POPULATION GENETICS OF *Rhizophora apiculata* IN PENINSULAR MALAYSIA USING MICROSATELLITE MARKERS

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POPULATION GENETICS OF *Rhizophora apiculata* IN PENINSULAR MALAYSIA USING MICROSATELLITE MARKERS

ABSTRACT

Rampant illegal logging, overharvesting and deforestation coupled with climate change, pose significant threats to the natural stands of *Rhizophora apiculata*, or locally known as Bakau Minyak; one of the most economically and ecologically important species of mangroves in Peninsular Malaysia. Despite being a dominant mangrove species, the reduction in the number of *R. apiculata* in its habitat has resulted in concerns over the long-term survival potential of the species. In Malaysia, genetic information to develop effective guidelines for the conservation and management of mangrove species has been lacking, and hence, further research should be conducted to fill this gap. The present study was therefore designed to generate novel genetic information for *R. apiculata,* aiming to facilitate the efforts to maintain the genetic diversity of the species in Peninsular Malaysia. A set of novel genic microsatellite markers was generated using an in-house transcriptome dataset of *R. apiculata* to assess its level of diversity and population differentiation throughout Peninsular Malaysia. A total of 22 identified polymorphic markers were validated and used to genotype 1,120 individuals collected from 39 natural populations of *R. apiculata*, uncovering its low genetic diversity (H_e: 0.3523) and high population differentiation (F_{st}: 0.3150). Low genetic diversity may indicate the occurrence of inbreeding or low levels of gene flow. Based on the microsatellite marker analysis, the populations were separated into two major clusters, corresponding to eastern and western regions of Peninsular Malaysia and coinciding with the Straits of Malacca and the South China Sea. The genetic information generated in this study will enable the formulation of *in situ* and *ex situ* conservation guidelines for *R. apiculata* in Peninsular

Malaysia. Additionally, the genic microsatellite markers generated from this study can be used for future research such as population genetic studies of other closely related species as well as for specific applications such as DNA profiling and forensic analysis.

Keywords: Conservation genetics, mangrove, Rhizophoraceae, SSR marker, transcriptome analysis.

POPULASI GENETIK *Rhizophora apiculata* DI SEMENANJUNG MALAYSIA MENGGUNAKAN PENANDA MIKROSATELIT

ABSTRAK

Pembalakan haram, penebangan berlebihan, pembasmian hutan dan perubahan cuaca merupakan ancaman utama terhadap *Rhizophora apiculata*, atau dikenali sebagai Bakau Minyak; adalah salah satu spesis bakau yang berkepentingan tinggi dari segi ekonomi dan ekologi di Semenanjung Malaysia. Walaupun *R. apiculata* merupakan sejenis bakau yang dominan, pengurangan bilangan spesis ini dalam habitatnya telah menzahirkan kebimbangan terhadap potensi spesis ini untuk terus hidup dalam jangka masa yang panjang. Di Malaysia, maklumat genetik untuk mewujudkan garis panduan konservasi dan pengurusan bagi spesis bakau ini adalah terhad dan penyelidikan selanjutnya perlu dijalankan untuk mengisi jurang yang ada. Oleh itu, kajian ini direka bentuk untuk menghasilkan maklumat genetik yang baharu untuk *R. apiculata*, dengan tujuan untuk memfasilitasi usaha pengekalan kepelbagaian genetik spesis ini di Semenanjung Malaysia. Satu set penanda mikrosatelit genik baharu telah dihasilkan melalui dataset transkriptom '*in-house*' *R. apiculata* untuk menilai tahap kepelbagaian dan perbezaan populasi spesis ini di Semenanjung Malaysia. Sejumlah 22 penanda DNA polimorfik yang dikenalpasti telah disahkan dan digunakan untuk mengenotip 1,120 individu dari 39 populasi asli *R. apiculata,* mendedahkan kepelbagaian genetiknya yang rendah (H_e: 0.3523) dan perbezaan genetiknya yang tinggi (F_{st}: 0.3150). Kepelbagaian genetik yang rendah mungkin disebabkan oleh pembiakbakaan dalam dan aliran gen yang terhad. Berdasarkan analisis penanda mikrosatelit, populasi *R. apiculata* di Semenanjung Malaysia dipisahkan kepada dua kluster iaitu kluster timur dan barat Semenanjung Malaysia, bertepatan dengan Selat Melaka dan Laut China Selatan. Maklumat genetik

yang dihasilkan daripada kajian ini membolehkan perumusan garis panduan pemuliharaan insitu dan eksitu bagi *R. apiculata* di Semenanjung Malaysia. Tambahan pula, penanda mikrosatelit genik yang dihasilkan boleh digunakan untuk penyelidikan masa depan seperti dalam kajian populasi genetik spesis lain yang berkaitan dan juga untuk aplikasi khusus seperti pemprofilan DNA dan analisa forensik.

Kata kunci: Pemuliharaan sumber genetik, bakau, Rhizophoraceae, penanda SSR, analisa transkriptom.

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LIST OF SYMBOLS AND ABBREVIATIONS

R_s	:	Allelic richness
T_a	:	Annealing temperature
°	:	Degree
°C	:	Degree celcius
H_e	:	Expected heterozygosity
D	:	Gene diversity
G_{st}	:	Genetic variation distributed among populations (Nei, 1987)
H_s	:	Genetic variation distributed within populations
F_{is}	:	Inbreeding coefficient
F_{it}	:	Inbreeding coefficient of an individual relative to the total population
T_m	:	Melting temperature
A	:	Number of alleles per locus
H_o	:	Observed heterozygosity
K	:	Optimal number of clusters
p	:	Probability of significance
n	:	Sample number
H_t	:	Total genetic diversity
D_{st}	:	Total genetic diversity distributed among populations
R_{st}	:	Total genetic variation in a subpopulation (Slatkin, 1995)
F_{st}	:	Total genetic variation in a subpopulation (Wright, 1951)
A_t	:	Total number of alleles
6-FAM	:	6-Carboxyfluorescein
AMOVA	:	Analysis of molecular variance
AEP	:	Atlantic East Pacific

bp	:	Base pair
cDNA	:	Complementary deoxyribonucleic acid
cm	:	Centimetre
CTAB	:	Cetyl trimethylammonium bromide
dNTP	:	Deoxynucleoside triphosphate
DNA	:	Deoxyribonucleic acid
dbh	:	Diameter at breast height
EST	:	Expressed sequence tag
GenAlEx	:	Genetic Analysis in Excel
GDA	:	Genetic Data Analysis
GPS	:	Global positioning system
g	:	Gram
g/cm^3	:	Gram per cubic centimetre
HWE	:	Hardy-Weinberg equilibrium
ha	:	Hectar
ha yr^{-1}		Hectar per year
HEX	:	Hexachlorofluorescein
IWP	:	Indo West Pacific
IUCN	:	International Union for Conservation of Nature
IBD	:	Isolation by distance
kg	:	Kilogram
kg/m^3	:	Kilogram per cubic metre
km	:	Kilometre
LD	:	Linkage disequilibrium
$MgCl_2$	:	Magnesium chloride
RM	:	Malaysian Ringgit

MCMC	:	Markov Chain Monte Carlo
m	:	Metre
µL	:	Microlitre
µM	:	Micromolar
MISA	:	Microsatellite Identification Tool
mL	:	Millilitre
mM	:	Millimolar
min	:	Minute
MEGA	:	Molecular Evolutionary Genetics Analysis
ng	:	Nanogram
nm	:	Nanometre
NGS	:	Next generation sequencing
ha^{-1}	:	Per hectar
PCA	:	Principal component analysis
g	:	Relative centrifugal force
rpm	:	Revolutions per minute
RNA	:	Ribonucleic acid
RIN	:	RNA integrity number
s	:	Second
SNP	:	Single nucleotide polymorphism
SSR	:	Simple sequence repeat
sq km	:	Square kilometre
Taq	:	*Thermus aquaticus*
TAE	:	Tris base, acetic acid and ethylenediaminetetraacetic acid
TE	:	Tris-Ethylenediaminetetraacetic acid
USD	:	United States Dollar

U	:	Units of activity
UPGMA	:	Unweighted pair group method
v	:	Version
V	:	Volt
yr	:	Year

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CHAPTER 1: INTRODUCTION

1.1 Research background

Mangrove forests are distributed in tropical and semi-tropical regions, covering up to 75% of tropical coastlines (Valiela et al., 2001; Alongi, 2018). They occur across two major biogeographic regions, the Atlantic East Pacific (AEP) and the Indo West Pacific (IWP) (Li et al., 2016). Mangrove forests make up a unique ecosystem for their ability to withstand strong currents and high water salinity (Parida & Jha, 2010; Saenger, 2013; Lewis III et al., 2016). Being the only woody haplotype that grows in the intertidal zone, mangrove forests have critical ecological responsibilities of forming the interface between land and sea, preventing coastal erosions and providing food and nursery areas for many fish and invertebrate species (Valiela et al., 2001; Alongi, 2002; Donato et al., 2011).

Globally, more than 35% of mangrove forest has been lost in the past two decades and this exceeds the destruction percentage of both rainforests and coral reefs combined (Valiela et al., 2001; Alongi, 2002). There is a growing body of scientific evidence which demonstrates that continuous destruction of mangrove forests due to various anthropogenic activities, such as land clearing and commercial logging, is disrupting many coastal ecosystems (Alongi, 2002; Ngo-Massou et al., 2016; Edi et al., 2017). More disturbingly, in Malaysia, a total of 21,417 ha of mangroves were destructed from 1990-2017 with an average deforestation rate of 793 ha yr^{-1} due to human encroachment in coastal areas (Omar et al., 2019). This may cause coastal species extinctions and reduced protection for coastal areas from storms, tidal waves and erosions. Furthermore, economic concerns for coastal communities that rely on mangrove forests for food and forest products have been raised (Polidoro et al., 2010; Dayalatha & Ali, 2018).

Some of the coastal shores in Malaysia consist of tropical mangrove species in the genus *Rhizophora*, including *R. apiculata* or locally known as Bakau Minyak (Polidoro et al., 2010). The species is favoured for its high quality wood, charcoal and fuel wood (Setyawan et al., 2014; Ismail et al., 2015; Lahjie et al., 2019). Consequently, *R. apiculata* has been threatened by overharvesting, particularly through frequent illegal logging activities in their natural habitats, which has caused a decline in their natural population (Duke, 2010; Omar et al., 2019). This species has been assessed as Least Concern (LC) with a decreasing trend in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Duke, 2010). More concerted efforts are therefore required to conserve this valuable species, especially in specific areas or regions where this species is commonly found such as Peninsular Malaysia.

Microsatellite markers, also known as simple sequence repeats (SSRs), are widely used in genetic diversity and population structure studies due to their co-dominant inheritance, high degree of polymorphism, and abundance in the genome (Morgante & Olivieri, 1993; Ashley & Dow, 1994; Vieira et al., 2016). These markers could provide valuable resource for understanding the population genetics of a species and ultimately assist effective conservation and management of the studied species.

1.2 Problem statement

The genetic information to develop effective guidelines for the conservation and management of mangrove species in Malaysia has been lacking, and thus, further research should be carried out to fill this gap. The current study was designed to generate essential genetic information in facilitating the efforts to maintain the genetic diversity of *R. apiculata* in Peninsular Malaysia.

1.3 Research objectives

1. To generate novel genic microsatellite markers from transcriptome data of *R. apiculata*;
2. To assess the genetic diversity within populations of *R. apiculata* in Peninsular Malaysia; and
3. To assess the genetic differentiation among populations of *R. apiculata* in Peninsular Malaysia.

1.4 Research hypotheses

1. Microsatellite markers can identify causal polymorphisms to investigate the genetic variation within and among the *R. apiculata* populations;
2. *R. apiculata* is a long-lived species that exhibits high levels of genetic diversity within populations; and
3. *R. apiculata* which is dispersed by sea water will exhibit lower levels of genetic differentiation among populations than those dispersed by gravity or animals.

CHAPTER 2: LITERATURE REVIEW

2.1 Mangrove forests

Mangrove forests which usually exist in extreme conditions, including high salinity, extreme tides, strong winds, hot climate and muddy, and anaerobic soils, are among the world's most productive ecosystems (Kathiresan & Bingham, 2001; Lewis III et al., 2016). Due to the extreme living conditions, mangroves and their inhabitants are often highly developed and physiologically adapted to changes in their environment (Kathiresan & Bingham, 2001). Mangroves are the only woody plants that are capable of thriving at the confluence of land and sea in tropical and sub-tropical latitudes (Alongi, 2002; Donato et al., 2011).

2.1.1 Distribution of mangrove forests

Globally, mangroves are distributed in approximately 112 countries and territories with an estimation of coverage varying from 10 million ha (Bunt, 1992) to 24 million ha (Twilley et al., 1992). Spalding (1997) reported that South and Southeast Asia had the highest mangrove area in the world (41.4%), followed by The Americas (27.1%) and West Africa (15.4%), with a total mangrove coverage of 181,399 sq km globally (Table 2.1). Mangrove forests stretch between latitudes 25°N and 30°S where warm oceanic currents are present and cover up to 75% tropical coastlines (Valiela et al., 2001). Their distributions are affected by temperature (Duke, 1992), moisture (Saenger & Snedaker, 1993), wind, coastal hydrology and geomorphology (Guisan & Thuiller, 2005).

Table 2.1: Areal coverage of mangrove forests. Source: Spalding (1997).

Region	Mangrove area (sq km)	Percent
South and Southeast Asia	75,172	41.4
The Americas	49,096	27.1
West Africa	27,995	15.4
Australasia	18,788	10.4
East Africa and the Middle East	10,348	5.7
Total	181,399	100.0

Southeast Asia is blessed with the best developed and the most species-diverse mangroves in the world (Giesen & Wulffraat, 1998). Out of 60 "true mangrove species" identified, 52 species can only be found in the mangrove habitat in Southeast Asia (Giesen et al., 2006). Indonesia is home to the largest mangrove forests with 23.5% occurrence in the whole world and 59.8% occurrence in Southeast Asia (Figure 2.1; Spalding, 1997; Giesen et al., 2006). Malaysia harboured 11.7% of Southeast Asia's mangroves with occurrences in Sabah (57%), Sarawak (26%) and Peninsular Malaysia (17%; Giesen et al., 2006). Malaysia is fortunate to have mangrove forests at all of its states, whereby the forests are highly concentrated in northeast Sabah, in the deltas in Sarawak and on the more sheltered west coast of Peninsular Malaysia where the climate is hot and humid with high precipitation (Figure 2.2; Kanniah et al., 2015). Nine mangrove genera comprising of 28 species can be found distributed throughout Malaysia (Spalding et al., 2010; Setyawan et al., 2014).

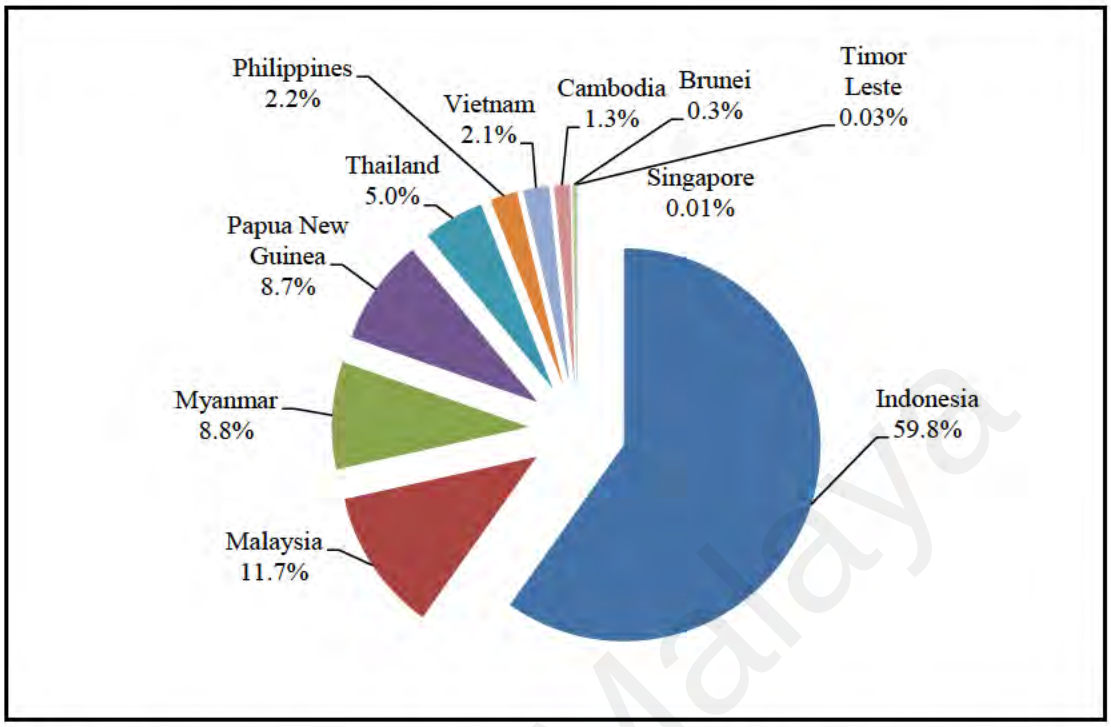


Figure 2.1: Mangrove forests distribution in Southeast Asia. Source: Giesen et al. (2006).

