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GENETIC VARIATIONS AMONG *AQUILARIA* SPECIES AND *GYRINOPS VERSTEEGII* USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM MARKERS

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ABSTRACT

Aquilaria sp. (Thymelaeaceae) is the most valuable non wood production of forestry plant in Indonesia. It produces a fragrant resin when subjected to fungal attack and has been traded internationally known as gaharu. Knowledge of genetic diversity and relationship among species and genus is important for breeding purposes and species conservation. In this study, genetic variability of six *Aquilaria* species were analyzed using the Amplified Fragment Length Polymorphism (AFLP) markers. Ten AFLP primer combinations amplified 1353 DNA fragments ranging in size from 100 to 350 bp of which 1285 (95%) of them were polymorphic. Genetic similarities among *Aquilaria* sp. consisted of *A. malaccensis*, *A. beccariana, A. microcarpa,* and *A. crassna* ranged from 63.90 to 72.00 % based on Dice coefficient. The dendrogram derived by the unweighted pair group method with arithmetic mean of germplasm analysis were clustered into two main groups. Hence, a genetic variation among species is quiet high. Bootstrap values for the groups supported 70% of the cluster using a linear relationship equation of ($r = 0.724$, $P < 0.0001$) was observed between known pedigrees and AFLP-derived genetic similarity for 136 pairwise comparisons of *Aquilaria* species. For example, *A. malacensis* and *A. microcarpa* have the highest genetic similarity (72.00%) compared with another *Aquilaria* species. Primer pairs E-ACG/M-CTA produced a specific fragment for *A. beccariana* (850 bp), *A. crasna* (550 bp, 180 bp, and 140 bp), *A. malaccencis* (1500 bp), *A. microcarpa* (250 bp) and *Gyrinops versteegii* (150 bp). Primer pairs E-ACG/M-CAA produced a specific DNA fragment only for *A. beccariana* (1500 bp and 100 bp). Primer pairs E-ACC/M-CAC also produced only specific fragment for *A. crassna* (1500 bp). Study showed the usefulness of AFLP analysis in *Aquilaria* sp. and its potential application for breeding and species conservation. Further, molecular diversity estimated in the present study combined with the datasets on other morphological/agronomic traits will be useful for selecting the appropriate accessions for plant improvement through conventional and molecular breeding approaches.

Key words: Gaharu, *Aquilaria* sp., *Gyrinops* sp., genetic diversity, molecular marker, amplified fragment length polymorphism (AFLP)

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INTRODUCTION

Gaharu is a fragrant resin of a rare tree species wood that develops after fungal infection. Gaharu has a high economic importance as it is needeed for production of incense and traditional medicines. Presently, this plant is placed on the Appendix II list of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

There is very limited information on the diversity of *Aquilaria* sp., *Gyrinops* sp. and fungi associated with agarwood in Indonesia. Another problem in gaharu, is the difficulty to differentiate it through morphological characters, except through the size of fruit and seeds (Hou 1960). However, the capability of several species in producing flowers and fruit are very low. Umboh *et al.* (2000) have tried to use some isozymes markers to differentiate *Aquilaria* species. The esterase isoenzyme marker could be utilized to identify *A. malaccensis* and *A. microcarpa*, as well as their genetic distances and variation. The results indicated that the polymorphism of this technique is very low around 20%, and could not differentiate both species with *A. beccariana, A. cummingiana, A. filaria,* and *A. hirta*. The CITES Plants Committee have a pilot study on the development of DNA identification tools (Eurlings & Gravendeel 2005).

Amplified fragment length polymorphism tend to generate dominant markers due to the differences in the DNA sequence in the selective 30 nucleotides immediately adjacent to the restriction enzyme site. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The AFLP technique provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity (Vos *et al.* 1995). AFLPs have been used to estimate genetic relationships in many studies including cotton (Murtaza 2006), *Sinopodophyllum hexandrum* (Xiao *et al.* 2006), *Vitis vinivera* (Ergül *et al.* 2006) and *Jathropha curcas* L. (Tatikonda *et al.* 2009).

The objective of this study was to evaluate the genetic diversities or relationships between *Aquilaria* and *Gyrinops,* and among species in the same genus by AFLP marker technology.

MATERIALS AND METHOD

Plant materials

Six agarwood species namely *A. malaccensis*, *A. microcarpa*, *A. beccariana*, *A. cumingiana*, *A. hirta* and *A. filaria* were used for the activity of discriminating *Aquillaria* species. *A. malaccensis* and *A. microcarpa* were collected from identified trees at Muara Fajar, Pekanbaru. *A. beccariana, A. cumingiana,* and *A. hirta* were collected from agarwood plantation of Mr. Gregorius Hambali at Bogor; while *A. filaria* and *Gyrinops versteegii* were collected from agarwood plantation of SEAMEO BIOTROP; Research Institute for Forestry, Bogor; and Bogor Botanical Garden.

