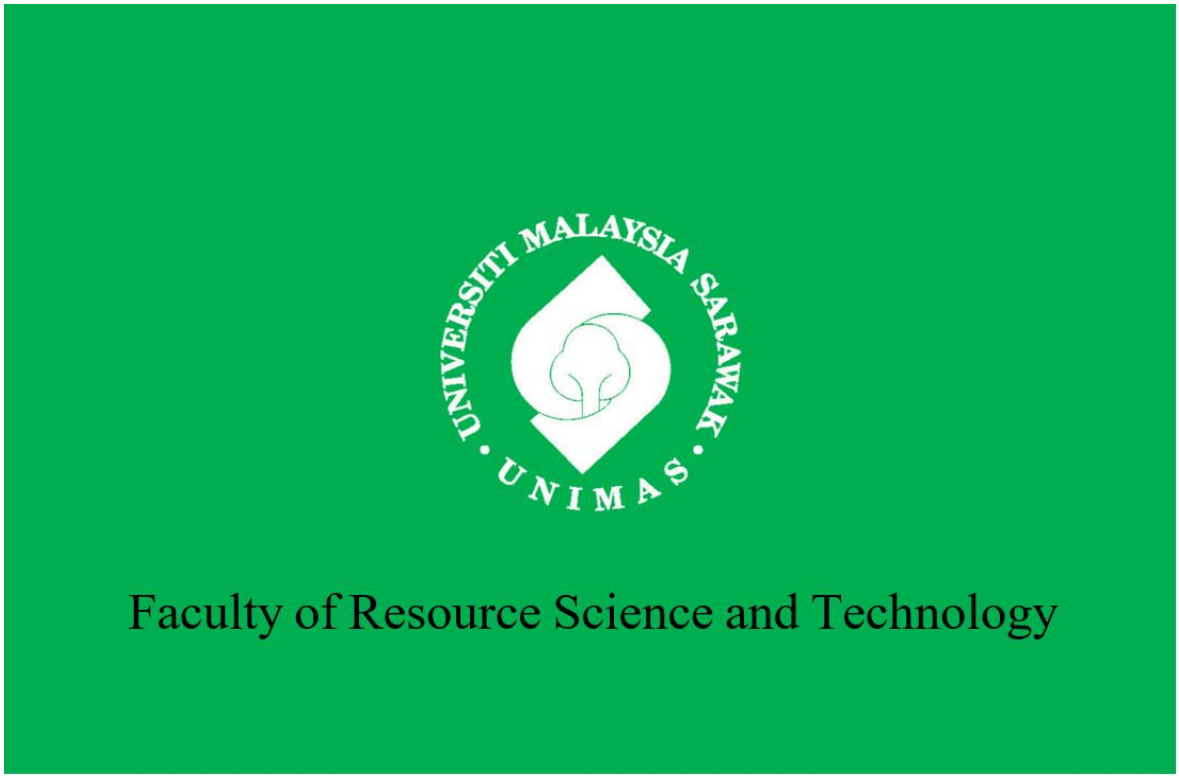

Molecular Profiling of *Shorea macrophylla* with other closely related *Shorea* species

Atirah Binti Abdul Majid (69155)

Bachelor of Science with Honours
(Resource Biotechonology)
2022

Molecular Profiling of *Shorea macrophylla* with other closely related *Shorea* species

Atirah Binti Abdul Majid

A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours
(Resource Biotechnology)

SUPERVISOR: PROFESSOR MADYA DR. WONG SIN YENG

Programme of Resource Biotechnology
Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2022

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Molecular profiling of *Shorea macrophylla* with other closely related *Shorea* species