2.1.2 Importance of mangrove forests

Mangrove forest is a unique and significant ecosystem which provides ecological, economical and community values. From an ecological perspective, mangrove forest is the habitat for hundreds of species at all levels of near shore food webs, including fish, crab, and shrimp (Spalding et al., 2010). Mangroves also provide numerous ecosystem functions including protection from strong winds and waves, stabilise land elevation by sediment accretion, preventing contamination of near shore waters, flood mitigation and sequestration of carbon dioxide (Hamdan et al., 2012). Wells and Ravilious (2006) estimated that the annual economic value of mangroves to be in the range of USD 200,000-900,000 ha^{-1}, while another report by Polidoro et al. (2010) revealed that mangrove forests generate at least USD 1.6 billion each year by providing ecosystem services worldwide.

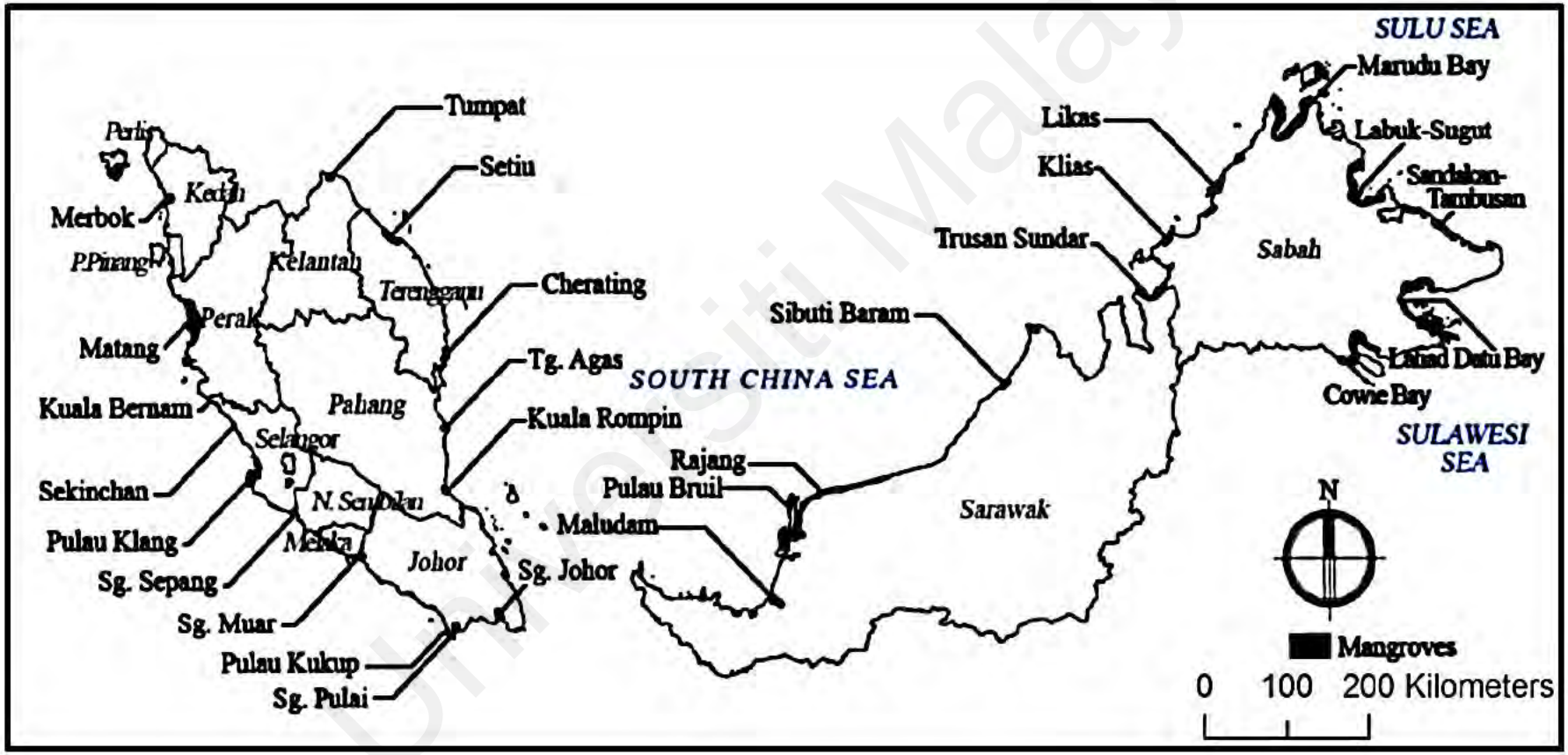


Figure 2.2: Mangrove forests distribution in Malaysia. Source: Kanniah et al. (2015).

Other than ecological importance, mangroves have significant economic values mainly from the wood-based industry and commercial fishing (Hamdan et al., 2012). Timber and poles are mostly made from species with hard and heavy wood such as *Rhizophora* spp., *Bruguiera gymnorrhiza*, *Lumnitzera* spp. and *Xylocarpus* spp. (Kusmana, 2018). Mangrove forests being the breeding ground for many marine species, had contributed to the fishery sector for prawns, mud crabs, barramundi and bream (Hamdan et al., 2012). The Malaysian Department of Fisheries reported that in the year 2009, 1,066,069 metric tonnes, equivalent to RM 5,005 million of fish was caught in Peninsular Malaysia whereby 68% of the entire commercial catch was composed of mangrove-dependent species (Hamdan et al., 2012).

As for community values, mangrove forests serve as ecotourism sites for fishing, bird-watching, photography and other recreation activities. Taking Larut Matang mangrove forest as an example, the location is well-known for bird-watching with more than 58 migratory species observed to have made stopovers in the area (Ahmad, 2009). Based on a study by Ahmad (2009), visitors were willing to pay around RM 41.18 per visit to Larut Matang mangrove forest, in which the total value of the mangrove forest to local recreationists is about RM 3.35 million per year. Other than that, mangroves also serve as valuable educational and research resources (Hamdan et al., 2012).

2.2 *Rhizophora apiculata*

Plants from the Rhizophoraceae family and the genus *Rhizophora* are common, hardy, fast-growing and have extensive distribution in tropical and subtropical coastal areas (Duke, 2006; Giesen et al., 2006; Polidoro et al., 2010). Plants from the genus *Rhizophora* appear to be self-compatible and are also easily reproducible through the dispersal by wind and insects (Coupland et al., 2006; Setyawan et al., 2014). The propagules of *Rhizophora* are dispersed by ocean currents (Inomata et al., 2009). Reported *Rhizophora* occurrences in Malaysia are *R. apiculata*, *R. mucronata*, *R. stylosa*, *R. x annamalayana* (hybrid between *R. apiculata* and *R. mucronata*), and *R. x lamarckii* (hybrid between *R. apiculata* and *R. stylosa*) (Sahu et al., 2015).

R. apiculata Blume or commonly known as Bakau Minyak (Figure 2.3 & Figure 2.4) is common and abundant in Malaysia (Polidoro et al., 2010). It grows on deep, soft and muddy soils, and generally avoids harder substrate mixed with sand (Setyawan et al., 2014). It can grow up to 30 m high with a diameter up to 50 cm (Setyawan et al., 2014). The arching stilt roots can be as high as 5 m tall and the bark covered in grayish spots (Setyawan et al., 2014). The root growth leads downwards (perpendicular) in waterlogged soil conditions but can also grow sideways in non-waterlogged conditions (Amaliyah et al., 2017). The leaf is dark green, sublanceolate, tip with shoot elongation and the undersurface with black or brown spots (Setyawan et al., 2014).

In South East Asia, leaves of *R. apiculata* emerge mostly around November-February (Duke, 2006). The petals are bisexual, glabrous, odourless and yellow in colour (Raju, 2016; Myint et al., 2019). Flowering period is during August-December in South East Asia (Duke,

2006). The seedling is viviparous, and is known as a hypocotyl (Raju, 2016). The hypocotyl is cylindrical, rounded and elongated with blunt ends (Setyawan et al., 2014). Fruiting, a phenomenon when a mature hypocotyl falls, usually occur from November-January in South East Asia (Duke, 2006).

Figure 2.3: *R. apiculata* plant collected during fieldwork in Perak. Photo courtesy of Dr. Lee Soon Leong.

(A)

(B)

Figure 2.4: *R. apiculata* vouchers collected from (A) Sg. Tinggi, Perak and (B) Dayang Bunting, Kedah.

The wood of *R. apiculata* is hard, strong and heavy with an air-dry density of 960-1,170 kg/m^3 and wood density of 0.60-0.77 g/cm^3 (Komiyama et al., 2005; Ismail et al., 2015). Comparative wood density studies demonstrated that *R. apiculata* was one of the mangrove species with the highest wood density (Table 2.2). The strength properties of the timber fall into Strength Group A (Burgess, 1961). The wood is mostly harvested for wood chips, poles, furniture and charcoal, and its bark is harvested for tannins (Setyawan et al., 2014). As the species is easily regenerated, it is often the species of choice for mangrove replantation programs (Hou, 1992).

Table 2.2: Wood density for mangrove species.

No.	Mangrove species	Wood density (g/cm^3)	
		Ismail et al. (2015)	Komiyana et al. (2005)
1	*Avicennia alba*	0.410	0.506
2	*Bruguiera cylindrical*	0.590	0.749
3	*Bruguiera gymnorrhiza*	0.560	0.699
4	*Brugueira parviflora*	0.540	-
5	*Ceriops tagal*	0.640	0.746
6	*Lumnitzera racemosa*	0.470	-
7	*Rhizophora apiculata*	0.600	0.770
8	*Rhizophora mucronata*	0.580	0.701
9	*Sonneratia ovata*	0.340	-
10	*Sonneratia alba*	0.410	0.475
11	*Sonneratia caseolaris*	0.330	0.340
12	*Xylocarpus granatum*	0.490	0.528

2.3 Population divergence and gene flow in *R. apiculata*

All natural populations are exposed to a number of genetic forces affecting the amount of genetic variations. Such forces are mutation, genetic drift, founder effect, selection, migration and mating system (Hedrick, 2000). These forces are responsible for the evolution and the genetic variation of the species (Hedrick, 2001). Gene flow, the movement of genetic material between populations is an important homogenising force that prevents different

populations to evolve independently (Slarkin, 1985). The absence of gene flow will cause population divergence and genetic differentiation (Andrews, 2010). The presence of population structure is ubiquitous in most wild populations in various species. Detecting genetic population structure and understanding its consequences for the evolutionary trajectories of a species is crucial in understanding the process of evolution. This delineation of subdivision within a population plays an important role in understanding the phylogeography, quantitative genetics, and population genetics of the species which ultimately are crucial for the conservation of the species (Komoroske et al., 2017). Changes in the size or number of populations may be indicators of the long-term impacts of anthropogenic influences on species persistence (Balmford et al., 2003).

In the case of mangroves, it is expected that these long-lived wood species to have high genetic variation and low genetic differentiation. However, many studies have proven otherwise (Duke, 2006; Takayama et al., 2013; Ng et al., 2014; Yahya et al., 2014; Chen et al., 2015). The population divergence and gene flow of many mangrove species have been found to be dependent on sea current movements, propagule dispersal potential, land masses and historical vicariance events (Duke, 2006; Yan et al., 2016). For example, a recent study on *R. apiculata*, *R. mucronata* and *R. stylosa* demonstrated that these species had low levels of genetic diversities attributed by high rates of inbreeding (Chen et al. 2015). These three species may practice self-crossing, leading to a deficiency in heterozygotes (Chen et al., 2015).

2.4 Major threats to mangrove forests in Malaysia and beyond

Malaysia came in second in the 'Top 10 countries with the highest annual total area of mangrove deforestation in 2000-2012' list as reported by Hamilton & Casey (2016). Another report by Omar et al. (2019) published that a total of 21,417 ha of mangroves were destructed in Malaysia from 1990-2017 with an average deforestation rate of 793 ha yr^{-1} due to various anthropogenic activities. Oil palm expansion was found to be the key driver to mangrove deforestation in Malaysia, however the threat was under-recognised (Richards & Friess, 2016). Over-exploitation and illegal logging of *R. apiculata* are wide-spreading because of its highly valued wood, causing the species to decline at an alarming rate.

Taking on a wider view, the increased human exploitation of coastal resources and habitats is putting mangrove forests at risk of significant population declination (Valiela et al., 2001). In 2001, it was reported that at least 35% of mangrove forests had been lost while another report indicated that 37.8% areas were deforested from 1996-2010 (Valiela et al., 2001; Thomas et al., 2017). Richards & Friess (2016) reported that between the year 2000 and 2012, Southeast Asia lost 0.18% of mangrove forests per year, with aquaculture being the biggest culprit, followed by rice agriculture and oil palm expansion (Figure 2.5). Authors predicted that the threat of oil palm to mangroves is likely to increase in the future especially in Indonesia (Richards & Friess, 2016). In addition, the ongoing global climate change which is linked to sea-level rise has been recognised as one of the greatest threats for mangrove forests worldwide (Field, 1995; Lovelock & Ellison, 2007; Gilman et al., 2008).

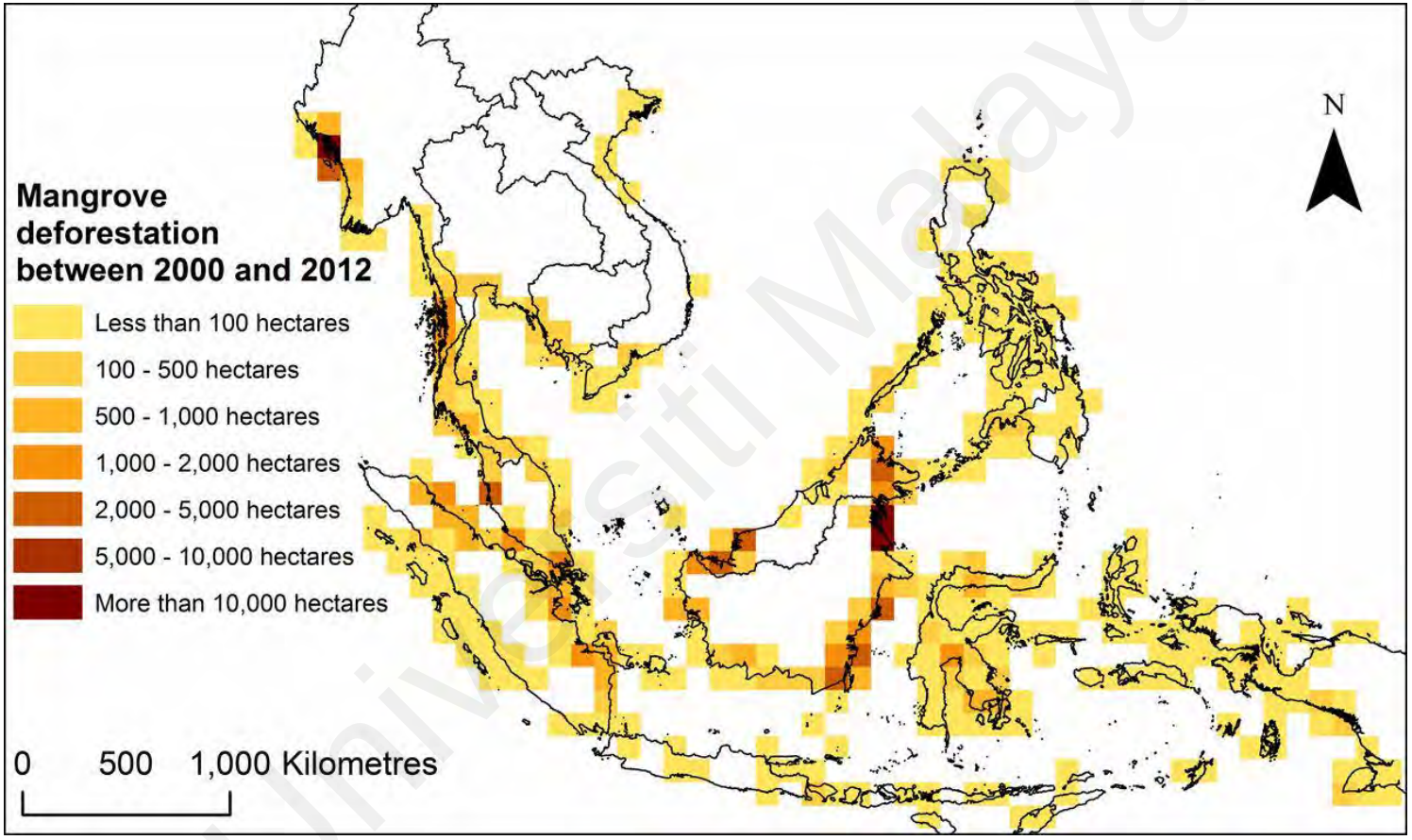


Figure 2.5: Mangrove deforestation in 2000-2012. Deforestation was summarised within each 1 decimal degree square. Hotspots of mangrove deforestation include Rakhine state in Myanmar, Indonesian Sumatra and Borneo; and Malaysia. Source: Richards & Friess (2016).

