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Genetic Relationship of Wickham and IRRDB 1981 Rubber Population Based on RAPD Markers Analysis

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Rubber hand pollination in Indonesian Rubber Research Institute program currently uses Wickham population which genetic analysis showed that genetic diversity of this population is narrow. The development of breeding activity has made the genetic base narrower by inbreeding. In order to solve this problem can use a new genetic resource that is the rubber germplasm IRRDB 1981 population. The genetic relationship between these populations is important to choose parents to avoid closely related genotypes in hand pollination. Therefore RAPD analysis was carried out using four selected primers i.e. OPH-03, OPH-05, OPH-18 and OPN-06. The result showed that Wickham and IRRDB 1981 population were separated into two different big groups with genetic similarity value of 0.64, and those big groups were separated further into many small sub groups with some genetic similarity level. The genetic similarity matrix showed that Wickham and IRRDB 1981 population has a range of genetic similarity 0.37– 0.98. The highest genetic similarity was found between RRIM 600 and PN 621, while the lowest was between BPM 1 and RRIC 100. Value in this matrix showed the genetic diversity between each clone. Based on this result, rubber genotypes of Wickham population could be crossed with genotypes of IRRDB 1981 population by choosing genotypes that have low genetic similarity.

Key words: *hevea brassiliensis*, germplasm analysis, molecular markers, parents selection

INTRODUCTION

One important effort to increase rubber productivity is by using the superior rubber clones as a planting material. Superior clones can be obtained by selecting the best plants from diverse genetic resources and preparing the new genetic diversity by hand pollination or natural pollination (Tan 1987). These limitation is both in terms of quality and quantity of the available genetic diversity.

Currently, obtaining superior clones by hand pollination is still a problem. Based on the experience hand pollination Sungei Putih Research Centre, success of hand crossing was about 2.5%. One limiting factor was the limited genetic resources that is used for rubber hand pollination. Generally, parents trees for rubber hand pollination use the Wickham population, obtained from the expedition in Boim, Brazilia in 1876. Genetic analysis showed that genetic diversity of this population is narrow (Chevallier 1988; Luo *et al*. 1995; Nurhaimi-Haris *et al*. 1998; Yeang *et al*. 1998). The development of breeding activity has made the genetic base narrower by inbreeding. Inbreeding will also increase the possibility of genetic erosion of many individual genes.

In order to solve the problem of limited genetic resources, the Indonesian Rubber Research Institute together with others countries of the International Rubber Research Development Board (IRRDB) conducted an expedition to Amazon valley to explore new genetic resources in 1981. From this expedition, Indonesia recieved 7,788 genotypes that was called IRRDB 1981 germplasm. Base on their growth and production, it has been known that the production potency of the IRRDB 1981 germplasm was low. Several genotypes produced more timber than rubber. This informations showed that there was no superior genotype for all characters, so these genotypes would be used as genotype resource as parents tree in a hand pollination program.

One factor that influences the succees of crossing is the genetic distance among clones. Using closely related genotypes could obtain small scion and bad (Allard 1960). The genetic distance could be studied for example from phenotype analysis, however this technique have a weakness because phenotypes are highly sensitive to environmental factors. Another alternative is to use moleculer markers.

Several moleculer markers which can be used to study the genetic relationship are isozymes, Simple Sequence Repeat (SSR), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), and Random Amplified Polymorphic DNA (RAPD). These techniques have advantages and weaknesses. RAPDs is moleculer technique that works base on amplification of DNA fragment by Polymerase Chain Reaction (PCR) using a random primer (William *et al*. 1990; Welhs & McClelland 1990). This technique is simpler than the others, does not use redioactivity, does not need a DNA specific probe, needs less DNA sample (McPerson *et al*. 1992; Yu *et al*. 1993) and was not influenced by

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environmental factors (Marn *et al*. 1996). The RAPD have been used to determine the genetic relationships of several plant species like coconut (Roslim *et al*. 2003), sago palm (Abbas *et al*. 2009), and mulberry (Hasan *et al*. 2009).

