Development of Random Amplified Polymorphic DNA Markers (RAPD) for Corynespora Leaf Fall Disease Resistance in Rubber (*Hevea brasiliensis*)

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ABSTRACT

One of the most destructive foliar diseases in rubber is Corynespora Leaf Fall (CLF) disease. The best method for control of this disease can be achieved by introducing resistant genotypes. In this respect, early identification of disease resistant and disease susceptible genotypes is important. Genetic markers provide valuable tools in this respect. This study was carried out to identify RAPD markers linked to CLF disease resistance.

The standard disease resistant clone RRIC 100 and the susceptible clone RRIC 103 and their selfed progenies were used in the study. Five highly polymorphic primers (OPA18, OPA10, OPC16, OPA6 and OPA20) giving polymorphism between RRIC 100 and RRIC 103 clones were selected to evaluate the progeny individuals. Three primers (OPA 18, OPC 16 and OPA 10) giving consistent bands with CLF disease resistant and susceptible individuals were selected. Among them the most informative bands for differentiating the CLF disease resistant and susceptible genotypes were observed as OPA18-09, OPC16-18, OPA10-25, OPA10-26, OPA10-28 and OPA10-29. These six marker loci were used to build up a tree diagram by using RAPDistance computer program. Two clusters were obtained and all the susceptible genotypes except one were clustered with the RRIC 103. Three of the resistant genotypes of RRIC 103 selfed progeny were also clustered in this group. Other cluster consisted mainly of the resistant genotypes, RRIC 100, RRIC 100 selfed progeny and four of the genotypes of RRIC 103 selfed progeny. The only exception in this cluster was one susceptible genotype of the RRIC 103 selfed progeny. Bands from A6 and A20 primers were also identified as suitable markers for further investigation.

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

INTRODUCTION

Sri Lanka is the 6th largest Natural Rubber (NR) producer in the world with 114000 ha of land under rubber (*Hevea brasiliensis*). It is the 3rd largest plantation crop in Sri Lanka, significantly contributing to the economy by providing raw materials to the domestic rubber based industries, providing employment and by earning foreign exchange. Around 61.7% of the local rubber production was consumed by the local industries and it has earned Rs.3718 million foreign exchange in the year 2004 (Anon, 2004). As a result of monoculture situation and the use of genetically uniform planting materials, more and more foliar diseases have become prominent, especially with the use of high yielding varieties. Among these, the most devastating foliar disease is the Corynespora Leaf Fall disease (CLF) (Liyanage *et al.*, 1986), which is caused by the fungus *Corynespora cassiicola*. A typical symptom of this disease is the railway track appearance or fish bone pattern on leaf blade. Repeated defoliation occurs on highly susceptible clones resulting the death of the plant. Main features of this disease are the selective nature in attacking different clones, occurrence throughout the year, ability of producing different types of symptoms depending on type of clone and ability of attacking all the development stages of plants (Jayasingha, 2004).

Disease was first reported in epidemic proportion in 1985 on the clone RRIC 103. As a result, 4500 ha representing more than 3% of the total area under rubber in Sri Lanka were uprooted (Jayasingha, 2003). Another outstanding clone succumbed to the disease was RRIC 110. During recent past more than a dozen of potential clones have been withdrawn from the experimental sites as they become highly susceptible to CLF. Different management practices have been launched by the Rubber Research Institute of Sri Lanka (RRISL) to safeguard the NR industry, such as chemical control measures. However, it is not economically feasible to the industry. The only solution is the production of resistant or tolerant clones to the disease by rubber breeding programs. Among the presently recommended clones RRIC 100 is proved to be resistant to the disease and RRIC 121, RRIC 102, RRIC 130, PB 28/59, PB 260 and PB 235 are also considered as disease tolerant. However, development of genetically improved rubber clones with resistance to the disease is a long term, time consuming and expensive procedure. It takes approximately 20 years for a new clone to be recommended for planting. In this procedure, identification of resistant clones at nursery level is necessary for the early selection of disease resistant plants.

