunakan DNA mitokondria "cytochrome oxidase DNA subunit-1 (*COI*)", kami percaya ia dapat menjadi sistem yang cepat, untuk mengakses kepelbagaian genetik dalam udang ternakan yang diambil dari sembilan lokasi yang di Semenanjung Malaysia. Keputusan AMOVA menunjukkan bahawa kedua-dua populasi liar dan buatan memperlihatkan perbezaan genetik yang tidak ketara, walau bagaimanapun, berdasarkan pokok "maximum-likelihood" dan nilai Tajima D Johor (populasi liar) adalah unik dan berbeza dari populasi lain. Populasi ternakan iaitu KKL, BLG and LGG menunjukkan kadar kepelbagaian genetik yang tinggi maka, populasi tersebut dapat menjadi populasi asas untuk program pembaikan genetik, daripada menggunakan populasi liar. Analisis temporal antara dua masa yang berbeza, 2004 dan 2013, mendedahkan bahawa terdapat pengurangan kepelbagaian haplotip dan kepelbagaian nukleotid. Analisis antara GMP dari Malaysia dengan negara-negara Asia Tenggara mendedahkan bahawa lokasi geografi GMP mungkin berasal dari Malaysia kerana haplotip 5, 14 dan 31 merupakan haplotip keturunan. Penemuan ini dapat menunjukkan bahawa penanda genetik *COI*, berguna untuk menilai struktur genetik populasi udang air tawar di Malaysia, dengan cepat dan membantu menguruskan stok secara lebih lestari. .

Kata kunci: *Macrobrachium rosernbergii,* kepelbagaian genetik*, COI*, akuakultur

ACKNOWLEDGEMENTS

I thank Allah for His blessings and for being my constant companion to go through this whole journey. I offer my sincerest gratitude to my supervisor, Associate Prof Dr Subha Bhassu who gave this opportunity and supported me during this project. I would like to express my deepest gratitude to my supervisor, Dr Hasmahzaiti Omar for her support throughout this project.

This research would never have happened without the financial support from High Impact Research (H-23001-G000006) and University of Malaya (PG103-2013A).

My special thank also goes to my colleagues in Animal Genetics and Genome Evolutionary Laboratory (AGAGEL); Dr. Sarasvathi Eawsvaran, Dr. Suma Tiruvayapati, Farhana, Mahtab, Ilamangai, Anees Fathima, Irda Yanti, Christie, Nurul Huda, Chan Chew Chin and Dann Chan.

Last but not least, I thanked my family Asril, Lutfiati, Habib, Icha and Hilman for their love and support throughout this journey. Thank you.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
>	:	More than
<	:	Less than
AMOVA	:	Analysis of Molecular Variance
AFRC	:	Anuenue Fisheries Research Center
bp	:	basepair
COI	:	Cytochrome oxidase subunit-1
DNA	:	Deoxyribonucleic acid
dNTP	:	Dinucleotide triphosphate
GMP	:	Giant Malaysia Prawn
ICZN	:	International Commission on Zoological Nomenclature
IHHNV	:	Infectious hypodermal and haematopoietic necrosis virus
kb	:	kilobytes
MEGA	:	Molecular Evolution Genetic Analysis
ML	:	Maximum-likelihood
mtDNA	:	Mitochondrial DNA
PCR	:	Polymerase Chain Reaction
PL	:	post-larvae
rRNA	:	ribomosal RNA

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CHAPTER 1: INTRODUCTION

1.1 General introduction

World fisheries and aquaculture production in 2014 was 73.9 million tonnes of seafood valued at US$160.2 billion (FAO, 2016). The aquaculture industry in Malaysia these days has turned out to be an important industry that has contributed to the economy of our nation. Aquaculture is viewed as one of the ways in providing a positive balance of trade to Malaysia. In this manner, commercialization of aquaculture industry is a priority of the government and private sectors as a means to increase the economy and standard of living of people in these sectors. Thus, fishes such as carp and tilapia, and crustacean groups like shrimp and prawn are being cultured tremendously to fulfil demand for seafood products and to maintain the food supply.

Giant Malaysian Prawn (GMP), *Macrobrachium rosenbergii*, also usually called as "udang galah" in Malaysia, belongs to the family Palaemonidae. GMP is the most prominent freshwater prawn commercially. GMP contains high protein and has a delicious taste which is favourable to the consumer contributing to the popularity of this prawn. In addition, the increasing popularity of GMP farming at the large-scale level is due to its easy method of culturing and high demand in global export (Whangchai et al., 2007). This species is fast-growing and inhabits freshwater and low brackish water conditions during its larval stages (Ling & Merican, 1961; Wowor & Ng, 2007). Hence, a substantial level of business success of GMP has been reported in Malaysia, Thailand and Southeast Asian countries. In 2009, Malaysia was among the top 15 GMP producers in the world which has recorded total production over 700 million tonnes (FAO, 2012).

In recent years, the industry of GMP aquaculture has shown significant interest by farmers in Malaysia. It is anticipated to keep expanding especially more new techniques are developed (Banu & Christianus, 2016). The demand for this species is high in nearby

and global markets empowering the increase of GMP cultivations in Malaysia. Also, the price is moderately higher in contrast to marine shrimp. However, there are declines in the production of GMP during grow-out, which means the phase of production where juveniles are grown to adult prawn, after several production cycles in culture (New, 2007; New & Nair, 2012). A few issues have been recognised to influence the productivity and profitability including the moderate rate of development, high rate of mortality and decrease of adult prawn's bodyweight, feed conversion ratio, survival rate and disease resistance (New, 2007).

Similarly, the decline in production for GMP farming has also been observed in other Southeast Asian countries. The significant drop of cultured GMP industries during the early 1990s was associated with inbreeding depression (Knibb et al., 2014). This case probably occurred the most because of the 'recycling of animals' practice at the hatcheries. This happened where GMP grow-out ponds which are inbred stocks, instead of wild stocks, become the broodstocks for the next breeding cycles. Repeating this process for many generations can reduce the genetic diversity in domesticated GMP stock (New et al., 2000). This practice could lead to problems such as inbreeding depression and reduce the GMP productivity, but analysis at the molecular level demonstrated no correlation between the inbreeding depression and GMP productivity (New & Kutty, 2010). However, a genetic comparison study between two hatcheries and wild stock populations of GMP revealed that there were significant genetic differentiation in both populations in Thailand (Chareontawee et al., 2007). High genetic differentiation between wild and hatchery products arise due to the differences of pond environment in relation to river ecosystems, which resulted in the fluctuation of GMP production from 2008 to 2014 as reported by Banu and Christianus (2016). Hence, the farmers faced problems such as slow growth rate, high mortality rate and reduction of body mass of GMP. This is because an organism which lives in a drastic environmental change will adapt to their

environment to survive and reproduce to ensure they will stay alive for the survival of the species. Natural selection will only keep the "fittest" or the population that will survive very well while the population that affected by environmental changes will die and the genetic variation in that population will be affected. In addition, domesticated prawns are the product of non-random mating where the same broodstock are used repeatedly for breeding. Natural selection and non-random mating cause evolution to occur and departure of genotype equilibrium distribution in a population. Therefore, restocking is very crucial to control the origin of broodstock location and ensure the broodstock used should be from the wild population.

The vast culture of domesticated seeds of GMP species around Southeast Asia originated from a Hawaiian strain which actually has its history in Malaysia. Thus, the freshwater prawn broodstock cannot be regarded as independent germplasm in fisheries yet. Germplasm is defined as a native organism which is capable to produce new pure line variety. It is debatable GMP is worthy as germplasm. Most of the writings which contain research on freshwater prawn broodstock do not acknowledge that the freshwater prawn broodstock from Malaysia is the best in the world. The lack of research on freshwater prawn broodstock in Malaysia is one of the many reasons why the government is not interested in developing further potential.

1.2 Problem statement

The development of the giant freshwater prawn farming is relatively well reported in the 1960s where modern aquaculture of the species began. In 1961, a fishery biologist at Marine Fisheries Research Institute in Penang, Malaysia, Shao-Wen Ling, discovered that GMP needs to live in brackish water to survive (Ling & Merican, 1961). This was a major milestone in GMP farming history. After a few years, in 1965, Takuji Fujimura and his team made a second big discovery where they started on research to produce post-

larvae (PL) on a commercial scale (Fujimura & Shang, 1977). This discovery later led to emergence of commercial farms of GMP in various countries in the 1970s (New, 2007). Therefore, there is a possibility that those Malaysian PL is distributed worldwide and Malaysia was the single geographical source of all the global populations. However, does the introduction make Malaysia the only geographic source to all domesticated breeding populations throughout the world? In this study, based on mitochondrial cytochrome oxidase subunit 1, we compare the genetic diversity of several populations of GMP in Malaysia and Southeast Asia with addition of Bangladesh, India and Brazil which has been reported previously by de Bruyn et al. (2005) and Iketani et al. (2011).

Several studies have found that investigating the genetic diversity of GMP populations assists in recognising strains that have good traits which can be used as candidates for possible breeding and selection program (Elsheikh et al., 2015). A study discovered a few stocks of GMP from Thailand and Myanmar were selected as candidates for breeding as the prawns were discovered as the meatiest. The selection was done to maximise survivability while maintaining desirable meat characteristics (New et al., 2000). In Malaysia, knowledge and research on population genetic structure of wild and domesticated GMP remains limited (Bhassu et al., 2008; Elsheikh et al., 2015). Additional work on GMP populations is not only providing the information on genetic structure but could also recognise quality differences among GMP populations sourced from various locations. This information on the prawn genetics can to some extent improved particular strains of GMP through breeding selection program.

In order to identify the sources of broodstock, molecular genetic analysis can be done using genetic markers by accessing variation based on the differences in amplified sequences in specific genes and based on the heterogeneity of the alleles. The genetic variation studies are very helpful to understand the patterns of breeding and the degree of

relatedness of genetic variation among them (Schierwater & Ender, 1993). In this experiment, the research question is to infer relationships among GMP and its genetic quality by contrasting the wild and cultured GMP. Another comparison were done between *COI* of GMP wild stocks collected in 2004 and 2013.The experimental design for all four riverine stocks was compared at temporal time because there was extensive overharvesting activities of brood stocks for PL production between those years (Bhassu et al., 2008) (See et al., 2009; Jaafar, 2014; Atin et al., 2017).. The null hypothesis for objective 2 is the genetic diversity of wild stocks collected between 2004 and 2013 remained the same

Information of the maternal parent is important as hatcheries typically used the same broodstock in the production of post-larvae. Mitochondrial cytochrome subunit 1 (*COI*) has been popularly used in genetic structure and diversity studies in prawn and shrimp (Xu et al., 2001; Liu et al., 2011; Hsu et al., 2013; Khedkar et al., 2013).

1.3 Research objectives

Specific aims of this study were:

1. To compare genetic variations between domesticated and wild GMP using cytochrome oxidase subunit 1 (*COI*) mtDNA gene.
2. To study the temporal genetic diversity of wild GMP from riverine sources collected at two different times namely 2004 and 2013.
3. To examine the molecular phylogenetics of GMP from Malaysia and Southeast Asian countries using cytochrome oxidase subunit 1 (*COI*) mtDNA gene.

CHAPTER 2: LITERATURE REVIEW

2.1 Utilising genetic data as tools of genetic resources management

2.1.1 Genetics effects of overharvesting in fisheries

Fisheries is an important strategy in harvesting wild fish, molluscs, crustaceans and any aquatic animals for its commercial value which involve capture fisheries or farmed fish (FAO, 2016). Huge demand in fisheries and human harvest of animals has caused overexploitation which could lead to demographic extinction (Burney & Flannery, 2005). Even though genetic changes due to exploitation has less effect than direct extinction, genetic changes could complicate the stock management for prolonged sustainable harvesting (Ratner & Lande, 2001; Walsh et al., 2006; Proaktor et al., 2007; Richard, 2007).

Excessive overharvesting has been proposed to cause different kinds of genetic changes includes alteration of population subdivision, reduction of genetic variation and exploitative selection (Allendorf et al., 2008). Overharvesting of wild population would disturb genetic subdivision among populations. Extirpation of some subpopulations could have resulted from a mixture of several subpopulations, which can lower overall productivity (Allendorf et al., 2008). Besides, the number of migrants among subpopulations might be reduced due to overharvesting and could eventually increase genetic drift and reduce genetic variation. The rate of gene flow among subpopulations might also increase and there might be a loss of local adaptations due to overharvesting.

Alteration of population subdivision has been discovered in marine and freshwater fish (Ryman & Utter, 1986; Dulvy et al., 2003). Four salmon species production in southern British Colombia has severely decreased between 1950 and 1980 due to prolonged subdivision caused by habitat destruction and failure in stock enhancement program (Walters & Cahoon, 1985). Generally, wild stocks with good traits usually face

greater exploitation and easily affected by exploitation might be the first to vanish. This phenomenon can be seen in the disappearance of lake trout *Salvelinus namaycush* from Lake Michigan as they have been overfished due to their high-fat content (Krueger et al., 1989).

Overharvesting could reduce population size which eventually reduces the genetic variation within subpopulations. Genetic variation could demonstrate the adaptation of evolution of a species and it is used to plan stock enhancement by conservationist (Schwartz et al., 2007). Neutral population genetic theory proposed that genetic variation within species is correlated with effective population size (N_e) (Frankham, 1996). A variety of other factors that may change levels of genetic diversity such as population size, mutation, inbreeding overfishing, distribution, abundances, bottlenecks, and founder effects (Amos & Harwood, 1998; Bromham, 2009; Leffler et al., 2012). Genetic diversity is measured by heterozygosity and allelic richness. Loss of heterozygosity is often being seen as a consequence of genetic drift. Loss of heterozygosity is estimated from N_e that is determined by demographic factors. Those demographic factors were census population size, N_c, sex ratio, and the mean and variance of a number of offspring produced by males and females (Waples, 2002).

Many studies reported a reduced level of genetic diversity in the overexploiting population. For example, excessive hunting in the 1990s resulted in slow bottleneck recovery of African elephant *Loxodonta africana* and showed reduced heterozygosity and low allelic diversity (Whitehouse & Harley, 2001). Harvest can also reduce the N_e and N_c ratio which could increase the loss of heterozygosity without showing an apparent effect in population size. Marine fish and invertebrates typically have higher N_c and N_e than the terrestrial vertebrates (Waples, 1998; Poulsen et al., 2006) but populations with larger N_c can lose heterozygosity as N_e is usually lesser than N_c in many marine species as they

occupied large areas and have high gene flow (Waples, 1998). It is estimated that the N_e/N_c ratios in marine species is 10^{-5} (Hauser & Carvalho, 2008).

2.1.2 The function of genetics research in fisheries management

Overexploitation of fish and shellfish stocks has caused negative genetic effects in animals' population. Effective measures need to be taken to establish fishery and aquaculture as an important source of healthy food to the world and at the same time are sustainable environmentally and economically. Reducing the harvest of animals to reduce long-term genetic effects is very hard to implement as it could affect the dependent communities economically (Walters & Martell, 2004). Genetics approach is a potential method to be implemented in fisheries management. However, many reports have discussed on lacks of implementation of genetics information into fisheries management, albeit the current progress and development in genetics and genomics area (Hauser & Carvalho, 2008; Kochzius & Nuryanto, 2008; Waples et al., 2008; Seeb et al., 2011; Zelenina et al., 2011) . A report by Ovenden et al. (2015) addressed that genetic approaches will apparently provide a valuable guideline, for example, species identification, fisheries stock structure, resolving mixed-stock fisheries, biomarker discovery, genetic diversity, and genetic effect of stock enhancement.

Genetics could provide a variety of helpful techniques and strategies in advising fisheries managers about problems that have a biological basis (Ovenden et al., 2015). There many things genetic analyses can offer to fisheries managers such as techniques of specimen identification and assessment of stock structure. The most common tools of genetic tools are allozymes, mitochondrial DNA, and microsatellites which have different properties. Those differences must be considered when describing and understanding gene frequency data obtained in stock structure research (Ward, 2000)

2.1.2.1 Fisheries stock structure

Fisheries stock structure symbolise groups of organisms which can be genetically isolated from other populations, as basic units of fisheries management demographically (Reiss et al., 2009). Genetic analysis has been extensively utilized to study the spatial range of fisheries stocks. A range of a species is divided into "stocks" by utilising genetic assays. Fisheries operators run stock boundaries in order to allocate quotas, model alternative harvesting and to plan stock enhancement program . One main constraint is whether the stock boundaries management can be implemented at a suitable spatial range. It is important to have good communication and understanding between researcher and fisheries managers in order to improve the genetic stock structure (Cadrin et al., 2014). In a long-term, genetic analysis will be focusing in measuring behaviour of individual species in ecological time frames instead of on the long-term average behaviours of entire populations (Hill, 2010). Moreover, to date, there are many of genomic resources for fisheries species which can be used in identifying genetic variants that undergo natural selection (Coyle, 1998). Hence, genetic markers become more powerful to identify stocks especially with unique adaptive characteristics (Nielsen et al., 2009). In one study, the need whether shark and snapper species, between northern Australia and Indonesia, require independent or joint management were investigated using genetic stock structure knowledge (Ovenden et al., 2009). Analysis using microsatellite loci on *P. monodon* populations showed there was a clear genetic differentiation between western population and those from the northern and eastern coasts populations, thus, revealed that western *P. monodon* is a unique genetic stock due to a reduction in genetic variation (Brooker et al., 2000).

