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Construction of Scaffold Genetic Linkage Maps for Two Hevea *Families: PB 5/51 X IAN 873 and RRIM 937 X RRIM 600*

SAFIAH ATAN*#

Four scaffold linkage maps were constructed for clones PB 5/51, IAN 873, RRIM 937 and RRIM 600 and two consensus maps (PB 5/51 × IAN 873 and RRIM 937 × RRIM 600) using AFLP markers. The loci for clones PB 5/51 and IAN 873 were spread across 18 linkage groups, corresponding to the basic chromosome number of Hevea *(n = 18) and cover between 269.6 cM to 272.3 cM, respectively. The average marker densities for these two clones were 4.4 to 4.5 cM per marker. Meanwhile, the coverage for clones RRIM 937 and RRIM 600 spanned 15 to 12 linkage groups respectively, with marker density between 4.7 cM for RRIM 937 and 11.3 cM for RRIM 600. The linkage groups for maps of clones RRIM 937 and RRIM 600 did not have the basic chromosome number of 18, because this population showed inbreeding in which RRIM 600 was a reoccurring parent in the population.*

Keywords: Amplified Fragment Length Polymorphism (AFLP); Genetic Linkage Map

Hevea brasiliensis has been cultivated in Malaysia for more than 100 years. This tree crop is the main producer of natural rubber in the world. *Hevea* has a long growing cycle and low fruit set per pollination (1 out of ten pollinations will develop into a fruit) along with other major limitations such as narrow genetic base, non-synchronous flowering, long gestation period, heterozygous nature, insufficient land for field experiments and the absence of reliable early selection parameters while genetic improvement has been slow and time consuming[1]. Cultivated rubber plants were initially seedling progenies from clonal parents that were randomly crossed. *Hevea* is still propagated today by grafting and breeding programmes that are based on hand pollination. However, clonal assessment of selected clones takes approximately 25-30 years which is both time consuming and labour intensive[2]. The conventional breeding programme at the Malaysian Rubber Board (MRB) involves several steps as summarised in *Figure 1*.

A number of advances in genetics and genomics has greatly enhanced the understanding of structural and functional aspects of plant genomes and the ability to improve crop plants *via* marker assisted selection (MAS)[4]. MAS is a concept making it possible to infer the presence of a gene of

*Rubber Research Institute of Malaysia, Malaysian Rubber Board, P.O. Box 10150, 50908 Kuala Lumpur, Malaysia.
Corresponding author (e-mail: safiah@lgm.gov.my)

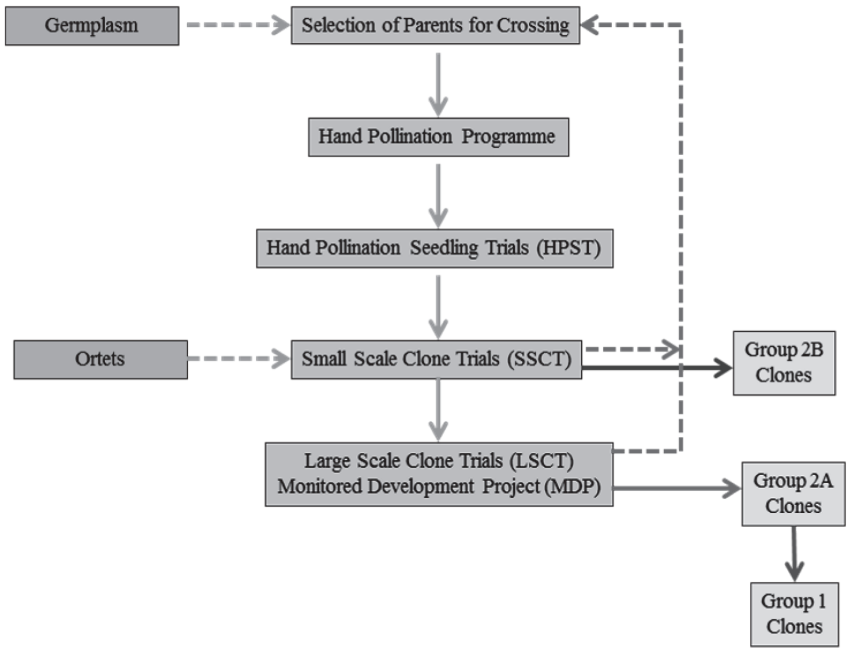

Figure 1. Flowchart of MRB's breeding programme[3].

Group 1 clones are clones with known track records based on at least 5 years of non-stimulated yield data as well as information on secondary characteristics in Large Scale Clone Trials (LSCT).

Group 2A clones consist of new clones which showed good early performance at least three years yield data in LSCT. Group 2B clones consist of newly released clones which are promising in the preliminary trials.

an organism from the presence of a marker (morphological, biochemical or one based on DNA/RNA variation) that is tightly linked to the gene or traits of interest. Therefore, a saturated genetic linkage map of the organism must be constructed first. Linkage maps indicate the position and relative genetic distances between markers along chromosomes. The most important use of linkage maps is to identify chromosomal locations containing genes and quantitative trait loci (QTL) associated with the traits of interest. The actual location of QTL involves an estimated genetic map with known distances between markers and evaluations of a likelihood function that is maximised over the established parameter space between molecular markers[5,6].

Lespinasse *et al.*[7] and Le Guen *et al.*[8] constructed the maps for clones PB 260 (an oriental clone) and RO 38 (an African clone) covering a distance of 2144 cM consisting of 18 linkage groups built with restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) molecular markers. This map was later used to locate the QTL for the South American Leaf Blight (SALB)[7]. At the MRB, two mapping populations were selected *i.e.* RRIM

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937 × RRIM 600 and PB 5/51 × IAN 873. RRIM 937, RRIM 600 and PB 5/51 are oriental clones while IAN 873 is a Brazillian clone. Initially in the process of map construction, RFLP and random amplified polymorphic DNA (RAPD) marker techniques were used but the majority of the fragments were monomorphic[9]. The AFLP marker technique was later adopted in the study of *Hevea*[10] and more polymorphic DNA fragments were elicited.

The usefulness of AFLP markers for genetic mapping of outbred crop species has been demonstrated for potato[11], apple[12] and rubber tree[13] as well as for mapping crop species with a large genome size such as the onion[14]. A higher number of selective nucleotides will amplify a smaller number of restriction fragments, resulting in less complex fingerprints to be visualised.

This paper presents the construction of four parental linkage maps (PB 5/51, IAN 873, RRIM 937 and RRIM 600) and two consensus maps (PB 5/51 × IAN 873 and RRIM937 × RRIM 600) using AFLP markers and comparing the map of each clone to the other.

MATERIALS AND METHODS

Description of Population

Population 1: PB 5/51 × IAN 873. Population 1 was from a cross between an oriental clone (PB5/51) and a Brazilian clone (IAN 873) (*Figure 2*). An oriental clone is a clone derived from the *Hevea* genetic materials collected by Henry Wickham in 1876 and introduced to the South East Asian region. Clone PB 5/51 is a clone with superior production of latex and is one of the most popular clones used in breeding programmes at the MRB due to its high General Combining Ability (GCA). IAN 873 is a Brazilian clone that was brought to Malaya in 1951 and was found to have a superior bole height and wood density but unfortunately this clone is susceptible to *Corynespora cassiicola*[15].

