Genetic diversity of *Aquilaria microcarpa* Baill. in Kalimantan using RAPD Markers

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**Abstract.** *Aquilaria microcarpa* is one of the agarwood producers species included in the endangered category based on the IUCN red list. Kalimantan is one of the natural distributions of this species. The high demand for agarwood causes this species to be commonly cultivated in community plantations. This study aimed to assess the genetic diversity of *A. microcarpa* using the RAPD markers. Forty-four leaf samples of *A. microcarpa* were collected from the local community in Gumbil (South Kalimantan) and Sanggau (West Kalimantan). The results showed that 9 out of 24 RAPD primers were stable in amplification, and polymorphic totally consisted of 49 polymorphic loci. The values of unbiased expected heterozygosity (uHE) were at a low level; they ranged between 0.152 (Gumbil II) to 0.249 (Gumbil I). The average genetic distance between Gumbil and Sanggau is 0.1733, related to their geographic distance. A private allele was only found at Gumbil at locus G18/540 and Sanggau at locus G18/550, respectively. A great DA value between Gumbil I and II showed that the plantations originated from different seed sources. AMOVA also verified the differences between the two populations. This finding has important implications in managing plantations and seeds transfer.

1. Introduction

*Aquilaria microcarpa* is a species included in the genus *Aquilaria* that is harvested for its highly valuable fragrant wood called "gaharu" or agarwood [1]. The economic value makes this species overharvested and has other threats such as habitat conversion [2]. High trading of agarwood product in the global market causing *A. microcarpa* as one of agarwood-producer has been listed in Appendix II Conservation on International Trade in Endangered Species of Wild Flora and Fauna (CITES) [3, 4].

*Aquilaria* spp mostly distributed in the western part of Indonesia includes Sumatera and Kalimantan. These species were naturally found in evergreen and semi-evergreen forests. An elevation of Aquilaria habitat ranged from 0-250 m above sea level (asl) [2, 5]. The population of *Aquilaria*, including *A. microcarpa*, has declined in their natural habitat, especially trees with ≥30 cm dbh in regions of Kalimantan [2]. Furthermore, the declining population of *Aquilaria* spp estimated up to 20.2% decline in forest cover (30% canopy density) in the species around 2000 until 2016 [2].

The decline of the natural population is caused by high market demand for agarwood, causing *A. microcarpa* to be conserved. The main goal of endangered plant conservation is to maintain their genetic diversity. One of *A. micoarca* conservation efforts is by mass planting that has been conducted in several locations in Kalimantan. However, genetic diversity at each location has not been well documented in this effort, so it is necessary to assess their genetic diversity.
Genetic analysis of plants has been carried out for many purposes, i.e., taxonomy, conservation and mating system. There are several methods for studying genetic, such as AFLP, SCAR, ISSR [1, 6-9]. Random Amplified Polymorphic DNA (RAPD) is one of the genetic analysis methods that is relatively inexpensive and easy [1]. Genetic information for agarwood-producing tree species is widely available. Research on phylogenetic relatedness, barcoding and species identification has been defined for agarwood-producing tree species in Indonesia and Malaysia [1, 6, 7]. Other information related to genetic agarwood-producing tree information is population genetic of *A. malaccensis* in India using AFLP and ISSR markers [8, 9]. This research is the first study of population genetic on *A. microcarpa* originating from a local community plantation in Kalimantan, Indonesia, based on the RAPD analysis.

