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Estimation of Pollination in Mahogany Revealed by Microsatellite Markers : Case in South Sulawesi, Indonesia

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ABSTRACT

Research Highlights: Pollen dispersal can be used to design and manage seed orchards of Mahogany to fit the breeding demand. Pollen dispersal and mating system in mahogany can be elucidated through pollen dispersal pattern analysis based on genetic structure.

Background and Objective: Microsatellite is a co-dominant genetic marker that has been widely applied for DNA analysis. The objective is this work were to to determine specific primers for parentage analysis, to calculate the distance of pollens travel from donor pollen to female recipient, to estimate the frequency of pollination, and to evaluate the proportion of selfing and outcrossing in mahogany.

Materials and Methods: The field activity was done by collecting leaf samples at genetic resource area of Sulawesi BPTH, South Sulawesi, Indonesia. Genetic molecular analysis were conducted at Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Universitas Hasanuddin, Indonesia.

Results: The study indicated that the suitable primers for parentage analysis were sm05, sm18, and sm48. Pollens could travel up to 83 m apart from the evaluated female parents, and the highest frequency of pollination was 22 at 20 to 30 m. The proportion of outcrossing was 95% which indicated that mahogany trees in this evaluated area tend to outcross pollinate.

Conclusion: Proportion of crossing was 95% which indicated crossing pollination dominating the mahogany population in genetic resource area Gowa. SSR primers screening generating polymorphic bands were sm03, sm18, and sm48. Highly variable microsatellite markers have facilitated a direct genetic approach to measuring gene flow based on parentage analysis.

Keywords: Gene flow; Mahogany; Mating system; Microsatellite; Pollen dispersal.

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INTRODUCTION

Mahogany is a protective tree mainly found as pedestrian wayside tree species. As protective tree, it performs heat resistant and high level of adaptability to different soil conditions. This species also has high economic value since the wood is quite hard, hefty, having a unique color and suitable for furniture and woodcraft. Mahogany is the most valuable hardwood species, and its conservation status has been the subject of increasing concern due to over-exploitation and habitat destruction [1]. Research about level carbon stock in mahogany investigated that how stand density would influence stem volume allometric equations as well as carbon stocks per unit area. This work also has implications on stand density management and the use of allometric equations in the estimation of stem volume and carbon stocks on mahogany [2].

Mahogany has monoecious inflorescence (having male and female reproductive organs together in an individual) which can increase the possibility of selfing in the species and may lead to inbreeding depression. If the level of inbreeding depression is high, genetic diversity will be low or reduce character value. [3] previously reported that genetic diversity of six mahogany provenances in Gowa, South Sulawesi, Indonesia, was high compared to other tree species and tended to decrease homozygosity in the species.

The mating system can be determined using pollen dispersal analysis. Gen flow via pollen in a population is used to predict whether the pollination is selfing or outcrossing [4]. Pollen dispersal pattern can be analyzed based on genetic structure of the plant in the population [5]. One of the molecular marker methods that have been widely applied for DNA analysis is microsatellite marker, also known as *Simple Sequence Repeat* (SSR) marker. SSR is DNA marker having simple sequences that consist of one to six repeat bases and abundant in plant genome [6]. Due to having high polymorphic level, SSR marker can distinguish individuals that have close relationship [7] and know xenia effect in kopyor nut yield [8].

Gene flow and mating system through pollen dispersal analysis using SSR marker has been applied in coconuts [9] and ebony [10]. Even though they are essential for supporting breeding and conservation strategies but they have not been investigated in mahogany. The study was aimed to determine specific primers for parentage analysis, calculate the distance of pollens travel from donor pollen to female recipient, estimate the frequency of pollination, and evaluate the proportion of selfing and outcrossing in mahogany.

MATERIAL AND METHOD

Sampling

Field activities were conducted (samples collection) at Sulawesi BPTH, Bittolo village, Gowa district, South Sulawesi, Indonesia (Figure 1). Molecular analysis was performed at Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Hasanuddin University, Makassar, Indonesia.

Trees surrounding the female parent trees (female recipients) were selected as male parent trees (pollen donors). Eighteen female parents and 66 progenies were assessed in the study. Female parents having seedlings grown under their canopy

(no more than 2 m from the tree trunk) were collected as many as three to five seedlings. Five leaves from each selected tree (parents and progeny trees) were cut and then used as DNA sample source.

