A genetic approach to study mating system on Jabon Merah (Anthocephalus macrophyllus Roxb.) from three different provenances in South Sulawesi

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Abstract. Jabon merah (Anthocephalus macrophyllus) is fast-growing species that endemic to Indonesia, particularly Sulawesi and Maluku. Studies on pollen dispersal and pollination type assist to determine whether the species suffering inbreeding depression in the population. This study investigated the mating system and pollen dispersal pattern on A. macrophyllus in its habitat distribution based on microsatellite marker (SSR). Here, we collected leaf samples from three different locations in South Sulawesi, Luwu, Wajo, and Sidrap, and conducted molecular analysis at Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Hasanuddin University, Makassar. The analyses indicated A. macrophyllus tended to do outcrossing in each evaluated population. Means of outcrossing and selfing rates observed in the populations were 76.9% and 23.1%, respectively. Distance pollen travel could reach up to 339 m, and the frequencies of pollinations were varied from 1 to 7 times. The findings indicate individuals of Jabon merah in the populations may not be threatened by inbreeding depression due to outcrossed pollination nature.

1. Introduction
Jabon merah (Anthocephalus macrophyllus Roxb.) is an endemic tree which thrives in Eastern Indonesia, i.e. Sulawesi and Maluku [1]. This species has monoecious inflorescence (both male and female within a single flower). Such inflorescence has high possibility to be self-pollinating and consequently having low genetic diversity in the populations or undergoing inbreeding depression. Study of pollen dispersal through a genetic approach can provide better information regarding the influence of pollination type on genetic diversity of a species.

The mating system of a species can be detected by analyzing its pollen dispersal pattern. [2] identified the chance of outcrossing among Melaleuca cajuputi in Gunungkidul orchard. Gene flow via pollens within a population can be used to infer whether the species receives pollen from the same (selfing) or different individual (outcrossing) [3]. The molecular marker that can be applied for this approach is microsatellite marker. Microsatellite and Single Nucleotide Amplified Polymorphism (SNAP) have been implemented for identifying pollen dispersal in coconuts [4] and ebony [5].
Information on mating system via genetic approach is crucial to support tree breeding and genetic conservation programs for Jabon merah. This study was aimed to determine pollination type of Jabon merah from three different provenances in South Sulawesi based on microsatellite markers.

2. Material and Method

2.1. Research location and plant material
Genetic materials were collected in three provenances of Jabon merah in South Sulawesi i.e. Luwu, Wajo, and Sidrap. Molecular analyses were carried out in Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Hasanuddin University, Indonesia.

2.2. Male and female Parents array and sample collection
As many as 75 leaf samples were collected in three Jabon merahs’ populations that consisted of 24 samples from Luwu district, 24 samples from Wajo district, and 27 samples from Sidrap district, respectively. Number of samples from each location were assigned as candidate male and female parents, i.e. 7 candidate female and 17 candidate male parents from Luwu, 9 candidate female and 15 candidate male parents from Wajo, and 13 candidate female and 14 candidate male parents from Sidrap, respectively.

The female parents were selected based on their progeny number, meanwhile, the adult trees surrounding the selected female parents were assigned as candidate male parents (pollen donor). Tree samples were labeled, and GPS coordinates were then recorded using Garmin 62S for mapping the research locations. The number of total samples was 278. Details of sample (tree) number from each location in South Sulawesi are presented in table 1.

Table 1. Locations of sample collection of Jabon merah in South Sulawesi.

| No. | Location (village) | Subdistrict | District | Number of trees | Number of progeny | Total |
|-----|-------------------|-------------|----------|-----------------|------------------|-------|
| 1.  | Tampumia village  | Bupon       | Luwu     | 24              | 49               | 73    |
| 2.  | Tangkoro village  | Pitumpanua  | Wajo     | 24              | 63               | 87    |
| 3.  | Bellawae village  | Pituriase   | Sidrap   | 27              | 91               | 118   |
|     |                   |             |          | 75              | 203              | 278   |

2.3. DNA analysis
DNA isolation was done using CTAB protocol as described by [6] and modified by [7]. Primer screening was performed using cross amplification method by selecting primer pairs on National Center for Biotechnology Information (NCBI) and then screening the primers with isolated DNA. The primers that produced clear bands would be selected as specific primer for Jabon merah [8]. The primer pairs that used in the screening are described in Table 2.

The specific primer pairs acquired in the screening process were then used to amplify all DNA samples. PCR amplification was carried out using 2 µl of DNA working 1.25 µl of each primer pair (0,625 µl of Forward and 0,625 µl of Reverse primers), 6,25 µl of PCR mix (KAPA Biosystem), and 3 µl of ddH2O for each reaction. The amplification was performed by the touchdown-PCR Sensoquest Thermocycler (Germany) with 12.5 µl of total volume.

