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Genetic Diversity of Parental and Offspring Population in Ebony (*Diospyros celebica* Bach) Revealed by Microsatellites Marker

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ABSTRACT: Ebony (*Diospyros celebica* Bakh) or known as Indonesia ebony is native species from Sulawesi and become one of the most valuable timber species in Indonesia. Illegal harvesting, excessive cutting, slow growth character and low natural regeneration has lead the population sharply decreased in recent decades. Due to this condition, IUCN in 1998 listing this species into vulnerable species. Conservation effort and breeding programs are among the urgent activities needed to prevent extinction. Genetic characteristics of the species and population is needed as basic consideration in formulating appropriate conservation strategy for the species. Our research was conducted to determined ebony genetic diversity at parental and offspring stages. Simple sequence repeat (SSR) markers were used to analyze 164 individuals (92 trees and 72 seedlings). Molecular data were processed using Gene Alexand DARw into analyze allelic frequencies and dendrograms clustering. Result showed that each population contained four to seven alleles, totalling 15 allels for all individual analyzed. Seventeen private alleles were found in this research, eight at the tree stage and nine at the seedling stage. Both stages combined possessed 5% genetic variation. Overall variation among individuals was 46%, and within each stage, individual genetic variation was 49%. The observed heterozygosity was 0.26. It concluded that genetic diversity of ebony in this research was low, genetic infusion was needed to prevent inbreeding process and decreased its genetic potential.

Keywords: Ebony, genetic diversity, microsatellite marker, private allele.

I. INTRODUCTION

The genus *Diospyros* (Ebenaceae) consists of about 400 species and is widely distributed from tropical to temperate regions of Asia, Africa, and Central-South America (Yonemori *et al.* 2000). Ebony (*Diospyros celebica*) or locally known as *kayu hitam* is native to central and northern Sulawesi Island of Indonesia. Ebony is widely distributed in lowland to an altitude of 540 m.asl (Soerianegara *et al.* 1995). Sutarno *et al.* (1997) stated that ebony grow well in primary forest of lowland. In Java, ebony have been cultivated together with teak. The occurrence of teak may act as good indicator for suitable ebony habitat.

Ebony wood is known as high quality and important source for building, bridge and ship manufacturer. The woods are categorized in 1st strength class, 1st durability class with specific gravity1.09 and classified into luxury wood species. The high economical value of its wood has led to excessive cutting and illegal harvesting of the wood in the wild. Recalcitrant seed character (Sutarno *et al.* 1997) and lower natural regeneration causing the population decreased every year. In 1998, IUCN listed this species into vulnerable status (World Conservation Monitoring Centre, 1998). Previous research conducted by Restu (2007) using genetic markerin five ebony provenance showed that Indonesia ebony has low genetic diversity and higher homozygosity. This condition will increase the possibility of self-pollination, which causes inbreeding depression. There is limited information on ebony population condition, therefore conservation strategies was needed for this species. Conservation based molecular tool was an important effort for threaten species such as ebony.

Simple sequence repeats (SSRs) are considered as the most reliable and reproducible molecular tools to assess genetic information in plant populations and collections (Fossati *et al.* 2005). SSRs have several advantages over other techniques in being faster, less costly, and better able to detect genetic diversity between genotypes. These microsatellites are abundant di-, tri- or tetra-nucleotide repeats that are dispersed throughout the eukaryotic genome and more polymorphic than other genetic markers. SSR resources are useful for cultivar identification, pedigree analysis, characterization of germplasm diversity and genetic mapping studies (Du, Zhang, & Luo, 2009). In coconuts, SSRs were used to genotype female parents, progeny arrays, and a number of potential male parents (Larekeng *et al.* 2015). Codominant simple sequence repeats (SSRs) are regarded as one of the most effective molecular markers for the examination of genetic diversity within and between populations, and they provide abundant genetic information. The importance of SSR marker as tool for genetic conservation of forest trees also has been reported in different plants like *Anthochepalus macropilus*

(Larekeng *et al.* 2018., Arif *et al.* 2019), *P. Merkusii* (Susilowati *et al.* 2013), *Ocotea*sp, (Martins *et al.* 2015), *Rhododenron ponticum* (Jane *et al.* 2015), *Manilkara maxima* (Silva-Junior *et al.,* 2016) and other species. Therefore, our objective was to determine the genetic diversity in ebony tree and seedling stages, thereby contributing to conservation and breeding efforts of this vulnerable species.

