UNIVERSITI PUTRA MALAYSIA

MOLECULAR DNA STUDIES OF THREE *Aquilaria* SPECIES IN MALAYSIA

LEE SHIOU YIH

FH 2011 20

MOLECULAR DNA STUDIES OF THREE *Aquilaria* SPECIES IN MALAYSIA

By

LEE SHIOU YIH

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

April 2011

DEDICATION

Give thanks unto the LORD; for he is good: because his mercy endureth for ever.

Specially Dedicated To,

My Late Grandfather,

LEE THIAN HEE

My Grandmother,

SIAW SOI CHOO

My Beloved Parents,

LEE MENG WAI & TING CHEK CHUI

My Sisters

LEE WAN SHUAN & LEE WAN TIEN

And Friends.

For encouragement, inspiration, understanding and constant prays throughout my study in Universiti Putra Malaysia.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

MOLECULAR DNA STUDIES OF THREE *Aquilaria* SPECIES IN MALAYSIA

By

LEE SHIOU YIH

April 2011

Chair: Rozi Mohamed, PhD

Faculty: Faculty of Forestry

There are five *Aquilaria* species reported from Malaysia: *A. beccarania*, *A. hirta*, *A. malaccensis*, *A. microcarpa* and *A. rostrata*. Although *A. malaccensis* is the most well-known gaharu-producing species which supplies bulk trades domestically and internationally, its genetic information is lacking. Establishment of *Aquilaria* plantation and sustainable gaharu production can be seen as an effort to diversify the gaharu industry without threatening the conservation effort in nature. Hence, the understanding of genetic diversity and variation of the different species is essential for the establishment of effective conservation practices for *Aquilaria* species in Malaysia.

In this study, Random Amplified Polymorphic DNA (RAPD) markers were used to measure genetic diversity of *A. hirta*, *A. malaccensis* and an incomplete known

species, *Aquilaria* sp.1. Initially, 60 RAPD primers were analyzed, yielding 23 RAPD showing clear and reproducible polymorphism results. A total of 368 bands were scored. Multi-populations Descriptive Statistics revealed that 333 (90.49%) polymorphic bands were found at species level: *A. malaccensis* had 107 (29.08%) bands, *A. hirta* had 56 (15.22%), and *Aquilaria* sp.1 had 11 (2.99%), for the percentage of polymorphic loci in a species. Nei's unbiased measurement indicates moderate similarities among species in this study. Out of the 23 RAPD primers, OPA02, OPA08 and OPB06 were found specific to *A. hirta*, OPA05 to *Aquilaria* sp.1, and OPA09 to *A. malaccensis*. PCR amplicons were cloned and sequenced to develop highly specific primers. Based on the sequence information, Sequence Characterized Amplified Region (SCAR) primers were designed. In *Aquilaria* sp.1, the SCAR marker OPA05AS yielded a 637 bp band. In *A. hirta*, OPA02AH yielded a 955 bp band, a SCAR marker named OPA08AH yielded a 866 bp band, and OPB06AH yielded a 826 bp band. In *A. malaccensis*, OPA09AM yielded a 566 bp band. These results showed successful detection in genetic variation among *Aquilaria* species in Malaysia using RAPD markers. SCAR markers were also successfully developed to help in distinguishing these three *Aquilaria* species.

In classical identification, reproductive parts are most important for species identification. However this is not easily done when examining their vegetative parts such as the leaf, branch and bark. In this study, general descriptions of three different *Aquilaria* species were recorded, crosschecked with previous literatures and botanical records: *A. hirta* through their heavily pubescent, large-sized leaves, *A. malaccensis* through elliptic-lanceolate shape leaves and *Aquilaria* sp.1 from the existence of strongly raised lateral veins below the leaves.

