Investigation on Random Amplified Polymorphic DNA Markers for the *Corynespora* Leaf Fall Disease Resistance in Rubber (*Hevea brasiliensis*)

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ABSTRACT

***Corynespora* Leaf Fall (CLF) disease is a devastative agent in the natural rubber industry in Sri Lanka, causing great impact on the economy. In order to facilitate the conventional screening methods used in selecting elite clones for field establishment specially for disease resistance molecular markers play a great role. In this study an attempt was made to identify RAPD markers for CLF disease resistance. Sixteen *Hevea* genotypes which have been previously screened for the CLF disease along with the standard resistant and susceptible clones RRIC100 and RRIC 103 respectively were analysed with six random primers in order to identify reliable RAPD markers for CLF resistance. A total of 51 bands showed polymorphism. Initial clustering using the RAPDistance separated RRIC 103 along with two other susceptible genotypes from the rest. In this analysis, the resistant clone RRIC 100 clustered with both resistant and susceptible clones. Nine bands which have more than 62% linkage values were then selected and used in the clustering by RAPDistance program. Then nine selected markers clearly separated the RRIC 103 and it's most related two genotypes separating from the rest, similar to earlier results. Though the standard resistant clone RRIC 100 was included in the main cluster with other resistant and susceptible genotypes, each sub cluster within the main cluster was clearly distinguished the resistant and susceptible clones. This study showed that the nine RAPD marker bands selected can clearly identify the genomic region of *Hevea* which might cause for the severe susceptibility to CLF disease in *Hevea* as found in the susceptible clone RRIC 103.**

KEY WORDS: *Corynespora* Leaf Fall Disease, *Hevea brasiliensis*, RAPD marker

INTRODUCTION

Rubber is the second largest plantation crop in Sri Lanka, next to tea. The total area under rubber cultivation is estimated to be 115,000 ha in 2005. The export earnings of the same year are Rs Mn 3718 and the value added percentage to GDP is 0.6 (Anon, 2005).

Therefore, it is important to secure the natural rubber production while increasing the productivity of the cultivation lands with proper management practices. The management of foliar diseases including *Corynespora* Leaf Fall (CLF) has become a major concern at present. CLF caused by *Corynespora cassicola* (Burk & Curt) is currently considered as the most destructive leaf disease of rubber (*Hevea brasiliensis*) in Asian & African continents (Jayasinghe, 2003).

It was revealed that dynamic changes of the pathogens might interfere with the long lasting resistance of this perennial host posing a great threat to the outstanding rubber clones in the world. Symptoms of CLF appear on both mature and immature leaves. The most characteristic diagnostic feature is described as railway track appearance or herringbone pattern as a result of browning or blackening of the veins adjacent to the lesions. Disease on polybag nursery could be seen on any clone leading to die – back including highly resistant clones in the field such as RRIC 100 and RRIC 121 (Jayasinghe, 2003).The control of disease can be done by using effective fungicides over a long period and by planting resistant clones. The chemical control of CLF is practiced only in nurseries. It is inefficient in the field because it waste not only the chemicals but also the time and labour. Therefore the identified effective method for control of CLF is a development of resistant clones through plant breeding. In *Hevea*, perennial plantation crop species with an economic life span of around 30 years and an immaturity period of seven years, identification of elite cultivars prior to field planting assumes much significance (Varghese *et al., 1997*).

Therefore, to reduce the time taken for breeding and selection cycle, use of effective DNA marker specific for the resistant trait are of paramount importance. The application of molecular marker technology in genotype identification, play important role as it avoids environmental influences.

More frequently, breeders use the Random Amplified Polymorphic DNA (RAPD) marker technology (Williams *et. al.*, 1990). Individual RAPD primers are able to anneal several sites within the target DNA, leading the production of number of Polymerase Chain Reaction (PCR) fragments. These fragments are usually generated from different regions of the genomes and hence multiple loci may be examined very quickly. Universal sets of primers are used without any need for sequence information. Therefore it is applicable to the analysis of most organisms. It is also useful in the detection polymorphisms, mapping of populations, isolation of markers linked to various traits or specific targeted intervals and analysis of

intervals and analysis of parentage. It is relatively inexpensive, fast, reliable and very appropriate to use in average plant breeders' laboratory.

The objective of the present investigation was to develop RAPD markers capable of identifying the CLF disease resistant or susceptible *Hevea* clones at early stage in the above selected progenies.