II. MATERIAL AND METHODS

A. DNA Isolation

Total 166 leaves sample were taken from Hasanuddin University education forest. The area is administratively located mostly in Limampocoe Village, Cenrana Subdistrict (previously known as Camba Subdistrict), Maros District, South Sulawesi Province, Indonesia. According to the Administration of Forestry, the area of experimental forest is part of Bulusaraung Forest, Bengo Police Forest, Lebbo Tengae Forest, Forest Service of Maros and Forest Service of South Sulawesi. Ninety four (94) parental trees leaf samples and seventy two (72) offspring leaf samples collected for this purpose. Not all parental trees in this location have seedling, only 11 of 94 adult trees had seedlings. Two to ten seedlings (total number 72) from each parental tree were evaluated for this research. Parental trees of ebony in this area originated from 5 (five) seed source those were Tappalang (Mamuju Regency), Malili (Luwu Timur Regency), Dua Pitue (Sidrap Regency), Amaro (Barru Regency) and Palanro (Maros Regency). Parental trees were numbered according the samples collecting, for example sample 11 (mean parent tree 11). The offspring sample was numbered according its parent, for example offspring 60.1 (mean that offspring number 1 from parent trees number 60.

Samples were stored in plastic bag containing silica gel and stored at -20°C before the isolation process. DNA isolation was performed by following the Genomic DNA Mini Plant (Geneaid) kit protocol. Extracted DNA quality was then tested with 8.0% agarose gel electrophoresis using TAE 1× buffer.

B. DNA Amplification

Seventeen SSR marker developed for *Diospyros kaki* Thumb (Liang *et al.* 2015) was used for screening polimorphic SSR primer that will be used forebony genetic diversity research (Table 1). According the screening process using polimorphicloci, targeted band and itreproducibility, three SSR marker were choosedthose were 1430DC588341, 8917DC591591, mDp17EF567410.

Table 1: Seventeen SSR marker used in this research.

S. No	Locus name/ Genbank accession no.	Repeat motif	Primer sequence (5′-3′)	Tm[1] (°C)
1	1430 DC588341	(GAG)5	F: TCAGTAAAGCTGCGGGCATC R: ACGGTT CTC CTGATC CTC ACG	56
2	1554 DC586537	(CAT)6	F: CACCGCATC CTC TTCGACATC C R: ACG CAT CCGTCAAATCACAAC A	56
3	4379 DC585084	(GAG)9	F: TGA CTC TGCTCCACAGGC ACT TC R: CTC GTCTGGCAATTCTGCTTC G	56
4	5553 DC585710	(GTAGTG)3	F: CCAGTT GAT GGCAATGGGAGG C R: GGTGCGATGTTG GAG GGA AGA G	56
5	6615 DC585737	(CTT)7	F: ACA CTC CAC TCT ACC CAA ATA CC R: GAC ATC ATA AGT CAA AGC ACG AA	55
6	6665 DC592790	(TA)9	F: TGACCAACCCCAAAGTGTGGG AG R:AGGTCC CTC TGGTGAGCA CAT GC	60
7	8125 DC592401	(GGC)4	F: TTATCC CAT CAAAGCAACCCA C R: CTGCCA ACT TCTTCTCCATCT CC	55
8	8917 DC591591	(AT)10	F: ACA CGT TCA GTA CCA GGA GGG A R: AGTACCACAAACCAC CAG TGG	55
9	9004 DC591297	(GCAGGA)3	F: GCCACAAACTTCACA GAG GAC C R: AGG CGA GTG CGA GTA AGA CGA A	55
10	DKs76 DC585435	(AGG)7	F: TCGGCTTCACCTATGTTG R: CGATTCCTTGGACCTTTG	52
11	DKs91 DC592713	(AG)7	F: CGGAAGAGGGAGAAATCG R: GAATCGGGAAAGCAAGTT	55
12	mDp17 EF567410	(GA)21	F: CCAAAT CAT TCGAAGCCA AT R: CCTTCACCGATGTCCTTT GT	52
13	ssrDK11 DQ097479	(GA)16	F: ATGTTTCAGGGGTTCCATTG R: TCACTCGTCTTTGCCTTTCC	53
14	ssrDK14 DQ097482	(AG)16	F: GTGAAGGAACCCCATAGAA R: CCATCATCAGGTAGGAGAGA	52
15	ssrDK16 DQ097484	(GA)12	F: ACTACAACGGCGGTGAGAAC R: GTCCTTCACTTCCCGCATT	55
16	ssrDK29 DQ097497	(CCTTT)8	F: ATCATGAGATCAGAGCCGTC R: CACGTTAACGTTACGGAACA	53
17	ssrDK31 DQ097499	(CT)15	F: AGTTCTTGCGATGGGATTTG R: GATGAGATGGGCTGATTGCT	60

