UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF CHLOROPLAST GENOME LIBRARY FOR DNA TRACKING AND CONSERVATION OF ENDANGERED AQUILARIA SPECIES NATIVE TO MALAYSIA

MUHAMMAD SYAHMI BIN HISHAMUDDIN

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By

MUHAMMAD SYAHMI BIN HISHAMUDDIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

January 2022

DEDICATION

In the name of ALLAH the Beneficent and the Merciful

Special dedication to:

Supervisory Committee

PROF. DR. ROZI MOHAMED

DR. SHAIRUL IZAN BINTI RAMLEE

DR. DHILIA UDIE LAMASUDIN

Parents

Kol. (B) JAMALUDDIN BIN AHAMAD & CHE ROHANA BINTI AHMAD

To my late father

Mej. (B) HISHAMUDDIN BIN HAJI KADIR

My wife and daughter

NURSUHAILI BINTI ZAHARI & NURSAKINAH MADEENA BINTI MUHAMMAD SYAHMI

and

My siblings and friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

DEVELOPMENT OF CHLOROPLAST GENOME LIBRARY FOR DNA TRACKING AND CONSERVATION OF ENDANGERED *AQUILARIA* SPECIES NATIVE TO MALAYSIA

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MUHAMMAD SYAHMI BIN HISHAMUDDIN

January 2022

Chair : Professor Rozi Mohamed, PhD
Faculty : Forestry and Environment

Aquilaria Lam., species [*Aquilaria beccariana* Tiegh., (*gaharu buaya*), *Aquilaria crassna* Pierre ex Lecomte, (*gaharu*), *Aquilaria hirta* Ridl., (*chandan bulu*), *Aquilaria malaccensis* Lam., (*ching karas*), *Aquilaria microcarpa* Baill., (*gaharu putih*), *Aquilaria rostrata* Ridl., (*candan gunung*), *Aquilaria sinensis* (*gaharu*) and *Aquilaria subintegra* Ding Hou, (*gaharu*)] is a naturally distributed in the Indomalesian region and are protected against over-exploitation. *Aquilaria* is highly prized for its unique scented resin, agarwood, which is often the subject of unlawful trade activities. Survival of the tree is heavily threatened by destructive harvesting and agarwood poaching, leading to its protection under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Ambiguous species delimitation and limited genetic information within *Aquilaria* are among the impediments to conservation efforts. In this study, a comparative analysis was performed on eight *Aquilaria* species complete chloroplast (cp) genomes, of which seven were newly sequenced using Illumina HiSeq X Ten platform followed by de novo assembly. All *Aquilaria* species utilised in this study were collected during fieldwork and the *Aquilaria* germplasm of Forest Research Institute of Malaysia (FRIM), and grown in the greenhouse of the Faculty of Forestry and Environment, Universiti Putra Malaysia. All *Aquilaria* species have been authenticated by experts. To conduct comparative cp genome analysis, the sequences of all eight *Aquilaria* cp genomes were aligned using MAFFT v7 and then imported into DnaSP v5.10.1 to determine nucleotide diversity in the total genome, LSC, SSC, and IR regions. The boundaries between the IR and SC regions were manually examined to determine the differences in length variation between *Aquilaria* cp genomes. *Aquilaria* cp genomes possess a typical quadripartite structure including gene order and genomic structure. The length of each of the cp genome is about 174 kbp and encoded between 89 and 92 proteins, 38 tRNAs, and 8 rRNAs, with 27 duplicated in the IR (inverted repeat) region. Besides, 832 repeats (forward, reverse, palindrome and complement repeats) and nine highly variable regions