2.1.2.2 Monitoring genetic diversity

Genetic diversity is defined as the diversity of alleles and genotypes in a species' genetic makeup (Frankham et al., 2002). It can be disturbed by continued reductions in

effective population size and changes to connections between populations (Toro & Caballero, 2005). Measurements of genetic diversity able to give knowledge on the abundance of a species and its ability for evolution due to changes in the environment (Ehrlén & Morris, 2015). Effective population size is a proper method to monitor and summarize changes in genetic diversity across taxa, which is relevant to time-scales and harvesting has occurred and it has the potential for indexing virgin biomass that predates harvesting (Ovenden et al., 2014). In addition, effective population size has a crucial role in detecting changes in genetic diversity attributed to fisheries stock enhancement. Usually, effective population size is the comparisons between estimates of effective size from genetic and demographic data will help to understand how they are related.

2.1.2.3 Genetic consequences of stock enhancement

Stock enhancement program needs to be done to combat interbreeding between the wild and domesticated individuals which could lead to hybrids (Bert et al., 2007; Ikhwanuddin & Abol-Munafi, 2016). Hybrids might have lower genetic diversity than that of the wild individual. Evolutionary processes such as natural selection could remove the unfit animals and reduce the production of the animals (Gregory, 2009). This effect can be prevented by introducing stocks that are compatible with the pond and wild environment. Here we can use genetics to monitor changes to genetic attributes and post-release survival where hybridization can be detected in hatcheries (Bert et al., 2007). We need a balance between the requirement for enhancement and its effects. Pond managers need to be guided to detect the possibility of genetic effects and learn how to minimise the genetic effects. Two important things are how to produce captive-bred animals with great fitness for survival upon releasing and to prolong the mixed spawning in cultured populations (Philippart, 1995).

2.2 *Macrobrachium rosenbergii* (de Man, 1879)

2.2.1 Nomenclature and taxonomy

Giant Malaysia Prawn (GMP), *Macrobrachium rosenbergii*, are called "udang galah" in Malaysia. The nomenclature of this species has a muddled history by which many taxonomists have proposed a different name for this species (New & Semusk, 1985). Previously, *M. rosenbergii* was known as *Palaemon carcinus* (de Man, 1879), *Palaemon dacqueti*, and *Palaemon rosenbergii* and until the name of a current scientific name, *M .rosenbergii* was introduced and accepted universally. Still, there are some taxonomists recognised two different type sub-species within *M. rosenbergii* separated by Huxley's Line namely western sub-species and eastern sub-species. Western sub-species lived in the east coast of India, the Bay of Bengal, Gulf of Thailand, Malaysia, Sumatra, Java and Kalimantan while eastern sub-species were found in the Philippines, the Indonesian regions of Sulawesi and Irian Jaya, Papua New Guinea and northern Australia (Johnson, 1960; Holthuis, 1995). Holthuis (1995) reported that those sub-species supposedly be recognised as different species which are *M. rosenbergii rosenbergii* (de Man, 1879) (eastern sub-species) and *M. rosenbergii dacqueti* (Sunier, 1925) (western sub-species) as both are distributed non-overlapping and the shape of the rostrum could distinguish both subspecies (Johnson, 1960). Nonetheless, naming those sub-species with different names had made a big nomenclature issue. This was because this species was extensively fished and cultured globally and should be known as *Macrobrachium dacqueti* and not *Macrobrachium rosenbergii* (Wowor & Ng, 2007).

In order to minimise this taxonomic confusion, Wowor and Ng (2008) preferred the name "*M. rosenbergii*" to be maintained commercially as it was more valuable and extensively researched species from Southeast Asia. Therefore, they had requested the International Commission on Zoological Nomenclature (ICZN) to revoke the previous naming for *P. rosenbergii* (de Man, 1879) a holotype female from Andai, New Guinea.

Later it was then designated to the lectotype specimen of *Palaemon dacqueti* (Sunier, 1925) as its neotype. The specimen conserved the name *Palaemon rosenbergii* (de Man, 1879), for the Southeast Asian species. The Commission agreed and ruled upon the naming of the species (ICZN 2010, Opinion 2253). According to Sunier (1925), the western sub-species is referred as *Macrobrachium rosenbergii dacqueti* while the eastern sub-species is referred to as *Macrobrachium rosenbergii rosenbergii* (de Man, 1879). Until now, there are some debates on the exact nomenclature of this species (Wowor et al., 2009). As *M. rosenbergii* was widely distributed into many another areas, the exact naming may have little relevance. Currently, the classification and taxonomy are stated as the following (Figure 2.1):

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Subclass: Eumalacostraca
Superorder: Eucarida
Order: Decapoda
Suborder: Pleocyemata
Infraorder: Caridea
Superfamily: Palaemonidae
Family: Palaemonidae
Subfamily: Palaemoninae
Genus: *Macrobrachium*
Species:*Macrobrachium rosenbergii* (De Man, 1879)
Synonyms:
Palaemon rosenbergii (de Man, 1879)
P. carcinus rosenbergii (de Man, 1879)
P. (*Eupalaemon*) *rosenbergii* (de Man, 1879)
P. whitei Sharp, 1893
P. spinipes Schenkel, 1902
P. dacqueti Sunier, 1925
Cryphiops rosenbergii (de Mann, 1879)
C. (*Macrobrachium*) *rosenbergii* (de Man, 1879)

Figure 2.1: Taxonomy and nomenclature of GMP. (Adapted from Holthuis and Ng (2009).

2.2.2 Morphology

GMP may look similar to other shrimp species. There are some significant characteristics to distinguish GMP to other species in the same genus. The rostrum is very long and has 11-14 dorsal teeth and 8-10 ventral teeth and the tip of the rostrum can reach posterior spines of the telson. The second cheliped of the adult male is very long and able to elongate and have blunt spines. The adult male also has a movable finger of the second chelipeds of that is covered by a dense velvet-like fur, but this fur is absent from the fixed finger and the rest of the cheliped. Reports showed that the adult male can have a total body length up to 33 cm while adult females up to 29 cm (Figure 2.2).

2.2.3 Distribution, habitat and lifecycle

The habitat of GMP is influenced by adjacent brackish water areas because its post-larvae (PL) grows in brackish water (Ling & Merican, 1961; Sandifer & Smith, 1975). New and Semusk (1985) reported that GMP could be found in turbid condition. The female prawn migrates downstream into estuaries then hatches their eggs as free-swimming larvae. The planktonic larvae undergo several zoea stages (Ling, 1969; Uno & Kwon, 1969). After PL metamorphosis, the PL enter benthic lifestyle and start to move to upstream towards freshwater (Figure 2.3).

The breeding of GMP had been done traditionally by collecting wild-juveniles from the river (New & Semusk, 1985). In 1960, the modern aquaculture of GMP has been successfully developed, where Ling & Merican (1961) discovered that PL of GMP required brackish water to grow. Their findings led to the culture activity of this species. After a few decades, the GMP farming has expanded and a number of hatcheries have been established (Banu & Christianus, 2016).

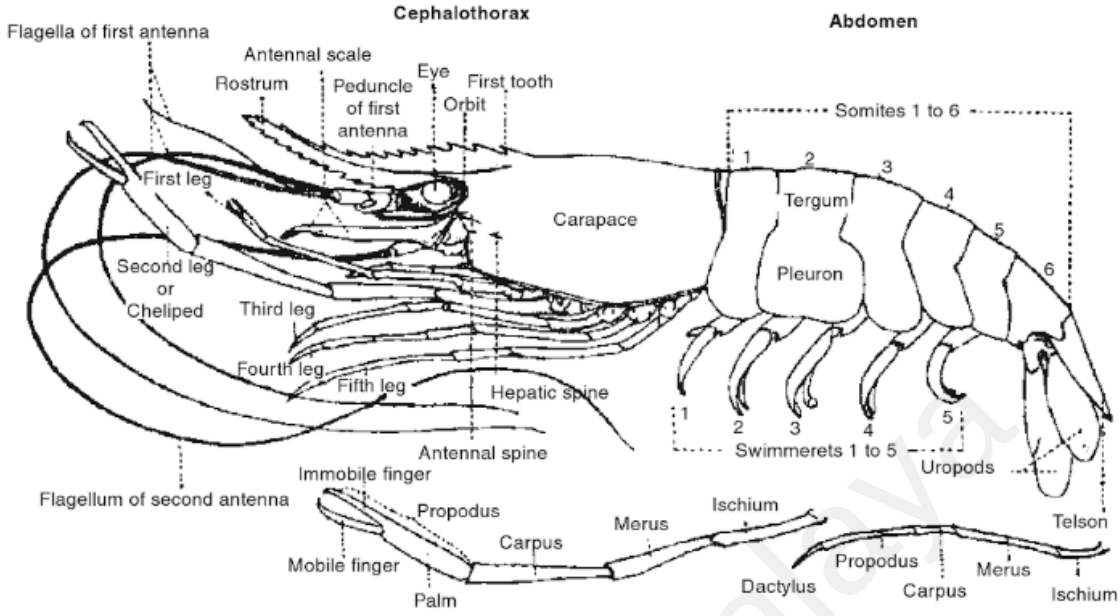

Figure 2.2: Morphology of *Macrobrachium rosenbergii* (de Man). Source: After Cowles (1914). This picture was taken from Freshwater Prawns: Biology and Farming by (New et al., 2009).

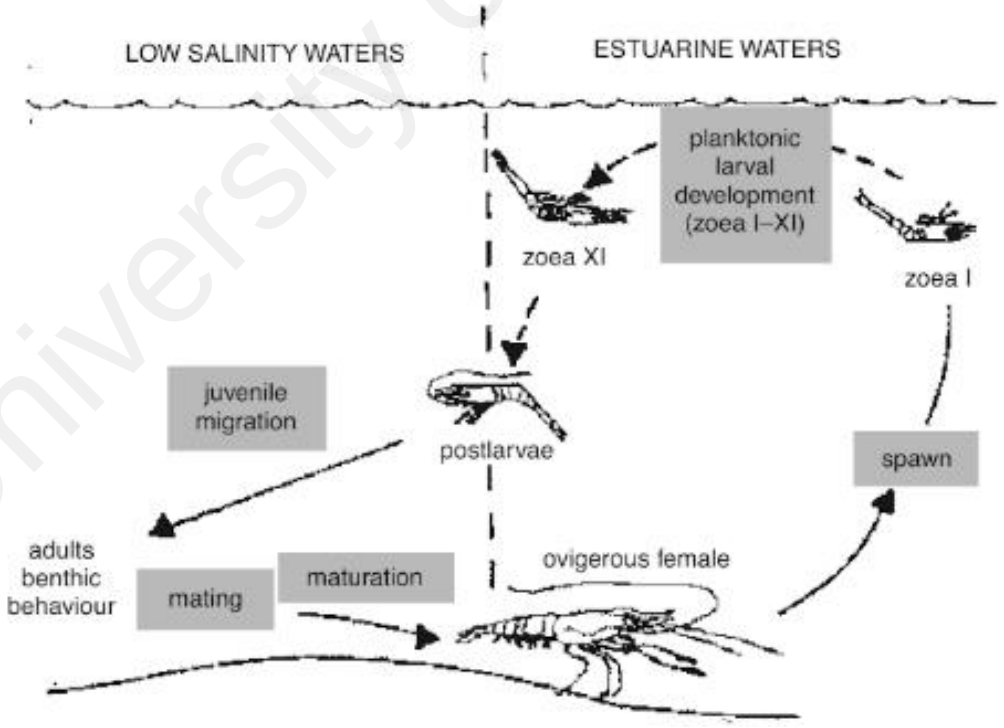

Figure 2.3: Lifecycle of Giant Malaysian Prawn. This picture was taken from Freshwater Prawns: Biology and Farming by (New et al., 2009).

Malaysian freshwater prawns are the most popular freshwater prawn for commercial culture. The reason lies on natural advantages that this species has. This species likely to have a fast growth rate and large size. The breeding of Malaysian freshwater prawn required well-oxygenated water and temperature between 27º-32º. Thus, the large-scale commercial culture of GMP succeeded in region Malaysia, Thailand and other parts of Southeast Asia. Today increased exploitation had reduced natural stocks of freshwater prawn which results in extinction and loss of genetic diversity. GMP has many advantages include high growth rate, ability to be cultured under freshwater to saline zone, ability to be cultured as the monoculture or mixed culture with carp fishes (New, 1990). In addition, the market rate is always higher compared to fishes (New, 1990). The low production is probably because GMP has been threatened with disease. GMP is prone to infection of virus-like infectious hypodermal and haematopoietic necrosis virus (IHHNV), white spot syndrome virus (WSSV), Taura syndrome virus (TSV), nodavirus diseases (whitetail diseases) and yellow head virus (YHV) (Arcier et al., 1999)

2.3 Current status of cultured freshwater prawn in Malaysia

In the early 1980s, the Malaysian government initiated the National Agriculture Policy (NAP). Current policy aims to increase food security by increasing production of GMP and also to increase GMP's export value as stated in the third NAP (1998-2010). *GMP* is very popular for export as they have high demand. The prawn size is relatively small to be compared with shrimp. However, the price in the market is relatively higher than that of shrimp making GMP farming is a high-income generator for the fisherman. The market price of freshwater prawn has increased from RM15/kg in the 1980s to RM65/kg now. The consumer demand for GMP locally and internationally is always high. The aquaculture of freshwater prawn is expected to increase food security and increase income to the locals. The tropical weather and temperature in Malaysia are

suitable for the growth of this species. These factors have created considerable interest among the hatchery operators and the farmers to do GMP farming. Nevertheless, the Department of Fisheries (DOF) and Persatuan Peladang Kecil (PPK) in each state have given modals and intensive to farmers to do prawn farming. There are many paddy fields which can be turned into prawn ponds. There are also programmes of aquaculture development from the government to help the small farmers starting their freshwater prawn farming. The government has provided financial assistance scheme to the farmers. Free workshop and seminar were done to teach those farmers the right technique to operate prawn farming.

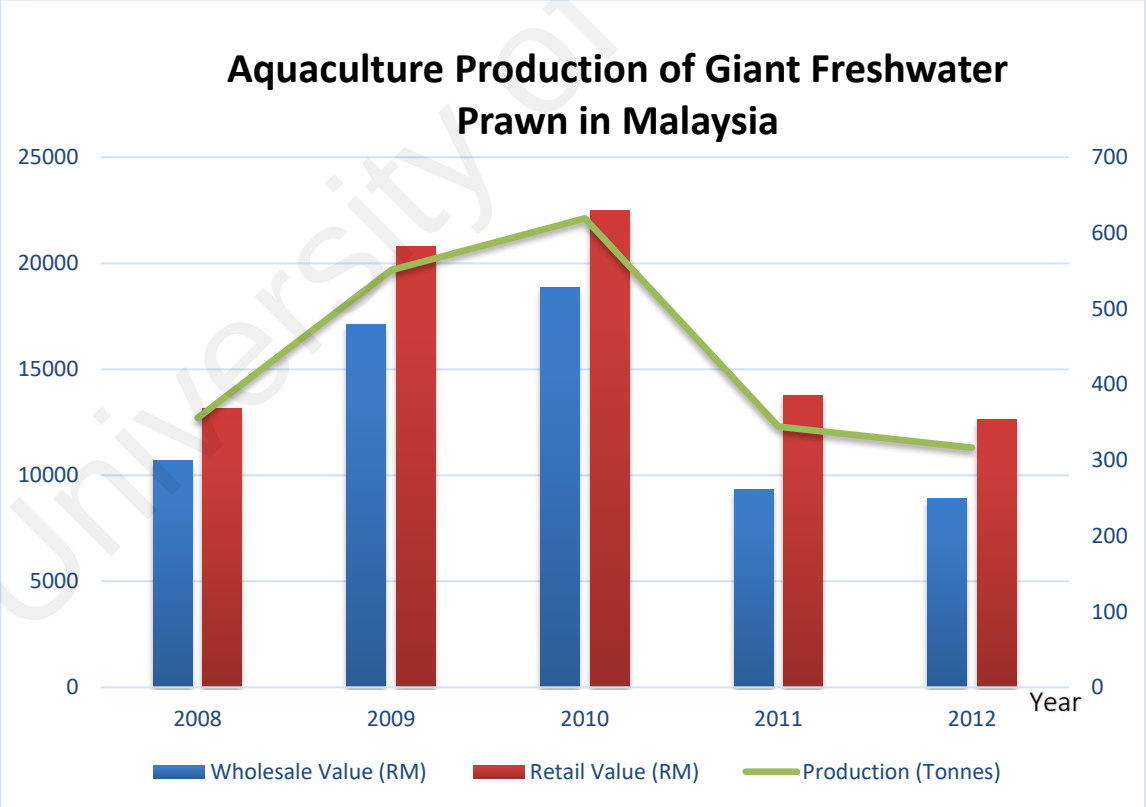

Figure 2.4: Aquaculture production of GMP in Malaysia from 2008-2012 which includes whole sale value, retail value and prawn production. (Adapted from (FAO, 2014)).