2. Material and Methods
We selected 44 individuals of *A. microcarpa* from four plantations; two plantations in Sanggau (1°10' - 0°35' S and 109°45' - 111°11' E), West Kalimantan namely Sanggau I and Sanggau II, and two plantations in Gumbil (2°58' N dan 115°38' E), South Kalimantan, namely Gumbil I and Gumbil II (Figure 1). Fresh leaves collected from those populations were preserved using silica gel to reduce leaves damages during transportation from the field to the Molecular Genetic Laboratory of the Center for Forest Biotechnology and Tree Improvement Research and Development (CFBTI) in Yogyakarta, Indonesia. The genomic DNA was isolated using a modified CTAB protocol [10], and DNA amplification was conducted using ready mix PCR buffer with Biotaq DNA polymerase (Bioline). The PCR process was carried out on a Thermocycler 9700 machine with PCR conditions described in the previous studies [11, 12]. The amplification products were visualized using 1.2% agarose gels in 1 X TBE buffer with ethidium bromide stain, and the DNA bands were observed and photographed under UV light using Quantity One software (Biorad).

![Figure 1. Sampling location of *A. microcarpa* in Kalimantan.](image)

We screened 24 RAPD primers to obtain polymorphism and stable primers for genetic analysis. We identified nine stable RAPD primers from the RAPD primer screening test. Amplified RAPD bands were scored as presence (1) or absence (0) for each sample to produce a binary data matrix. The genetic diversity and genetic distance of *A. microcarpa* were calculated using GenAlex 6.5 program [13]. These measurement values were based on 1971 Nei's Gene Diversity and 1971 Nei's Original Measures of Genetic Distance [14]. Principal Coordinate Analysis (PCoA) and Analysis of molecular variance (AMOVA) were used to analyze the overall distribution of molecular variation within and among *A. microcarpa* population. We calculated both analyses using the GenAlex ver 6.4 program [9, 14, 18].
3. Results and Discussion
This study showed that the RAPD marker for *A. microcarpa* in Kalimantan successfully amplifies 49 polymorphic alleles from 9 stable primers. Each primer has 4 to 7 polymorphic loci. Amplicon position detected in the gel electrophoresis ranged between 490-1200 bp. The detailed characters of the RAPD marker are presented in Table 1. All primers were found to be polymorphic and produced different percentages of polymorphisms [1].

| No | Primer name | Sequence 5’-3’ | Number of polymorphic loci | Locus sizes (bp) |
|----|-------------|----------------|-----------------------------|-----------------|
| 1  | OPA 10      | GTGATCGCAG     | 4                           | 590, 630, 700, 1050 |
| 2  | OPA 11      | CAATCGCCGT     | 4                           | 510, 520, 590, 610 |
| 3  | OPA 12      | TCGGCGATAG     | 5                           | 420, 570, 660, 720, 800 |
| 4  | OPA 13      | TCGGCGATAG     | 7                           | 830, 840, 1010, 1020, 1050, 1070, 1250 |
| 5  | OPA 18      | AGGTGACCGT     | 4                           | 490, 510, 680, 730 |
| 6  | OPG 06      | GTGCTAACC      | 6                           | 600, 630, 790, 870, 1000, 1240 |
| 7  | OPG 07      | GAACCTGCGG     | 5                           | 500, 550, 700, 790, 800 |
| 8  | OPG 12      | CAGCTCACGA     | 7                           | 500, 580, 1000, 1050, 1110, 1150, 1200 |
| 9  | OPG 18      | GGCTCATGTG     | 7                           | 500, 540, 550, 600, 700, 780, 790 |
|    | TOTAL       |                | 49                          |                 |

Table 2 showed that based on 49 polymorphic loci, the uHE values of the plantations ranged between 0.169 (Gumbil II) to 0.258 (Gumbil I). It was in moderate level (mean uHE = 0.216± 0.015). The mean uHE value obtained in this study was lower than that of *A. malaccensis* species from natural stands in Assam, India (0.25 ± 0.17) [9]. The moderate value of genetic diversity in this study indicated that the agarwood plantations in Kalimantan are well managed.

| Population | N   | Na | Specific Locus | Ne   | uHe       |
|------------|-----|----|----------------|------|-----------|
| Gumbil I   | 15  | 2  | 1              | 1.430±0.054 | 0.258±0.030   |
| Gumbil II  | 5   | 1  | 0              | 1.270±0.054 | 0.169±0.033   |
| Sanggau I  | 10  | 2  | 1              | 1.404±0.056 | 0.244±0.030   |
| Sanggau II | 14  | 1  | 1              | 1.390±0.052 | 0.239±0.030   |
| Mean       |     |    |                | 1.413±0.050 | 0.216±0.015   |

Table 2. Genetic diversity parameters of *A. microcarpa* in Kalimantan using RAPD marker.