[1]T_m: annealing temperature

DNA amplification was performed in reaction volumes of 25 µl, containing PCR mix Kappa 2 G fast, DNA template, 10 ng/ µl ofeach primer pair, and dd H_2O. A Labcycler® Thermocycler (Sensoquest, Göttingen, Germany) was used for performing amplifications with PCR protocols following KAPA Biosystem kit. The amplification condition was 94℃ at 180 s, 35 cyclesof 94℃ at 30s, 30 s, 72℃ at 60 s (according the annealing temperature of marker), and final extention 72℃ at 300 s. Amplification products were separated using electrophoresis on 3% Super Fine Resolution (SFR) gel in 1 × TAE bufferat 100 V for 90 minutes (Seng *et al.* 2013). Allel were sized using 100bp DNA marker (Geneaid).

PCR products were subsequently separated using horizontal electrophoresis with 3% of Super Fine Resolution (SFR) agarose and TAE 1x buffer. The fluorescent stain GelRed was added once SFR agarose dissolved. Electrophoresis separation was done for 90 minutes at 100 volt (Seng *et al.* 2013). The electrophoresis process later visualized and documented using Geldoc (Biostep).

C. Data analysis

Only clear, well-defined and reproducible bands were recorded. Band pattern from amplification processs cored and then scoring pattern analyzed using so megenetic software. Based on the scoring result, measured of genetic variability was conducted. The value of alleles number (Na), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), PcoA, and Analysis of Molecular Varians (AMOVA) was perform using GeneAlex 6.5 software (Peakall and Smouse, 2012). The clustering pattern was performed in DAR Win 6.0 using the Neighbor Joining (NJ) method (Perrier *et al.* 2003). PIC value was calculated using Polymorphic Information Content Calculator an Online Program (Naggy et al., 2012).

$$\mathbf{PIC} = 1 - \sum_{i=1}^{l} P_i^2 - \sum_{i=1}^{l-1} \sum_{j=i+1}^{l} 2\, P_i^2\, P_j^2$$

III. RESULTS AND DISCUSSION

A. Genetic diversity of parental population

Fifteen (15) alleles were detected in this research and the numbers of alleles for each locus was varied from 4 to 7. The highest allele was found with mDp17EF567410. The highest number of effective alleles (Ne) was 2.96(8917DC591591). The mean number of effective alleles, mean observed heterozygosity (Ho), and mean expected heterozygosity (He) were 0.75 0.22, and 0.50, respectively. The highest PIC value was 0.06(8917DC591591), while the lowest value was 0.20(mDp17EF567410). The mean PIC for all marker loci was 0.46 (Table 2). This value indicated that it harbors reasonable levels of genetic diversity.

Table 2: Number of alleles (Na), number of effective alleles (Ne), observed heterozygosity (Ho), expected heterozygosity (He), and Polymorphic Information Content (PIC) of parental and offspring population.

