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A genetic diversity on Jabon Merah (*Anthocephalus macrophyllus* Roxb.) from three different provenances in South Sulawesi

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Abstract. Information on genetic diversity has a vital role in supporting quantity and quality improvement in plant breeding process of a species. In this study, we investigated the genetic diversity of Jabon merah (*Anthocephalus macrophyllus*) in three provenances in South Sulawesi. The analysis revealed the genetic diversity in Luwu, Wajo, and Sidrap was low, with heterozygosity (He) ranged from 0.05 up to 0.29. The number of allele was ranged between two and four alleles with 0.27 and 0.2 of PIC means. The low genetic diversity of Jabon merah in the evaluated provenances was as the result of the genetic mixture from the same parents (selfing pollination), and subsequently produced progenies with similar traits and close genetic relationship.

1. Introduction

Jabon merah (*Anthocephalus macrophyllus* Roxb.) is a local tree which thrives in Eastern Indonesia, such as Sulawesi and Maluku. This species is categorized as a pioneer tree having fast growth (fast growing species) and high economic value that potentially developed in natural and plantation forests. Moreover, it can be planted in critical areas as conservation tree at watershed areas due to its high capability in absorbing and holding water. Another superiority of Jabon merah is possessing straight and cylindrical trunk that suitable for raw material for the wood industry. Its height can reach up to 45 m with 30 m trunk length without branch and 160 cm in diameter.

Information on genetic diversity plays a vital role in tree breeding program in order to improve the quality and quantity of the tree. [1] stated that information on genetic diversity is applied to select the desired genotype. An approach for estimating genotype variability is molecular analysis method. Molecular development on DNA analysis has been implemented for characterization of genetic variation and relationship in the genus, species, cultivar, and accession. In some studies, leaves were commonly utilized as the source of DNA in genetic diversity analysis.

A genetic marker that has been widely used in DNA analysis is microsatellite marker. Microsatellite, also known as *simple sequence repeat* (SSR), has simple sequence consists of one to six repeated base pairs and abundant in plant genome [2]. High polymorphic level owned by microsatellite nature can



distinguish individuals having genetically related [3]. It is also beneficial for gene flow and mating system studies as it usually presents considerable variation among individuals.

Finkeldey (2009) reported that many studies had utilized microsatellite markers on tropical trees, e.g., *Eucalyptus* spp., *Shorea* spp., and *Dipterocarpaceae* [4]. A study by Nurtjahjaningsih *et al.* [5] resented 8, 10, and 12 of 13 evaluated microsatellite markers were polymorphic and could amplify DNAs from three different *Eucalyptus* species, *E. deglupta*, *E. urophylla*, and *E. pellita*, respectively. They indicated the low genetic diversity in *E. deglupta* compared to *E. urophylla* and *E. pellita* was influenced by no sufficient conserved of the markers on different subgenus or fragmented natural distribution in *E. deglupta* population. [6] proved that microsatellite markers could be used in evaluating the genetic diversity of *Agathis* sp. She showed as many as five of eight evaluated microsatellite primers were able to amplify with two up to four alleles. The application of microsatellite on Jabon merah has never been published, yet information on genetic diversity of this species is needed for supporting breeding and genetic conservation programs. Here, this study was aimed to determine the genetic diversity of Jabon merah from three provenances in South Sulawesi.

2. Research Methodology

2.1. Research location

Research field was conducted in Jabon merahs' populations in South Sulawesi, Luwu district (Bupon subdistrict, Tampumia village), Wajo district (Pitumpanua subdistrict, Tangkoro village), and Sidrap district (Pituriase subdistrict, Bellawae village). Molecular analysis was carried out at Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Hasanuddin University, Makassar.

2.2. Populations and samples

Populations evaluated in this study were trees of Jabon merah from three provenances, Luwu, Wajo, and Sidrap districts. Seventy-five trees consisted of 24 trees from Luwu, 24 trees from Wajo, and 27 samples from Sidrap were used as the samples. The selected trees had superior phenotypes, such as straight trunk, large diameter, few branches, and healthy. The leaf samples collection was done on each tree sample from every evaluated provenance for molecular analysis.