The PCR amplification was conducted in 35 cycles under the following steps: one cycle of initial denaturation at 95 °C for 180 seconds and followed by 35 cycles of template denaturation at 95 °C for 15 seconds, primer annealing at each specific primer annealing temperature for 50 seconds, primer elongation at 72 °C for 60 seconds, and final extension at 72 °C for 600 seconds as recommended by KAPA Biosystem kit.

PCR products were separated on 3% of SFR agarose gel [9] using 0.5x TAE buffer at 100V for 1 hour [10] and stained by gel red. The gels were then visualized by UV transilluminator and documented using digital camera. Scoring was done for each sample.
### Table 2. Primer of Jabon Merah for primer screening

| No. | Primer | Accession No. | Repeat Motif | Primer (5’-3’) |
|-----|--------|---------------|--------------|---------------|
| 1.  | M302   | AM408775      | (GT)<sup>9</sup> | F: CAAAAGTAAATAAACGATGGACGA  
                   R: AAGAGGTAAAAATCAAATCCCAAG |
| 2.  | M306   | AM408777      | (TG)<sup>9</sup> (AG)<sup>7</sup> | F: AAGCAACATTTCACCAGTCAA  
                   R: GACGGGAAATTTCCTTTGATTC |
| 3.  | M309   | AM408738      | (GT)<sup>9</sup> | F: CGATTTGGTTTGGCATACTATCT  
                   R: GCCAGATATAGTTGCTTGC |
| 4.  | M321   | AM408748      | (GT)<sup>8</sup> | F: ATTACTCTGCTCGTGGAC  
                   R: CATCAGATGATGGAGAG |
| 5.  | M325   | AM408751      | (A)<sup>5</sup>GGGC(A)<sub>6</sub> | F: CACCTTTTGAGTTTTGAGTTGG  
                   R: AAAATAAACCCCTTCGTCG |
| 6.  | M326   | AM408752      | (GA)<sup>8</sup> | F: GGCTAAAATCCACTGTCACAC  
                   R: CTAGGATCGTGGCAGAAGAAG |
| 7.  | M327   | AM231546      | (GT)<sup>8</sup> | F: CTGGTTTTGAGTTTTGAGTTGG  
                   R: GACGGGAAATTTCCTTTGATTC |
| 8.  | M328a  | AM408753      | (AT)<sup>6</sup>(GT)<sup>8</sup> | F: AACGGGTGGTCTCATTTTATC  
                   R: TTTCTTGTAGTGGTTTTGTCTCC |
| 9.  | M329   | AM231547      | (GT)<sup>9</sup> | F: CACCTTTTGAGTTTTGAGTTGG  
                   R: GACGGGAAATTTCCTTTGATTC |
| 10. | M333   | AM408757      | (AT)<sup>6</sup>(GT)<sup>8</sup> | F: AACGGGTGGTCTCATTTTATC  
                   R: TTTCTTGTAGTGGTTTTGTCTCC |

### 2.4. Data analysis

#### 2.4.1 Identification of candidate male parent
Each progeny has a known female parent and unknown male parent (pollen donor). Candidate male parent could be one of the female parents in the adult tree population. This step was conducted to predict pollen donor among the candidate male parents for every progeny.

Identification of the pollen donor was performed by analyzing genotype of the progeny versus all candidate male parents. All candidate male and female parents were evaluated, considering they could potentially be the pollen donors. Molecular analysis was done using Parentage Analysis CERVUS ver 2.0 software. The outputs of the analysis presented frequency of the alleles, Polymorphic Information Content (PIC), heterozygosity, and homozygosity. The analysis was then followed by simulation and parentage analysis. By doing Parentage analysis, candidate male parents would be obtained with marks of “*” for 95% of confidence level, “+” for 80% of confidence level, and “-” for <80% of confidence level, respectively [4].

#### 2.4.2 Analysis of distance and pollen dispersal pattern
The locations of female and male parents from Cervus analysis were plotted using Garmin Map Source GPS ver. 76C5x. The distance between male and female parents was measured by the same software. Position and distance of both female and male parents were then used to illustrate pollen dispersal pattern in the research location. Selfing pollination is if the identified male parent is the same individual as the female parent, and otherwise called as outcrossing [7].

### 3. Results

#### 3.1. Primer screening
Primer screening on 10 SSR primer pairs showed 3 primer pairs were able to be amplified clearly and produced polymorphic bands (M306, M309, and M326). M321 could also generate bands, but monomorphic. Whilst, M302, M325, M327, M328a, M329, and M333 produced unclear bands, and
consequently, they could not be used in the analyses. The visualization of primer screening results is exhibited in figure 1.

