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Polymorphism of Simple Sequence Repeat Regions of Sulawesi Ebony (*Diosphyros celebica* Bakh.) in Experimental Forest of Hasanuddin University Provenance

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Abstract

Polymerase Chain Reaction (PCR)-based molecular techniques have been used to detect the polymorphism in plants. The utilization of molecular markers plays essential role in germplasm characterization and plant breeding since the information of DNA marker technology can be exchanged between laboratories and should have standard method to be reproducible. The molecular aspect has been commonly linked to DNA isolation protocol and polymorphic molecular marker, thus can be used for molecular research recommendation purposes. The objectives of this study were to evaluate the capability of microsatellite marker of Ebenaceae Family for amplifying Ebony DNA, and to determine the appropriate PCR annealing temperatures. The DNA isolation of Ebony leaves from Experimental Forest of Hasanuddin University Provenance was carried out using Genomic DNA Mini Kit (Plant) Geneaid protocol. Nine of seventeen selected primers from the Genus Diospyros were able to amplify Ebony DNA. Amplification products produced polymorphic bands with different annealing temperatures (ranged from 53 to 56°C). These nine polymorphic primers will be recommended to use for future studies in genetic diversity as well as pollen dispersal pattern analyses.

Keywords: polymorphism, microsatellite marker, ebony, annealing temperature, primer screening

A. Introduction

Ebony (*Diospyros celebica* Bakh.) known as "black-wood" is endemic to Indonesia that distributes from Northern Sulawesi (i.e in Minahasa, Bolaang, Mongondo) and Central Sulawesi (i.e in Parigi, Poso, Donggala, Toli-Toli, Kolonedale, Luwuk) to Southern Sulawesi (i.e in Barru, Luwu, Mamuju) (Hartati and Kamboya 2007). Its wood durability and strength levels are classified to class I which mean the wood can be usable more than five years under moist soil condition (Departemen Kehutanan 2013; Akbar and Rusmana 2013).

Ebony belongs to the Ebenaceae Family and is categorized as vulnerable species. Prevention of Ebony extinction requires the species conservation through breeding program which is supported by genetic-based molecular information. Molecular data would be valuable to shorten and facilitate the future breeding programs in Ebony.

Molecular data can be obtained through a molecular marker. Molecular markers are DNA fragments that representing differences in genome level. The molecular marker should be polymorphic, evenly distributed in the genome, able to distinguish genetic differences, easy to apply, less time and less DNA sample required for processing, has lingkage to discriminate phenotypes and not require any prior information of the organisms' genome (Agarwal *et al.* 2008). One comonly used molecular marker is SSR or microsatellite marker.

Ease of SSR marker in amplifying and detecting DNA fragments as well as high polymorphism level causing this method capable of analysing genetic diversity with numerous number of DNA samples. Moreover, PCR technique in SSR markers only requires less amount of DNA with amplification region ranged 100 to 300 base-pair (bp) from whole genome. SSR method can be applied without damaging the plants because only need less leaf tissue sample or other tissue parts, such as seed and pollen, in DNA extraction process (Kalia *et al.,* 2011). SSRs markers are also multiallelic and easily repeatable, thus their applications are more interesting for analysing genetic diversity among different genotypes (Rao *et al*., 2011). Another advantage of this method is PCR products can be separated not only by agarose gel but also by acrylamide gel, in particular for allele of a character that has low polymorphic level (Macaulay *et al*., 2001).

Some of the other considerations that SSR markers have been widely used in genetic diversity analyses are abundantly and evenly distributed in the genome, very high variability level (multiple alleles per locus) and co-dominant nature with the known genome location. Therefore, microsatellite markers are useful tools that present high reproducibility and accuracy used in distinguishing genotypes, testing seed purity, mapping gene as well as providing efficient tool for selection strategy, genetic population study and genetic diversity analysis. Recently, numerous published studies have broadly used this approach, for instance, genetic diversity analysis and characterization in coconut and teak (Larekeng, 2016; Kumar *et al*. 2011), mating system and study of xenia effect in Kopyor coconut (Maskromo *et al*. 2016, Larekeng *et al.* 2015a).