DNA extraction

DNA was extracted from fresh young leaves using a modified extraction protocol (Orozco-Castillo *et al.* 1995). Young plant leaf tissue was collected on ice, from one plant of each species and ground into a fine powder in liquid nitrogen. The purity and concentration of the extracted DNA were assessed by electrophoresis on a 1% agarose gel (Sambrook *et al.* 1998).

The AFLP procedure (Vos *et al.* 1995) was used to access the genetic diversity. DNA of 250 ng was extracted from all the five species of *Aquilaria* sp. and *G. versteegii* . The DNA was digested with *Eco*RI and *Mse* I at 37ºC for 2 hrs. A small aliquot of the digested DNA was run on a 1.5% (w/v) agarose gel to check if the DNA digestion was complete. The digested samples were incubated at 70ºC for 15 min to inactivate the restriction endonucleases. *Eco*RI and *Mse*RI adapters were ligated to the digested DNA samples to generate template DNA for amplification. Pre-amplification was carried out with +1-primers each carrying one selective nucleotide (*Eco*RI + A, *Mse*RI + C) in a thermocycler for 20 cycles set at 94ºC denaturation (30 sec), 56ºC annealing (60 sec), and 72ºC extension (60 sec). The initial denaturation was done at 94ºC for 30 sec and the final extension at 72ºC for 8 min.

The amplification products were diluted 20-folds in TE buffer and stored at -20ºC. Selective AFLP amplification was carried out with *Eco*RI +10 primers and *Mse*RI + 10 primers and 5 µL of the diluted PCR products from the pre-amplification. Four primer pair combinations of *Eco*RI + 3 (E-plus three nucleotides) and *Mse*RI + 3 (M-plus 3 nucleotides) were tested (Table 1). The PCR amplifications were carried out as follows: one cycle at 94ºC for 30 sec, 65ºC for 30 sec, and 72ºC for 60 sec; followed by 12 cycles of touchdown PCR in which the annealing temperature was decreased to 0.7ºC every cycle until a touchdown annealing temperature of 56ºC was reached. Once reached, another 20 cycles were conducted as described above for pre-amplification. The reaction product (2 µL) was mixed with an equal volume of formamide loading buffer (98% [v/v] formamide, 10 m*M* EDTA,0.005% [v/v] of each of xylene cyanol and bromophenol blue) denatured by incubating at 90ºC for 5 min and quickly cooled on ice.

Table 1. Sequence of 10 primer pairs of AFLP used in gaharu genetic variation analysis

No.	Sequences of selective primer (*Eco*RI+AX & *Mse*I+CY)
1	GACTGCGTACCAATTC-AAC & GATGAGTCCTGAGTAA-CAA
2	GACTGCGTACCAATTC-AAG & GATGAGTCCTGAGTAA-CAA
3	GACTGCGTACCAATTC-ACA & GATGAGTCCTGAGTAA-CTC
4	GACTGCGTACCAATTC-ACC & GATGAGTCCTGAGTAA-CAG
5	GACTGCGTACCAATTC-ACG & GATGAGTCCTGAGTAA-CTA
6	GACTGCGTACCAATTC-ACG & GATGAGTCCTGAGTAA-CAA
7	GACTGCGTACCAATTC-ACC & GATGAGTCCTGAGTAA-CAC
8	GACTGCGTACCAATTC-ACA & GATGAGTCCTGAGTAA-CAA
9	GACTGCGTACCAATTC-ACT & GATGAGTCCTGAGTAA-CTA
10	GACTGCGTACCAATTC-AAC & GATGAGTCCTGAGTAA-CAC

The products were analyzed on 6% (w/v) denaturing polyacrylamide gels. The gel was run at constant power (50-55 W) until the xylene cyanol was about two-thirds down the length of the gel. The gel was silver stained.

AFLP analysis - Detection of bands

Amplified products were separated by means of electrophoresis with 6% denaturing polyacrylamide gels in 1 × TBE buffer (Sambrook *et al.* 1989). Gels were dried, and AFLP bands were visualized following autoradiography (Kodak Biomax MR) according to the standard conditions (Zhu *et al.* 1998).

Estimates of genetic distance

Estimation of similarity based on the number of non-shared bands between each pair of plant was recorded for those fragments and subjected to the cluster analysis, and the dendogram was constructed by the unweighted pair-group method with arithmetic average (UPGMA) using NTSYS-pc program (Rohlf 1993). Fragments smaller than 80 bp were excluded from the data matrix. Distance matrices for all pairs of genotypes were constructed from the AFLP data matrix using the Euclidean distance method Nei and Lie (1979).