2.4 Mitochondrial DNA (mtDNA) in population genetics

Mitochondrial DNA (mtDNA) is utilised in this study as it has the ability to trace broodstock populations lineages through maternal inheritance. Mitochondria are intracellular organelles that have the sites for the formation of ATP by oxidative phosphorylation and has double-stranded circular mitochondrial DNA (mtDNA) (Kucuktas & Liu, 2007). Animal mtDNA contains 37 genes in a very small genome that is approximately 16 kb (Boore, 1999). Animal mtDNA genome studies revealed that, generally, the invertebrate mtDNA genome contains more or less the same number of genes, includes GMP (Pereira et al., 2000; Miller et al., 2005). The mtDNA gene encodes 13 protein subunits, two ribosomal RNAs and 22 transfer RNAs (Boore, 1999; Miller et al., 2005) (Figure 2.5). There is also a large noncoding control region which known as the D-loop (Pereira et al., 2000)

Unlike nuclear DNA, mtDNA is maternally inherited and has haploid nature (Avise, 1995). The haploid nature of mtDNA with no recombination is advantageous as the effective population size of the mtDNA is 1/4th of nuclear DNA (nDNA). Therefore theoretically, mtDNA is more sensitive to genetic drift hence mtDNA can be very sensitive in detecting population structure. Lack of recombination in mtDNA means that the mtDNA evolve as a single lineage (Boore, 1999). Hence mtDNA could differentiate between historical and contemporary structure process that may have influenced any observed population structure.

Although mtDNA genomes typically evolve rapidly, some mtDNA genes, such as *COI*, *COII* and *COIII*, evolve very slowly. Therefore, those genes usually be used in deeper lineage phylogeny study (Stock, 2009) The cytochrome oxidase subunit-1 mitochondrial DNA (*COI* mtDNA) gene has been popularly used as a genetic marker in molecular genetics work (Medina & Walsh, 2000; Long et al., 2003; Stoeckle, 2003; Cui

et al., 2018). There are several studies utilising mitochondrial DNA gene to investigate genetic structure. Study of populations of *Penaeus monodon* along Andhra Pradesh coast using D-loop mtDNA gene showed high genetic diversity with mean genetic diversity 0.191 suggesting no overexploitation has occurred in present time (Khedkar et al., 2013). The utilisation of the *COI* gene between wild and farmed mitten crab *Eriocheir sinensis* among 6 localities showed low genetic differentiation based on AMOVA but revealed potential genetic hybrids of *E. sinensis* (Zhang et al., 2017). Several studies using *COI* gene on GMP has been reported in Malaysia, Asian countries and Brazil (Zhang et al., 2009; Iketani et al., 2011; Elsheikh et al., 2015; Nguyen-Thanh et al., 2015; Maidin et al., 2017).

2.5 Population and phylogeographic genetics studies in Malaysia

There were several study of population and phylogeographic genetic study of wild GMP in Malaysia using both nuclear markers and mitochondrial markers. See et al. (2008) has conducted a study where they utilised random amplified polymorphism markers to investigate the genetic diversity of wild GMP among 11 populations in Malaysia and revealed almost 100% polymorphism in all populations. In the following year, they successfully developed and characterized seven novel polymorphic microsatellites marker from GMP using 5' anchored techniques (See et al., 2009; See et al., 2011).

In 2011, University of Malaya research team has performed mining and validation of Expressed Sequence Tag – Simple Sequence Repeat (EST-SSR) using transcriptomic analysis where 14 potential polymorphic EST-SSR has been identified (Mohd-Shamsudin et al., 2013). The EST-SSR then was utilized to do genetic diversity analysis in riverine

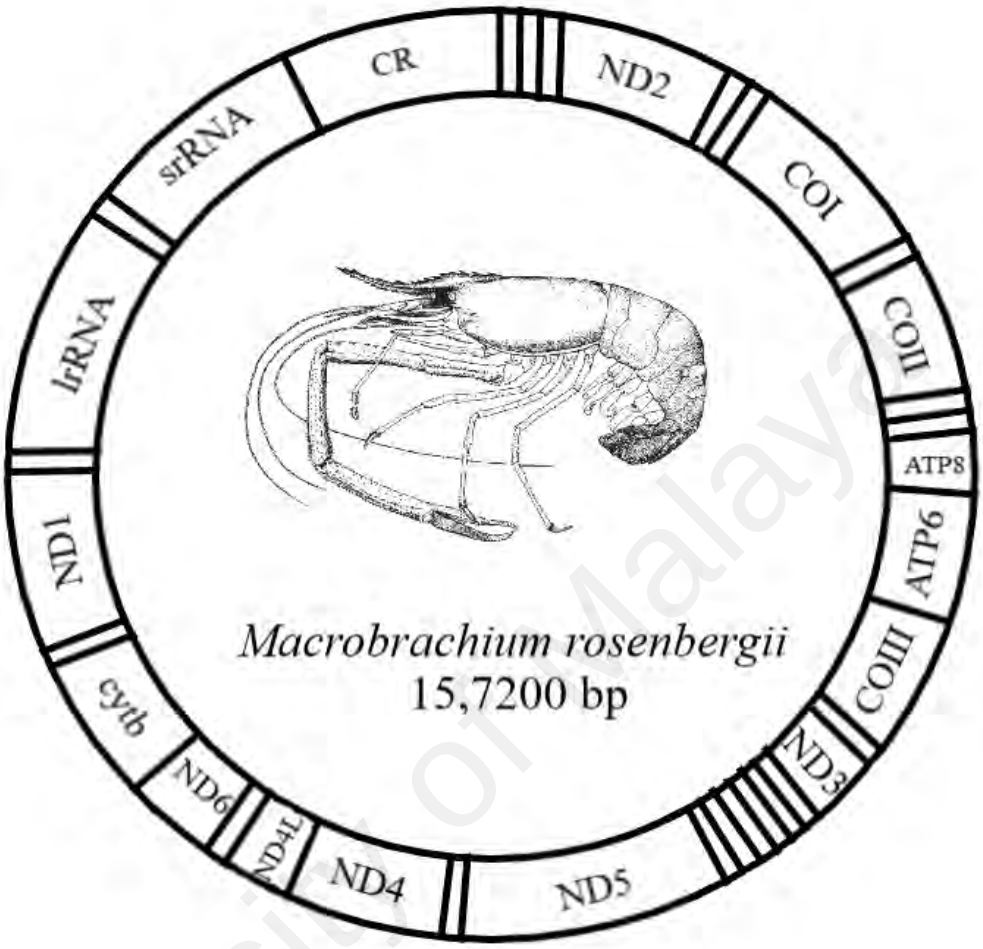

Figure 2.5: Gene map of mitochondrial genomes of GMP. Adapted from (Miller et al., 2005).

sources and low variation in the genetic structure were detected suggesting that all river populations could be considered as a single panmictic population (Shairah, 2011; Atin et al., 2017). However they found that the Sungai Penarik and Sungai Johor populations were genetically isolated in pairwise analysis and suggested crossing program could be done to the other populations for stock enhancement program (Shairah, 2011; Atin et al., 2017).

A few stock enhancement programs of GMP have been done in Malaysia to prevent depletion of genetic resources of GMP. Jaafar (2014) has investigated the effectiveness of stock enhancement program of GMP from Sungai Timun, Negeri Sembilan with Kampung Acheh, Perak using microsatellites and mitochondrial marker. It was concluded that the stock enhancement program was a success based on population structure produce an increment of allelic richness in first, second and third generation population from the wild and parental populations indicating the occurrence of hybridization between wild and parental populations (Jaafar, 2014). A similar study has been done in Sungai Petagas, Sabah using mtDNA *COI* gene. However, they found the very low genetic distance between the BMRI shrimp hatcheries and wild Sungai Petagas population (Maidin et al., 2017). Another mtDNA *COI* gene analysis was done to study the performance of cycling mating in preserving the genetic diversity of GMP. There were significant pairwise F_{ST} comparison between the progeny (86%) and parental populations (67%) showing this strategy is effective in preventing inbreeding (Elsheikh et al., 2015).

CHAPTER 3: MATERIALS AND METHOD

3.1 Genetic variations between domesticated and wild Giant Malaysian Prawn (GMP) using cytochrome oxidase subunit-1 (*COI*)

3.1.1 Collections of samples

The prawn samples used in this study were collected from Peninsular Malaysia. A total of nine populations were collected (Figure 3.1); 4 wild populations (Sungai Bernam, Selangor, Sungai Johor, Johor, upstream and lower stream of Sungai Perak, Perak) and 5 domesticated populations (Tanjung Tualang, Lenggong, Kampung Kelian Gunung, Baling and Jelebu ponds). Thirty wild stocks were collected from four wild riverine systems. The reasons chosen to obtain samples from the above riverine system were based on the current breeding schemes applied by industry and DOF to obtain brood stocks for breeding and domestication as reported by Atin et al. (2017) and Elsheikh et al. (2015). Perak wild sample had been screened and detected previously with infectious hypodermal and haematopoietic necrosis virus by Nita et al. (2012) where she found two populations; IHHNV infected populations and IHHNV non-infected population. Wild Perak sample was sampled in 2010 and the muscle tissues were preserved in 70% ethanol and were stored in -20℃ freezer. It is found that the haplotype frequency of Perak 2010 and 2013 (Elsheikh, 2015) are almost similar. Both of them has same major haplotypes. Therefore, we combine 2010 of wild Perak sample with wild Sg. Bernam, Selangor and Kota Tinggi,,Johor.

270 individuals of wild and domesticated GMP were used to investigate and compare the genetic variation of wild and domesticated GMP in Malaysia. The collection of prawn samples was divided into wild and domesticated populations. Table 3.1 shows the location, tag, sample size and year of sampling of GMP. The history and source of broodstocks of domesticated prawn were investigated and recorded (Table 3.2).

3.1.2 Sample storage and preservation treatment

The pleopod and muscle tissues of each freshwater prawn were dissected using a pair of sterile scissors and forceps and were stored in separate 1.5 ml microcentrifuges tube contained 1000 µl of absolute ethanol. Ethanol was used as it would not affect the quality of DNA and a high concentration of ethanol could denature protein that degrades DNA (Flournoy et al., 1996). Each ethanol-preserved tissue sample was stored at room temperature.

Table 3.1: Population, tag label, number of sample and year of sampling for GMP.

GMP	Population	Tag	No. of sample (n)	Year of sampling
Wild	Perak (IHHNV infected)	IP	30	2010
	Perak (IHHNV non-infected)	NP	30	2010
	Sg Bernam, Selangor	SBN	30	2013
	Kota Tinggi, Johor	KTJ	30	2013
Domesticated	Lenggong, Perak	LGG	30	2013
	Tanjung Tualang, Perak	TTL	30	2013
	Kg Kelian Gunung, Perak	KKL	30	2013
	Baling, Kedah	BLG	30	2013
	Jelebu, Negeri Sembilan	JLB	30	2013

Table 3.2: Source of broodstock for domesticated prawn.

Farms	**Source of fry & broodstock**
Lenggong, Perak	Bukit Gantang, Taiping & Teluk Intan
Tanjung Tualang, Perak	Teluk Intan & Sabah
Kg Kelian Gunung, Perak	Telok Intan
Baling, Kedah	Tg Piandang, Sitiawan and Merbuk
Jelebu, Negeri Sembilan	Tg Piandang & Telok Intan

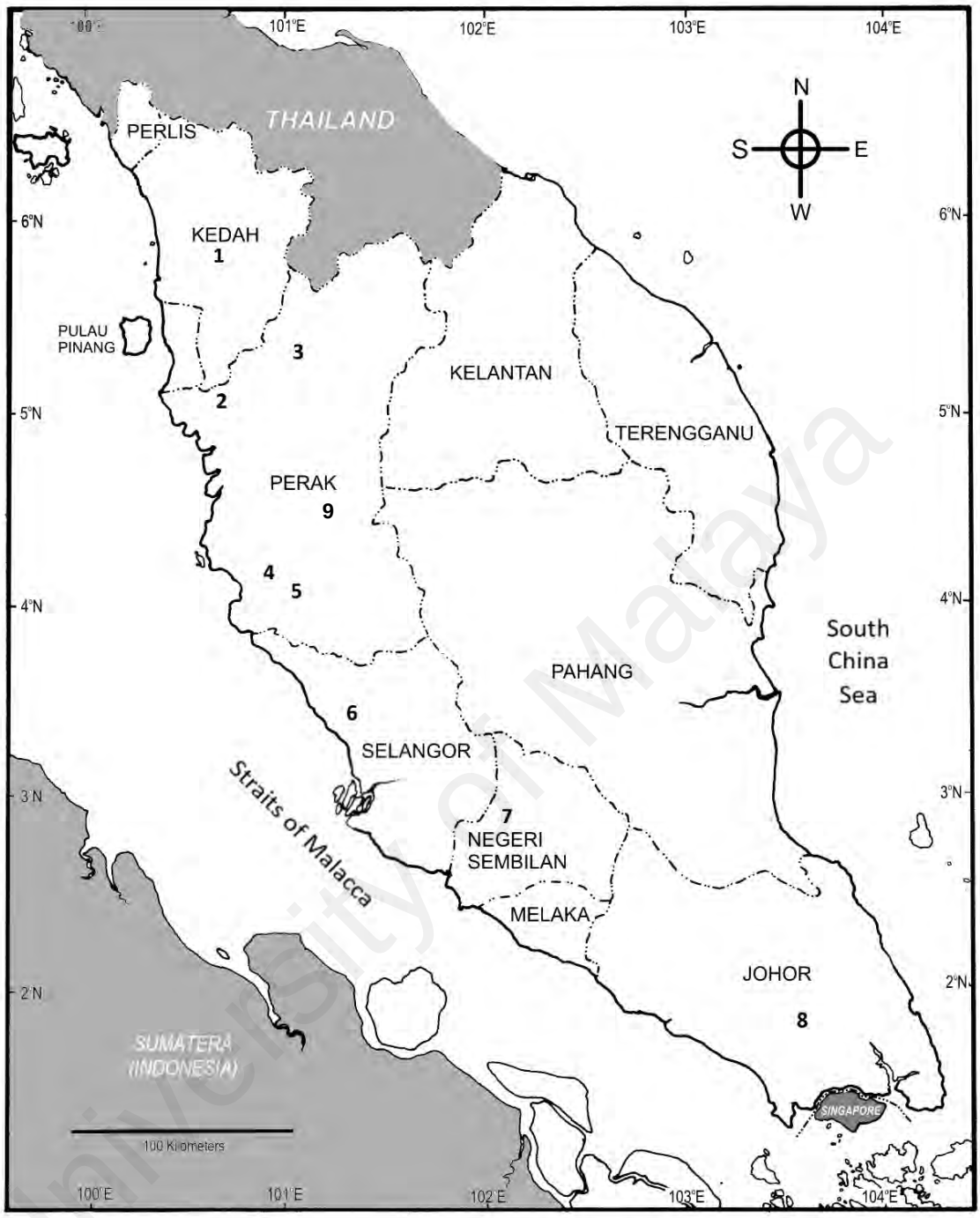

Figure 3.1: Location of selected sampling sites. 1. Baling; 2. Lenggong; 3. Kg Kelian Gunung; 4. IHHNV-infected Perak; 5. IHHNV-non-infected Perak; 6. Sungai Bernam; 7. Jelebu; 8. Kota Tinggi; 9. Tanjung Tualang.

3.1.3 Genomic DNA extraction and DNA storage

DNA was extracted from GMP muscle tissues based on the protocol provided by the DNA extraction kit (Qiagen DNA Extraction Blood & Tissue Kit) with some modified steps. Since the tissue was kept in ethanol, each tissue was rehydrated by rinsing it with distilled water. Approximately 20 mg of prawn tissue was cut and wad placed in a 1.5 ml microcentrifuge tube. Sample lysis was done by adding 180 µl ATL buffer and 20 µl Proteinase K was added to the tube. The homogenisation and incubation of the sample were done at 56ºC overnight. After the sample was entirely lysed, 200 µl of AL buffer was added and the incubation of the sample was done again at 56ºC for another 10 minutes. Precipitation of the DNA was done by adding 200 µl of absolute ethanol. The mixture was then vortexed briefly. For each sample, the DNeasy Mini spin column was put into a collection tube and the sample mixture was pipetted into the column. The sample was then centrifuged for 1 minute at 8,000 rpm. The flow-through was removed and the column was placed back into a new collection tube. 500 µl Buffer AW1 was added and was centrifuged for 1 min at 8000 rpm and again the flow-through was removed. The sample was then replaced with a new collection tube. 500 µl Buffer AW2 was added and the sample centrifuged at 14000 rpm for 1 minute. Flow through and collection tube was removed. The spin column was placed to a new 1.5 ml microcentrifuge tube. 50µl pre-heated Buffer AE was pipetted to the middle of the spin column membrane and was incubated at room temperature for 30-40 minutes to elute the DNA. Then the column was centrifuged for 2 min at 8000 rpm. To prevent chemical and enzymatic degradation, the extracted DNA was stored at -20ºC freezer prior to use.

3.1.4 Quantification and quality check of genomic DNA

NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to measure the concentration and the purity of extracted genomic DNA at optical density (OD) reading of A260/280. 1 µl of DNA was carefully pipetted into the lower measurement

pedestal of the spectrophotometer. The spectral measurement was started once the sampling arm was lowered. The concentration and the purity of DNA obtained were recorded. The desired DNA concentration used for this study was 50 ng/µl. The OD reading of A260/280 that lies 1.8 to 2.0 was considered to have a high purity of DNA.

