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Genetic Diversity of Wild Germplasm and Cultivated Clones of Hevea brasiliensis *Muell. Arg. Detected by RAPD Analysis*

F. OKTAVIA*#, M. LASMININGSIH* AND KUSWANHADI*

Information about genetic diversity of a population is essential in breeding programmes. Characterisation of rubber germplasm for providing genotype diversity can be accomplished by morphological or genotypic assessment. The objective of this study was to obtain information about the genetic diversity of rubber based on DNA analysis. The random amplified polymorphic DNA (RAPD) technique was used as a tool for assessing diversity and the genetic relationships among 41 wild germplasms and 45 cultivated clones of rubber. DNA polymorphisms among germplasm were scored in binary form based on the presence (1) and absence (0) of each band. A dendogram was assembled through a UPGMA cluster analysis using the NTSys programme. From the RAPD analysis using 14 random primers, a total of 86 polymorphic amplified products (81.9 % total) were obtained from both populations, wild germplasm and cultivated clones, with the levels of polymorphic fragments ranging from 66.7% to 88.9%. The level of polymorphism of the wild germplasm was higher than the cultivated clones. The dendrogram showed that the 86 accessions were separated into five clusters with different degrees of genetic similarity, while the wild germplasm was separated from the cultivated clones with a genetic similarity of 0.56. This information could be used as a guide in the selection of wild germplasms for enlarging the genetic resource in rubber breeding programmes.

Keywords: *Hevea brasiliensis*; RAPD; genetic diversity; wild germplasm; clone; polymorphism; breeding

Rubber tree (*Hevea brasiliensis*) belongs to the family of Euphorbiaceae and is the main source of natural rubber which are cultivated in Indonesia, Malaysia, Thailand and many other countries. The rubber clones cultivated in the world today known as Wickham clones were originated from a few seeds collected by Wickham from a specific location in the Amazon basin, which is the area of origin of *Hevea brasiliensis*. These clones showed a very narrow genetic base[1].

The genetic base of the cultivated clones is not sufficient to carry out a breeding programme to obtain substantial genetic improvement in rubber production or disease resistance. For this reason, an expedition was carried out in 1981 by International Rubber Research Development Board (IRRDB) member countries to three different Brazilian states namely Acre, Rondonia and Mato Grosso. These genotypes were referred to as wild germplasms and Indonesia recieved

*Sembawa Research Centre, Indonesia Rubber Research Institute, PO.BOX 30001 Palembang, Indonesia.
Corresponding author (e-mail: fetrina_oktavia@yahoo.com)

7,788 genotypes from this expedition. Exploitation of these genetic resources would benefit from information on the genetic diversity of the germplasm. Usually, a study on the genetic diversity can be based on a morphological/ phenotype assessment, but this approach has many weaknesses due to the influence of environmental factors. At the same time molecular markers analyses have been developed to detect the genetic variation of rubber germplasm.

Several molecular markers could be used to study genetic variability. They are Simple Sequence Repeat (SSR), Inter Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD). Each of these techniques have their own advantages and weaknesses. The RAPD is a molecular technique based on amplification of DNA fragments by Polymerase Chain Reaction (PCR) using a random primer[2,3]. This technique is simpler than the others because it could be done at anytime, uses no radioactive substances, needs no DNA probe, needs less DNA sample[4,5] and is not influenced by environmental factors and developmental stages[6].

RAPD has been used to determine the genetic relationships of several plant species like thymus[7], sago palm[8], wormwood[9], mulberry[10] and carrot[11]. Besides that, this method could also be used to identify markers resistant to certain diseases in coffee[12,13] and tea[14]. In rubber, a number of RAPD markers have been used to identify clones[15–17], markers related to disease[18], markers related to character of dwarf genome-specific[19] and a sequence having partial homology with proline-specific permease gene[20].

This study reports the application of RAPD markers to study the genetic diversity among 86 genotypes of *H. brasiliensis* germplasm consisting 45 genotypes of wild germplasm and 41 genotypes of Wickham clones. The information obtained will be used as a basis for the selection of appropriate wild genotypes for enlarging the genetic resource in rubber breeding programmes.

MATERIALS AND METHODS

Planting Material

The trial was done on 86 genotypes that consist of 41 Wickham clones from several countries and 45 genotypes of wild germplasm from three states and different districts in Brazil (*Table 1*). These genotypes have been conserved in a germplasm garden of Sembawa Research Centre, Indonesia Rubber Research Institute. Young rubber leaves measuring about 3 – 5 cm in length and 1.5 – 1.7 cm in width were used as a source of DNA.