Three progenies from this cross were recommended for planting by the MRB. They are RRIM 2020, RRIM 2015 and RRIM 2016[16]. These clones have high latex and timber yields and are called Latex-timber clones (LTC). Clone RRIM 2020 was initially found to be resistant to the fungus *C. cassiicola* when screened in the laboratory and nursery and was highly recommended for replanting schemes both in small holdings as well as in the plantations. Unfortunately after planting this clone in the field, it was devastated by the fungus[17]. Clone RRIM 2016, as with RRIM 2020, was found to be resistant to *C. cassiicola* in laboratory and nursery screenings, but its performance in the field is unknown. Clone RRIM 2015, on the other hand was found to be susceptible to *C. cassiicola* in the laboratory and nursery. Its resistance to the fungus in the field is unknown.

Hevea budsticks from the original 20 year old hand pollinated selection (HPS) collection of population 1 in the Rubber Research Institute Malaysia (RRIM) Experimental Station, Kota Tinggi, Johor were bud grafted to mix *Hevea* rootstocks in Field 68, RRIM Experimental Station, Sg. Buloh, Selangor.

Due to its outbreeding habit, *Hevea* is generally assumed to have a high level of heterozygosity, either in natural populations or commercial clones. Therefore, each progeny family is assumed as F2 between two F1 parents[8].

Population 2: RRIM 937 × RRIM 600. The second population was a cross between two oriental clones (RRIM 937 × RRIM

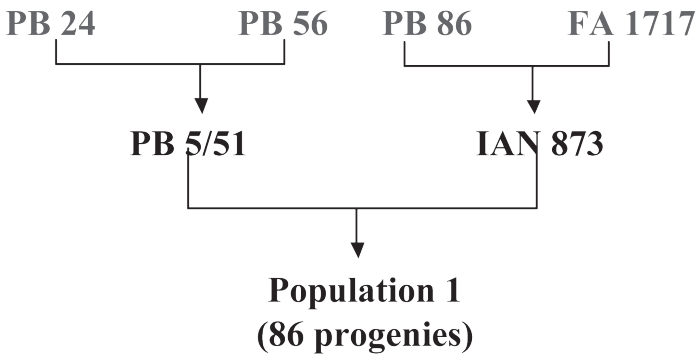

Figure 2. Pedigree map of Population 1 (86 progenies). The progenies of the original Hand Pollinated Selection (HPS) collection included RRIM 2020, RRIM 2015 and RRIM 2016.

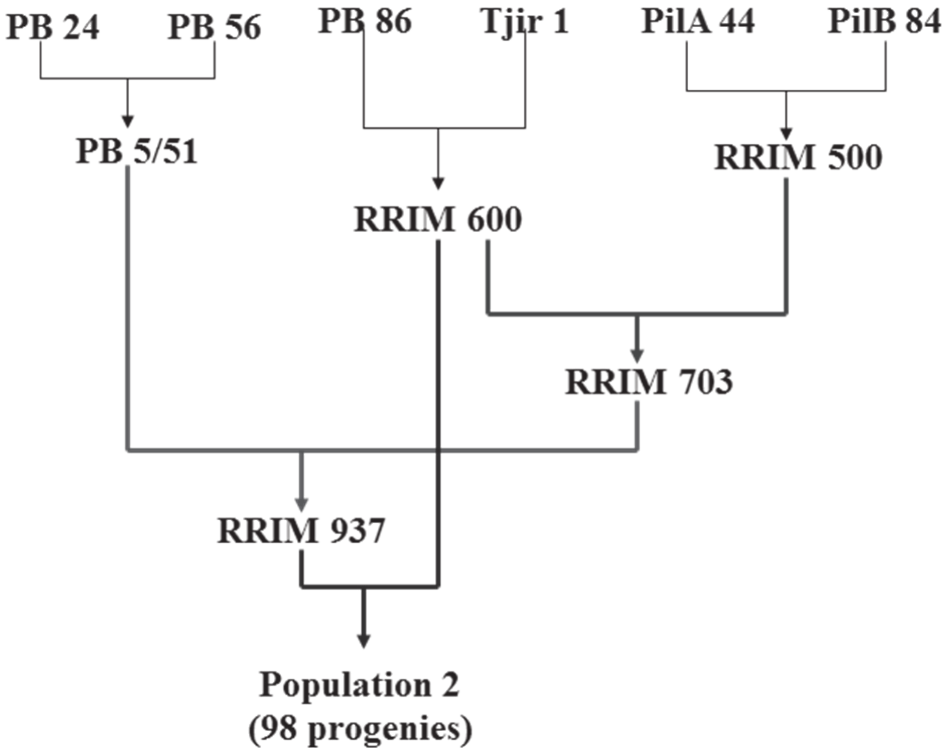

Figure 3. Pedigree map of Population 2 (98 progenies). PB 56, PB 24, PB 86, Tjir 1, PilA 44 and PilB 84 are the primary clones.

600) (*Figure 3*). The aim of this cross was to create a population that was resistant to *C. cassiicola*, a fungus that causes Corynespora leaf fall (CLF) in *Hevea*. The clone RRIM 937 was found to be resistant to the fungus during disease screening in the laboratory. RRIM 600 on the other hand, is known to be highly susceptible to the fungus. RRIM 600 clone was a widely planted clone in Malaysia up to the 1980s and is susceptible to race 1 of the *C. cassiicola* fungus[18,19]. The leaves from this population were collected from tree stands in Field 59, RRIM Experimental Station, Sg. Buloh, Selangor, Malaysia.

Plant Material and Extraction of DNA

Soft, pale green leaf samples *i.e.* leaves at stage C[20] were obtained from two *Hevea* mapping populations *i.e.* PB 5/51 × IAN 873 and RRIM 937 × RRIM 600 in the MRB nursery of Field 68 and Field 59, RRIM Experimental Station, Sg. Buloh, Selangor, Malaysia respectively. These plants are routinely pollarded to keep them low and to encourage the production of dense new growth.

The leaves from the two mapping families were rinsed thrice in distilled water and plunged into liquid nitrogen immediately after collection. They were stored at –70ºC till further use.

Genomic DNA was extracted from 1.5 g leaf samples using a modified protocol as mentioned by Safiah[21]. DNA concentrations were checked against standard lambda DNA. An aliquot of newly extracted DNA (5 µL) was electrophoresed along standard phage lambda DNA of 5, 10, 15, 20, 30 and 40 ng/µL on a 1% gel in 1X TBE, 100 V for 15 min, stained with EtBr and viewed using the Alpha Innotech gel imager. The genomic DNA was stored at –20ºC prior to further usage.

Amplified Fragment Length Polymorphism (AFLP)

The AFLP technique was performed according to general steps as described by Vos *et al*.[22]. AFLP kits bought from ABI Biosystems, USA, were used throughout the project.

Evaluation of the AFLP Reaction *via* Gel Electrophoresis

The AFLP reactions were analysed by separating the amplified DNA fragments on a 4% denatured polyacrylamide gel (4% polyacrylamide mix, 1 M urea, 1× TBE) ran on the 377 ABI Prism Automated Sequencer (Applied Biosystems) at 1500 V for 2.5 hours.

Prior to loading the sample on to the polyacrylamide gel, 1.2 µL loading buffer (180 µL formamide, 70 µL blue loading dye, 50 µL GS-500 ROX-labeled size standard) was added to each AFLP reaction. Multiplexing the reactions was done by adding to a 0.2 mL PCR tube 0.3 µL FAM-labeled (blue flourescent) product, 0.4 µL JOE-labeled (green fluorescent) product and 0.8 µL TAMRA-labeled (yellow fluorescent) product, quickly mixing and spinning down briefly before denaturing the reaction in the thermocycler at 90ºC for 3 min followed by a quick chill on ice.