Threats caused by human encroachment from infrastructure, urban development, aquaculture, agriculture and development of tourism industries also have led to the degradation of mangrove forests (Sarmin et al., 2016). The degradation of mangroves have devastating impacts including habitat loss, biodiversity loss, decline in water quality, increased negative impacts of coastal disasters such as tsunami, increased of atmospheric carbon dioxide and disruption to forest productivity (Nobre, 2011; Sarmin et al., 2016; Alongi, 2018; Sharma et al., 2020).

Various studies have observed low genetic diversities in *R. apiculata* mainly due to high rate of inbreeding, limited seed dispersal and demographic history (Inomata et al., 2009; Yahya et al., 2014; Ng et al., 2014). Genetic diversity is important for a species to cope with environmental changes and to ensure long-term response to selection (Waldvogel et al., 2020). The loss of genetic diversity often decreases the fitness of a species, which may lead to an increased risk of extinction (Keller & Waller, 2002; Charlesworth & Willis, 2009; Michaelides et al., 2016). Conserving the genetic diversity of *R. apiculata* is therefore vital for its survival and long-term persistence.

2.5 Mangrove research and conservation

Publications spanning the past three decades have demonstrated the importance and significance of mangrove research. Globally, the amount of mangrove studies had increased exponentially from 1980-2017 with a total of 14,741 records with the keyword “mangroves” found on Web of Science (Sharma, 2020). The rising popularity of mangrove research is largely due to the uniqueness of mangrove’s ecosystem and its significant functions, such as protecting shorelines from damaging current and waves (Vannucci, 2001).

Despite their importance, many forest areas have been destroyed by various anthropogenic activities, making them one of the most threatened ecosystems worldwide (Sharma, 2020). Conservation of mangrove forests is essential to ensure the survival of a diverse range of mangroves inhabitants, and to reduce the impacts of disasters in the coastal areas. In many countries, including Malaysia, the conservation and management of mangroves are very challenging due to insufficient research, lack of awareness and politics (Friess et al., 2016; Dharmawan et al., 2016).

Conservation research based on scientific evidence is essential to help preserve mangrove species and enhance their ability to deal with environmental changes (Frankham et al., 2002; Burivalova et al., 2019). Generally, there are two main strategies of conservation, namely *in situ* and *ex situ* conservations. *In situ* conservation focuses on safeguarding the species and their genetic material in their natural habitats, while *ex situ* conservation manages the preservation of species outside their natural habitats, for example keeping their genetic material in specific places such as gene banks and botanical gardens (Koski et al., 1997; Rotach, 2005; O'Donnell & Sharrock, 2017). For long-term storage, plant cells, tissues or organs are usually preserved in gene banks under suitable conditions. Seed preservation is often preferred because it is the most convenient, affordable and safe method (Bangarwa, 2017).

2.6 Microsatellite markers and their application in population genetics

Microsatellite markers, also known as simple sequence repeats (SSRs), are short tandem repeats commonly used for ecological and evolutionary studies. Along with single nucleotide polymorphism (SNP), they are often the preferred markers because of their unique characteristics, including being highly polymorphic, abundant, co-dominant, and easily amplified by polymerase chain reaction (PCR) (Morgante & Olivieri, 1993; Ashley & Dow, 1994; Vieira et al., 2016). The high rate of polymorphism in microsatellites is due to the evolution of the marker which roughly follows a stepwise mutation model (Ohta & Kimura, 1973), where mutations result in the addition or deletion of one or more repeat units due to slippage (Figure 2.6).

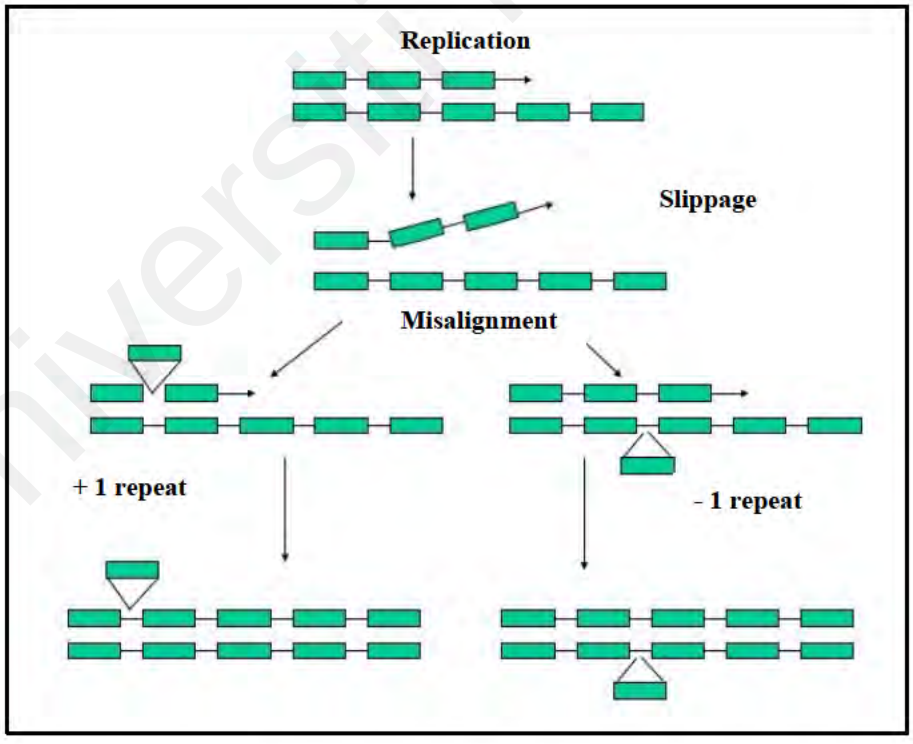


Figure 2.6: Slippage during DNA replication. Slippage leads to the formation of shorter (-1 repeat) or longer (+1 repeat) allele, depending on the strand containing the polymerase error. Modified from Goldstein & Scholotterer (1999).

A significant number of microsatellite markers have been developed especially since the inception of next generation sequencing (NGS). Expressed sequence tags (ESTs) or genic microsatellites (also known as genic SSRs) are generated from sequencing data of cDNA libraries (Kalia et al., 2010). These markers are known to offer advantages over genomic microsatellites because they detect variation thus perfect for marker-trait associations, and they are more transmissible among closely related species (Davey et al., 2011; Zalapa et al., 2012; Sakiyama et al., 2014). However, since DNA sequences of genic microsatellites are more conserved, these markers have lower polymorphism, making them less informative for fingerprinting and varietal identification studies (Kalia et al., 2010).

Molecular markers have been developed for many mangrove species across different genera such as *Avicennia*, *Bruguiera*, *Kandelia*, *Rhizophora*, *Sonneratia*, *Ceriops*, *Aegiceras*, *Excoecaria*, *Acanthus*, *Xylocarpus* and *Heritiera* (Sahu & Kathiresan, 2012). The markers have been widely used in population genetic studies, for example to infer gene flow and to deduce population divergence (Sahu & Kathiresan, 2012). Despite being one of the dominant species in the Indo West Pacific (IWP) region, suitable microsatellite markers have yet to be developed for *R. apiculata* until today (Lo et al., 2014). The absence of suitable microsatellite markers for *R. apiculata* has led to the difficulty in understanding the population genetic structure of the species in the IWP region.

CHAPTER 3: MATERIALS AND METHODS

The methods utilised in this study were the standard methods used to study population genetics which included sample collection, nucleic acid extraction, RNA sequencing, isolation and characterisation of transcriptome microsatellite markers, microsatellite genotyping and statistical analyses.

3.1 Sample collection

Extensive sample collections of *R. apiculata* from June 2017 to June 2018 were carried out in the present study, resulting in a large final sample size of 1,120 individuals from nine states in Peninsular Malaysia (Table 3.1; Figure 3.1). Each location was recorded using a Global Positioning System (GPS) receiver Garmin 60CSX.

Leaf samples of *R. apiculata* from 39 natural populations were collected from multiple sites in Kedah, Pulau Pinang, Perak, Selangor, Negeri Sembilan, Melaka, Johor, Pahang and Terengganu, where nine to 31 individuals were sampled from each population (Table 3.1). Around five to ten leaves were collected from each individual, cleaned, and placed in individual plastic bags with identification tags. The samples were collected randomly, with preference given to trees that were taller and had bigger diameter at breast height (dbh). To avoid collecting closely related individuals, collection between sampled individual trees was done with a distance of more than 5 m.

Table 3.1: Information on *R. apiculata* sampling locations from 39 natural mangrove forests throughout Peninsular Malaysia.

No.	State	Population	Code	*n*	Latitude	Longitude
1	Kedah	Kubang Badak	KBa	30	06°24'	99°43'
2	Kedah	Kisap	Kis	30	06°23'	99°51'
3	Kedah	Dayang Bunting	DBu	29	06°13'	99°49'
4	Kedah	Merbok	Mer	28	05°40'	100°23'
5	Pulau Pinang	Balik Pulau	BPu	28	05°18'	100°11
6	Perak	Pulau Gula	PGu	30	04°55'	100°29'
7	Perak	Teluk Kertang	TKe	30	04°50'	100°38'
8	Perak	Trong	Tro	30	04°42'	100°41'
9	Perak	Sg. Tinggi	STi	30	04°35'	100°40'
10	Perak	Sg. Batang	SBa	28	03°50'	100°46'
11	Selangor	Banjar Utara	BUt	29	03°21'	101°14'
12	Selangor	Pulau Klang	PKl	26	03°03'	101°19'
13	Selangor	Pulau Ketam	PKe	26	03°01'	101°15'
14	Selangor	Pulau Tengah	PTe	29	02°58'	101°14'
15	Selangor	Pulau Pintu Gedong	PPi	29	02°56'	101°15'
16	Selangor	Pulau Che Mat Zin	PCh	28	02°58'	101°17'
17	Selangor	Telok Gedong	TGe	31	02°58'	101°22'
18	Selangor	Sepang Besar	SBe	26	02°40'	101°44'
19	Selangor	Sepang Kecil	SKe	28	02°37'	101°40'
20	Negeri Sembilan	Jimah	PBJ	30	02°36'	101°43'
21	Negeri Sembilan	Sg. Linggi	SLi	30	02°23'	101°59'
22	Melaka	Pulau Besar	PBe	9	02°06'	102°19'
23	Melaka	Merlimau Tambahan	MTa	30	02°07'	102°25'
24	Johor	Muar	Mua	30	01°58'	102°36'
25	Johor	Pulau Kukup	PKu	29	01°19'	103°26'
26	Johor	Tanjung Piai	TPi	29	01°16'	103°30'
27	Johor	Sg. Pulai	SPu	30	01°26'	103°35'
28	Johor	Pulau Juling	PJu	30	01°30'	104°00'
29	Pahang	Endau	End	30	02°45'	103°30'
30	Pahang	Kuantan	PBK	30	03°47'	103°18'
31	Pahang	Peramu	Per	30	03°48'	103°20'
32	Pahang	Balok	Bal	30	03°56'	103°22'
33	Pahang	Cherating	Che	30	04°07'	103°23'
34	Terengganu	Kuala Kemaman	KKe	30	04°17'	103°24'
35	Terengganu	Kuala Paka	KPa	30	04°38'	103°24'
36	Terengganu	Sg. Pimpin	SPi	30	04°47'	103°24'
37	Terengganu	Merchang	Mrc	28	05°01'	103°18'
38	Terengganu	Kuala Terengganu	KTe	30	05°16'	103°09'
39	Terengganu	Pengkalan Gelap	PGe	30	05°40'	102°43'

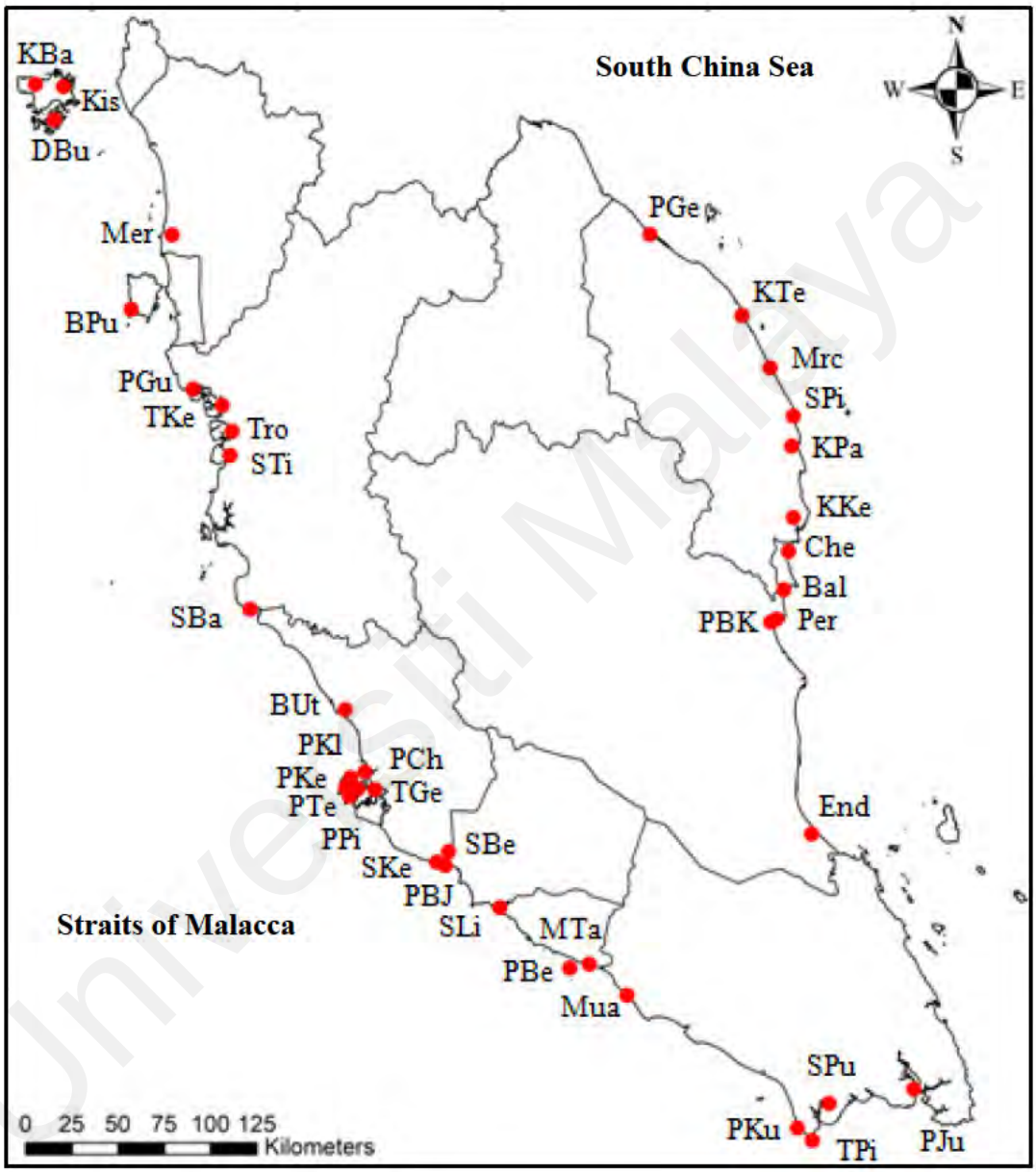


Figure 3.1: Map showing 39 sampling sites in Peninsular Malaysia. Population code corresponds to Table 3.1.

(A)

(B)

(C)

(D)

(E)

Figure 3.2: (A) measurement of tree diameter at breast height (dbh); (B) sample collection from a boat using a cutting pole; (C) sample collection by foot in a mangrove forest; (D) leaf sample was individually packed with identification tag; and (E) leaf sample being cleaned, cut and weighed before storage in liquid nitrogen, prior to DNA extraction.

