

Selection of parent trees for Rubber (*Hevea brasiliensis*) breeding based on RAPD analysis

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Abstract. Oktavia F, Lasminingsih M, Kuswanhadi. 2011. Selection of parent trees for Rubber (*Hevea brasiliensis*) breeding based on RAPD analysis. *Nusantara Bioscience* 3: 124-129. The parent trees' clones usually originate from the previous generation having the potential of high production with better agronomical characters. Although, phenotype characters can determine the genetic variability among accessions, they are highly sensitive to environmental factors, so it is often difficult to identify the difference between closely related clones. The genetic variability or phylogenetic relationships among rubber clones can be analyzed using RAPD method, and based on the result, the parent trees can be selected. This research was aimed to analyze the genetic distance among rubber clones using RAPD method. Analysis was conducted on 45 rubber clones with 12 random primers. Pair-wise comparisons of unique and shared polymorphic amplification products were used to generate similarity coefficients. These coefficients were employed to construct a dendrogram by using an Unweighted Pair-Group Method with Arithmetical Averages (UPGMA). The amplification of genomic DNA from 45 clones yielded 2408 DNA fragments, ranging in size from 250 bp to 3000 bp. The range of genetic similarity matrix was very wide (59.18%-94.23%). It indicated that most of the clones have a low level of polymorphism. The lowest genetic similarity (59,18%) was found between RRIC 110 and AVROS 352 clones, while the highest (94,23%) was between IRR 41 and IRR 42 clones. Cluster analysis showed that 45 clones of rubber were divided into two groups, the biggest group consisted of 30 clones, while the other one consisted of 15 clones with a genetic similarity value of 0,73.

Keywords: rubber, RAPD, hand pollination, hevea breeding, parents trees.

Abstrak. Oktavia F, Lasminingsih M, Kuswanhadi. 2011. Pemilihan pohon induk untuk pemuliaan karet (*Hevea brasiliensis*) berdasarkan analisis RAPD. *Nusantara Bioscience* 3: 124-129. Klon-klon yang digunakan sebagai pohon induk biasanya berasal dari generasi sebelumnya yang memiliki potensi produksi tinggi dengan karakter agronomi yang lebih baik. Karakter fenotipe dapat menentukan variabilitas genetik di antara aksesori, namun sangat sensitif terhadap faktor-faktor lingkungan, sehingga sering kali sulit untuk mengidentifikasi perbedaan antar klon. Variabilitas genetik atau hubungan kekerabatan antar klon karet dapat analisis dengan menggunakan metode RAPD, dan berdasarkan hasil analisis tersebut klon-klon tua dapat dipilih. Penelitian ini bertujuan untuk menganalisis jarak genetik antar klon karet dengan menggunakan metode RAPD. Analisis dilakukan pada 45 klon karet dengan 12 primer acak. Perbandingan pita polimorfik hasil amplifikasi digunakan untuk menghasilkan koefisien kesamaan. Koefisien ini berguna untuk menyusun dendrogram dengan menggunakan *Unweighted Pair-Group Method with Arithmetical Averages* (UPGMA). Amplifikasi DNA genom dari 45 klon menghasilkan 2408 fragmen DNA yang berukuran 250-3000 bp. Kisaran matriks kesamaan genetik cukup luas (59,18%-94,23%). Hal ini menunjukkan bahwa sebagian besar klon memiliki tingkat polimorfisme yang rendah. Kesamaan genetik terendah (59,18%) ditemukan antara klon RRIC 110 dan AVROS 352, sedangkan yang tertinggi (94,23%) antara klon IRR 41 dan IRR 42. Analisis pengelompokan menunjukkan bahwa 45 klon karet terbagi menjadi dua kelompok, kelompok terbesar terdiri dari 30 klon, sedangkan yang lain terdiri dari 15 klon dengan nilai kesamaan genetik 0,73.

Kata kunci: karet, RAPD, persilangan buatan, pemuliaan karet, pohon induk.