(HVR) were also identified. The phylogenetic analysis performed using Maximum likelihood (ML) and Bayesian inference (BI) suggests that the topology structure of *Aquilaria* cp genomes were well presented with strong support values based on the cp genomes data set and matches their geographic distribution pattern. Five of the nine HVR (*mat*K-*rps*16, *ndh*F-*rpl*32, *psb*J-*pet*A, *trn*D, and *trn*T-*trn*L) were selected based on a cut off value of >0.01. These regions were further analyzed using the neighbor-joining (NJ) method to assess their ability at discriminating the eight species. Coupled with in silico primer design, two potential barcoding regions, *psb*J-*pet*A and *trn*T-*trn*L, were identified. Their strengths in species delimitation were evaluated individually and in combination, via DNA barcoding analysis. The results showed that the combined dataset, *psb*J-*pet*A+*trn*T-*trn*L, effectively resolved members of the genus *Aquilaria* by clustering all species into their respective clades. In addition, this study show great potential for agarwood identification that the newly proposed DNA barcode was capable at identifying the species of origin of six commercial agarwood samples that were included as unknown samples. Such achievement offers a new technical advancement, useful in the combat against illicit agarwood trades and in assisting the conservation of these valuable species in natural populations. The identification of simple sequence repeats (SSR) across complete cp genome sequences from eight *Aquilaria* species has never been reported before. Perhaps because developing these genetic are regarded as laborious and expensive. To overcome this issue, the current work also aims to find SSRs in *Aquilaria* cp genomes using silico methods. For identification of the simple sequence repeats (SSRs), MISA PERL script which had a repeat length of 12 for mononucleotides (mono-), 6 for dinucleotides (di-), 4 for trinucleotides (tri-), 3 for tetranucleotides (tetra-), pentanucleotides (penta-), and hexanucleotides (hexa-), respectively, along with frequency were utilized. From a total of 312 SSRs that were discovered, merely 50 (16%) were found localized within the coding region while the majority (84%) were within the intergenic regions, with an average of one SSR per 4.5 kb. The mean length of the SSRs were 11.63 bp. Mono- repeats were the predominant motifs (29.2%), followed by tetra- (28.8%), di- (20.5%), tri- (19.9%), and penta- (1.6%). Whereas the most recurring motifs were A/T (97.8%) for mono-, AT/AT (87.5%) for di-, AAT/ATT (48.4%) for tri-, and AAAT/ATTT (45.6%) for tetra-. GO analysis using the REVIGO software identified four molecular functions, six biological processes and three cellular components. In conclusion, findings of this SSR offer a scientific foundation for future phylogenetics, evolutionary genetics, diversity studies and breeding programs on *Aquilaria* species.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN PERPUSTAKAAN DATA GENOM KLOROPLAS BAGI MENJEJAKI DNA DAN PEMULIHARAAN SPESIS *AQUILARIA* (TERANCAM) DI MALAYSIA

Oleh

MUHAMMAD SYAHMI BIN HISHAMUDDIN

Januari 2022

Pengerusi : Profesor Rozi Mohamed, PhD
Fakulti : Perhutanan dan Alam Sekitar

Aquilaria spesis [*Aquilaria beccariana* Tiegh., (*gaharu buaya*), *Aquilaria crassna* Pierre ex Lecomte, (*gaharu*), *Aquilaria hirta* Ridl., (*chandan bulu*), *Aquilaria malaccensis* Lam., (*ching karas*), *Aquilaria microcarpa* Baill., (*gaharu putih*), *Aquilaria rostrata* Ridl., (*candan gunung*), *Aquilaria sinensis* (*gaharu*) and *Aquilaria subintegra* Ding Hou, (*gaharu*)] tersebar secara semula jadi di wilayah Indomalesia dan dilindungi daripada eksploitasi berlebihan. *Aquilaria* sangat berharga kerana resinnya yang unik dan berbau wangi, gaharu, yang sering terlibat dalam aktiviti perdagangan yang tidak sah. Kelangsungan hidup pokok ini sangat terancam dengan penuaian yang merosakkan dan perburuan gaharu secara besar-besaran, menyebabkan ianya dilindungi dan disenaraikan dibawah Konvensyen Perdagangan Antarabangsa Spesies Fauna dan Flora Liar yang Terancam (CITES). Persempadanan spesies yang kurang jelas dan maklumat genetik yang terhad dalam *Aquilaria* adalah antara halangan usaha pemuliharaan. Dalam kajian ini, analisis perbandingan dilakukan terhadap lapan urutan genom kloroplas lengkap (cp) spesies *Aquilaria*, di mana tujuh daripadanya baru disusun mengikut urutan menggunakan platform Illumina HiSeq X Ten diikuti dengan pemasangan de novo. Semua spesies *Aquilaria* yang digunakan dalam kajian ini telah diperolehi semasa kerja lapangan dan juga daripada germplasma *Aquilaria* Institut Penyelidikan Perhutanan Malaysia (FRIM), dan ditanam di rumah hijau Fakulti Perhutanan dan Alam Sekitar, Universiti Putra Malaysia. Semua spesies *Aquilaria* telah disahkan oleh pakar akan ketulenan spesisnya. Untuk menjalankan analisis perbandingan genom kloroplas, jujukan kesemua lapan genom kloroplas *Aquilaria* telah diselaraskan menggunakan MAFFT v7 dan kemudian diimport ke dalam DnaSP v5.10.1 untuk menentukan kepelbagaian nukleotida dalam keseluruhan genom, LSC, SSC dan wilayah IR. Sempadan antara kawasan IR dan SC telah diperiksa secara manual untuk menentukan perbezaan variasi panjang antara genom kloroplas *Aquilaria*.Genom kloroplas *Aquilaria* mempunyai struktur kuadripartit termasuk susunan gen dan struktur genom. Panjang setiap genom kloroplas adalah kira-