The objective of the research is explore the genetic relationship between rubber genotypes of the Wickham population and the IRRDB 1981 population by using RAPD markers, to give a basis for selection of parent rubber tree in a hand pollination program.

MATERIALS AND METHODS

Planting Material. This trial used 10 clones from the Wickham population that originated from several countries, and 15 genotypes of IRRDB 1981 population that originated from three states in Brazil from different districts (Table 1). These populations have been conserved in the germplasm garden of the Sembawa Research Centre, Indonesia Rubber Research Institute.

DNA Extraction. DNA extractions were performed according to the procedure described by Orozco-Castillo *et al*. (1994) which was modified by the addition of 0.1% polivinylpolipyrolidon (PVPP) to each sample at the time of grinding in liquid nitrogen to fine powder by using pestle and mortar. The powder then was transferred to an Eppendorf tube using a 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-mercaptoetanol) was added immediately. The mixture was homogenized by gentle shaking, and incubated at 65 °C for 30 min. An equal volume of chloroform-isoamylalcohol (24:1) was added, and then centrifuged at 11,000 rpm for 3 min. The supernatant was transferred to a new Eppendorf tube. To precipitate DNA, an equal volume of isopropanol was added and the mixture was refrigerated at 4 °C for at least 30 min. The DNA was pelleted by centrifugation at 11,000 rpm for 10 min. The pellet then was washed with ice cold 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 TBE buffer (40 mM Tris-acetate pH 8.1, 1 mM EDTA). The samples were loaded onto the 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 and 280 nm wave length. DNA concentration was based on the value of OD at 260 nm (1 OD unit = 50 µg/ml DNA; Sambrook *et al*. 1989).

PCR Analysis. Four primers were used for DNA amplification i.e. OPH-03, OPH-05, OPH-18, and OPN-06. These were from test of 40 primers on the GT 1 clone. GT 1 is a superior rubber clone that used commonly as a control in progeni test. Each primer consisted of 10 bases and contained 60-70% G and C bases. DNA amplification was carried out following the method of William *et al.* (1990). The PCR reaction were in 25 µl volume reaction mixture containing 1.0 µl DNA template, 1.5 µl MgCl2 25 mM, 2.5 µl PCR 5x buffer, 0.5 µl dNTP mix, 0.2 µl tag DNA polymerase (5 U/µl), 1.0 µl 10 mM primer, with demineralized water was to give a total volume of 25 µl. PCR amplification using a Biometra machine programmed for 45 cycles of denaturation for 2 minute at 94 °C, annnealing for 1 minute at 53 °C, and extention for 2 minute at 72 °C. The last cycle was followed by incubation for 4 minute at 72 °C.

DNA amplification products were separated on 1% agarose gels in 1x TBE buffer (0.04 M Tris-acetic in 1 mM EDTA) with 5 µl loading dye. DNA migration was conducted for 1 hour and 15 minutes at 50 volt. The gel then was stained in 0.5 µg/ml ethidium bromide, and washed with distilled water. DNA fragments were visualized using a UV transiluminator and a picture of DNA fragment in the gel was taken using a polaroid camera. The molecular weight of DNA was estimated by the migration of DNA marker (1 kb DNA ladder) from Sigma.

Data Analysis. The DNA fragments used in RAPD analysis were the fragments that could be clearly identified, allowing presence (1) or absence (0) of the fragment to be recorded. Based on the DNA fragment analysis, genetic distances were estimated by a dendogram which was constructed using the UPGMA method, and a similarity matrix among clones was generated by using the NTSYSpc program (Rohlf 1993).