INTRODUCTION

Rubber tree (*Hevea brasiliensis* Muell. Arg.) belongs to the family of Euphorbiaceae. It is an important crop-producing natural rubber which have been cultivated in South-East Asia. The plant is indigenous to the Amazon basin of South America, and has a high heterozygotic genetic base. Recently high yielding clones have been produced as a result of selection program conducted by Rubber Research centers.

High yielding clones are generally obtained through longterm breeding programs by crossing between clones

having special characters. The goal of rubber breeding is to obtain superior clones which have a high production of latex or wood, and are resistant to diseases (IRRI 2005). The selected parent clones usually originate from the previous generation having a high production potential and better agronomical characters. Although, phenotype characters are helpful in determining the genetic variability among accessions, they are highly sensitive to environmental factors, so it is often very difficult to identify the difference among closely related clones. The information on genetic variability is required to select the parent in order to avoid the use of closely related clones. That Information can also

describe correctly the level of genetic difference among clones. Crossing of the clones having high genetic distance will increase the possibility of obtaining a heterosis hybrid vigor.

Molecular markers such as isozymes (Chevallier 1988; Chaidamsari et al. 1993; Seguin et al. 1995; Yeang et al. 1998), restriction fragment length polymorphism (RFLP) (Besse et al. 1994; Luo et al. 1995), and microsatellite (Lekawipat et al. 2003) have already been applied to investigate the polymorphism among rubber tree clones and used in varietal identification. Another technique which has been developed with detailed results is the marker of Random Amplified Polymorphic DNA (RAPD). According to Williams et al. (1990), RAPD was one of the techniques of DNA analysis based on random amplified DNA sequences in polymerase chain reaction (PCR) by using an arbitrary primer. Among techniques for DNA polymorphism analysis, PCR-based RAPD is a relatively simple and efficient method. Here, only a small quantity of DNA is required to develop DNA fingerprints. Besides, knowledge of the targeted plant genome is not necessary and it can distinguish the closely related genotypes.

RAPD technique has already been applied in research with several aims. The RAPD has been used to determine genetic relationships for several plant species like coffee (Toruan-Mathius et al. 1998) and cocoa (Wilde et al. 1992; Toruan-Mathius et al. 1997). RAPD can also be used to identify markers related to resistance to certain diseases in coffee (Toruan-Mathius et al. 1995; Agwanda et al. 1997) and tea (Sriyadi et al. 2002). In rubber, a number of RAPD markers have been used to identify clones (Nurhaimi-Haris et al. 1998; Venkatachalam et al. 2002; Zewei et al. 2005), to identify markers related to diseases (Toruan-Mathius et al. 2002), to identify markers related to character of dwarf genome-specific (Venkatachalam et al. 2004) and to identify a sequence having partial homology with proline-specific permease gene (Venkatachalam et al. 2006).

The objective of the present research was to use RAPD markers to estimate the genetic distance among rubber clones in germplasm of Sembawa Research Station, Indonesian Rubber Research Institute. The result will be used in parents trees selection for hevea breeding program.

MATERIAL AND METHODS

Planting material

This trial was done on 45 cultivated clones, which consisted of elite rubber clones in Indonesia. As a source of DNA, young rubber leaves measuring about 3-5 cm long and 1.5-1.7 cm wide were used. All of the 45 accessions have been planted in hand pollination garden of Sembawa Research Station, Indonesia Rubber Research Institute.

DNA extraction and RAPD analysis

DNA extractions were performed according to the procedure described by Orozco-Castillo et al. (1994) which was modified, specifically by the addition of polyvinyl-pyrrolidone (PVPP), in each sample at the time of grinding in liquid nitrogen to fine powder using pestle and

mortar. The powdered was transferred to Eppendorf tube using spatula and 5 mL of DNA extraction buffer (1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 30 mM β -mercaptoethanol) was added immediately. The mixture was homogenized by gentle shaking, and incubated at 65°C for 30 minutes. An equal volume of chloroform-isoamyl alcohol (24:1) was added, and then spun at 11.000 rpm for 3 minutes. The supernatant was transferred to a new Eppendorf tube. To precipitate DNA, an equal volume of isopropanol was added and the mixture was refrigerated 4°C for at least 30 minutes. The DNA was pelleted by centrifugation at 11.000 rpm for 10 minutes. The pellet was then washed with ice-cold of 70% (v/v) ethanol and dried. Finally, the DNA pellet was dissolved in 1 mL TE (10 mM Tris-HCl pH 8,0; 1 mM EDTA) and stored at -20°C, until it was used as DNA template in PCR.