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ABSTRACT

Shorea macrophylla (de Vriese) P.S. Ashton, locally known as the '*Engkabang Jantong*', is endemic to Sarawak as well as other parts of the island of Borneo. It is a lowland indigenous riparian species that comes from the Dipterocarpaceae family, which is one of Southeast Asia's important tropical rainforest families. To date, the rapid decline in the population of *S. macrophylla* in Borneo's tropical lowland rainforest has been alarming. Conservation measures are urgently needed to protect the genetic diversity of this species. Prior to the implementation of conservation measures, genetic diversity data is significant. However, the lack of documentation and genetic diversity data of *S. macrophylla* has made it difficult for its preservation efforts. Our knowledge and understanding of the relationship between *S. macrophylla* and other *Shorea* spp. are also lacking. Therefore, the purpose of this study is to determine the genetic variability of *S. macrophylla* with other closely related *Shorea* spp. using DNA profiling. Four *Shorea* spp. were selected for this study, namely, *S. macrophylla, S. stenoptera* Burck, *S. seminis* (de Vriese) P.S. Ashton, and *S. splendida* (de Vriese) P.S. Ashton. Maturase K (*matK*), a universal chloroplast DNA barcoding marker, was used in the polymerase chain reaction (PCR). The SEPa Plant DNA Isolation kit was used to extract the DNA. However, the extraction produced a low yield of DNA. Gel electrophoresis was used to determine the success of the DNA extraction and purified PCR products. The concentration and quality of the DNA were determined using spectrophotometric analysis. The results revealed an insufficient amount of DNA concentration; hence, analysis of the sequences to show genetic variability of *S. macrophylla* and other *Shorea* species was not achieved.

Key words: *Shorea macrophylla,* Dipterocarpaceae, genetic diversity, Maturase K (*matK*), conservation

ABSTRAK

Shorea macrophylla (de Vriese) P.S. Ashton, yang dikenali sebagai 'Engkabang Jantong', adalah endemik di Sarawak serta bahagian lain di pulau Borneo. Ia adalah spesies riparian asli tanah rendah yang berasal dari keluarga Dipterocarpaceae, yang merupakan salah satu keluarga hutan hujan tropika penting di Asia Tenggara. Sehingga kini, penurunan pesat populasi S. macrophylla di hutan hujan tanah pamah tropika Borneo telah membimbangkan. Langkah pemuliharaan amat diperlukan untuk melindungi kepelbagaian genetik spesies ini. Sebelum pelaksanaan langkah pemuliharaan, data kepelbagaian genetik adalah penting. Namun begitu, kekurangan dokumentasi dan data kepelbagaian genetik S. macrophylla telah menyukarkan usaha pemeliharaannya. Pengetahuan dan pemahaman kami tentang hubungan antara S. macrophylla dan Shorea spp yang lain. juga kurang. Oleh itu, tujuan kajian ini adalah untuk menentukan kebolehubahan genetik S. macrophylla dengan Shorea spp lain yang berkait rapat. menggunakan pemprofilan DNA. Empat Shorea spp. telah dipilih untuk kajian ini, iaitu, S. macrophylla, S. stenoptera Burck, S. seminis (de Vriese) P.S. Ashton, dan S. splendida (de Vriese) P.S. Ashton. Maturase K (matK), penanda barcoding DNA kloroplas universal, digunakan dalam tindak balas rantai polimerase (PCR). Kit Pengasingan DNA Tumbuhan SEPa telah digunakan untuk mengekstrak DNA. Walau bagaimanapun, pengekstrakan menghasilkan hasil DNA yang rendah. Elektroforesis gel digunakan untuk menentukan kejayaan pengekstrakan DNA dan produk PCR yang telah dipurifikasikan. Konsentrasi dan kualiti DNA ditentukan menggunakan analisis spektrofotometri. Hasilnya mendedahkan jumlah konsentrasi DNA yang tidak mencukupi; Oleh itu, analisis jujukan untuk menunjukkan kebolehubahan genetik S. macrophylla dan spesies Shorea lain tidak tercapai.