DNA Extraction and RAPD Analysis

DNA was extracted according to the procedure described by Orozco-Castillo[21] which was modified specifically by the addition of 0.1 g polyvinylpolypyrrolidone (PVPP), in each sample at the time of sample grinding in liquid nitrogen to fine powder using pestle and mortar. The powder was transferred to an Eppendorf tube using a spatula and immediately 5 mL of DNA extraction buffer (1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 30 mM ß-mercaptoethanol) was added. The mixture was homogenised by gentle shaking and incubated at 65ºC for 30 min. An equal volume of chloroform-isoamylalcohol (24:1) was added, and then spinned at 11,000 r.p.m. for 3 min. The supernatant was transferred to a new Eppendorf tube. An equal volume of isopropanol was added to precipitate the DNA and the mixture

TABLE 1. LIST OF RUBBER GENOTYPES AND THEIR ORIGIN

Wickham Germplasm			Wild Germplasm		
No	Name	Country of Origin	No	Name	Country of Origin /State/District
1	BPM 24	Indonesia	42	PN 88	Brazil/Rondonia/Calama
2	BPM 1	Indonesia	43	PN 452	Brazil/Rondonia/Calama
3	PR 300	Indonesia	44	PN 560	Brazil/Rondonia/Calama
4	IRR 24	Indonesia	45	PN 93	Brazil/Rondonia/Calama
5	IRR 39	Indonesia	46	PN 262	Brazil/Rondonia/Calama
6	IRR 44	Indonesia	47	PN 386	Brazil/Rondonia/Calama
7	IRR 118	Indonesia	48	PN 448	Brazil/Rondonia/Calama
8	IRR 104	Indonesia	49	PN 451	Brazil/Rondonia/Calama
9	BPM 107	Indonesia	50	PN 223	Brazil/Rondonia/Calama
10	TJIR 1	Indonesia	51	PN 67	Brazil/Rondonia/Calama
11	IRR 100	Indonesia	52	PN 561	Brazil/Rondonia/Calama
12	IRR 105	Indonesia	53	PN 99	Brazil/Rondonia/Calama
13	GT 1	Indonesia	54	PN 265	Brazil/Rondonia/Calama
14	LCB 1320	Indonesia	55	PN 305	Brazil/Rondonia/Calama
15	BPM 109	Indonesia	56	PN 232	Brazil/Rondonia/Calama
16	IRR 42	Indonesia	57	PN 591	Brazil/Rondonia/Calama
17	IRR 41	Indonesia	58	PN 545	Brazil/Rondonia/Jiparana
18	IRR 32	Indonesia	59	PN 365	Brazil/Rondonia/Jiparana
19	TM 9	Indonesia	60	PN 485	Brazil/Rondonia/Jiparana
20	BN 1	Indonesia	61	PN 124	Brazil/Rondonia/Jiparana
21	TM 5	Indonesia	62	PN 120	Brazil/Rondonia/Jiparana
22	IRR 220	Indonesia	63	PN 177	Brazil/Rondonia/Jaru
23	IRR 204	Indonesia	64	PN 502	Brazil/Rondonia/Jaru
24	FX 351	Indonesia	65	PN 316	Brazil/Rondonia/Pimenta Bruno
25	TM 8	Indonesia	66	PN 333	Brazil/Rondonia/Pimenta Bruno
26	IRR 18	Indonesia	67	PN 441	Brazil/Rondonia/Aracatuba
27	BPPJ 3	Indonesia	68	PN 412	Brazil/Rondonia/Aracatuba
28	AVROS 352	Indonesia	69	PN 667	Brazil/Mato Grosso/Ita Uba
29	PR 303	Indonesia	70	PN 724	Brazil/Mato Grosso/Ita Uba
30	PR 228	Indonesia	71	PN 534	Brazil/Mato Grosso/Ita Uba
31	PR 107	Indonesia	72	PN 677	Brazil/Mato Grosso/Ita Uba
32	RRIM 600	Malaysia	73	PN 494	Brazil/Mato Grosso/Ita Uba
33	RRIM 712	Malaysia	74	PN 235	Brazil/Mato Grosso/Ita Uba
34	PB 260	Malaysia	75	PN 666	Brazil/Mato Grosso/Ita Uba
35	PB 217	Malaysia	76	PN 621	Brazil/Mato Grosso/Ita Uba
36	PB 235	Malaysia	77	PN 295	Brazil/Mato Grosso/Ita Uba
37	RRIC 101	Sri Lanka	78	PN 186	Brazil/Mato Grosso/Ita Uba
38	RRIC 100	Sri Lanka	79	PN 77	Brazil/Mato Grosso/Ita Uba
39	RRIC 110	Sri Lanka	80	PN 23	Brazil/Mato Grosso/Ita Uba
40	RRIC 102	Sri Lanka	81	PN 406	Brazil/Mato Grosso/Ita Uba
41	RRII 105	India	82	PN 171	Brazil/Mato Grosso
			83	PN 142	Brazil/Mato Grosso
			84	PN 309	Brazil/Mato Grosso
			85	PN 373	Brazil/Acre/Feijo
			86	PN 604	Brazil/Acre/Feijo