The Quality of DNA was confirmed by agarose gel electrophoresis (0.8% agarose) with ethidium bromide in TAE buffer (40 mM Tris-acetate pH 8.1, 1 mM EDTA). The samples were loaded into agarose gel with 0.25% bromophenol blue, 0.25% Xylene cyanol FF and 30% glycerol in water, as loading buffer. The DNA purity was determined by using a spectrophotometer based on the ratio of optical density (OD) value between 260 nm and 280 nm wavelength. DNA concentration was determined, based on the value of OD at 260 nm (1 OD unit = 50 μ g/mL DNA) (Sambrook et al. 1989).

In PCR analysis, arbitrary primers selection was based on its capability to produce different DNA fragments in various clones, in order to obtain polymorphic bands. Each primer consists of 10 bases and contains 60-70% G and C base (Table 1). The Primer used was 20 kinds of Kit-N primers produced by Operon Technologies (Alameda, USA), which had been selected randomly.

Table 1. RAPD primer nucleotide sequence

Primer	Primer sequences (5' → 3')	Primer	Primer sequences (5' → 3')
OPN-01	5'-CTCACGTTGG-3'	OPN-11	5'-TCGCCGCAAA-3'
OPN-02	5'-ACCAGGGGCA-3'	OPN-12	5'-CACAGACACC-3'
OPN-03	5'-GGTACTCCCC-3'	OPN-13	5'-AGCGTCACTC-3'
OPN-04	5'-GACCGACCCA-3'	OPN-14	5'-TCGTGCGGGT-3'
OPN-05	5'-ACTGAACGCC-3'	OPN-15	5'-CAGCGACTGT-3'
OPN-06	5'-GAGACGCACA-3'	OPN-16	5'-AAGCGACCTG-3'
OPN-07	5'-CAGCCCAGAG-3'	OPN-17	5'-CATTGGGGAG-3'
OPN-08	5'-ACCTCAGTCT-3'	OPN-18	5'-GGTGAGGTCA-3'
OPN-09	5'-TGCCGGCTTG-3'	OPN-19	5'-GTCCGTA CTG-3'
OPN-10	5'-ACA ACTGGGG-3'	OPN-20	5'-GGTGCTCCGT-3'

DNA amplification was carried out following the method of Williams et al. (1990). The PCR reaction was in 25 μ L volume reaction mixture containing 1.0 μ L DNA template, 1.5 μ L MgCl₂ 25 mM, 2.5 μ L PCR 5x buffer, 0.5 μ L dNTP mix, 0.2 μ L tag DNA polymerase (5 unite), 1.0 μ L primer 10 mM and demineralized water was added until the volume was 25 μ L. PCR amplification by using Biometra machine was programmed for 45 cycles of denaturation for 2 minutes at 94°C, annealing for 1 minute

at 53°C, and extension for 2 minutes at 72°C. The last cycle was followed by incubation for 4 minutes at 72°C.

DNA amplification products were separated by 1.4% agarose gel in 1x TAE buffer (0.04 M Tris-acetic in 1 mM EDTA) and added 5 µL loading dye. DNA migration was conducted for 1 hour and 15 minutes at 50 volts. The gel was then stained in 0,5 µg/mL ethidium bromide, and washed with aquadest. DNA fragments were visualized by UV transilluminator and a picture of DNA fragment in the gel was taken by Polaroid camera. Molecular weight of DNA was determined by the migration of DNA marker (1 Kb DNA ladder).

Data analysis

The DNA fragments used in RAPD analysis were the one which could be clearly identified by determining its presence (1) or absence (0). Based on the data of DNA fragment, genetic distances were estimated by a dendrogram which was constructed following the UPGMA method, and the similarity matrix among clones was analyzed by using NTSYSpc program (Rohlf 1993).