Kata kunci: Shorea macrophylla, Dipterocarpaceae, kepelbagaian genetik, Maturase K (matK), pemuliharaan

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LIST OF ABBREVIATIONS

CIA	Chloroform: isoamyl alcohol
cpDNA	Chloroplast DNA
DNA	Deoxyribonucleic Acid
IUCN	International Union for Conservation of Nature
matK	Maturase K
PCR	Polymerase Chain Reaction
RPM	Rotation per Minute
UNIMAS	Universiti Malaysia Sarawak

CHAPTER 1

INTRODUCTION

1.1 Study Background

The recent breakthroughs in human forensic genetics have contributed to the development of molecular DNA technology by leaps and bounds over the past decades, facilitating the application of this technology to a wider range of non-human organisms, such as plants (Ng et al., 2017). Genetic data has demonstrated the high efficiency and usefulness of molecular DNA technology such as DNA-based markers in identifying and characterising levels of diversity, even at lower taxonomic levels (Surabhi et al., 2017). The use of genetic data has grown increasingly important in molecular systematics, especially in understanding the genetic diversity and evolutionary relationships between taxa. It is currently a key focus of various research in evolutionary and systematic biology.

Nowadays, plant taxonomists are depending on genetic data, in addition to morphological characteristics, to classify various plant species. Molecular techniques such as DNA profiling investigations are crucial in determining the degree of diversity in conjunction with DNA-based molecular markers. Numerous studies have been conducted on the subject of how reliable molecular data may be in supporting the plant identification system (Finkeldey et al., 2010). Hence, the main goal of this study was to accurately identify one of the sought-after tree species from the family Dipterocarpaceae, *Shorea macrophylla* (de Vriese) P.S. Ashton, through its genetic data. In this study, DNA profiling was attempted using the widely used molecular marker, chloroplast Maturase K, or *matK* gene sequences,

to provide adequate information capable of identifying and resolving the genetic diversity of *S. macrophylla*.

1.2 Problem Statement

Shorea macrophylla (de Vriese) P.S. Ashton is a member of the family Dipterocarpaceae, and the genus *Shorea* (Purwaningsih & Kintamani, 2018). As a result of its numerous economic and ecological values, the tree is acknowledged as a prime candidate for restoration and reforestation programmes. To date, the population of *S. macrophylla* is seen to have declined significantly. Additionally, the distribution area abundance of this tree species in the tropical lowland rainforest, particularly in Borneo, has noticeably shrunk due to over-exploitation of its valuable light red *meranti* timber, land changes, and climatic factors (Perumal et al., 2021). Back in 1998, *S. macrophylla* was listed under the Red List of Threatened Species as a "Vulnerable (VU)" plant species by the International Union for Conservation of Nature (IUCN) due to these external threats. Nonetheless, it was re-evaluated in 2019 and since then has been listed under the category of "Least Concern (LC)". Although *S. macrophylla* is not suspected to have reached the threshold of being an endangered species, there is cause for concern for its genetic diversity loss in the future.

Conservation measures are urgently needed to preserve the population of *S. macrophylla*. The genetic diversity of a species must first be evaluated for species identification before efficient conservation and sustainable use of genetic resources can be introduced (Surabhi et al., 2017). However, the pattern of genetic diversity has received little attention in comparison to other *Shorea* species. Additionally, insufficient genetic data of *S. macrophylla* has set limitations on understanding the relationship of *S. macrophylla* with other *Shorea* species, which is important for assessing the genetic variability to distinguish the species from one another.

1.3 Objectives

Molecular techniques based on DNA profiling investigations may have the potential to provide an approach to measuring the genetic diversity of *S. macrophylla* using DNA marker sequences, as well as to resolve the taxonomic relatedness of *S. macrophylla* with other closely related *Shorea* species which have not been studied extensively. Generating a genetic database for *S. macrophylla* will be helpful in preserving the genetic diversity of this species in future conservation.

Therefore, the objectives of this study were to: 1) assess the genetic diversity of *Shorea macrophylla* using *matK* gene sequences; and 2) determine the relatedness of S*horea macrophylla* with other closely related *Shorea* species.

CHAPTER 2

LITERATURE REVIEW

2.1 Family Dipterocarpaceae

The family Dipterocarpaceae (often known as “dipterocarps”) is one of the most important families in Southeast Asia’s tropical rainforests. It is home to over 680 species and 17 genera, which are further divided into three subfamilies: Monotoideae, Pakaraimoideae, and Dipterocarpoideae (Kamiya, 2005). They are commonly found at elevations lower than 1,000 metres above sea level and often occupy lowlands with soil that is yellow or red in colour, has a deep solum, and good drainage. Trees from this family thrive in tropical regions that receive an annual rainfall of more than 1,000 millimetres and have a climate that is dry for fewer than six months out of the year. More than half of the total tree population of the family Dipterocarpaceae inhabits Southeast Asian forests, and the island of Borneo is the hotspot of biodiversity for this family (Finkeldey et al., 2009).