RESULTS AND DISCUSSION

Ten primer-pair combinations were used to assay six *Aquilaria* sp. and *G. versteegii* plants for AFLP analysis. An average of 30 to 47 scorable bands was detected after selective PCR amplification with each primer combination (Fig. 1). The bands ranged in size from 100 to 2000 bp. The results of agglomeration schedule (Table 1) as well as the dendrogram of genetic relationships from UPGMA cluster analysis from 370 fragments (Fig. 2) resulted in two major genetic groups with genetic similarity of (I) 63.90 % and 64.05 % (II). The genetic similarity between species of *Aquilaria* and *G. versteegii* is around 0.59-0.72%. The highest genetic similarity was obtained from *A. microcarpa* and *A. malaccencis* (72 %), while *A. crasna* and *G. versteegii* with genetic distance 64.05%. The *Aquilaria* sp. was clustered into two major groups depending upon their species. The first group consisted of *A. becariana, A. microcarpa* and *A. malaccensis* in one sub group, while the second group consisted of *A. crassna* and *G. Versteegii.*

From ten selective primer pairs used to amplify DNA of five *Aquilaria* sp. and *G. versteegii* three primer pairs were obtained and produced specific fragments. These fragments can be used to differentiate among species. Primer E-ACG/M-CTA produced a specific fragment for each *Aquilaria* species such as *A. beccariana* (850 pb), *A. crasna* (550 bp, 180 bp, and 140 bp), *A. malaccencis* (1500 bp), *A. microcarpa* (250 bp) and *G. versteegii* (150 bp). Primer E-ACG/M-CAA produced specific primer only for *A. beccariana* (1500 and 100 bp). Primer E-ACC/M-CAC also produced specific fragment for *A. crasna* (1500 bp). To apply those DNA fragments as a specific marker for each species of *Aquilaria* sp., more detailed informations are needed. These data indicate that considerable intra-population genetic diversity exists in *Aquilaria* sp., in agreement with its life history traits and geographical distribution. *Aquilaria* sp. and

G. versteegii are long-lived, insect-pollinated woody perennial and spread throughout the tropical and subtropical regions of the Pacific. The high genetic diversity observed indicates that these populations are able to adapt to environmental changes. This is reflected in the current adaptive capacity of *Aquilaria* sp. and *Gyrinops* sp. to various habitats.