Molecular markers have now been proved as very useful genetic markers for the selection of disease resistance. These markers, especially the DNA markers, have many advantages, such as, they are not affected by the environment, developmental stage or by gene interactions. DNA markers could be detected with a little amount of the sample. Considering the above advantages it is envisaged in this study to develop Random Amplified Polymorphic DNA Markers (RAPD) for early selection of CLF disease resistant genotypes.

MATERIALS AND METHODS

The study was carried out at the Genetics and Plant Breeding Department of the Rubber Research Institute Sub-station at Nivithigalakele, Matugama.

Plant Materials

Leaf materials from CLF disease resistant clone RRIC100 and CLF disease susceptible clone RRIC103, and leaf materials from their selfed progenies were obtained from an experimental site of the Genetics and Plant Breeding Department. Disease ratings on each of the progeny individual are given in table 1.

Table 1: Details of the progenies used for the study

Cross	Progeny size	Plant number and their disease rating
RRIC100x100	7	2-R, 3-R, 6-R, 8-R, 9-R, 11-R, 12-R.
RRIC103x103	10	13-R, 14-R, 15-R, 16-R, 17-R, 18-R, 20-S,21-S, 23-S,24-S.

R Resistant to the disease; S Susceptible to the disease.

PCR Primers

Thirty-eight primers from 10-mer primer series of OPA, OPB, OPC and OPE produced by Operon technologies, Almeda, USA were used.

DNA Isolation

DNA isolation was done using the method available at RRISL (Withanage *et al.*, 2005). In this method, extraction buffer (100 mM, Tris HCl pH 8.0, 50 mM EDTA, 500 mM NaCl) and 10% SDS was mixed in 9:1 proportion to prepare the solution for the extraction procedure. Ten milligrams of leaf materials were ground with 800 µl of the prepared solution on ice. The ground mixture was pipetted out in to an eppendorf tube to which an equal volume of chloroform was added. The solution was mixed for 10 minutes and centrifuged for 4 minutes and 30 sec at 7500 rpm. Then, the supernatant was pippetted out to a new tube and 1 µl of RNAase was added to the supernatant and kept for 10-15 minutes at room temperature. A volume of 650-700 µl of 100% ethanol was added to the tube and centrifuged for 4 minutes and 30 sec at 7500 rpm. The DNA pellet was washed twice with 100 µl of 70% ethanol for 1 minute at 1000 rpm. The pellet was air dried and resuspended in 50 µl of deionized distilled water.

Assessment of the DNA Quality and Quantity

Purity and the quantity of the DNA isolates were tested by agarose gel electrophoresis. Gel contained with 0.8% agarose in 0.5x TBE buffer. Following electrophoresis DNA was visualized under UV transilluminator. According to the intensity and the width of the DNA bands, concentrations of the samples were estimated with compared to the λ DNA standard.

Dilution of DNA Samples

The concentrated DNA samples were diluted to give 50-100 ng/2µl. Diluted samples were tested for the concentration by agarose gel electrophoresis.

Polymerase Chain Reaction (PCR)

PCR was performed in 20 µl reaction volume. It contained 50-100 ng template DNA with 1x PCR buffer(Mg^{2+} free), 2.5 mM $MgCl_2$, 2 mM dNTPs and one unit of *Taq* polymerase (Promege) and 16.5 ng primer. The reaction mixture was covered by a drop of mineral oil (Sigma). Amplification was performed using Amplitron II cycler for 45 cycles, each cycle consisting of 94 °C for 1 minute, 36 °C for 1 minute and 72 °C for 2 minutes. The amplified products were observed by gel electrophoresis at 70 v in 0.8% agarose gel in 0.5x TBE buffer and visualized by Ethidium bromide staining on a UV transilluminator.

Selection of Polymorphic Primers

Thirty-eight primers were initially tested for the selection of polymorphic primers between the standard resistant and susceptible genotypes *i.e.* RRIC100 and RRIC103. Selected primers giving reproducible polymorphic bands were used to evaluate the individuals of the two progenies.

Preparation of λ Digested DNA

λ digested DNA was used as a size marker in the gel electrophoresis process. DNA was digested in 75 µl volume containing 7.5 µl of 1x RE buffer 0.75 µl of BSA, 15 µl of λ DNA, 4.25 µl of *Hind* III and 47.5 µl of distilled water. All the components were mixed in a microfuge tube and it was placed in a water bath at 37°C for 2 hours to complete the digestion. Finally, the reaction mixture was heated at 65°C for 10 minutes.