Over the years, dipterocarps have contributed significantly to the economic growth of many Southeast Asian countries, providing international timber supplies as well as essential domestic needs (Indrioko et al., 2021). However, external factors such as extensive logging, land change, and climatic factors have greatly impacted the distributions of this family, especially on the island of Borneo. Additionally, conversion of forested to non-forested areas has gradually resulted in the permanent loss of this family’s habitat and eventually threatens its biodiversity (Montagnini et al., 1997). Concerns were raised about the susceptibility of many endemic riparian species from this family, including *Shorea macrophylla*.

It is imperative that corrective actions be taken in order to preserve the genetic resources. In order to effectively preserve the genetic variety of this family, conservation measures, both ex-situ and in-situ, are necessary. Identification of dipterocarps has become a priority for researchers for the purpose of conserving its existing diversity and further understanding its ecological and evolutionary relationships, but the process can be a challenging task, although this family has garnered a lot of attention for its commercial value. The lack of genetic information regarding the relatedness among species within the family is a limitation for the implementation of conservation measures (Tsumura et al., 2010). Most of the time, these trees lack reproductive structures due to the huge variety of dipterocarps as well as the occasional mass flowering and fruiting that takes place (Finkeldey et al., 2009). In addition, species that are genetically related to one another and have a similar appearance might coexist, which adds another layer of complexity to this problem, particularly when it comes to ecological research. As a result, conservation measures necessary for the practise of sustainable forestry have become significantly more complicated due to these limitations.

2.2 Genus *Shorea*

Shorea, *Rubroshorea*, *Richetioides*, and *Anthoshorea* are the names of the four sections that make up the genus *Shorea*. These sections correspond to the four commercially important wood species that are distinguished by their respective colours. According to Ashton (1982), the genus *Shorea* is made up of 10 parts, each of which is further classified into one of four groupings that each contain several sections. Each of these sections is further subdivided into many subsections. The four most important colour-based groups of timber are the colour type *Balau* (Sections *Shorea*, *Pentacme*, and *Neohopea*), the colour type Yellow *Meranti* (Sections *Richetioides* and *Anthoshorea*), the colour type White *Meranti*

(Section *Anthoshorea*), and the colour type Red *Meranti* (Sections *Rubella*, *Brachypterae*, *Mutica*, and *Ovalis*). The wood of the *S. macrophylla* tree is referred to as "light red *meranti*". *S. macrophylla* wood is in high demand in the timber industry due to its numerous economic and domestic applications. It can be utilised in the production of veneer and plywood, as well as in the construction of light-weight structures, the manufacturing of furniture and musical instruments, and the packaging of crates (Istomo & Hidayati, 2012).

2.3 Taxonomy and Botanical Features of *S. macrophylla*

S. macrophylla is classified under the kingdom Plantae, phylum Tracheophyta, class Magnoliopsida, order Malvales, and family Dipterocarpaceae, and the genus *Shorea*. The tree can easily be distinguished from other *Shorea* species by observing the botanical features of the leaves, which are elliptic and oblong in shape with an obtuse base, subcordate with 13 to 18 pairs of conspicuous, hairy midrib above a well-spaced nerve, and petiole stout, while the bark is smooth yet shallowly scaly, and has a short bole (Newman et al., 2022).