Table 1. List of origin of Wickham and IRRDB 1981 populations

Wickham population		IRRDB 1981 population	
Name	Country of origin	Name	Country/state/distric of origin
BPM 24	Indonesia	PN 677	Brazil/Mato Grosso/Itauba
BPM 1	Indonesia	PN 667	Brazil/Mato Grosso/Itauba
PR 300	Indonesia	PN 124	Brazil/Rondonia/Jiparana
SBW 2004	Indonesia	PN 545	Brazil/Rondonia/Jiparana
SBW 2020	Indonesia	PN 316	Brazil/Rondonia/Pimenta Bruno
RR IM 600	Malaysia	PN 560	Brazil/Rondonia/Calama
SBW 901	Indonesia	PN 485	Brazil/Rondonia/Jiparana
SBW 911	Indonesia	PN 452	Brazil/Rondonia/Calama
PB 260	Malaysia	PN 93	Brazil/Rondonia/Calama
RRIC 100	Srilangka	PN 666	Brazil/Mato Grosso/Itauba
		PN 494	Brazil/Mato Grosso/Itauba
		PN 373	Brazil/Acre/Feijo
		PN 621	Brazil/Mato Grosso/Itauba
		PN 295	Brazil/Mato Grosso/Itauba
		PN 604	Brazil/Acre/Feijo

RESULTS

Amplification of 25 rubber clones using four selected primers produced 529 DNA fragments which formed 40 DNA fragment patterns with the size of DNA fragments from 250 to 3000 bp. Each primer formed between 7 and 14 DNA fragment patterns. The result of amplification with the OPH-03 primer yielded 74 DNA fragments with 7 fragment patterns (Figure 1a), one pattern was monomorphic and the other six patterns were polymorphics. Amplification by the OPH-05 primer produced 122 DNA fragments with 9 patterns, with one monomorphic and 8 polymorphic patterns (Figure 1b). The OPH-18 primer amplified 169 DNA fragment with 10 fragment patterns, which consisted of 2 patterns monomorphic and 8 polymorphic patterns (Figure 1c). 164 DNA fragments with 14 fragment patterns were obtained from amplified with the OPN-06 primer, with 3 monomorphic and 11 polymorphic patterns (Figure 1d). Out of all primers that have been used to amplify 25 clones, OPN-06 primer obtained the highest fragment pattern, 11 polymorphic of 14 total fragment patterns, thus having 78.6% polymorphism.

The coefficient of genetic similarity which was close to 1 showed the closer relationship among the member of the group, whereas values closer to 0 means the further relationship. Genetic similarity levels have been calculated from 40 DNA fragment patterns, ranging between 0.37-0.98 (Table 2). The lowest genetic similarity value was 0.37, obtained from pair of RRIM 600 and PN 621, whereas the highest genetic similarity value was 0.98, obtained from pair of BPM 1 and RRIC 100. The cluster analysis of clones by using 4 primers is shown in dendogram of 25 clones (Figure 2). The dendogram showed that Wickham and IRRDB 1981 population separated in to two different big groups (group I and II) with a genetic similarity value of 0.64 (63%) or the genetic diversity of these population was 0.36 (36%). These two big groups were separated further into some small groups with different genetic similarity coefficients. Group II was separated in to two groups i.e. group IIA that consists of six clones and group IIB that consists of nine clones with a genetic similarity value of 0.69. In the group I, from the Wickham population, there was a pair of clones that have the same parent in the same group, that is SBW 2004 and SBW 901. These clones

