UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF SELECTED Aquilaria SPECIES THROUGH DNA BARCODES AND IDENTIFICATION OF WOUND-RESPONSE PROTEINS OF Aquilaria malaccensis Lam.

LEE SHIOU YIH

FH 2016 8

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By

LEE SHIOU YIH

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

July 2016

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DEDICATION

Praise be to the name of God forever and ever; wisdom and power are his.

(Daniel 2:20)

Special dedication to:

Supervisory Committee

ASSOC. PROF. DR. ROZI MOHAMED

ASSOC. PROF. DR. MOHD NAZRE SALEH

DR. DHILIA UDIE LAMASUDIN

Parents

LEE MENG WAI & TING CHEK CHUI

and

Sisters

LEE WAN SHUAN & LEE WAN TIEN

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

CHARACTERIZATION OF SELECTED *Aquilaria* SPECIES THROUGH DNA BARCODES AND IDENTIFICATION OF WOUND-RESPONSE PROTEINS OF *Aquilaria malaccensis* Lam.

By

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July 2016

Chairman: **Associate Professor Rozi Mohamed, PhD**
Faculty: **Forestry**

Over-exploitation in search of its valuable non-wood fragrance product, the agarwood, has put pressure on the survival of the endangered *Aquilaria* trees in the wild. The lack of genetic information on these species rendered conservation efforts a tough task. Agarwood species identification is challenging as conventional techniques alone are unable to ascertain the species origin. In this work, the genetic variation within *Aquilaria* species in Peninsular Malaysia, and their relationship to several *Aquilaria* species of foreign origins were studied using molecular approaches. The internal transcribed spacer (ITS) of the nuclear region of 19 wild *Aquilaria* populations from different states in Peninsular Malaysia were sequenced and compared to the same species residing outside of Malaysia using fresh leaf samples. Single nucleotide polymorphisms (SNPs) were identified when comparisons were made between *A. malaccensis* from different countries, suggesting geographical segregation is a contributing factor toward genetic variation in *A. malaccensis*. The phylogenetic analysis conducted on the nuclear ribosomal ITS and the intergenic spacer region *trn*L-*trn*F regions of selected agarwood-producing species further revealed that both sequences were able to separate two important genera of agarwood tree species (*Aquilaria* and *Gyrinops*) into two clades, indicating they are paraphyletic. In addition, during this study, a critically endangered species, *Aquilaria rostrata*, endemic to Peninsular Malaysia, was rediscovered and compared to other *Aquilaria* species using DNA sequence and taxonomic treatments. For the identification of agarwood species of origin, a reference DNA barcode library was developed using eight candidate barcode loci (*mat*K, *rbc*L, *rpo*B, *rpo*C1, *psb*A-*trn*H, *trn*L-*trn*F, ITS, and ITS2) amplified from 24 leaf accessions of seven *Aquilaria* species obtained from living trees. The combination of *trn*L-*trn*F+ITS and *trn*L-*trn*F+ITS2 yielded the greatest species resolution using the least number of loci combination, while *mat*K+*trn*L-*trn*F+ITS showed potential in detecting the geographical origins of *Aquilaria* species. However the *trn*L-*trn*F+ITS2 was proposed as the best candidate barcode for *Aquilaria* because

the ITS2 is shorter in sequence length compared to ITS. This eases PCR amplification especially when using degraded DNA samples. In an attempt to identify processed agarwood products, real-time PCR technique coupled with species-specific primers derived from SNP positions in the *mat*K and *trn*L-*trn*F sequences successfully targeted three commercial *Aquilaria* species: *A. crassna*, *A. malaccensis*, and *A. sinensis*, demonstrating their specificity for the purpose of DNA tracing. For identification of wound-response related proteins from *A. malaccensis* tree stem, 16 protein spots were identified reproducible between biological replicates under 2D-PAGE, with only two protein spots showing regulation in expression after wounding treatment. The two proteins were predicted as malate synthase and NADPH quinone oxidoreductase subunit 2B. Both proteins were reported to be directly and indirectly related to wounding treatments in plants, and thus may be involved in agarwood formation mechanism. In conclusion, the molecular information obtained from this study will serve as a useful reference in designing in-situ programs to conserve this threatened species, contributes to the international timber trade control by providing an effective method for species identification and agarwood product authentication, and contributes in preliminary information on protein expressions related to agarwood formation in *Aquilaria*.

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

PENCIRIAN BAGI SPESIES *Aquilaria* TERPILIH MELALUI BARKOD DNA DAN PENGENALPASTIAN UNTUK PROTEIN TINDAKBALAS LUKA *Aquilaria malaccensis* Lam.