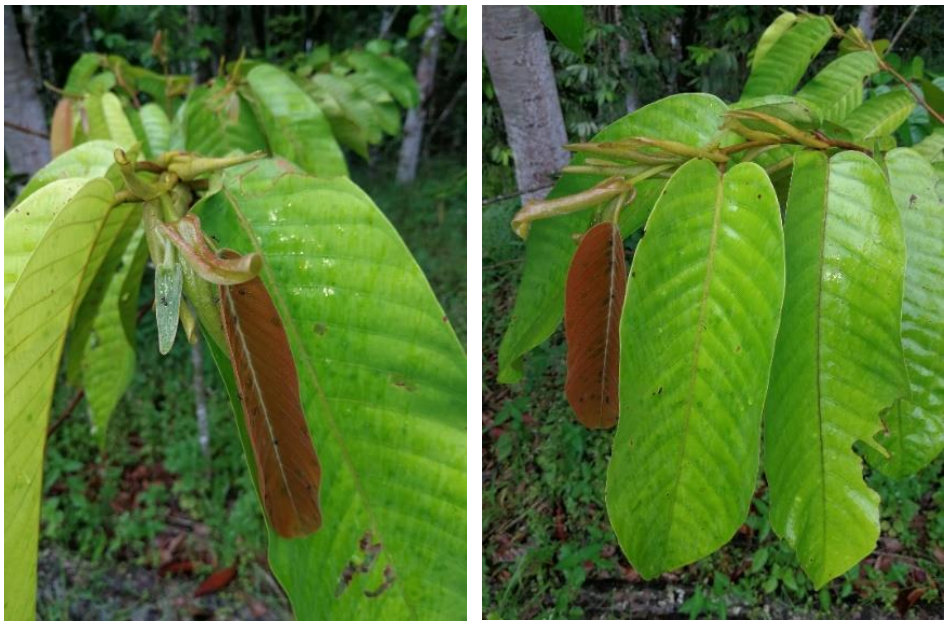

Figure 2.3 The leaf laminae of *S. macrophylla* (de Vriese) P.S. Ashton. Photos taken at Semenggoh Research Centre, Kuching, Sarawak @ Wong SY

The sapwood and heartwood have the same colour and cannot be distinguished from one another. The wood's primary colours are pale cream, with a hint of pink, and yellow brown, with a straw-coloured tone. The texture of the wood is moderately coarse to coarse to even, exhibiting an interlocked grain. The presence of artificial growth rings on the plane surface makes the wood appear lustrous, but the tangential or flat-sawn surfaces can sometimes have feathery tracery (Coolen, 2014).

The fruits of the *S. macrophylla* tree, also known as *illipe* nuts, are exceptionally large and of high quality, with an average size of 4.5 cm x 3.0 cm for the fruits it bears. The winged fruit is shaped in an allusion to the Malay word '*jantung*', which means heart.

Figure 2.4 Wings of the *S. macrophylla* fruit aids in wind-borne germination. Photos taken by Ahmad (2019).

2.4 DNA Barcoding

DNA barcoding has seen extensive application for the purpose of identifying plant material by making use of short DNA sequences, also known as DNA markers, which are located in a specific region of a chloroplast genome (Thakur et al., 2019). DNA markers are well-known for their efficiency in distinguishing between a wide variety of plant species (Arif et al., 2010). Extraction of DNA, amplification of DNA using PCR, sequencing of DNA, and analysis of DNA are the primary steps involved in the DNA barcoding process.

The use of genetic data, which is an alternative to identifying a species based on its botanical characteristics, is becoming an increasingly widespread practise. The resemblance of the DNA sequences to one another, as well as their homology within their respective species, is used as a basis for the identification process. It is essential, for this reason, to have access to suitable DNA markers to use as molecular identifying tools (Jiang, 2015).

2.5 Chloroplast Genome, Maturase K (*matK*) Region

In phylogenetic studies, the Maturase K (*matK*) enzyme, which is encoded by the chloroplast gene, is one of the genes that is utilised most frequently in DNA-based identification of plant species, especially in dipterocarps (Barthet & Hilu, 2007). Species discrimination of angiosperms at a high level is known to be contained in the chloroplast of the gene (Lahaye et al., 2008). After many years, researchers were able to successfully amplify the *matK* gene by employing a pair of primers from the conserved regions of the *trnK*, *Rps16*, and *PsbA* genes (Müller et al., 2006). Today, the *matK* gene is one of the important DNA markers in plant systematics. The *matK* gene provides a high phylogenetic signal, which is an advantage in evolutionary analysis because it can provide fundamental information on the evolutionary history and determine phylogenetic relationships between species (Harnelly et al., 2018).