was refrigerated at 4ºC for at least 30 min. The DNA was pelleted by centrifugation at 11 000 r.p.m. for 10 min. The pellet was then washed with 70% cold 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 -20ºC until it was used as a template in PCR.

The quality of DNA was confirmed using agarose gel electrophoresis (0.8 % agarose) with ethidium bromide staining in TAE buffer (40 mM Tris-acetate pH 8.1, 1 mM EDTA). The samples were loaded into an agarose gel with loading buffer (0.25 % bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water). The DNA purity was determined by using a spectrophotometer based on the ratio of optical density (OD) value between 260 nm and 280 nm wave length. The DNA concentration was calculated based on the value of OD at 260 nm (1 OD unit = 50 µg/mL DNA)[22].

For PCR analysis, arbitrary primers were selected based on their capability in producing different DNA fragments in various clones, in order to obtain polymorphic bands. Each primer consists of 10 bases that contains 60–70% G and C bases. A total of 14 primers produced by Operon technologies (Alameda, USA) which had been selected randomly were used for this study (*Table 2*).

DNA amplification was carried out following the method of William *et al*[23]. The PCR reaction was prepared in a in 25 µL volume reaction containing 1.0 µL DNA template 25 ng/µL, 1.5 µL $MgCl_2$ 25 mM, 2.5 µL PCR buffer 5×, 0.5 µL dNTP mix, 0.2 µLDNA taq polymerase (5 U/µL), 1.0 µL primer 10 mM, and demineralised water. PCR was conducted by using the Biometra T-Personal machine (Germany) which was programmed for 45 cycles which were denaturated for 2 min at 94ºC, annealing for 1 min at 53ºC, and an extention cycle for 2 min at 72ºC. The last cycle was followed by incubation for 4 min at 72ºC.

The amplification products of 10 µL DNA were separated in 1.4% agarose gel with ethidium bromide staining in 1× TAE buffer (0.04 M Tris-acetic in 1 mM EDTA). DNA migration was conducted for 1 h and 15 min at 50 volt. DNA fragments were visualised by UV transiluminator and DNA fragments in the gel was photographed with a polaroid camera. The molecular weight of the DNA fragments were identified by the migration of DNA marker (1 kb DNA ladder).

Data Analysis

The DNA fragments used in the RAPD analysis were the fragments which could be clearly identified by determining its presence (1) or absence (0). Based on those data the genetic distances were estimated by a dendrogram which was constructed following the UPGMA method. The similarity matrix among clones was analysed by using the NTSys programme[24].

RESULTS

Level of Polymorphism

The 14 primers used in the RAPD analysis produced 106 DNA fragment patterns that ranged from 5 to 9 bands with an average of 7.5 DNA fragments per primer. The highest percentage of polymophism was obtained by the OPH-19 primer (88.9%) and the lowest was obtained by the OPH-03 primer (66.75%). For each primer, the wild germplasm has a higher polymorphism than the Wickham clones (*Table 2*). *Figure 1* shows the distribution of 9 DNA fragment patterns amplified with the OPH-19 primer.