Genomic DNA quality check was run on 1% agarose gel electrophoresis where 5µl of DNA sample with 1 µl loading dye was added in each well. The gel electrophoresis was carried out at 100V, 350mA in 1 × TBE running buffer for 45 minutes. Electrophoresis was completed once the yellow tracking dye had migrated to the end of the agarose gel and then was stained with ethidium bromide before visualising under ultra-violet (UV) light. After that, the photograph of the image was captured using AlphaImager™ 2200 gel documentation system, (Alpha Innotech, Kasendorf, Germany).

3.1.5 MtDNA *COI* gene amplification and sequencing

3.1.5.1 Amplification of mtDNA *COI* by polymerase chain reaction

In this study, LCO1490 and HCO2198 primer that was adapted from Folmer et al. (1994) were used for the amplification of mitochondrial cytochrome oxidase subunit I gene (*COI*). The *COI* gene was approximately 710 bp in length. The primer sequences used to amplify in this study are as follows:

LCO1490 (Forward primer)	5'-GGTCAACAAATCATAAAGATATTGG-3'
HCO2198 (Reverse primer)	5'-TAAACTTCAGGGTGACCAAAAAATCA-3

The polymerase chain reaction (PCR) amplifications were done in a 50µl reaction containing 100 ng DNA template, 1× PCR reaction buffer (Promega), 200 µM each dNTP, 0.08 µM of each forward and reverse primer, 2.5 mM of $MgCl_2$, 1.25 U of Taq polymerase (Promega, USA) and the remaining was ultrapure water. The PCR profile was initial denaturation at 94ºC for 7 min, followed by 29 cycles at 94ºC for 30 s, 45 ºC

annealing temperature for 30 s, extension at 72ºC for 30 s, and final extension at 72ºC for 5 min, in a master cycler machine (Eppendorf, Germany). The PCR products were stored in -20ºC freezer to avoid DNA degradation.

3.1.5.2 Gel Electrophoresis

After PCR amplification, the presence of PCR products was checked and validated by using 1% agarose gel electrophoresis. The pre-stained technique was used by adding PeqGreen Dye into the agarose gel. The gel electrophoresis was carried out at 80 V, 160 mA in 1 × TBE Running Buffer for 30 minutes. The gel was then visualised under ultraviolet light and the photograph of the image was captured using AlphaImager™ 2200 gel Documentation System, (Alpha Innotech, Kasendorf, Germany).

3.1.5.3 Purification of PCR products

PCR purification was performed by adding 200µl of Buffer NT to the PCR products and were incubated for 10 minutes at 50°C. The PCR products were pipetted into the NucleoSpin® column which was placed in a collection tube. The column was centrifuged for 1 minute at 11,000×*g* speed and the flow through was removed. Next, 600 µl of Buffer NT3 was placed into the column and again was centrifuged at 11,000×*g* for 1 minute After the flow through was removed, 200µl of Buffer NT3 was pipetted into the column and was centrifuged at 11,000×*g* for 1 minute. After that, the columns were centrifuged at 11,000×*g* for 2 minutes for the drying process. Then, the column was placed into a new 1.5 ml microcentrifuge tube. 50 µl of pre-heated elution buffer were pipetted to the center of the column. The columns were then incubated for 1 hour in the room temperature and were centrifugated for 1 minute at 11,000×*g*. The elution contained the purified DNA fragment which was then stored at -20 °C freezer for sequencing service.

3.1.5.4 Sequencing of purified PCR product

The purified PCR products were sequenced by MyTACG Bioscience where conventional Sanger sequencing method was applied. Sequencing of both forward and reverse directions was performed using the universal *COI* primer namely LCO1490 and HCO2198 (Folmer et al., 1994) on ABI Prism Genetic Analyser 3130xl (Applied Biosystem, USA).

3.1.6 mtDNA *COI* gene analysis

3.1.6.1 *COI* gene analysis of wild and domesticated prawns

All the sequences were viewed and checked using Chromas Software (Technelysium Proprietary Limited, Australia). The nucleotide sequences were trimmed to a length of 676 bps and were aligned using MEGA Software version 6.0 (Tamura et al., 2011). The number of conserved and variable sites were calculated using MEGA Software Version 6.0 (Tamura et al., 2011). Maximum likelihood tree was calculated using Tamura-Nei parameter (Tamura et al., 2013) with gamma distribution model of 5 categories using MEGA 6.0. Then haplotype data was generated using the software DnaSP 5.0 (Librado & Rozas, 2009). The demographic signature of mtDNA haplotype variation was evaluated with the pairwise mismatch distribution using DnaSP 5.0 software (Librado & Rozas, 2009). A haplotype network which connects all the haplotypes by mutational step was performed using software Network 5.0 (Bandelt et al., 1999). MEGA 6.0 is used to measure the mean pairwise genetic distance between populations using Kimura-2-parameter model (Kimura, 1980). The pairwise F_{ST} values for each combination of populations were also calculated and significance of this estimate was calculated using 1000 permutations of the data at P = 0.05 using Arlequin 3.5 (Excoffier & Lischer, 2010). The deviation of neutrality in GMP were computed by Arlequin 3.5 (Excoffier & Lischer, 2010) using Tajima's D and Fu's Fs statistics.

3.2 Genetic diversity of GMP by performing a temporal analysis between two different times which are 2004 and 2013

3.2.1 Source of sample

Here we utilised previous data from GMP populations collected in 2004 (Che Harun, 2013) and 2013 (Elsheikh et al., 2015). Temporal analysis was done to examine genetic differentiation between a total of 4 wild river populations of GMP in Che Harun and Elsheikh's report. All sample used in this analysis were recorded in the following table (Table 3.3). There was a total of 8 populations and 203 individuals used for temporal analysis. The Johor population in Elsheikh's report is the same set of population (KTJ) used in section 3.1 (see table 3.1).

3.2.2 Temporal analysis of GMP collected at 2004 and 2013

The AMOVA test (Excoffier et al., 1992) was run using the Arlequin Version 3.5 (Excoffier & Lischer, 2010) where all data were divided into two groups; 2004 data and 2013 data. The number of conserved and variable sites were calculated using MEGA 6.0 (Tamura et al., 2013). The relationships between haplotypes were illustrated in a maximum likelihood tree (Felsenstein, 1981) generated also by MEGA 6.0 and calculated with 1000 bootstrap replicates (Felsenstein, 1985). Then haplotype data was generated using the software DnaSP 5.0 (Librado & Rozas, 2009). A haplotype network which connects all the haplotypes by mutational step was performed using software Network 5.0 (Bandelt et al., 1999). Software Arlequin 3.5 was used to measure the pairwise F_{ST} values for each combination of populations were also calculated and significance of this estimate was calculated using 1000 permutations of the data at P = 0.05.

Table 3.3: Sampling location, years of collection and the total number of samples for temporal analysis between two different years of collection.

Year of collection	Sampling locations	n	Total	Sources
2004	Sungai Muda, Kedah	13	86	(Che Harun, 2013)
	Sungai Perak, Perak	30		
	Sungai Johor, Johor	30		
	Sungai Linggi, Negeri Sembilan	13		
2013	Sungai Muda, Kedah	28	117	(Elsheikh et al., 2015)
	Sungai Perak, Perak	29		
	Sungai Johor, Johor[1]	30		
	Sungai Linggi, Negeri Sembilan	30		

[1]Sungai Johor population is the same population (KTJ) used in section 3.1

3.3 Phylogenetic of GMP from Malaysia and Southeast Asian country using cytochrome oxidase subunit 1 (*COI*) mtDNA gene

3.3.1 Phylogenetic analyses using maximum likelihood

Molecular phylogenetics was examined based on analysis of mitochondrial *COI* of GMP from Malaysia, selected from the present study and 2004 data, and Southeast Asian. Additional sequence samples from Brazil, India and Bangladesh were added to the analysis to understand the pattern of Malaysian and Southeast Asia countries in comparison to other countries from non-SEA that also produced GMP (Brazil, India and Bangladesh) in large-scale (Table 3.4). All haplotypes from all populations in previous objectives are used with addition COI sequences from Che Harun, 2013 (Table 3.5).

3.3.2 Indices of diversity and haplotype network

Seven wild GMP populations used from objective 1 and objective 2 were used to compare with 35 haplotypes (GenBank accession numbers: GQ995505-GQ995518) that have been recorded by de Bruyn et al. (2005) and two haplotypes (GenBank accession numbers: GQ995505-GQ995518) that have been recorded by Iketani et al. (2011). This dataset was used to find the origin location of GMP in Southeast Asia. There were 672 *COI* sequences used to generate haplotype data. A haplotype network which connects all the haplotypes was performed using Network 5.0 (Bandelt et al., 1999).

Table 3.4: *COI* sequences used in phylogenetic tree analysis.

Locality	Region	Accession no	Reference
Irian Jaya	West Papua, INA	FM958079	Wowor et al. (2009)
Musi	Sumatra, INA	AY554317	de Bruyn et al. (2005)
Barito	Kalimantan, INA	AY554310	de Bruyn et al. (2005)
Raimangal	Bangladesh	AY554296	de Bruyn et al. (2005)
Meghna	Bangladesh	AY554298	de Bruyn et al. (2005)
Dongnai	Vietnam	AY554322	de Bruyn et al. (2005)
Mekong	Vietnam	AY554319	de Bruyn et al. (2005)
Kraburi	Southern Thailand (west coast)	AY554313	de Bruyn et al. (2005)
Tapi	Southern Thailand (east coast)	AY554324	de Bruyn et al. (2005)
Gujarat	Northwest India	KF499324	Hurwood et al. (2014)
Kerala	Southwest India	KF499337	Hurwood et al. (2014)
Orissa	Northeast India	KF499351	Hurwood et al. (2014)
Augusto Correa	Brazil	GQ995517	Iketani et al. (2011)
Tracuateua	Brazil	GQ995518	Iketani et al. (2011)

Table 3.5: Haplotypes sequence used in phylogenetic tree analysis.

Wild Population	Sequence Used
KTJ	KTJ 14, KTJ 16, KTJ18, KTJ 29
SBN	SBN 3, SBN 5, SBN 10, SBN, 16, SBN 20, SBN 24
IP	IP 26, IP 27, IP 28, IP 30
NP	NP 10, NP 11, NP, 17, NP, 27
Kedah	K 2, K 5, K 9, K21
Negeri Sembilan	N 1, N 15, N 16, N 24
Perak	N 10, N 17, N 18, N 19, N 29

CHAPTER 4: RESULT

4.1 Genetic variations between domesticated and wild GMP using cytochrome oxidase subunit-1 (*COI*)

4.1.1 DNA extraction and quantification analysis

DNA of 270 individuals of GMP was extracted. The extracted DNAs were quantified using Thermo Scientific NanoDrop Spectrophotometer. The quality of the extracted DNA is checked by running agarose gel electrophoresis.

Based on NanoDrop Spectrophotometer, the average DNA yield is 500ng/µl with the purity of A260/280 ranged 1.8-2.0 (Appendix A). The DNA concentration is high and the purity is also very good. The DNA is then diluted into working concentration (100 ng/µl).

All genomic DNA was subjected to 1 % agarose gel electrophoresis to visualise the band intensity of the extracted DNA. All extracted DNA produces bands indicating the sizes are more than 10 kb. The thickness of the bands indicated the amount of DNA. From figure 4.1, generally all genomic DNA produce high-intensity band indicating a high amount of DNA.

4.1.2 *COI* gene PCR amplification and Sequencing

PCR amplification of mitochondrial DNA of cytochrome oxidase subunit 1 (*COI*) gene on all 270 individuals of GMP was performed using the optimised condition of PCR master mix and temperature profile. The forward and reverse primer was adapted from Folmer et al. (1994).

4.1.2.1 PCR Amplification

The partial *COI* gene of 270 individuals of GMP was successfully amplified with size approximately 710 bp (Figure 4.2).

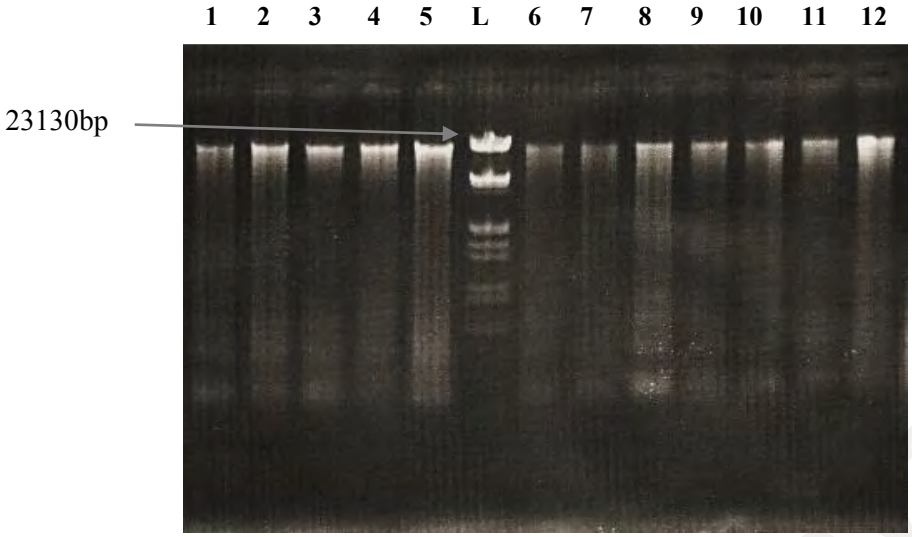

Figure 4.1: Image showing the presence of genomic DNA for 12 individuals in Kg Kelian Gunung population. Lambda DNA ladder (Promega, USA) was loaded into the gel as a ruler.

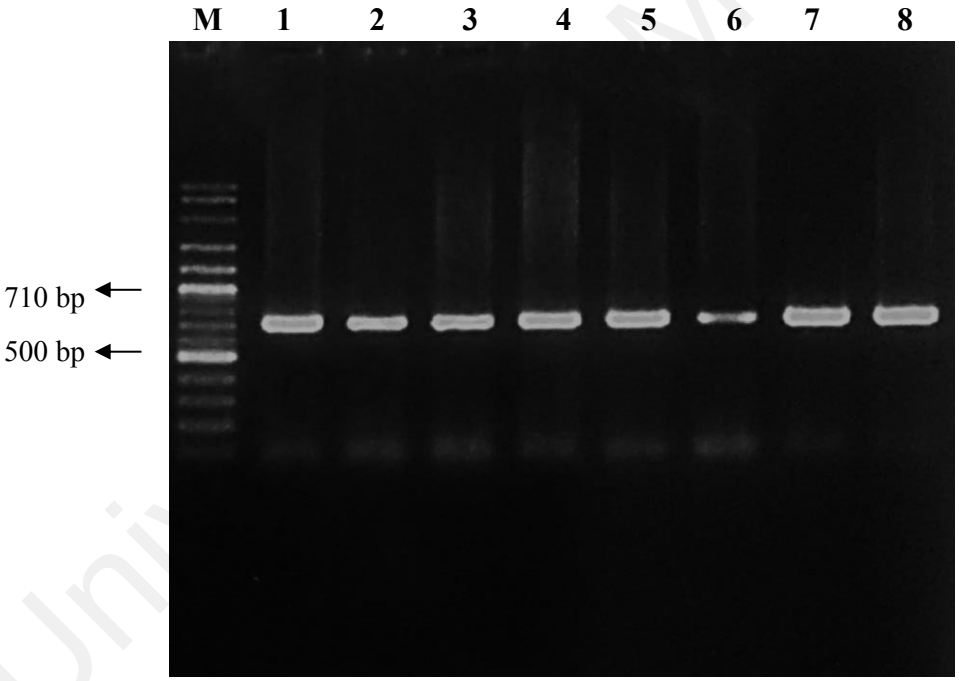

Figure 4.2: Image showing amplification of *COI* gene on 8 individuals selected from Tanjung Tualang farm. 100 bp DNA ladder (Promega, USA) was loaded into the gel as a ruler. The expected size of amplified products is 710 bp.

4.1.2.2 Sequencing of purified PCR products

All 270 sequences were checked based on clear peaks and the lack of baseline 'noise' on the sequence chromatogram. The average length of the sequences obtained is approximately 710 bp. Basic Local Alignment Search Tool (BLAST) was used to verify the sequences obtained were *COI* from GMP. The E-value is 0.0 with 100% query cover (Genbank accession no: NC006880.1).

4.1.3 Analysis of Partial MtDNA *COI* Gene of GMP

4.1.3.1 Information of partial *COI* sequence

After multiple sequence alignment, there are 699 bp of partial *COI* gene from 270 individuals of GMP with a total of 44 haplotypes. The sequences contained 608 conserved sites, 60 variable sites and 31 singleton sites where the rest included substitutions in only a single sequence (Table 4.1). The average nucleotide composition was T: 28 % A: 26.7 % C: 25.7% G: 19.6%. The average haplotype diversity (*H*) of all populations was 0.632 ($p < 0.05$) and nucleotide diversity (π) was 0.00684 ($p < 0.05$), of which the *H* of the wild population ($H = 0.667$, $p < 0.05$) has slightly higher of haplotype diversity than that of the domesticated population ($H = 0.605$, $p < 0.05$). Similarly, the nucleotide diversity of the wild population ($\pi = 0.00749$, $p < 0.05$) was slightly higher than that of domesticated populations ($\pi = 0.00634$, $p < 0.05$).