In Southeast Asia, numerous studies on the genetic phylogeny of the Dipterocarpaceae family have been conducted, with the *matK* gene serving as the key molecular marker (Barthet & Hilu, 2007). The results from *matK* sequencing have been utilised very successfully, which has allowed for the resolution of connections at the generic and even species-level. This is one of the many reasons why *matK* gene is famous in phylogenetic studies (Müller & Borsch, 2005). Since not much is known about the genetics of *S. macrophylla*, *matK* genes may be a beneficial approach to assess its genetic diversity.

2.6 DNA Database of the Genus *Shorea*

Molecular techniques, which are compatible with the DNA database in the presence of appropriate DNA marker sequences, are commonly employed in DNA profiling of timbers belonging to the genus *Shorea* (Nuroniah et al., 2010). For many decades, the phylogeny of *Shorea* has been successfully determined using molecular methods and sequence analysis using the chloroplast DNA (cpDNA) regions (Cao et al., 2006). Primers designed specifically for cpDNA sequences can be used in the molecular classification database of the genus *Shorea* for species identification, including nucleotide substitution detection and other molecular techniques. The DNA sequencing method can easily extract information for identifying *Shorea* trees then classify them into their respective groups. This enables the database to be used to classify *Shorea* spp. based on their molecular properties (Tsumura et al., 2010).

2.7 Challenges in Preserving the Genetic Diversity of *S. macrophylla*

The purpose of this effort is to preserve and conserve the genetic diversity of *S. macrophylla*. It is imperative that a swift conservation measure be put into action in order to prevent this species from being placed on the list of endangered species. However, the

population of *S. macrophylla* has noticeably become less abundant in its natural habitat across the Malesian region. Borneo's tropical rainforest has shrunk in terms of area and biodiversity due to high industrial demands for wood products from trees belonging to the family Dipterocarpaceae (Widiyatno et al., 2020). The resilience of *S. marcophylla* highly depends on the effectiveness of the strategies proposed in rehabilitation programmes and sustainable forest management. Unfortunately, the threats to its biodiversity have restricted these strategies and management.

Concerns have been expressed regarding the rate of intensive logging of its light red *meranti* wood, as well as the conversion of natural ecosystems into agricultural land and the deforestation of the tropical rainforest forests for palm oil planting. Additionally, external influences such as land use changes and climatic factors have also impacted the population and distribution of this species (Rikando et al., 2019). Conservation measures are required to be implemented to protect this species, but it is crucial to accurately identify the species before the measures can be set. Without reliable and efficient identification techniques, it will be difficult to distinguish *S. macrophylla* in the tropical rainforests of Southeast Asia, which is known to contain half of the world's tree population.

The application of botanical-based identification may be useful, but it may be time-consuming and requires the expertise of highly trained and experienced professionals (Ogata et al., 2008). Information for the identification of dipterocarps that spans taxonomic groupings and geographical regions is sorely lacking at present in molecular plant systematic studies. Implementation of an effective conservation measure requires detailed evaluations of the genetic diversity of *S. macrophylla* and its composition. Unfortunately, very little attention is given to this tree species, and the present genetic data is still lacking.

CHAPTER 3

METHODS AND MATERIALS

3.1 Sample Collection and Preparation

Four *Shorea* species were selected for this study, namely, *S. macrophylla, S. stenoptera* Burck, *S. seminis* (de Vriese) P.S. Ashton, and *S. splendida* (de Vriese) P.S. Ashton. The fresh young leaf samples were obtained from Bau, Kuching, Sarawak. The first step in the preparation of the leaf samples was to cut them into small pieces before placing them inside the mortar, then covered with aluminium foil. The samples were then left to freeze in the freezer overnight. After that, the samples were then ground using a mortar and pestle to obtain pulverised plant tissues.