TABLE 2. NUMBER OF DNA FRAGMENTS OBSERVED IN 41 WICKHAM CLONES AND 45 WILD GERMPLASM OF *H. BRASILIENSIS*

No	Primer	Number of DNA Fragments								
		Wickham+Wild germplasm			Wickham			Wild germplasm		
		T	P	% P	T	P	% P	T	P	% P
1	OPN-06	8	7	87.5	7	6	85.7	8	7	87.5
2	OPN-08	9	7	77.8	8	7	87.5	9	7	77.8
3	OPN-10	7	6	85.7	6	5	83.3	7	6	85.7
4	OPN-17	8	7	87.5	6	5	83.3	8	7	87.5
5	OPH-01	7	6	85.7	6	4	66.7	7	6	85.7
6	OPH-03	9	6	66.7	7	5	71.4	9	6	66.7
7	OPH-05	7	6	85.7	7	5	71.4	7	6	85.7
8	OPH-18	6	5	83.3	5	4	80.0	6	5	83.3
9	OPH-19	9	8	88.9	9	8	88.9	9	8	88.9
10	OPA-17	9	6	66.7	9	6	66.7	9	5	55.6
11	OPA-18	6	5	83.3	5	4	80.0	6	5	83.3
12	OPB-12	8	7	87.5	8	7	87.5	7	6	85.7
13	OPC-05	5	4	80.0	5	3	60.0	5	4	80.0
14	OPG-04	7	6	85.7	6	4	66.7	7	6	85.7
	Total	105	86	81.9	94	73	77.6	104	84	80.7

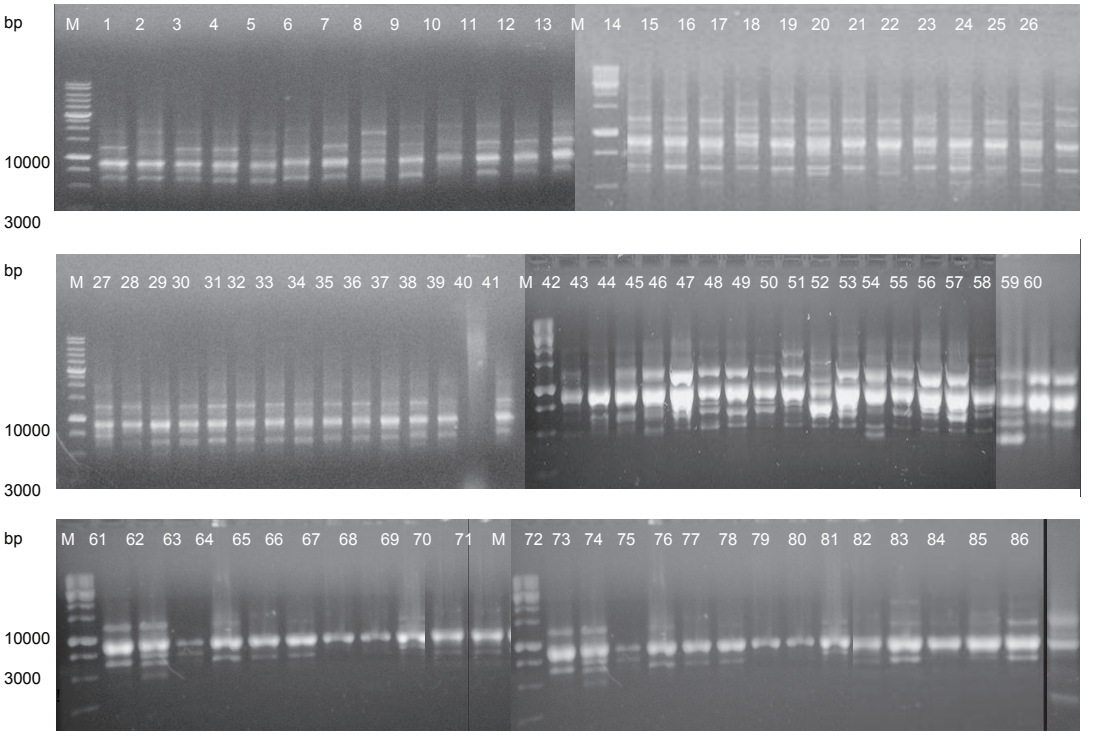
T : Total of DNA fragments
P : Polymorphic DNA fragments
%P : Percentage of DNA polymorphic fragments

Relationship of Wickham and Wild Germplasm

Pair-wise comparisons were made between all germplasms and average similarity values were calculated based on RAPD polymorphic data. Cluster analysis between wild germplasm was carried out by using the UPGMA method and resulted in a dendrogram as shown in *Figure 2*. The genetic distance for wild germplasm ranged from 0.69 to 0.94 and 0.66 to 0.98 for the Wickham clones. The dendrogram discriminated all of the wild germplasm and revealed three distinct big clusters (I, II and III). The first cluster (I) comprised 25 genotypes from Rondonia and 2 from Mato Grosso. This cluster is closely related with cluster II formed by 2 genotypes from Mato Grosso. Cluster III consists of 12 genotypes from Mato Grosso and genotypes each from Rondonia and 2 Acre. These big clusters were separated further to small clusters with various genetic distances.

When Wickham clones were added to the analysis, these clones were separated in two different clusters (IV and V). Cluster IV consists of 26 clones, while cluster V consists of 15 clones. The genetic distance for all of the germplasm ranged from 0.56 to 0.58.