However, the information about microsatellite marker in Ebony has not been reported before. Eventhough no prior information on DNA marker of ebony is available, molecular data of this species can still be obtained. DNA markers from other species either in same genus or family are applicable to use to acquire molecular data. The closer taxonomic relation between evaluated individuals with selected markers, the higher success rate of markers to produce amplified DNA products (Nurtjahjaningsih *et al*., 2012). In this study, our objective was to obtain SSR primers of Ebony from selected SSR primers from the genus *Diospyros* using primer screening method. By this study, we would like to support the conservation and breeding programs of ebony.

B. Material and Methods

1. Genetic Material

Ebony leaf samples were collected from experimental forest of Hasanuddin University, Maros, South Sulawesi, Indonesia. As many as 12 leaf samples were harvested from different trees and then wrapped in plastic before stored at -20^{0}C until needed for extraction.

2. DNA Isolation and Primer Screening

DNA isolation was carried out following the Genomic DNA Mini Plant (Geneaid) kit protocol. Extracted DNA quality was tested with 2% agarose gel electrophoresis and then followed by amplifying the DNA using 17 SSR primers from the Genus *Diospyros* (Liang *et al.,* 2015). The 17 primer sequences used in this study are describe in Table 1.

Table 1. SSR Primers of The Genus Diospyros used in Primer Screening

No	Locus name/genbank accession no.	Repeat motif	Primer sequence (5'-3')	Tm* (oC)	Allele size (bp)
1	1430 DC588341	(GAG)5	F: TCA GTA AAG CTG CGG GCA TC R: ACG GTT CTC CTG ATC CTC ACG	56	190 –250
2	1554 DC586537	(CAT)6	F: CAC CGC ATC CTC TTC GAC ATC C R: ACG CAT CCG TCA AAT CAC AAC A	56	190 –223
3	4379 DC585084	(GAG)9	F: TGA CTC TGC TCC ACA GGC ACT TC R: CTC GTC TGG CAA TTC TGC TTC G	56	208 –235
4	5553 DC585710	(GTAGTG)3	F: CCA GTT GAT GGC AAT GGG AGG C R: GGT GCG ATG TTG GAG GGA AGA G	56	226 –254
5	6615 DC585737	(CTT)7	F: ACA CTC CAC TCT ACC CAA ATA CC R: GAC ATC ATA AGT CAA AGC ACG AA	55	244 –268
6	6665 DC592790	(TA)9	F: TGA CCA ACC CCA AAG TGT GGG AG R:AGG TCC CTC TGG TGA GCA CAT GC	60	171 –209
7	8125 DC592401	(GGC)4	F: TTA TCC CAT CAA AGC AAC CCA C R: CTG CCA ACT TCT TCT CCA TCT CC	55	189 –207
8	8917 DC591591	(AT)10	F: ACA CGT TCA GTA CCA GGA GGG A R: AGT ACC ACA AAC CAC CAG TGG	55	166 –197
9	9004 DC591297	(GCAGGA)3	F: GCC ACA AAC TTC ACA GAG GAC C R: AGG CGA GTG CGA GTA AGA CGA A	55	251 –272
10	DKs76 DC585435	(AGG)7	F: TCGGCTTCACCTATGTTG R: CGATTCCTTGGACCTTTG	52	111 –138
11	DKs91 DC592713	(AG)7	F: CGGAAGAGGGAGAAATCG R: GAATCGGGAAAGCAAGTT	55	191 –207
12	mDp17 EF567410	(GA)21	F: CCA AAT CAT TCG AAG CCA AT R: CCT TCA CCG ATG TCC TTT GT	52	128 –168
13	ssrDK11 DQ097479	(GA)16	F: ATGTTTCAGGGGTTCCATTG R: TCACTCGTCTTTGCCTTTCC	53	155 –195
14	ssrDK14 DQ097482	(AG)16	F: GTGAAGGAACCCCATAGAA R: CCATCATCAGGTAGGAGAGA	52	158 –192
15	ssrDK16 DQ097484	(GA)12	F: ACTACAACGGCGGTGAGAAC R: GTCCTTCACTTCCCGCATT	55	136 –172
16	ssrDK29 DQ097497	(CCTTT)8	F: ATCATGAGATCAGAGCCGTC R: CACGTTAACGTTACGGAACA	53	112 –152
17	ssrDK31 DQ097499	(CT)15	F: AGTTCTTGCGATGGGATTTG R: GATGAGATGGGCTGATTGCT	60	191 –207

*Tm : Melting temperature

The DNA amplification protocol was conducted using 96 well-PCR(Sensoquest Thermocycler, Germany) with these following steps : one cycle of pre-amplification at 94°C for 180 seconds, 35 cycles of amplification steps at 94 °C for 30 s (template denaturation), annealing at ± 5oC from the given melting temperature (Table 1) for 30 s (primer annealing), and 72 °C for 60 s (primer extension), and one cycle of final primer extension at 72 °C for 300 s. Amplification products were then separated using 3% Super Fine Resolution (SFR) agarose with TAE 1x buffer at 100 V for 90 minutes (Seng *et al* 2013).