The fractionated AFLP fragments were then visualised and recorded by GeneScan® software Ver. 2.0 and subsequently analysed using the Genotyper® software (ABI Biosystems, USA).

Analysis of Digital AFLP Gel Images

Digital AFLP gel images were processed for data extraction by "scoring" using the ABI

377 software Genotyper Ver. 2.0 to obtain quantitative data. The following criteria were set to include genotyping the bands/fragments (present = 1; absent = 0) in the scoring process: i) fragment size range had to be between 50 and 500 bp, ii) intensity had to be at least threefold higher than the local background intensity and iii) the fragments had to be at least 0.5 bp from other fragments of the same lane. Subsequent images with AFLP lanes produced with the same primer combination were automatically scored and combined through the Genotyper software.

The band sizes, intensities and lane names were exported directly to an Excel format for further analysis. AFLP loci were coded with P1 or P2 at the beginning to denote the parental origin, E and M for identity of the *Eco*RI and *Mse*I primers and finally the molecular weight markers. For example P1E6M1337 signifies this marker originated from Parent 1 (maternal parent), primers *Eco*RI-6 and *Mse*I-1 were the primer pair used and the molecular weight of this marker was 337 bp.

Linkage Analysis

Markers were subdivided into two groups according to their segregation patterns. The first group comprised markers that presented a 1:1 segregation ratio in the progeny (heterozygous in one parent and homozygous null in the other). The second group comprised markers that were heterozygous in both parents, therefore segregating in a 3:1 ratio in the progeny. Mendelian segregation of the markers was tested by the chi-square test ($P \leq 0.05$). The few distorted 1:1 and 3:1 markers were discarded from further analysis.

In the preliminary data analysis, segregating data were used to construct a map for each parent, PB 5/51, IAN 873 and RRIM 937 and RRIM 600. The segregation ratio of each marker was tested with a chi square test for goodness of fit to the expected 1:1 ratio when the marker was present in one of the two parents or a 3:1 ratio when the marker was present in both parents. Parental maps were then established using the JoinMap software version 4.0[23]. The cross pollinator function (CP) and the Kosambi mapping functions were applied to convert recombination frequencies into map distances[24].

A preliminary grouping of the 1:1 markers was performed for each parent using the JoinMap software version 4.0 with LOD 5.0. Markers segregating in the 3:1 ratio were later added onto the base map. Consensus maps of both populations were also constructed using bridge markers.

RESULTS AND DISCUSSION

Construction of PB 5/51 and IAN 873 Maps

Of a total of 256 AFLP primer combinations tested (16 *Eco*RI and 16 *Mse*I primers, *Table 1*), 35 were retained because of the large number of polymorphic bands amplified among the two parents. All polymorphic AFLP markers (*Table 2* and *3*) were evaluated by chi-square tests for suitability of fit against the 1:1 and 3:1 ratio ($P \leq 0.05$). In constructing the parental maps, markers with Mendelian ratios of 1:1 and 3:1 were used. In addition, only the markers with ratios of 3:1 were used for construction of consensus maps.

The inferred linkage maps are shown in *Figure 4* (PB 5/51) and *Figure 5* (IAN 873). For the PB 5/51 clone, the selected 35 primer pairs were able to elicit 277 markers (60 markers had 1:1 ratio; 167 markers had 3:1 ratio) of which 63 markers (27%) segregated into 18 linkage groups. The skeleton map was obtained at LOD 5.0 spanning 276.8 cM. Meanwhile, 164 markers remained unlinked.

TABLE 1. AFLP PRESELECTIVE *EcoR*I AND *Mse*I PRIMER SEQUENCES
(the *EcoR*I primers were labeled with fluorescent dyes TAMRA or FAM or JOE)

Primer Sequence (Core + 3nt)	Restriction Enzyme	Primer Name/ Flourescent Label
5'-GAC-TGC-GTA-CCA-ATT-CAA-C	*EcoR*I	E1 / TAMRA
5'-GAC-TGC-GTA-CCA-ATT-CAA-G		E2 / FAM
5'-GAC-TGC-GTA-CCA-ATT-CAC-A		E3 / FAM
5'-GAC-TGC-GTA-CCA-ATT-CAC-C		E4 / TAMRA
5'-GAC-TGC-GTA-CCA-ATT-CAC-G		E5 / FAM
5'-GAC-TGC-GTA-CCA-ATT-CAC-T		E6 / FAM
5'-GAC-TGC-GTA-CCA-ATT-CAG-C		E7 / JOE
5'-GAC-TGC-GTA-CCA-ATT-CAG-G		E8 / TAMRA
5'-GAC-TGC-GTA-CCA-ATT-CAA-A		E9 / FAM
5'-GAC-TGC-GTA-CCA-ATT-CAA-T		E10/ FAM
5'-GAC-TGC-GTA-CCA-ATT-CAG-A		E11/ TAMRA
5'-GAC-TGC-GTA-CCA-ATT-CAG-T		E12 / FAM
5'-GAC-TGC-GTA-CCA-ATT-CAT-A		E13 / FAM
5'-GAC-TGC-GTA-CCA-ATT-CAT-C		E14 / JOE
5'-GAC-TGC-GTA-CCA-ATT-CAT-G		E15 / TAMRA
5'-GAC-TGC-GTA-CCA-ATT-CAT-T		E16 / FAM
5'-GAT-GAG-TCC-TGA-GTA-ACA-A	*Mse*I	M1
5'-GAT-GAG-TCC-TGA-GTA-ACA-C		M2
5'-GAT-GAG-TCC-TGA-GTA-ACA-G		M3
5'-GAT-GAG-TCC-TGA-GTA-ACA-T		M4
5'-GAT-GAG-TCC-TGA-GTA-ACT-A		M5
5'-GAT-GAG-TCC-TGA-GTA-ACT-C		M6
5'-GAT-GAG-TCC-TGA-GTA-ACT-G		M7
5'-GAT-GAG-TCC-TGA-GTA-ACT-T		M8
5'-GAT-GAG-TCC-TGA-GTA-ACC-A		M13
5'-GAT-GAG-TCC-TGA-GTA-ACC-C		M14
5'-GAT-GAG-TCC-TGA-GTA-ACC-G		M15
5'-GAT-GAG-TCC-TGA-GTA-ACG-A		M16
5'-GAT-GAG-TCC-TGA-GTA-ACG-C		M17
5'-GAT-GAG-TCC-TGA-GTA-ACG-G		M18
5'-GAT-GAG-TCC-TGA-GTA-ACG-T		M44
5'-GAT-GAG-TCC-TGA-GTA-ACC-T		M45

TABLE 2. CHI-SQUARE TESTS FOR SUITABILITY OF FIT AGAINST 1:1 AND 3:1 RATIO ($P < 0.05$) FOR 63 AFLP MARKERS THAT WERE PLACED ON 19 LINKAGE GROUPS OF THE MAP OF CLONE PB 5/51
(These markers were amplified using 35 AFLP primer pair combinations mentioned earlier)