2.3. Methods

Research activities were initiated by collecting leaves samples in the field and then followed by DNA extraction, primer screening, PCR amplification, and microsatellite analysis. Leaves samples used in the study were leaves of *Anthocephalus macrophyllus* collected from each from three different populations. The leaves were stored into a plastic clip and then placed in a cold box containing ice gel. At the laboratory, the samples were then stored at -20°C freezer until DNA extraction. DNA extraction was carried out using modified Cationic Hexadecyl Trimethyl Ammonium Bromide (CTAB) method with modification [7]. Primer screening was conducted using *cross amplification* method. Before primer screening started, the primers were firstly selected from *National Center for Biotechnology Information* (NCBI). As Jabon merah has the same family with coffee (*Rubiaceae*), thus we decided to do the screening on microsatellite primers that previously used in genetic identification on coffee (*Coffea canephora*). The primers that could produce clear and polymorphic bands would be used in genetic diversity analysis. The detail primers used in the primer screening is presented in Table 1.

Table 1. SSR primer used in the primer screening

No.	Primer	Accession No.	Repeat Motif	Primer (5'-3')
1.	M302	AM408775	(GT) ₉	F: CAAAAGTAAATAAACGATGGACGA R: AAGAGGTAAAAATCAAATCCCAAG
2.	M306	AM408777	(TG) ₉ (AG) ₇	F: CTCGTTTGTGCTCTTTTTG R: TTTGTTAGTTTCTCTCCACCA
3.	M309	AM408738	(GT) ₉	F: AGCAACATTTCCCAGTCAA R: GACCGCAATTTTCTTGTTC
4.	M321	AM408748	(GT) ₈	F: TCGATTGGTTTTGCATACATCT R: GCCAAGATAATGGTTGTGTGG
5.	M325	AM408751	(A) ₅ GGGC(A) ₆	F: ATTACTCTGCTTCGTTTGAC R: CAGTTGCCTGAGATTGAC
6.	M326	AM408752	(GA) ₈	F: GCTTTCTTGCCTTTCTTTTCC R: CATCCACTTACCTCTCCCAAA
7.	M327	AM231546	(GT) ₉	F: GGCTCAAATCACCTTTGT R: CTAGGATCGTGGCAGAAGAAG
8.	M328a	AM408753	(AT) ₆ (GT) ₈	F: CACCTTTTGAGTTTGAGTTGG R: AAAATAAACCCCTTCGTTCA
9.	M329	AM231547	(GT) ₁₀	F: ACTCAGACAAACCCTTCAAC R: GATGTTTTGCATCTATTTGG
10.	M333	AM408757	(AT) ₉ (GT) ₈	F: AACGGGTGGTCTCATTTTATC R: TTTCTGTAGTGGTTTGTCTCC

After screening the primers, the selected primers were used to amplify all DNA samples. PCR amplification was done using 2 µl of DNA working, 0,625 µl of forward primer, 0,625 µl of reverse primer, 6,25 µl of PCR mix (Kappa 2G fast PCR kit), and 3 µl of ddH₂O for each reaction. PCR amplification was conducted by PCR Sensoquest machine, and PCR steps was based on KAPA Biosystem protocol that consisted of one cycle of preliminary denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 15 seconds, specific primer attachment for 50 seconds (at specific temperature of each primer pairs), primer annealing at 72 °C for 60 second, and one cycle of final extension at 72 °C for 10 minutes.

PCR products were separated using 3% of Super Fine Resolution (SFR) gel agarose using 0,5x TAE buffer at 100V for one hour. Electropherograms were visualized using UV transilluminator and documented by a digital camera. Scoring on each genotype was carried out on each sample.