The distance matrix shows that the genetic similarity level among the clones ranging between 0.36 – 0.99 (data not shown). The lowest genetic similarity value was 0.36 between IRR 41 and PN 406 whereas the highest genetic similarity value was 0.99 between IRR 24 and IRR 39.



Note:

1. IRR 24
2. IRR 39
3. IRR 104
4. IRR 118
5. IRR 105
6. RRIM 600
7. Tjir 1
8. F 351
9. PB 217
10. IRR 44
11. IRR 100
12. BPM 107
13. PR 228
14. RRII 105
15. BPM 109
16. BPM 24
17. BPM 1
18. PR 300
19. PB 260
20. GT1
21. PR 303
22. LCB1320
23. RRIC 100
24. RRIC 110
25. RRIC 101
26. RRIM 712
27. IRR 42
28. IRR 41
29. TM 5
30. TM 8
31. IRR 18
32. H. Sp
33. IRR 220
34. PB 235
35. IRR 204
36. RRIC 101
37. IRR 32
38. TM 9
39. BPPJ 3
40. BN 1
41. AV. 352
42. PN 365
43. PN 177
44. PN 724
45. PN 88
46. PN 502
47. PN 591
48. PN 534
49. PN 677
50. PN 667
51. PN 124
52. PN 545
53. PN 316
54. PN 560
55. PN 485
56. PN 452
57. PN 93
58. PN 666
59. PN 494
60. PN 235
61. PN 373
62. PN 99
63. PN 265
64. PN 621
65. PN 262
66. PN 386
67. PN 295
68. PN 305
69. PN 171
70. PN 604
71. PN 232
72. PN 448
73. PN 333
74. PN 451
75. PN 120
76. PN 441
77. PN 142
78. PN 223
79. PN 412
80. PN 186
81. PN 23
82. PN 561
83. PN 67
84. PN 309
85. PN 77
86. PN 406

Figure 1. Polymorphism of 41 Wickham clones and 45 wild germplasm amplified by OPH-19 primer.

Figure 2. Similarity among the 86 H. brasiliensis germplasm.

DISCUSSION

Many studies on the genetic diversity of plants based on RAPD markers have been conducted, for instance, on thymus[7], sago palm[8], wormwood[9], mulberry[10] and carrot[11]. For *H. brasiliensis*, RAPD has been used to study genetic diversity[15–17] by many workers using different genetic materials.

The comparison between wild germplasm and Wickham clones showed unambiguously that the wild germplasm has a higher genetic diversity. A similar result has already been revealed by RFLP[25] and microsatellite markers[26–28]. The higher genetic diversity in wild germplasm was expected, although it did not have a close relationship to desirable agronomic breeding characters. The wild germplasm will be introduced in the rubber tree breeding, so that it will enlarge the genetic base of the cultivated clones[29].

The wild germplasm was separated into three clusters based on genetic diversity. Although not all of the genotypes of the wild germplasm were clustered based on their origin of geographical collection areas. In general, clustering clearly described their origins. For example, in cluster I, 25 out of 27 genotypes came from Rondonia and only 2 genotypes came from Mato Grosso. All genotypes of Cluster II came from Mato Grosso. Among the 16 genotypes in cluster III, 12 genotypes came from Mato Grosso, 2 from Rondonia and 2 from Acre. This clustering result showed that the genotypes from Rondonia were separated from the Mato Grosso while the genotypes from Acre have a closer relationship to Mato Grosso. The same result was obtained from the microsatellite study that showed the Acre genotypes had a closer relationship with that of Rondonia[27]. However, a different result from a RFLP study showed that the genotypes from Acre were closer to Rondonia compared to Mato Grosso[25]. Based on the geographic map, the position of Rondonia is next to Mato Grosso, while Acre is further away from the other states (*Figure 3*). This RAPD analysis could be improved if more primers are used in order to reveal more polymorphisms among these germplasm.

Figure 3. Map of wild germplasm origin.

Despite the narrow genetic base and high degree of inbreeding, the cultivated Wickham clones still showed a high level of polymorphism. Clustering showed that the cultivated clones were separated into two clusters (IV and V). Clustering of clones were not exactly based on their origin. Clones from Indonesia were placed in both clusters, but all clones from Malaysia, Sri Lanka and India were in cluster IV.

CONCLUSION

This study revealed that the wild germplasm has a higher genetic diversity than the cultivated clones. This result could be used as a guide in selection of appropriate wild genotypes to be included in the rubber germplasm and used in breeding schemes.

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