



**CROSS-SPECIES AMPLIFICATION OF *Aquilaria* MICROSATELLITES
ACROSS THIRTEEN AGARWOOD-PRODUCING SPECIES AND GENETIC
STRUCTURE OF *Aquilaria Beccariana* TIEGH. IN MALAYSIA**

By

PERN YU CONG

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
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Master of Science**

October 2019

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

CROSS-SPECIES AMPLIFICATION OF *Aquilaria* MICROSATELLITES ACROSS THIRTEEN AGARWOOD-PRODUCING SPECIES AND GENETIC STRUCTURE OF *Aquilaria Beccariana* TIEGH. IN MALAYSIA

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Tree species in the Aquilarieae tribe produce agarwood, a natural product, highly valued for its compound-rich fragrance. However, these resources in the wild are under threat from agarwood demand in the international market. DNA profiling approaches have been utilized for tree conservation purposes. Unfortunately, this effort is hampered by the lack of suitable molecular markers and the challenges to obtain resources due to the endangered status of the species. In several *Aquilaria* species, microsatellite markers have been developed but cross-species amplification and evaluation has yet to be examined. In this study, 30 microsatellite markers, developed for *Aquilaria malaccensis*, *Aquilaria crassna* and *Aquilaria sinensis*, were selected and cross-amplified in ten *Aquilaria* and three *Gyrinops* species, all from the Aquilarieae tribe. Cross-species amplification of 18 perfect dinucleotide microsatellite markers (Ama040, Ama067, Ama101, Ama114, Ama131, Ama144, Ama263, Ama264, Ama331, Ama338, 16pa17, 71pa17, BMX1, BMX3, BMX6, BMX7, BMX8, and AQEGMS-53) on the 13 species revealed a high probability of amplification success (86%) and identified a total of 300 alleles with PIC ranged from 0.5770 to 0.9244. The mean gene diversity (H_E) and heterozygosity (H_o) were 0.8598 and 0.3641, respectively. Species-specific alleles and genotype were tabulated as a substitute for DNA fingerprinting profile. The high cross-species amplification rate of the selected microsatellite markers displays high genetic variability among the species. Among the *Aquilaria* species native to Malaysia, *Aquilaria beccariana* is found distributed in the Malay Peninsula and Borneo Island. To study their phylogeographical relationship and genetic relatedness, fresh leaf samples of 47 individuals from five different wild populations (Mersing in Johor; Long Banga, Ba'lai, and Lawas in Sarawak; Sungai Kangkawat in Sabah, and Kalimantan, Indonesia) were collected. Using PCR, seven non-coding chloroplast DNA (cpDNA) regions and a nuclear ribosomal DNA (nrDNA) region were amplified and sequenced. Phylogenetic,

median-joining, and Principal Coordinate Analysis (PCoA) analyses assembled the five populations into two major clusters, Malay Peninsula and Borneo. Trees in Borneo were further clustered into Central, Northern, and Southern populations. DNA barcode data generated from this study serve as important reference to support species identification. In addition, a population distribution map was generated for several *Aquilaria* species based on published herbarium data, previous reports and this study. In conclusion, this work provides useful information about marker cross ability and genetic relationships of several agarwood-producing species, both of which may help in the management and conservation of agarwood resources.



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

**AMPLIFIKASI SILANG SPESIES *Aquilaria* MIKROSATELIT ANTARA TIGA
BELAS SPESIES MENGHASILKAN GAHARU DAN STRUKTUR GENETIK
Aquilaria Beccariana TIEGH. DI MALAYSIA**

