

Genomic validation of PB 260 clone of rubber (*Hevea brasiliensis*) at Cikumpay Plantation by SSR marker

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Abstract. Rubber from *Hevea brasiliensis* is the only commercial natural rubber in the world. Propagation of rubber trees usually done by grafting and seed germination. BPPT had been producing rubber tree by *in vitro* technique with embryo somatic methods. Validation of mother plant for *in vitro* propagation is important to compare between mother plant and propagated plants. The aim for this research was to validation of PB 260 clone that planted at Cikumpay Plantation by SSR marker. Sampling of 10 rubber leaves were done at Cikumpay Plantation based on GPS position from the area of PB 260 clone. Rubber leaves were isolated with CTAB modification method to obtained DNA. Four of SSR primers from rubber, i.e.: *hmac* 4, *hmac* 5, *hmct* 1, and *hmct* 5, were used as primers to amplification of rubber DNA. The result showed that no band that different from 10 rubber of PB 260 clone at Cikumpay Plantation. This research will continue to compare genomic validation between mother plant and propagated plants that had been produced from BPPT.

1. Introduction

Rubber from *Hevea brasiliensis* is the only one resource for commercial natural rubber in the world due to its high rubber content, good yield, and have the excellent physical properties of the rubber products and high quality [1]-[2]-[3]. Natural rubbers from *Hevea brasiliensis* cannot be replaced by synthetic alternatives because have unique properties, such as high elasticity, flexibility, resilience, impact and abrasion resistance, impermeable to liquids, efficient heat dispersion and malleability at cold temperature [4] which is difficult to be replaced by synthetic rubber in many applications

Now, Indonesia is the country with the largest rubber plantation in the world, almost 3.5 million hectares [5] but for productivity Indonesia still under Thailand with 26% or 3.2 thousand tons/year at 2016 [6]. Rubber in Indonesia grown by more than 1 million farmers, but most of plantation used of unselected seeds, almost 40% of all small holders' rubber plantations use of the low quality of seedlings. These made the production of rubber plantation in Indonesia still low.

Genomic validation with molecular marker now became the routine procedure to validate the plants and propagated plant especially when plants propagated by tissue culture and embryo somatic. Genomic validation for plants usually used for genetic diversity, authentication of plants, and genetic variation. For micropropagated plants, genomic validation used for identification and selection of mutation, confirm the uniformity, validation of somaclonal variation and epigenetic.

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Molecular markers used for genomic validation were random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), MSAP, amplified fragment length Polymorphism (AFLP), simple sequence repeat or microsatellite (SSR) and single nucleotide polymorphism (SNP) markers. Application of each methods based on availability, simplicity, cost, accurately, efficiency, highly reliable, and reproducibility [7]. The genomic validation of micropropagated plant by molecular marker had been reported by [8] for *Cassia angustifolia* Vahl with RAPD marker, [9] for *Dendrobium nobile* Lindl with RAPD and SCOT marker, [10] for *Nothapodytes nimmoniana* (Graham) Mabb with ISSR marker, [11] for *Brassica oleracea* L.var. italic with RAPD marker, [12] for *Pilosocereus robinii* with ISSR marker and [13]-[14] for *Hevea brasiliensis* with AFLP and SSR.

Validation of micropropagated plants, even by morphologic, genomic, pytochemical or with another character and method, will compared with mother plants to ensure that micropropagated plants true to type with mother plants. This point is very important to commercial utilization of miropropagated plants, especially for rubber clone.

The aim for this research was to validation of PB 260 clone of rubber tree at Cikumpay Plantation Purwakarta as a source of mother plants on micropropagated rubber at BPPT by genomic

2. Materials and methods

2.1 Materials

The materials used for this research were 10 of rubber leaves from PB 260 clone belongs to PT Perkebunan Nusantara VIII that planted at Cikumpay Plantations Purwakarta. Location of rubber tree sample by GPS position can see at Table 1.

Table 1. Location of rubber leaves sampling

Sample	Location		Altitude
	(East)	(South)	
1	06°30.297'	106°43.791'	326
2	06°29.020'	107°29.455'	311
3	06°28.993'	107°29.427'	314
4	06°28.967'	107°29.421'	309
5	06°28.964'	107°29.439'	310
6	06°28.964'	107°29.458'	313
7	06°28.954'	107°29.476'	320
8	06°28.939'	107°29.494'	313
9	06°28.914'	107°29.509'	322
10	06°28.968'	107°29.512'	331

Primers used for this research were 4 primers based on GenBank with accession numbers are: AY135656, AY135657, AY135651 and AY135653 for the loci i.e. *hmac4*, *hmac5*, *hmct1*, *hmct1*, and *hmct5* [15] as seen at Table 2.

2.2 Sampling and DNA isolation

Sampling of 10 rubber leaves from PB 260 clone was done at Cikumpay plantation Purwakarta based on GPS position. 10 rubber leaves were taken from 11 Ha of rubber plantation from PB 260 clone that planted since 2006. Young leaves were placed in to plastic clip, labeling and stored at cool box then bring to the Lab.