Remarks: N = population number, Na = number of allele, Ne = number of effective allele, uHe = unbiased expected heterozygosity.

The range of genetic diversity values in this study is related to the number of populations analyzed. This is consistent with previous research on *A. malaccensis* that a large population tends to show high genetic diversity [9]. Another factor that can affect the value of genetic diversity is the mating system. Simultaneous flowering in agarwood plants will increase the random mating (outcrossing) rate; therefore, genetic diversity will increase [13, 16, 17]. In plantations, genetic diversity can also be affected by the number of mother trees of the seed/seedling. The larger number of collected mother trees can produce higher genetic diversity of seed/seedling.
Figure 2. Principal Coordinates Analysis (PCoA) of individual *A. microcarpa* from plantation forests in Kalimantan.

PCoA analysis of *A. microcarpa* from Kalimantan resulted in three clusters. The individual of *A. microcarpa* from Gumbil I and II was in different ordinate. Furthermore, the individuals from Sanggau I and II were in the same ordination (Figure 1). It means that plantation at Gumbil I and II originated from different seed sources; Sanggau I and II originated from seed sources that closed genetic relationship.

Table 3. Nei's genetic distance of *A. microcarpa* among four plantations in Kalimantan.

| Plantation  | Gumbil I | Gumbil II | Sanggau I | Sanggau II |
|-------------|----------|-----------|-----------|------------|
| Gumbil I    | -        |           |           |            |
| Gumbil II   | 0.395    | -         |           |            |
| Sanggau I   | 0.133    | 0.364     | -         |            |
| Sanggau II  | 0.172    | 0.391     | 0.053     | -          |

The genetic distance of the four populations studied was large (mean DA = 0.251). Sanggau I closed to and Sanggau II (DA = 0.053). In contrast, Gumbil I and Gumbil II had the highest genetic distance between populations (DA = 0.395). The great DA values between Sanggau and Gumbil showed that genetic distance was concordance with the geographical distance between the populations. Moreover, the great DA value between Gumbil I and Gumbil II might differ in the origin of genetic material when the plantations were established.
Table 4. Analysis of molecular variance (AMOVA) for the population of *A. microcarpa* in Kalimantan.

| Source              | df | SS   | MS   | Est. Var. | %   | P-value     |
|---------------------|----|------|------|-----------|-----|-------------|
| Among plantations   | 3  | 119.833 | 39.944 | 3.241     | 36% | 0.001***    |
| Within plantation   | 40 | 226.827 | 5.671  | 5.671     | 64% | 0.001***    |
| Total               | 43 | 346.659 | 8.911  |           | 100%|             |

Remarks: df = degrees of freedom; SS = sum of squared deviation; MS = mean squared deviation; Est Var. = estimated variance; % = percentage of total variance

The differentiation among populations was verified with AMOVA (Table 4), in which 36% explained variation among populations and 64% explained variation within-population. The percentage of total variance value indicates strong population differentiation [18]. This finding is also similar to population differentiation in *A. malaccensis* from Assam, India [9]. The great genetic differentiation among populations in this study might be that there is no gene flow among the originated populations.

4. Conclusion
Finding from this study has an important implication in plantations management of agarwood. The genetic diversity was at a low to moderate level. Thus to maintain the level, it should be strictly managed number of individual trees, spacing among individual trees and stimulating flowering, then a randomly mating system might occur. In addition, we found strong population differentiation due to differences in genetic structure. The consequence arising from these findings is the need to be careful in transferring seeds between populations. This is to keep the genetic uniqueness of a certain population from being lost.