Data analysis

Amplified bands were manually scored. If the band was visible (DNA was amplified), the score was 1. However, if the band did not appear, it would score as 0. Each DNA band using microsatellite marker was presented allele position in the locus. In another word, one marker is one locus [8]. reported that heterozygosity in genetic diversity analysis is calculated using:

$$He = 1 - \sum_{i=1}^n X_i^2$$

Where: He= heterozygosity, n= number of alleles, X_i= frequency of the ith of the genes, heterozygosity is ranged from 0 to 1. If heterozygosity is close to 0, it means low heterozygosity and vice versa. The

analysis was followed by calculating Polymorphic Information Content (PIC) for determining an informative level of the primer. The PIC was calculated using:

$$PIC = 1 - (\sum x_i^2)^2$$

3. Results and discussion

3.1. Primer screening

Primer screening on ten SSR primer pairs showed there were three primer pairs that could be used for amplifying DNA Jabon merah and produced bands or polymorphic alleles. Those primers were M306, M309, and M326. M321 could also amplify DNA, but the bands were monomorphic. Whereas M302, M325, M327, M328a, M329, and M333 generated smear bands, and thus they could not be used in the analysis as they would be misinterpreted in the scoring process. The small differences on amplicons (amplified products) size resulted overlapping (smear) the bands on agarose gel and consequently the size of each fragment could not be determined properly.

3.2. Polymorphism analysis

Band pattern of the amplicons generated from the evaluated primers on 75 samples revealed M309 produced the highest number of allele (four alleles), whilst M306 and M326 generated two and three alleles, respectively. The polymorphic, monomorphic, and smear bands, as well as no bands produced by the evaluated primers are presented in Figure 1.

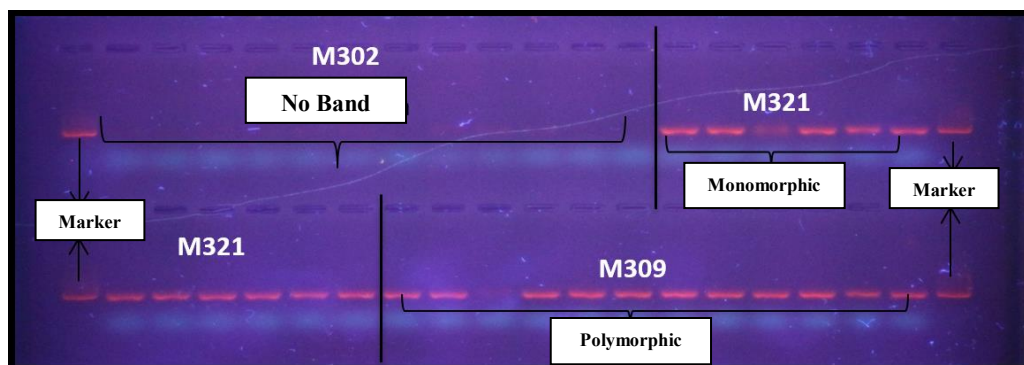


Figure 1. Polymorphic, monomorphic, and no bands generated by M302, M321, and M309.

Figure 1 depicts M309 was able to produce clear polymorphic bands. Contrary to M309, M321 can only generate monomorphic band. Meanwhile M302 did not amplify any band (no band).