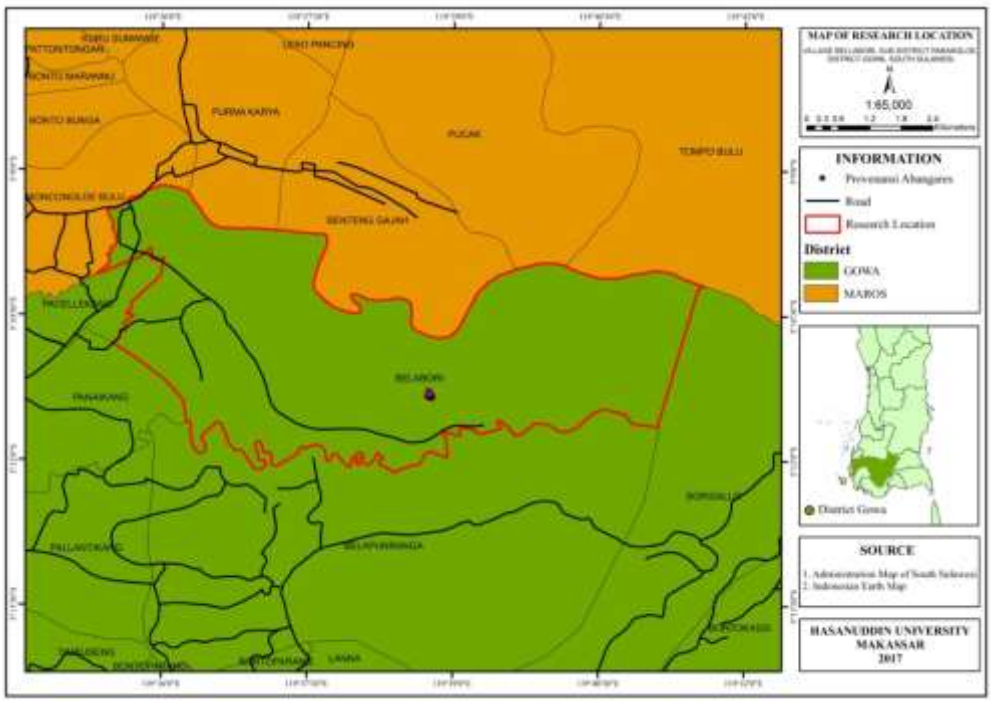

Figure 1: Map of research location at genetic resource area in Gowa district, South Sulawesi, Indonesia.

Microsatellite Analysis

DNA isolation was conducted using the CTAB method [11] with modification [12]. Young leaves of each sample were weighted at 200-300 mg without their costa and later used as DNA samples. The samples were crushed and added 500 µl of CTAB buffer and then placed into 2 ml of tube. The tubes were incubated in 65 °C water bath for 90 min. The incubated samples were added 100 µl of chloroform:isoamyl-alcohol and centrifuged at 11.000 rpm for 10 min. Supernatant was then transferred to a new microtube and added 800 µl of isopropanol. The supernatant was centrifuged at 1.000 rpm for 5 min and then removed. DNA pellet was dried for one night.

The DNA pellet was added 500 µl of TE buffer and centrifuged at 10.000 rpm for 10 min. The solution was transferred into new microtube and added 100 µl of chloroform and 800 µl of isopropanol. The tube was centrifuged at 10.000 rpm for 10 min. The solution was then removed and dried for one night. As much as 100 µl of ddH2O was added into dried tube and centrifuged at 10.000 rpm for 1 min. The solution contained extracted DNA was added 4 µl of RNase and centrifuged at 10.000 rpm for 1 min. The solution was then stored at –20 °C in the freezer.

Primer screening was done using ten SSR primers Table 1 of *Swietenia macrophylla* [13]. It was aimed to determine the primers that obtained polymorphic and clear bands. The selected primers were then used in pollen dispersal pattern analysis. The process was conducted by amplifying twelve randomly selected DNA samples. In this process, the gradient temperature was performed using ± 5 °C from annealing temperature written on primer label. It was done to get suitable annealing temperature of each primer that can obtain clear bands. The primers are described in Table 2.

Table 1: SSR primers used for primer screening

NO	Locus	Motif Repeat	Nucleotida Sequence	Temperature melting (°C)	Allele size (bp)
1	sm05	(AG) 17 GG(AG) 6	F:GCATGAGCTTGAGAGAATC R:CAGAGGACTGAAGTAGCTGA	60	240 – 262
2	sm12	(AG) 12 TT(AG) 7	F :AGAGTGTTCGAGAGCCTCAA R : AGAGCCGAATTCACCGAT	56	196-224
3	sm18	(AG) 19	F: CTGTCATGCATATCGTTGGA R:GGGCAGATAAAGAGGAACAAG	56	196 – 232
4	sm43	(CT) 18	F: TAGGAACCAACCACCAAC R: GTTCTCCTGCTCTCTTTGA	56	210 – 238

5	sm48	(AG) 20	F:TCAGGAATGGAAGGTACAGG R:CAGTCATGGAGCGTAGCTAA	56	264 – 310
6	sm49	(AG) 19	F:GAACTGGCAATGTGCTGACT R:TCGGCAATAGCAAGACATTC	64	136 – 174
7	sm01	(AG) 19	F: GCGCGATTGATTGACTTC R: GCGCTTAGCATTATTCTCC	56	258 – 280
8	sm40	(AG) 19	F: TGTACTGTCAAGAGTGTAT R: GACAAACATGTACCACAAG	55	120-146
9	sm46	(AG) 20	F: GCAGTACTCGCCTATCTTCA R: TGAGAACTGCAGAATCCTTT	56	190-226
10	sm51	(AG) 22	F: GCAATTTCCAGAAGAAACC R: CTGTAGGCGATAACAATCAG	55	138-182

Table 2: SSR primers used for progeny analysis

No	Locus	Repeat Motif	Nucleotida Sequence (5'-3')	Temperature Annealing (^{0}C)	Allele size (bp)
1	Sm05	(AG) 17 GG(AG) 6	F: GCATGAGCTTGAGAGAATC R: CAGAGGACTGAAGTAGCTGA	56.5	200-300
2	Sm18	(AG) 19	F: CTGTCATGCATATCGTTGGA R: GGGCAGATAAAGAGGAACAAG	57.4	150-300
3	Sm43	(CT) 18	F: TAGGAACCAACCACCAAC R: GTTCTCCTGCTCTCTTTGA	56.3	200-300

DNA amplification was performed using the following steps: one cycle of pre-denaturation at 95 °C for 3 min, and 35 cycles of denaturation at 95 °C for 30 s, primer annealing (annealing temperature) for 50 s, primer extension at 72 °C for 60 s, and one cycle of final extension at 72 °C for 5 min.

Products of DNA amplification were separated using horizontal electrophoresis. Electrophoresis process was performed using 3% SFR agar and 1x TAE buffer at 100 V for 90 min [14]. The SFR agar was then visualized using UV transilluminator and documented by a digital camera. Data were scored based on the band size of the evaluated samples.

Identification of Candidate Male Parent

Each progeny has known female parent and unknown donor pollen (male parent). The candidate male parent could be one of the female parents in the population. This analysis was aimed to predict the donor pollen for each progeny among all evaluated candidate male parents.