Oleh

LEE SHIOU YIH

Julai 2016

Pengerusi: **Profesor Madya Rozi Mohamed, PhD**
Fakulti: **Perhutanan**

Eksploitasi berlebihan untuk mencari produk wangian bukan kayu yang berharga, gaharu, telah memberikan tekanan kepada kebolehidupan pokok terancam *Aquilaria* yang tumbuh liar. Kekurangan informasi genetik terhadap spesies ini mengakibatkan usaha pemuliharaan merupakan suatu tugas yang sukar. Pengenalpastian spesies gaharu adalah mencabar kerana penggunaan teknik konvensional sahaja tidak dapat menentukan asal-usul spesies dengan tepat. Dalam kajian ini, variasi genetik bagi spesies *Aquilaria* di Semenanjung Malaysia, serta hubungannya dengan beberapa spesies *Aquilaria* luar negara dikaji menggunakan pendekatan molekul. Penjarak jujukan dalam (ITS) di kawasan nuklear bagi 19 populasi liar *Aquilaria* dari beberapa negeri di Semenanjung Malaysia telah dijujuk dan dibandingkan dengan spesies yang sama yang bertempat di luar Malaysia dengan menggunakan sampel daun segar. Polimorfisme nukleotida tunggal (SNP) telah dikenalpasti semasa perbandingan dijalankan dengan *A. malaccensis* dari negara yang berbeza, yang mana ia mencadangkan bahawa pengasingan geografi merupakan satu faktor yang menyumbang ke arah variasi genetik dalam *A. malaccensis*. Analisis filogenetik yang dijalankan ke atas ITS ribosom nuklear dan rantau antara penjarak gen *trn*L-*trn*F bagi spesis gaharu yang terpilih juga mendedahkan bahawa kedua-dua jujukan tersebut dapat mengasingkan dua genera penting pokok gaharu (*Aquilaria* dan *Gyrinops*) kepada dua klad, justeru menunjukkan bahawa mereka adalah parafili. Di samping itu, spesies terancam *Aquilaria rostrata* yang endemik kepada Semenanjung Malaysia telah dijumpai semula dan dibandingkan dengan spesies *Aquilaria* lain menggunakan jujukan DNA serta taksonomi. Untuk pengenalpastian spesies asal gaharu, satu perpustakaan barkod DNA rujukan telah dibangunkan dengan menggunakan lapan calon lokus barkod (*mat*K, *rbc*L, *rpo*B, *rpo*C1, *psb*A-*trn*H, *trn*L-*trn*F, ITS, dan ITS2) yang diamplifikasi daripada 24 aksesi daun dari tujuh spesies *Aquilaria* yang diperoleh daripada pokok hidup. Gabungan *trn*L-*trn*F+ITS dan *trn*L-*trn*F+ITS2 telah menghasilkan resolusi spesies terbesar dengan menggunakan bilangan kombinasi lokus

yang sedikit, manakala *mat*K+*trn*L-*trn*F+ITS menunjukkan potensi dalam pengesanan asal-usul geografi spesies *Aquilaria*. Walaubagaimanapun, *trn*L-*trn*F+ITS2 telah dicadangkan sebagai calon barkod terbaik untuk *Aquilaria* kerana ITS2 mempunyai jujukan yang lebih pendek berbanding ITS. Hal ini memudahkan amplifikasi PCR terutamanya bagi sampel DNA yang terurai. Dalam usaha untuk mengenalpasti produk gaharu yang telah diproses, teknik PCR masa nyata telah digabungkan dengan primer yang spesifik kepada spesies tertentu hasil daripada terbitan kedudukan SNP dalam jujukan *mat*K dan *trn*L-*trn*F, telah berjaya mensasarkan tiga spesies *Aquilaria* komersial: *A. crassna*, *A. malaccensis*, dan *A. sinensis*, justeru menunjukkan kekhususan mereka untuk tujuan pengesanan DNA. Bagi mengenalpasti protein berkaitan dengan tindakbalas luka daripada batang pokok *A. malaccensis*, 16 tompok protein telah dikenalpasti melalui ulangan biologi di bawah 2D-PAGE, dengan hanya dua tompok protein yang menunjukkan perbezaan ekspresi setelah pokok dicederakan. Protein tersebut dijangkakan adalah malat synthase dan NADPH quinona oxidoreduktas subunit 2B. Kedua protein ini dilaporkan berkait secara langsung dan tidak langsung dengan rawatan pencederaan dalam tumbuh-tumbuhan, dan oleh demikian ia mungkin terlibat dalam mekanisma pembentukan gaharu. Kesimpulannya, informasi molekular yang diperolehi daripada kajian ini akan menyumbang sebagai satu rujukan yang berguna dalam perancangan program in-situ bagi memelihara spesies terancam ini, menyumbang kepada kawalan perdagangan kayu antarabangsa melalui kaedah pengenalpastian spesies yang berkesan dan pengesahan produk gaharu, serta menyumbang kepada informasi awalan mengenai ekspresi protein yang berkaitan dengan pembentukan gaharu dalam pokok *Aquilaria*.