A total of 44 haplotypes were identified among 9 populations (Table 4.2). The main haplotype is haplotype 1 (160/270 individuals) that shared among all populations in Peninsular Malaysia. The other three major haplotypes; haplotype 2, haplotype 3 and haplotype 4 were observed in almost equal frequency. The wild populations recorded quite a high number of haplotypes, 9-10 haplotypes, compared to the domesticated populations, 3-7 haplotypes, except, the Lenggong populations that have 11 haplotypes. The haplotype number is the lowest in Jelebu population with 3 haplotypes only. Among

44 haplotypes, only 6 haplotypes were shared among both wild and domesticated populations, 22 haplotypes were shared among wild populations and 16 haplotypes generated from the domesticated populations only.

Pairwise genetic distance between 9 population showed less than 1% with the highest genetic distance is between KKL and LGG populations (0.9%) (Table 4.3). The values are too low indicating there no divergence between each population. The highest haplotype diversity (H) and nucleotide diversity (π) in wild GMP population is from SBN population (H= 0.7172, π = 0.009206, $p < 0.05$), while the highest H and π in domesticated populations are from LGG population (H= 0.7770, π = 0.009966, $p < 0.05$) (Table 4.4). It is noted that three populations of domesticated; LGG, BLG and KKL have significant high H and π and higher than wild populations (IP and KTJ).

Table 4.1: Number of haplotypes and the number of variable, conserved and singleton sites, where Wild populations; IP=IHHNV infected Perak, NP=IHHNV non-infected Perak, KTJ=Sg. Johor, SBN=Sg. Bernam, domesticated populations; TTL=Tanjung Tualang, BLG=Baling, JLB=Jelebu, KKL=Kg Kelian Gunung, LGG=Lenggong, n=number of individuals, Z=number of haplotype.

Group	Pop	n	Z	Number of sites		
				Variable	Conserved	Singleton
Wild	**IP**	30	9	60/699	608/699	31/699
	NP	30	9			
	KTJ	30	9			
	SBN	30	10			
Domesticated	**TTL**	30	5			
	BLG	30	7			
	JLB	30	3			
	KKL	30	6			
	LGG	30	11			
	Total	**270**	**44**			

Table 4.2: Haplotype frequencies in wild and domesticated GMP populations, where H=haplotype, C=Category of haplotype (Wild, domesticated or both), Wild populations; IP=IHHNV infected Perak, NP=IHHNV non-infected Perak, KTJ=Sg. Johor, SBN=Sg. Bernam, domesticated populations; TTL=Tanjung Tualang, BLG=Baling, JLB=Jelebu, KKL=Kg Kelian Gunung, LGG=Lenggong.

H	C	Wild populations				Domesticated populations					Total
		IP	NP	KTJ	SBN	TTL	BLG	JLB	KKL	LGG	
1	Both	17	16	19	16	24	14	26	14	14	160
2	Both	1	5	1	2	2	1	3	3	3	21
3	Both	4	1	4	0	0	10	0	1	2	22
4	Both	3	3	0	1	0	0	0	10	2	19
5	Wild	1	0	0	2	0	0	0	0	0	3
6	Wild	1	0	0	0	0	0	0	0	0	1
7	Wild	1	0	0	0	0	0	0	0	0	1
8	Wild	1	0	0	0	0	0	0	0	0	1
9	Wild	1	0	0	0	0	0	0	0	0	1
10	Wild	0	1	0	0	0	0	0	0	0	1
11	Both	0	1	0	0	0	0	0	0	1	2
12	Wild	0	1	0	0	0	0	0	0	0	1
13	Wild	0	1	0	0	0	0	0	0	0	1
14	Wild	0	1	0	0	0	0	0	0	0	1
15	Dom	0	0	0	0	0	0	0	1	0	1
16	Dom	0	0	0	0	0	0	0	1	0	1
17	Dom	0	0	0	0	0	0	0	0	1	1
18	Dom	0	0	0	0	1	0	0	0	2	3
19	Dom	0	0	0	0	0	0	0	0	1	1
20	Dom	0	0	0	0	0	0	0	0	1	1
21	Dom	0	0	0	0	0	0	0	0	1	1
22	Dom	0	0	0	0	0	0	0	0	1	1
23	Dom	0	0	0	0	0	0	0	0	1	1
24	Dom	0	0	0	0	1	0	0	0	0	1
25	Both	0	0	0	1	1	0	0	0	0	2
26	Dom	0	0	0	0	1	0	0	0	0	1
27	Dom	0	0	0	0	0	1	0	0	0	1
28	Dom	0	0	0	0	0	2	0	0	0	2
29	Dom	0	0	0	0	0	1	0	0	0	1
30	Dom	0	0	0	0	0	1	0	0	0	1
31	Dom	0	0	0	0	0	0	1	0	0	1
32	Wild	0	0	1	0	0	0	0	0	0	1
33	Wild	0	0	1	0	0	0	0	0	0	1
34	Wild	0	0	1	0	0	0	0	0	0	1
35	Wild	0	0	1	0	0	0	0	0	0	1
36	Wild	0	0	1	0	0	0	0	0	0	1
37	Wild	0	0	1	0	0	0	0	0	0	1
38	Wild	0	0	0	2	0	0	0	0	0	2
39	Wild	0	0	0	1	0	0	0	0	0	1
40	Wild	0	0	0	1	0	0	0	0	0	1
41	Wild	0	0	0	1	0	0	0	0	0	1
42	Wild	0	0	0	1	0	0	0	0	0	1
43	Wild	0	0	0	1	0	0	0	0	0	1
44	Wild	0	0	0	1	0	0	0	0	0	1
Total		30	30	30	30	30	30	30	30	30	270

Table 4.3: Mean pairwise genetic distance (%) of the wild and domesticated population of GMP based on *COI* sequence using Kimura-2-parameter model, (Wild populations; IP=IHHNV infected Perak, NP=IHHNV non-infected Perak, KTJ=Sg. Johor, SBN=Sg. Bernam, domesticated populations; TTL=Tanjung Tualang, BLG=Baling, JLB=Jelebu, KKL=Kg Kelian Gunung, LGG=Lenggong).

Between group mean distance	Wild populations				Domesticated populations				
	IP	NP	KTJ	SBN	TTL	BLG	JLB	KKL	LGG
IP									
NP	0.81								
KTJ	0.64	0.74							
SBN	0.84	0.89	0.76						
TTL	0.61	0.71	0.43	0.72					
BLG	0.60	0.70	0.38	0.73	0.40				
JLB	0.49	0.61	0.25	0.62	0.25	0.22			
KKL	0.91	0.93	0.94	0.97	0.88	0.91	0.83		
LGG	0.89	0.93	0.83	0.96	0.80	0.80	0.71	0.99	

Table 4.4: Haplotype and nucleotide diversity of the wild and domesticated population of GMP based on *COI* sequence, where, *N*=number of individuals, *Z*=number of haplotypes, *S*=polymorphic sites, *H*=Haplotype diversity and π=nucleotide diversity. (Wild populations; IP=IHHNV infected Perak, NP=IHHNV non-infected Perak, KTJ=Sg. Johor, SBN=Sg. Bernam, domesticated populations; TTL=Tanjung Tualang, BLG=Baling, JLB=Jelebu, KKL=Kg Kelian Gunung, LGG=Lenggong.

Group	Pop	*N*	*Z*	*S*	*H*	*π*
Wild	IP	30	9	21	0.6667	0.003211
	NP	30	9	18	0.6943	0.008521
	SBN	30	12	28	0.7172	0.009206
	KTJ	30	9	29	0.5931	0.004178
Domesticated	KKL	30	6	16	0.6805	0.009089
	LGG	30	12	29	0.7770	0.009966
	TTL	30	6	16	0.3632	0.003885
	JLB	30	3	14	0.2460	0.003724
	BLG	30	7	16	0.6851	0.003211

4.1.4 Interpopulation genetic variability

4.1.4.1 Phylogenetic tree: Maximum likelihood (ML) analysis

Maximum likelihood tree has divided the 44 haplotypes into 2 main clusters (Figure 4.3). Based on the tree, there is no clear difference showed between the wild and domesticated prawn as both groups contain both wild and domesticated populations. Interestingly, haplotype 35 which originated from Kota Tinggi, Johor is not clustered to any of the group. The two *COI* sequences *Macrobrachium asperulum* formed the outgroup in this tree.

4.1.4.2 Pairwise F_{ST} values among the wild and domesticated populations

Pairwise F_{ST} values for population differentiation in GMP among 9 populations have been generated by Arlequin 3.5 (Table 4.5). Mean pairwise F_{ST} values were observed ranging from 0.0058 (p-value < 0.05) to 0.2346 (p-value < 0.05). There are 17 of 36 pairwise F_{ST} between populations were significant. BLG populations showed significant F_{ST} differentiation among all wild and domesticated populations except KTJ and JLB populations. The highest pairwise F_{ST} value was recorded between KKL (domesticated) and KTJ (wild) population (F_{ST} = 0.2671, p < 0.01).

4.1.4.3 Analysis of Molecular Variance (AMOVA)

AMOVA showed that the highest variation was within populations, 93.31% with p-value < 0.05 (Table 4.6). However, the results from AMOVA revealed non-significant genetic structure between wild and domesticated based on F_{CT} value (F_{CT} = 0.0907, p > 0.05). AMOVA among populations within group of sample collection also showed significant structure (F_{SC} = 0.06689, p < 0.05)

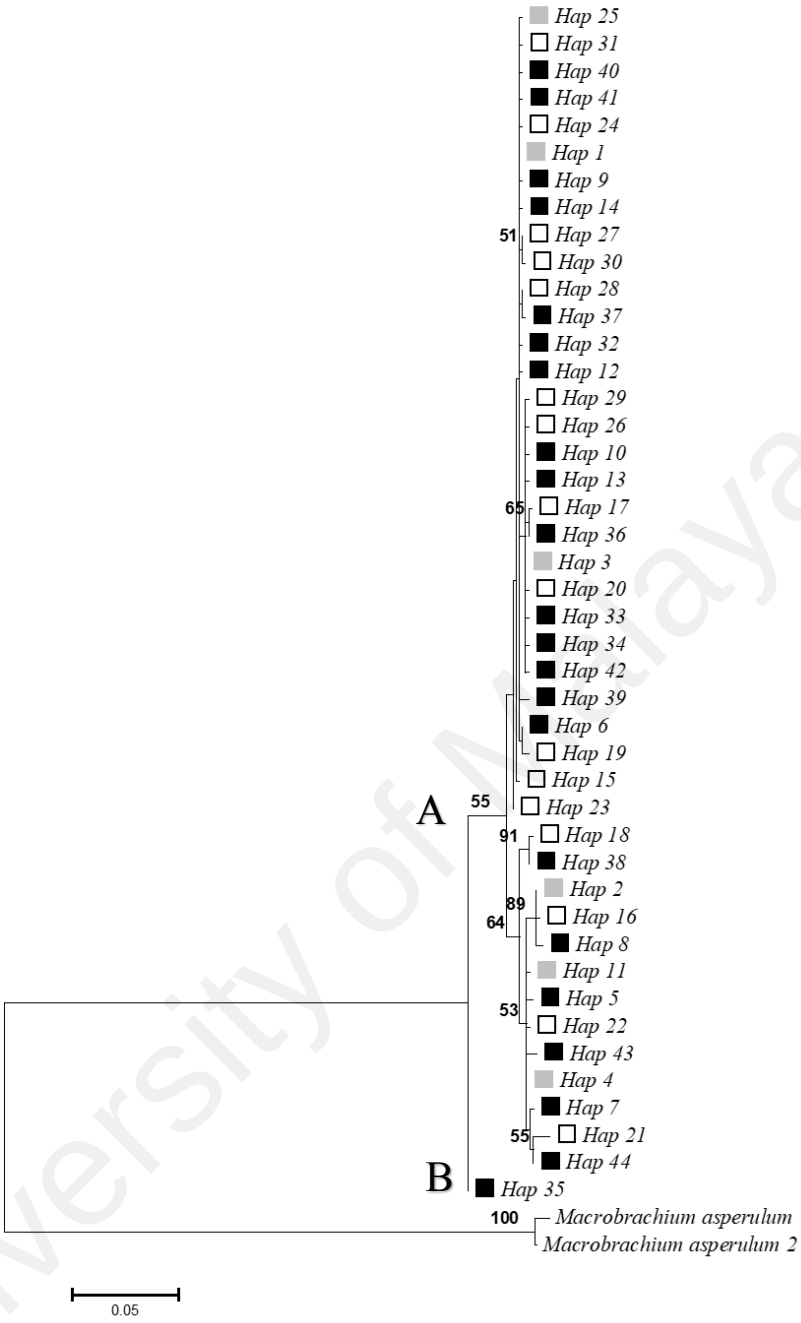

Figure 4.3: Phylogenetic tree from maximum likelihood analysis of the 44 haplotypes, with bootstrap 1000, Tamura-Nei parameter model, complete deletion. The outgroup *COI* sequence is *Macrobrachium asperulum*. (Key: ■=wild population, □=domesticated populations and ■= combination of wild and domesticated populations.

Table 4.5: Pairwise differentiation F_{ST} for *COI* gene sequence among GMP as computed by Arlequin 3.5, where the below diagonal: Pairwise differentiation between populations; the above diagonal: p-value of $F_{ST.}$ (Wild populations; IP=IHHNV infected Perak, NP=IHHNV non-infected Perak, KTJ=Sg. Johor, SBN=Sg. Bernam, domesticated populations; TTL=Tanjung Tualang, BLG=Baling, JLB=Jelebu, KKL=Kg Kelian Gunung, LGG=Lenggong.

	WILD				**DOMESTICATED**				
	IP	**NP**	**KTJ**	**SBN**	**TTL**	**BLG**	**JLB**	**KKL**	**LGG**
IP		0.7117	0.4955	0.6486	0.0090	0.0270	0.1351	0.0540	0.5315
NP	-0.0199		0.0360	0.6756	0.0090	0.0270	0.0540	0.2342	0.3423
KTJ	0.0577	0.1114*		0.0875	0.0360	0.4955	0.1351	0.0000	0.0000
SBN	-0.0174	-0.0180	0.0975		0.0360	0.0000	0.0900	0.1171	0.7387
TTL	0.0405*	0.0841*	0.0187*	0.0621*		0.0099	0.8108	0.0180	0.0090
BLG	0.0881*	0.1411*	-0.0086	0.1340**	0.0829**		0.0881	0.0000	0.0000
JLB	0.0352	0.0747	0.0249	0.0625	-0.0275	0.0881		0.0000	0.0180
KKL	0.0629	0.0171	0.2671**	0.0297	0.2346*	0.2346**	0.2346**		0.8198
LGG	-0.0112	-0.0238	0.1182**	0.1488	0.0987**	0.0829**	0.0963*	0.0059	

*Significant of F_{ST} *p-values < 0.05, **p-value < 0.01

Table 4.6: Result of AMOVA between wild and domesticated populations, as computed by Arlequin 3.5.

Source of variation	d.f.	Variance components	Percentage of Variation (%)	Fixation Indices	P-value
Among wild and domesticated	1	-0.06172	-2.62	F_{CT}: 0.0907	0.9980
Among populations within groups	7	0.21923	9.31	F_{SC}: 0.0669*	0.0000
Within populations	261	2.19719	93.31	F_{ST}: -0.02621*	0.0000
Total	269	2.35470			

Note: Significant *p-values < 0.05

4.1.4.4 Minimum spanning network based on median joining

Minimum spanning network (Figure 4.4) revealed four major haplotypes, Hap 1 and Hap 2 consist of all 9 populations. The larger the circle the higher its haplotype frequency and the longer the connecting lines the higher the number of mutational values between haplotypes. The distribution of each population was represented by the patterns of the pie.

4.1.4.5 Neutrality Test Tajima D's and Fu's Fs Test

Neutrality test was performed using Tajima's D and Fu' Fs statistics to evaluate the goodness-of-fit data in this populations. The Fu's statistics revealed no significant result in all populations (Table 4.7). Based on Tajima's test, KTJ, TTL and BLG populations showed negative value (-2.229), (-1.58) and (-1.59) respectively with significant P-value ($p < 0.05$) which suggested that there is recent population demographic expansion after a bottleneck event.