3.2 Nucleic acid extraction

Total RNA was extracted from the fresh leaf of *R. apiculata* using cetyl trimethylammonium bromide (CTAB) method (Murray & Thompson, 1980), with minor modifications. Plant sample weighing around 0.02-0.03 g was ground using tissue lyser together with 250 µL of 2X CTAB buffer containing 2% of β-mercaptoethanol. The slurry was incubated at 65 °C for an hour. Next, 500 µL of chloroform was added and mixed well. Subsequently, the tube was centrifuged at 6,000 rpm for 5 min in room temperature. The upper aqueous phase of the mixture was transferred into a new tube and 330 µL of isopropanol was added. The tube was centrifuged at 6,000 rpm for 5 min at room temperature. The aqueous phase of the mixture was discarded and 500 µL of 70% ethyl alcohol was added. The tube was centrifuged at 8,000 rpm for 5 min at room temperature. Lastly, 50 µL of DNase-free water was added.

The extracted RNA was purified using TURBO DNA-free kit (Ambion, Life Technologies, Gaithersburg, MD) and Qiagen RNeasy kit (Qiagen, USA). The quality of the extracted RNA was checked using NanoDrop 2,000 (Thermo Fisher Scientific, USA) and 1% agarose gel in 1X TAE buffer at 100 V for 60 min, and subsequently quantified using Qubit 2.0 fluorometer (Life Technologies, USA). The RNA integrity number (RIN) was obtained using Plant Nano chip of Agilent 2,100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Total DNA was extracted from the fresh leaves of *R. apiculata* using CTAB method (Murray & Thompson, 1980) with slight modifications. First, 2% of β-mercaptoethanol was added to 20 mL of 2X CTAB extraction buffer in a 50 mL Falcon tube. The buffer was

preheated in a 65 °C water bath. Five g of fresh leaves were ground with liquid nitrogen to a fine powder using a grinder. The fine powder was transferred into the preheated extraction buffer to form a homogeneous slurry. The slurry was incubated at 65 °C for 30 min and then cooled to ambient temperature. Equal volume of chloroform-isoamyl alcohol (24:1) was added into the tube and was gently mixed for 15 min. Next, the mixture was centrifuged at 2,700 rpm for 10 min. The upper aqueous phase of the mixture was transferred to a clean tube and added with 2/3 volume of cold (-20 °C) isopropanol. The tube was gently mixed to precipitate the nucleic acid. The nucleic acid was spooled out using a Pasteur pipette and placed into 1 mL of wash buffer (76% ethanol and 10 mM ammonium acetate) in a 1.5 mL tube. The nucleic acid was left in the wash for a few hours to a few days. The supernatant was poured off onto a clean kitchen towel and the pellet was dried using a desiccator. Lastly, the dried nucleic acid was dissolved in 800 µL of TE buffer and the tube was rotated overnight in a dual hybridisation oven. The extraction products were then stored in 4 °C prior to purification.

DNA purification was done using High Pure PCR Template Preparation Kit ver. 20 with minor modifications (Roche, Applied Science, IN, USA). First, 200 µL of sample was transferred into a 1.5 mL tube. Then, 2 µL or RNAse A was added and the tube was incubated at 65 °C for 15 min. Forty µL of Proteinase K and 200 µL of Binding Buffer were added into the tube. The tube was then incubated at 70 °C for 10 min. Next, 100 µL of cold isopropanol was added and mixed well by tilting the stand or shaking the tube. The mixture was poured into a filter tube and centrifuged at 8,000 x g for 1 min. The collection tube with flowthrough was discarded and a new collection tube was placed. A total of 500 µL of Wash Buffer was added and the tube was centrifuged at 8,000 x g for 1 min. The collection tube with

flowthrough was discarded and a new collection tube was placed. The previous steps were repeated where 500 µL of Wash Buffer was added and the tube was centrifuged at 8,000 x g for 1 min. The collection tube with flowthrough was discarded and a new collection tube was placed. The tube was centrifuged at 12,000 x g for 1 minute. The collection tube with flowthrough was discarded and replaced with a capped tube. Lastly, 200 µL of Elution Buffer (incubated at 70 °C) was added and centrifuged at 8,000 x g for 1 min.

The concentration and quality of the extracted DNA were checked using NanoDrop 2,000 (Thermo Fisher Scientific, USA). The samples were measured at UV absorbance wavelength of 230, 260 and 280 nm. The qualities of the extracted samples were determined by the absorbance ration of 260/230 nm and 260/280 nm. Gel electrophoresis was conducted using 1% agarose gel at 100 V for 25 min. The gel was viewed and documented using Alphalmager Mini (Cell Biosciences, USA).

3.3 Transcriptome sequencing and microsatellite marker identification

RNA sample was sent to Beijing Novogene Bioinformatics Technology Cp., Ltd to be sequenced using Illumina HiSeq 4,000 (Illumina, Inc, CA, USA). The raw data underwent quality checking, trimming and assembling using FastQC (Andrews, 2010), Trimmomatic v0.32 (Bolger et al., 2104) and Trinity v2.4.0 (Grabherr et al., 2011), respectively. *De novo* assembled sequences were used for microsatellite identification using MISA program (Varshney et al., 2002) and the repeats set for di- and trinucleotides were ≥8 while tetranucleotides were ≥6. Primer3 (Rozen & Skaletsky, 2000) was used to design primers for the amplification of the target regions. The best primers were selected based on the repeat lengths of less than 30 bp, GC content around 50%, melting temperature around 55-60 °C,

product size of around 100-400 bp, perfect microsatellite repeats and not more than four and six continuous mono repeats in primer sequence and amplicon, respectively.

3.4 Polymerase chain reaction (PCR) and microsatellite genotyping

The designed primers and three nuclear microsatellite primers developed by Shinmura et al. (2012) underwent initial primer screening where primers were tested on four unrelated samples through polymerase chain reaction (PCR) amplification using GeneAmp PCR System 9,700 (Applied Biosystems, USA). The PCR cocktail comprised of approximately 1 ng of template DNA, 1X GoTaq Flexi Buffer, 1.5 mM of $MgCl_2$, 0.3 μM of each primer, 0.2 mM of dNTP, and 0.5 U of GoTaq Flexi DNA polymerase (Promega Corporation, USA) for an initial denaturing step of 3 min at 94 °C, 40 cycles of 94 °C for 1 min, 55 °C annealing temperature for 30 s, and 72 °C for 40 s, followed by 30 min at 72 °C. The PCR products were electrophoresed on 1.5% agarose gel in 1X TAE buffer at 100 V for 25 min.

Primer pairs that resulted in specific-single bands were selected for 5' end fluorescent labelling using either HEX or 6-FAM. PCR was conducted using the same PCR program as mentioned above. The PCR products were subjected to fragment analysis using ABI 3,130xl Genetic Analyzer (Applied Biosystems, USA) with ROX400 as the internal size standard (Applied Biosystems, USA). The alleles were scored using GeneMarker (SoftGenetics, 2010).

Primers that resulted in tall and clean peaks were chosen for genotyping on all 1,120 samples. Multiplex PCR was conducted in an 8 µL reaction mixture, consisting of approximately 1 ng of template DNA, 1 x 2X Type-it Multiplex PCR Master Mix (Qiagen, Germany) and 0.8 µM of primer mix for an initial denaturing step of 5 min at 95 °C, 35 cycles of 95 °C for 30 s, 55 °C annealing temperature for 1 min 30 s, and 72 °C for 30 s, followed by 30 min at 60 °C. The PCR products were subjected for fragment analysis using ABI 3,130xl Genetic Analyzer (Applied Biosystems, USA) with ROX400 as the internal size standard (Applied Biosystems, USA). The allele sizes were scored using GeneMarker (SoftGenetics, 2010).

3.5 Data analysis

MICRO-CHECKER (Van Oosterhout et al., 2004) was used to detect genotype scoring errors and the presence of non-amplified alleles (null alleles). Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were tested using Fisher's exact test in GDA v1.1 (Lewis & Zaykin, 2002). A Bonferroni correction was used to compensate for multiple comparisons between loci (Rice, 1989). Low quality and problematic samples that resulted in ≥50% genotyping failures were excluded. It is crucial to exclude problematic samples, loci and populations before proceeding to other genetic analyses.

3.5.1 Levels of genetic diversity and population differentiation

Microsatellite Toolkit (Park, 2001) was used to determine vital genetic variation parameters including the observed (H_o) and expected heterozygosities (H_e, or gene diversity, D) (Nei, 1987), number of alleles per locus (A) and allele frequency by locus for each population. H_e can be calculated as follows:

$$He = \sum_{i=1}^{k} pi^2 \quad (3.1)$$

where p_i is the frequency of the i^{th} of k alleles.

Allelic richness (R_s) which is a standardised measure of the number of alleles per locus independent of the sample size was computed using FSTAT v2.9.3 (Goudet, 2002) while private alleles in the populations were obtained using GDA v1.1 (Lewis & Zaykin, 2002).

Wright's F-statistics (Wright, 1951) and its anologue R-statistics (Slatkin, 1995) were used to determine the indirect estimates of gene flow and population structure. F-statistics measures F_{is} (inbreeding coefficient of an individual relative to the subpopulation), F_{st} (effect of subpopulations compared to the total population) and F_{it} (inbreeding coefficient of an individual relative to the total population). They can be calculated as follows:

$$(1-F_{is})\,(1-F_{st}) = 1-F_{it} \quad (3.2)$$

FSTAT v2.9.3 (Goudet, 2002) was used to obtain *F*-statistics (Weir & Cockerham, 1984) coefficients, R_{st} (Goodman, 1997) and Nei's genetic diversity statistics (Nei, 1973, 1977). The significance of F_{is} was measured using GDA v1.1 (Lewis & Zaykin, 2002) based on 1,000 randomisations and 95% confidence interval.

Mantel test was used to evaluate the relationship between geographic distance and genetic divergence that drives population structure. The isolation by distance (IBD) analysis using Mantel test was conducted in GenAlEx v6.5 (Peakall & Smouse, 2006) using Nei's genetic distance data from POWERMARKER v3.25 (Liu & Muse, 2005) and was tested for significance by 9,999 permutations.

3.5.2 Relationship among populations

Three approaches were used to determine the relationship among the populations: (1) principal component analysis; (2) cluster analysis based on Nei's genetic distance; and (3) cluster analysis using a Bayesian approach. All three analyses delineate groupings based on individual's genotypes.

(1) Principal component analysis

Principal component analysis (PCA) using PCAGEN v1.2 (Goudet, 1999) was carried out to visualise genetic distance and relatedness between populations in a two dimensional standard plot. Estimations were based on the correlation matrix of population allele frequency. PCA was performed on all the 39 populations in Penisular Malaysia and to test for significance, 1,000 randomisation tests were carried out.

(2) Cluster analysis based on Nei's genetic distance

Nei's D_A was calculated and the average distance was estimated across all loci using POWERMARKER v3.25 (Liu & Muse, 2005). Nei's D_A genetic distance 1983 (Nei et al., 1983) was selected because of its reputation to give reliable population trees for microsatellite data. Subsequently, a dendogram was constructed using the Unweighted Pair Group Method (UPGMA) (Michener & Sokal, 1957) using the same software and viewed in MEGA v5.0 (Tamura et al., 2011). UPGMA assumes an ultrametric tree or a 'molecular clock hypothesis' in which it deduces the same evolutionary speed on all lineages. Bootstrap resampling of 1,000 times was applied to get a reliable tree with correct branch topology.

(3) Cluster analysis using a Bayesian approach

The STRUCTURE (Pritchard et al., 2000) was used for cluster analysis using the admixture model. Twenty independent runs were performed for all populations with simulations of 250,000 burn-in iterations and 850,000 Markov Chain Monte Carlo (MCMC). Then the StructureSelector (Li & Liu, 2018) was used to select and visualise the optimal number of clusters (*K*). The program calculated six statistics together (Ln Pr(X|*K*), Δ*K*, MEDMED*K*, MEDMEA*K*, MAXMED*K* and MAXMEA*K*) and reported the best *K* for each estimator. Subsequently, the clumped cluster was viewed based on the selection of the best *K*.

After identifying population groups from the three analyses, GenAlEx v6.5 (Peakall & Smouse, 2006) was used to carry out analysis of molecular variance (AMOVA, Excoffier et al., 1992). AMOVA evaluated the level of genetic differentiation within and among populations and regions. The significance of the differentiation was determined by permutation of 1,000 replicates.

3.6 Optimum population size

The optimum population size was determined by pooling all the genotype data (total 1,120 individuals) for a simulation analysis based on Lee et al. (2013) using Cutting Simulation 1+2. To determine the optimum population size required to maintain the total number of alleles (A_t), a total of 1,110 out of 1,120 samples were sampled without replacement 1,000 times using a computerised algorithm. The samples were reduced in a 10-sample reduction interval from 1,110 to 10 samples and A_t was calculated during each reduction. The percentage means of A_t with standard errors were plotted against sample sizes to reveal trends. The goal of this study was to maintain at least 95% of the current genetic diversity, thus 95% A_t was marked on graph.

CHAPTER 4: RESULTS

This population genetics study incorporated a total of 1,120 individuals of *R. apiculata* that were successfully collected from 39 natural mangrove forests distributed from 9 states in Peninsular Malaysia. All the individuals were genotyped using 19 novel transcriptomic and 3 published nuclear microsatellite markers. The genetics information generated from this study is crucial for the conservation and management of *R. apiculata* in Peninsular Malaysia.

4.1 Transcriptome sequencing

Paired-end transcriptome sequences of *R. apiculata* were obtained and their qualities were checked using FastQC. Both forward and reverse strands had 25,938,686 total number of sequences in each strand with zero poor quality strand and satisfactory per base quality graphs (Appendix A and B). The sizes of the sequences were around 150 bp.

Trimmomatic v0.32 was used to trim adapters and low quality sequences to improve the quality of the raw next generation sequencing data. The total number of sequences for both forward and reverse strands dropped to 25,627,792 (-1.20%) and the lengths of sequences became shorter ranging around 36-140 bp (Appendix A and B). Improvements in per base quality could be observed at the beginnings and ends of both forward and reverse strands. Only good quality data should be used to ensure problem-free downstream analyses. After trimming the sequences, Trinity v2.4.0 assembled the forward and reverse sequences into full length transcripts.

4.2 Microsatellite marker isolation and characterisation

De novo assembled sequences which were constructed by Trinity v2.4.0 were subsequently used as the input for MISA and Primer3 for microsatellite identification and primer design, respectively. Details of MISA's microsatellite search results are presented in Table 4.1. MISA identified a total of 18,674 microsatellites (Figure 4.1) with dinucleotides having the highest distribution (15,898, 85.13%), followed by trinucleotides (2,403, 12.87%) and tetranucleotides (373, 2%). From the analysis, dinucleotide repeats of CT (3,138, 16.80%), AG (3,116, 16.68%) and TC (2,513, 13.45%) had the highest microsatellite motifs frequencies (Appendix C). On the other hand, TTC (196, 1.05%) and AAAG (30, 0.16%) had the highest frequencies for tri- and tetranucleotide repeats, respectively (Appendix C).

The microsatellite information generated by MISA was used to design the forward and reverse primers for the targeted regions. Using Primer3, 60 primers were designed (labelled as *Rap*T01 to *Rap*T60) and screened, whereby 22, 28, and 10 primers had di-, tri- and tetranuclueotide microsatellite motifs, respectively (Appendix D). From the 60 primers screened for DNA amplification, 48 primers except *Rap*T03, *Rap*T07, *Rap*T11, *Rap*T12, *Rap*T14, *Rap*T28, *Rap*T29, *Rap*T40, *Rap*T47, *Rap*T49, *Rap*T52 and *Rap*T55 showed clear, single bands on the agarose gels (Figure 4.2). Out of the 48 primers, 46 (95.83%) primers were chosen and were fluorescently-labelled at the forward primer with HEX or 6-FAM for fragment analysis (Appendix D).

Table 4.1: Results of microsatellite search by MISA.