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Spesies pokok dalam suku Aquilarieae menghasilkan gaharu, sejenis resin wangi semulajadi yang bernilai. Walau bagaimanapun, sumber-sumber alam semulajadi ini terancam disebabkan permintaan gaharu yang semakin meningkat. Pendekatan profil DNA telah digunakan untuk tujuan pemuliharaan pokok. Walau bagaimanapun, usaha ini terhalang oleh kekurangan penanda molekul yang sesuai dan cabaran yang dihadapi oleh para penyelidik untuk mendapatkan sumber daya kerana status spesies yang terancam. Dalam beberapa spesies-spesies *Aquilaria*, penanda mikrosatelit telah dibangunkan tetapi amplifikasi dan penilaian silang spesies masih belum dikaji. Dalam kajian ini, 30 penanda mikrosatelit yang dihasilkan daripada *Aquilaria malaccensis*, *Aquilaria crassna* and *Aquilaria sinensis*, telah dipilih. Keputusan amplifikasi silang spesies dalam 18 mikrosatelit sempurna dinucleotide (Ama040, Ama067, Ama101, Ama114, Ama131, Ama144, Ama263, Ama264, Ama331, Ama338, 16pa17, 71pa17, BMX1, BMX3, BMX6, BMX7, BMX8, dan AQEGMS-53) telah dipamerkan. Amplifikasi silang spesies dalam 13 spesies dari Aquilarieae telah menunjukkan kadar kebarangkalian yang tinggi (86%). Sejumlah 300 allele telah dikenalpasti dengan PIC di antara 0.5770 hingga 0.9244. Purata keelbagaian genetik (H_E) dan heterozygositi (H_o) 0.8598 and 0.3641 masing-masing. Alel dan genotip khusus spesies telah ditabulasikan sebagai rujukan profil cap jari DNA. Kadar ampifikasi silang spesies penanda mikrosatelit yang terpilih telah menunjukkan variasi genetic yang tinggi antara spesies. Antara *Aquilaria* spesies yang wujud secara semula jadi di Malaysia, *Aquilaria beccariana* telah dijumpai Semenanjung Malaysia dan Pulau Borneo. Untuk mengkaji keterkaitan mereka, 47 individu sampel daun segar dari 5 populasi liar (Mersing di Johor; Long Banga, Ba'lai, dan Lawas di Sarawak; Sungai Kangkawat di Sabah dan Kalimantan) telah dikumpul. Dengan menggunakan cara PCR, tujuh kawasan kloroplas bukan kod dan satu kawasan nuklear ribosom telah diamplifikasi dan

dijujuk. Analisis filogenetik, gabungan median, dan kordinat utama telah menghimpunkan lima populasi menjadi dua kluster majoriti iaitu Semenanjung Malaysia dan Borneo. Populasi di Borneo dicabangkan dengan lebih lanjut menjadi populasi pertengahan, utara dan selatan. Data barkod DNA telah dihasilkan sebagai rujukan penting untuk pengecaman spesies. Peta pengedaran populasi beberapa spesies *Aquilaria* telah dihasilkan berdasarkan data herbarium yang telah diterbitkan, laporan, dan kajian ini. Kesimpulannya, kerja ini menyumbangkan informasi yang berguna mengenai keupayaan serta hubungan genetic antara beberapa *Aquilaria* spesies dan dijangkai membantu dalam pengurusan dan konservasi sumber gaharu.



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This thesis was submitted to the Senate of the 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:

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microlitre
µM	Micromol
AFLP	Amplified fragment length polymorphism
BBG	Bogor Botanical Garden
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
bp	Base-pair
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CoC	Chain of custody
cp	Chloroplast
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization
FASTA	Fast alignment
FGR	Forest genetic resource
FORDA	Forest Research and Development Agency
FRIM	Forest Research Institute Malaysia
IMPLAD	Institute of Medicinal Plant Development
ISSR	Inter-simple Sequence Repeat
ITS	Internal transcribed spacer
ITTO	The International Tropical Timber Organization
IUCN	International union for Conservation of Nature

mg	Miligram
min	Minute
mL	Mililitre
NCBI	National Center for Biotechnology Information
NJ	Neighbor-joining
nr	Nuclear ribosome
NTSYS-pc	Numerical Taxonomy and Multivariate Analysis System
PCR	Polymerase chain reaction
QSBG	Queen Sirikit Botanical Garden
RAPD	Random amplified polymorphic DNA
SCAR	Sequence characterized amplified region
SRAP	Sequence related amplified polymorphism
SSLPs	simple sequence length polymorphisms
SSR	Simple tandem repeat
STR	Short Tandem Repeat
T_a	Annealing temperature
t_a	Annealing time
TRAFFIC	The wildlife Trade Monitoring Network
UPGMA	Unweighted pair Group Method with Arithmetic Mean
VNTRs	Variable number of tandem repeats