Analysis

The amplified bands were scored as 1 and 0 for the presence and absence of bands, respectively. RAPDistance computer program was used for the analysis of the data (Armstrong *et al.*, 1995)

RESULTS AND DISCUSSION

Initially thirty-eight primers were used to select the best polymorphic primers between CLF disease resistant and CLF disease susceptible genotypes, RRIC 100 and RRIC 103, respectively. Only eleven primers showed polymorphism between the two clones and others were monomorphic. Table 2 summarizes the results obtained with respect to each of the eleven primers.

It was observed that, eight primers *viz.* A10, A16, C10, A18, A6, A20, A12 and C16 produced more than 30% polymorphic bands. After checking the reproducibility, five of them namely, A18, A10, A20, A6 and C16 were used for the detailed analysis. Though all the five primers were tested in PCR, only three primers A18, C16 and A10 produced a clear and consistent banding pattern with all the nineteen genotypes used in this study.

Table 2. Characteristics of selected primers and behaviour on RRIC 100 and RRIC 103 in PCR

Primer	Sequence 5'-3' prime.	Total number of bands	Number of polymorphic bands	Polymorphism in %
A10	GTGATCCCAC	10	5	50
A16	AGCCAGCGAA	6	3	50
C10	TGTCTGGGTG	6	3	50
A18	AGGTGACCGT	10	4	40
A6	GGTCCCTGAC	5	2	40
A20	GTTGCGATCC	5	2	40
C16	CACACTCCAG	9	4	33
A12	TCCCCCATAC	3	1	33
A7	GAAACGGGTG	4	1	25
B18	CCACAGCAGT	4	1	25
B7	GGTGACGCAG	7	1	14

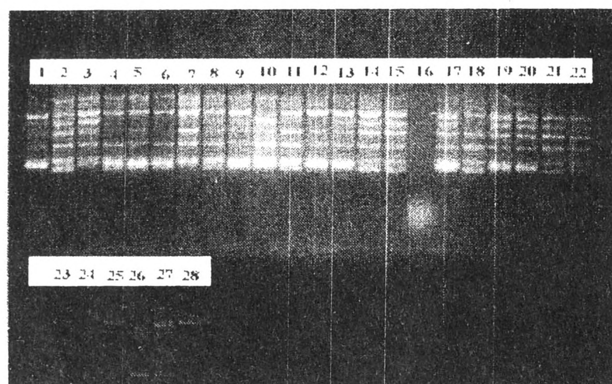

Figure 1. Amplification of genomic DNA with primer A18: *Lane 1 and 2 RRIC100 and RRIC103. Lane 4,5,8,10,11,13, and 14. RRIC100 selfed progeny individuals of 2,3 6,8,9,11 and 12. Lane 15,19,20,21,22 and 23; The genotypes of 13 14,15,16,17 and 18 in RRIC 103 selfed* progeny. Lane 17,18,27 and 28;. The susceptible genotypes of 20,21,23, and 24 in RRIC 103 selfed progeny.

Figure 1 indicates the RAPD bands obtained from the primer A18 for nineteen genotypes

RAPD Analysis of RRIC 100 Selfed Progeny

All the seven individuals of the RRIC 100 selfed progeny proved to be resistant to the CLF disease. RAPD analysis using the selected three primers was carried out on RRIC 100, RRIC 103 and the selfed progeny of RRIC 100 with the objective of identifying the highly heritable PCR bands specific to the CLF disease resistant genotype RRIC 100 (Table 3).

Fourteen bands were identified showing polymorphism for CLF disease resistant and CLF disease susceptible genotypes RRIC 100 and RRIC 103. Segregation pattern of these bands and their degree of association in the RRIC 100 selfed progeny are shown in table 4. An index for the marker- trait association was calculated as (1-Number of recombinant genotypes/Total progeny) X 100 for each of the marker loci.

