Genetic Variation of *Agathis loranthifolia* Salisb. in West Jawa Assessed by RAPD

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ABSTRAK

Variasi Genetika *Agathis loranthifolia* Salisb Jawa Barat Menggunakan Analisis RAPD. *Agathis loranthifolia* Salisb merupakan salah satu tumbuhan hutan yang menghasilkan hasil hutan bukan berupa kayu yaitu berupa getah. Untuk mengetahui derajad variasi genetika dari jenis ini maka dicoba dianalisis menggunakan RAPD. Sampel daun tumbuhan ini di peroleh dari Perum Perhutani Cianjur dan Garut. Variasi genetika populasi tumbuhan ini dari Cianjur sebesar He = 0.1952 atau lebih tinggi dari populasi asal Garut (He = 0.1125). Namun berdasarkan produksi copalnya menunjukkan bahwa variasi genetika pohon Lanang paling tinggi He = 0.2105

Kata kunci: RAPD, kopal, *Agathis loranthifolia*

INTRODUCTION

Agathis loranthifolia Salisb. is one of the forest trees which produces wood and non wood products in the form of resin (copal). Forest products in the form of wood from agathis trees are commonly used as raw materials for plywood, good quality veneer, and as knot connecting plate in wood roof construction (Rilatupa *et al*. 2004). On the other hand, the copals being produced are commonly used as paint materials, spirit, red lacquer, and varnishes (Nurhasybi & Sudrajat 2002).

Based on data from Directorate General of Forestry Production Development, production of copal in the year 2000 was as large as 647 tons, year 2001 as large as 428 tons, year 2002 as large as 442 tons, year 2003 as large as 403 tons, and year 2004 as large as 318 tons (Dephut 2004). In the year 2007 the price of copal reached Rp. 284 200/ton. The high price of copal creates a bright prospect for copal as industrial raw materials, so that the role of agathis forest as supplier for copal industries should be maintained by pursuing the stand sustainability and high production of copal.

In general, production of copal is determined by external factors (environment) and internal factors. The external factors are: quality of growth site, tree density, climate, and sun light intensity. On the other hand, internal factors which influence the production of agathis resin are among other things tree age, tree diameter, number and size of resin canal,

condition of nutrient supply, and condition of water supply, particularly in the wounded part (Suharlan 1983). Other factor which influences copal production is the existence of agathis trees which are less productive in producing resin (dry of resin). Trees which are "dry of resin" existing in the field are known and identified only after the first resin tapping, because it is physically difficult to distinguish them from trees which are "abundant of resin" or from trees which are more productive in producing resin (Soesilotomo & Harbagung 1992).

One effort which can be conducted for increasing the productivity for agathis resin is by tree improvement activity in the form of provision of superior seeds, so that plantation forests which use quality planting stocks (superior planting stocks) could be developed. Genetic improvement program for obtaining superior planting stocks with desirable traits could be started with genetic exploration activities through morphological analysis for distinguishing the morphological character differences between trees which produce abundant resin and trees which do not produce resin, and conducting genetic improvement activities which utilize technology of molecular marker.

According to Finkeldey (2005), content of particular secondary metabolism production which constitutes the result of complex metabolic flow is often controlled only by one or a small number of gene locus, so that by conducting DNA (deoxyribonucleic acid) analysis, particular marker gene locus could be obtained. Activities of DNA analysis could be conducted by utilizing technology of molecular marker, which among other things is RAPD (Random Amplified Polymorphic DNA). Technique of RAPD (Random Amplified Polymorphic DNA) has superiority because it can rapidly detect DNA polymorphism, is relatively easy to be performed, and requires only a small number of DNA (Weising *et. al*. 2005). RAPD marker could be used to discriminate among the clones, characterization of varieties for salinity tolerance, develop a genus-specific probe, and estimation of genetic divergence among cultivars-genotypes (Roberts & Crawford 2000, Rahman *et. al.* 2002, Kurup *et. al*. 2009). Benefit which is expected to be obtained from this research result is the collection of information concerning genetic variability as the basis of genetic conservation and genetic improvement program.