The identification of donor pollen was done by analyzing the genotype of all progenies and candidate male parents. Parentage analysis was performed using Cervus 2.0 software [15]. The outputs of Cervus were frequency of the allele, polymorphic information content (PIC), heterozygosity, and homozygosity. The simulation was conducted to determine the male parents with confidence interval of 95% (*), 80% (+), and <80% (-) [9].

Analysis of Pollen Dispersal Pattern

Locations of the selected female and male parents from Cervus 3.03 analysis were plotted using Quantum GIS software. The distance and position of both female and male parents were then used for illustrating the pollen dispersal pattern in this research site. Selfing was defined if the pollen donor was the same as the female parent. Otherwise, they were assigned as outcrossing [9].

RESULT AND DISCUSSION

Primer Screening

Primer screening using ten SSR primer pairs showed there were three primer pairs, sm05, sm18, and sm43, that could be used for parentage analysis (Table 2). Figure 2 presents the electropherogram of DNA amplification products using sm05, sm12, and sm18 primers. Bands produced by these primers were clear and polymorphic so that they could distinguish between the individuals. It also exhibited different size of alleles generated by the primers. Primer sm12 amplified monomorphic bands that were shown on their uniform band size. This kind of primer could not be used in further analysis.

Electropherogram of PCR products generated by sm43, sm48, and sm49 primers is presented in figure 3. The figure shows that primer sm43 amplified polymorphic and clear bands, whereas primer sm43 did not obtain any DNA band. Primer sm49 only amplified three out of twelve evaluated DNA samples. It assumed that even though we used primers developed from mahogany, it was not guaranteed that they could amplify other mahogany DNA samples.

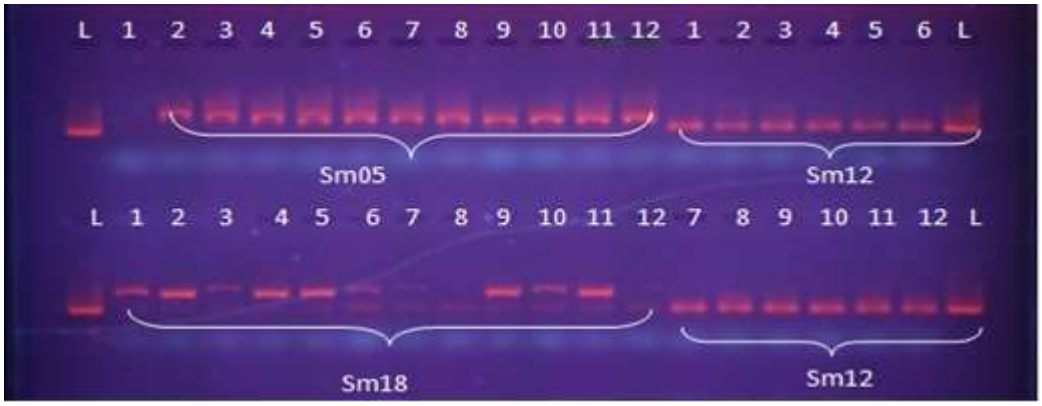

Figure 2: Electropherogram of PCR products. Note: 1-12 = DNA bands, L = marker 100 bp.

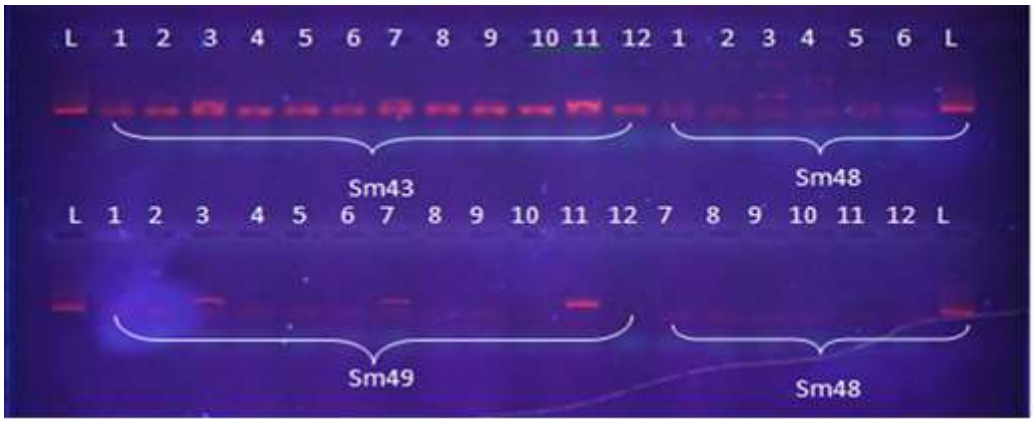

Figure 3: Electropherogram of PCR amplification products. Note: 1-12 = DNA band, L = marker 100 bp.

Genotyping parents and progeny

Ninety-nine mahogany trees were analyzed, and 18 trees were selected as female parens as well as candidate male parents in parentage analysis. Based on primer screening, there were only three primers that could be used in pollen dispersal analysis. The visualization of PCR products using primer sm43 is shown in figure 4.

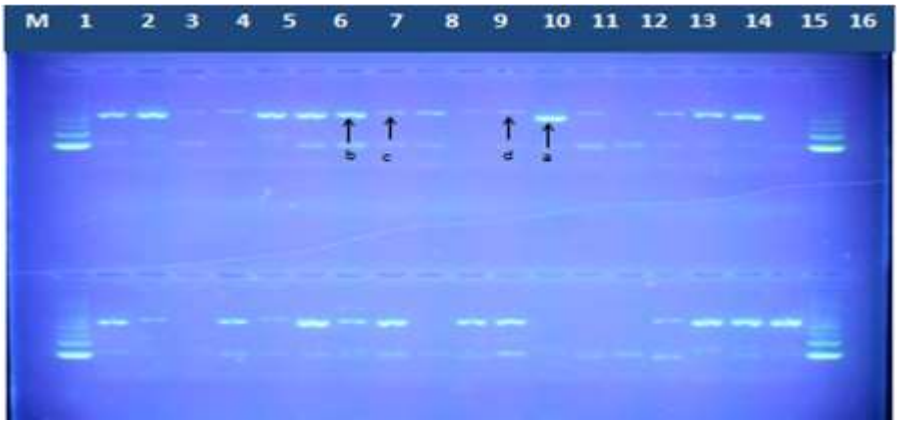

Figure 4: Visualization of PCR products using primer sm43. Note: M = DNA marker 100bp, 1-18 = DNA sample. Arrows indicated variation of the alleles, (a-d) allele.