C. Result and Discussion

The DNA quality test showed that not all individuals in each family consistently obtained DNA band. The DNA quality test is presented in Figure 1. The quality test in the extracted DNA rubber plant was done using 2% agarose. Ebony DNA isolation procedures was conducted using Genaid kit protocol. Ying and Zaman (2006) stated DNA isolation using kit protocols have widely used in plants and tended to show high success level. This method is practically easy to implement and produces high quality of extracted Ebony DNA like this rubber DNA (Ain 2011). Haris *et al* (2003) previously reported that DNA quality affects amplified DNA fragment

products. Less concentration of DNA would visually produce thin or no fragment on agarose, and vice versa.

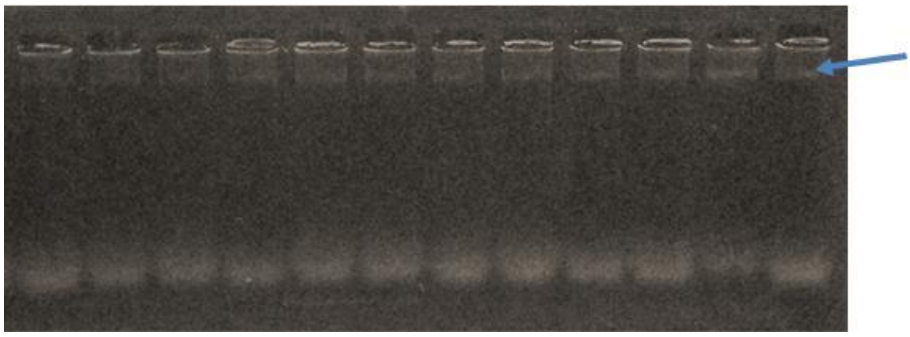

Figure 1. The extracted DNA quality of Ebony from experimental forest of Hasanuddin University. Arror : DNA band position.

The separation by agarose electrophoresis showed the extracted DNAs were good, eventhough almost all DNA samples displayed slight smear on bands. Smear is frequently shown due to contaminations either by RNA or incorrect DNA isolation protocol (i.e centrifugation, incorrect temperature or solution treatment). Smear may also affected by too much concentration of DNA or unstable electrical voltage during electrophoresis process (Ausubel *et al. 1990)* and purity level of extracted DNA. Purity level of more than 2.0 means the DNA samples were contaminated by RNAs, whereas that of lower than 1.8 were contaminated by proteins (Yuwono 2006). In this study, smear did not affect subsequent analysis since PCR process in microsatellite markers analysis required less amount DNA sample (even with low quality of DNA) (Acquaah 2007). Another advantage of SSR markers is capable of amplifying DNAs with intermediate quality and relatively less concentration of DNA (Gupta *et al.* 1996).

Table 2. SSR Primer Names of the Genus Diospyros that succeeded to amplify Ebony DNA