RESULT AND DISCUSSION

RAPD analysis

Forty primers have been used to amplify the DNA of GT 1 clone to select the best primer. The amplification could obtain 181 fragments with the range of 0-8 fragments per primer. Primers were selected according to the number of DNA fragments obtained in PCR. From 40 kinds of primers used, twelve primers (OPN-05, OPN-06, OPN-08, OPN-10, OPN-11, OPN-12, OPN-17, OPH-01, OPH-03, OPH-05, OPH-18, and OPH-19) produced the highest number of DNA fragment. These primers were then used to amplify 45 rubber clones.

DNA amplification of 45 rubber clones by 12 primers produced 2408 DNA fragments which formed 95 DNA

fragment patterns with the size of DNA fragment of 250-3000 bp. The size of DNA fragments amplified depend on the DNA region surrounded by two primers (McPherson et al. 1992). In general, the fragment pattern obtained on all 45 clones rubber tree was still relatively the same (monomorphic). When a similar pattern was obtained from different clones by using a primer, it showed that primer could not be used to track genetic difference among those analyzed clones.

Among the 78 DNA fragment patterns obtained, 2 specific DNA fragments were found on certain clone i.e. fragment no. 1 which was found only on GT 1 and fragment no. 11 on PN 177 which were amplified by OPN-08 (Figure 1). Beside many specific DNA fragments found only on certain clones, many fragments with certain size were also only found in a small group of clones, for example, the fragment with the size of 850 bp which was amplified by OPN-10 primer could be observed on IRR 39 and IRR 44 clone only. These fragments were assumed to be related to a specific genetic character that was inherited by their parents or a specific character that is formed genetically in an individual. It could be shown in IRR 39 and IRR 44 clone which had the same character in one of their parents, that is LCB 1320. We expect those specific DNA fragments can be furthermore analyzed, cloned and sequenced. It may be used as a specific marker like SCAR or CAPS.

To know the relationship between specific DNA fragments with a certain character, a more detailed molecular study is needed. This study can be carried out by taking into account the agronomical characters found in plant groups which have the same specific DNA fragment, and then doing DNA hybridization using these fragments as a probe. Another method can be applied using more specific molecular techniques such as analysis at mRNA level related to the already known agronomical characters of each clone like high production or resistance to a certain disease.

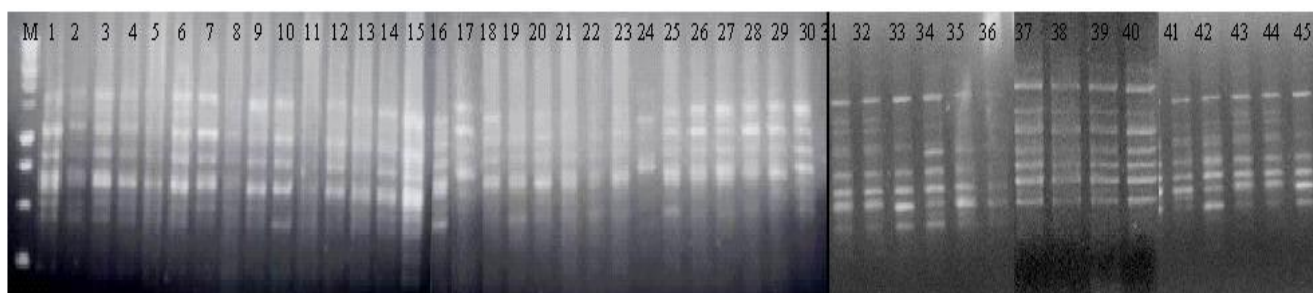


Figure 1. Amplification products generated from 45 clones of rubber by using OPN-08 primer.