MATERIALS AND METHOD

Plant materials used for genetic analysis were in the form of leaves originating from three locations (populations), namely: 1). Year 1963 dammar plantation forest as large as 25 ha in Subdivision of Forest Management Sub Units (RPH) Cibatu, Forest Management Sub Units (BKPH) Cibatu, KPH (Forest Management Unit) Garut, 2). Year 1959 dammar plantation forest as large as 5 ha in RPH Cijedig, BKPH Cianjur-Gede Timur, KPH Cianjur, and 3). Year 1957 dammar plantation forest as large as 3.3 ha in RPH Puncak, BKPH Cianjur-Gede Timur, KPH Cianjur.

Average resin production of population of KPH Garut was as much as 1.18 gram/ tree / day, and of KPH Cianjur as

much as 2.29 gram/ tree / day. Selection of plus tree was conducted by using scoring system with minimum resin production of 2 grams/ tree / day. Numbers of trees obtained in selection of plus trees are as follows: 1). In KPH Garut there were selected as many as 16 plus trees in compartment 11b, RPH Cibatu, BKPH Cibatu, KPH Garut; and 2). In BKPH Cianjur, KPH Cianjur there were selected 9 plus trees, namely in RPH Puncak (3 plus trees) and RPH Cijedil (6 plus trees). Number of samples for genetic analysis could be seen in Table 1 and description on categorization of trees on the basis of resin production can be seen in Figure 1.

Activities of DNA isolation from leaves was conducted by using method of CTAB (Cetyl Trimethyl Ammonium Bromide) which had been modified for obtaining DNA which were sufficiently pure (Siregar *et. al*. 2008). Sample of leaves (2 cm x 2 cm) were ground by using liquid nitrogen in clean pestle. The ground products were afterwards transferred to 1.5 mL tube, and were subsequently added with 500-700 uL buffer exstract solution and 100 uL PVP 2%. Afterwards, the ground products were subjected to vortex to make them homogeneous. Afterwards, there was incubation process in waterbath for 45 minutes-1 hour at temperature of 65oC.

For binding the DNA, there were addition of 500 uL chloroform and 10 uL phenol. Afterwards the mixture was shaken to make it homogeneous. Centrifugation was conducted for separating liquid phase and organic matter phase at speed of 13 000 rpm for 2 minutes (supernatant). Afterwards, the supernatant was taken and transferred to new tube. Toward the supernatant, there were addition of 500 uL cold isopropanol and 300 uL NaCl, and then, shaking was conducted. Afterwards, mixture of supernatant and cold isopropanol and NaCl was stored in freezer for duration ranging between 45 minutes and 1 hour for obtaining pellet of DNA.

Further activity was washing process of DNA by adding of 100% ethanol as much as 300 uL, which was subsequently centrifuged at speed of 13 000 rpm for 2 minutes. Ethanol liquid was discarded away carefully in order to prevent the DNA pellet being also discarded away. Afterwards, the DNA pellet was kept in desiccators for ± 15 minutes and was afterwards added with 20 uL buffer TE solution. Afterwards, they were subjected to vortex and being centrifuged again.

Table 1. Number of samples for genetic analysis.

Location of KPH (Forest Management nit)	Categories				Sum of sub total	Total sum
	Plus trees	Comparison trees	Random trees	Lanang	Resin leaking	
Garut	7	10			24	42
Cianjur	3		10	2	18	

Primer is short chain of DNA which is artificially produced and usually consist of between 10-25 nucleotides (Finkeldey 2005). Primer functions as initial point for occurrence of amplification. Segments of DNA between the two primer meeting points will be amplified in the PCR reaction. In RAPD technique, generally, the primer being used are in the form of oligonucleotide which possess length of 10-mer, selected randomly and possess five bases of G and C. Primer which possess length of less than 9-mer could be used, but it will produce less amplification product, and there is a need for more sensitive staining method to detect it.