CHAPTER 1

INTRODUCTION

1.1 General

Aquilaria is a genus of endangered woody tree in Malaysia from the Thymelaeaceae family and is endemic in the Indomalaysia region (Mabberley, 1997). It produces highly valuable resinous wood and there are 21 species that have been recognised (Nguyen *et al.*, 2017; Lee & Mohamed, 2016). Agarwood has been used historically as incense in religious ceremonies for its pleasant smell and as an ingredient in traditional herbal medicines for its sedative, aphrodisiac and carminative effects (Naef, 2011). Due to increasing demand, agarwood has become very rare in the wild (Newton & Soehartono, 2001). As a result of illegal harvesting by agarwood smugglers and illegal logging, many species of *Aquilaria* are being threatened with extinction. As a result, all species of *Aquilaria* are placed on CITES Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora in 1995, in order to control trade of agarwood around the world. This matter has attracted the attention and interest of scientist to find out means to prosecute illegal logging and to identify the legality of the wood-based product. One of the approaches to curb illegal logging is via DNA genotyping. This is reliable as the DNA between species and even individuals have differences. By using DNA as the source, the species of the log can be identified. However, DNA genotyping has its own limitations. Some of the current DNA genotyping technique such as Inter Simple Sequence Repeat (ISSR) can only find the interspecific variation of different species. It is found that there are only minor variations and no intraspecific sequence variation that showed high genetic variation among the same species (Eurlings & Gravendeel, 2005). The specific geographical origin of the tree individual cannot be detected. This has created a problem in the wood forensic investigation and timber certification. Microsatellite markers approach has been developed by a group of scientists to alleviate this problem (Motamayor *et al.*, 2008).

Highly polymorphic microsatellites are suitable for the assessment of genotypic variation from populations down to the individual level (Eurlings *et al.*, 2010). Microsatellite or simple sequence repeat (SSR) loci, which have been referred to in the literature as variable number of tandem repeats (VNTRs) and simple sequence length polymorphisms (SSLPs), are found throughout the nuclear genomes of most eukaryotes and to a lesser extent in prokaryotes (Vaughan & Lloyd, 2003). It is highly polymorphic between species and even between individuals within species and population, implying that the microsatellite is extremely precise in identifying the individual. (Gupta *et al.*, 1996). In terms of working process, microsatellite is shorter in length (1 to 6 base pairs) when compare to other similar molecular markers such as minisatellites (11 to 60 base pairs). Hence, it is easier to clone, sequence and amplify microsatellites through

PCR. Due to its highly polymorphic and handy character, it is now frequently being used in wood forensic (Finkeldey, 2010).

There are five reported *Aquilaria* species available in Malaysia, namely *A. beccariana*, *A. hirta*, *A. malaccensis*, *A. microcarpa* and *A. rostrata*. (Lee & Mohamed, 2016a). Three species (*A. beccariana*, *A. malaccensis*, and *A. microcarpa*) are distributed in both East and West Malaysia, which are separated by the South China Sea. Of the three species, *A. beccariana* was found naturally populates in the southern part of the Malay Peninsula Malaysia and East Malaysia (Faridah-Hanum et al., 2009; Forest Department of Peninsular Malaysia, 2015). To date, researchers have covered several aspects (reproductive ecology, distribution pattern, field assessment) in effort to biological conservation and sustainable reproduction of this species (Soehartono & Newton, 2001). Being one of the known agarwood-producing species, agarwood produced from *A. beccariana* were harvested in the wild by Malaysian aborigines (Kanazawa 2017) and cultivation attempts for sustainable agarwood production were reported in several areas in Indonesia (Turjaman et al. 2017).

Phylogeographical study on the Aquilarieae tribe has always been an interesting topic but remains unknown because of the scarcity of the resources and sparsely distributed populations in Southeast Asia. The particularity of agarwood species being endemic to certain areas because of the geographical barriers, such as the left with *A. beccariana*, has yielded interest to conduct this study by using nuclear ribosomal DNA (nrDNA) and chloroplast DNA (cpDNA) molecular markers, which also concurrently serve as barcode candidates for species identification

1.2 Problem Statement

Aquilaria species is an endangered species, whereby its endangered status ranges from vulnerable to critically endangered under Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 1995). Several microsatellite markers have been developed in *Aquilaria* for wood forensic purposes, however their cross-transferability between species and among species have to be tested before they can assist illegal logging prosecution. From phylogeography aspect, the intraspecific genetic variation of the different *A. beccariana* populations may provide some insights on the genetic isolation happening within the same species. Findings from this study may contribute to both species-level and genus-level research in the future and provides an identification tool for conservation efforts of *Aquilaria*.

1.3 Objectives

- i. To examine cross-transferability of 30 selected microsatellite markers among 13 agarwood-producing species in the Aquilarieae tribe
- ii. To determine the phylogeographical structure of *Aquilaria beccariana* using nuclear and chloroplast DNA markers



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