The amplified DNA using three selected primers derived four to five alleles for each primer, and the mean of alleles number was 4.7. Primer sm43 generated four alleles, whilst primer sm05 and primer sm18 showed five alleles, respectively. The number of allele, PIC, number of heterozygous and homozygous individual, and heterozygosity are presented in table 3.

Table 3: Number of allele and individual, number of heterozygous and homozygous individual, observed heterozygosity (Ho), expected heterozygosity (He), and polymorphic information content (PIC) using three SSR markers on mahogany population.

Locus Name	Number of Individual	Number of Alleles	Number				
			Heterozygous Individual	Homozygous Individual	Ho	He	PIC
Sm05	162	5	0	141	0	0,658	0,591
Sm18	162	5	0	149	0	0,782	0,743
Sm43	162	4	0	144	0	0,373	0,343
Average	162	5	0	145	0	0,604	0,559

Identification of Candidate Male Parent

Parentage analysis detected the success rate of confidence level was lower than 80% with positive Likelihood of odds (LOD). Eighteen individuals having three to five progenies were selected as female parents (Figure 5). By identifying the male parents, we determined how many time male parent pollinate the female recipients. The number of pollination and the pollination distance are presented in figure 6 and 7.

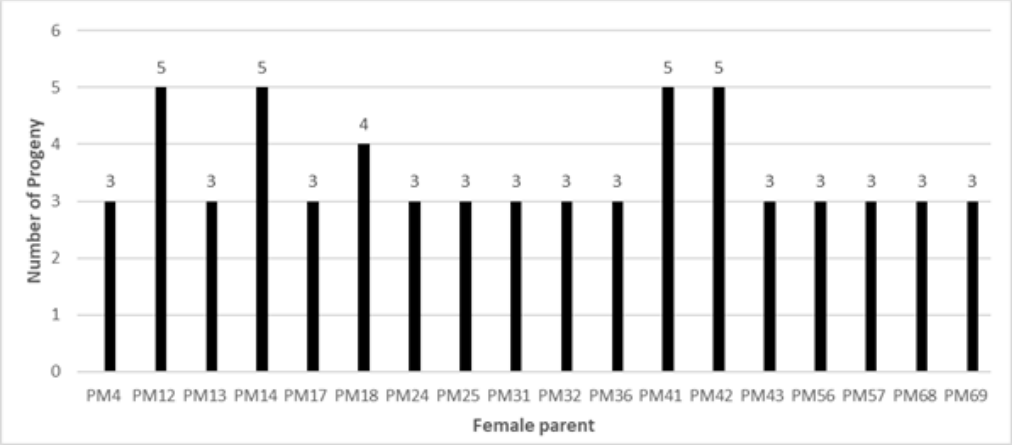

Figure 5: Number of progeny collected from each female parent in mahogany population at Genetic Resources Area of BPTH in Gowa.

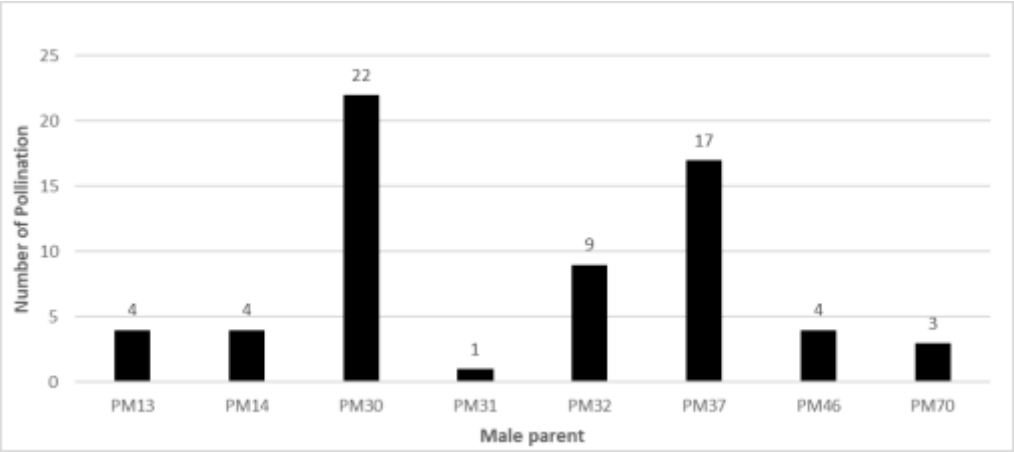

Figure 6: Number of pollinations conducted by male parent over female parent in mahogany population at Genetic Resources Area of BPTH in Gowa.