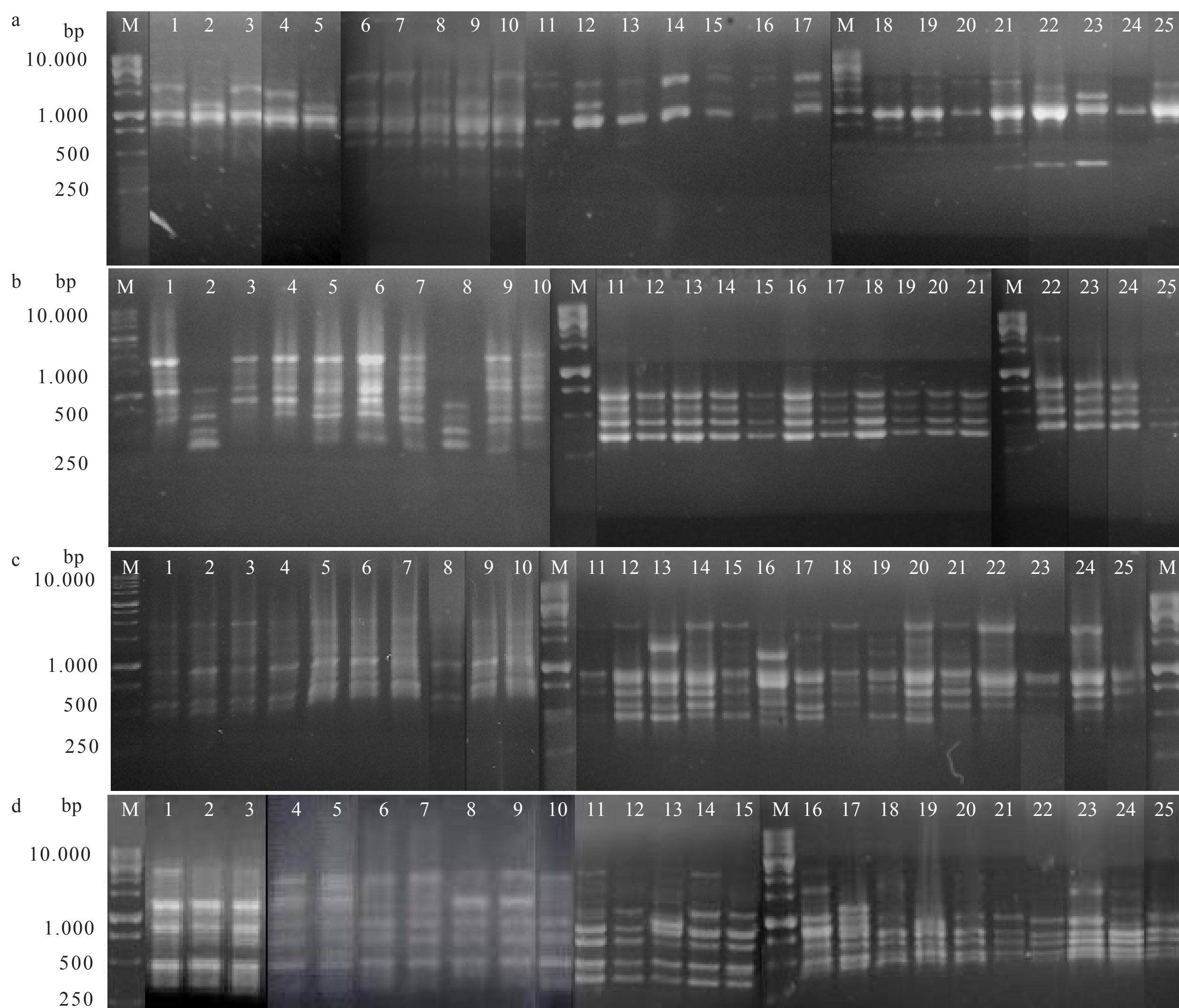

Figure 1. Amplification products generated from 25 clones of rubber using. a. OPH-03, b. OPH-05, c. OPH-18, and d. OPN-06. primers. M: 1 kb Ladder, 1: RRIM 2004, 2: RRIM 2020, 3: RRIM 600, 4: RRIM 901, 5: RRIM 911, 6: BPM 24, 7: BPM 1, 8: PR 300, 9: PB 260, 10: RRIC 100, 11: PN 677, 12: PN 667, 13: PN 124, 14: PN 545, 15: PN 316, 16: PN 560, 17: PN 485, 18: PN 452, 19: PN 93, 20: PN 666, 21: PN 494, 22: PN 373, 23: PN 621, 24: PN 295, 25: PN 604.