S. No.	Primer Name	Na		Ne		Ho		He		PIC	
		P	O	P	O	P	O	P	O	P	O
1	1430DC588341	4	5	2.88	1.95	0.20	0.10	0.65	0.49	0.59	0.45
2	8917DC 591591	4	5	2.96	3.74	0.25	0.58	0.66	0.73	0.60	0.68
3	mDp17EF567410	7	5	1.27	1.24	0.23	0.21	0.21	0.19	0.20	0.18
	Average	5	5	0.75	2.31	0.22	0.26	0.50	0.47	0.46	0.37

Note: P: parental population; O: offspring population

Based on microsatellites data, genetic distance among individual parental was generated using neighbor joining cladogram (Fig. 1). The cladogram showed that parental population divided into three major group. The group consist of individual from different seed source. Most of parental was joined in first group, it conclude that parental trees of ebony in Hasanuddin University education forest was not follow their recorded origin, from five seed source origin they clustered only into 3 cluster.

B. Genetic Diversity of offspring

The total allele found in offspring population was 15 (Table 2). The mean of effective allele number was 2.30. Whereas Ho and He values were 0.37 and 0.26, respectively. The expected value of heterozigosity was slighty similar with it parental. The highest PIC value (0.68) was obtained from 8917DC591591 and the lowest (0.18) obtained from mDp17EF567410 (Table 2). The mean of PIC value for all marker loci was 0.44.

Based on neighbor joining cladogram, offspring population divided into three major clade (Fig. 2). Some of individual offspring population is clustered based on it parental trees, but some of them separated from other. For example, offspring from parent tree 67. The seedling of parent 67 was separated into two group. Individual number 67.15, 67.8,67.6, 67.13, 67.12 clustered into one clade, while individual of 67.7,67.9 clustered in sperated clade. Previous study on seed dispersal in the same location conducted by Restu *et al.* (2017) found that ebony parental trees in Hasanuddin education forest have opportunity for donate the pollen through self-pollination occurred in 8 events (11.1%) and cross pollination occurred in 64 events (88.9%). This condition might cause seedling of parental trees number 67 distributed into different clade. Based on those research, we can concluded that parental tree number 67 has selfing rate value 28.6% while out crossing rate was 71.4%.

C. Genetic Diversity of parental and offspring

Private alleles were found in parental population (eight) and offspring population (seven). AMOVA analysis determined that the highest percentage of the total genetic diversity was distributed within group, although a significant percentage of the diversity is attributed to differences between groups. AMOVA result showed that the variation and F_{ST} valuesin both stages were 5% and 0.05, respectively (Fig. 3). Between-individual variation and F_{IT} were 46% and 0.50, respectively. Within-individual variation and F_{IS} were 49% and 0.48.