Total number of sequences examined	141,915
Total size of examined sequences (bp)	202,216,115
Total number of identified SSR	18,674
Number of SSR containing sequences	16,182
Number of sequences containing more than 1 SSR	2,270
Number of SSRs present in compound formation	1,266

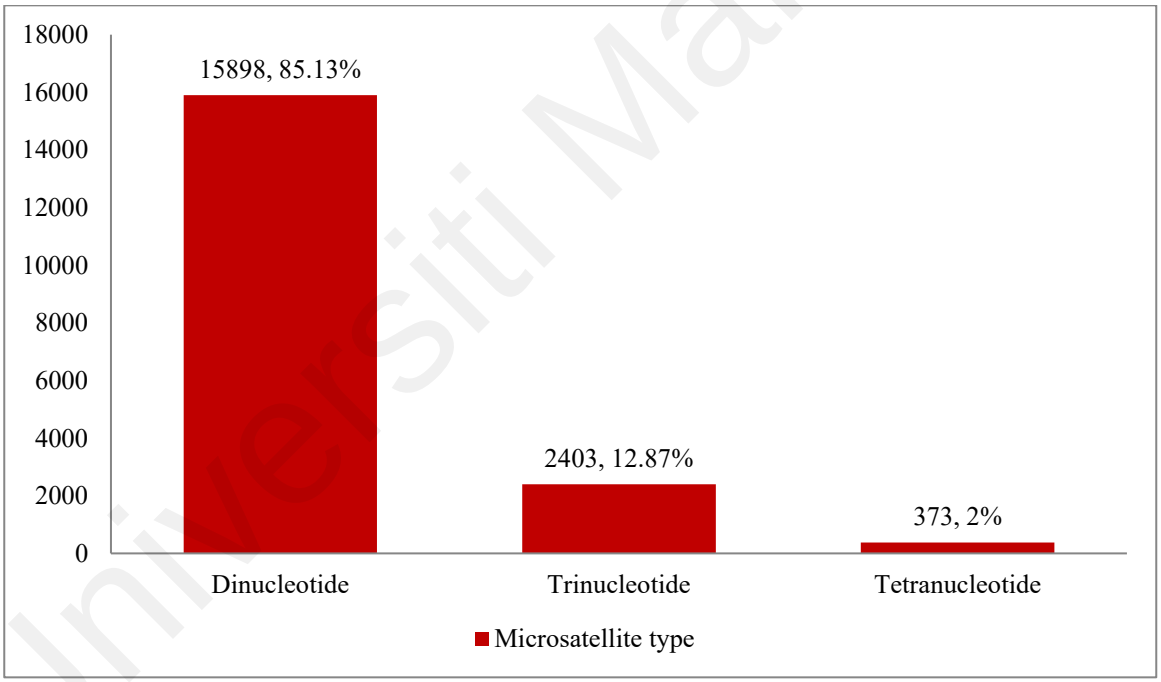
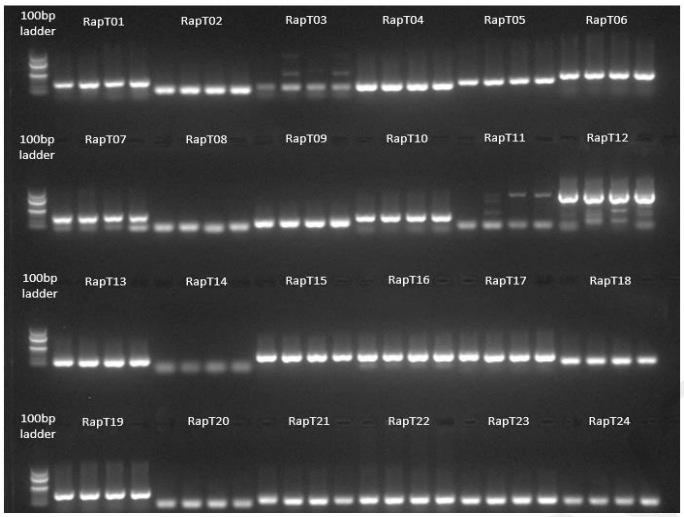
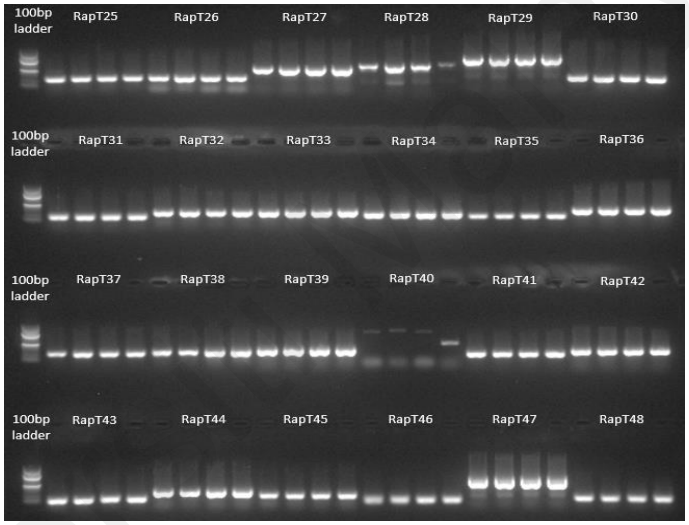


Figure 4.1: Distribution of different microsatellite repeat types.

(A)



(B)



(C)

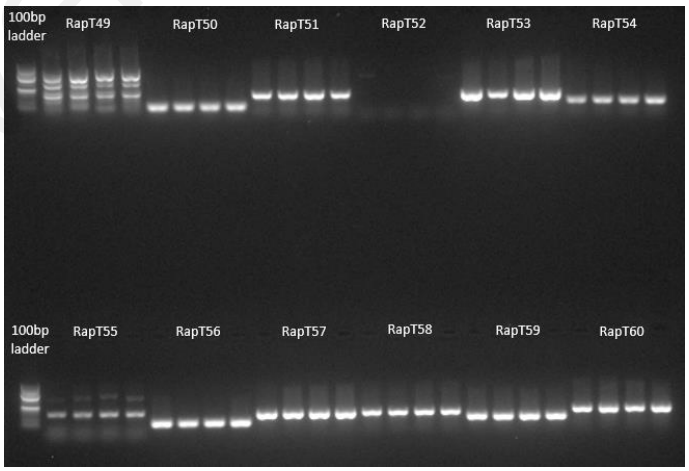


Figure 4.2: Gel electrophoresis results using primers (A) *Rap*T01 to *Rap*T24; (B) *Rap*T25 to *Rap*T48; and (C) *Rap*T55 to *Rap*T60 on 1.5% agarose gels at 100 V for 25 min.

4.3 Fragment analysis

All the 46 labelled primers were screened on 24 individuals from Teluk Gedong by PCR and fragment analysis. After excluding monomorphic and problematic (resulted in multiple and confusing peaks) primers, a total of 19 (39.58%) primers were selected to genotype all the 1,120 samples collected throughout Peninsular Malaysia. Three nuclear microsatellite markers (*RM*111, *RM*116, *RM*121) developed by Shinmura et al. (2012) were included in this study to increase the amount of polymorphic markers for genotyping. The 22 primers were divided into six sets (M1 to M6) for multiplex PCR (Table 4.2). After multiplex PCR and fragment analysis, allele scoring was carried out using GeneMarker (Appendix E).

Table 4.2: Multiplex sets for 22 primers subjected for Multiplex PCR.

Multiplex sets	Primer	Label	Expected size (bp)	T_a (°C)
M1	*Rap*T08	HEX	102	55
	*Rap*T43	6-FAM	153	
	*Rap*T51	HEX	359	
	*RM*121	HEX	174-183	
M2	*Rap*T02	HEX	138	55
	*Rap*T25	6-FAM	267	
	*Rap*T31	HEX	217	
	*Rap*T53	HEX	338	
	*RM*111	6-FAM	141-157	
M3	*Rap*T17	HEX	281	55
	*Rap*T18	6-FAM	165	
	*Rap*T46	HEX	116	
M4	*Rap*T06	HEX	349	55
	*Rap*T09	HEX	170	
	*Rap*T20	HEX	102	
	*Rap*T23	6-FAM	148	
M5	*Rap*T16	HEX	296	55
	*Rap*T21	6-FAM	156	
	*Rap*T60	HEX	369	
	*RM*116	HEX	137-167	
M6	*Rap*T01	6-FAM	298	55
	*Rap*T38	HEX	347	

4.4 Levels of genetic diversity and population differentiation

Null alleles were detected in 21 (95.45%) loci from 32 (82.05%) populations (Table 4.3). No null allele was detected in locus *Rap*T20 and from seven populations (Balik Pulau, Pulau Gula, Pulau Tengah, Pulau Che Mat Zin, Sungai Besar, Pulau Besar and Merlimau Tambahan). The frequency of null allele occurrence ranged from 0 (*Rap*T20) to 33.33% (*Rap*T38) with a mean of 14.92%. Population Dayang Bunting had the highest null allele occurrence at 15 loci. Fisher's exact test detected deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) ($p < 0.05$) after Bonferroni adjusted at $\alpha = 0.05/22 = 0.0023$ and $\alpha = 0.05/ [(22*21)/2] = 0.0002$, respectively (Appendix F). Even though some loci and populations deviated from HWE and LD, all loci and populations were included in further genetic analyses because *R. apiculata* engages in non-random mating (Yahya et al., 2014; Wee et al., 2014).

Genetic diversity analysis (Table 4.4) using 22 polymorphic microsatellite markers on 1,120 *R. apiculata* individuals from 39 populations throughout Peninsular Malaysia revealed low mean number of allele (*A*) and allelic richness (R_s) of 3.21 and 2.65, respectively. *A* ranged from 2.32 (Kuala Paka) to 4.59 (Pulau Ketam). Kuala Kemaman had the lowest R_s (2.07), while Pulau Ketam had the highest R_s (3.63). The mean observed heterozygosity (H_o, 0.2985) was lower than the expected heterozygosity (H_e, 0.3523). H_o and H_e ranged from 0.1938 (Sungai Batang) to 0.4833 (Merlimau Tambahan) and 0.2469 (Balik Pulau) to 0.5027 (Muar), respectively. A total of 44 private alleles were detected in some of the populations. Of the 39 populations, 14 (35.90%) had zero private allele while the other 25 had one to five private alleles. Pulau Gula had the highest amount of private alleles. There was an observable trend that most populations at eastern Peninsular Malaysia had lower *A* and R_s as compared

to the populations in western Peninsular Malaysia. However, such trend was not observed for H_o, H_e and the availability of private allele.

Table 4.3: Null allele occurrence in 22 loci and 39 populations of *R. apiculata*. Locus with null allele marked with (●).

Locus/ Pop.	*RapT* 01	*RapT* 02	*RapT* 06	*RapT* 08	*RapT* 09	*RapT* 16	*RapT* 17	*RapT* 18	*RapT* 20	*RapT* 21	*RapT* 23	*RapT* 25	*RapT* 31	*RapT* 38	*RapT* 43	*RapT* 46	*RapT* 51	*RapT* 53	*RapT* 60	*RM* 111	*RM* 116	*RM* 121	Total
KBa											●								●				3
Kis						●					●	●			●		●		●			●	7
DBu		●	●			●				●	●	●	●	●	●		●		●	●		●	15
Mer																					●		1
BPu																							0
PGu																							0
TKe			●																	●			2
Tro			●																				1
STi					●	●																	2
SBa			●	●		●						●			●	●		●			●		8
BUt	●				●							●									●		3
PKl					●																●		2
PKe		●	●		●			●		●	●						●	●					8
PTe																							0
PPi					●												●						2
PCh																							0
TGe							●														●		2
SBe																							0
SKe	●			●																			2
PBJ														●									1
SLi														●									1
PBe																							0
MTa																							0
Mua		●		●	●						●	●	●	●			●	●	●	●		●	12
PKu		●			●			●		●	●		●	●	●	●							9
TPi								●					●								●		3
SPu			●					●						●									3
PJu														●									1
End			●											●							●		3
PBK								●				●		●									3
Per								●															1
Bal								●		●													2
Che								●				●											2
KKe								●						●									2
KPa														●									1
SPi				●																			1
Mrc		●		●	●	●				●	●	●	●	●			●		●		●		12
KTe														●						●			2
PGe	●																						1
Total	3	5	7	5	8	5	1	9	0	5	7	8	5	13	4	4	6	4	5	4	7	3	118

Table 4.4: Genetic diversity parameters of *R. apiculata*, including number of samples (*n*), number of alleles (*A*), observed (H_o) and expected (H_e) heterozygosities, allelic richness (R_s), number of private alleles and inbreeding coefficient (F_{is}). Values in parentheses denote standard deviations.

No.	Population	State	Code	*n*	*A*	H_o	H_e	R_s	Private allele	F_{is}
1	Kubang Badak	Kedah	KBa	30	2.73 (1.58)	0.2194 (0.0162)	0.2608 (0.0492)	2.30	0	0.161*
2	Kuala Kisap	Kedah	Kis	30	4.32 (1.43)	0.3212 (0.0182)	0.4035 (0.0417)	3.19	3	0.207*
3	Dayang Bunting	Kedah	DBu	29	3.95 (1.50)	0.2419 (0.0171)	0.4361 (0.0355)	3.17	3	0.45*
4	Merbok	Kedah	Mer	28	2.73 (1.32)	0.2776 (0.0180)	0.2900 (0.0481)	2.32	0	0.044*
5	Balik Pulau	P. Pinang	BPu	28	2.64 (1.43)	0.2338 (0.0171)	0.2469 (0.0469)	2.13	1	0.054*
6	Pulau Gula	Perak	PGu	30	2.91 (1.63)	0.3123 (0.0181)	0.3216 (0.0519)	2.34	5	0.029
7	Teluk Kertang	Perak	TKe	30	2.95 (1.89)	0.3045 (0.0179)	0.3274 (0.0544)	2.39	0	0.071
8	Trong	Perak	Tro	30	2.82 (1.74)	0.2500 (0.0169)	0.3079 (0.0557)	2.31	1	0.191*
9	Sg. Tinggi	Perak	STi	30	2.86 (1.49)	0.2742 (0.0174)	0.3011 (0.0497)	2.24	1	0.091
10	Sg. Batang	Perak	SBa	28	3.05 (1.09)	0.1938 (0.0159)	0.3022 (0.0449)	2.43	0	0.363*
11	Banjar Utara	Selangor	BUt	29	2.95 (1.36)	0.2273 (0.0166)	0.2805 (0.0514)	2.35	1	0.193*
12	Pulau Kelang	Selangor	PKl	26	3.41 (1.44)	0.3655 (0.0202)	0.3747 (0.0443)	2.86	0	0.025
13	Pulau Ketam	Selangor	PKe	26	4.59 (1.56)	0.3575 (0.0201)	0.4781 (0.0383)	3.63	2	0.256*
14	Pulau Tengah	Selangor	PTe	29	3.68 (1.55)	0.4091 (0.0195)	0.3954 (0.0475)	2.87	0	-0.035
15	Pulau Pintu Gedong	Selangor	PPi	29	3.68 (1.55)	0.3527 (0.0189)	0.3551 (0.0463)	2.83	1	0.007
16	Pulau Che Mat Zin	Selangor	PCh	28	3.55 (1.57)	0.3773 (0.0195)	0.3858 (0.0457)	2.89	0	0.022
17	Teluk Gedong	Selangor	TGe	31	3.59 (1.44)	0.3215 (0.0179)	0.3639 (0.0495)	2.76	2	0.118*
18	Sepang Besar	Selangor	SBe	26	3.23 (1.72)	0.3689 (0.0202)	0.3923 (0.0484)	2.77	0	0.061*
19	Sepang Kecil	Selangor	Ske	28	3.05 (1.68)	0.3393 (0.0191)	0.3765 (0.0511)	2.68	0	0.101*
20	Jimah	N. Sembilan	PBJ	30	3.50 (1.63)	0.4045 (0.0191)	0.4114 (0.0504)	2.94	2	0.017
21	Sg. Linggi	N. Sembilan	SLi	30	3.73 (1.75)	0.4545 (0.0194)	0.4630 (0.0498)	3.07	1	0.019
22	Pulau Besar	Melaka	PBe	9	2.91 (1.54)	0.4040 (0.0349)	0.4551 (0.0585)	2.91	0	0.118*

Table 4.4, continued: Genetic diversity parameters of *R. apiculata*, including number of samples (*n*), number of alleles (*A*), observed (H_o) and expected (H_e) heterozygosities, allelic richness (R_s), number of private alleles and inbreeding coefficient (F_{is}). Values in parentheses denote standard deviations.