Note:

1. IRR 24	8. RRIM 600	15. PN 177	22. PR 300	29. RRIC 101	36. H. benthamiana	43. BPPJ 3
2. IRR 39	9. Tjir 1	16. RRIM 901	23. PB 260	30. RRIM 712	37. IRR 220	44. BN 1
3. IRR 104	10. PN 138	17. RRIM 911	24. GT1	31. IRR 42	38. PB 235	45. AV 352
4. IRR 118	11. PB 217	18. PN 680	25. PR 303	32. IRR 41	39. IRR 204	
5. IRR 105	12. IRR 44	19. BPM 109	26. LCB1320	33. TM 5	40. RRIC 101	
6. RRIM 2004	13. IRR 100	20. BPM 24	27. RRIC 100	34. TM 8	41. IRR 32	
7. RRIM 2020	14. BPM 107	21. BPM 1	28. RRIC 110	35. IRR 18	42. TM 9	

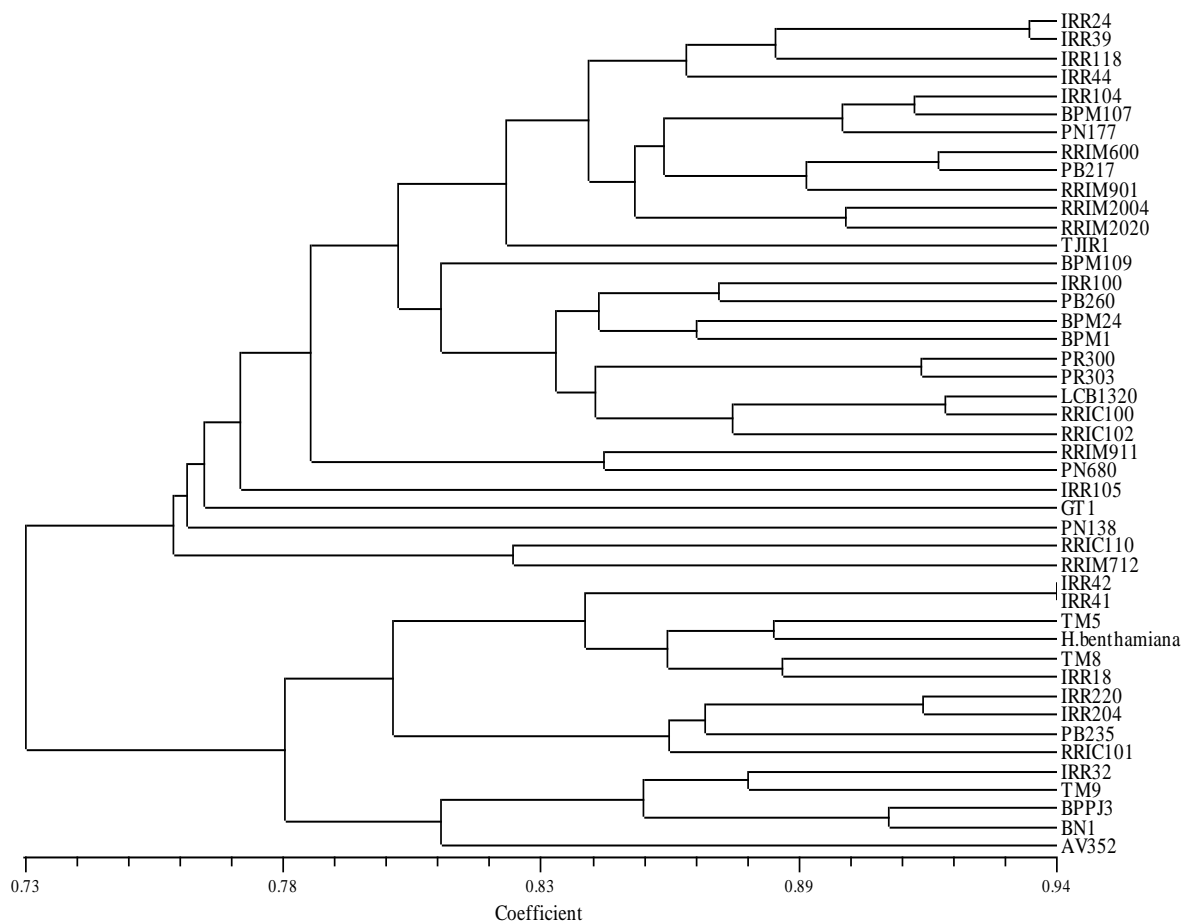


Figure 2. Dendrogram of 45 rubber clones based on the UPGMA method

Genetic relationship

The genetic similarity matrix based on UPGMA method (Table 2) indicated that the proportion of the same DNA fragments among clones was quite high, ranging between 59.18% and 94.23%. The lowest genetic similarity (59.18%) was found between RRIC 110 and AVROS 352 clone, while the highest (94.23%) was between IRR 41 and IRR 42 clone. This showed that the genetic variability of clones analyzed by using OPN-05, OPN-06, OPN-08, OPN-10, OPN-11, OPN-12, OPN-17, OPH-01, OPH-03, OPH-05, OPH-18 dan OPH-19 primers was low. It might be caused by the limited number of DNA marker which was used to distinguish it, so that it could not differentiate the analyzed clones yet. Some publications showed that in genetic analysis to know the relationship number genetic among population need a minimum number of 200 different patterns of DNA fragments. If every primer can produce 5-9 different DNA fragments, it means that on polymorphism observation or analysis of genetic relationship among clones can use 22-40 primers to track genetic difference of these clones. While on this research we used 12 primers only and obtained a total of 95 DNA fragments, so that it still obtained a low carefulness level.

Cluster analysis of clones by using 12 primers was shown in dendrogram of 45 clones (Figure 2). According to the similarity level of 0.73, 2 groups were separated, a big group consisting of 30 clones and a small one of 15 clones.

These groups could be divided further into many subgroups with different genetic distances. The dendrogram showed that many clones which had the same character in one of their parents and came into the same group, as IRR 41 and IRR 42 clone with LCB 1320 and F 351 clone as their parent, have a genetic similarity of 0.94. This could also be observed between IRR 24 and IRR 39 clone that have that same parent of LCB 1320 with genetic similarity 0.93, so as