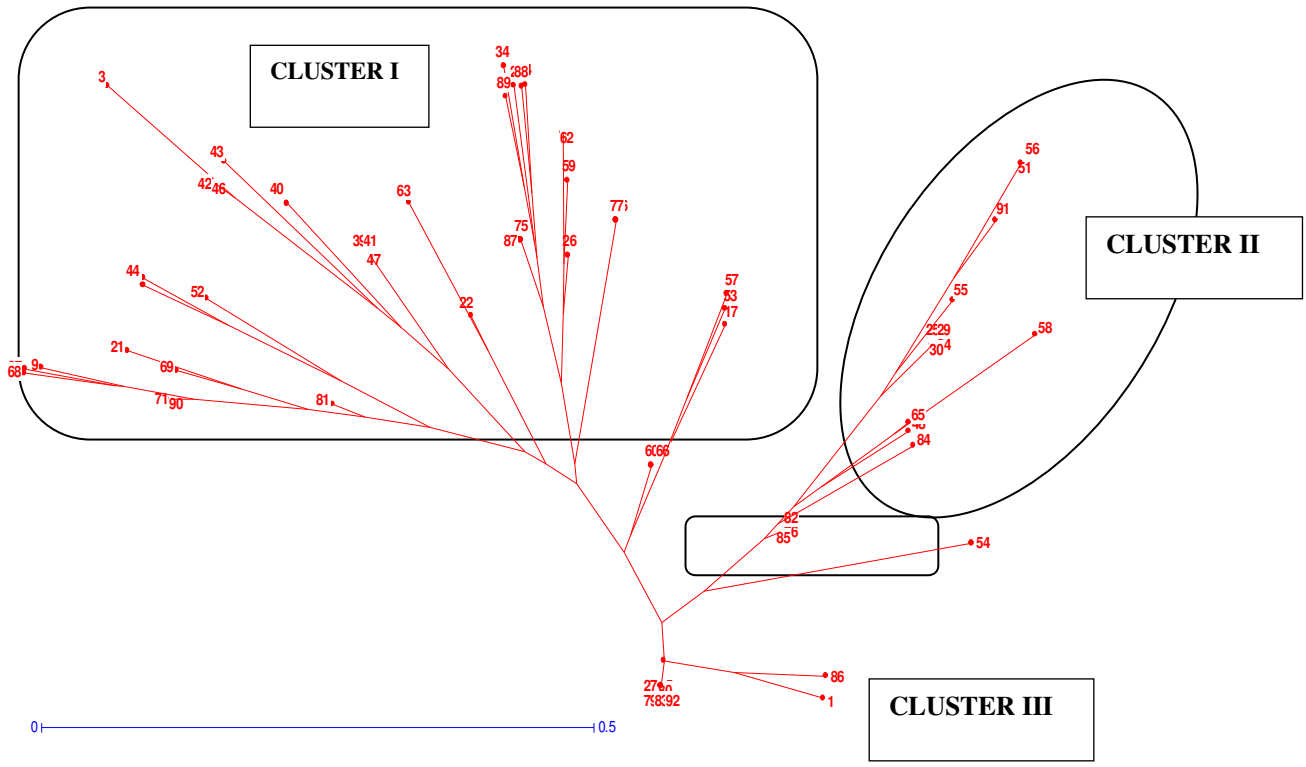

Fig. 1. Cladogram obtained by Neighbor-joining of 94 parental trees, using three microsatellite markers.