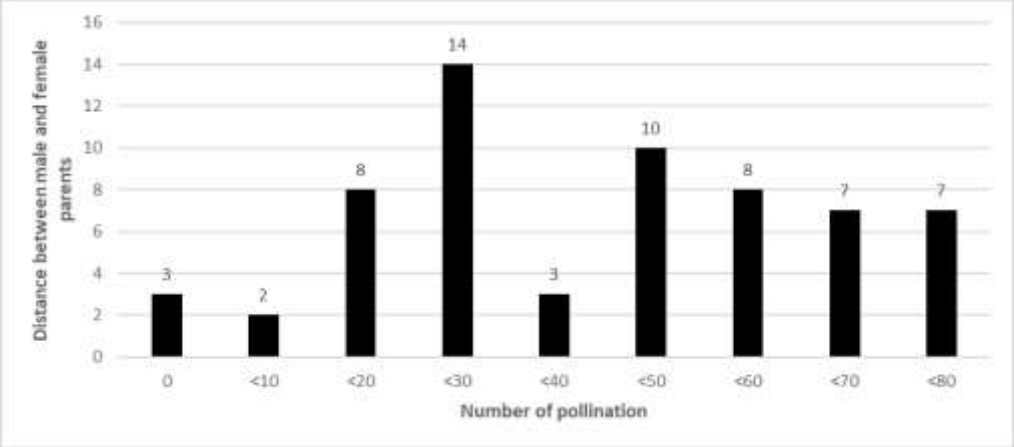

Figure 7: Number of pollination occurred at certain distances between male and female parents in Mahogany at genetic resource area of BPTH in Gowa.

Pattern of pollen dispersal

The furthest distance from male parent to female parent is displayed in figure 8, and the optimum distance is presented in figure 9. The furthest distance of pollination observed was 83 m (PM30 male parent to PM12 female parent), whilst the nearest one was 0 m (self-pollination). The distance between male parents and female parent were ranged from 20 up to 30 m (Figure 9).

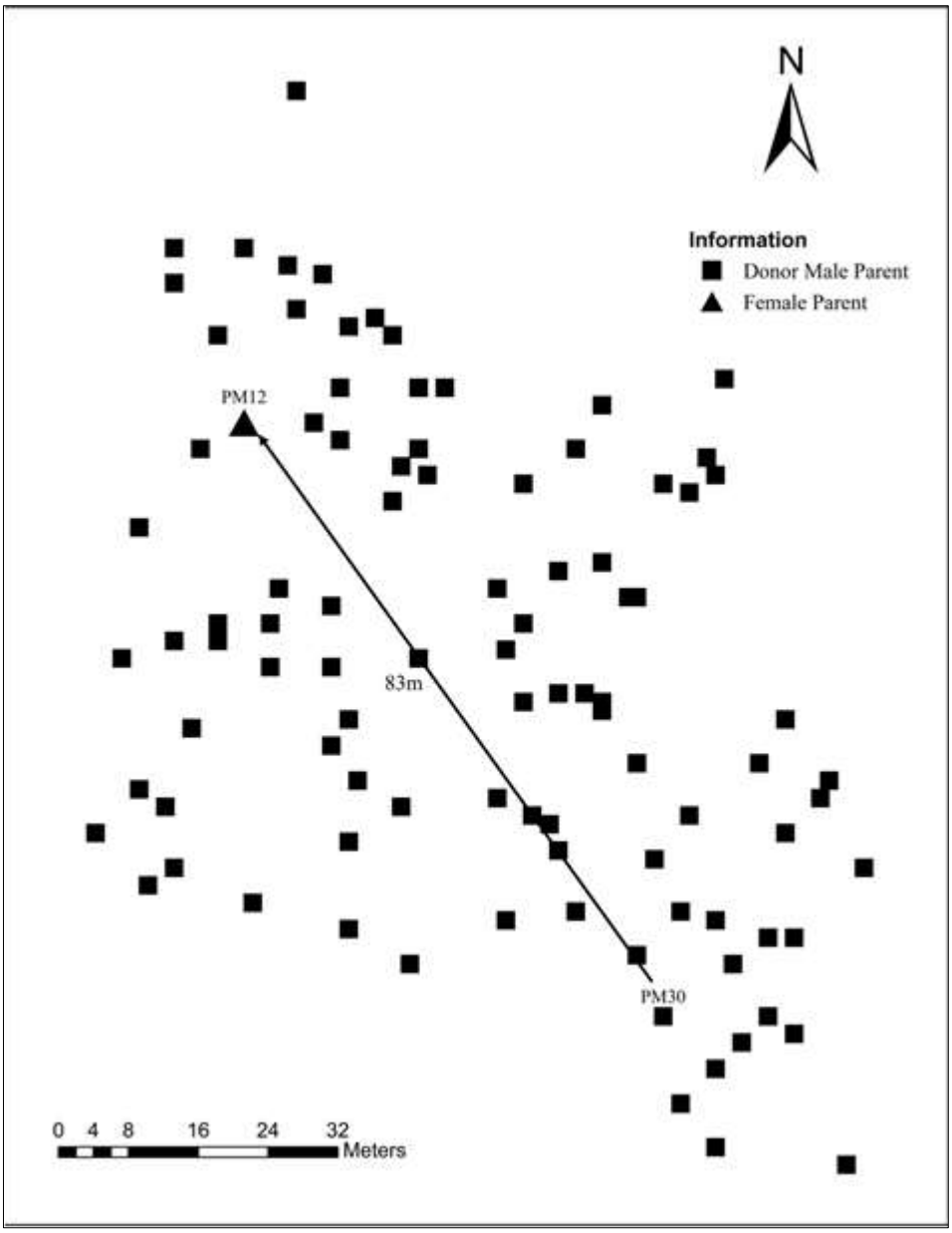

Figure 8: The furthest distance of pollination observed in mahogany population at genetic resource area of BPTH.