Linkage Group	Nr	Locus	Position (cM)	X^2	[a]Significance ($P < 0.05$)	Linkage Group	Nr	Locus	Position (cM)	X^2	[a]Significance ($P < 0.05$)
grp1	1	P1E3M13371	0.00	2.60	-	grp2	23	P1E2M27275	0.00	1.91	-
	2	P1E6M14364	5.92	0.02	-		24	P1E1M4177	9.99	0.00	-
	3	P1E3M28145	8.65	0.84	-		25	P1E1M4126	20.53	0.38	-
	4	P1E3M13140	11.73	0.02	-	grp3	26	P1E4M1865	0.00	3.42	*
	5	P1E3M13220	15.16	0.02	-		27	P1E4M1879	7.24	1.69	-
	6	P1E6M14250	19.67	0.99	-	grp4	28	P1E2M13130	0.00	3.69	*
	7	P1E3M13159	21.65	1.74	-		29	P1E3M1777	19.23	2.80	*
	8	P1E5M14131	23.81	5.84	**	grp5	30	P1E2M1777	0.00	3.66	*
	9	P1E3M13209	25.05	4.84	**		31	P1E6M14113	17.92	1.52	-
	10	P1E3M28129	26.20	4.78	**	grp6	32	P1E2M17209	0.00	0.02	-
	11	P1E1M1596	26.48	4.78	**		33	P1E3M17209	16.26	4.29	**
	12	P1E3M28220	28.22	3.63	*	grp7	34	P1E1M16119	0.00	0.89	-
	13	P1E3M28141	28.56	3.63	*		35	P1E1M16128	12.93	1.48	-
	14	P1E3M28180	30.89	2.63	-	grp8	36	P1E6M1862	0.00	3.42	*
	15	P1E1M27169	31.11	1.25	-		37	P1E6M1872	9.36	4.51	**
	16	P1E5M14103	34.06	0.51	-	grp9	38	P1E6M14111	0.00	0.18	-
	17	P1E3M18427	37.21	0.13	-		39	P1E6M1496	8.44	0.02	-
	18	P1E5M14206	39.40	0.99	-	grp10	42	P1E3M28228	0.00	0.73	-
	19	P1E1M13230	41.54	0.43	-		43	P1E6M14128	3.70	0.97	-
	20	P1E3M16101	44.86	5.84	**		44	P1E1M27210	9.54	0.53	-
	21	P1E3M16371	45.00	4.55	**		45	P1E1M13179	15.44	0.40	-
	22	P1E3M16248	49.85	3.41	*		46	P1E5M27152	23.52	4.07	**
grp 11	47	P1E4M2472	28.46	0.69	-	grp15	56	P1E1M13235	0.00	0.40	-
	48	P1E5M14118	34.48	0.97	-		57	P1E1M23235	17.33	0.71	-
	49	P1E3M2891	41.04	0.37	-	grp16	58	P1E6M23271	0.00	3.02	*
grp12	50	P1E6M18130	0.00	2.07	-		59	P1E6M23295	1.91	5.26	**
	51	P1E6M18141	14.20	6.43	**	grp17	60	P1E1M452	0.00	0.86	-
grp13	52	P1E2M17332	0.00	2.30	-		61	P1E3M461	15.48	2.38	-
	53	P1E3M17332	9.84	3.22	*	grp18	62	P1E4M14126	0.00	0.60	-
grp14	54	P1E1M13418	0.00	0.26	-		63	P1E4M24126	12.70	0.04	-
	55	P1E3M13418	7.16	0.54	-						

NB: [a]Significance levels ($P<0.05$): - 0.0, *0.1, ** 0.05; denotes segregation distortion.

TABLE 3. CHI-SQUARE TESTS FOR SUITABILITY OF FIT AGAINST 1:1 AND 3:1 RATIO ($P < 0.05$) FOR 63 AFLP MARKERS THAT WERE PLACED ON 17 LINKAGE GROUPS OF THE MAP OF CLONE IAN 873.
(These markers were amplified using 35 AFLP primer pair combinations mentioned earlier)

Linkage Group	Nr	Locus	Position (cM)	X^2	[a]Significance ($P < 0.05$)	Linkage Group	Nr	Locus	Position (cM)	X^2	[a]Significance ($P < 0.05$)
grp1	1	P2E1M13284	0.00	1.92	-	Group 2	23	P2E6M27103	0.00	1.43	-
	2	P2E2M13205	11.37	7.23	***		24	P2E6M24271	7.09	0.00	-
	3	P2E2M13380	13.52	7.23	***		25	P2E6M14271	11.14	0.06	-
	4	P2E3M13371	17.26	3.63	*		26	P2E6M24450	21.11	4.63	**
	5	P2E3M13140	21.75	0.02	-	Group 3	27	P2E6M2784	0.00	5.71	**
	6	P2E2M13192	22.40	0.15	-		28	P2E2M27206	5.82	1.91	-
	7	P2E3M13209	26.36	4.84	**		29	P2E6M1887	14.02	1.43	-
	8	P2E2M13198	27.05	4.74	**		30	P2E6M1865	18.86	0.17	-
	9	P2E3M13220	28.83	0.02	-	Group 4	31	P2E4M1887	0.00	0.02	-
	10	P2E2M1377	30.17	1.33	-		32	P2E6M18102	16.71	0.93	-
	11	P2E3M13148	32.19	2.32	-	Group 5	33	P2E6M1862	0.00	4.29	**
	12	P2E1M13230	32.60	0.43	-		34	P2E6M1872	9.14	5.50	**
	13	P2E6M24441	36.76	2.80	*	Group 6	35	P2E1M29176	0.00	0.06	-
	14	P2E3M13159	37.37	1.74	-		36	P2E1M29247	15.33	3.88	**
	15	P2E1M27169	40.40	1.25	-		37	P2E1M29292	16.73	2.44	-
	16	P2E6M2464	41.24	0.17	-		38	P2E1M29187	30.72	0.24	-
	17	P2E6M18444	43.60	0.23	-	Group 7	39	P2E4M1862	0.00	6.91	***
	18	P2E4M18444	46.31	0.00	-		40	P2E6M18130	0.69	1.54	-
	19	P2E2M27275	53.31	1.91	-		41	P2E6M18141	14.66	5.50	**
	20	P2E3M2796	57.63	1.47	-	Group 8	42	P2E2M23204	0.00	6.06	**
	21	P2E2M27307	60.62	1.19	-		43	P2E3M2367	4.28	0.31	-
	22	P2E2M27141	62.68	4.59	**						
grp 9	44	P2E6M14111	0.00	0.18	-		54	P2E5M27285	22.04	2.80	*
	45	P2E6M1496	8.15	0.02	-	grp 14	55	P2E6M14242	0.00	0.24	-
grp 10	46	P2E3M27106	0.00	1.47	-		56	P2E6M24342	4.63	0.23	-
	47	P2E6M1453	5.72	4.55	**	grp 15	57	P2E4M14126	0.00	0.60	-
grp 11	48	P2E5M14110	0.00	2.18	-		58	P2E4M24126	14.24	0.17	-
	49	P2E6M14143	15.90	0.06	-	grp 16	59	P2E1M13418	0.00	0.26	-
grp 12	50	P2E1M27129	0.00	0.53	-		60	P2E3M13418	5.36	1.05	-
	51	P2E3M27389	8.92	0.53	-	grp 17	61	P2E6M14205	0.00	0.06	-
grp 13	52	P2E4M27285	0.00	0.06	-		62	P2E6M24205	9.34	0.51	-
	53	P2E6M27285	10.63	0.23	-						

NB: [a]Significance levels ($P<0.05$): - 0.0, *0.1, ** 0.05, *** 0.01; denotes segregation distortion.

On the other hand, for the IAN 873 map, 174 AFLP markers (86 markers had 1:1 ratio; 88 markers had 3:1 ratio) were generated with 35 primer pairs. Of these, 60 markers (34%) segregated into 17 linkage groups. The skeleton map was obtained at LOD 5.0 spanning 272.3 cM with 60 linked markers and 114 markers that remained unlinked. The details of maps for PB 5/51 and IAN 873 are summarised in *Table 4*.