kira 174 kbp dan dikodkan antara 89 dan 92 protein, 38 tRNA, dan 8 rRNA, dengan 27 pengulangan di wilayah IR (ulangan terbalik). Selain itu, 832 pengulangan (ke depan, bertentangan, palindrom dan pengulangan pelengkap) dan sembilan kawasan yang sangat berubah (HVR) juga dikenal-pasti. Analisis filogenetik yang dilakukan menggunakan kaedah kemungkinan maksimum (ML) dan Inferens Bayesian (BI) menunjukkan bahawa struktur topologi genom kloroplas *Aquilaria* dibentangkan dengan baik dengan nilai sokongan yang kuat berdasarkan set data genom kloroplas dan sepadan dengan corak taburan geografi mereka. Lima daripada sembilan HVR (*mat*K-*rps*16, *ndh*F-*rpl*32, *psb*J-*pet*A, *trn*D, and *trn*T-*trn*L) dipilih berdasarkan nilai pemotongan > 0.01. Kawasan-kawasan ini selanjutnya dianalisa menggunakan kaedah hubung-kait jiran (NJ) untuk menilai kemampuan mereka dalam mendiskriminasi lapan spesies. Ditambah dengan reka bentuk *primer* yang dihasilkan secara *in-silico*, dua wilayah barkod yang berpotensi, *psb*J-*pet*A dan *trn*T-*trn*L, dikenal-pasti. Kekuatan mereka dalam penghadan sempadan spesies dinilai secara individu dan gabungan, melalui analisis barkoding DNA. Hasil kajian menunjukkan bahawa kumpulan data gabungan antara, *psb*J-*pet*A + *trn*T-*trn*L, berjaya menyelesaikan anggota genus *Aquilaria* dengan mengkelaskan semua spesies ke dalam kelompok masing-masing. Di samping itu, kajian ini menunjukkan bahawa barkod DNA yang dicadangkan mampu mengenal-pasti spesies asal enam sampel gaharu komersial yang dimasukkan sebagai sampel yang tidak diketahui. Pencapaian ini memberikan kemajuan teknikal baru, berguna dalam memerangi perdagangan gaharu secara haram dan dapat membantu pemuliharaan spesies berharga ini dalam populasi semula jadi. Pengenalpastian ulangan jujukan mudah (SSR) merentasi jujukan genom kloroplas lengkap daripada lapan spesies *Aquilaria* tidak pernah dilaporkan sebelum ini. Mungkin kerana membangunkan genetik ini dianggap rumit dan melibatkan kos yang mahal. Untuk mengatasi isu ini, kajian ini dilakukan bertujuan untuk mencari SSR dalam genom kloroplas *Aquilaria* menggunakan kaedah silico. Untuk mengenal pasti ulangan jujukan mudah (SSR), skrip MISA PERL yang mempunyai panjang ulangan 12 untuk mononukleotida (mono-), 6 untuk dinukleotida (di-), 4 untuk trinukleotida (tri-), 3 untuk tetranukleotida (tetra-).), pentanukleotida (penta-), dan heksanukleotida (hexa-), telah digunakan. Daripada sejumlah 312 SSR yang ditemui, hanya 50 (16%) ditemui dalam kawasan pengekodan manakala majoriti (84%) berada dalam kawasan intergenik, dengan purata satu SSR setiap 4.5 kb. Purata panjang SSR ialah 11.63 bp. Mono- ulangan adalah motif utama (29.2%), diikuti oleh tetra- (28.8%), di- (20.5%), tri- (19.9%), dan penta- (1.6%). Manakala motif yang paling berulang ialah A/T (97.8%) untuk mono-, AT/AT (87.5%) untuk di-, AAT/ATT (48.4%) untuk tri-, dan AAAT/ATTT (45.6%) untuk tetra- . Analisis GO menggunakan perisian REVIGO mengenal pasti empat fungsi molekul, enam proses biologi dan tiga komponen selular. Kesimpulannya, penemuan SSR ini menawarkan asas saintifik untuk filogenetik, genetik evolusi, kajian kepelbagaian dan program pembiakan spesies *Aquilaria*.