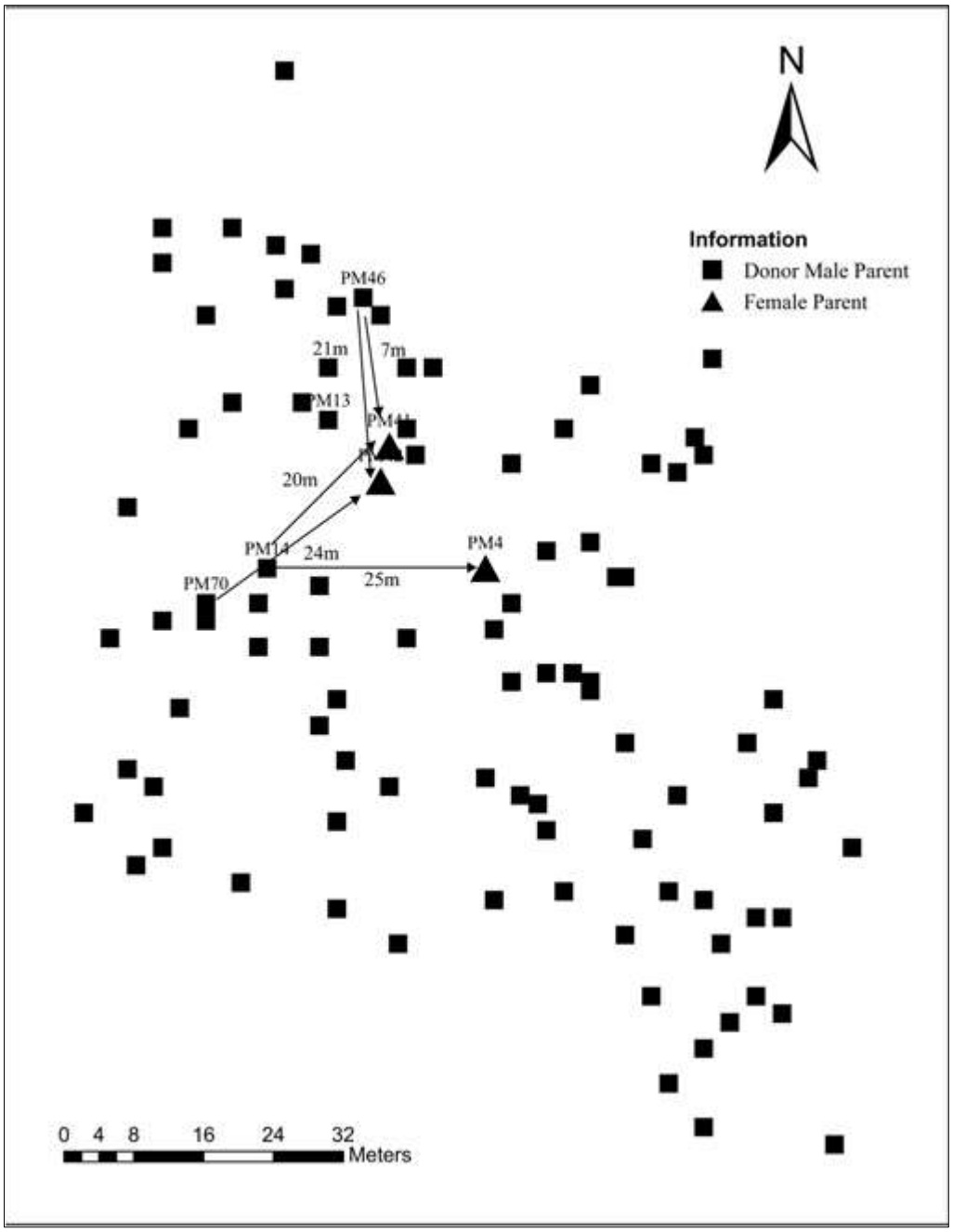

Figure 9: The optimum distance of pollination observed in the mahogany population at genetic resource area of BPTH.

PM30 male parent had the highest pollination frequency (22 times) to 14 different female parents, and one female parent could be pollinated up to 3 times. PM14 and PM12 were female parents that were pollinated the most by PM30 male parent, 3 times, respectively (figure 10). On the other hand, PM31 was male parent having the lowest pollination frequency (1 time) to PM42 female parent (figure 11). Parentage analysis indicated that selfing and outcrossing pollinations were occurred three times (5%) and 60 times (95%), respectively. The recapitulation of pollinations is shown in Table 4.