Genetic diversity and genetic similarity

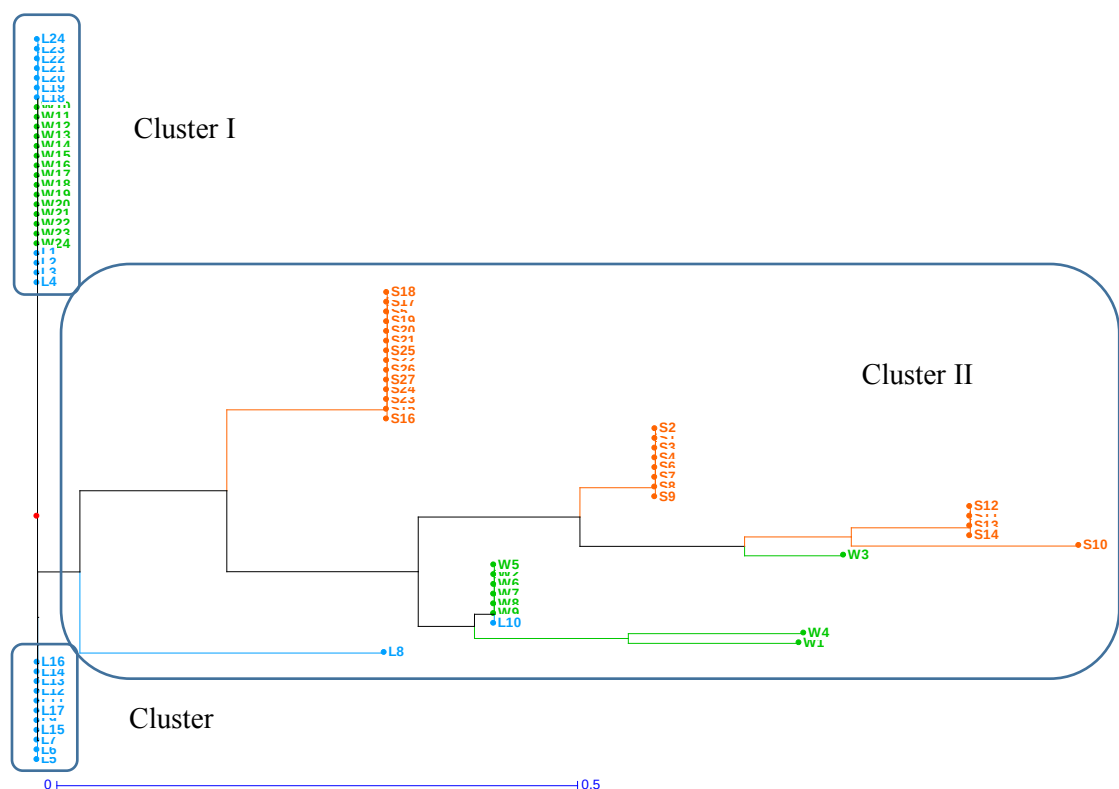
Based on allele frequency observed on each primer in each provenance, the highest PIC was 0,37 obtained using M304 in Sidrap provenance. Meanwhile, M206 generated the lowest PIC (0,07) also in Sidrap provenance. M206 detected 1 of PIC in Wajo and Luwu provenances as both provenances produced only one allele (monomorphic). M206 amplified monomorphic bands in Wajo and Luwu provenances. Moreover, Means of PIC for M306 and M309 were 0,27 and 0,2, respectively. M306 was the only primer that categorized as moderate informative, whereas the other primers were low informative. Classified PIC into three classes, where $PIC > 0,5$ means highly informative, $0,25 < PIC < 0,5$ means moderate informative, and $PIC < 0,25$ means low informative. Overall, the PIC of each primer in each provenance is summarized in Table 2.

Table 2. PIC of each primer in each provenance

No	Provenance	Primer		
		M306	M309	M206
1	Wajo	0,36	0,22	1 (monomorphic)
2	Luwu	0,08	0,08	1 (monomorphic)
3	Sidrap	0,37	0,26	0,07

The highest H_e was observed in Sidrap provenance (0,29) and that of the lowest one was in Luwu (0,05). H_e in Wajo provenance was 0,23. According to the H_e , the genetic diversity of the evaluated Jabon merah was low. [5] explained that genetic diversity can be defined as high if H_e is more than 0,5 ($H_e \geq 0,5$). The objective of genetic reserve location is to determine which areas or sites containing the target species are the most important in terms of genetic diversity for the creation of a genetic reserve or a network of genetic reserves. Since the focus is on genetic diversity within a single taxon, the basic management unit for conservation must be below the species level like *Anthocephalus macrophylus* and teak [9,10], study mating system report in Jabon Merah and Ebony [11,12].