Construction of the Consensus Map PB 5/51 x IAN 873

Two hundred and fourteen (214) AFLP markers that segregated in PB 5/51 and/or IAN 873 were used for the construction of the consensus map. These markers had a ratio of 3:1 and common alleles in both parents. Only 75 markers (35%) were placed on the map and the rest remained unlinked at LOD 5.0 spanning 241.9 cM (*Figure 6*).

Linkage group 1 had the largest number of markers (22 markers) with the longest genetic distance of 49.9 cM, while linkage group 12 had the shortest genetic length at 0 cM. The details of the maps are summarised in *Table 4*.

Construction of RRIM 937, RRIM 600 and the RRIM 937 x RRIM 600 Consensus Maps

Maps of RRIM 937 and RRIM 600 were similarly constructed using the 35 pairs of the AFLP primer (*Figures 7, 8* and *9*). The details of the map are summarised in *Table 5*.

Map Descriptions

The basic chromosome number for *Hevea* is 18. Therefore ideally all maps should have 18 linkage groups. Out of six maps, the map for clone PB 5/51 was the only one which had 18 linkage groups. As summarised in *Tables 4* and *5*, many of the markers were not mapped in any of the linkage groups. Therefore, the total map lengths obtained were still insufficient in covering the complete genome. The length of the *Hevea* genome constructed by Lespinasse *et al*.[13] was 2000 cM for the PB 260 clone and 2145 cM for the RO38 clone. The estimated map lengths for all maps were difficult to calculate due to the paucity of polymorphic markers generated, though it could be assumed that the map length is between 2000 to 3000 cM. Thus, on average the current maps covered only 7.2% to 13% of the estimated genome length.

The paucity of segregating and mapped loci in both mapping populations was obvious. This could be due to the fact that three of the clones *i.e.* PB 5/51, RRIM 937 and RRIM 600, came from Malaysia and their parentage can be traced back to the limited number of ancestors that were used as the foundation to the rubber industry in the country (*Figures 2* and *3*). Even the maternal parent of clone IAN 873, PB 86, came from the Malaysian collection. The *Hevea* clones in Malaysia have been selected for accumulating superior traits such as high latex and timber yields. These selected clones were then vegetatively propagated and used as parents in subsequent crosses, thus, explaining the narrow genetic base.

The distribution of the mapped AFLP markers was uneven for all maps in which the largest group was Group 1 (*Table 6*). A few widely spaced marker intervals were detected in the current map in which most groups had a maximum of two loci indicating that the maps were not saturated which suggests that the AFLP markers identified are not randomly sampled throughout an entire chromosome[25]. The current genetic map can be made denser by adding more markers genome wide.

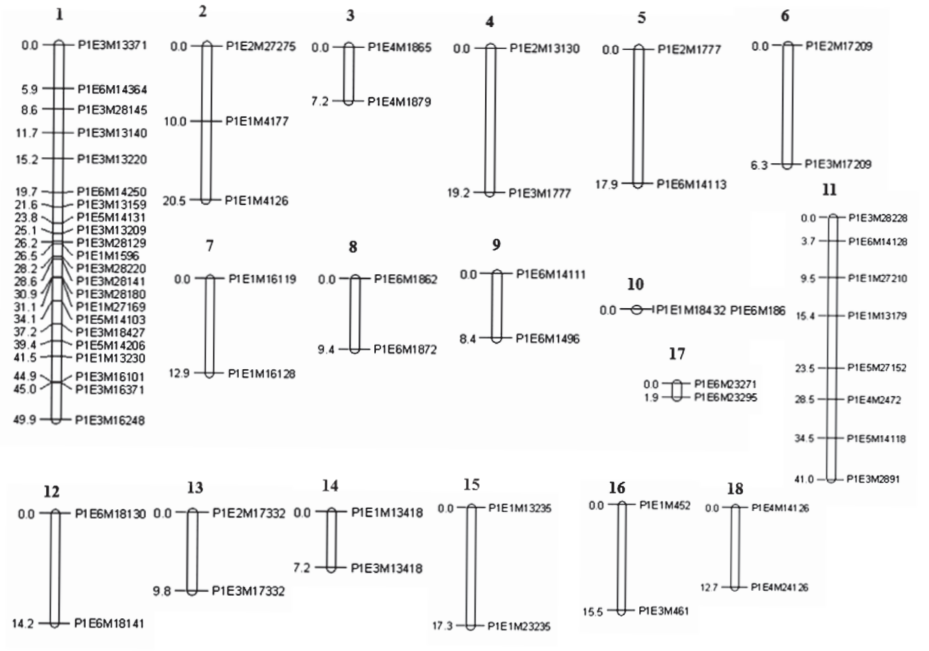

Figure 4. Genetic linkage map of PB 5/51(LOD 5.0, Kosambi mapping function).
The majority of the markers were located on linkage group 1. The other linkage groups had only two markers each with exception of group 11.

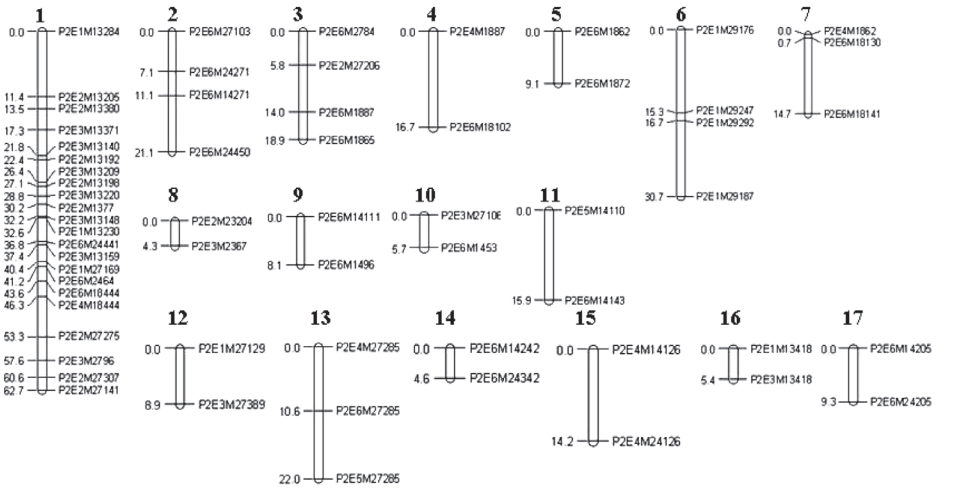

Figure 5. Genetic linkage map of IAN 873 (LOD 5.0, Kosambi mapping function).
The majority of the markers were located on linkage group 1. The other linkage groups had only two markers each with exception of groups 2, 3, 6, 7 and 13.

TABLE 4. SUMMARISED DESCRIPTION OF THE MAP DATA FOR PB 5/51, IAN 873 AND CONSENSUS MAP PB 5/51 × IAN 873
(The consensus map was constructed using bridge markers *i.e.* 3:1 segregating markers)

	PB 5/51	IAN 873	Consensus Map
Number of markers	275	174	214
Number of groups	18	17	18
Number of markers with 1:1 ratio	60	86	-
Number of markers with 3:1 ratio	167	88	75
Number of linked markers	61	60	75
Number of unlinked markers	216	114	139
Map length (cM)[a]	269.6	272.3	241.9
Marker density (cM)	4.4	4.5	3.2

[a]Corresponds to the sum of linkage group sizes, established with JoinMap ver 4.0.