No	Locus name/genbank accession no.	Repeat motif	Primer sequence (5'-3')	Tm *($_o$C)	Allele size (bp)
1	1430 DC588341	(GAG)5	F: TCA GTA AAG CTG CGG GCA TC R: ACG GTT CTC CTG ATC CTC ACG	56	190 –250
2	1554 DC586537	(CAT)6	F: CAC CGC ATC CTC TTC GAC ATC C R: ACG CAT CCG TCA AAT CAC AAC A	56	190 –223
3	4379 DC585084	(GAG)9	F: TGA CTC TGC TCC ACA GGC ACT TC R: CTC GTC TGG CAA TTC TGC TTC G	56	208 –235
4	5553 DC585710	(GTAGTG)3	F: CCA GTT GAT GGC AAT GGG AGG C R: GGT GCG ATG TTG GAG GGA AGA G	56	226 –254
5	6665 DC592790	(TA)9	F: TGA CCA ACC CCA AAG TGT GGG AG R:AGG TCC CTC TGG TGA GCA CAT GC	60	171 –209
6	8125 DC592401	(GGC)4	F: TTA TCC CAT CAA AGC AAC CCA C R: CTG CCA ACT TCT TCT CCA TCT CC	55	189 –207
7	ssrDK11 DQ097479	(GA)16	F: ATGTTTCAGGGGTTCCATTG R: TCACTCGTCTTTGCCTTTCC	53	155 –195
8	8917 DC591591	(AT)10	F: ACA CGT TCA GTA CCA GGA GGG A R: AGT ACC ACA AAC CAC CAG TGG	55	166 –197
9	DKs76 DC585435	(AGG)7	F: TCGGCTTCACCTATGTTG R: CGATTCCTTGGACCTTTG	52	111 –138

*Tm : Melting temperature

Primer is a short DNA fragment produced artificially and consisted of 10 to 25 nucleotides (Finkeldey 2005). The sequences of primer pair that have been determined to be able to anneal with the sequence DNA targets are used during PCR process. DNA fragment that is located between two meeting points of primers will be amplified in PCR process. Primers function as starting points of synthesis, called as *DNA polymerase* which was derived from *Thermus aquaticus*. This enzyme is also known as *Taq DNA polymerase.* It is suitable for amplification process because it can withstand the high temperature up to 95$_0$C, though the optimal heat of

this enzymes' activity is 72$_0$C. After primers annealing are done, the primers extension process begin at 72$_0$C.

Primer screening is required to obtain strong and clear polymorphic-band pattern of PCR products by selecting random primers that can produce amplification products, as not all tested nucleotide primer pairs may turn out to have amplified products (positive primer) and, other than that, not all positive primer pairs yield good polymorphic DNA fragments (Siregar *et al*. 2008). The results concluded that there were seventeen out of twenty RAPD primers showed good amplification products and polymorphisms for Kayu Kuku DNA amplification along with specific PCR temperature in each primer. In future studies of Kayu Kuku, weanticipate increasing the number of RAPD charactersand the number of species to provide a greater understanding of the genetic resources of the genus*Pericoopsis* (Larekeng *et al,* 2015b).

In this recent study, the PCR amplification test was done by 17 SSR primers from *Diospiros kaki* (Table 1). The result showed that nine of 17 SSR primers were able to produce polymorphic, strong and clear alleles on Ebony DNA from Maros provenance. These selected primer pairs are displayed in Table 2. Moreover, there was one primer pair that produced monomorphic band, primer 6615 DC585737.

Polymorphic primer (Figure 2) is primer that can distinguish one individuals' genetic material from the others by detecting alleles in evaluated population. Primer pair is categorized as polymorphic primer if it can detect the variation in alleles at least 1% (Emrani and Arbabe 2011). The primers used in genetic diversity analysis should be not only polymorphic, but also able to obtain strong and clear PCR-band products. Primers which performed unclear or smear bands were discarded, in order to avoid miscoring of alleles resulting errors in the analysis. For instance, a primer pair which generated either unclear or smear bands using primer 9004 DC591297 is presented in Figure 3. Only for primers that produced unclear and smear bands, the PCR amplification processes need to be repeated by more specific annealing temperature and then validated by Qiaxcell fragment analysis for more specific base-pair differences.

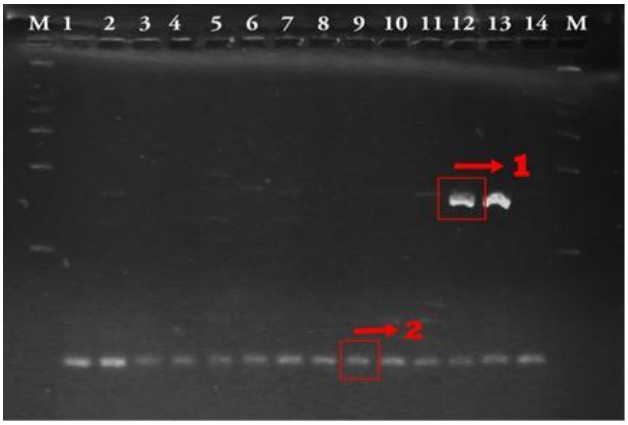

Figure 2. Electropherogram of allelic scoring variation in amplified DNA fragments using primer 8917 D591591. Column 1-14 : DNA samples, column M : DNA ladder (100 bp of *DNA ladder*). Position 1-2 indicate alleles in the evaluated primer.