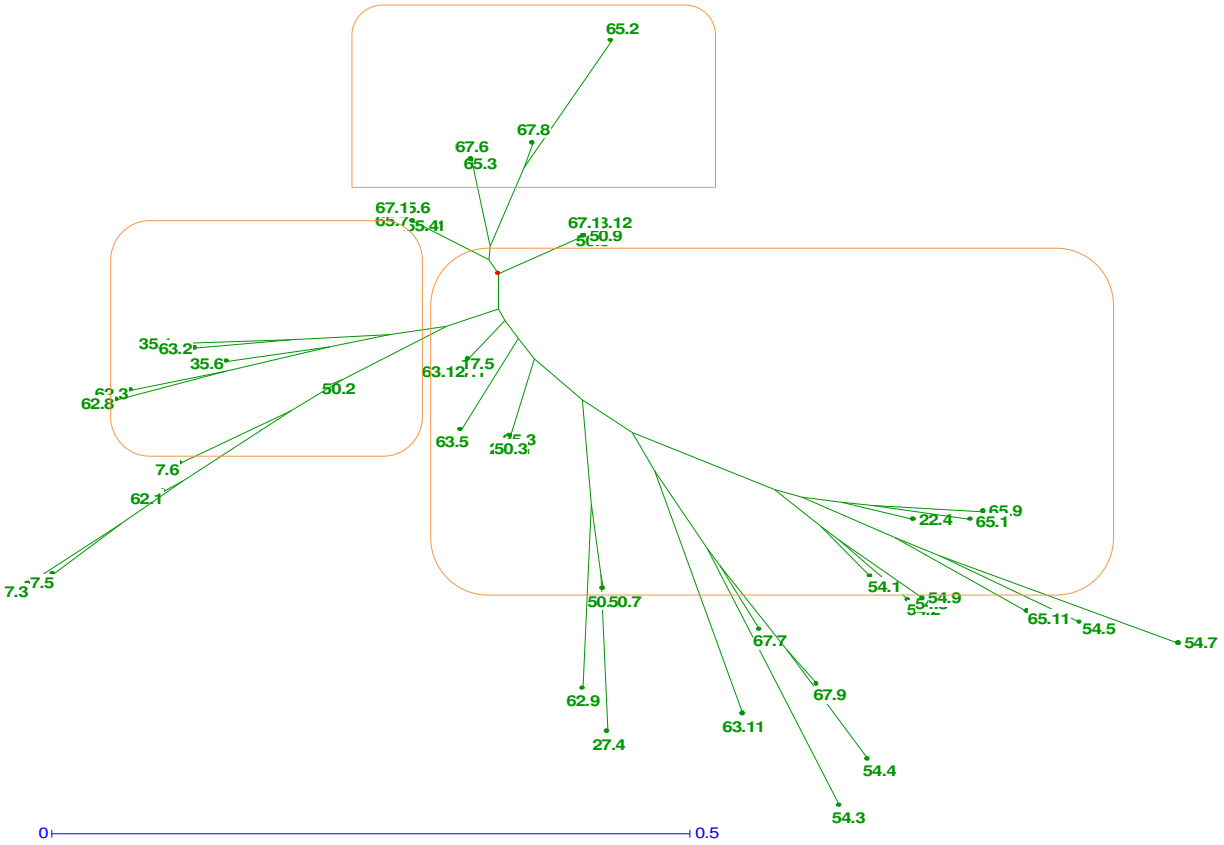

Fig. 2. Cladogram obtained by Neighbor-joining of 72 offspring, using three microsatellite markers.

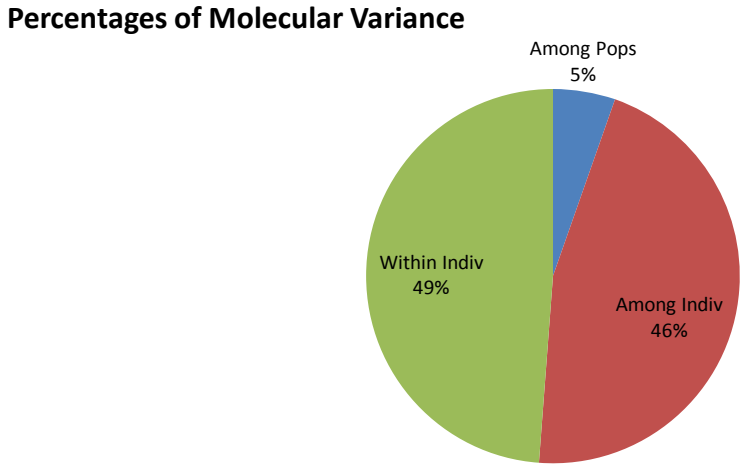

Fig. 3. Diagram of AMOVA (analysis of molecular variance) values in ebony parental and offspring based on three microsatellite markers. Pops, populations; indiv, individuals.

Principal coordinate analysis (PCoA) further helped to describe the variability among these accessions in a 2-dimensional mode (Jing, *et al.,* 2013). PCoA was completed using the genetic similarity matrix and aimed to better understand the relationships between individual population (Fig. 4).

Individual of parental and offspring population were equally distributed in all four quadrants and did not grouped according the population. Neighbor-joining clustering yielded similar results, with all individuals equally distributed across groups (Fig. 5).

Since their discovering, microsatellites have been the most popular markers used for parentage analysis (Hulata 2001; Wilson and Ferguson 2002; Jones and Ardren 2003; Chistiakov *et al.* 2006). Microsatellites are codominant markers (heterozygotes can be distinguished from homozygotes) and are therefore far more informative for genotyping individuals and for linkage mapping than dominant markers such as RAPDs.

The utility of microsatellites is due to their high variability, together with the ability to semi-automate their analysis and scoring. Interestingly, microsatellites has been the marker of choice for assessment of genetic variability in many species such as *A. sativum* (Kumar *et al.,* 2019), *P. merkusii* (Nurtjahjaningsih*et al.* 2007; Susilowati*et al.* 2013), Saccharum (Ali *et al.* 2019), *A. macrophyllus* (Arif *et al.* 2019), *O. europaea* (Bahmani *et al.,* 2016) and *T. yunnanensis* (Miao *et al.* 2015).

Our result found 18 alleles, whereas sixth alleles was private. The number of private allele will determined the value of expected heteozigosity (He). Private alleles found in Loci 1 (1 private allele), loci 2 (1 private allele) and loci 3 (1 private allele) in offspring population. Three private alleles were found in Loci 3 of parental population. It indicated that new alleles were found in progeny population, but three of parental alleles not inherited on offspring population.