Table 2. Genetic similarity matrix between 25 clones of rubber based on the propotion of shared RAPD fragments

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1.00																								
0.80	1.00																							
0.84	0.80	1.00																						
0.89	0.77	0.86	1.00																					
0.80	0.80	0.72	0.81	1.00																				
0.81	0.78	0.78	0.87	0.86	1.00																			
0.84	0.80	0.72	0.81	0.92	0.94	1.00																		
0.74	0.88	0.69	0.71	0.75	0.80	0.83	1.00																	
0.79	0.84	0.75	0.80	0.92	0.89	0.96	0.82	1.00																
0.85	0.82	0.73	0.83	0.90	0.96	0.98	0.85	0.93	1.00															
0.72	0.68	0.63	0.65	0.69	0.66	0.69	0.71	0.68	0.70	1.00														
0.65	0.80	0.60	0.61	0.66	0.59	0.62	0.73	0.65	0.63	0.76	1.00													
0.60	0.70	0.55	0.61	0.62	0.54	0.57	0.68	0.60	0.59	0.80	0.78	1.00												
0.75	0.80	0.71	0.68	0.68	0.60	0.63	0.74	0.66	0.65	0.81	0.85	0.85	1.00											
0.75	0.85	0.66	0.68	0.68	0.69	0.72	0.83	0.71	0.73	0.77	0.85	0.80	0.90	1.00										
0.70	0.66	0.57	0.59	0.64	0.57	0.64	0.69	0.62	0.61	0.76	0.74	0.69	0.75	0.71	1.00									
0.62	0.76	0.57	059	0.63	0.60	0.68	0.79	0.71	0.65	0.68	0.80	0.70	0.71	0.76	0.71	1.00								
0.62	0.71	0.61	0.59	0.63	0.60	0.68	0.74	0.71	0.65	0.68	0.65	0.70	0.71	0.71	0.75	0.80	1.00							
0.62	0.71	0.61	0.58	0.59	0.51	0.59	0.70	0.61	0.55	0.53	0.64	0.70	0.71	0.71	0.66	0.76	0.82	1.00						
0.62	0.71	0.61	0.63	0.63	0.60	0.68	0.79	0.71	0.65	0.68	0.70	0.75	0.71	0.71	0.75	0.85	0.85	0.82	1.00					
0.57	0.71	0.61	0.53	0.63	0.55	0.63	0.70	0.66	0.60	0.68	0.64	0.64	0.66	0.66	0.76	0.71	0.87	0.72	0.82	1.00				
0.60	0.70	0.65	0.57	0.66	0.59	0.66	0.68	0.69	0.63	0.66	0.63	0.63	0.65	0.65	0.69	0.75	0.80	0.75	0.85	0.91	1.00			
0.40	0.59	0.37	0.41	0.61	0.48	0.57	0.63	0.60	0.53	0.66	0.68	0.74	0.59	0.59	0.65	0.70	0.64	0.64	0.70	0.76	0.74	1.00		
0.53	0.68	0.52	0.55	0.69	0.57	0.65	0.71	0.68	0.61	0.80	0.77	0.77	0.73	0.68	0.73	0.78	0.73	0.62	0.78	0.80	0.77	0.84	1.00	
0.47	0.62	0.51	0.43	0.55	0.46	0.55	0.61	0.57	0.51	0.64	0.66	0.72	0.62	0.62	0.68	0.74	0.80	0.75	0.80	0.87	0.84	0.80	0.83	1.00

1: SBW 2004, 2: SBW 2020, 3: RRIM 600, 4: SBW 901, 5: SBW 911, 6: BPM 24, 7: BPM 1, 8: PR 300, 9: PB 260, 10: RRIC 100, 11: PN 677, 12: PN 667, 13: PN 124, 14: PN 545, 15: PN 316, 16: PN 560, 17: PN 485, 18: PN 452, 19: PN 93, 20: PN 666, 21: PN 494, 22: PN 373, 23: PN 621, 24: PN 295, 25: PN 604.