3.2 DNA Extraction

The DNA of all four *Shorea* spp. were extracted with the SEPa Plant DNA Isolation kit. In 2 mL microcentrifuge tubes, 200 mg of pulverised plant tissues were placed. Each tube was then filled with 1 mL of pre-warmed Plant Lysis Buffer, 25 µL of Binding Enhancer, and 20 µL of β-mercaptoethanol. The tubes were vortexed briefly before incubating in the water bath for 30 minutes at 65°C. Each tube was inverted several times at an internal of 10 minutes After incubation, the tubes were centrifuged at a temperature of 4°C for 10 minutes at a speed of 14,800 rpm. Following the centrifugation of the tubes, approximately 900 µL of the supernatants were poured into fresh 2 mL tubes. After adding an equivalent volume of chloroform: isoamyl alcohol (CIA) at a ratio of 24:1, the new tubes were turned upside down twenty times to ensure that the supernatant and the CIA were well mixed together.

After centrifuging the tubes at a temperature of 4°C for 15 minutes at a speed of 13,000 rpm, about 600 µL of the supernatant were poured into fresh 2 mL tubes. After this, there was an addition of three hundred millilitres of diluent solution to the tubes that contained the supernatant. To prevent any liquid from spilling, the tubes were carefully turned upside down. After that, 5 µL of the RNase solution was added, and the tubes were turned upside down once again before being spun down. After that, each tube was put inside the incubator for half an hour at 37°C.

After half an hour, or 30 minutes, the tubes were removed from the warm water bath, and approximately 900 µL of CIA were added to the mixture in order to wash the samples of any leftover contaminants. After placing them on the Rotary Mixer at a speed of RPM 45 for 20 minutes, the tubes were turned upside down 20 times. After ensuring that the liquids were thoroughly combined, the test tubes were centrifuged once again for 14 minutes at a speed of 13,000 rpm in a temperature of 4°C. The next step is to transfer around 750 µL of the supernatant into fresh 2 millilitre tubes, and then follow it up by adding 900 µL ice-cold isopropanol. After turning the tubes upside down and incubating them at a temperature of minus 20°C for 10 to 20 minutes. Finally, the tubes were centrifuged at 13,000 rpm for in 4°C for about 10 minutes.

The upper layer known as the supernatant was thrown away. A volume of 1 mL of iced-cold ethanol with 70% concentration was added. After giving the tubes a light shake to mix the liquid contained therein, the mixture was centrifuged once again at a speed of 10,200 rpm for a period of 5 minutes while the temperature was set at 4°C. Once more, the supernatants were discarded, and this time they were placed upside down on a paper towel. The tubes were left to air dry for 3 minutes. Following an incubation period of three minutes, an amount

ranging from approximately 100 to 200 µL of Diluent solution was added using a micropipette to resuspend all DNA pellets.

3.3 Agarose Gel Preparation

First and foremost, 15 mL of TAE buffer was added into an empty beaker followed by 1% of agarose gel powder (0.15 g of powder). The TAE buffer and powder were heated in the microwave for 30 seconds to melt. Following that, approximately 3 µL of SYBR® Safe DNA Gel Stain was dropped into the beaker. The warm gel was transferred into the electrophoresis slide and was cooled down at room temperature for 10 to 15 minutes. After the gel cools down and solidifies, the combs were removed slowly from the slide in a straight angle to avoid any damage to the gel.

3.4 Gel Electrophoresis

The slide was transferred into the electrophoresis machine with TAE buffer flooding the gel. 4 µL of PrimeWay 100 kB DNA Ladder was transferred into the first well on the slide using a pipette, followed by of 1 µL of yellow dye mixed with 3 µL of DNA samples of all four isolated genomic DNA from the *Shorea* spp. Each DNA samples were placed gently into the well without penetrating the bottom of the gel. The electrophoresis machine was run for 15 minutes.

3.5 PCR Amplification

For the DNA amplification, the PCR mix contained 1 µL DNA, 1 of primers, 4 µL of 5X Colorless GoTaq® Flexi Buffer, 0.2 µL of dNTP mix (10 mM), 2 µL of $MgCl_2$ (25 mM), 0.2 µL of GoTaq® Flexi DNA polymerase, and 10.6 µL of nuclease-free water. The *matK* regions were amplified using two pairs of primers: *matK* 5F: 5’-GAAATGGGTTCGACA-3’ (Gamage et al., 2006), with *matK* 990R primers: 5’-GGACAATGATCCAATGGC-3’