TABLE 5. SUMMARISED DESCRIPTION OF THE MAP DATA FOR RRIM 937, RRIM 600 AND CONSENSUS MAP FOR RRIM 937 × RRIM 600
(The consensus map was constructed using bridge markers *i.e.* 3:1 segregating markers)

	RRIM 937	RRIM 600	Consensus Map
Number of markers	125	123	122
Number of groups	15	12	11
Number of markers with 1:1 ratio	33	21	–
Number of markers with 3:1 ratio	92	102	122
Number of linked markers	38	10	34
Number of unlinked markers	87	113	88
Map length (cM)[a]	180.3	36.2	241.9
Marker density (cM)	4.7	11.3	7.1

[a]Corresponds to the sum of linkage group sizes, established with JoinMap ver 4.0.

TABLE 6. SUMMARISED DESCRIPTION OF THE MAP DATA RRIM 937, RRIM 600, PB 5/51, IAN 873 AND CONSENSUS MAPS FOR PB 5/51 × IAN 873 AND RRIM 937 × RRIM 600

	PB 5/51	IAN 873	PB 5/51 × IAN 873	RRIM 937	RRIM 600	RRIM 937 × RRIM 600
Total number of markers	277	174	214	125	123	122
Number of markers in Group 1	22	22	34	13	3	11
Length of Linkage Group 1(cM)	49.9	62.7	56.4	59.0	15.4	21.6

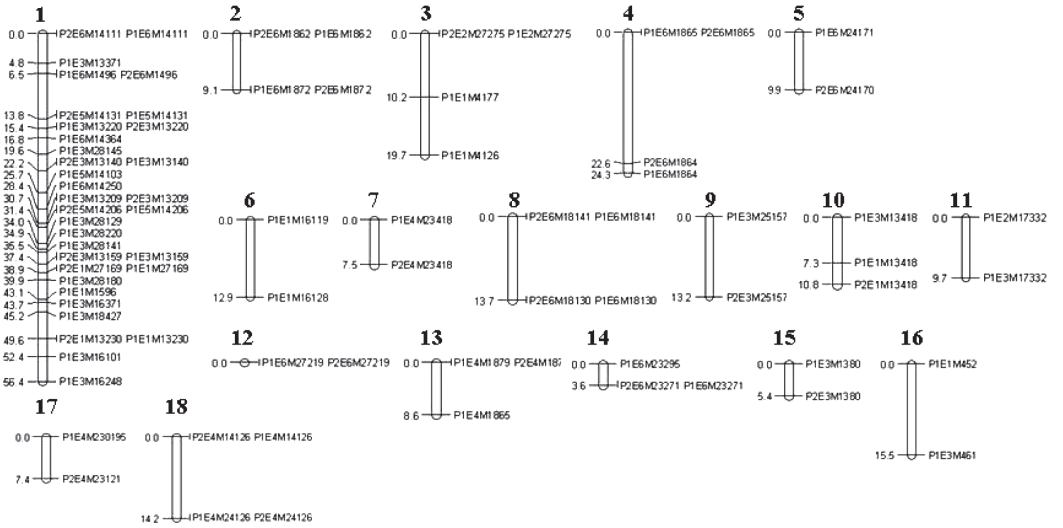

Figure 6. Consensus Map: PB 5/51 × IAN 873 (LOD 5.0, Kosambi mapping function) was constructed using 3:1 segregating markers. The majority of the markers were found to cluster in the centromere area of Group 1. The other linkage groups have between 2 to 4 markers linked in each group.

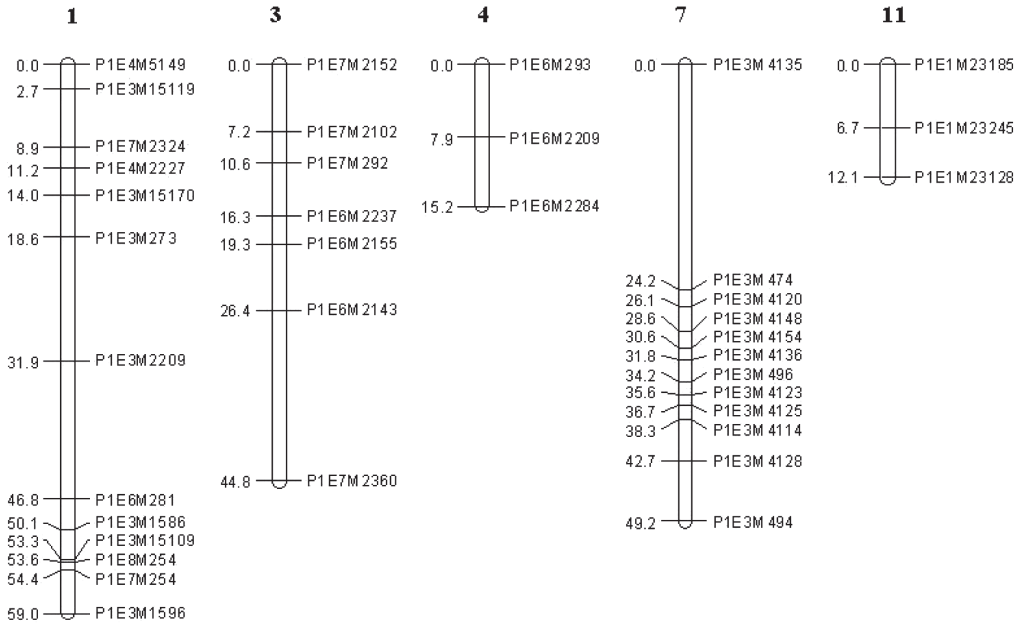

Figure 7. Genetic linkage map of RRIM 937-LOD 5.0 (Kosambi mapping function). The majority of the markers were located on linkage groups 1 and 7.

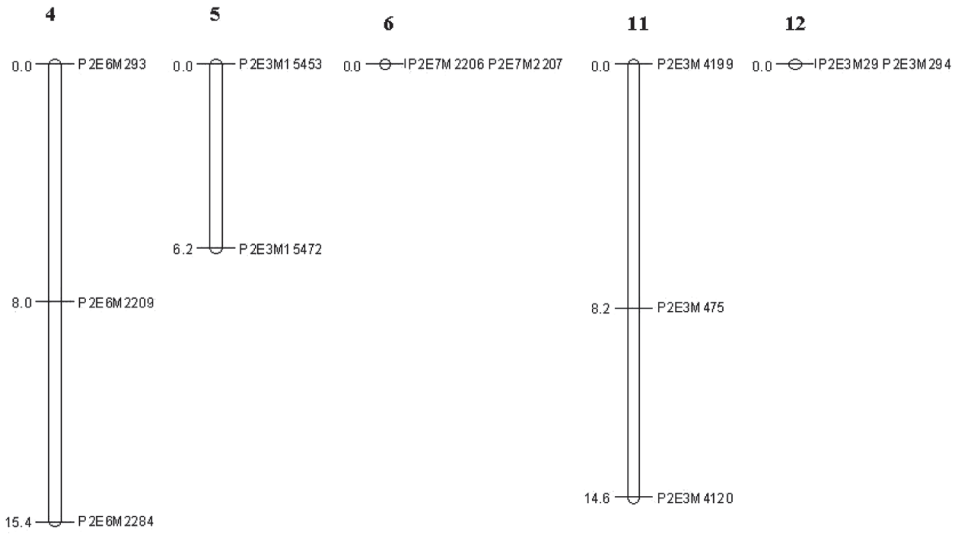

Figure 8. Genetic linkage map of RRIM 600 (LOD 5.0, Kosambi mapping function). RRIM 600 is the recurrent parent for the population RRIM 937 × RRIM 600 causing inbreeding depression and thus less segregating markers.