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Praise the Lord for His mercy and love. Amen.

I certify that a Thesis Examination Committee has met on 20 July 2016 to conduct the final examination of Lee Shiou Yih on his thesis entitled "Characterization of Selected *Aquilaria* Species through DNA Barcodes and Identification of Wound-Response Proteins of *Aquilaria malaccensis* Lam." in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microlitre
µM	Micromol
1-DE	1st dimension electrophoresis
2-D PAGE	Two-dimensional polyacrylamide gel electrophoresis
2-DE	2nd dimension electrophoresis
A.S.L	Above sea level
AFLP	Amplified fragment length polymorphism
BLAST	Basic Local Alignment Search Tool
bp	Base-pair
CBOL	Consortium for the Barcode of Life
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
cm	Centimeter
*CO*I	Cytochrome c oxidase I
CR	Critically endangered
CTAB	Cetyl trimethylammonium bromide
DD	Data deficient
DNA	Deoxyribonucleic acid
FASTA	Fast alignment
FBL	Forest Biotechnology Laboratory
FDPM	Peninsular Malaysia Forestry Department
FORDA	Forest Research and Development Agency
FRI	FRIM herbarium
FRIM	Forest research Institute Malaysia
GC	Guanine-cytosine
HR	Hypersensitive response
IEF	Isoelectric focusing
IMPLAD	Institute of Medicinal Plant Development
IPG	Immobilized pH gradient
ISSR	Inter-simple sequence repeat
ITS	Internal transcribed spacer

IUCN	International Union for Conservation of Nature
km	Kilometer
km^2	Kilometer square
LC	Loci combination
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
*mat*K	Maturase K
MEGA	Molecular evolutionary genetics analysis software
mg	Miligram
min	Minute
mL	Mililitre
MS	Mass spectrometry
NADPH	reduced Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnolog Information
ng	Nanogram
NJ	Neighbor-joining
NTFPs	Non-timber forest products
NTSYS-pc	Numerical Taxonomy and Multivariate Analysis system
PCD	Programmed cell death
PCR	Polymerase chain reaction
pI	Isoelectric point
PM	Peninsular Malaysia
PMF	Peptide mass fingerprinting
PTM	Post-translation modification
PVP	Polyvinylpyrolidone
Q-Q-TOF	Hybrid quadruple time-of-flight
RAPD	Random amplified polymorphic DNA
*rbc*L	Ribulose-bisphosphate carboxylase
rpm	Revolutions per minute
*rpo*B	Beta subunit of bacterial RNA polymerase
*rpo*C1	DNA-directed RNA polymerase subunit beta’
s	Second
SCAR	Sequence characterized amplified region
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SING	Herbarium of Singapore Botanic Gardens
SNP	Single nucleotide polymorphism
SRAP	Sequence related amplified polymorphism
SSR	Simple sequence repeat
STR	Short tandem repeats
T_a	Annealing temperature
ToF-ToF	Tandem time-of-flight
TRAFFIC	The Wildlife Trade Monitoring Network
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UPM	Universiti Putra Malaysia
VU	Vulnerable

CHAPTER 1

INTRODUCTION

1.1 General

Aquilaria, from the Family of Thymelaeaceae, is an evergreen tropical forest tree, which is endemic to the Indomalesia region. The tree is well-known for its rare and valuable fragrance non-wood product, the agarwood. The natural production of agarwood on *Aquilaria* trees is commonly associated to exterior wounding on the stem and branches due to natural disasters such as wind and lightning, then with its open wounds further affected by fungi attacks. These fungal attacks trigger the defense mechanism of the tree, produced fragrance resin which creates a wall to prevent further penetration against foreign objects. Among 21 accepted species name in the genus *Aquilaria*, only 13 species were reported able to produce agarwood (Ding Hou, 1960; Ng *et al.*, 1997; Compton and Zich, 2002; Kiet *et al.*, 2005). However, not all *Aquilaria* individuals were able to produce agarwood naturally in the wild. Agarwood formation in *Aquilaria* is believed to relate to the genotype and environment of the tree. It was reported that not all trees in a population produce agarwood, and only a small part of the tree itself contains agarwood (Ng *et al.*, 1997). As agarwood resources are rare, while its demand is high in the market, agarwood of different qualities can fetch incredible prices. This drives agarwood collectors to search for agarwood to make a fortune from these trees. Trees in the wild were mechanically wounded using machetes, creating wounds to allow fungal infections to form agarwood resins. The continuous routine to wound the stem and collect the agarwood on the tree had slowly threatened the life of the tree.