Though the primer bands A18-1, A18-2, A18-10, C16-15 and A10-21 were polymorphic between the resistant and susceptible clones RRIC 100 and RRIC 103, the RAPD results of the progeny individuals showed low association values of the parental banding pattern with the CLF disease resistant individuals of the progeny. The primer band A10-21 was not detected among any of the individuals of the RRIC 100 selfed progeny. The primer bands C16-11, C16-13 and C16-19 showed around 70% association with the resistant genotypes. Six PCR bands, A18-9, C16-18, A10-25, A10-26, A10-28 and A10-29 were shown to have a considerable association with the resistant clone RRIC 100 (85%-100%). A18-9, C16-18 and A10-28 were selected as most linked markers for the CLF disease resistant genotypes of RRIC 100 selfed progeny. A18-9, C16-18, A10-25, A10-26, A10-28 and A10-29 were further tested using the selfed progeny of the susceptible clone RRIC 103.

RAPD Analysis of RRIC 103 Selfed Progeny

Both CLF disease resistant and susceptible genotypes were present in the selfed progeny of RRIC 103, suggesting the segregation of CLF disease resistant genes in the progeny. RAPD analysis using the same selected three primers was carried out on RRIC 100, RRIC103 and selfed progeny of RRIC103, to observe the segregation of the six selected RAPD markers A18-9, C16-18, A10-25, A10-26, A10-28 and A10-29 between the CLF disease susceptible and CLF disease resistant genotypes of the RRIC103 selfed progeny. Total number of bands observed for the three primers are given in table 5.

The RAPD polymorphism with respect to the selected six primer bands obtained from RRIC100, RRIC103 and selfed progeny RRIC 103 and their degree of association are given in the table 6. Within the progeny, plant numbers 13-18 were resistant and the plant numbers 20-24 were susceptible to the disease.

No strong relationship could be established between the CLF disease resistant genotypes and the inheritance of the selected RAPD marker loci. The degree of association of the selected markers with CLF disease resistance varied between 30%-70% suggesting that these markers are not tightly linked to the CLF disease resistance. Only three RAPD loci (A10-28, A10-29 and A10-25) have shown more than 50% association.

A cluster analysis was performed to examine the usefulness of the selected six RAPD loci in distinguishing the CLF disease resistant and CLF disease susceptible genotypes (Figure 2).

Table 3. RAPD banding patterns obtained for RRIC 100 and RRIC 103 and the genotypes of RRIC 100 Selfed progeny for the selected three primers

Band No		RRIC 100	RRIC 103	RRIC 100 Selfed progeny						
				2	3	6	8	9	11	12
	1	-	+	+	+	+	+	+	-	-
	2	-	+	+	+	+	-	+	-	+
	3	+	+	+	+	+	+	+	+	+
	4	-	-	-	-	-	-	+	-	-
A18	5	+	+	-	-	+	+	+	+	+
	6	+	+	-	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+
	9	+	-	+	+	+	+	+	—	-
	10	-	+	+	+	+	-	+	-	+
	11	-	+	+	+	-	-	-	-	-
	12	+	+	+	+	-	-	+	—	+
	13	+	-	+	+	-	-	+	+	+
	14	+	+	+	+	+	+	+	+	+
C16	15	-	+	+	+	+	+	+	-	-
	16	-	-	+	-	-	+	+	-	-
	17	+	+	+	+	+	+	+	+	+
	18	+	-	+	+	+	+	+	+	-
	19	-	+	-	-	-	-	+	+	-
	20	+	+	+	+	+	+	+	+	-
	21	+	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	+	-	-
	23	-	-	-	-	-	-	+	-	-
	24	+	+	-	-	-	-	+	-	-
A10	25	-	+	-	-	-	-	-	-	-
	26	-	+	+	-	-	-	-	-	-
	27	+	+	-	-	-	-	+	-	-
	28	+	-	+	+	+	-	+	+	+
	29	-	+	-	-	-	-	-	-	-

- *Bands absent ;* + *Bands present*

Table 4. Summery of the banding pattern obtained for the clones of RRIC100, RRIC103 and the genotypes of RRIC100 selfed progeny.