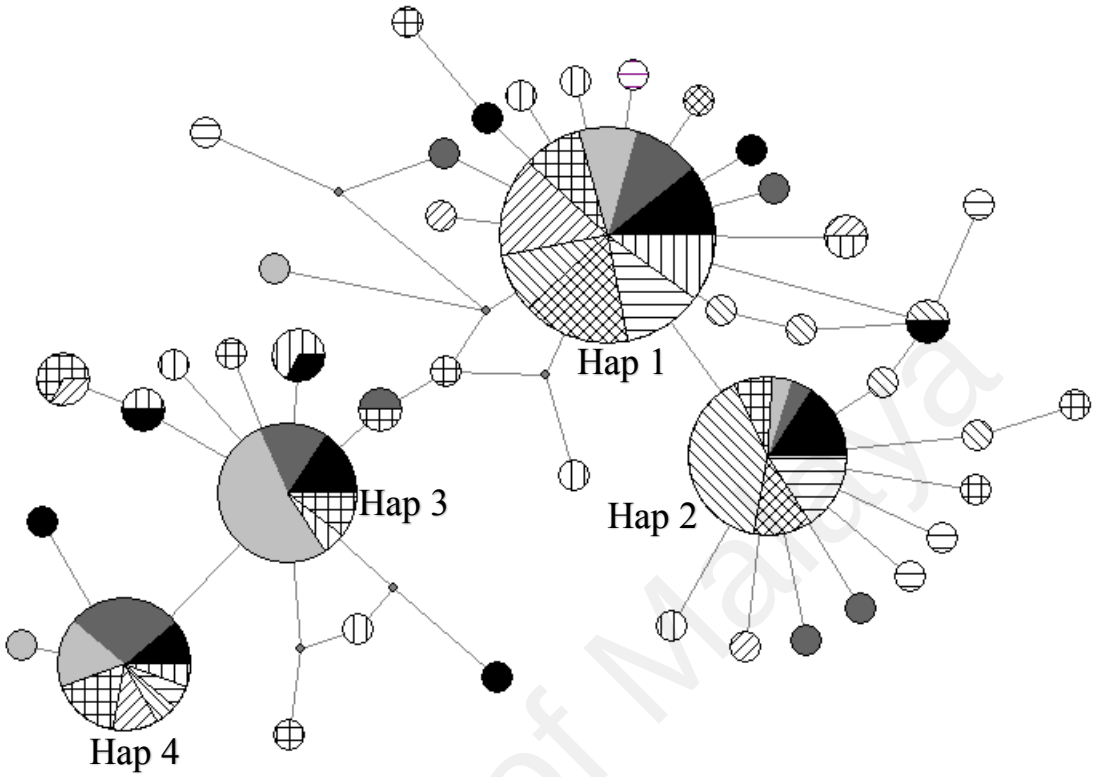

Figure 4.4: Median-joining haplotype network computed by Network 5.0 visualising the 44 haplotypes. Each pie represents haplotype and each pattern represent location of sampling.

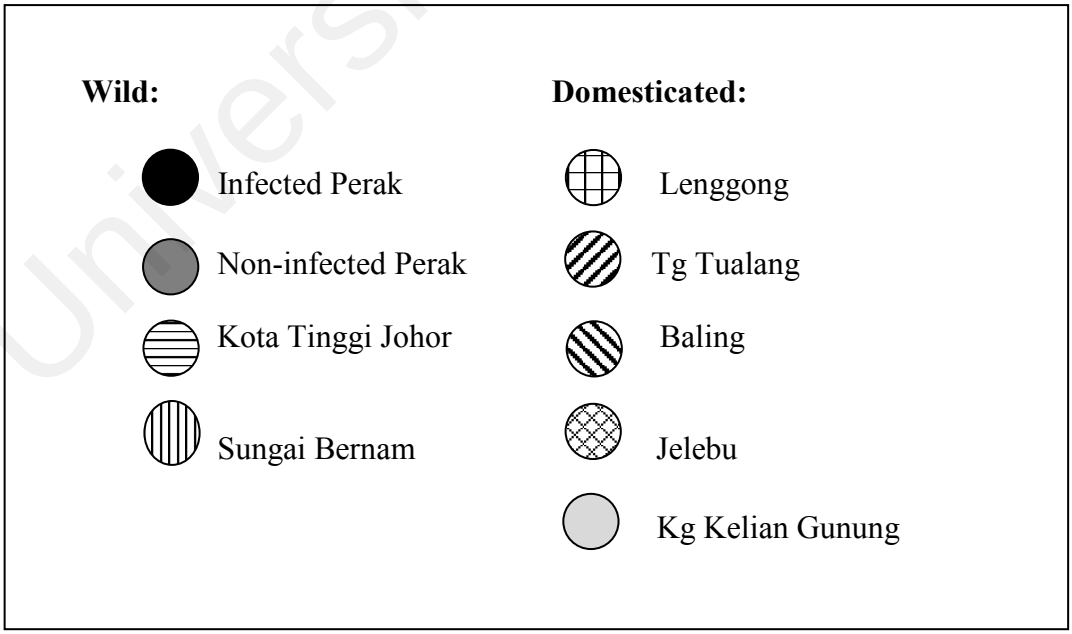

Table 4.7: Table of neutrality test using Tajima's D and Fu's Fs test (Wild populations; IP=IHHNV infected Perak, NP=IHHNV non-infected Perak, KTJ=Sg. Johor, SBN=Sg. Bernam, domesticated populations; TTL=Tanjung Tualang, BLG=Baling, JLB=Jelebu, KKL=Kg Kelian Gunung, LGG=Lenggong).

	Wild				**Domesticated**					**Mean**	**s.d.**
Statistics	IP	NP	KTJ	SBN	TTL	BLG	JLB	KKL	LGG		
Sample size	30	30	30	30	30	30	30	30	30	30	0.00
S	21	18	29	28	19	16	14	16	29	21.1	6.00
Pi	4.96	5.69	2.79	6.14	2.59	2.14	2.48	6.07	6.65	4.39	1.85
Tajima's D	-0.22	0.87**	-0.46	-0.32	-1.58*	-1.59*	-0.98	1.70	-2.22	-0.53	1.24
Tajima's D p-value	0.46	0.84	0.00	0.36	0.04	0.04	0.14	0.84	0.43	0.36	0.35
Fu's FS	1.13	1.69	-0.94	-0.15	1.24	-0.22	4.94	5.25	0.16	1.45	2.22
Fu's Fs p-value	0.73	0.76	0.35	0.51	0.78	0.47	0.97	0.96	0.53	0.68	0.22

Note: *p-value < 0.05, **p-value < 0.01

4.1.4.6 Mismatch distribution

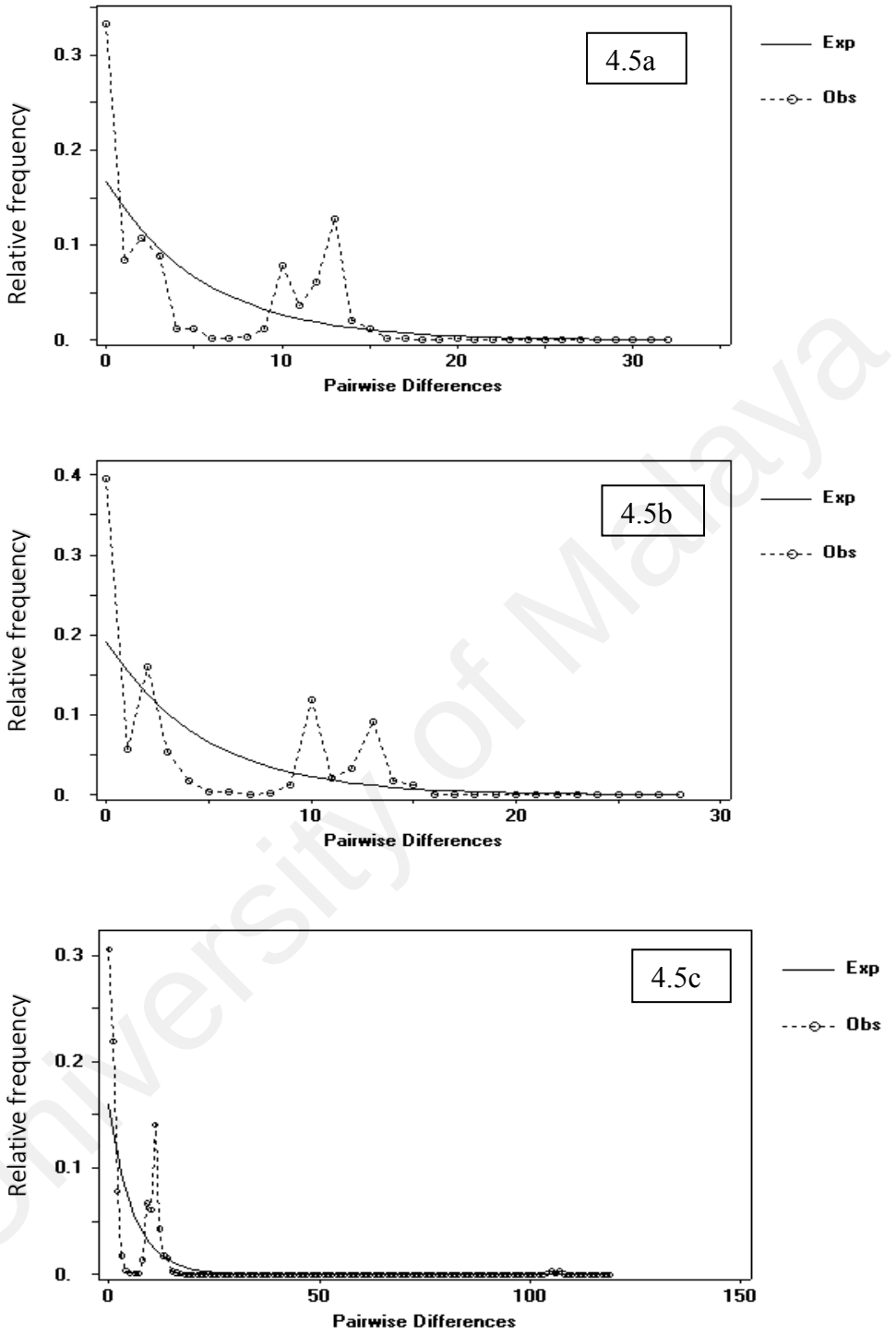

Figure 4.5: Observed and expected mismatch distribution of *COI* sequence in wild population (Figure 4.5a), domesticated population (Figure 4.5b) and combined wild and domesticated population (Figure 4.5c).

The demographic signature was measured with the pairwise mismatch distribution using DNAsp 5.0 software (Librado & Rozas, 2009). Mismatch distribution was performed using wild data, domesticated data and combined of wild and domesticated data (Figure 4.5).

The mismatch distribution test showed the wild and domesticated GMP have a multimodal distribution which indicates the absence of population expansion. In addition, the wild and domesticated populations data showed bimodal distribution of sharp peaks, which also suggests no population expansion, thus showing the populations have been constant over time (Rogers & Harpending, 1992).

4.2 Temporal analysis of GMP collected at 2004 and 2013

4.2.1 Information about *COI* sequence

Total 38 haplotypes were generated from both 2004 and 2013 data with the length of 574 bp sequence (Table 4.8 and Appendix D). Significant reduction of H was found from 2004 ($H = 0.7086$, $p < 0.05$) to 2013 ($H = 0.6618$, $p < 0.05$). There was a significant reduction of π from 2004 ($\pi = 0.01526$, $p < 0.05$) to 2013 ($\pi = 0.00825$, $p < 0.05$). Based on table 4.9, Sungai Kedah population recorded the highest H in both years, $H = 0.7949$ (2004), $p < 0.05$ and $H = 0.8571$ (2013), $p < 0.05$. There was a large reduction of H in Sungai Johor population from 0.6023 (2004) $p < 0.05$ to 0.1931 (2013), $p < 0.05$. The highest π in 2004 were observed in Sungai Johor population ($\pi = 0.024945$, $p < 0.05$) while the highest π in 2013 were observed in Sungai Kedah ($\pi = 0.010753$, $p < 0.05$).

Table 4.8: Haplotype frequencies of *COI* sequences in each GMP population of 2004 and 2013, where H=haplotype, C=Category of haplotype (2004, 2013 or both), 2004 data; JH=Sungai Johor, KD= Sungai Kedah, NS=Sungai Linggi, PR=Sungai Perak, 2013 data; J=Sungai Johor, K= Sungai Kedah, N=Sungai Linggi, P=Sungai Perak.

Hap	Category	2013				2004				Total
		P	K	N	J	PR	KD	NS	JH	
1	Both	12	8	20	27	12	6	8	19	112
2	2013	1			1					2
3	2013				1					1
4	2013				1					1
5	2013		4							4
6	Both	1	6	1		4				12
7	2013		1							1
8	Both	3	3	2		6	1			15
9	Both	1	1			1				3
10	2013		2							2
11	2013	1	2							3
12	2013		1							1
13	2013			1						1
14	2013			1						1
15	2013			1						1
16	Both	4		2		3	2	3	2	16
17	Both			1					1	2
18	2013	1		1						2
19	2013	1								1
20	2013	1								1
21	2013	1								1
22	2013	1								1
23	2013	1								1
24	2004					1	1			2
25	2004						1			1
26	2004						1			1
27	2004						1			1
28	2004								1	1
29	2004								2	2
30	2004								1	1
31	2004							1	1	2
32	2004								1	1
33	2004								1	1
34	2004								1	1
35	2004							1		1
36	2004					1				1
37	2004					1				1
38	2004					1				1
Total		29	28	30	30	30	13	13	30	203

Table 4.9: Haplotype and nucleotide diversity of the wild and domesticated population of GMP based on *COI* sequence, where, *N*=number of individuals, *Z*, number of haplotypes, *S*=polymorphic sites, *H*=Haplotype diversity and π= nucleotide diversity. 2004 data; JH=Sungai Johor, KD= Sungai Kedah, NS=Sungai Linggi, PR=Sungai Perak, 2013 data; J=Sungai Johor, K= Sungai Kedah, N=Sungai Linggi, P=Sungai Perak.

Years collected	Pop	*N*	*Z*	*S*	*H*	π
2004	JH	30	10	110	0.6023	0.024945
	KD	13	7	17	0.7949	0.009565
	NS	13	4	3	0.6026	0.001212
	PR	30	9	17	0.7931	0.005577
2013	J	30	4	3	0.1931	0.000350
	K	28	9	23	0.8571	0.010753
	N	30	9	16	0.5580	0.004239
	P	29	13	26	0.8153	0.009257

4.2.2 Population genetic structure and haplotype analysis

Genetic differentiation was performed between GMP collected at 2004 and 2013 (Che Harun, 2013) by standard AMOVA performed in Arlequin 3.5 (Excoffier & Lischer, 2010). AMOVA (Table 4.10) revealed that there is no significant structure between 2004 and 2013 data (F_{SC} = 0.0561, p > 0.05). However, AMOVA results revealed significant structure among populations within the two years of collection (F_{SC}= 0.2080, p < 0.05). The most percentage of variation observed was within the populations (83.64%) with significant structure (F_{ST}= 0.1636, p < 0.05).

Computed pairwise F_{ST} genetic differentiation revealed that Sg. Johor (2004) population has significant differentiation between all populations in 2004 and 2013 with F_{ST} ranging from 0.0365 to 0.5973 (p < 0.05) (Table 4.11). Sungai Kedah (2004) population showed significant differentiation with all populations except with Sg. Perak (2013) population. Sg. Linggi (2013) population showed significant differentiation with two populations only; Sg. Johor (2004) and Sg. Kedah (2004) population. The highest significant F_{ST} value was recorded between Sg. Johor (2004) and Kedah (2004), F_{ST} = 0.5973, p-value < 0.05, between Sg. Kedah (2004) and Sg. Linggi (2013), F_{ST} = 0.4790,

p-value < 0.05 and between Sg. Kedah (2004) and Sg. Linggi (2004), F_{ST} = 0.4283, p-value < 0.05 while the lowest significant F_{ST} value was recorded between Sg. Johor (2004) and Kedah (2004), F_{ST} = 0.5973, p-value < 0.05. There was significant F_{ST} value in Sg. Johor (2004) – Sg. Johor (2013), F_{ST} = 0.0365, p-value < 0.05, and Sg. Kedah (2004) – Kedah (2013), F_{ST} = 0.1577, p-value < 0.05, indicating Sg. Johor and Sg. Kedah became genetically different structure after 9 years. However, non-significant F_{ST} value was observed in Sg Linggi (2004) – Sg. Linggi (2013) and Sg. Perak (2004) – Sg. Perak (2013).

A maximum-likelihood (ML) tree of 38 haplotypes was generated using the Tamura-Nei parameter, bootstrap = 1000 between 2004 and 2013 data (Figure 4.6). *P. monodon* formed as an outgroup here. The ML tree revealed lack of genetic structure between 2 different years as the haplotypes were evenly distributed. The biggest clade, clade A, consisted of 2004 haplotypes, 2013 haplotypes and shared haplotypes between 2004 and 2013. Only haplotype 32 and 33, which both belonged to Sg. Johor (2013) population, were branched out from the major clade.

Similar results were shown in the minimum spanning network using medium-joining calculation (Figure 4.7). Two groups (group A and B) showed no genetic structure among all four populations from different years and there was no pattern geographic pattern was observed. Only haplotype 1, the major haplotype consists of all 8 populations (2004 and 2013). In line with the ML tree result, haplotype 32 and 33 were clustered in one group. The absence of these two haplotypes in 2013 data, suggesting there was a loss of genetic diversity happened here.

Table 4.10: Result of AMOVA between GMP collected in 2004 and 2013.