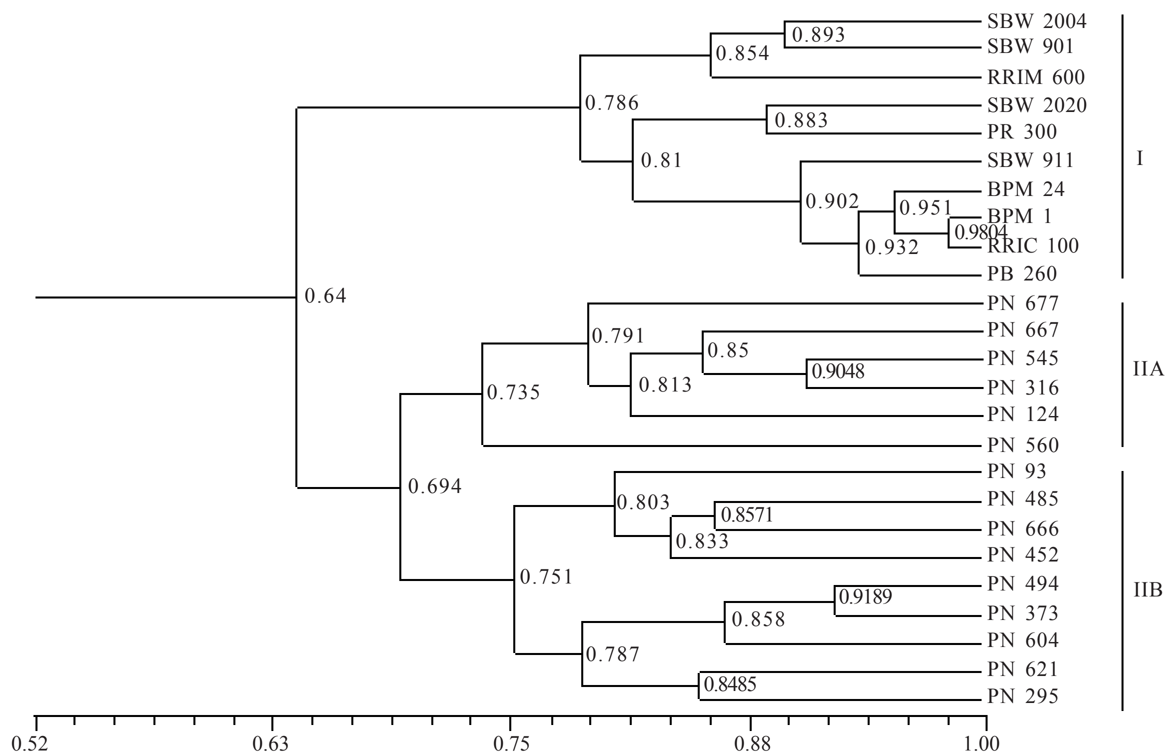

Figure 2. Dendogram of genetic relationship among 25 rubber clones from the Wickham and IRRDB 1981 populations generated using the UPGMA method.

came from parent of PB 5/51 clone with genetic similarity value of 0.89. Based on genetic similarity value of less than 0.5, we can be selected seven pair of clones as parents in hand pollination program i.e SBW 2004 with PN 621, RRIM 600 with PN 621, SBW 901 with PN 621, BPM 24 with PN 621, SBW 2004 with PN 604, SBW 901 with PN 604, and BPM 24 with PN 604 (Table 3).

Table 3. Pairs of rubber clones that have genetic similarity values less than 0.50

♂ ╲ ♀	SBW 2004	RRIM 600	SBW 901	BPM 24
PN 621	+	+	+	+
PN 604	0.47	-	+	+

♀ : Female parent, ♂ : male parent, +: used as parent, -: did not use as parent.

DISCUSSION

The genetic relationship of Wickham and IRRDB 1981 population was determined by polymorphism of DNA fragment patterns using RAPDs. The highest number of polymorphisms was yielded by OPN-06 primer, showing that the OPN-06 primer can detect more genetic variation than other primers. Polymorphisms indicate genetic variation in a population. Polymorphisms are generated during DNA replication and are changes in the nucleotide sequence of DNA (McGregor *et al.* 2000). Polymorphisms showed different characters among the clones and can be used to determine genetic relationships between the analyzed clones.