![Figure 1. SSR primer screening on Jabon merah using M302, M309, and M321](image)

M306, M309, and M326 were suitable to be utilized as markers for analyzing mating system and pollen dispersal pattern on Jabon merah because the primers were capable to amplify DNA samples and produced clear and polymorphic bands, and thus the scoring process would be done easily. Those primers had PIC ranged from 0.74 to 0.87 for all provenances.

### 3.2. Mating system in Luwu provenance

Parentage analysis resulted 21 samples (42.86%) with “+” mark, 13 samples (26.53%) with “*”, and 15 samples (30.61%) with “-”, respectively. All LOD values were positive. The parentage analysis on 49 evaluated progenies from Luwu showed 14 progenies (28.6%) and 35 progenies (71.4%) were from selfing and outcrossing pollinations, respectively. One of pollen dispersal patterns which had been done by male and female parents is illustrated in figure 2.

Seven progenies from female parent L11 received pollen from 2 different male parents, L15 and L19. Pollination distance from male parent to female parents and frequency of pollen donors in Jabon merah population in Luwu are presented in figure 3.

Figure 3 illustrates that L11 received pollen from L15 for 5 times that located at 255 m apart, meanwhile L19 were pollinated for 2 times at 34 m apart. L1 only received pollen from L9 for 7 times at 95 m apart. L8 and L10 were not pollinated by other pollen donors, but from its own pollen (selfing) for 7 times, respectively.

![Figure 2. Pollen dispersal pattern of Jabon Merah L15 and L19 in Luwu provenance](image)
The farthest distance of pollen travel in Luwu was 255 m. L8 as pollen donor donated pollen with the highest frequency (14 times), 7 times at 0 m (selfing) and 7 times at 140 m to L3 (outcrossing).

3.3. Mating System in Wajo provenance

Parentage analysis showed that numbers of samples with mark “+”, “*”, and “-” were 17 samples (26.98%), 24 samples (38.10%), and 22 samples (34.92%), respectively. The observed LOD value in this provenance was negative. The parentage analysis for 63 evaluated progenies presented 18 progenies (28.6%) derived from selfing and 45 (71.4%) progenies from outcrossing. The pollen dispersal pattern in Wajo provenance is depicted in figure 4.

Seven progenies belong to female parent W6 received pollens from W2 (4 times) and W9 (3 times). Pollination distance from male to female parents and frequency of pollen donors are displayed in figure 5.

Figure 5 shows W6 was pollinated by W2 for 4 times at 86 m and W9 for 3 times at 125 m. W9 only received pollen from W3 for 7 times at 209 m apart. W1 and W4 were selfing for 7 times, respectively. W3 was outcross-pollinated by W4 for twice at 52 m and self-pollinated for 5 times.

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**Figure 3.** Diagram of pollination distance and frequency of pollen donors in Luwu provenance

**Figure 4.** Patterns of pollen dispersal Jabon Merah on W2 and W9 in Wajo Provenance
Figure 5. Pollination distance and frequency of pollen donors in Wajo provenance

The farthest pollen travel from male to female parents in Wajo was 209 m and the highest frequency of pollen donors was done by W9 (24 times). W9 pollinated W2, W5, W6, and W7 for 7 times, 7 times, 3 times, and 7 times, respectively, at the range of 109 to 161 m apart.

3.4. Mating System in Sidrap provenance
Parentage analysis presented 18 samples (19.78%) with “+”, 43 samples (47.25%) with “*”, and 30 samples (32.97%) with “-“. LOD value was positive. The analysis also showed the 91 evaluated progenies consisted of 11 progenies from selfing (12.1%) and 80 progenies from outcrossing (87.9%). The pollen dispersal pattern in Sidrap provenance is presented in figure 6.

Figure 6. Patterns of pollen dispersal Jabon Merah on S1, S4, S5, and S6 in Sidrap provenance

Seven evaluated progenies from female parent S12 were pollinated by S1, S4, S5, and S6 and the distances from S12 to the pollen donors, respectively, were 164 m, 161 m, 99 m, and 108 m. S1 donated pollens for 4 times, whilst S4, S5, and S6 were once, respectively. The pollination distance and frequency of pollen donors from male to female parents are presented in figure 7.
Figure 7. Pollination distance and frequency of pollen donors in Sidrap provenance

Figure 7 shows female parent S8 received pollen from six different individuals (outcrossing) and was pollinated once by S1, S4, S5, S6, and S7 and twice by S10. The distances from S8 to S1, S4, S5, S6, S7, and S10, respectively, were 181 m, 131 m, 114 m, 157 m, 84 m, and 109 m. S10 experienced the selfing pollinations for 7 times. Whereas, S4 was self-pollinated 4 times and outcrossing-pollinated by S6 for 3 times at 84 m.