No.	Population	State	Code	*n*	*A*	H_o	H_e	R_s	Private allele	F_{is}
23	Merlimau Tambahan	Melaka	MTa	30	3.86 (1.78)	0.4833 (0.0195)	0.4728 (0.0496)	3.20	1	-0.023
24	Muar	Johor	Mua	30	4.50 (1.65)	0.3696 (0.0188)	0.5027 (0.0434)	3.44	1	0.268*
25	Pulau Kukup	Johor	PKu	29	4.55 (1.68)	0.3725 (0.0192)	0.4984 (0.0428)	3.61	2	0.256*
26	Tanjung Piai	Johor	TPi	29	3.73 (1.72)	0.4227 (0.0196)	0.4666 (0.0503)	3.11	1	0.096*
27	Sg. Pulai	Johor	SPu	30	3.68 (1.67)	0.4041 (0.0192)	0.4739 (0.0513)	3.07	1	0.150*
28	Sg. Johor	Johor	PJu	30	2.91 (1.44)	0.3276 (0.0184)	0.3658 (0.0584)	2.49	1	0.106*
29	Endau	Pahang	End	30	2.68 (1.46)	0.3119 (0.0181)	0.3696 (0.0580)	2.36	1	0.159*
30	Kuantan	Pahang	PBK	30	2.45 (1.06)	0.2734 (0.0174)	0.3233 (0.0503)	2.16	2	0.157*
31	Peramu	Pahang	Per	30	2.77 (1.23)	0.3093 (0.0181)	0.3692 (0.0507)	2.33	0	0.165*
32	Balok	Pahang	Bal	30	2.45 (1.34)	0.2742 (0.0174)	0.3121 (0.0540)	2.16	1	0.123
33	Cherating	Pahang	Che	30	2.77 (1.85)	0.3015 (0.0179)	0.3292 (0.0546)	2.25	4	0.085*
34	Kuala Kemaman	Terengganu	KKe	30	2.41 (1.18)	0.2262 (0.0163)	0.2835 (0.0533)	2.07	0	0.205*
35	Kuala Paka	Terengganu	KPa	30	2.32 (1.09)	0.2924 (0.0177)	0.3192 (0.0528)	2.12	1	0.085*
36	Sg. Pimpin	Terengganu	SPi	30	2.59 (1.40)	0.3030 (0.0179)	0.3124 (0.0520)	2.21	0	0.031
37	Merchang	Terengganu	Mrc	28	3.55 (1.60)	0.2557 (0.0176)	0.3990 (0.0536)	2.87	0	0.363*
38	Kuala Terengganu	Terengganu	KTe	30	2.64 (1.18)	0.3091 (0.0180)	0.3430 (0.0550)	2.31	2	0.101*
39	Pengkalan Gelap	Terengganu	PGe	30	2.68 (1.32)	0.3137 (0.0181)	0.3271 (0.0555)	2.32	3	0.042
	Mean				3.21 (1.50)	0.2985 (0.0200)	0.3523 (0.0500)	2.65	1.13	

* Significant at 95% confidence interval

Nearly all populations (94.87%) had excess of homozygotes and positive inbreeding coefficient values (F_{is}) ranging from 0.007 to 0.450. Pulau Tengah and Merlimau Tambahan had negative F_{is} values of -0.035 and -0.023, respectively, but were not significant ($p < 0.05$). A total of 26 (66.67%) populations were significant at $p < 0.05$ (Table 4.4). Most of the total genetic diversity ($H_t = 0.532$) was partitioned within genetic population ($H_s = 0.370$; $D_{st} = 0.162$). The proportion of genetic variation distributed among populations (G_{st}) was estimated at 0.305, implicating that 30.5% of genetic variability was distributed among populations (Table 4.5). The mean F_{st} (0.315) estimate was slightly higher than G_{st} and was significantly greater than zero ($p < 0.05$), while the mean R_{st} (0.242) was lower than F_{st} (Table 4.5).

Table 4.5: Genetic diversity of 22 loci in 1,120 individuals of *R. apiculata.*

Locus	H_t	H_s	D_{st}	G_{st}	F_{st}	R_{st}
*Rap*T01	0.489	0.403	0.086	0.176	0.180	0.249
*Rap*T02	0.532	0.283	0.250	0.469	0.487	0.617
*Rap*T06	0.648	0.430	0.218	0.337	0.350	0.305
*Rap*T08	0.615	0.564	0.052	0.084	0.089	0.083
*Rap*T09	0.818	0.645	0.173	0.212	0.221	0.400
*Rap*T16	0.512	0.198	0.314	0.614	0.638	0.402
*Rap*T17	0.842	0.683	0.159	0.189	0.195	0.145
*Rap*T18	0.633	0.395	0.239	0.377	0.392	0.042
*Rap*T20	0.518	0.456	0.063	0.121	0.126	0.070
*Rap*T21	0.656	0.396	0.260	0.396	0.412	0.528
*Rap*T23	0.439	0.166	0.273	0.622	0.616	0.090
*Rap*T25	0.529	0.217	0.312	0.590	0.609	0.581
*Rap*T31	0.560	0.432	0.129	0.230	0.229	0.270
*Rap*T38	0.584	0.460	0.124	0.212	0.222	0.038
*Rap*T43	0.257	0.237	0.020	0.079	0.079	0.071
*Rap*T46	0.404	0.183	0.221	0.548	0.564	0.070
*Rap*T51	0.470	0.275	0.195	0.415	0.431	0.273
*Rap*T53	0.357	0.188	0.169	0.474	0.480	0.508
*Rap*T60	0.201	0.180	0.021	0.103	0.106	0.117
*RM*111	0.694	0.603	0.092	0.132	0.134	0.275
*RM*116	0.690	0.526	0.164	0.237	0.249	0.094
*RM*121	0.259	0.222	0.036	0.140	0.147	0.070
Mean	0.532	0.370	0.162	0.305	0.315*	0.242

* Significant at 95% confidence interval

The mantel test (Figure 4.3) tested for significance by 9,999 permutations showed that there was positive correlation between geographical distance and genetic differentiation (Nei's 1983 distance method: $Rxy = 0.510$; $p = 0.000$).

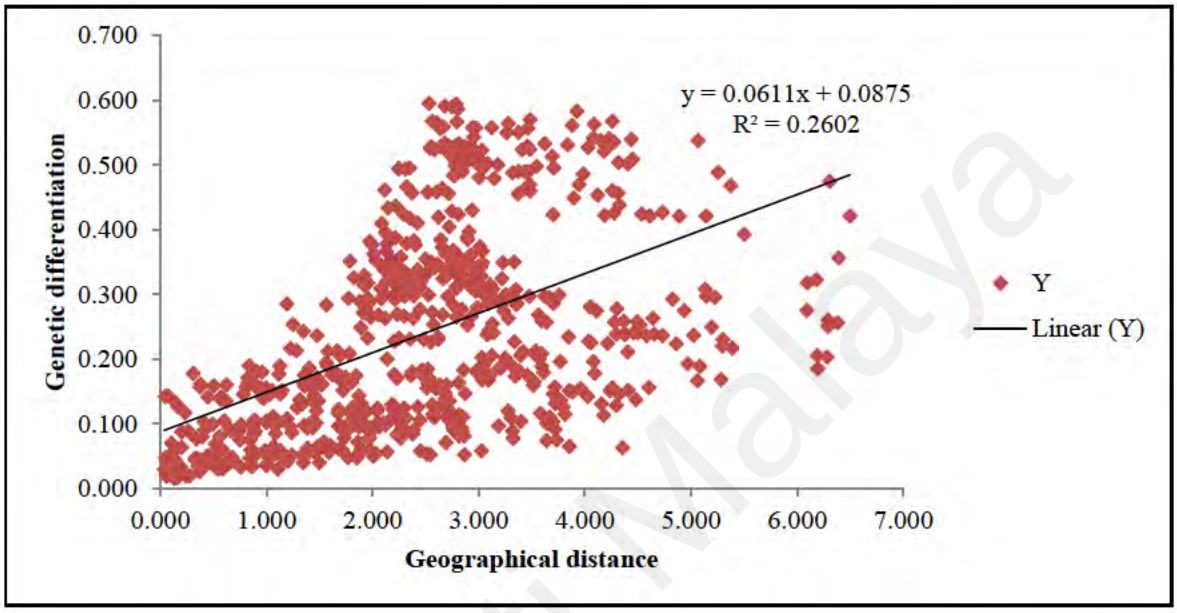


Figure 4.3: Mantel test for isolation by distance (IBD) using Nei's 1983 distance. The graph showed positive correlation between geographical distance and genetic differentiation.

4.5 Relationship among populations

Principal component analysis (PCA) was carried out to determine the relationship of all 39 populations of *R. apiculata* in Peninsular Malaysia. The analysis showed that the populations were divided into two main clusters where populations 1 to 24 were in one cluster and populations 25 to 39 were in another cluster (Figure 4.4). It can be observed that the clusters corresponded with eastern and western Peninsular Malaysia, coinciding with the Straits of Malacca and South China Sea.

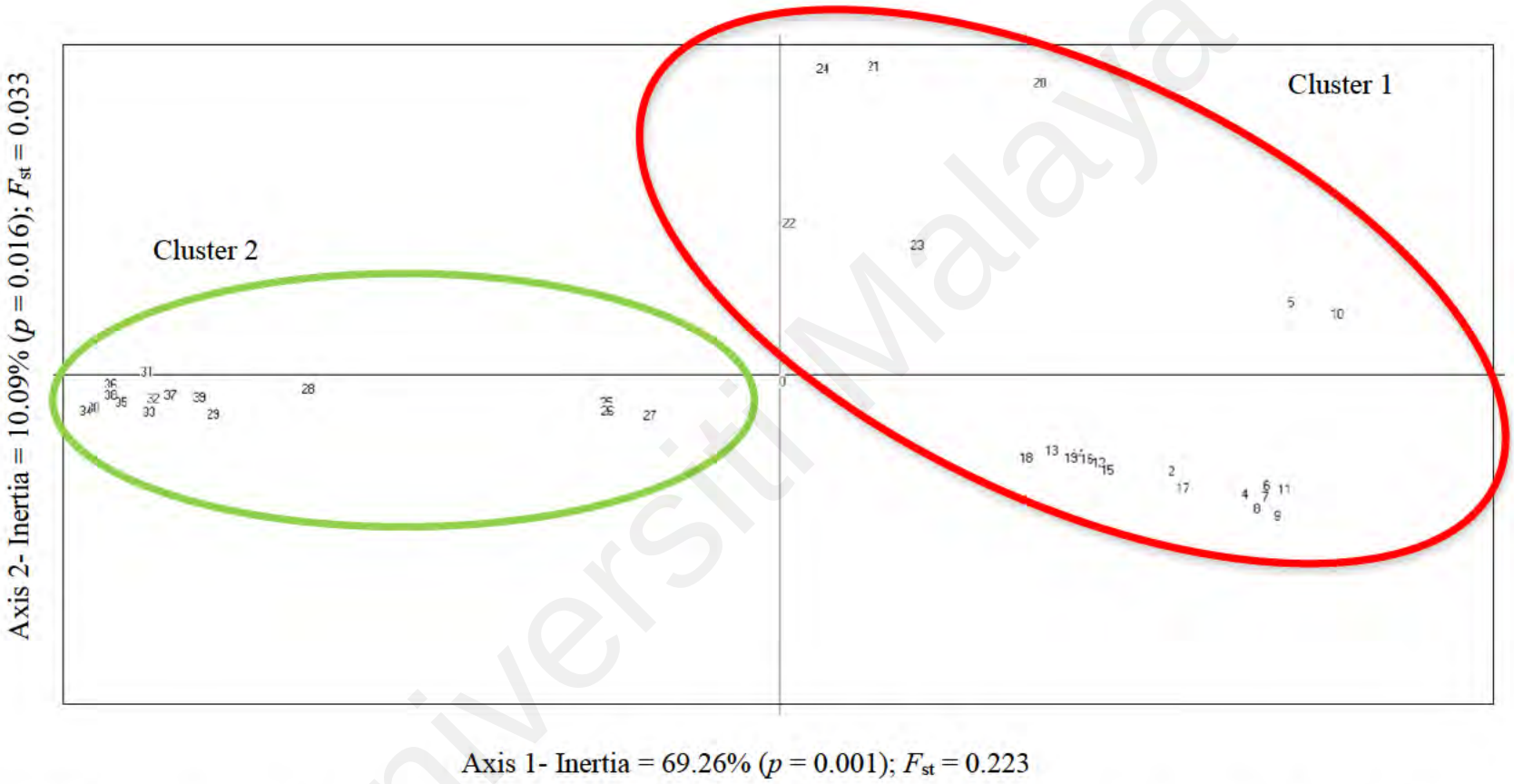


Figure 4.4: Principal component analysis (PCA) based on pairwise F_{st} of 39 *R. apiculata* populations. Both axes were significant. The populations were separated into two main clusters: (1) western Peninsular Malaysia; circled in red; and (2) eastern Peninsular Malaysia; circled in green. Population number corresponds to Table 3.1.

Pairwise F_{st} values based on Nei's genetic distance can be referred at Appendix G. Clustering based on Nei's genetic distance and construction of a dendogram using the Unweighted Pair Group Method (UPGMA) with 1,000 times bootstrap produced a phylogenetic tree with two main branches (Figure 4.5). Bootstrap values ranged from 16-93% and were shown on nodes. The two main branches divided eastern populations (1 to 27) from western populations (28 to 39) in Peninsular Malaysia with high bootstrap values of 92% on both nodes. Nearly similar observation was obtained such as in PCA in which the populations were assigned to two main clusters which coincided with the populations' geographical regions.

The analysis of individual multilocus genotypes of 1,120 samples using STRUCTURE algorithm showed the best clustering solution was at $K = 2$ based on Evanno method (Figure 4.6; Table 4.6). Clustering of individuals according to $K = 2$ provided good biological explanation since the clusters corresponded to geographical groups where Cluster 1 (populations 1 to 27) and Cluster 2 (populations 28 to 39) coincided with western and eastern Peninsular Malaysia, respectively. In addition, western and eastern Peninsular Malaysia corresponded to the Straits of Malacca and South China Sea, respectively. A gradually increasing level of admixture was observed in populations 12 to 27 (Figure 4.7).

All three analyses had nearly similar results where the 39 populations were grouped into two clusters (Cluster 1: populations 1 to 27; and Cluster 2: populations 28 to 39) that corresponded to the geographical regions coinciding with the Straits of Malacca and South China Sea. The determination of sub regions or clusters is important for the development of effective conservation and management guidelines for the species.

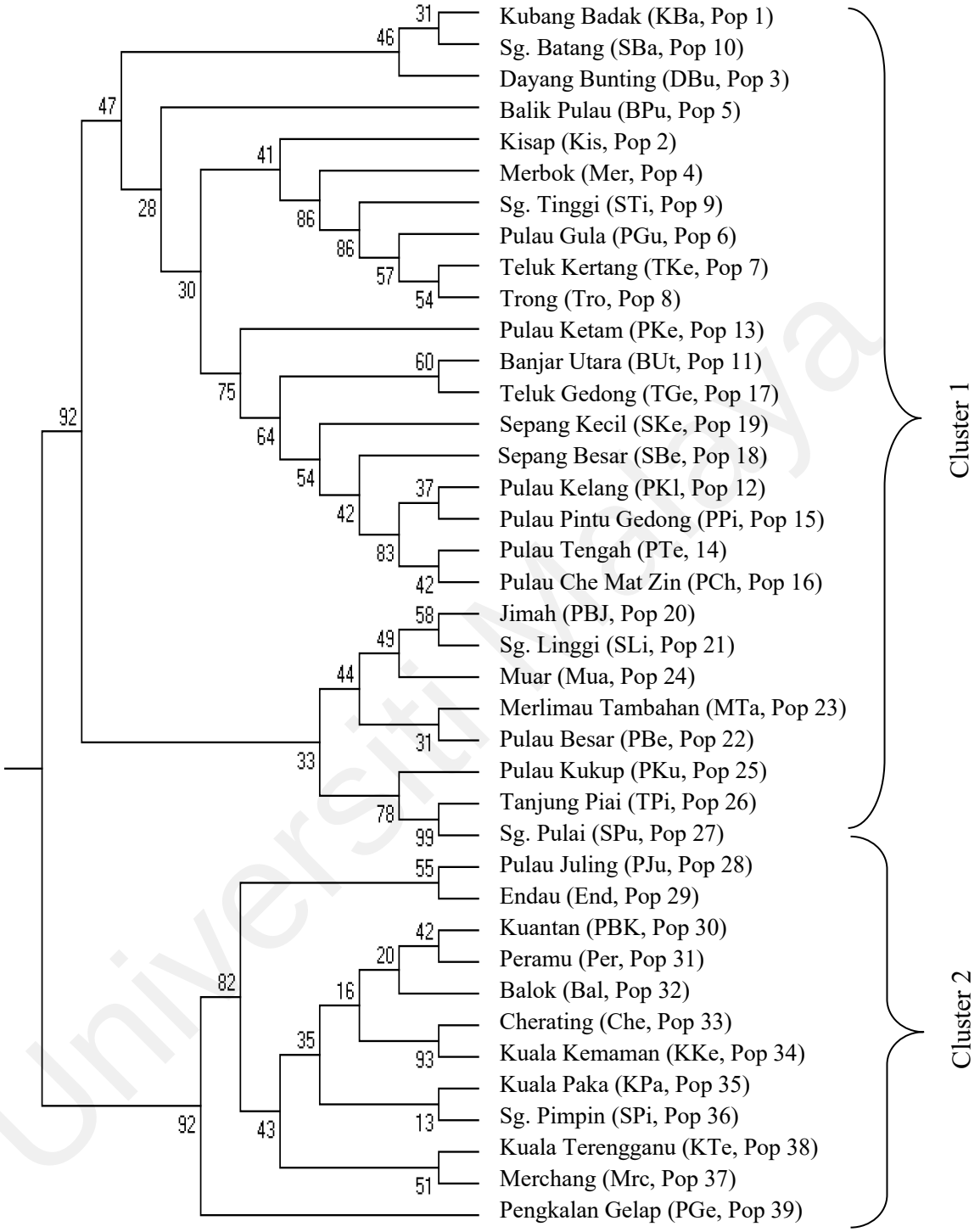


Figure 4.5: Phylogenetic tree of 39 populations of *R. apiculata* constructed using UPGMA with 1,000 times bootstrap. Cluster 1 and 2 consisted of populations that coincided with western and eastern Peninsular Malaysia, respectively.