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SPECIALLY DEDICATED TO MY LATE FATHER,
Mej. (B) Haji Hishamuddin Bin Haji Kadir

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

UPM	Universiti Putra Malaysia
°C	degree Celsius
+ F	base frequencies
+ G4	Gamma model with default 4 rate categories
%	Percentage
s	second
min	minutes
Ta	Annealing Temperature
Tm	Melting Temperature
ng	Nanogram
µL	Microliter
µmol/L	Micromole per litre
w/v	Weight per Volume
AFLP	Amplified Fragment-Length Polymorphism
AS	Agarwood Stick
AT	Agarwood Tea
AWB	Agarwood Wood Block
AWC	Agarwood Wood Chips
Bar-HRM	Barcoding-High-Resolution Melting
BI	Bayesian inference
bp	Base Pair
C	Complement
CBOL	The Consortium for the Barcode of Life-Plant Working Group
CDS	Coding Sequence

cemA	chloroplast envelope membrane protein
CE	Capillary Electrophoresis
CO1	cytochrome c oxidase 1
cp	Chloroplast
cpDNA	Chloroplast DNA
cpSSRs	Chloroplast SSRs
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CTAB	cetyltrimethylammonium bromide
di-	Dinucleotide
DNA	Deoxyribonucleic Acid
DNASP	DNA Sequence Polymorphism
F	Forward
FASTA	Fast Alignment
FDR	False Discovery Rate
FRIM	Forest Research Institute of Malaysia
Gb	Gigabyte
GC content	guanine-cytosine content
GO	Gene Ontology
h	Hour
HVRs	Highly Variable Regions
IGS	Intergenic Spacer
IUCN	International Union for Conservation of Nature and Natural Resources
ITS	Internal Transcribed Spacer
ITS1	Internal Transcribed Spacer 1
ITS2	Internal Transcribed Spacer 2

IR	Inverted Repeat
ISSR	Inter-Simple Sequence Repeat
K2P	Kimura Two-Parameter
kb	Kilobases
kbp	Kilobase pairs
LSC	Large Single Copy
MAFFT	Multiple Sequence Alignment
matK	maturaseK
Mb	Million Base
MCMC	Markov chain Monte Carlo
MISA	Microsatellite Finder
mg	Milligram
mL	Millilitre
ML	Maximum likelihood
mono-	Mononucleotide
mtDNA	Mitochondrial DNA
mtSSRs	Mitochondrial SSRs
nSSRs	Nuclear SSRs
NCBI	National Center for Biotechnology Information
NJ	Neighbor Joining
NGS	Next Generation Sequencing
OGDRAW	OrganellarGenomeDRAW
ORFs	Open Reading Frames
P	Palindrome
PCR	Polymerase Chain Reaction
Pi	Nucleotide Variability

PIS	Parsimony Informative Sites
PIS2V	Parsimony Informative Sites with Two Variants
PIS3V	Parsimony Informative Sites with Three Variants
PVP	Polyvinylpyrolidone
R	Reverse
rRNA	Ribosomal RNA
RAPD	Random Ampified Polymorphic DNA
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SH-aLRT	SH-like Approximate Likelihood-Ratio Test
SNP	Single Nucleotide Polymorphism
spp	Species
SRAP	Sequence-Related Amplified Polymorphism
SSC	Small Single Copy
SSR	Simple Sequence Repeat
SVS	Singleton Variable Sites
SV2V	Singleton Variable with Two Variants
SV3V	Singleton Variable with Three Variants
tri-	Trinucleotide
tRNA	Transfer Ribonucleic Acid
TVM	Transversion Model
UFboot	Ultrafast Bootstrapping Algorithm