Table 2. SSR primers used for rubber DNA amplification [15]

Primers	Sequences	Size (bp)
<i>hmac4</i>	5'-GTTTTCCTCCGCAGACTCAG-3' (L) 5'-ATCCACCAAATAAGGCATGA-3' (R)	216-272
<i>hmac5</i>	5'-TCGGTTGGTTTACCATGACA-3' (L) 5'-ACATCACATGAGTGTATCTGATCTC-3' (R)	270-286
<i>hmct1</i>	5'-AACCAGAAGGGTGTCTATGCT-3' (L) 5'-GGAATCCCATGACAATCCAC-3' (R)	196,198
<i>hmct5</i>	5'-ATGTATGTGTGCGCAGGAAG-3' (L) 5'-CTGTAGTCATGGCAGCAGGA-3' (R)	187-211

DNA isolation was done using a modified CTAB (*Cetyl trimethylammonium bromide*) method. The extraction buffer contains 10% CTAB, 5M NaCl, 0.5M EDTA pH 8.0, 1M Tris HCl pH 8.0 and purified water. Young leaves from PB 260 clone were weighted as much as 200 mg, cut out became slice and grounded with liquid nitrogen with mortar and pestle. Every sample was repeated 3 times for optimization method and collecting of DNA samples.

The purity and concentration of the extracted DNA were checked by electrophoresis with 1% gel agarose and measured by a Nanodrop spectrophotometer at 260/280 absorbance.

2.3 Amplification of DNA rubber with SSR marker

DNA amplification was running with composition as seen at Table 3. The condition for DNA amplification steps following a protocol described by [14] with initial denaturation step for 1 minute at 95°C, followed by elongation step with 35 cycles at 94°C for 30 s, 52°C for 60 s and 72°C for 120 s and a final elongation step at 72°C for 8 min. Composition of DNA amplification of rubber can see at Table 3. PCR products were visualization at electrophoresis with 1.2% gel agarose to analyze DNA bands.

Table 3. Composition of reagent for DNA amplification

No.	Reagent	Final Concentration
1.	Taq Polymerase Mix, 2X	1 X
2.	Forward Primer	0,1-1,0μM
3.	Reverse Primer	0,1-1,0μM
4.	Template DNA	0,3 μM
5.	Purified water	0,3 μM
Total Volume		25μL

2.4 Analysis of DNA bands

Analysis of DNA bands based on scoring from monomorphic and polymorphic bands that appears after visualization of PCR products on gel agarose. When DNA bands appear scored as absent (0) or present (1) to obtain binary data.

3. Results and discussions

3.1 DNA isolation

The result showed that isolation of DNA with modified CTAB successful to obtain DNA with the average of concentration between 1400-4000 μg/μL with quality of DNA was 1.15-2.05 at absorbance 260/280. Protocol from [16] said that DNA not contaminated with protein and RNA if the absorbance

from 260/280 is 1.8-2.0. For this research, DNA with high purity was used as template for amplification.

3.2 DNA amplification with SSR marker

DNA profile from 10 rubber trees from PB 260 clone had been amplified with 4 SSR primers from [15]. The result showed that all of samples have the same bands when amplified with each primer. Primer *hmac4* has 1 single DNA band for each sample with size among 100-200 bp, this DNA bands different size with [15] that reported among 216-272 bp. For primer *hmac5*, the DNA profiles showed size among 200-300 bp with single band for each samples. They have same bands from 10 samples and same size with experiment reported by [15]. Primer *hmct1* showed that DNA band from 10 sample of PB 260 clone have the same size and single band. This is different with [15] that reported with *hmct1* primer, there were 2 alleles appear in the DNA profile. Amplification with primer *hmct5* showed that DNA profiles from 10 samples of PB 260 clone have 2 alleles with size among 100-200 bp and 600-700 bp and have the same DNA bands.

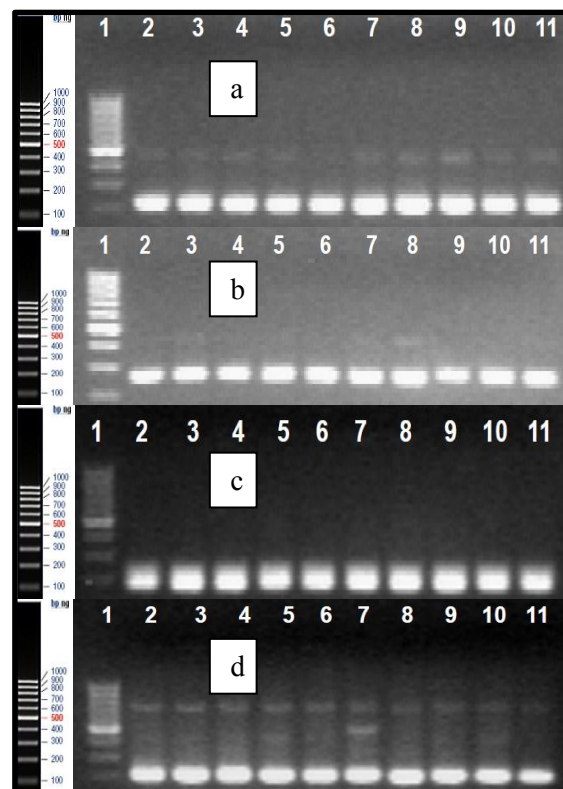


Figure 1: Genomic validation of 10 rubber trees from PB 260 clone, amplification with 4 SSR primers, (a) *hmac4*, (b) *hmac5* (c) *hmct1* and (d) *hmct5*

DNA profiles of 10 samples from PB 260 clone at Cikumpay Plantation showed that no different clone and no genetic change even have been planted since 10 years ago. This research ensures that PB 260 clone used as a source of mother plant on micropropagated plants at BPPT same as genetic. This is the first report of genomic validation of rubber tree after a long planted at rubber plantations. After certain that no change genetically of mother plant, this research will continue to compare genomic validation between mother plant and micropropagated plants that had been produced from BPPT.

4. Conclusions

4 SSR primers successful to amplified 10 rubber of PB 260 clone. Genetic validation of 10 rubber of PB 260 clone showed no different and genetic change of clone.

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