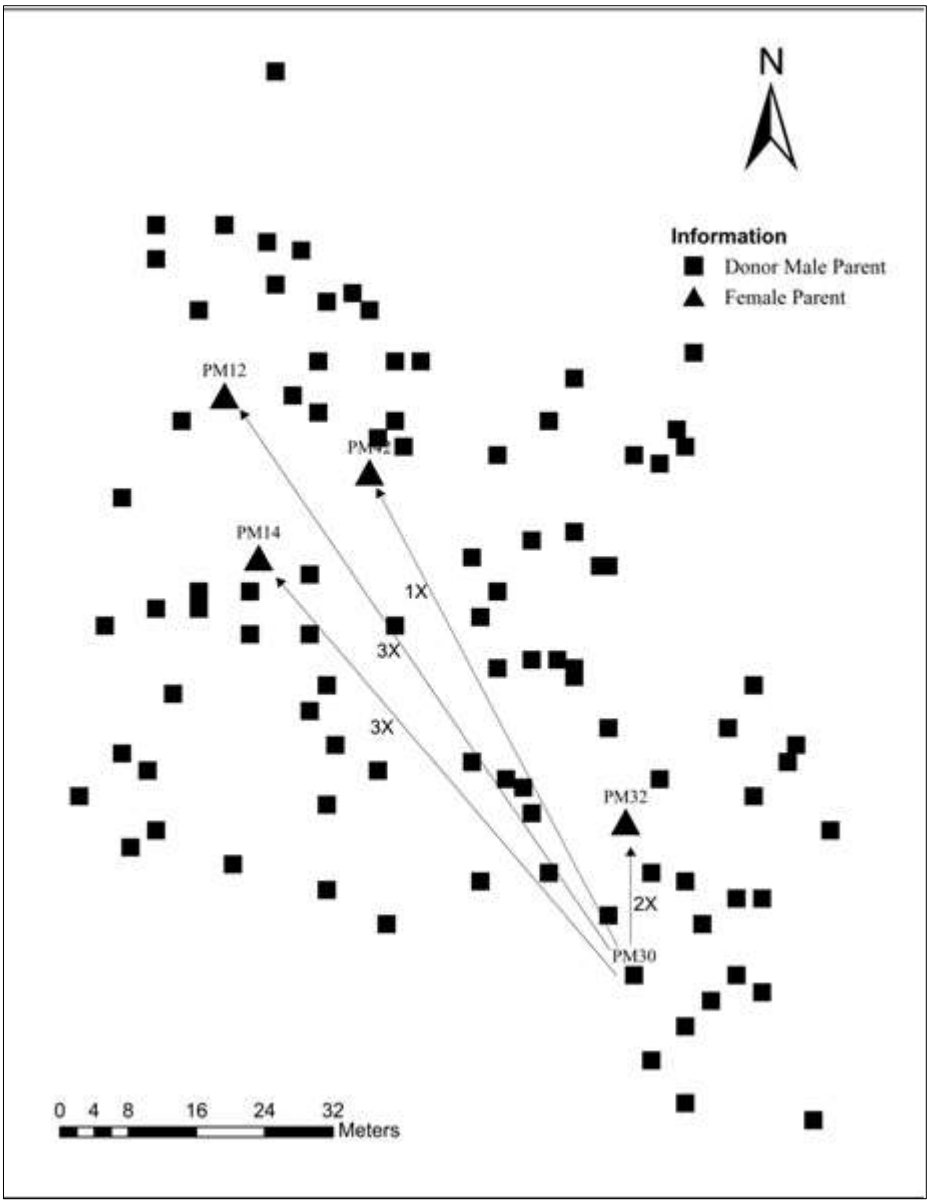

Figure 10: Pollination frequency of PM30 male parent to several female parents in mahogany population at genetic resources area of BPTH.

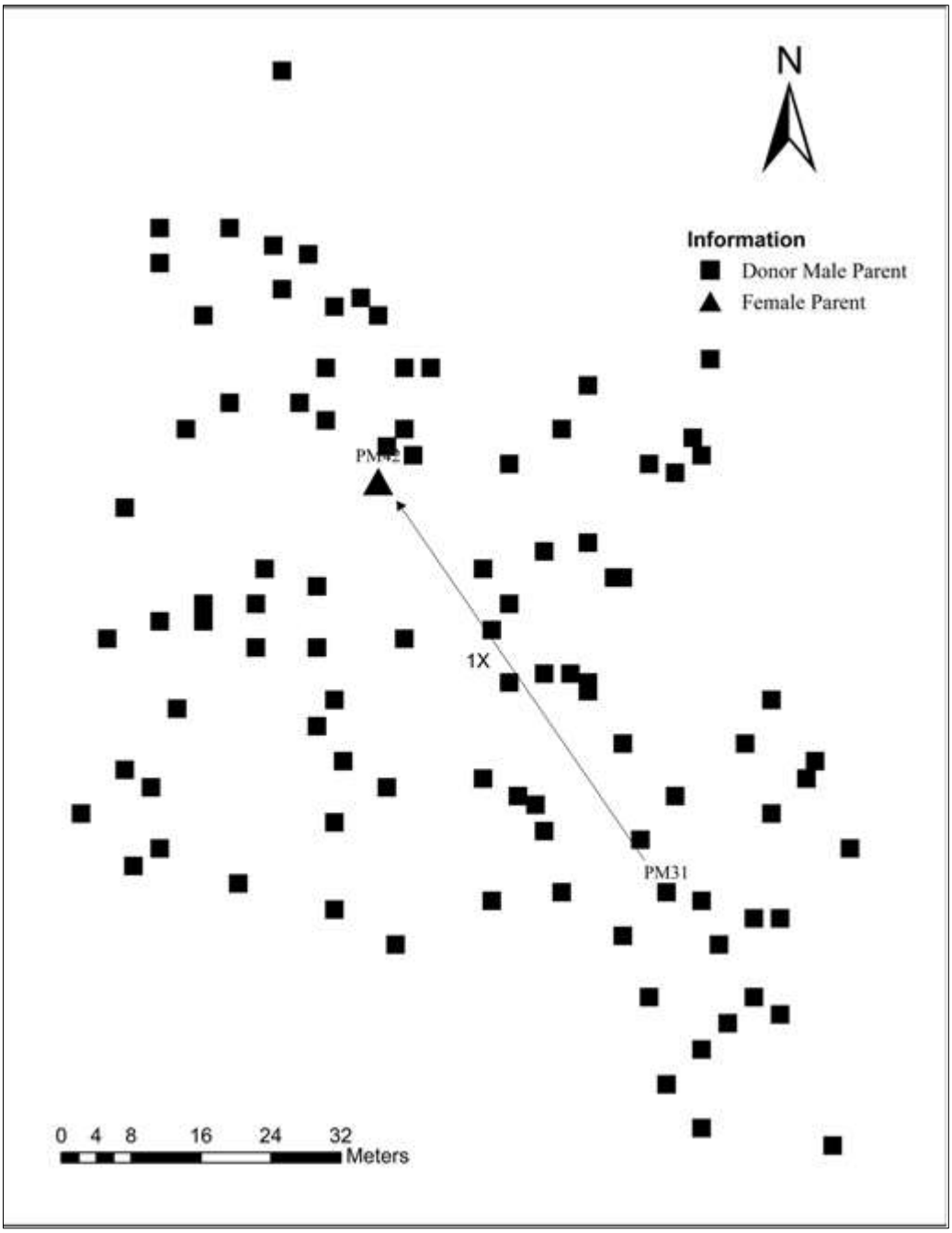

Figure 11: The lowest pollination frequency occurred between PM31 male parent and PM42 female parent in mahogany population at genetic resources area of BPTH.