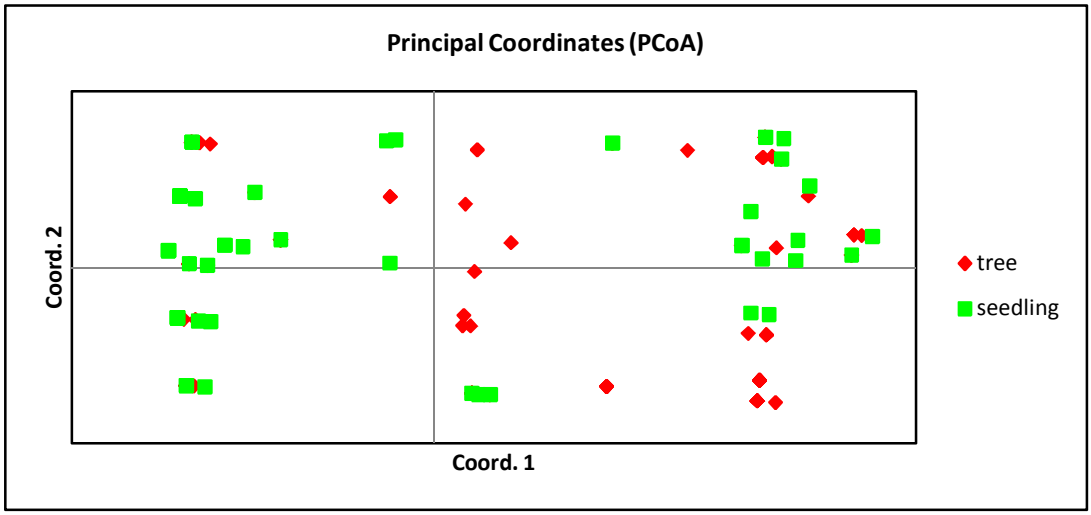

Fig. 4. Results of principle coordinates analysis (PcoA) showing distribution ofindividual ebony parental and offspring based on three microsatellite markers.