References
[1] Lee S Y, Weber J and Mohamed R 2011 Genetic variation and molecular authentication of selected aquilaria species from natural populations in Malaysia using RAPD and SCAR Markers Asian J. Plant Sci. 10(3) 202-11
[2] Harvey-Brown Y 2018 *Aquilaria microcarpa* The IUCN Red List of Threatened Species 2018
[3] Anonim 2004 CITiES: Consideration of Proposals for Amendment of Appendices I and II- Aquilaria spp and Gyrinops spp. (Bangkok, 13th meeting conf.) CoP17
[4] Turjaman M and Hidayat A 2017 Agarwood-planted tree inventory in *Indonesia IOP Conf. Series: Earth and Environmental Science* 54 012062
[5] Faridah-Hanum I, Mustapa M Z, Lepun P, Tuan Marina T I, Nazre M, Alan R and Mohamed R 2009 Notes on the distribution and ecology of *Aquilaria* Lam. (Thymelaeaceae) in *The Malaysian Forester* 72(2) 247-59
[6] Lee S Y, Turjaman M and Mohamed R 2018 Phylogenetic Relatedness of Several Agarwood-Producing Taxa (Thymelaeaceae) from Indonesia *Trop. Life Sciences Research* 29(2) 13-28
[7] Pern Y C, Lee S Y, Kamarudin N and Mohamed R 2020 Genetic variation and DNA barcoding of the endangered agarwood-producing *Aquilaria beccariana* (Thymelaeaceae) populations from the Malesia Region *Forestist* 70(2) 85-94
[8] Singh P, Nag A, Parmar R, Ghosh S, Singh Bhau B and Sharma R 2015 Genetic diversity and population structure of endangered *Aquilaria malaccensis* revealed potential for future conservation *J. Genet.* 94 697-704
[9] Banu S, Baruah D, Bhagwat R M, Sarkar P, Bhwmick A and Kaoo N Y 2015 Analysis of genetic variability in *Aquilaria malaccensis* from Bramhaputtra Valley, Assam, India using ISSR marker *Flora* 217 24-32
[11] Shiraishi S and Watanabe A 1995 Identification of chloroplast genome between Pinus densiflora SIEB. EtZUCC. and P. thunbergii PARL. based on the polymorphism in rbcL Gene J. Jap. For. Sci. 77429-36

[12] Nurtjahjaningsih I L G, Herawan T and Rimbawanto 2018 Random amplified polymorphism DNA marker test to assess genetic stability of teak (Tectona grandis) clones JPHT 12(2) 127-34

[13] Nurtjahjaningsih I L G, Haryjanto L, Sulistyawati P and Widyatmoko A Y P B C 2020 Mating system of two priority species for ecosystem restoration at Mount Merapi, AIP Conference Proceedings 2020

[14] Peakall R and Smouse P E 2006 GENALEX6: Genetic Analysis in Excel, Population genetic software for teaching and research Molecular Ecology Notes 6 288-295

[15] Sulistyawati P and Widyatmoko A Y P B C 2017 Keragaman genetik populasi kayu merah (Pterocarpus indicus Willd) menggunakan penanda Random Amplified Polymorphism DNA Jurnal Pemuliaan Tanaman Hutan 11(1) 67-76

[16] Nurtjahjaningsih I L G, Saito Y, Tsuda Y and Ide Y 2007 Genetic diversity of parental and offspring populations in a Pinus merkusii seedling seed orchard detected by microsatellite markers Bulletin-Tokyo University Forest 118 1-14

[17] Nurtjahjaningsih I L G, Widyatmoko A Y P B C, Haryjanto L, Yuliah and Hadiyan Y 2020 Genetic diversity of Aquilaria malaccensis from western Bangka and its implication for manage seed stands JPHT 14 (2) 121-128

[18] Hartl D L and Clark A G 1997 Principles of Population Genetics, Third Edition, Sinauer Associates Inc. Publisher, Sunderland, Massachusetts

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Authors’ contribution
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