Selection of primer was intended to seek random primer which produced polymorphic marker, because not all nucleotide primer were able to produce amplification products (positive primer). And of the positive primer, not all of them produced polymorphic DNA fragments. In this research, survey was conducted on 35 primers, namely primer from the category OPO and OPY which were produced by Operon Technology. Primer from category OPO were those which possessed primer code O.1, O.2, O.4, O.5, O.6, O.7, O.8, O.9, O.10, O.11, O.12, O.13, O.14, O.15, O.16, O.18, O.19 and O.20. On the other hand, primer from category OPY possessed primer code Y.1, Y.2, Y.3, Y.4, Y.5, Y.6, Y.8, Y.9, Y.11, Y.12, Y.13, Y.14, Y.15, Y.16, Y.17, Y.18 and Y.20. From the result of selection, there were only 3 primers being selected, namely O.6, O.9 and Y.2. Sequences of nucleotide of each primer were OPO.6 (5'-CCACGGGAAG-3'), OPO.9 (5'-TCCCACGCAA-3') and OPY.2 (5'-CATCGCCGCA-3').

According to Bernard (1998) PCR is a technique for multiplying specific DNA segment. There are 4 main components required for conducting the PCR process, namely: 1). DNA target, 2). Primer, 3). DNA polymerase, and 4). 4 dNTP. In principle, PCR is a short term (30-60 seconds) cycle with three changes of temperature which change rapidly. Technique of RAPD does not need initial information concerning base sequence of a species, and those needed are DNA which are relatively pure and

(a) (b) (c)

Figure 1. Description of tree categorization on the basis of resin production. Explanation: (a) Resin leaking; (b) Ordinary, in Gunung Walat University Forest; (c) Lanang, in KPH Cianjur.

in relatively small amount as compared with method of Restriction Fragment Length Polymorphysm (RFLP). Therefore, RAPD can be applied in nearly all plant species (Rimbawanto *et. al*. 2004). Beside that, the amount of DNA needed in the process of PCR-RAPD is very small, namely around 1 µL or ≤ 10 ng/µL (Promega 2003).

Reaction of PCR-RAPD was conducted by using 13 uL volume of solution which consisted of H20 2 uL, primer 1.5 uL, GoTaq® Green Master Mix 7.5 uL (Promega), and 2 uL of genomic DNA. Amplification of DNA was conducted by using machine of PTC-100 Progammable Thermal Cycler (MJ Research, Massachussetts, USA). Process of RAPD was conducted by using primer resulting from selection. Temperature regulation in PTC-100 machine for PCR reaction was based on research by Ratih *et. al*. (1998) which had been modified (Table 2).

Characteristics of DNA resulting from extraction and RAPD could be observed by performing electrophoresis using agarose gel in buffer solution 1x TAE. Characteristics of DNA band, resulting from extraction could be observed in agarose gel with concentration of 1% (w/v). On the other hand, results of RAPD were analyzed by performing electrophoresis using 2.0 % (w/v) agarose gel.

Results of RAPD which have undergone electrophoresis were afterwards photographed and analyzed by performing scoring on the emerging band pattern. Band pattern which emerged (positive) was given score 1 and band pattern which did not emerge (negative) was given score 0. Scoring results were then analyzed for learning the frequency and variability within and between populations of A. loranthifolia by using software POPGENE Version 1.2 (Yeh *et. al*. 1997). Estimation of kinship relation was conducted on the basis of number of polymorphic bands which are jointly possessed, whereas grouping of relatives was based on method of UPGMA (Unweighted Pair Group with Arithmatic Average) with software NTSYS Version 2.0 (Rohlf 1998).

RESULTS

Based on amplification results by using RAPD method, of the 3 primers being used, namely OPO-09 (Figure 2a), OPO-06 (Figure 2b) and OPY-02, there were 57 locus being produced. The

Table 2. Steps in the RAPD process.

Steps	Temperature	Time	Number of cycle
Pre-denaturation	95^0C	2 minutes	1
Denaturation	95^0C	1 minute	
Annealing	37^0C	2 minutes	45
Extension	72^0C	2 minutes	
Final Extension	72^0C	10 minutes	1

greatest numbers of locus being produced are those in primer OPO-06, as many as 27 locus

Parameters being studied from variation between population were number of expected alleles (Na), number of observed alleles (Ne), variability of gene (He), and percent of polymorphic locus (PLP). Results of data processing on the parameters mentioned above are presented in Table 3 and Table 4.