In conclusion, the identification of *Aquilaria* species with higher level of confidence can be achieved by performing both botanical observations and molecular authentication with the aid of molecular markers. Molecular information can support species identification for *Aquilaria* species even with the absence of their reproductive parts. This will help in reducing the occurrence of species misidentification caused by phenotype changes due to environment factors. These results are useful information for conservation and molecular breeding purposes in the future.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KAJIAN DNA MOLEKUL ANTARA TIGA SPESIS *Aquilaria* DI MALAYSIA

Oleh

LEE SHIOU YIH

April 2011

Pengerusi: Rozi Mohamed, PhD

Fakulti: Fakulti Perhutanan

Terdapatnya lima spesies *Aquilaria* dilaporkan dari Malaysia: *A. beccariana*, *A. hirta*, *A. malaccensis*, *A. microcarpa* dan *A. rostrata*. Walaupun *A. malaccensis* merupakan spesies pengeluaran gaharu yang paling dikenali ramai dalam penyediakan bekalan secara banyak secara domestik dan antarabangsa, maklumat genetiknya masih kekurangan. Pembukaan ladang *Aquilaria* dan pengeluaran gaharu secara mampan dapat dilihatkan sebagai usaha untuk mempelbagaikan industri gaharu tanpa mengancam usaha pemuliharaan alam sekitar. Oleh itu, pengetahuan terhadap kepelbagaian dan variasi genetik untuk pelbagai spesies adalah penting untuk pembentukan usaha pemuliharaan secara efektif bagi spesies *Aquilaria* di Malaysia.

Dalam kajian ini, penanda DNA Amplifikasi Polimorfik Secara Rawak (RAPD) telah digunakan untuk mengukur kepelbagaian genetik *A. hirta*, *A. malaccensis* dan spesis yang tidak lengkap diketahui, *Aquilaria* sp.1. Permulaannya, 60 pencetus RAPD

telah dianalisa, menghasilkan 23 RAPD yang menunjukkan keputusan polimorfisme yang jelas dan dapat diperoleh-semula. Sejumlah 368 jalur telah dicatatkan daripada 23 pencetus RAPD yang dianalisis. Statistik Deskriptif Multi-populasi, 333 (90.49%) jalur polimorfik telah didedahkan pada peringkat spesies, di mana *A. malaccensis* mempunyai 107 (29.08%) jalur, *A. hirta* mempunyai 56 (15.22%) dan *Aquilaria* sp.1 mempunyai 11 (2.99%), bagi peratus lokus polimorfik dalam sejenis spesies. Ukuran tanpa prejudis oleh Nei menunjukkan persamaan sederhana di antara spesies dalam kajian ini. Daripada 23 pencetus RAPD, OPA02, OPA08 dan OPB06 telah didapati spesifik terhadap *A. hirta*, diikuti dengan OPA05 spesifik terhadap *Aquilaria* sp.1 dan OPA09 terhadap *A. malaccensis*. Amplikon-amplikon PCR tersebut telah diklon dan dijujuk bagi menghasilkan pencetus yang sangat spesifik. Berdasarkan maklumat jujukan, pencetus Kawasan Amplifikasi Jujukan Berciri (SCAR) yang menghasilkan jalur spesies berspesifik, telah direka. Bagi *Aquilaria* sp. 1, OPA05AS telah menghasilkan jalur bersaiz 637 bp. Bagi *A. hirta*, OPA02AH telah menghasilkan jalur bersaiz 955 bp, OPA08AH telah menghasilkan jalur bersaiz 866 bp, dan OPB06AH yang menghasilkan jalur bersaiz 826 bp. Keputusan menunjukkan kejayaan dalam pengesanan kepelbagaian genetik di antara spesies *Aquilaria* di Malaysia dengan menggunakan kaedah RAPD. Penanda baru SCAR ke atas *Aquilaria* sp.1, *A. hirta* dan *A. malaccensis* turut membantu dalam pembezaan antara spesies *Aquilaria* yang lain.