Source of variation	d. f.	Variance components	Percentage of Variation	Fixation indices	p-value
Among 2004 data and 2013 data	1	-0.180	-5.61	F_{CT}=0.0561	0.76442± 0.00000
Among populations within groups	6	0.704	21.97	F_{SC}= 0.2080*	0.00000± 0.00000
Within populations	195	2.681	83.64	F_{ST}=0.1636*	0.00000± 0.00000
Total	202	3.205			

Note: *p-value < 0.05

Table 4.11: Pairwise differentiation (F_{ST}) for *COI* gene sequence among GMP as computed by Arlequin 3.5, where the below diagonal: Pairwise differentiation between populations; the above diagonal: p-value of $F_{ST.}$ 2004 data; JH = Sungai Johor, KD = Sungai Kedah, NS = Sungai Linggi, PR = Sungai Perak, 2013 data; J = Sungai Johor, K = Sungai Kedah, N = Sungai Linggi, P = Sungai Perak.

	GMP collected at 2013				GMP collected at 2004			
	J	K	N	P	JH	KD	NS	PR
J		0.0270	0.0000	0.0180	0.0000	0.0180	0.0000	0.0000
K	0.5973*		0.0000	0.0000	0.0000	0.0090	0.0000	0.0901
N	0.0640*	0.4283*		0.0901	0.1711	0.1351	0.5135	0.0090
P	0.2061*	0.2139*	0.0448		0.0090	0.9009	0.0901	0.1351
JH	0.0365*	0.2844*	0.0284	0.0608*		0.0360	0.6036	0.000
KD	0.3471*	0.1577*	0.0775	-0.0430	0.0443*		0.0630	0.3784
NS	0.1257*	0.4790*	0.0039	0.0988	-0.0087	0.1679		0.0090
PR	0.4061*	0.0574	0.2138*	0.0373	0.1569*	-0.0035	0.2836*	

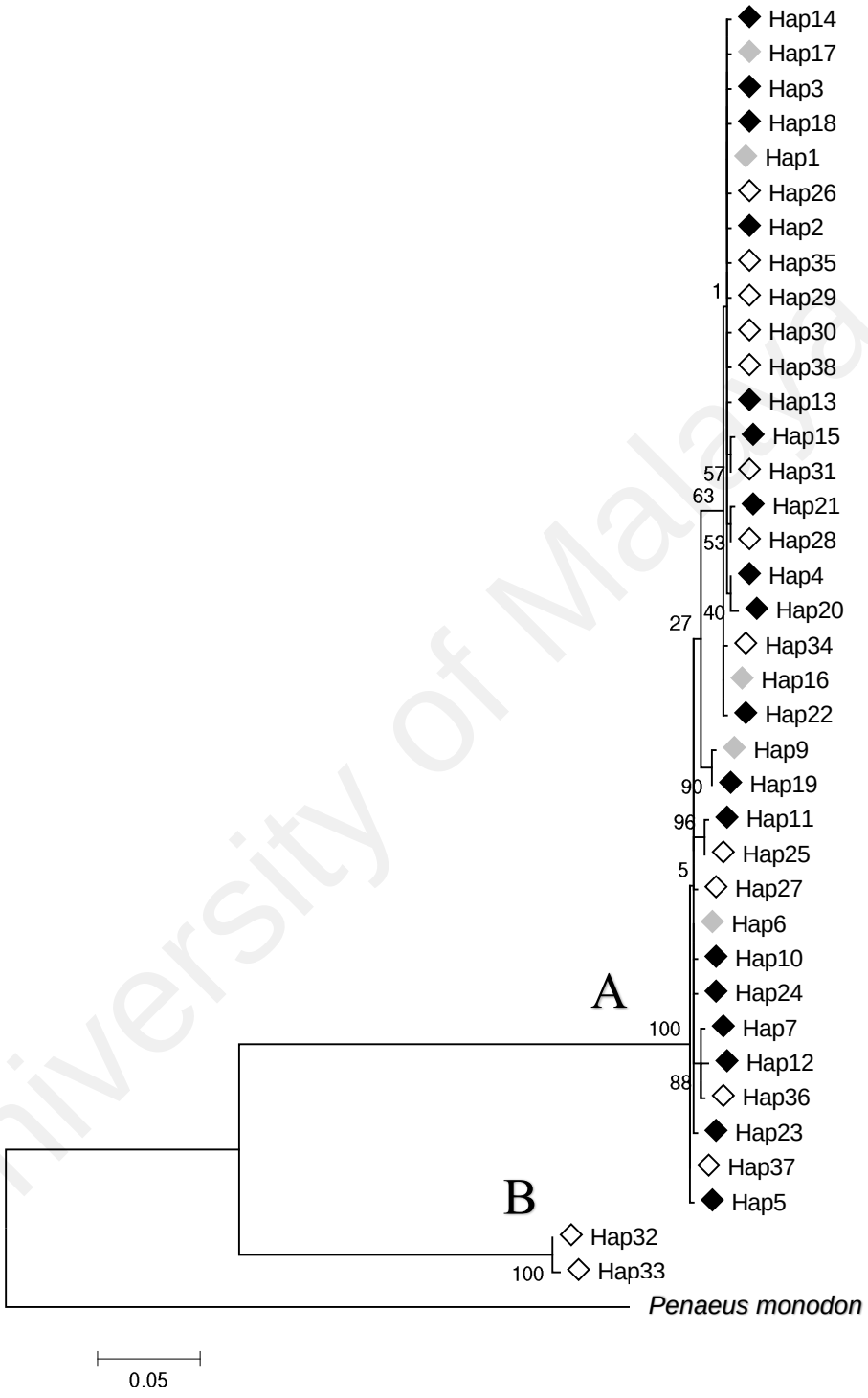

Figure 4.6: Phylogenetic tree from maximum likelihood analysis of 38 haplotypes, bootstrap 1000, Tamura-Nei parameter model and complete deletion. The outgroup is *COI* sequence of *P. monodon*, where, ◇ = 2004 haplotypes, ◆ = 2013 haplotypes and ◆ = haplotypes shared by 2004 and 2013.

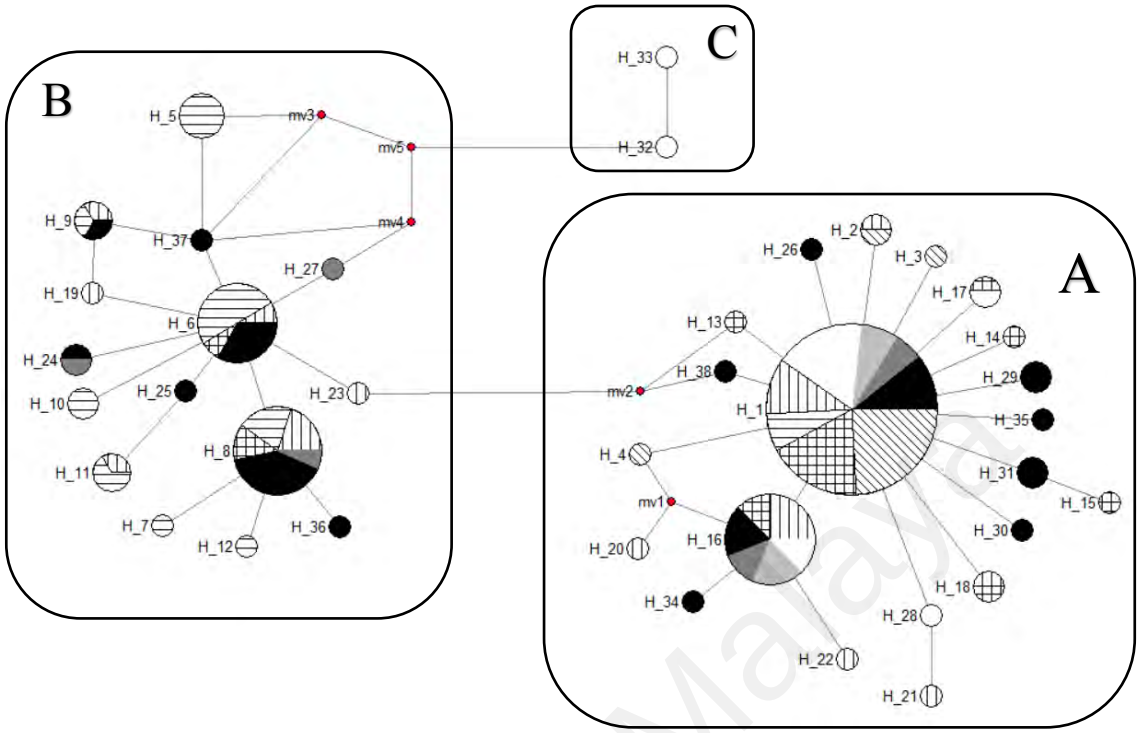

Figure 4.7: Median-joining haplotype network computed by network 5.0 visualising the relationship of 38 haplotypes for 2004 and 2013 data (Key; solid colour pie represents 2004 populations; black = Sg. Perak, dark grey = Sg. Kedah, light grey = Sg. Linggi, white = Sg. Johor; patterned pie represents 2013 populations; vertical = Sg. Perak, horizontal = Sg. Kedah, cross = Sg. Linggi, diagonal = Sg. Johor).

4.3 Phylogenetics of GMP from Malaysia and Southeast Asian country using cytochrome oxidase subunit 1 (*COI*) mtDNA gene

4.3.1 Phylogenetic tree based on maximum likelihood analysis

The dataset is used to infer the phylogenetics of GMP from Malaysia with Southeast Asia countries. Phylogenetics relationship based on mitochondrial gene *COI* showed that all populations seem mixed except the Irian Jaya populations. There are three clades in the phylogenetic tree (Figure 4.8). Clade A is a big clade consists of all populations except Irian Jaya and Sabah can be divided to A1 and A2. Clade A1 consists mainly of GMP from Peninsular Malaysia, Sarawak population with the addition of Orissa, Barito, Raimangal and Brazil populations. In clade A2, there is a mixture of Peninsular Malaysia, Sarawak, Vietnam, India, Indonesia and Thailand populations. Interestingly, East Malaysia countries, Sabah and Sarawak population are not clustered in one group. Sabah populations formed clade B with Brazil and Gujarat populations, while Irian Jaya population is the only one in clade C.

4.3.2 Indices of diversity and haplotype network

A total of 75 haplotypes (Appendix C) were generated from 672 sequences where haplotype 1-35 (GenBank Accession no: AY554293-AY554327) have been reported by de Bruyn et al. (2005). Haplotype 36 and 37 (GenBank Accession no: GQ985387-GQ985388) reported by Brazil team (Iketani et al., 2011) who investigated the geographic origins of GMP in Brazil. There is no haplotype were shared among all sixteen populations. The major haplotype is haplotype 29 (135/525) that were shared by all Southeast Asian countries; Malaysia, Vietnam and Indonesia. The additional of Malaysia populations used in this study resulted to more 28 haplotypes (hap 38-75) which are not present in the de Bruyn et al. (2005) and Iketani et al. (2011)(Appendix C).

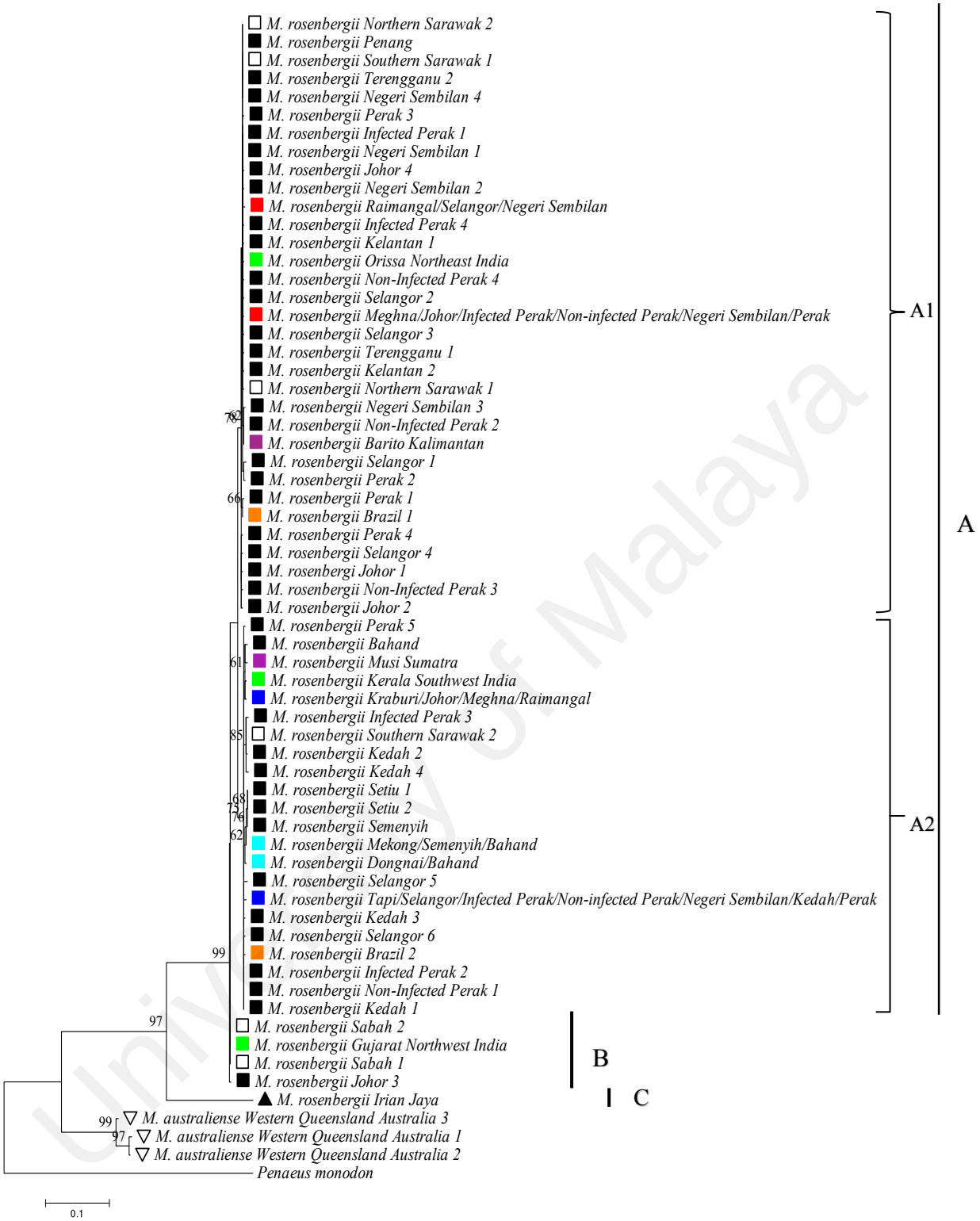

Figure 4.8: Phylogenetic tree of maximum likelihood analysis of *COI* sequence based on the Tamura-Nei model (Tamura, 1994). *M. australianse and P. monodon* formed the outgroup in this tree. (Key: Southeast Asia countries, ■ = Peninsular Malaysia, ○ = East Malaysia, ■ = Thailand, ■ = Vietnam, ■ = Indonesia, ▲ = Irian Jaya; Non-southeast Asia countries as comparison, ■ = Bangladesh, ■ = India, ■ = Brazil).

Table 4.12: Haplotype and nucleotide diversity of GMP from Southeast Asian countries, Bangladesh and Brazil based on *COI* sequence, where, N=number of individuals, Z, number of haplotypes, S=polymorphic sites.

Country	Location	*N*	*Z*	*S*	*H*	π
Bangladesh	Raimangal	32	7	11	0.7601	0.00597
	Meghna	34	6	8	0.6809	0.00399
Thailand	Kraburi	43	10	24	0.6246	0.01167
	Tapi	40	2	1	0.0500	0.00008
Malaysia	Setiu	15	2	5	0.1333	0.00111
	Semenyih	46	1	0	0.0000	0.00000
	Bahand	42	3	7	0.0941	0.00086
Vietnam	Dongnai	49	5	12	0.5391	0.00375
	Mekong	45	6	8	0.5788	0.00419
Indonesia	Musi	23	3	7	0.1700	0.00155
	Barito	36	3	14	0.3984	0.00852
Brazil	Augusto Correa	21	3	14	0.5524	0.01111
	Colares	18	3	16	0.3856	0.00717
	Taracuateua	21	3	16	0.3381	0.00740
Malaysia (wild)	Johor	30	9	28	0.5931	0.00453
	Selangor	30	12	29	0.7172	0.00991
	Infected Perak	30	9	20	0.6667	0.00813
	Non-infected Perak	30	8	17	0.6920	0.00934
	Kedah	28	10	26	0.8704	0.01162
	Negeri Sembilan	30	11	24	0.6437	0.00540
	Perak	29	13	28	0.8153	0.01022

*p-value < 0.05

According to table 4.12, the distribution of haplotype ranging from 1 to 13 haplotypes in each population. The Perak population recorded the highest number or haplotype (Z= 12), followed by Selangor with 10 haplotypes. Each location in Indonesia and Brazil countries have three haplotypes while Semenyih population was represented with one haplotype only. The highest haplotype diversity among all population is from Kedah population (H = 0.8704) with high nucleotide diversity (π = 0.01162).