The farthest pollen travel in this provenance was 339 m and the highest frequency of pollen donors was 18 times by S4 and S10, respectively. S4 donated pollen to 7 different female parents (S2, S8, S9, S11, S12, S13, and S14), respectively, for 7, 1, 3, 2, 1, 3, and 1 times at 52 m, 131 m, 167 m, 105 m, 161 m, 151 m, and 206 m.

4. Discussion

4.1 Primer screening and Polymorphism analysis

Primer screening is applied to determine primer pairs that are able to produce bands, both clear and polymorphic bands. Polymorphism of the alleles is considerably affected by primer screening process because each primer pair has specific annealing sites in the term of numbers of bases and identified alleles.

The variations in amplified DNA fragments (DNA polymorphisms) are caused by distributions of bases in the genome where the primers attached. The differences between profile of amplified DNA bands (both number and size) extremely influence level of genetic diversity in the population. Setting the annealing temperature in the PCR amplification procedure is a vital step and thus 1-degree temperature change causes failure in primers attachment [11].

Genetic identification via molecular approach is required in plant breeding program in order to obtain superior genotypes. Molecular marker becomes a tool to overcome issues in conventional breeding. An ideal molecular marker must be evenly distributed in the genome, has high up to intermediate level of polymorphism, and requires less tissue and DNA samples [12].

The primers evaluated in the analysis had 0.80 of PIC mean. It explained that M306, M309, and M326 showed high informative in mating system analysis [13] in [14] stated PIC value is divided into 3 classes, high informative (PIC>0.5), moderate informative (0.25<PIC<0.5), and low informative (PIC<0.25).

4.2 Mating System in Jabon merah

Parentage analysis provides candidate male parents with 3 different levels of confidence (+, *, and -). Even though the candidate male parents with “-” mark possess <80% of confidence level, yet the results are still valid if LOD value is positive. The higher LOD value, the higher the possibility of a candidate
male parent to be the pollen donor [15]. All the candidate male parents in Luwu and Sidrap showed positive LOD, whereas that of in Wajo was negative. Those results revealed that all candidate male parents in Luwu and Sidrap were the true pollen donors for the evaluated progenies, whilst negative LOD observed in Wajo indicated the identified pollen donors from the analysis could not completely presume to be the male parents.

Most of the evaluated progenies were derived from outcrossing (76.9%) and only 23.1% from selfing. If the pollen donor has the same individual as the female parent, the pollination is defined as selfing, and vice versa as outcrossing [7]. Outcrossing produces high genetic diversity if the pollination is performed by the same species. The progeny formed by this pollination commonly has mixed traits depending on the dominance of the traits owned by both parents that inherited randomly [16].

4.3 Pollen dispersal pattern

Pollen dispersal patterns illustrated in Figure 3, 5, and 7 show the farthest pollens travel from male to female parents were 339 m for 6 times. The highest frequency of pollen donor was 24 times to female parents at 109 up to 161 m. The distances among evaluated trees affected frequency of pollination in the populations. The nearest pollination distance was ranged from 0 to 52 m and had the highest percentage of contributed pollens.

Pollen can be transferred from the male to female parents via wind due to the differences in altitude between donor and recipient trees that subsequently allowing pollen flies and pollinates trees in the lower areas.[17] stated the maximum distance of pollen travel in Oenocarpus bataua was 2363 m through insect pollinator. Insect has an important role in pollination as it can carry the pollen farther than by wind [18]. [5] proved that topography in Barru provenance caused pollens of Diospyros celebica could be flown by pollinators up to 269 m. Other studies on pollination reported [19] that investigated the existence of selfing pollination in Hymenaea courbaril, in contrast to the previous study [20]. They confirmed H. courbaril has self-incompatibility. Non-selective logging on H. Courbaril in forest areas strongly reduces the number of reproductive trees. This situation increases the percentage of selfing as the individual trees become isolated [18]. [21] reported in coconut plantation in Dukuh Seti, the distance of dispersal pollen range from 0 m to 54 m.

Conclusion

The findings deduce Jabon merah is likely to be outcrossing in each population, with the means of outcrossing and selfing were 76.9% and 23.1%, respectively. The pollen travel from male to female parents could reach up to 339 m and frequency of pollination was varied from 1 to 7 times. The distance and pollinator are the vital factors in pollination of a species.

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