Dalam pengecaman klasikal, bahagian pembiakan adalah penting dalam pengecaman spesies. Walau bagaimanapun, ia bukan kerja senang apabila mengkaji bahagian vegetatif seperti daun, ranting, dan kulit. Dalam kajian tersebut, gambaran am bagi tiga jenis spesies *Aquilaria* yang berlainan telah direkodkan, dengan pemeriksaan

ulangan daripada literatur dan rekod botani sebelum ini. *A. hirta* melalui daunnya yang bersaiz besar serta berbulu lebat, *A. malaccensis* melalui bentuk daunnya yang bulat memanjang dan berhujung tajam, dan *Aquilaria* sp.1 dengan kehadiran vena lateralnya yang menimbul tinggi di bawah daun.

Secara keseluruhan, pengecaman spesies *Aquilaria* dengan lebih tinggi keyakinan dapat dicapai dengan melaksanakan kedua-dua pemerhatian secara botani dan pengesahan secara molekul dengan bantuan penanda molekul. Maklumat molekul dapat menyokong pengecaman spesies *Aquilaria* walaupun tanpa bahagian pembiakannya. Hal ini akan membantu dalam mengurangkan kejadian salah dalam pengecaman spesis yang cenderung terhadap perubahan fenotip akibat faktor alam sekitar. Keputusan-keputusan tersebut adalah maklumat yang berguna untuk tujuan pemuliharaan dan pembiakan molekul pada masa depan.

ACKNOWLEDGEMENTS

First, I would like to extend my warmest gratitude to everyone who, in one way or another, made the completion of my Master's thesis possible: to Dr. Rozi Mohamed, my supervisor, for her patience and effort in guiding me throughout the duration of my project and her perseverance in proofreading my manuscript; to Prof. Datin Dr. Faridah Hanum Ibrahim and Assoc. Prof. Dr. Mohamad Azani Alias, my co-supervisors, for their technical proficiency and valuable feedback throughout my project; Dr. Jean Weber for his generosity in providing information for the molecular works, Dr. Teo Guan Young for his effort in guiding and advice about the protocols and methodologies of basic molecular applications in my research; to Nancy Liew Woan Charn, Science Officer of the Molecular Biomedicine Lab at Institute Bioscience, UPM, for her kindness, lab members from the Genetics Laboratory from Department of Biology, Faculty of Science, UPM, for their patience in guiding and information sharing for the molecular applications; the staff from the Faculty's herbarium, for their help in providing information in terms of botany studies; to all my laboratory colleagues and friends, Wong Mun Theng, Liong Yan Yee, Jong Phai Lee, Siah Chai Har, Jency Jenuai Anak Tiun, Tan Hui Rus, Chong Yi Way, Lim Kian Lam, How Chee Wun, Goh Su Hua, for their help and advice directly or indirectly in my research even though they were working on other projects; to Mr. Jelani Alias, the faculty's driver, for bringing me to field trips and sample collections and to UPM, for giving me the privilege of university education through the Graduate Research Fellowship.

My special and deepest thanks are forwarded to the Forest Department of Peninsular Malaysia, especially the Forest Department of Terengganu State and the forest ranger in-charge Mr Salleh bin Endot , the Highest Commission Forest Ranger of the State Forest Department whom provided help and assistance in sourcing seedlings and making visits in various plantations and forest reserves in both Terengganu and Kelantan states; the Herbarium of Forest Research Institute Malaysia (FRIM), the curator Dr. Richard Chong and all the herbarium staffs, who provided the services and facilities in sample collection and botany identifications; the National Seed and Planting Material Procurement Centre, Lentang, and the Botanical Research Centre of Sarawak Forestry Corporation, Semenggoh, which both provided saplings and botanical information for my project.

Last but not least, I would like to display my sincere appreciation towards my beloved parents, siblings and girlfriend who persistently pray for me, and my relatives and friends who always support and motivate me throughout my studies. May the Almighty God in Heaven bless all of you with joy and happiness in your future undertakings.