RRIM 2004 and RRIM 2020 clone with the same parent of PB 5/51 clone, come that into the same group with genetic similarity about 0.90. However, not all clones with the same parent come into the same group. This could be observed on PB 260 and PB 5/51 clone was not in the same group with PB 217 and RRIM 901 clone. This case was also found for IRR 24 and IRR 39 clone that came into different groups with their parent LCB 1320 clone. Nurhaimi-Haris et al. (1998) and Toruan-Mathius et al. (2002) reported the same condition between RRIC 100 and RRIM 600 clone which had the same clone, PB 86, as one of their parents. The analysis showed that RRIC 100 and RRIM 600 were in different groups. That could also be observed between PPN 2447 and PPN 2444 clone which originated from LCB 1320 illegitimate, come into different group (Nurhaimi-Haris et al. 1998).

Some clones had high genetic similarity but really they did not have genealogy relationship such as IRR 104 and BPM 107 clone had genetic similarity of 0,91; 0,915 for

RRIM 600 and PB 217 clone; 0,9 for PR 300 and PR 303 clone1; 0,915 for RRIC 100 and LCB 1320 clone. Varghese et al. (1997) reported that it could happen because generally, the rubber tree was a crossed pollination plant where F1 hybrid multiplied by a vegetative method and also these clones were very heterozygous. Segregation caused proportion of hybrid alleles from parents to vary. This may be able to explain why the parents and hybrid come into different groups.

From the dendrogram obtained by UPGMA method, we could know the genetic distance between 45 clones analyzed. This genetic distance can be used as a consideration in selecting the parent clones for hand pollination. To obtain a heterosis effect, the clones crossed should have a wide genetic distance (low similarity level).

CONCLUSION

The DNA polymorphism of rubber clones based on RAPD analysis could be produced using OPN-05, OPN-06, OPN-08, OPN-10, OPN-11, OPN-12, OPN-17, OPH-01, OPH-03, OPH-05, OPH-18, and OPH-19 primers. The genetic similarity among the analyzed clones was quite high, i.e. between 59.18%-94.23%. The lowest genetic similarity (59,18%) was found between RRIC 110 and AVROS 352 clones, and the highest (94.23%) was found between IRR 41 and IRR 42 clones. UPGMA with cluster analysis showed that 45 clones of rubber were divided into two groups, the first one consisted of 30 clones, while the other one consisted of 15 clones with a genetic similarity value of 0.73.

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