MATERIALS AND METHODS

The study was conducted at the Genetic and Plant Breeding Department of the Rubber Research Institute Substation at Niwithigalakale, Matugama.

Plant materials

Leaf materials from CLF disease resistant clone RRIC 100 and susceptible clone RRIC 103 along with clones from RRISL 200 series, and selected six genotypes from 1978 and 2005 hand pollination program were obtained from the experimental fields of genetic and plant breeding Department.

Extraction of DNA

Genomic DNA was extracted from fresh leaves using the procedure described by Withanage *et al,* (2005). According to that protocol, nine volumes of extraction buffer (100 mM Tris HCI, PH 8.0, 50 mM EDTA, 500 mM NaCl) were mixed with one volume of 10 % SDS to prepare the extraction solution. Ten milligrams of apple green colour leaf materials were ground quickly with 800µl extraction solution in a mortar. Then the liquid phase was transferred to 2 ml ependorf tube which has been placed in ice. This was mixed well by slowly inverting the tubes with equal volume of chloroform for 5-10 minutes and spun at 7500 rpm, for 4 minutes and 30 seconds. After centrifugation, the supernatant was pipetted out to another tube and 1µl of RNAase was added to the supernatant. After mixing, it was kept at room temperature for 10-15 minutes. Then 600-700 µl of 100% Ethanol was added, mixed well and spun at 7500 rpm for 4 minutes and 30 seconds. After removing the supernatant the DNA pellet was washed twice with 100 µl of 70 % Ethanol for 1 min at 1000 rpm and dried at room temperature. The DNA pellet was re-suspended in 50 µl of autoclaved ultra purified water and stored at 4^0C.

Analysis of DNA Quality & Quantity

Purity and the concentration of DNA samples were determined using the spectrophotometer and electrophoresing on 0.8 % agarose in 0.5x TBE buffer. After electrophoresis the intensity and the width compared to the γ DNA standard under UV transilluminator. DNA samples were diluted to give 50-100 ng/µl concentration to perform PCR.

RAPD Assay

Decamer primers from Operon Technologies, Almeda, USA were used for amplification. PCR was performed in 20µl reaction volumes. It consists of 50-100 ng template DNA with 1x PCR buffer (Mg^{+2} free), 25 mM $MgCl_2$, 2 mM dNTPs and one unit of Taq DNA polymerase (promege) and 16.5 ng primer.

Table 1 - Details of the genotypes used for the study:

Plant number	Clone	Pedigree	ADSI value	Description
1	RRIC 100	RRIC 52* PB 86	0	no disease
2	RRIC 103	RRIC 52* PB 86	4	very severe
3	2005 H/P	RRIC 103 * RRIC 103	4	very severe
4	2005 H/P	RRIC 103 * RRIC 103	4	very severe
5	211	RRIC 101 * RRIM 600	0	no disease
6	208	RRIC 101 * RRIM 600	2.86	severe
7	210	RRIC 101 * RRIM 600	observe under bud wood nursery	resistant
8	207	RRIC 101 * RRIM 600	3	severe
10	216	illegimate	0	no disease
11	225	RRIC 102 * PB 28/59	observe under bud wood nursery	resistant
12	223	RRIC 100 * RRIC 100	1	severe
13	215	illegimate	0	no disease
14	226	RRIC 102 * PB 28/59	0	no disease
15	219	RRIC 102 * PB 28/59	0	no disease
16	203	RRIC 100 * RRIC 101	0	no disease
18	78 H/P	RRIC 103 * RRIC 103	3	severe
19	78 H/P	RRIC 103 * RRIC 103	3	severe
20	78 H/P	RRIC 103 * RRIC 103	3	severe

Source: Proceedings of the First Symposium on Plantation Crop Research (2004), TRISL

PCR was performed in Amplitron II cycler for 45 cycles with 1min at 94 ^{0}C, 1min at 36^{0}C and 2 min at 72^{0}C. The amplified products were separated by electrophoresis with 1% Agarose gel at 60 V and visualized under UV-transilluminator with Ethidium Bromide staining.

Selection of Polymorphic Primers

Previously proven ten primers were used for testing polymorphism between CLF resistant genotype RRIC 100 and susceptible genotype RRIC 103 and primers giving reproducible bands were used for the evaluation of the study.