CHAPTER 1

INTRODUCTION

1.1 General

Aquilaria Lam. (Thymelaeaceae) is an evergreen tropical forest tree endemic to the Indomalesia region (Whitmore, 1972). This tree is well-known for producing aromatic, rare and precious wood, commonly known as agarwood (*gaharu)* that has significant industrial values. The woodchip of agarwood is processed into incense powder, sticks, cubes, cosmetic accessories like bracelets and pendants, and hygienic goods, such as shampoo and soap and scented balms, and perfumes essential oil (Snelder and Lasco, 2008). There are 21 accepted species in the genus *Aquilaria*. Five of them, namely *Aquilaria beccariana* Tiegh., *Aquilaria hirta* Ridl., *Aquilaria malaccensis* Lam., *Aquilaria microcarpa* Baill., and *Aquilaria rostrata* Ridl., are reported to be native to Malaysia (Lee and Mohamed, 2016a). *Aquilaria rostrata* Ridl., an endemic species to Peninsular Malaysia, was previously thought to be extinct until it was rediscovered in 2015, nearly 100 years after its first record in 1911 (Lee and Mohamed, 2016b).

Currently, all the species in this genus are listed in Appendix II of CITES and are also classified as vulnerable to extinction on the Red List of the International Union for Conservation of Nature (UNEP-WCMC, 2019). Consequently, agarwood trade is strictly controlled by international legislation, as these species are listed in Appendix II. Commercial activities require specific permits, and traders frequently attempt to circumvent these regulations through different means, including illegal ways. Similar to other plant species, the identification of *Aquilaria* species is mostly based on its physical and morphometric traits, specifically on the floral reproductive structures (Lee and Mohamed, 2016a). However, due to its appearance and phenotypic plasticity, agarwood is relatively easy to be separated from wood from other genera by hand lens or microscopy, but difficult or impossible for species identification using wood anatomy (Yin et al., 2016). In general, the identification of agarwood species using the naked eye is a nearly impossible and difficult task.

The DNA sequence of an organism provides an effective and efficient tool for species identification (Hollingsworth et al., 2011). As a result, several researchers have attempted to develop a library of useful gene sequences for *Aquilaria* species delineation. Previous studies showed that the *trn*L-*trn*F region is useful in examining the phylogenetic relationships between different *Aquilaria* species (Eurlings and Gravendeel, 2005). Thus, this genomic region is potentially useful as biomarker in distinguishing degraded agarwood products by using a real-time polymerase chain reaction (PCR) approach (Mohamed et al., 2012). It was also reported that the DNA nuclear ribosomal internal transcribed spacer 1 (ITS1) region and the intergenic spacer region *psb*C-*trn*S could differentiate between different *Aquilaria* species (Ito and Honda, 2005). In

addition to the identification techniques based on gene markers, the advent of the DNA barcoding technique shed light on *Aquilaria* species identification and bolstering traditional wood identification. However, this initiative is not always attainable at the species level. The feasibility of DNA barcoding on *Aquilaria* wood samples was tested by confirming the limitation of extracting useful genomic information through the amplification process performed on heat-treated samples (Jiao et al., 2014). Furthermore, the combination loci of *trn*L-*trn*F+ITS and *trn*L-*trn*F+ITS2 were reported as potential DNA barcoding loci for *Aquilaria* species discrimination and identification of agarwood-related products (Lee et al., 2016a). Despite the potential use of single gene markers, there are still limitations in identifying very closely related species, necessitating further investigation by using alternative DNA resources, such as the chloroplast (cp) genome.

The chloroplast genomes exist in a semi-autonomous state relative to the nuclear genome, having their own genome and gene expression system. Chloroplast genes are derived from subsets of endosymbiont genes, the products of which regulate their own gene transcription. These genes remain confined to a single membrane-bound compartment, together with their protein products (Allen, 2015). In addition, the cp genomes also comprise a separate transcription and transport mechanism encoded by ribosomal proteins related to photosynthesis (Bungard, 2004). Although the cp genome is significantly smaller than the nuclear genome, each cell has around 400 to 1600 copies. These numbers are enough to provide an important foundation for the research of species relationships, evolutionary and genetic polymorphisms. The comparative study of the cp genome is crucial for the evolution of chloroplasts and the discovery of their basic variation (Bennett, 1987). To date, most of the study on the cp genome were one-sided and focused only on individual area or were based on specific species. The comparative researches on the cp genome levels between related species are not widely studied and available.