I certify that a Thesis Examination Committee has met on 25th April 2011 to conduct the final examination of **Lee Shiou Yih** on his **Master** thesis entitled "**Molecular DNA studies of three *Aquilaria* species in Malaysia**" 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 students be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Ahmad Ainnuddin Nuruddin, PhD
Associate Professor
Faculty of Forestry
University Putra Malaysia
(Chairman)

Mohd Nazre Saleh, PhD
Senior Lecturer
Faculty of Forestry
Universiti Putra Malaysia
(Internal Examiner)

Azmy Haji Mohamed, PhD
Associate Professor
Faculty of Forestry
Universiti Putra Malaysia
(Internal Examiner)

Choong Chee Yen, PhD
Senior Lecturer
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

_____
NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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

Rozi Mohamed, PhD
Senior Lecturer
Faculty of Forestry
Universiti Putra Malaysia
(Chairman)

Faridah Hanum Ibrahim, PhD
Professor
Faculty of Forestry
Universiti Putra Malaysia
(Member)

Mohamad Azani Alias, PhD
Associate Professor
Faculty of Forestry
Universiti Putra Malaysia
(Member)

_____

HASANAH MOHD. GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____

LEE SHIOU YIH

Date: 25 April 2011

TABLE OF CONTENTS

		Page
DEDICATIONS		ii
ABSTRACT		iii
ABSTRAK		vi
ACKNOWLEDGEMENT		ix
APPROVAL		xi
DECLARATION		xiii
LIST OF TABLES		xvi
LIST OF FIGURES		xvii
LIST OF ABBREVIATIONS		xviii
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	4
	2.1 Forest Genetic Conservation	4
	2.2 Conservation Status of *Aquilaria*	6
	2.3 DNA Molecular Markers in Plant Conservation	7
	2.3.1 Polymerase Chain Reaction (PCR)	8
	2.3.2 Random Amplified Polymorphism DNA (RAPD)	10
	2.3.3 Sequence Characterized Amplified Region (SCAR)	11
	2.4 Thymelaeaceae	13
	2.4.1 *Aquilaria*	14
	2.4.2 *Aquilaria* in Malaysia	14
	2.4.3 Agarwood	24
	2.4.4 Agarwood Cultivation	25
3	**DESCRIPTION OF SELECTED *Aquilaria* SPECIES IN MALAYSIA**	27
	3.1 Introduction	27
	3.2 Materials and Methods	29
	3.2.1 Plant Materials	29
	3.2.2 Herbarium Collection	29

3.3 Results and Discussion 32
3.3.1 *Aquilaria sp.1* 36
3.3.2 *Aquilaria hirta* Ridl. 40
3.3.3 *Aquilaria malaccensis* Lam. 44
3.4 Conclusion 48

4 **RAPID DETECTION IN GENETIC VARIATION AND SPECIES AUTHENTICATION OF SELECTED WILD *Aquilaria* SPECIES IN MALAYSIA USING RAPD AND SCAR MARKERS** 49
4.1 Introduction 49
4.2 Materials and Methods 52
4.2.1 Plant Material 52
4.2.2 Genomic DNA Isolation 53
4.2.3 Determination of DNA Concentration and Quality 55
4.2.4 Primers 57
4.2.5 RAPD Amplification 57
4.2.6 Agarose Gel Electrophoresis 59
4.2.7 Statistical Analysis 59
4.2.8 Selection of RAPD Primers for SCAR 61
4.2.9 Isolation of Polymorphic Marker Bands 62
4.2.10 Purification of PCR Products 62
4.2.11 Gene Cloning 63
4.2.12 Specific Primer Sequences Development 66
4.2.13 SCAR-PCR Amplification 66
4.3 Results and Discussion 68
4.4 Conclusion 85

5 **SUMMARY, CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH** 87

BIBLIOGRAPHY 89
APPENDICES 97
BIODATA OF STUDENT 101