Genetic similarity values obtained from 40 DNA fragment pattern ranged from 0.37-0.98. These values showed the percentage of genetic diversity in the analyzed population (Sriyadi *et al.* 2002). The genetic diversity matrix was based on character similarity that was represented by the number of DNA fragments (Nurhaimi-Haris *et al.* 1998). Based on the similarity genetic matrix, it can be seen that the genetic diversity between each clone is low. This is due to the high level of genetic similarity between most of the paired clones. Only seven pairs of clones has genetic similarity value under of 0.50, and ranged 0.37-0.48. High of genetic similarity values showed that these clones genetically have a close genetic relationship compared to clones that have a low genetic similarity value (Sriyadi *et al.* 2002).

High genetic similarity between the 25 clones analyzed might be due to the genomes of the 25 have low diversity, so the primers only amplified similar fragments. Therefore, more primers are needed to obtain polymorphic DNA fragment. The more primers used, the more percentage of plant genome would be represented (Karsinah *et al.* 2002), presumably giving more detected variation. In genetic analysis, the genetic relationship between an individual could be revealed by generating up to 200 distinctive fragment patterns, and the more distinctive fragment patterns would increase the validity of the analysis. There have been several publications on genetic relationships between rubber clones which used various numbers of primers to obtain distinctive fragment pattern e.g. 8 primers used to analyze 37 clones which obtained 132 DNA fragment with 68 fragment pattern (Venkatachalam *et al.* 2002) and 11 primers to analyze 79 clones which obtained 85 fragment (Nurhaimi-Haris *et al.* 1998).

Moreover, the dendogram showed that Wickham and IRRDB 1981 population were separated into two different groups and these groups were separated further from some small groups with different genetic similarity coefficients. Coefficients of genetic similarity between populations showed the genetic similarity of these populations was high which means that these populations have close genetic relationship.

In the Wickham population, there was a pair of clones that have same parent in the same group, that is SBW 2004 and SBW 901 that came from parent of PB 5/51 clone. However, not all clones that have the same parent were categorized in the same group. It is showed by SBW 2020, SBW 911, and PB 260 clones that came from the same parent PB 5/51. Likewise RRIC 100 and RRIM 600 clones that came from PB 86 were not categorized in the same group. The same result was also found by Nurhaimi-Haris *et al.* (1998) and Mathius *et al.* (2002). This might be caused by Hevea is a crossing pollinating plant and tends to be a heterozygote. As the result of free segregation and grouping of gene alleles, that caused the proportion of alleles in hybrid of each parents to be varied. Therefore, parents and hybrids might not in the same group (Varghese *et al.* 1997).

Grouping members of IRRDB 1981 population in to small groups did not reflect the origin of IRRDB 1981 population i.e. Acre, Rondonia and Moto Groso. In the first sub group (PN 677, PN 667, PN 545, PN 316, PN 124, and PN 560), clones from Mato Groso are mixed with clones from Rondonia, and this also happens within the second sub group. Although the first small sub group were dominated by clones from Rondonia (PN 93, PN 485, and PN 452), there were also clones from Mato Grosso (PN 666). In the second small sub group clones from Mato Grosso (PN 494, PN 621, and PN 295) were mixed with clones from Acre (PN 373 and PN 604). This kind of grouping could happen because these states were geographically close.

The clones from Mato Grosso and Rondonia showed the furthest relationship compared to clones which from Acre-Mato Grosso or Acre-Rondonia, as seen by polymorphism levels in this pair of groups being higher than other groups. Besse *et al.* (1994) reported that Mato Grosso and Rondonia population were the most variable populations. These populations have high polymorphism because they have more specific fragments compare to the Acre population.

In conclusion, this study indicated the genetic relationship among rubber genotypes of Wickham and IRRDB 1981 population using RAPD analysis. These populations divided into two different large groups. This will be of great help in guiding the choice of convenient populations to be used in recurrent breeding schemes being currently developed. In addition, the high resemblance of Wickham and Mato Grosso or Wickham and Acre clones has to be taken into account in Wickham x IRRDB 1981 crosses.

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