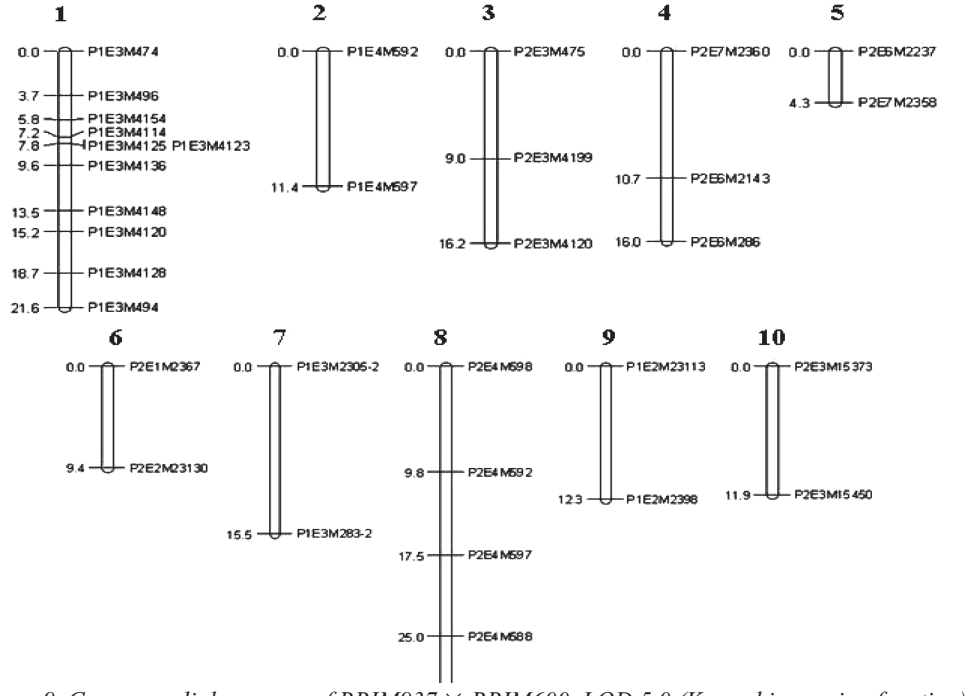

Figure 9. Consensus linkage map of RRIM937 × RRIM600, LOD 5.0 (Kosambi mapping function) was constructed using 3:1 segregating markers. The majority of the markers were found to cluster in the centromere area of Group 1.

There was no duplication of loci within each map but some loci were present in both maternal and paternal maps (*Figures 10* and *11*). Seven loci were duplicated in linkage group 1 in both PB 5/51 and IAN 873 clones in which five loci (E3M131371, E3M13140, E3M13220, E3M13159, E1M 27169) maintained the same arrangement in both clones whilst two loci (E3M13209 and E1M13230) maintained the arrangement between them but are in a different position.

It appears that the EcoRI-based AFLP markers localise preferentially in centromeric regions. These markers are expected to cluster in certain regions in the genome such as in the centromere[26,27]. EcoRI-based AFLP markers are insensitive to methylation and markers developed with this restriction enzyme combination tend to identify with hypermethylated polymorphic regions of the genome *i.e.* the centromere. AFLP markers characteristically cluster in the centromeric regions in plant species with a large genome[13,28] as with many other plants with smaller genomes like *Arabidopsis thaliana* (L.) Heynh[27], *Petunia hybrida* (Hook) Vilm[29] and *Coffea arabica* L.[30].

In order to attain better coverage of the genome, it would be better to utilise *Pst*I/*Mse*I primer combinations. The *Pst*I/*Mse*I primer combination would be more efficient in detecting polymorphism than *EcoR*I/*Mse*I primers, as *Pst*I recognises unmethylated euchromatic regions and is more randomly distributed across the chromosome regions[31,32]. However, Gubliti-Onarici *et al.*[33] showed that *Pst*I/*Mse*I primer combinations produced a smaller number of polymorphic bands in Alfalfa (*Medicago sativa* L.). The reduction in fragments, and hence polymorphic markers, is consistent with the lesser number of restriction fragments generated by *Pst*I genomic digests. The *Pst*-AFLP markers generally mapped to regions containing a lower density of markers similar to that of the RFLP markers[34]. With this in mind along with data from an earlier mapping study using the RFLP marker technique in *Hevea*[9,35], the application of this primer combination may not be able to produce the quantity of polymorphic markers needed to construct dense genetic maps of *Hevea*.

The maps for RRIM 937 and RRIM 600 did not have the basic number of linkage groups of 18 chromosomes. One explanation is that both clones are closely related, whereby RRIM 600 is not only the paternal parent for the population, but also the maternal grandparent for RRIM 937. The *EcoR*I/*Mse*I primer combinations generated 125 loci for clone RRIM 937 which were placed on 15 linkage groups (*Figure 7*). Unfortunately only linkage groups 1, 3, 4, 7 and 11 were mapped after linkage analysis. Linkage groups 2, 5, 6, 8, 9, 10, 12, 13, 14 and 15 had only two loci which could not form pairwise estimate data and were rejected. As for RRIM 600, only linkage groups 4 (3 loci), 5 (2 loci), 6 (2 loci), 11 (3 loci) and 12 (2 loci) were mapped (*Figure 6*) while the other markers remained unlinked.

The RFLP marker technique was able to distinguish interspecific and intraspecific *Hevea* clones using hybridisation probes from the *Hevea* genomic DNA[36,37]. By a combination of appropriate probes and enzymes, some of the popular clones could distinguish between some siblings, *i.e.* cultivars which share two common parents. Sibling pairs such as PR 255 and PR 261; RRIM 937 and RRIM 938 which are progenies of crosses between Tjir 1 × PR 107 and between PB 5/51 × RRIM 703, respectively, were distinguishable by their RFLPs. However, this marker technique was unable to generate enough polymorphic markers that can be used to construct genetic linkage maps when applied to the mapping populations that are being studied.

Since the process involved in generating RFLP markers is long, tedious and time consuming, PCR-based markers were employed in the study. One such marker was RAPD, but it was observed that this population had 1/20th of the amount of polymorphic markers when compared to *Pinus radiata*[9]. After the observation made here with the inclusion of AFLP markers, it is obvious that even with the use of other markers like microsatellites and using *Pst*I/*Mse*I AFLP primer pairs, no such improvements can be done.

It is imperative for MRB to create new mapping populations to study primary (latex and wood yield) and secondary (disease resistance) characteristics. This new population(s) must use parental clones that have as divergent a background as possible. Clones such as FORD 351 (a Brazilian clone) and RRIM 938 (Malaysian clone, progeny of PB 5/51 × RRIM 703) are being considered as parents for the future mapping population as Gouvêa *et al*.[38] discovered that Amazonian genotypes had a higher genetic diversity than the Asian genotypes.

Comparisons between Parental Maps

Due to the paucity of markers and the low coverage of the maps, duplication of markers on the same clone was not detected as was reported in apple[12] and in the PB 260 × RO 38 *Hevea* map[13]. It was observed that some of the markers were linked together but on different clones (*Figures 10* and *11*). Group 1 for PB 5/51 and IAN 873 had the same 7 markers in which E3M13371, E6M14364, E3M13140, E3M13220, E3M13159 and E1M27169 were linked and maintained the same arrangements in PB 5/51 and IAN 873. Meanwhile E3M13209 and E1M13230 are linked to each other but at different positions on the linkage groups in each clone. Other linked markers and their locations are summarised in *Table 7*.