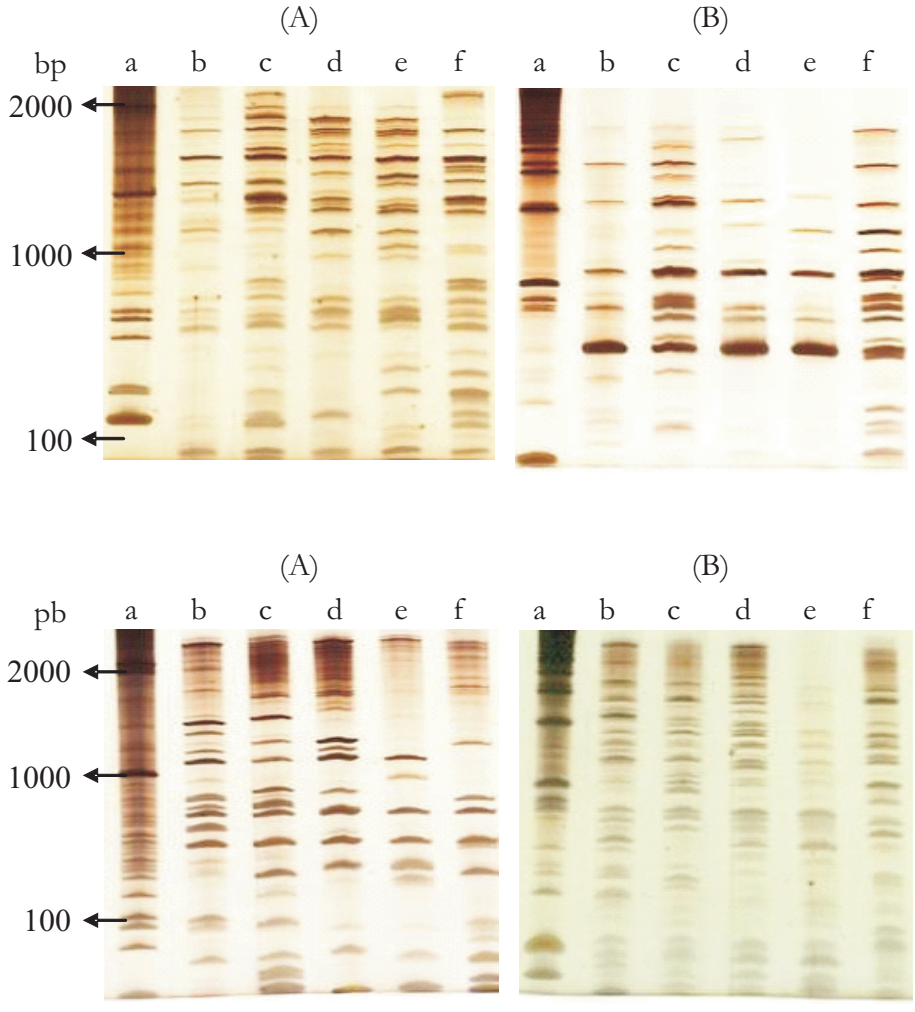

Figure 1. Electrophoresis of selective amplification with (A). E-ACA/M-CTC, (B). E-ACC/M-CAG, (C) E-ACG/M-CTA, and (D) E-ACG/M-CAA. (a) marker 1000 bp DNA ladder ; (b) *A. beccariana*, (c) *A. crasna*, (d) *A. malaccensis*, (e) *A. microcarpa*, and (f) *G. Verstegii*

Table 1. Matrix of genetic similarities among species of *Aquilaria* sp. and *G. versteegii.*

	A. beccariana (Bc)	*A. crassna* (Ac 8)	*A. microcarpa* (P8)	*A. malacencis* (B27)	*G. versteegii* (Af27)
A. beccariana	1.00				
A. crassna	0.63	1.00			
A. microcarpa	0.63	0.63	1.00		
A. malaccensis	0.65	0.62	0.72	1.00	
G. versteegii	0.59	0.64	0.62	0.59	1.00

Indonesia has a National program for sustainable agarwood production through planting this species in several provincies through government project and community participation. There are six *Aquilaria* species in Indonesia, namely *A. malaccensis, A. hirta, A. beccariana, A. microcarpa, A. cumingiana* and *A. filaria* (Hou 1960). The first four species are found in Sumatera and Kalimantan, while the fifth in Maluku and the latter species in Maluku and Irian Jaya. The taxonomic status of *Aquilaria* and *Gyrinops* (Thymelacaceae, Malvalves) as a separate genera is doubted as they are only distinguished by the number of stamens. According to Eurling and Gravendeel (2005) *Trn*L-*trn*F sequence data showed the paraphyletic of *Aquilaria* and *Gyrinops*. Molecular diversity determined in the present study combined with the datasets of other morphological/agronomic traits will be very useful for selecting the appropriate accessions for plant improvement through conventional as well as molecular breeding approaches.

A better understanding of the relationships amongs the genus and species is needed for plant improvement. In this study, DNA analysis by AFLP can be used to evaluate genetic relatedness between two genus and species of *Aquilaria* and *Gyrinops*. A comprehensive inventory is also required to determine the distribution of the species within the country and characteristics of the trees and stands. Genetic resources of agarwood represent the raw material used to create novel varieties that are more reproductive, better adapted to the regions of cultivation. Together with the related wild species, they represent the repository of potential genetic variability for improvement programs of the cultivated plants. It is therefore necessary to provide breeders with information on the genetic diversity of agarwood in Indonesia, which is still little known and exploited. This can and must be done by means of the characterization of these genetic resources. Suhartono (1999) has reported on the status distribution and made the database of several species of *Aquilaria* sp. in Indonesia. But, these data need to be completed for another *Aquilaria* sp. and *Gyrinops* sp., in attempt to obtain a comprehensive understanding of the whole genus of gaharu.

CONCLUSION

Genetic similarities among *Aquilaria* sp., *i.e. A.malaccensis*, *A. beccariana, A. microcarpa,* and *A. crassna* were around 63.90 up to 72.00 %. It means that genetic variations among species are quite high. *A. malacensis* and *A. microcarpa* have the highest genetic similarity *i.e.* 72.00% as compared to other *Aquilaria* species.

Primer pairs of E-ACG/M-CTA gave a specific DNA fragment for *A. beccariana* at 850 bp, *A. crasna* at 550 bp, 180 bp, and 140 bp, *A. malaccencis* at 1500 bp, *A. microcarpa* at 250 bp and *G. versteegii* at 150 bp. Primer pairs E-ACG/M-CAA gave specific fragment only for *A. beccariana* at 1500 bp and 100 bp, while primer pairs E-ACC/M-CAC only gave a specific primer for *A. crasna* at 1500 bp.

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