Previous researcher had reported the challenges in using universal DNA barcoding genes to distinguish and identify highly evolutionarily related *Aquilaria* species (Lee et al., 2016a). Therefore, this study is proposing to sequence the cp genome as this genome is smaller than the whole genome, therefore the sequencing data are more manageable. In addition, the genome is potentially valuable in giving numerous highly polymorphic loci for discriminating between various *Aquilaria* species. In other words, this study aims to develop a cp genome library based on five native *Aquilaria* species in Malaysia. The suitable regions from each species will be identified to develop DNA barcodes, and a DNA tracking system will be developed. The identification of potential DNA barcode sites is essential in developing conservation plans for each species. Furthermore, the library will provide a solid platform for future researches, such as by identifying *Aquilaria* evolutionary history and ecophysiology.

1.2 Problem Statement

The demand for natural agarwood on the international market has continued to rise over the years (Azren et al., 2019). The high prices range from US$100/kg for low grade to US$100,000/kg for good grade, and the lucrative profits from the sales of wild agarwood have resulted in massive illegal hunting and harvesting activities of *Aquilaria* trees (Ismail et al., 2015). These actions further lowered the number of wild *Aquilaria* that can reproduce naturally and, as a consequence, gradually reduced their population. Due to the rapid reduction in wild *Aquilaria* species populations, nearly all the *Aquilaria* species are now being placed on the Appendix II (CITES), including five Malaysian *Aquilaria* species, namely *A. beccariana*, *A. hirta*, *A. malaccensis* Lam., *A. microcarpa*, and *A. rostrata* Ridl.. Three of these five *Aquilaria* species have been listed as endangered (*A. microcarpa*) and critically endangered (*A. malaccensis* and *A. rostrata*). Even though most nations have stringent cross-border monitoring, there are still reports of illegal international agarwood commencing and cross-border smuggling. One main reason contributing to this is that different forms of agarwood, ranging from powders to hardwood beams, are easily blended with non-protected species, thus foiling the detection. The development of a complete cp genome of *Aquilaria* sequence database, especially from five *Aquilaria* species native to Malaysia, can aid in enhancing species-specific molecular techniques for differentiating between various *Aquilaria* species. This initiative is critical for the agarwood sector to ensure consumers right, authenticate market products, and effectively combat agarwood trade fraud.

1.3 Justification

The decreased population of natural *Aquilaria* and the listing of *Aquilaria* as an endangered species have prompted the researchers, naturalists, and conservationists to enhance species gene pool resources to conserve and protect this genus from extinction. To successfully establish programmed reproduction and characterize the genetic information from the natural *Aquilaria* population, the developments of a complete cp genome sequences database and the research on data utilization from this database are critical for conservation efforts. With the availability of a complete cp genome, the researchers can increase the efficacy of traditional methods for identifying *Aquilaria* species that are based on a partial genome sequence. The complete cp genome contains a huge quantity of genetic information, thus is ideal for studying genetical and structural varieties of natural populations. DNA-based studies on *Aquilaria* species began in 2005 after TRAFFIC emphasized the importance of conserving the natural *Aquilaria* resources in the wild, while the studies based on the cp genome of *Aquilaria* were started only in 2016.

The study of the complete cp genome can assist in the understanding of tree life, evolution, taxonomy, and genetic diversity. Cp genome is widely used in phylogenetic studies, as it is maternally inherited and is recombination-free. The highly conserved structure of cp genome facilitates sequencing and identification

of potential barcodes useful for plant species identification. In this study, genetic information of these endangered tree species are sequenced as a necessary step towards conserving their natural habitat and genetic diversity. Adequate genetic information and an understanding of species identification techniques will aid in the conservation efforts, specifically by assisting in the establishment of appropriate breeding programs and timber trade controls.

1.4 Research Objectives

The general objective of this study is to develop a complete sequences database of chloroplast genomes retrieved from five *Aquilaria* species that are native to Malaysia

Specific objectives:

1. To conduct a comparative analysis between the chloroplast genomes of eight *Aquilaria* species, including the characterization of chloroplast genomes, the identification of interspecies sequence variations and highly polymorphic regions in the library, followed by the determination of their molecular placements in phylogenetic trees;

2. To generate DNA barcodes from complete chloroplast genomes of *Aquilaria* species and demonstrate their utility for agarwood species tracking;

3. To identify the cross-transferability of simple sequence repeats (SSRs) in the chloroplast genomes between eight *Aquilaria* species.

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