Meanwhile, only 3 markers were found to be linked in group 4 of both RRIM 937 and RRIM 600 (E6M293, E6M2209, E6M2284) (*Figure 12*). It was obvious here that the loci seem to have been generated by the same *EcoR*I/*Mse*I primer sets but the molecular weights differed slightly. It is possible that the polymorphisms in both populations were only a few nucleotides apart.

CONCLUSION

Comparing all four parental maps, it is observed that RRIM 937 and RRIM 600 exhibit an inbreeding depression as seen by the lack of loci located in linkage groups and the reduced number of linkage groups. In normal cases, inbreeding depression will cause reduction in the fitness of the organism such as reduced timber yield[39], low fruit set[1,40] and susceptibility to diseases. Eventhough this was the case, the reduction in fitness can be overcome by good agronomic practices as seen in the case of RRIM 600. In its heyday, RRIM 600 was able to produce 1,866 kg/ha/yr[41]. This clone was once the most sought one

TABLE 7. SUMMARY OF LINKED MARKERS IN PB 5/51 AND IAN 873

	Loci	Linkage Group/ Clone
1.	E6M1862/E6M172	9 (PB 5/51); 5 (IAN 873)
2.	E4M14126/E4M24126	19 (PB 5/51); 15 (IAN 873)
3.	E6M18130/E6M18141	13 (PB 5/51); 7 (IAN 873

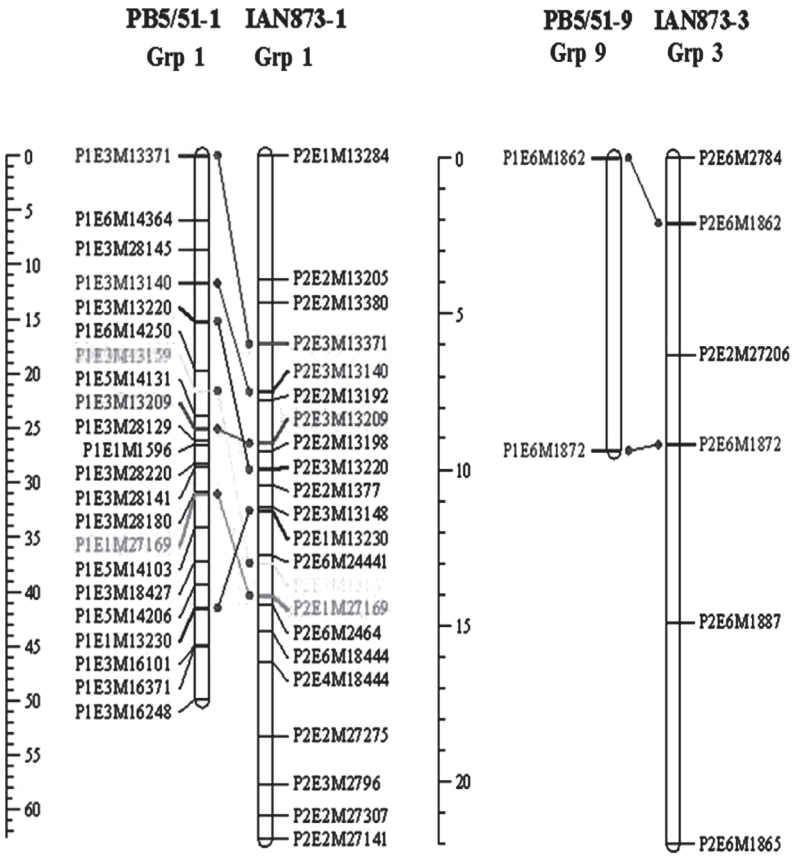

Figure 10. Genetic linkage map of PB 5/51 × IAN 873 (LOD 5.0, Kosambi mapping function). Allelic bridges are indicated by lines. No duplications of markers were observed within the clones but some existed between clones (coloured markers).

in Malaysia in the 1970's and 1980's, before it was replaced by the RRIM 900 and RRIM 2000 series. Whilst in Thailand, India and Indonesia, RRIM 600 is still widely planted.

The effect of inbreeding of *Hevea* has not been thoroughly examined. From the construction of the genetic linkage maps for both populations, it is observed that the Malaysian clones are in danger of inbreeding. Recent breeding programme at MRB has introduced new parental clones, mainly from the 1981 and 1995 IRRDB germplasm collection with the objective to produce *Hevea* clones with high timber yields. This step had inadvertently broadened the genetic pool for *Hevea* in Malaysia[42,43].

It is necessary to develop and utilise a larger number of molecular markers such as cDNA-AFLP, single nucleotide polymorphic (SNP) markers and SSR to obtain a denser and saturated map with distance of markers at 1 cM to 5 cM apart. A denser map will enable the detection of important QTL such as resistance to *C. cassiicola*.

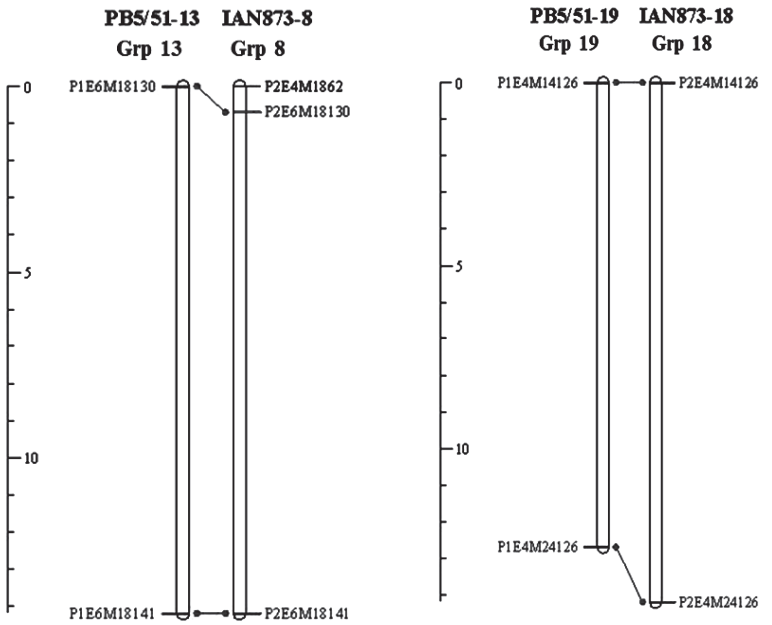

Figure 11. Genetic linkage map of PB 5/51 × IAN 873 (LOD 5.0, Kosambi mapping function). Allelic bridges are indicated by lines. No duplications of markers were observed within the clones but some existed between clones (coloured markers).

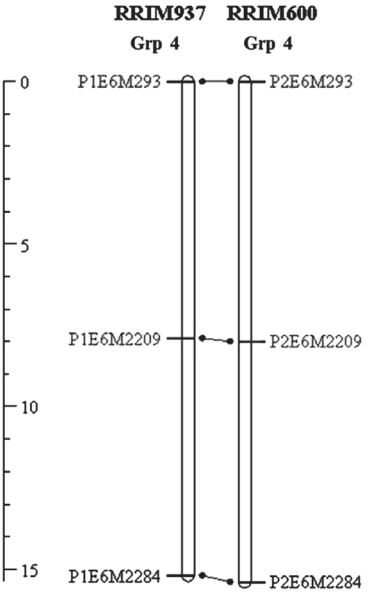

Figure 12. Genetic linkage map of RRIM 937 × RRIM 600 (LOD 5.0, Kosambi mapping function). Allelic bridges are indicated by lines. No duplication of markers within clone was observed.

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