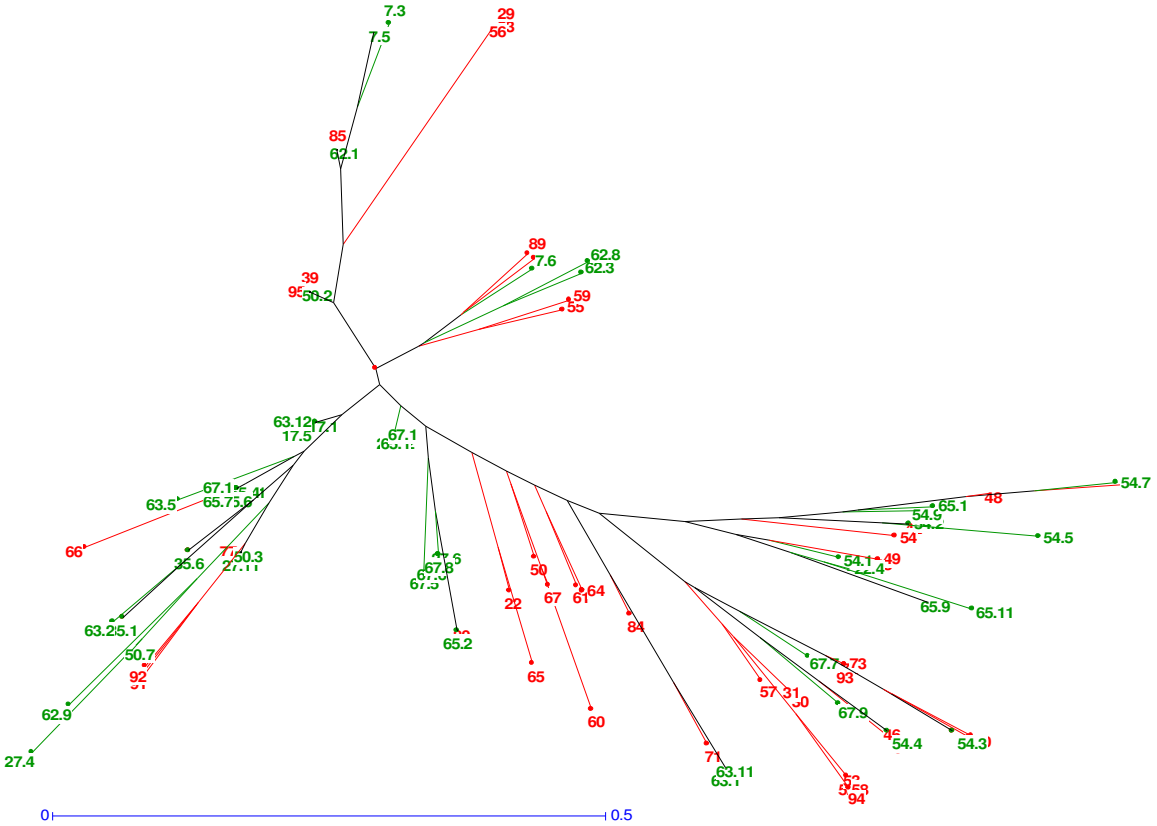

Fig. 5. Neighbor-joining phylogenetic tree of ebony in parental (red lines) and offspring (green lines) stages using three microsatellite markers.

From this result we also assumed that the decrease of genetic diversity on ebony population was not occurred due to the occurrence of new alleles in offspring population. The capability of long pollen dispersal that originate outside the Hasanuddin University education forest may contributed to the occurrence of private alleles in offsprings population. It is in accordance with Restu *et al.* (2017) that stated Ebony pollen dispersal may reached 166 m. Other research in coconut (Larekeng *et al.,* 2018) that the pollen dispersal range 0 m to 54 m and out crossing frequency at least 95%.

The number of private allele will determine the value of expected heterozigosity (He). The mean value of Ho and He was slightly higher in parental compared offspring, it migh caused from the parental origin which were came from different seed soure, whereas the offspring population obtained from limited parental seed source in Hasanuddin University education forestand closely related although it cross polinated species. Hardy Weinberg Equilibrium Law states that in large populations where no selection, migration, mutation, and mating between individual constituents occur randomly, the allele frequency will remain the same from generation to generation (Yeh, 2000). Deviations in the proportion of allele frequencies and genotypes of Hardy Weinberg Equilibrium law can occur due to mutations, recombination, selection, isolation, migration, and genetic drift.

AMOVA results showed only a 5% variation between the parental and offspring population with an F_{ST} of 0.05. An F_{ST} of 0 indicates identical allele frequencies in a subpopulation, whereas an F_{ST} of 1 indicates fixed and genetically differentiated subpopulation (Mohammadi and Prasanna, 2003). Thus, these data suggest that parental and ebony offsping have high genetic similarity and not genetically structured.

This study also clarified which microsatellite marker was most suitable for the analysis of ebony genetic variation. Here, we used the PIC to quantify a primer's ability to detect an allele. This metricis determined via allele frequency (rather than observed allele count; DeVicente and Fulton, 2003) and can be grouped into three classes: very informative (PIC>0.5), moderately informative 0.50<PIC<0.25, and less informative (PIC<0.25) (Bostein *et al.* 1980). We found that primer 1430DC588341 was the most effective (highest PIC: 0.60) and primer mDp17EF567410, the least (lowest PIC: 0.20). The high PIC value for 1430DC588341indicate an isolation of highly polymorphic microsatellites. The importance of allele frequency rather than allele number explains why mDp17EF567410-amplified tree samples had highest allele count, but the lowest PIC value. Similarly, seedling samplesdid not differ considerably in alleles numbers across primers, but did differin PIC. Overall, primer mDp17EF567410 detected seven alleles, including many private alleles, but allelic frequency was consistently low; these factors resulted in its low PIC value.

The two-dimensional graph of accessions differentiation was revealed by PCA. This type of graphical illustration enables the assessment of the population structure and geometric distances among all of the accessions in the study. The distribution of the accessions in the two-dimensional graph based on the first two principle components was similar to that

obtained from cluster analysis, where all parental collected from closely related seed source.

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