The agarwood is an essential resource in the production of traditional medicines. *Aquilaria sinensis* and *A. malaccensis* are well recognized for their medicinal usage such as for relieving spasms and blockage in digestive and respiratory systems. The application of agarwood in medicines was backdated since more than 2000 years ago and is still strongly practiced in several communities, such as the Chinese, Indian, Tibetan and Greek-derived Islamic society (Burfield and Kirkham, 2005). Besides as traditional medicines, the captivating fragrance from the burning agarwood was made as meditation tools for religious ceremonies. In the Chinese and Indian societies, incenses made from agarwood were used for worshiping their Gods, and as for the Japanese and Buddhist, incense were burnt for meditation purposes as it promotes a relaxing environment through the release of fragrance in the air. Agarwood chips are processed for its oil which is commercially known as *oud* for the Arabs. Being a natural ingredient in perfumery, the agarwood oil is able to provide a distinct and long-lasting smell (Compton and Ishihara, 2004; Lim and Awang Anak, 2010). Due to the booming growth of the agarwood market in the early 21st century, agarwood traders and entrepreneurs began to investigate for sustainable agarwood production by establishing *Aquilaria* tree plantations. This is to lift-off reliance on the natural stands, which may led to breaching the law in illegal agarwood (Edy Komar *et al.*, 2014). The effort in producing sustainable agarwood resources was aided by various agarwood induction techniques for agarwood formation in these cultivated trees. *Aquilaria* trees

cultivation may help in reducing the exploitation of *Aquilaria* natural stands and at the same time promotes the growth of the agarwood industry to an even competitive market for both quality and sustainable agarwood production (Pojanagaroon and Kaewrak, 2005).

1.2 Problem Statements

Indiscriminate felling was reported in the wild due to illegal harvesting in search of agarwood, threatening the natural reproduction cycle in the nature, resulting in population decline over the years. The first species in *Aquilaria* to be reported endangered and listed in the CITES Appendix II was *A. malaccensis* in 1995. The list expanded in 2005 to all species under *Aquilaria*, together with the inclusion of a closely –related agarwood-producing genus, *Gyrinops*. The continuously increasing demand for agarwood drives traders to locate for alternative sources, indirectly posing a threat towards its habitat. At the same time, the International Union for Conservation of Nature and Natural Resources (IUCN) had taken the initiative to list nine *Aquilaria* species under the IUCN Red List of Threatened Species since 1998, highlighting the endangered status of these species in the wild and possible of extinction if conservation measurements are not taken seriously.

1.3 Justification

The decline in natural *Aquilaria* populations has raised the alarm among naturalists and conversationists to protect and conserve its gene pool resources. In order to develop effective breeding programmes and to identify the genetic structure of these natural *Aquilaria* populations, studies on the genetic diversity through DNA approaches is an important first step towards conservation efforts. Molecular markers that utilize DNA polymorphism have always been the first choice for most researchers in investigating genetic diversity and genetic structure of natural populations. DNA-based studies on *Aquilaria* species started to pick-up from 2005 onwards after TRAFFIC had pointed out the need to secure the natural *Aquilaria* resources in the wild.

Molecular markers and DNA fingerprinting techniques have developed rapidly over the years and have been applied widely in plant identification. The advantages of using DNA in analyzing the plant samples is that they provide rich, reliable, high polymorphic genetic information which is not easily influenced by environment changes. In this study, genetic information of these endangered tree species will be generated as it is a requisite step towards conserving their natural habitat and genetic diversity. Sufficient information on genetic variation and useful species identification techniques will help conservation efforts such as in setting up suitable breeding programmes, and in assisting in timber trade controls.

1.4 Objectives

The general objective of this study was to characterize selected *Aquilaria* species using molecular techniques.

The specific objectives of this study include:

1. To identify the genetic variation within two *Aquilaria* species in Peninsular Malaysia using gene markers from nuclear ribosomal internal transcribed spacer (ITS) region and chloroplast intergenic spacer region *trn*L-*trn*F.

2. To determine the genetic diversity of agarwood-producing trees of different species and related genera using using gene markers from ITS and *trn*L-*trn*F regions at taxa level.

3. To create DNA barcodes for selected *Aquilaria* species and demonstrate their use in tracing agarwood species.

4. To characterize wound-response peptides from *Aquilaria malaccensis* using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).

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