Table 2 - Characters of the selected primers:

Primer	Sequence 5'-3'	Total number of bands	Number of polymorphic bands
A10	DTGATCCCAC	8	2
A12	TCCCCCATAC	9	5
A16	AGCCAGCGAA	4	3
A18	AGGTGACCGT	12	6
A20	GTTGCGATCC	9	7
B7	GGTGACGCAG	9	4

Analysis

The amplified and reproducible bands were scored as 1 and 0 for the presence and absence of bands. RAPDistance computer program was used for analysis of data (Amstrong *et al.,* 1992). Linkage values were calculated for each polymorphic marker loci as below.

Linkage % = (total progeny size - number of recombinant genotypes/total progeny)*100.

RESULTS AND DISCUSSION

Initially ten selected primers (OPA 10, OPA 12, OPA 16, OPA 18, OPA 20, OPB 7, OPB 18, OPC 4, OPC 10, and OPC 16) which were tested previously were used to evaluate the best polymorphic primers between CLF disease resistant genotype RRIC100 and susceptible genotype RRIC103. Six out of that (OPA 10, OPA 12, OPA 16, OPA 18 , OPA 20 and OPA 7) , showed clearly distinguishable polymorphic amplification products with all the 18 genotypes where as the others produce poor amplification products. Example for amplification of selected clones is given in Figure1.

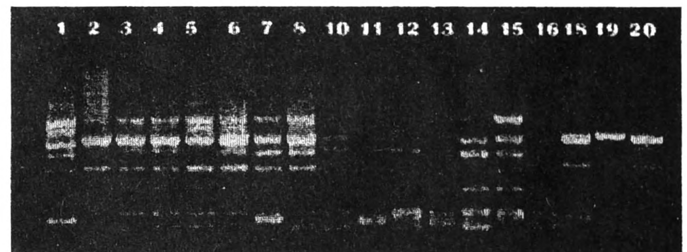

Figure 1 - Amplification of genomic DNA with primer A18. Numbers 1-20 refer to plant numbers as listed in Table 1.

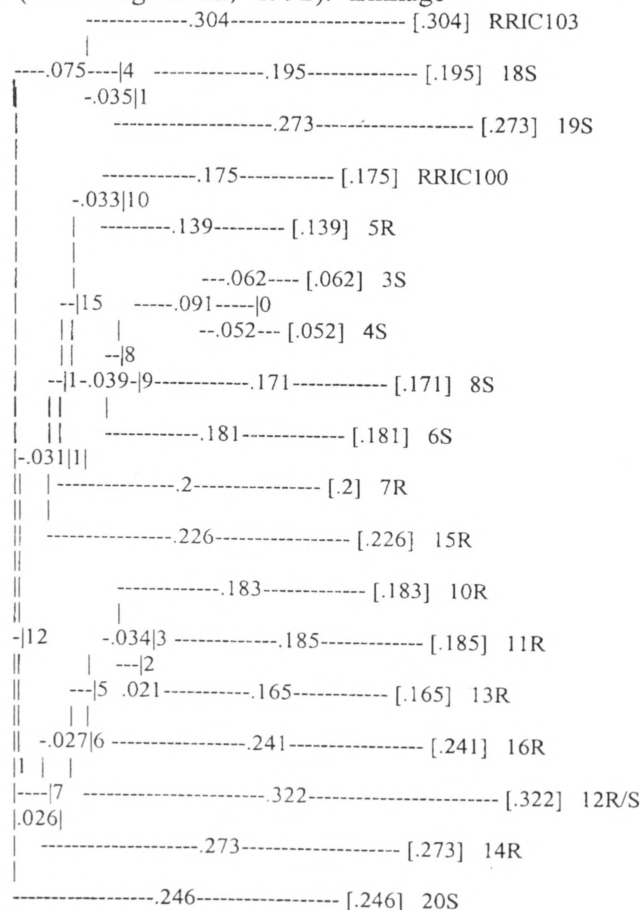

Figure 2 - Tree diagram for the eighteen genotypes:

Table 3 – Genetic distance between eighteen genotypes:

Clone	100	103	5	7	10	11	12	13	14	15	16	3	4	6	8	18	19	20
100	0.000																	
103	0.676	0.000																
5	0.314	0.632	0.000															
7	0.414	0.597	0.394	0.000														
10	0.568	0.638	0.522	0.541	0.000													
11	0.632	0.655	0.587	0.577	0.357	0.000												
12	0.655	0.715	0.662	0.598	0.601	0.590	0.000											
13	0.586	0.681	0.569	0.560	0.402	0.350	0.566	0.000										
14	0.539	0.780	0.549	0.568	0.513	0.588	0.612	0.504	0.000									
15	0.441	0.665	0.388	0.441	0.532	0.539	0.616	0.466	0.530	0.000								
16	0.577	0.655	0.558	0.548	0.486	0.447	0.552	0.439	0.588	0.539	0.000							
3	0.435	0.630	0.306	0.465	0.525	0.559	0.707	0.517	0.550	0.429	0.612	0.000						
4	0.422	0.648	0.285	0.453	0.514	0.549	0.701	0.507	0.541	0.416	0.604	0.114	0.000					
6	0.485	0.607	0.361	0.383	0.519	0.587	0.638	0.595	0.577	0.507	0.587	0.373	0.356	0.000				
8	0.478	0.624	0.356	0.447	0.541	0.548	0.655	0.532	0.539	0.500	0.577	0.329	0.310	0.343	0.000			
18	0.577	0.553	0.558	0.577	0.588	0.566	0.659	0.577	0.650	0.596	0.600	0.530	0.519	0.587	0.548	0.000		
19	0.642	0.592	0.620	0.642	0.692	0.681	0.717	0.707	0.665	0.687	0.716	0.647	0.638	0.623	0.642	0.469	0.000	
20	0.485	0.635	0.496	0.485	0.577	0.616	0.577	0.568	0.548	0.535	0.587	0.500	0.489	0.522	0.542	0.557	0.623	0.000
Avg	0.525	0.646	0.480	0.511	0.537	0.555	0.637	0.537	0.579	0.519	0.569	0.484	0.475	0.509	0.501	0.571	0.647	0.550

Those selected six primers (Table 2) clearly exhibited 51 bands while 27 bands showing polymorphism between RRIC 100 and RRIC 103. Using the 51 RAPD markers scored, genetic distances were estimated (Table 3) and a dendrogram was formed to see the grouping of resistant and susceptible clones (Figure 2).

Genetic distance among eighteen genotypes was detected (Table 3) and it showed a range between 0.475 and 0.647. Plant number 19 was identified as the most distant (0.647) followed by RRIC 103 and plant number 18 with genetic distance values 0.646 and 0.571 respectively.

In the cluster analysis (Figure 2), also these three plants clustered separately showing strong susceptible characters with a similar pedigree (Table1). The cluster analysis with all the bands of all six primers (Fig 2) formed three main clusters, one main cluster containing susceptible genotypes RRIC 103, 18 and 19. This shows the uniqueness of these genotypes. Although RRIC100 is resistant it has clustered with other resistant and susceptible clones. Plant number 20 which is a self product of RRIC103 (Table1) clustered separately. The cluster analysis using all the bands therefore was capable of clustering RRIC 103 and related genotypes. As this cluster analysis was not adequately separate the resistant and susceptible genotypes, another cluster analysis was performed based on the linkage values of each RAPD loci of polymorphic bands. The bands which showed linkage value more than 62 % (Table 4) were used for this analysis.

Except primer B 7, other 5 primers showed linkage values more than 62 % in nine polymorphic bands (A10 – 5 , A10-7, A 12 -8 , A16 -2 , A16 – 4 , A 18 – 9 , A 18 -10 , A18 – 11 , A 20 -5) while A16-2 and A18-11 showing value of 81%.

According to the results obtained, there are two main clusters (Fig 3). Plant number 18 and 19 were clustered together with the standard for susceptible genotype, RRIC 103. These plants were observed clustering together even in the cluster analysis of all bands of all primers. These cluster also proves the genetically relatedness of three clones where the 18, 19 plants are inbreds of RRIC 103. Therefore the selected nine bands would adequately identify the genomic regions for CLF disease susceptible as found in susceptible checked clone RRIC 103.