Based on results of data processing (Table 3), it could be inferred that agathis species in KPH Cianjur possessed average values of variability parameters (Na = 1.8246, Ne = 1.2986, PLP = 82.46%, and He = 0.1952) which were greater as compared with that of agathis species in KPH Garut. This phenomenon showed that genetic variability of agathis species in KPH Cianjur was greater if compared with genetic variability of that in KPH Garut. This phenomenon had positive relation with average production of resin of the two regions, where the average daily resin production of agathis tree in KPH Cianjur was greater than that of resin production of agathis tree in KPH Garut.

On the other hand, on the basis of resin production (Table 4), population of Lanang possessed values of variability parameters (Na = 1.6667, Ne = 1.3490, PLP = 66.67% and He = 0.2105) which were greater as compared with other populations, followed in rank by population of comparison trees, plus trees, and random trees. Population of "resin leaking" possessed the smallest value of variability parameters (Na = 1.0877, Ne = 1.0620, PLP = 8.77% and He = 0.0363). This phenomenon was possibly due to the small number of samples being analyzed.

Variation among populations showed genetic distances between populations or kinship relation between two different

Table 3. Variation within population on the basis of place of origin.

Place of Origin	Number of Samples	Na	Ne	PLP	He
Cianjur	16	1.8246	1.2986	82.46%	0.1952
Garut	26	1.5965	1.1774	59.65%	0.1125

Table 4. Variation within population based on resin production.

Category	Number of samples	Na	Ne	PLP	He
"*Lanang*"	10	1.6667	1.3490	66.67%	0.2105
Plus	10	1.4561	1.1704	45.61%	0.1075
Check tree	10	1.5263	1.2146	52.63%	0.1285
Random	10	1.3158	1.1185	31.58%	0.0756
"Resin leaking"	2	1.0877	1.0620	8.77%	0.0363

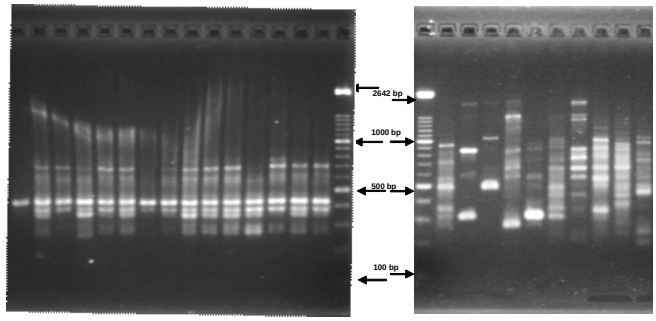

Figure 2 a) Result of RAPD amplification with primer OPO-09 and b). Result of RAPD amplification with primer OPO-06.

populations. Result of grouping among populations on the basis of resin production can be seen in Figure 3.

From the dendrogram among populations on the basis of resin production (Figure 3), it can be seen that Lanang population separates and forms its own group. Likewise, population of resin leaking forms the last cluster. These phenomena showed that population of "Lanang" and "Resin Leaking" has far (distant) kinship relation with other populations (plus, comparison and random trees). Based on resin production, it could also be seen that, population of plus trees and comparison trees occur in the first cluster and form the second cluster with population of random trees, and this shows that the three populations are genetically identical.

DISCUSSION

Resin production constitutes a part of tree physiological process. Therefore, all factors which influence physiological process would also influence resin production. Resin production is influenced by internal and external factors. Internal factors which influence resin production were tree age, tree diameter, number and size of resin canals, condition of nutrient supply, and condition of water supply (particularly in the wounded parts). On the other hand, external factors (environment) were in the form of growth site quality, tree density, climate, and sunlight intensity (Dharmawan 2004).