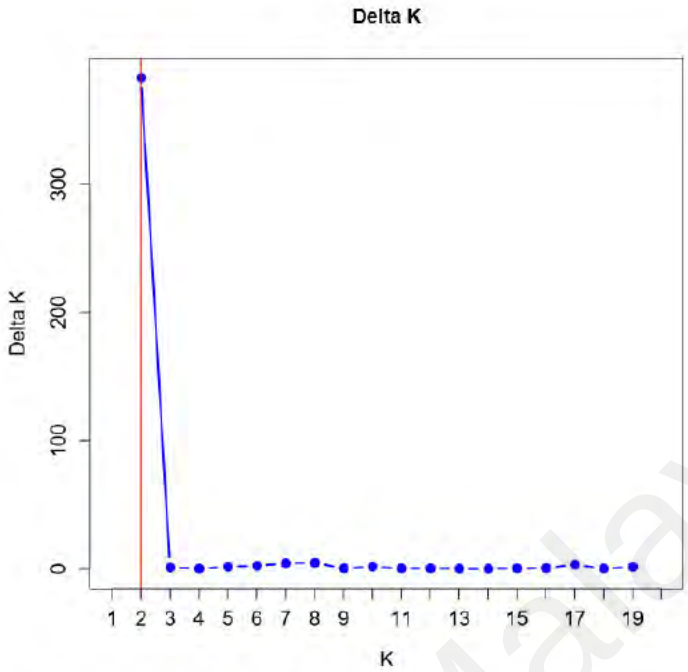


Figure 4.6: Graphical plot of the Bayesian analysis using Evanno method (2005) to determine the true number of *K* of the 39 *R. apiculata* populations using Delta *K*. The true number of *K* = 2.

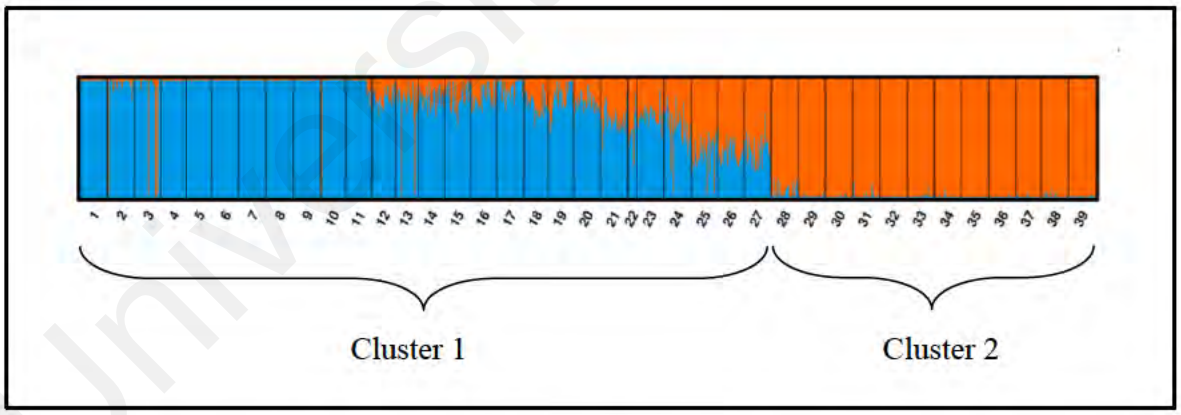


Figure 4.7: The result of STRUCTURE showing the bar plot with individual assignments into two clusters (*K* = 2): (1) individuals from populations 1 to 27 (blue colour); and (2) individuals from populations 28 to 39 (orange colour), coincided with western and eastern Peninsular Malaysia, respectively. Population number corresponds to Table 3.1.

Table 4.6: Bayesian analysis using Evanno method (2005) to determine the true number of *K*. *K* = 2 had the highest Delta *K* value thus selected as the best *K* to represent the relationship of the 39 *R. apiculata* populations.

No. of *K*	Reps	Mean LnP(*K*)	Stdev LnP(*K*)	Ln'(*K*)	|Ln"(*K*)|	Delta *K*
1	20	-50481.2	0.08751	NA	NA	NA
2	20	-39430.3	25.14832	11050.880	9628.505	382.86880
3	20	-38007.9	204.48511	1422.370	250.575	1.22539
4	20	-36836.1	374.14346	1171.795	80.915	0.21627
5	20	-35583.4	382.99061	1252.710	642.605	1.67786
6	20	-34973.3	61.54133	610.105	145.480	2.36394
7	20	-34508.7	52.88199	464.625	230.700	4.36254
8	20	-34274.8	33.53206	233.925	158.385	4.72339
9	20	-34199.2	313.55339	75.540	145.525	0.46412
10	20	-33978.2	75.29117	221.065	134.935	1.79218
11	20	-33892.0	58.28943	86.130	22.590	0.38755
12	20	-33783.3	98.45141	108.720	41.610	0.42265
13	20	-33716.2	44.74904	67.110	7.325	0.16369
14	20	-33656.4	62.37264	59.785	5.770	0.09251
15	20	-33602.4	61.67289	54.015	27.760	0.45012
16	20	-33576.1	87.48366	26.255	56.015	0.64029
17	20	-33493.9	30.75776	82.270	100.115	3.25495
18	20	-33511.7	283.71046	-17.845	78.580	0.27697
19	20	-33451.0	221.39855	60.735	366.745	1.65649
20	20	-33757.0	794.23160	-306.010	NA	NA

AMOVA 1 revealed that 44% of observed variation was due to differences among populations and 56% within populations (Table 4.7). When the populations were grouped based on geographical regions, AMOVA 2 revealed that 45% of the variation was apportioned between the western and eastern regions of Peninsular Malaysia, 13% among populations within region, and 42% within populations (Table 4.6). All partitions were significant at $p < 0.005$.

Table 4.7: Results of analysis of molecular variance (AMOVA) performed by grouping all 39 populations together (AMOVA 1) and separating the populations into geographical regions (AMOVA 2).

Source of variation	Degree of freedom	Sum of squares	Variance component	Percentage of variance
AMOVA 1				
Among populations	38	8393.434	7.368	44
Within populations	1081	10125.069	9.366	56
Total	1119	18518.504	16.734	100
AMOVA 2				
Among regions	1	4950.041	9.966	45
Among populations within regions	37	3443.394	2.918	13
Within populations	1081	10125.069	9.366	42
Total	1119	18518.504	22.250	100

4.6 Optimum population size

The simulation analysis predicted that to maintain 95% current genetic diversity, the optimum population size needed was 860 individuals, with standard errors ranging from 710-960 individuals (Figure 4.8).

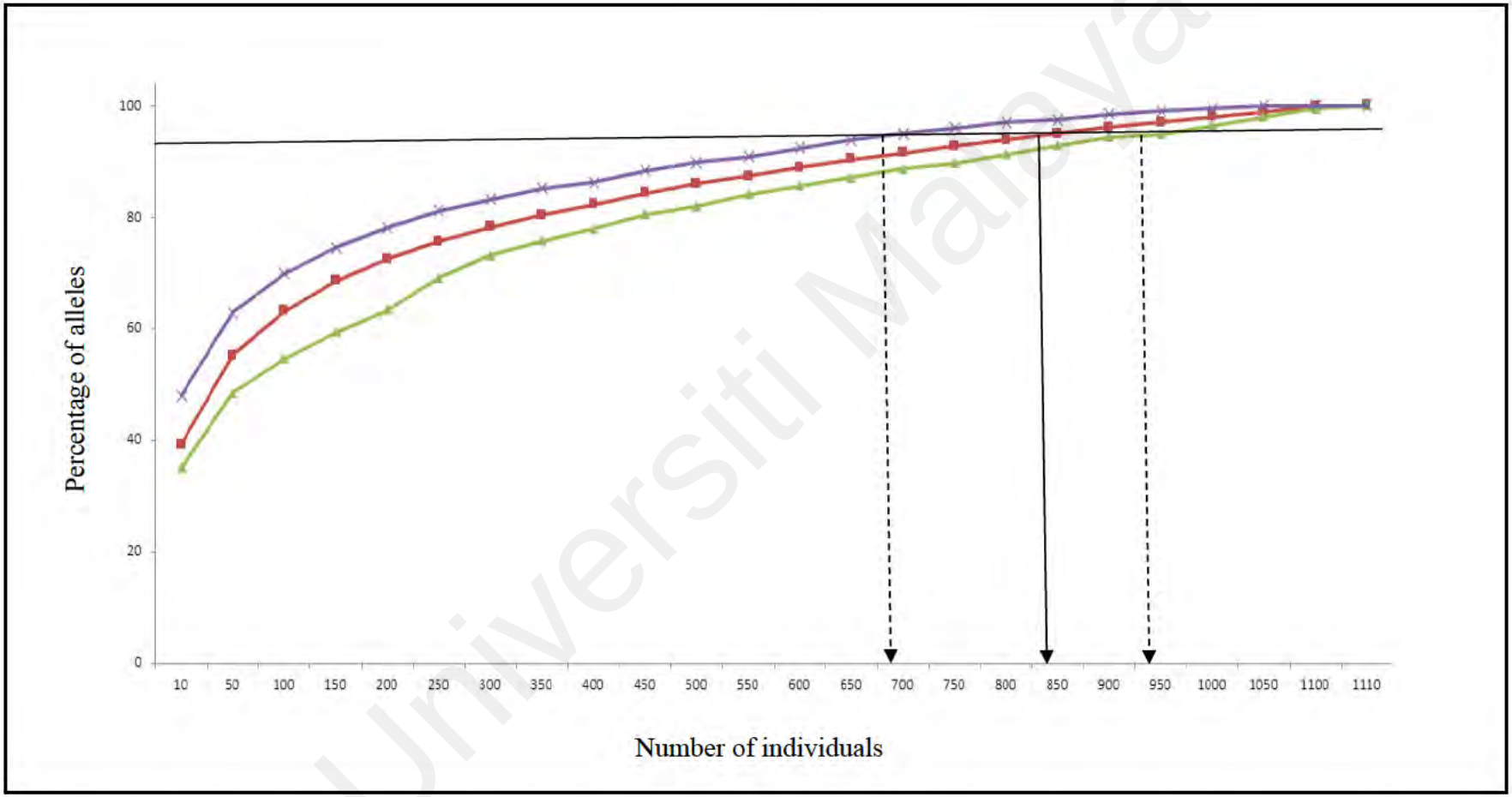


Figure 4.8: Relationship between the percentage of alleles against sample size. All values were based on 1,000 resampling from 1,110 of the 1,120 individuals with standard errors. Points for 95% number of alleles were marked on graph. The optimum population size was 860 individuals with standard errors ranging from 710-960 individuals. Dotted lines represent standard errors.

CHAPTER 5: DISCUSSIONS

5.1 Microsatellite marker development

Next generation sequencing (NGS) is a revolutionary method which can produce millions of sequences in a relatively fast and inexpensive manner (Zalapa et al., 2012). Transcriptome sequencing using NGS platform could reveal the genic sequences of an organism which can subsequently be used to develop transcriptomic microsatellite markers (Yue et al., 2014; Torales et al., 2018). Isolation of microsatellites using NGS has been carried out in various plants such as pigeon pea (*Cajanus cajan*) (Dutta et al., 2011), downy oak (*Quercus pubescens*) (Torre et al., 2014) and long jack (*Eurycoma longifolia*) (Lee et al., 2018). In this study, the Illumina HiSeq 4,000 was used to generate 25,938,686 raw sequences which were then assembled into 141,915 contigs harbouring a total of 18,674 microsatellites. The amount of microsatellite regions found would be different depending on the parameters set during MISA search. If mono-, penta- and hexanucleotides as well as longer microsatellite repeats were included in the parameter, more microsatellite regions will be discovered (Zalapa et al., 2012). In this study, the repeats set for di- and trinucleotides were ≥8 while tetranucleotides were ≥6 because there's a strong positive correlation between total microsatellite length and polymorphism (Temnykh et al., 2001; Vieira et al., 2016).

R. apiculata possessed higher number of di- (85.13%) than tri- (12.87%) and tetranucleotides (2%). Similar observation was seen in grey mangrove (*A. marina*) (Maguire et al., 2000), downy oak (*Q. pubescens*) (Torre et al., 2014) and elephant grass (*Pennisetum purpureum* Schumach) (López et al., 2018). Dinucleotides were reported to be abundant in most species, however, less frequent in coding regions because there is a predominance of

tri- and hexanucleotides (Wang et al., 1994; Li et al., 2002; Kalia et al., 2010). Tri- and hexanucleotides are neutral in nature and are controlled by stronger mutation pressure in coding regions (Vieira et al., 2016). Contrary to that, this study found that dinucleotides were the most abundant in the coding regions of *R. apiculata*. Similar observations whereby dinucleotides were the most abundant in the transcriptome sequences were observed in other mangrove species including *S. alba* (Chen et al., 2011), *A. marina* (Huang et al., 2014) and *A. corniculatum* (Fang et al., 2016). Varshney et al. (2005) suggested that the relative abundance of the types of motifs were highly dependant of genome composition, dataset sizes as well as the searching parameters.

The number of polymorphic loci and sample sizes used in an experiment can influence the statistical power of microsatellite-based statistics. Koshinen et al. (2004) reported that at least 30 microsatellite loci are required for accurate inference and estimation in population genetics. However, the simulation study conducted by Mariette et al. (2002) demonstrated that about 20 microsatellite loci or between 100 and 200 dominant markers could yield adequate results for within-population diversity assessment. More recently, Arthofer et al. (2018) reported that eight microsatellites were sufficient to assign individuals back to their clusters and only two highly polymorphic microsatellites were needed to determine major population structure. As regard to sample size per population, the golden standard to assess genetic diversity is 25-30 individuals and small sample sizes should be avoided (Hale et al., 2012).

In the present study, a total of 22 polymorphic microsatellite loci were used to study the population genetics of *R. apiculata*. Since *R. apiculata* is common and abundant in Peninsular Malaysia, nearly all populations had adequate number of individuals except for those in Pulau Besar, Melaka because *R. stylosa* was found to be the dominant *Rhizophora* species in the island.

5.2 Levels of genetic diversity

Microsatellite null alleles were observed at almost all (95.45%) loci. These alleles are commonly found in population genetic studies, which could be caused by various events such as genetic mutation (causing defective allele at a particular locus), preferential amplification of short alleles and Taq DNA polymerase slippage during PCR (Gagneux et al., 1997; Chapuis & Estoup, 2007). Null allele may affect the estimation of population differentiation and cause deviation to Hardy-Weinberg equilibrium (HWE). In this study, deviation of HWE was observed in *R. apiculata*. It is important to note that the deviation may be caused by non-random mating of the species rather than the presence of null alleles (Yahya et al., 2014; Wee et al., 2014).

Despite being one of the most abundant mangrove species in Peninsular Malaysia, *R. apiculata* was found to have a low genetic diversity (H_e = 0.3523) in the current study. The result is comparable with the genetic diversities of other mangrove species such as *R. mucronata* (H_e = 0.354, Yan et al., 2016), *R. stylosa* (H_e = 0.321, Yan et al., 2016) and *S. alba* (H_e = 0.280, Wee et al., 2017). In addition, *R. apiculata* from the greater Sunda Islands also had low genetic diversity (H_e = 0.378, Yahya et al., 2014). Low genetic diversity is common in mangroves and this could be attributed to various factors including ocean current

movement, propagule dispersal potential, land masses, historical vicariance and inbreeding (Duke, 2006; Takayama et al., 2013; Ng et al., 2014; Yahya et al., 2014; Chen et al., 2015). Yan et al. (2016) reported that high null allele occurrence in *Rhizophora* might also contribute to the positive inbreeding coefficient value in the species. Nearly all populations (94.87%) in this study had excess of homozygotes and positive inbreeding coefficient values (F_{is}). Inbreeding, which facilitates the colonisation of distant locations, is one of the forms of non-random mating that is widespread in mangroves (Wee et al. 2015). The limited availability of pollinators may also lead to greater selfing in *R. apiculata* (Brys & Jacquemyn, 2011).