Molecular analysis of the three SSR primer pairs using Darwin software indicated a high genetic similarity within individuals in the same population. It is shown in Figure 2 which illustrated the individuals from a population tended to arrange in a line. It also presents that the individuals were grouped into three clusters. The first cluster consisted of individuals from Wajo and Luwu, the second one was dominated by individuals from Sidrap, nine individuals from Wajo, and two individuals from Luwu, and the last one was all individuals from Luwu. Similar results were also found by AMOVA (Molecular Variance) which shown variation within individuals in each population was 0% (Figure 3). Between populations presented the highest molecular variation and then was followed by variation between the individual in all populations.

**Figure 2.** Genetic similarity of the three provenances based on SSR markers

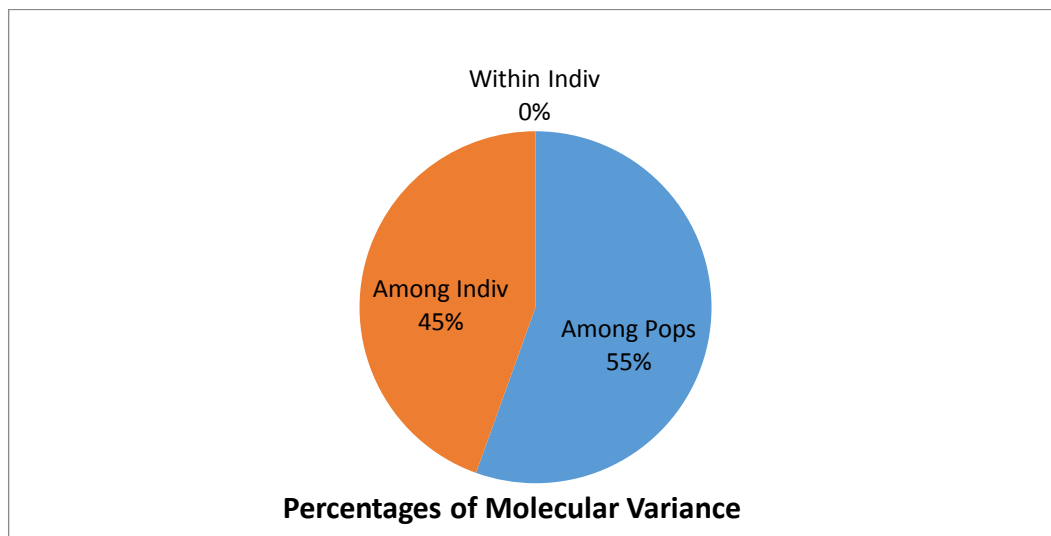


Figure 3. Percentages of Molecular Variance

Genetic diversity of the three provenances was low. That can be explained by two factors. Firstly, the evaluated individuals were from the same population that spread to other regions. The same genotype will tend to group into the same cluster, and thus the level of genetic similarity will be high. Secondly, the primers used in the study had low PIC. Only one primer showed moderate informative for comparing the individuals. It can thus be assumed that variation analysis in the evaluated individuals had not been done correctly. Reported that three SSR primers from Teak were able to analyse the genetic diversity of *Vitex cofassus*. She observed three up to eight alleles with 0,62 of PIC mean. Those findings demonstrated that although the primers were not originated from the same species, they can be utilized to detect variation from different species.

4. Conclusion

In general, we have obtained three primers that produced clear and polymorphic bands. The number of allele was ranged between two and four alleles with 0.27 and 0.2 of PIC means. The He for Luwu, Wajo, and Sidrap were 0.05, 0.23, and 0.29, respectively. The highest genetic diversity was observed on variation between populations (55%). In addition, these Jabon merahs' populations had high genetic similarity, or in another word, they had low genetic diversity.

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