According to Munajat (2004) production of copal per tree was influenced very much by various factors such as: quality of growth site, tree age, stand density, genetic properties, elevation of growth site above sea level, thickness of bark, stem diameter, topography, crown quality and direction of resin tapping. Manuputty (1955) explained that copal flow during resin tapping was influenced by several factors, such as: species, condition of growth site, tree diameter, number of wounding within one tree, time interval for wound recovery, schedule / time of resin tapping and treatment on wound surface of resin tapping. Each species of dammar has different capability in producing copal.

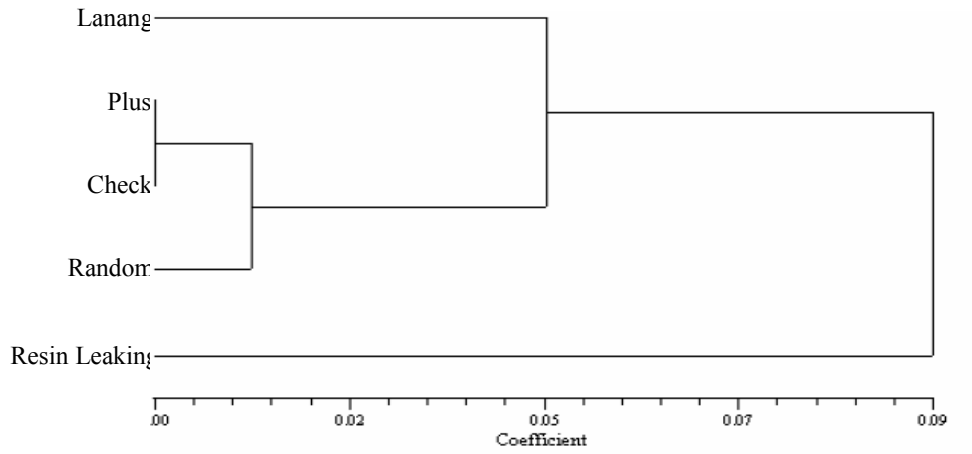

Figure 3. Dendrogram among population on the basis of resin production

Environment of the growth site influences copal production. Dammar stand does not require specific kind of soil in order to grow properly, but the soil should have good drainage. Age class also influences copal production. The presence of stand age variation resulted in varying production of copal. The older the age of the tree, the higher was the production of copal, but after a certain age, the copal production will decrease.

Plant genetic improvement could be defined as an art and science which studies exchange and improvement of plant traits which are passed on to new population with new genetic properties. Art and science which support the attempt of improvement of a plant trait through plant improvement program, comprise two phases, namely: (1). Evolutionary phase, which has the objective of establishing or increasing genetic variability, and (2). Evaluation phase, where selection is conducted on desirable genotypes from several available populations. Besides that, the objective of forest tree improvement is modifying (increasing) average phenotype appearance of traits which have high economic value in commercial plantation forests of forestry tree species (Finkeldey 2005). Characters which generally constitute the selection target are among other things production, production quality, resistance toward pest and diseases, and tolerance toward marginal environment. It has been known that appearance of a character (phenotype) is determined by genetic factor and environment factors, or even determined also by interaction between genetic and environment factors. Therefore, choice of plant on the basis of this phenotype has several weaknesses or drawbacks, particularly if the character is influenced more by environmental factors, which in plant improvement science is categorized as character which has low heritability. Discovery on the technique of gene identification which control a character as marker or molecular marker, is very helpful in selection process in terms of effectiveness of selection which will be con-

ducted (Widodo 2003).

For obtaining stand with similar character to the parent, plant propagation could be conducted by vegetative propagation so that stand with genetic characters similar to its parent, could be obtained. Establishment of stand with high production of resin will encourage increase of economic profit.

CONCLUSION

Based on amount of copal production, population of "Lanang" trees had values of variability parameters (Na = 1,6667, Ne = 1,3490, PLP = 66,67% and He = 0,2105) which were greater than those of other populations, while population of "resin leaking" had smallest values of variability parameters (Na = 1,0877, Ne = 1,0620, PLP = 8,77% and He = 0,0363). On the other hand, on the basis of genetic parameter of place of origin, population of KPH Cianjur (Cianjur FMU) could be made as the base for activities of conservation and genetic improvement of *A. loranthifolia* species.

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