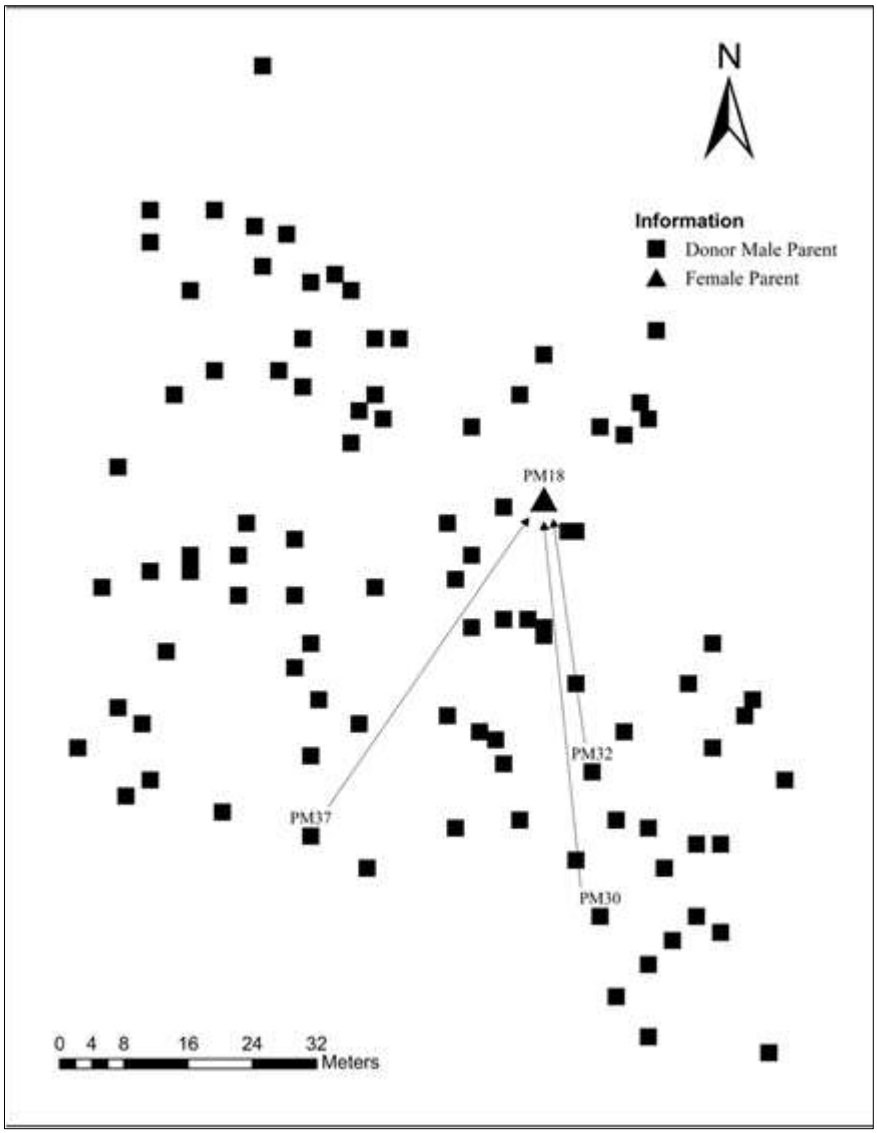

Figure 12: Cross-pollination occurred in PM18 female parent with three different male parent in mahogany population at Genetic Resource Area of BPTH in Gowa.

Figure 13: Selfing pollination of PM13 that occurred three times in mahogany population at genetic resources area of BPTH in Gowa.

Table 4: Recapitulation of pollination and pollination type based on parentage analysis in mahogany population.

Scheme of pollination	Pollination type	Number of pollination	Percentage
Mahogany	Selfing	3	5%
Mahogany	Outcrossing	60	95%
Total		63	100%

DISCUSSION

sm05, sm18, and sm43 molecular marker were selected because they produced polymorphic and clear bands. Study on selected primer in ebony [16], cocoa [17] , Katokkon pepper [18] showed selected markers had clear bands to continous genetic diversity study. The PIC mean for these primers was 0.55. It was higher than previously reported by [19] on Ebony populations using SSR markers (0.47). The PIC measures the effectiveness of the primers used for parentage analysis. It was also supported by [9] that stated SSR marker is very effective for analyzing the mating system and predicting the distance of pollen dispersal on tree species. We convinced that they were definitely the candidate male parents, even though they had a low confidence level. LOD was more than 0 and positive. Positive LOD indicated that all candidate male parents were the right pollen donors. The higher LOD is detected, the higher possibility of candidate male parent becomes pollen donor [20]. Paternity assignment was used to calculate the realised outcrossing

rate,multiple paternity per capsule and per tree and realised pollen dispersal distances [21].

Distance, frequency, and proportion of pollination could be determined using pollen dispersal pattern analysis. Pollination distance evaluated the distance from male parent to female parent, and the number of pollination occurred in the population. The pollination frequency determines which male parent that pollinated the most female parent. Moreover, it also could predict the direction of pollen dispersal. The proportion of pollination showed the percentage of selfing and outcrossing pollination. Mahogany have effect for antihiperglicemic for diabetes mellitus [22], like Ficus religiosa all the parts of the plants have medicinal properties [23].

The learning of mating system, spatial hereditary, and family structure are key for effective tree species conservation, management, breeding, and natural reforestation. In *Prosopis flexuosa*, one of semi-arid species having relatively reduced pollen-mediated gene dispersal distance (4.56–20.35 m), which favors inbreeding as a consequence of mating between related individuals [24].

CONCLUSION

Proportion of crossing was 95% which indicated crossing pollination dominating the mahogany population in Gowa (genetic resource area). SSR primers screening generating polymorphic bands were sm03, sm18, and sm48. Highly variable microsatellite markers have facilitated a direct genetic approach to measuring gene flow based on parentage analysis.

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