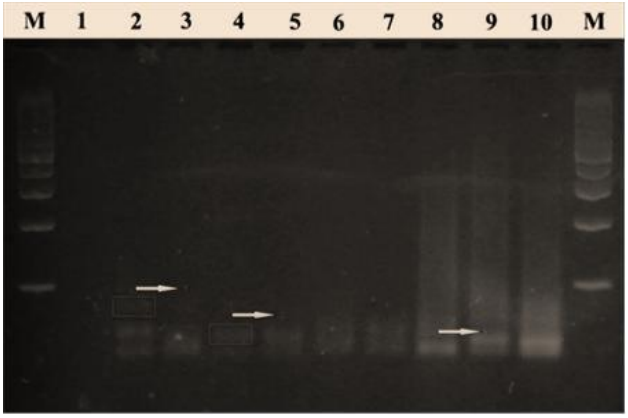

Figure 3. Electropherogram of allelic scoring variation in amplified DNA fragments using primer 9004 DC591297. Column 1-10 : DNA samples, column M : DNA ladder (100 bp of *DNA ladder*). Position 1-3 indicate alleles in the evaluated primer.

The disappeared band pattern can be caused either by less DNA volume used during electrophoresis process, unbalanced mixture between DNA solution and other solutions (primer and PCR mix solutions), less DNA extracted from leaf samples, or DNA wasted during extraction process. Another cause is the contamination of DNA by protein, RNA, phenol or organic compounds. To do the molecular genetic analysis, high purity and concentration of DNA are required,but extracted DNA from plant tissues with high DNA purity level is often difficult to achieve.

The presence of primers without any amplified DNA products can be induced by unmatched primer pairs with DNA sequence that used as DNA template. The SSR marker locus is single strain nucleotide having at least 15 nucleotides in length. Primers are required as starting point of DNA amplification during PCR process. According to Acquaah (2007), PCR processes using SSR marker loci do not require large amount of DNA (approximately 50-5000 µg of DNA), hence, do not influence subsequent analysis. It is proved by the emergence of SSR band pattern shown at agarose and polyacrylamid gels.

The result showed that only nine (53%) of 17 SSR primers from Diospyros could generate strong, clear and polymorphic bands at the 12 tested random samples of Ebony from Maros provenance. It revealed higher number of polymorphic primers compared to that of reported by Nurtjahjaningsih *et al* (2012). In their study, they used as many as 12 SSR primers from three pine species (*Pinus densiflora*, *Pinus pinaster* and *Pinus taeda*) to amplify *Pinus merkusii* DNA, but failed to produce polymorphic allele. In contrast to previous report by Yang *et al* (2015), 13 (93%) of 14 SSR primers from *Diospyros kaki* could amplify DNA from *Diospyros glaucifolia*, *Diospyros lotus*, *Diospyros oleifera* and *Diospyros* sp, and only primer mDP08 could not generate PCR-product in *Diospyros glaucifolia.*

DNA amplification of SSR markers in Ebony from experimental forest of Hasanuddin University provenance becomes an initial step on the usage of specific primer for the molecular genome research. SSR markers are intensively used in various molecular analyses, such as genetic diversity analysis between and among populations, genetic stability analysis in clonal plants derived from in vitro somatic embryogenesis, parents and progeny arrays and pollen dispersal analysis. Alcalá *et al* (2014) previously used eight microsatellite primer pairs to evaluate genetic structure and diversity of Mahagony (*Swietenia macrophylla*) in Mexicos' forest ecosystem. Marum *et al.* (2009) also reported the genetic stability analysis in Pine (*Pinus pinaster*) derived from somatic embryogenesis using seven microsatellite marker loci. Another previous study by Prabha *et al.* (2011) investigated mating system and gene flow analysis of Teak in natural forest using microsatellite primers.

D. Conclusion

Microsatellite primers from *Diospyros kaki* species could be used for obtaining molecular data in Maros provenance of Ebony. Based on alleles visualization, nine of 17 evaluated SSR primers were recommended to be used for amplifying all Ebony DNA samples for subsequent genetic diversity analyses.

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