Based on the median-joining network, haplotype 5, 14, 24 and 31 showed star-burst structure indicating that the three haplotypes are the central haplotype (Figure 4.9). Haploype 5, 14 and 24 consist of Malaysian population while haplotype 14 only consist of Vietnam population. Haplotype 5 consists mainly of Malaysia populations which connected to haplotype 14 that emerged one haplotype that consists of Bangladesh and Thailand populations. Haplotype 31 mainly are Thailand populations and give descendant haplotypes from Vietnam, Indonesia and Brazil. Generally, the network can be clustered into two groups (group I and II) where both groups consist of all countries except Vietnam population that only exist in group II.

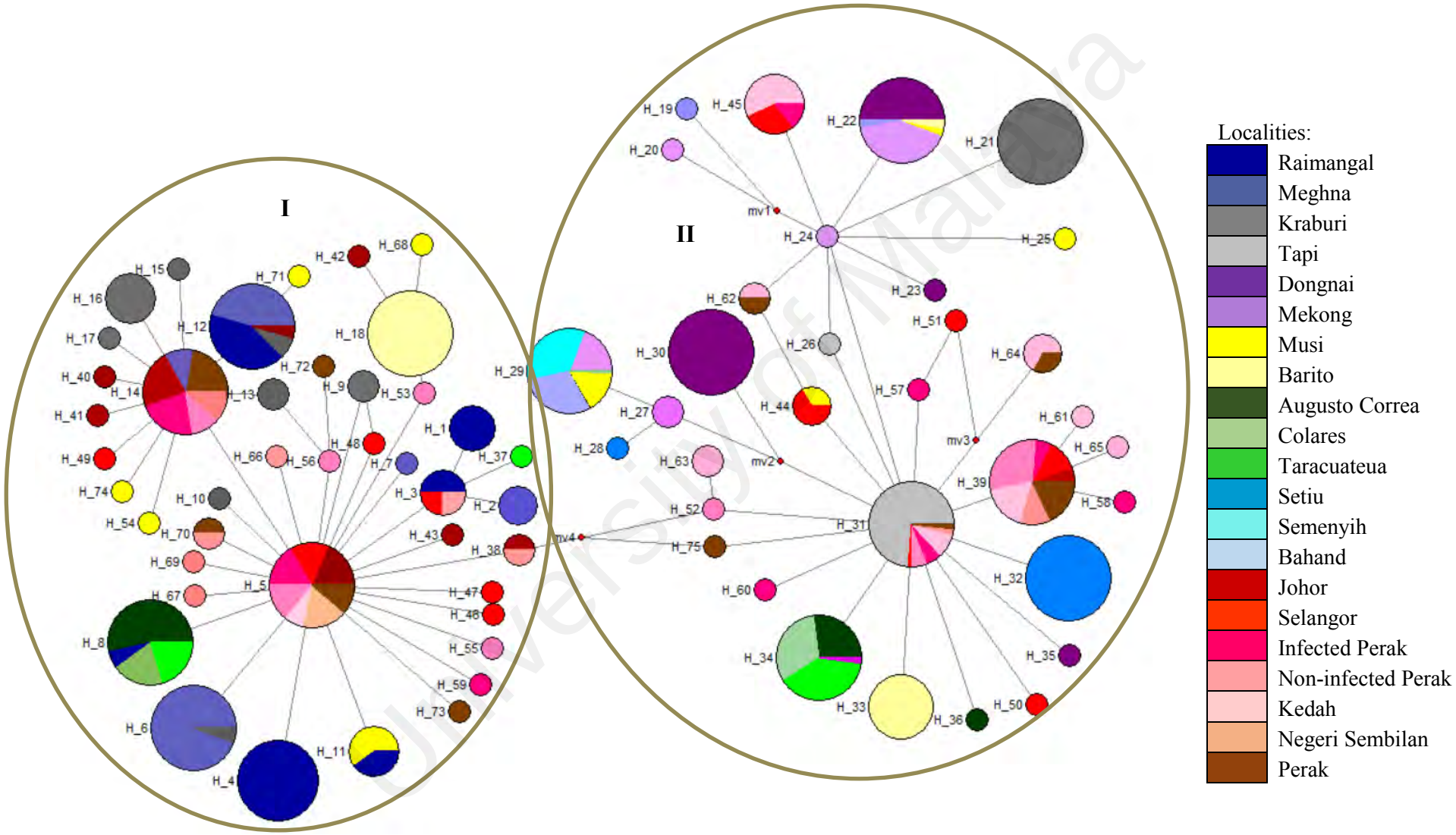

Figure 4.9: Minimum spanning network of Malaysian and populations from other countries.

CHAPTER 5: DISCUSSION

The Giant Malaysian Prawn (GMP) is a profitable and a popular freshwater species in Malaysia, Thailand, Bangladesh and several Southeast Asian countries (FAO, 2016). The farming activity of GMP has widely distributed around the world, however, there is limited knowledge on the levels of genetic diversity in wild, domesticated or both. The GMP industry is important to maintain food supply and increase the socio-economy status of the pond farmers and hatchery operators. Recently, the inconsistency of GMP size, as well as low production, has constrained the growth and sustainability of the GMP industry.

5.1 Genetic variations between domesticated and wild using cytochrome oxidase subunit-1 (*COI*)

For the first objective, the *COI* gene was utilised to investigate the genetic variation between wild and cultured GMP in Peninsular Malaysia. Forty-four haplotypes were generated from 270 *COI* gene sequences and were further analysed. Haplotype diversity (H) and nucleotide diversity (π) of wild population ($H = 0.667$, $\pi = 0.00749$, p-value < 0.05) were slightly higher than those of domesticated population ($H = 0.605$, $\pi = 0.00634$, p-value < 0.05). The wild population consisted of 28 haplotypes while there were 22 haplotypes in domesticated populations. The Fu's F of neutrality test showed positive value for all populations however the values are not significant. The neutrality test based on Tajima's D test revealed one wild population (KTJ) and two domesticated populations (TTL and BLG), showed negative value significantly ($p < 0.05$). Negative values in Tajima's test showed that those populations might undergo purifying selection or demographic expansion (Tajima, 1996; Martel et al., 2004). The other populations showed positive value indicating that there is no demographic expansion. This is consistent with mismatch distribution result where bimodal and multimodal pattern was observed suggesting the populations have been constant over time (Ray et al., 2003)

Maximum likelihood phylogenetic tree has divided into three clades. Two of three clades consist of both wild and domesticated GMP. Similar results were shown in the minimum spanning network analysis that the wild and domesticated GMP does not diverge. In addition, the AMOVA test also shows no genetic variation between the wild and domesticated GMP but only showed significantly high genetic variation within populations. This lack of genetic variation between wild and domesticated population probably because the collected domesticated GMP was an F1 generation of the broodstock. The utilisation of F2, F3 or higher generation may show genetic divergence by reducing the genetic diversity. Another reason that can explain why there is no significant genetic variation between wild and domesticated populations is due to restocking activity that releases domestic GMP into natural river populations which might cause adaptation to the GMP population. Besides that, accidental release, for example, flooding might release the domesticated GMP into the riverine that contributing to the lack of differentiation or structure between the wild and domesticated GMP. Otherwise, opposite phenomenon could happen as there might be the introduction of wild GMP into some hatchery stocks due to preferences (Schönhuth et al., 2003).

One more clade in the ML tree is represented by only one haplotype (Hap 35) which is from the natural river of Johor populations. This is supported by the negative of Tajima's D value of Johor population (-2.229) and the p-value is highly significant. The negative value signifies that rare alleles are present in low frequencies suggesting there is population expansion after a recent bottleneck (Maruyama & Fuerst, 1985). This result is supported by a study using EST SSR which revealed that there are unique alleles which suggesting isolation happened in that population (Hedayati, 2014; Elsheikh et al., 2015).

There is no clear genetic structure were discovered between wild and domesticated of GMP population based on the AMOVA analysis. There are signs of gene flow and

population admixture between the two groups. The lack of genetic structure between wild and domesticated GMP populations showed that difference of productivity between wild and domesticated may not due to genetic factors but could be due to the management factors. However, three domesticated population which are KKL, BLG and LGG showed higher genetic variation than a few wild populations. KKL, BLG and LGG domesticated populations showed higher *H* and π rather than the wild populations. This is probably due to high mutation rate and big effective population size in the population causing maintenance of neutral alleles , thus, help in maintaining high genetic variation in a population (Kimura & Crow, 1964; McMillen-Jackson & Bert, 2004). As the domesticated population able to maintain high genetic variation, these three domesticated populations can be used as base population for genetic stock improvement (Ren et al., 2018) to prevent overharvesting of wild GMP population. This strategy has been done in Latin American shrimp (Goyard et al., 2008), GMP in Thailand and *Penaeus vannamei* in China (Ren et al., 2018). Utilising highly genetic variability of the domesticated population as a base population is advantageous so that the wild stocks can be preserved and to prevent genetic disturbance in wild stocks population. Therefore, in this study, we suggest that the domesticated populations of KKL, BLG and TTL could be a good candidate as base population for stock improvement GMP program or selective breeding in Malaysia.

There are a few weaknesses in this objective. We have used Perak wild population, which were sampled three years earlier in 2013 as a geographical sample. They might not be comparable. Four or five generations would have passed within the three years as the generation time of GMP is approximately 6 months (Kumar et al., 2017). Due to the lack of sample in wild populations of Perak we combined the 2010 samples with 2013 samples for comparison to domesticated samples. Therefore, in future, we hope we can run the

proper analysis using same years with addition of more wild and domesticated populations.

5.2 Temporal analysis of GMP collected at 2004 and 2013

The temporal analysis was done between GMP collected at 2004 and 2013 (Che Harun, 2013). The results from AMOVA showed that there was no significant structure among populations that collected at 2004 and 2013. However, there is reduction of haplotype diversity and nucleotide diversity from 2004 populations to 2013. The absence of cluster C, haplotype 32 and 33 (see Figure 4.7) after nine years suggesting that there is a loss of genetic diversity which may lead to extinction due to overfishing (De Grave et al., 2015). This is consistent with computed pairwise F_{ST} genetic differentiation which revealed that there is significant F_{ST} value in Sg. Johor (2004) – Sg. Johor (2013), F_{ST} = 0.0365, p-value < 0.05, and Sg. Kedah (2004) – Kedah (2013), F_{ST} = 0.1577, p-value < 0.05, suggesting that Sg. Johor and Sg. Kedah become genetically different structured after 9 years. (2013).

The reduction of genetic diversity could be explained by sex-biased dispersal. Dispersal influenced the evolution of an organism. We could trace the sex-biased dispersal patterns in a random mating riverine system (Fraser et al., 2004). However, in this study, we did not record the sex of each GMP involved, thus, tracing of the dispersion is impossible. Since mtDNA is maternally inherited, the results shown here only reflected the translocation of female broodstocks to other populations. Utilising nuclear DNA, which is biparentally inherited, such as microsatellites and RAPD markers, could give different results than we have reported here. In future, it would be interesting to investigate the mechanism of sex-biased dispersal in male and female wild population.

Conservation of wild stocks of GMP needs to be done fast to prevent the extinction of this species. Although the IUCN stated that GMP as the least concern in Red List

Category and Criteria (De Grave et al., 2013), GMP requires special attention in conservation activity due to a drastic decline in genetic diversity (Ibrahim & Ilias, 2006). A few GMP stock enhancement program has been done in Sungai Timun, Negeri Sembilan (Jaafar, 2014) and Sungai Petagas, Sabah (Maidin et al., 2017). Here, we propose extensive conservation steps are important to prevent the extinction of GMP in Malaysia.

5.3 Phylogenetics of GMP from Malaysia and Southeast Asian country using cytochrome oxidase subunit 1 (*COI*) mtDNA gene.

In the third objective, we want to investigate the geographic origin of GMP the by studying the phylogenetic relationship of GMP from Malaysia and Southeast Asian countries. In 1965, a number of specimens of GMP were taken to Hawaii and research continued in producing GMP at large-scale. This led to the introduction of GMP to other parts of the world but this activity was not fully documented. Studies on the movement and management of GMP from different geographical regions is important as wild Malaysian species can be regarded as an important germplasm of fisheries management in Malaysia

Total 75 haplotypes were generated from 672 sequences where haplotype 1-35 has been reported by de Bruyn et al. (2005) (GenBank Accession no: AY554293-AY554327). Haplotype 36 and 37 reported by Brazil team (Iketani et al., 2011) where previously they concluded that their GMP was not originated from Malaysia, based on *COI* sequences from Setiu, Semenyih and Bahand only. They proposed that the GMP from Brazil are descendant from Bangladesh, Thailand or Vietnam populations. The two wild populations of GMP from Malaysia populations collected from Selangor and Johor in 2012 resulted to another 37 haplotypes (hap 38-75) which are not present in the earlier reports. Surprisingly the GMP from Malaysia in this study does not share any haplotype with Malaysia populations that reported by de Bruyn et al. (2005) which only represented

three populations in Peninsular Malaysia. The discrepancy occurred in de Bruyn et al. (2005) and (Iketani et al., 2011) report is due to the lack of populations used which did not represent the gene pool of giant freshwater prawn in Malaysia. Current study which involved additional populations from west-coast (Selangor) and south-coast (Johor) of Peninsular Malaysia will give a good coverage and better representative of the gene pool of GMP in Malaysia. In future, *COI* sequences from East Malaysia; Sabah and Sarawak should be added to give more information of the genetic structure of GMP in Malaysia.

Haplotype 5, 14, 24 and 31 showed a star-like cluster of haplotype network. This indicated that haplotype 5, 14, 24 and 31 are the dominant haplotypes where many descent haplotypes derived from the dominant haplotypes (Ferreri et al., 2011; Teixeira et al., 2011). This pattern could suggest the most haplotypes originate recently and as an indicator of population expansion during the recent history of taxa *Macrobrachium* (Ferreri et al., 2011). In cluster 1, haplotype 5 and 14 which comes from Malaysia populations and surrounded by haplotypes from which suggested that Malaysia could be the possible origin of GMP. Haplotype 5 give rise to multiple descendant haplotypes which represent Thailand, Bangladesh and Brazil. In cluster 2 of minimum spanning network showed haplotype 31 as central haplotype which consists of Thailand and Malaysia populations. Haplotype 31 give rises to descendent haplotypes which represent Vietnam, Indonesia and Brazil.

On that note, we also establish significant haplotype that clusters stocks from specific locations in Malaysia which are grouped to other Southeast Asian countries. We also faced difficulties in using all the Malaysian *COI* data we have to infer the genetic relationships and infer the possible origin of stocks of the GMP. We could not include few locations such as Kedah, Perak, Endau, Sabah and Sarawak that was sampled in 2004 (Che Harun, 2013), in our final analysis as the length of the sequences is much shorter

with more than 100 bp differences and multiple alignments of these stocks with reference haplotypes of other countries will change the generated haplotype. Due to the constraint and problems faced, it is proposed all 5 locations need to be resequenced to obtain better coverage which is more than 600bp in length, and in future, could be reanalysed and incorporated to give a better representation of the actual situation. It is also hoped to include additional wild stocks especially from India, Bangladesh, Sri Lanka, Cambodia, Vietnam, Sumatera, Java Island, Kalimantan, Sulawesi, Madagascar, Philippines, Papua New Guinea to resolve the important question which is to find the actual geographic origin of GMP.

CHAPTER 6: CONCLUSION

In conclusion, the utilisation of mitochondrial DNA cytochrome oxidase subunit 1 (*COI*) could reveal the genetic structure of GMP in Malaysia. We discovered the genetic variation of wild and domesticated GMP are non-significantly different. There are signs of gene flow and population admixture occurring between these two groups. However, three domesticated GMP populations; KKL, BLG and LGG showed higher genetic variation than the wild populations. We propose these three populations could be used as the base population in GMP genetic improvement program signs of gene flow and population admixture occurring between these two stocks. Temporal analysis between GMP collected at 2004 and 2013 revealed that there is a reduction in genetic diversity in wild population, especially in Sg. Johor and Sg. Kedah. Precautionary measures need to be taken as the haplotype diversity has reduced from temporal timescale over 9 years span which means there is an indication of overharvesting of stocks and reduction of effective population size. Johor populations still exhibit unique alleles which means this populations can still be differentiated from the other stocks and can be managed in a sustainable manner to avoid further actions of genetic depression. Analysis between GMP from Malaysia with Southeast Asian countries revealed that that geographic origin location could be from Malaysia based on the haplotype network. However further investigation should be done by comparing GMP from Malaysia with more populations within Southeast Asian countries and nearby region to validate it. Wild Malaysian stocks, Johor and Selangor have high haplotype diversity and managing stocks from those populations should be done to avoid a problem such as inbreeding and reduction of gene diversity. The utilisation of nuclear markers such as microsatellites marker could give more insights and discovery about the current status of wild and domesticated GMP in Malaysia and Southeast Asia.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

i. **Publication 1 (co-author)**

Atin, K., Christianus, A., Fatin, N., **Lutas, A. C**., Shabanimofrad, M., & Bhassu. S. (2017). Genetic diversity analysis in Malaysian giant prawns using expressed sequence tag microsatellite markers for stock improvement program. *Genetics and Molecular Research, 16*(3).

ii. **Papers presented**

Lutas, A. C. & Bhassu, S. (2014, February) *Utilization of EST-SSR in Population Studies in Giant Freshwater Prawn, Macrobrachium rosenbergii*. Poster presented at Asian Fisheries Biodiversity Conference, Penang, Malaysia.