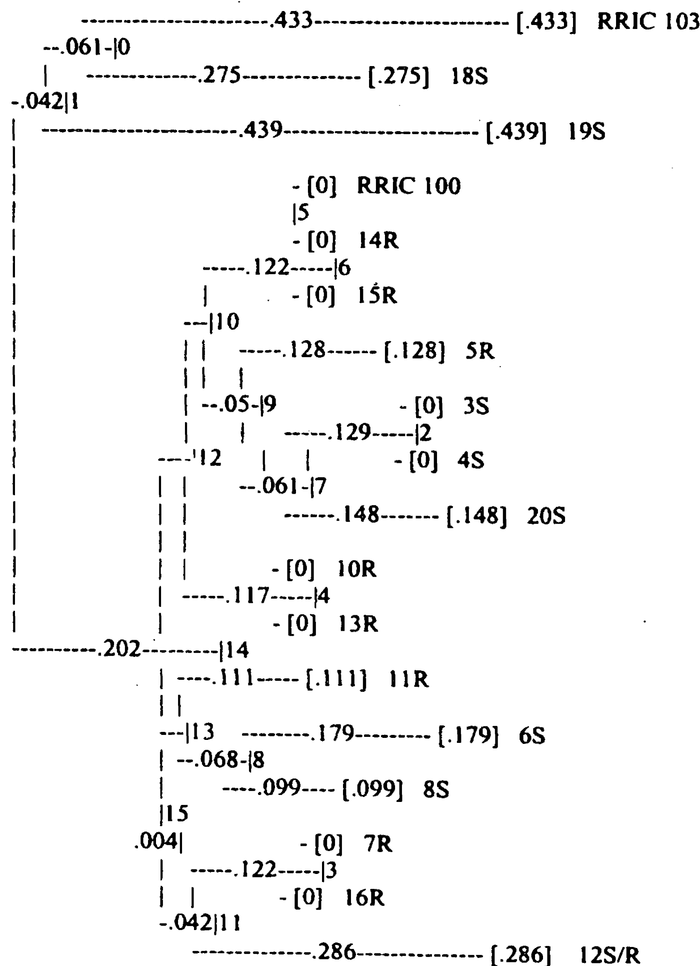

Figure 3 -Tree diagram for the eighteen genotypes with selected bands:

Table 4 - RAPD banding patterns obtained for all the genotypes with selected markers:

Primer	Band	100	103	5 R	7 R	10 R	11 R	13 R	14 R	15 R	16 R	3 S	4 S	6 S	8 S	12 S	18 S	19 S	20 S	Linkage %
A10	5	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	62
	7	1	0	1	0	1	0	0	0	1	0	1	1	0	0	0	0	0	1	62
A12	8	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	69
A16	2	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	81
	4	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	63
A18	9	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	63
	10	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	63
	11	1	0	0	1	1	1	1	1	1	1	0	0	0	0	1	1	0	0	81
A20	5	0	1	0	0	1	1	1	0	0	0	1	1	1	1	0	1	0	0	63

RRIC 100 clustered separately with a mixture of resistant and susceptible genotypes. But within that main cluster, 10 sub clusters were contained, either resistant or susceptible. Plant number 3 and 4 which are susceptible and have similar parents, clustered together and it shows relatedness to the clone 20 which also have the same pedigree, from those three clones , plant number 5, resistant to CLF disease separated clearly. Two illegimate clones, 10 and 13 and also the plant number 12 which had shown resistant to CLF disease earlier also clustered separately. With regard to the ADSI (Average Disease severity Index) values (Table 1),the plants which are in similar ADSI ranges,clustered together at sub cluster levels such as plant number 3 and 4, plant number 6 and 8, plant number 10 and 13, plant number 14 and 15 and plant number 18 and 19.

Plant number 7 and 11 which have been observed as resistant plants in bud wood nursery also grouped with resistant clones. But the fact that they have not tested in the field suggest that the clone number 11 likely to be prove as susceptible in the field.

Although limited number of primers and clones were used in this study, these RAPD makers shows their usefulness in distinguishing the CLF resistant and susceptible *Hevea* genotypes, each sub cluster differentiating resistant or susceptible genotype. By using these clones as standard genotypes, bulk of *Hevea* genotypes can be differentiated into resistant and susceptible sub clusters.

CONCLUSIONS

Selected nine marker loci were not adequate to differentiate the CLF resistant and susceptible genotype. But the markers are capable of differentiating CLF disease resistant and susceptible genotypes separately at the sub cluster level, each sub cluster containing only resistant or susceptible genotypes. However the selected RAPD loci identified the potential genomic region of the RRIC 103 which would determine CLF susceptibility in this clone and it's related genotypes.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to all the staff members of the Department of Plant Genetics and Breeding, RRISL for all the support given throughout the study.

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