It was observed that most populations from the central region of Peninsular Malaysia (Selangor, Negeri Sembilan, Melaka and northern Johor) harboured higher genetic diversities as compared to populations from other parts of Peninsular Malaysia. This could be due to the ocean currents along the Straits of Malacca and South China Sea (Figure 3.1). The ocean current movements of both oceans are highly dependent on the monsoon winds during the north-east (December-January) and south-west (June-July) monsoons (Figure 5.1, Wee et al., 2014). Some of the propagules that detach from the parent tree would float in ocean water for an extended period and follow the ocean currents to transport the population from the source to the sink (Nathan et al., 2008). A circulation simulation conducted by Rizal et al. (2012) demonstrated that water mass flowed from the South China Sea to the Straits of Malacca, resulting in a hydrological connection between the two oceans. The connection has allowed the mangrove propagules from both coasts to disperse and get mixed in Selangor, Negeri Sembilan, Melaka and Johor populations.

5.3 Differentiation and relatedness among populations

F-statistics (Wright, 1951) is one of the most commonly used methods to estimate the level of heterozygosity in a population. The F_{st} is more sensitive to detect intraspecific differentiation as compared to its analogue, the R_{st} (Slatkin, 1995; Goodman, 1997) which assumes the stepwise mutation model. The R_{st} however, is thought to better reflect the mutation patterns of microsatellites and is a better predictor of interspecific divergence (Moulin et al., 1996). Both models have been widely used in population genetics because none of the models are suited to analyse all microsatellite loci (Balloux & Lugon-Moulin, 2002).

This similar approach was adapted in the present study and the tested 39 populations of *R. apiculata* across Peninsular Malaysia revealed a strong genetic structure (F_{st} = 0.315, R_{st} = 0.242). Partitioning of genetic variability indicated that 68.5% and 31.5% of microsatellite variations were distributed within and among *R. apiculata* populations, respectively. High population differentiations were also observed in other mangroves including *A. marina* (F_{st} = 0.410, Maguire et al., 2000), *C. tagal* (G_{st} = 0.529, Ge & Sun, 2001), *A. germinans* (G_{st} = 0.410, Dodd et al., 2002) and *R. apiculata* from the greater Sunda Islands (F_{st} = 0.381, Yahya et al., 2014). The high genetic differentiation between *R. apiculata* populations in Peninsular Malaysia can also be explained by the positive correlation between geographic distance and genetic differentiation demonstrated in the Mantel test for isolation by distance (Figure 4.3).

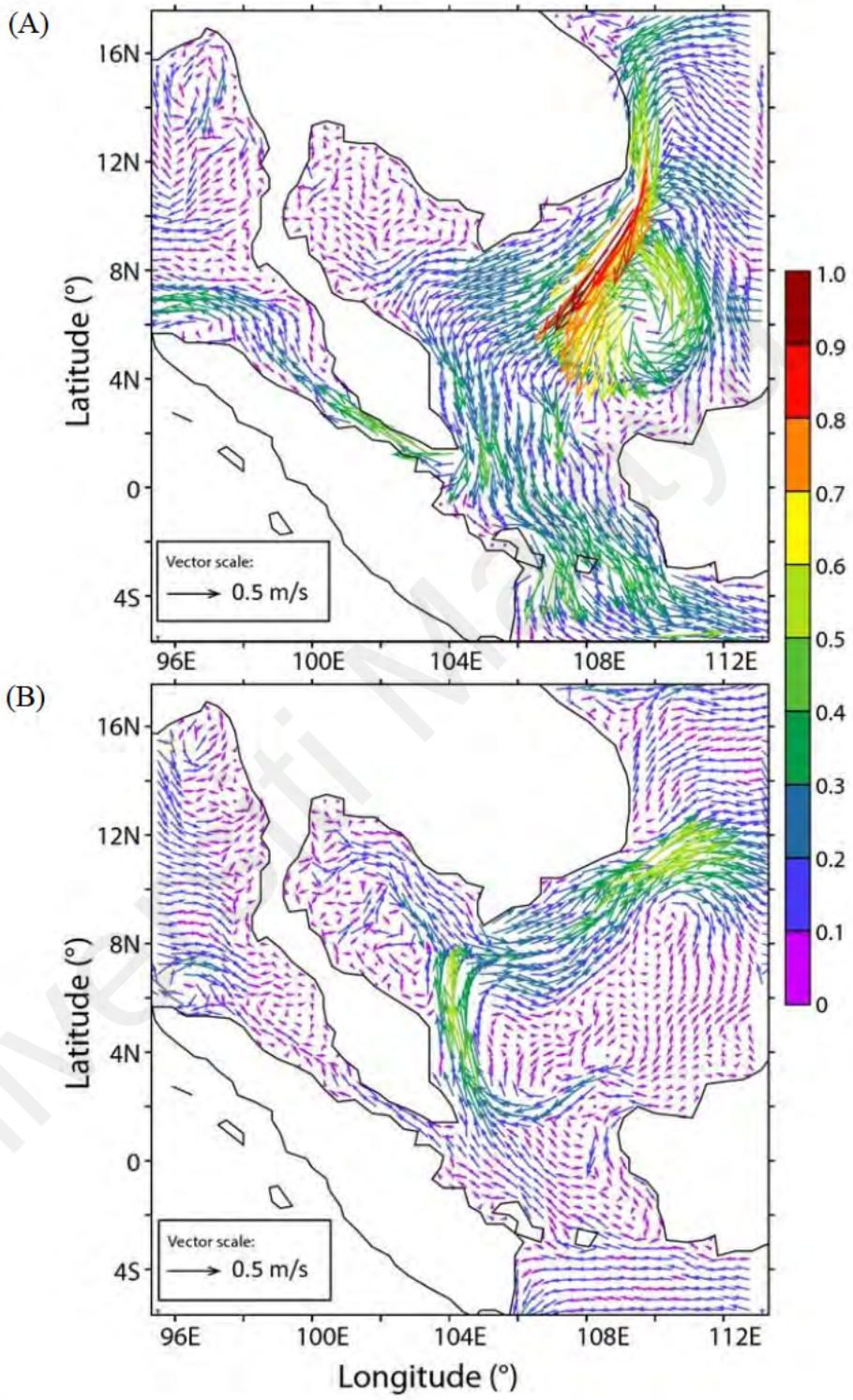


Figure 5.1: Ocean current simulation during (A) north-east and (B) south-west monsoons. The colour gradient indicates the current speed in m/s. Source: Wee et al. (2014).

Cluster analyses in this study defined the 39 tested populations into two geographical clusters (Figure 4.4, 4.5 and 4.7). These clusters corresponded to the populations' geographical origins and coincided with the connecting oceans (i.e., the Straits of Malacca and the South China Sea). Bayesian analysis (Figure 4.7) showed that there were admixture events happening at the central to southern Peninsular Malaysia. The mixing of alleles from western and eastern Peninsular Malaysia can be explained by the ocean current movements whereby water mass flows from the South China Sea into The Straits of Malacca resulting in a hydrological connection between the two oceans (Rizal et al., 2012). Based on fossil and morphological records, Duke et al. (2002) proposed that *R. apiculata* first migrated into Southeast Asia from the north and travelled south from opposite sides of the Malay Peninsula, a peninsula in Southeast Asia that stretches approximately 1,100 km between the Straits of Malacca and the South China Sea. Repeated sea level fluctuations then forced *R. apiculata* to retreat back north and form two isolated refuge populations on opposite sides of the Malay Peninsula. In recent studies, the Malay Peninsula has been identified as the land barrier to gene flow among mangrove populations (Liao et al., 2007; Li et al., 2016). While the study conducted by Wee et al. (2014) demonstrated that ocean currents instead of land masses were responsible in maintaining genetic differentiation in sea-dispersed mangroves.

Interestingly, *R. apiculata* populations in this study and some other commonly found mangrove species reported in previous studies appeared to have low genetic diversities and high genetic differentiations. These are perhaps due mainly to their ecological characteristics, historical vicariance and ocean current movement (Yahya et al., 2014; Wee et al., 2014). Despite high levels of inbreeding and selfing in *R. apiculata*, considerable genetic diversity was partitioned within rather than among populations (Table 4.7). This could be because *R.*

apiculata has the ability to reproduce with the help of wind and pollinators (such as insects) or efficient gene flow by long-distance dispersal (Duke et al., 1998; Ng et al., 2014; Yan et al., 2016). The low genetic diversity of the species together with the loss of genetic diversity from anthropogenic activities can decrease the fitness of a species, which may lead to an increased risk of extinction (Keller & Waller, 2002; Charlesworth & Willis, 2009; Michaelides et al., 2016). The increasing over-exploitation and illegal logging of *R. apiculata* from natural forests in Peninsular Malaysia are wide-spreading because of its highly valued wood, causing the species to decline at an alarming rate. Conserving the genetic diversity of *R. apiculata* is therefore vital for its survival and long-term persistence.

5.4 Implications for conservation

According to Kuhn (1996), habitat quality and conservation genetics are the main paradigms that threaten biodiversity. The main goal of the latter paradigm which relates to this project is to preserve the evolutionary potential of a species by maintaining sufficient levels of genetic diversity so that the species is capable of responding to future environmental changes (Ouborg et al., 2006). Genetics and genomics are major aspects of conservation science to conserve and manage the three components of biodiversity: ecosystems, species and genes (Byrne, 2018). Genetic studies provide information to produce appropriate guidelines for conservation and management strategies of a targeted species (Birkeland et al., 2017; Byrne, 2018). The genetic data enables identification of populations which are important for conservation, given that not all populations have equal adaptive capacities (Petit et al., 1998; Melville & Burchett., 2002). In the present study, molecular tools have contributed in defining the distribution of genetic variations within and among populations

of *R. apiculata* in Peninsular Malaysia. The generated information can be used to formulate *in situ* and *ex situ* conservation guidelines for the species.

In situ conservation is a viable approach to conserve genetic diversity of a species by maintaining reproducing populations within the species' natural habitat (Flower et al., 2018). One important criteria set for *in situ* conservation is to determine the minimum number of populations that are sufficient to cover the maximum preservation of the species' gene pool and identify which populations to select (Prance, 2006). Suitable population representation and viability would influence the effectiveness of the genetic conservation (Thomson et al., 2001). The cluster analyses in this study partitioned the studied populations into two genetic clusters corresponding to two geographical regions in Peninsular Malaysia. This indicates that these two regions should be considered independently for the selection of *in situ* conservation areas. Since *R. apiculata* harboured an F_{st} value of >30% (0.315), approximately five to ten populations per region would be adequate for the conservation of genetic resources of *R. apiculata* in Peninsular Malaysia (Hamrick, 1993). The selection of these populations should be based on the presence of unique alleles and high genetic variations in the populations (Table 5.1). Based on the optimum population size analysis, a total of 860 individuals are needed to avoid inbreeding depression and to ensure 95% of genetic diversity is conserved effectively (Figure 4.8; Lee et al., 2013). The determination of the optimum population size is crucial for successful *in situ* conservation programmes (Lee et al., 2006).

Table 5.1: Appropriate populations for *in situ* conservation in Peninsular Malaysia.

Cluster	State	Population
Western Peninsular Malaysia	Kedah	Dayang Bunting
	Kedah	Kuala Kisap
	Perak	Teluk Kertang
	Perak	Pulau Gula
	Selangor	Pulau Ketam
	Selangor	Pulau Tengah
	Negeri Sembilan	Sg. Linggi
	Melaka	Merlimau Tambahan
	Johor	Muar
	Johor	Pulau Kukup
Eastern Peninsular Malaysia	Pahang	Peramu
	Pahang	Endau
	Pahang	Cherating
	Terengganu	Pengkalan Gelap
	Terengganu	Merchang

The *in situ* conservation areas should have a central core area, surrounded by a buffer zone and peripheral to this, a transition zone (Tan & Tan, 2001; Lee et al., 2006). The core zone is the central area with stable habitat while the presence of a buffer zone will protect the populations in the central core area from edge effects and other factors that might threaten their viability (Lee et al., 2006). Tan & Tan (2001) suggested that the buffer zone is suitable for research applications while the transition zone may be made available for sustainable harvesting activities. Regular inventories can be carried out every two years and previous records of the inventory should be compared with present records (Tan & Tan, 2001). The *in situ* sites should be actively monitored and managed for at least five years or until the survival, reproduction and quality of the offspring are acceptable (Lee et al., 2006).

Ex situ conservation is another key component of conservation for *R. apiculata*. *Ex situ* conservation is conserving a species or all levels of biological diversity outside their natural habitats. Even though conservation of a species in its native habitat is the way to go, *ex situ* conservation is crucial in providing insurance against catastrophic events and as an offsite breeding ground (such as botanical gardens) for the species to facilitate the possibility of reintroduction in the future (Lee et al., 2013). Additionally, individuals in *ex situ* populations can act as ambassadors for endangered wild populations which play a major role in conservation education, raising awareness and gaining public and political support for conservation actions (Kasso et al., 2013; Ferrie, 2016).

Similar to the selection of *in situ* conservation areas, the two regions for *R. apiculata* in the current study should be considered independently for the selection of the mother trees for seed collections. Considering that the species exhibited a high selfing rate, at least 50 unrelated mother trees from each region should be considered for germplasm collections to capture the maximum levels of genetic diversity (Lee et al., 2006). By using 40 progenies from each mother tree, each region would have a total of 2,000 individuals for the establishment of a field gene bank. It has been recommended for mixed strands of *R. apiculata* and *R. mucronata* to have spacing of 1.8 x 1.8 m (Singh & Odaki, 2004). However, a more recent study on *ex situ* conservation of *R. mucronata* demonstrated that the growth performance of the species is better on 2.5 x 2.5 m compared to 2 x 2 m and 1.5 x 1.5 m spacing in moderate saline zone in Bangladesh (Rahman, 2016). If the *R. apiculata* individuals are line-planted at a spacing of 2.5 x 2.5 m, a minimum of 2.5 ha is required, however, the minimum 10 ha is usually stipulated (Theilade et al., 2005).

CHAPTER 6: CONCLUSIONS

6.1 Conclusions

The present study aimed to facilitate the efforts to maintain the genetic diversity of *R. apiculata* in Peninsular Malaysia, mainly through the generation of novel genetic information. The three main objectives designed at the beginning of the study have been achieved, whereby a total of 22 microsatellite markers have been valida ed to assess the genetic diversity within populations and genetic differentiation mong populations of *R. apiculata* found throughout Peninsular Malaysia. Of these, 19 of t e markers were developed *de novo* in this study. These polymorphic mark rs rev aled that *R. apiculata* harboured relatively low levels of genetic diversity but high levels of population differentiation, which could be due mainly to inbreeding and ocean c rrents. The 39 studied populations were defined into two major geographical clusters, corresponded to the populations' geographical origins and coincided with the top g aphy of Peninsular Malaysia where the peninsula may act as the barrier to gene flow

The new genetic information generated in this study will enable the formulation of *in situ* and *ex situ* conservation guidelines for *R. apiculata* in Peninsular Malaysia. The newly developed microsatellite markers can be used in future population genetic studies of *R. apiculata* and its closely related species. Considering that two major clusters were being defined for *R. apiculata* in this study, these two regions can be considered independently for the selection of *in situ* conservation areas in Peninsular Malaysia. It is important to note that the current study was the first extensive population genetics study of *R. apiculata* in Malaysia, covering nearly all natural mangrove stands in Peninsular Malaysia.

6.2 Recommendations for future research

Further sampling of *R. apiculata* from Sabah and Sarawak will yield a complete picture of the population genetics of the species in Malaysia. It would also be interesting to carry out genetic studies on other mangroves living in the same niche as this would allow for comparative analyses with *R. apiculata*. Also, phylogeography study using chloroplast DNA markers could expose the principles and processes governing the geographical distribution of genealogical lineages, especially those within and among closely related species. All these suggested future studies, together with the results from the current study can provide data for long-term conservation management of *R. apiculata* in the whole of Malaysia.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of publications

1. **Azman, A**., Ng, K. K. S., Ng, C. H., Lee, C. T., Tnah, L. H., Zakaria, N. F., … & Lee, S. L. (2020). Low genetic diversity indicating the threatened status of *Rhizophora apiculata* (Rhizophoraceae) in Malaysia: declined evolution meets habitat destruction. *Scientific Reports, 10*, Article#19112**.**

Papers presented

1. **Azman, A**., Lee. S. L., Ng. K. K. S., Ng. C. H., Lee, C. T., Zakaria, N. F., … & Cheng. A. (2019). *Population genetics of Rhizophora apiculata (Bakau Minyak) and its implication for conservation in Peninsular Malaysia*. Oral presentation presented at the Biological Sciences Graduate Congress, 19-21 December 2019. Kuala Lumpur, Malaysia.

2. **Azman, A**., Ng. K. K. S., Ng. C. H., Lee, C. T., Tnah, L. H., Zakaria, N. F., … & Lee, S. L. (2018). *Novel genic simple sequence repeats (SSRs) markers developed from the transcriptome of Rhizophora apiculata (Rhizophoraceae)*. Poster presented at the Malaysian Society of Applied Biology Symposium. 30 June-2 July 2018. Melaka, Malaysia.