Band No:		RRIC 100	RRIC 103	RRIC 100 selfed progeny							Degree of association
				2	3	6	8	9	11	12	
	1	-	+	+	+	+	+	+	-	-	28%
A18	2	-	+	+	+	+	-	+	-	+	28%
	9	+	-	+	+	+	+	+	+	-	85%
	10	-	+	+	+	+	-	+	-	+	28%
	11	-	+	+	+	-	-	-	-	-	71%
	13	+	-	+	+	-	-	+	+	+	71%
C16	15	-	+	+	+	+	+	+	-	-	28%
	18	+	-	+	+	+	+	+	+	-	85%
	19	-	+	-	-	-	-	+	+	-	71%
	21	+	-	-	-	-	-	-	-	-	No association
A10	25	-	+	-	-	-	-	-	-	-	100%
	26	-	+	+	-	-	-	-	-	-	85%
	28	+	-	+	+	+	-	+	+	+	85%
	29	-	+	-	-	-	-	-	-	-	100%

- Bands absent ; + Bands present

Two main clusters were obtained. All the susceptible genotypes (20, 21, 24 and RRIC103) except the plant number 23 were clustered in to one main group with three of the resistant genotypes of the RRIC 103 selfed progeny. All the other resistant genotypes RRIC 100, RRIC 100 selfed progeny and three resistant genotypes of RRIC 103 selfed progeny were grouped in to a separate cluster. The only susceptible individuals clustered in this resistant group have shown 60% bands common with the RRIC 100.

Though a limited number of RAPD markers and progeny individuals has been used in this study, the cluster diagram has adequately differentiated the CLF disease resistant and susceptible genotypes. Inclusion of all the RRIC 100 selfed individuals

Table 5. Banding pattern for genotypes of RRIC103 selfed progeny for A18, C16 and A10 primers.

				Resistant genotypes of RRIC103 selfed progeny					Susceptible genotypes of RRIC 103 selfed progeny				
Band No:		RRIC 100	RRIC 103	13	14	15	16	17	18	20	21	23	24
	1	-	+	-	-	-	-	-	-	-	-	-	-
	2	-	+	-	-	-	-	-	-	-	-	-	-
	3	+	+	+	+	+	+	+	+	+	+	+	+
	4	-	-	-	-	-	-	-	-	-	+	-	-
A18	5	+	+	+	+	+	+	+	+	+	-	+	+
	6	+	+	+	+	+	+	+	+	+	+	+	+
	7	+	+	+	+	+	+	+	+	+	+	+	+
	8	+	+	+	+	+	+	+	+	+	+	+	+
	9	+	-	-	+	+	-	-	+	+	-	+	-
	10	-	+	+	-	-	+	+	+	-	+	-	+
	11	-	+	+	-	+	+	+	-	-	-	-	-
	12	+	+	+	+	-	+	-	+	+	-	+	-
	13	+	-	+	-	+	+	-	+	-	+	+	-
	14	+	+	-	+	-	+	+	+	+	-	-	-
C16	15	-	+	+	-	-	-	-	-	-	+	-	-
	16	-	-	-	+	-	-	-	-	+	-	+	+
	17	+	+	+	+	+	+	-	+	+	-	+	+
	18	+	-	-	-	-	-	-	-	-	+	-	-
	19	-	+	+	-	-	+	-	-	-	+	-	-
	20	+	+	+	+	+	+	-	+	+	+	+	+
	21	+	-	+	+	+	+	-	-	+	+	-	-
	22	-	-	-	-	-	-	-	-	-	+	-	-
	23	-	-	-	-	+	-	-	-	+	-	-	-
	24	+	+	+	+	+	+	-	-	+	+	-	-
A10	25	-	+	+	-	-	-	-	-	+	-	-	-
	26	-	+	-	+	+	+	-	-	+	+	-	-
	27	+	+	+	-	+	+	+	+	-	-	+	+
	28	+	-	-	-	+	+	+	-	-	-	-	-
	29	-	+	-	+	-	+	-	-	+	+	-	-

+ *Bands present ;* - *Bands absent*

Table 6. Summery of the banding pattern obtained for the RRIC 100 and RRIC 103 clones and the genotypes of RRIC103 selfed progeny.

				Resistant Genotypes					Susceptible Genotypes					
Band No:		RRIC 100	RRIC 103	13	14	15	16	17	18	20	21	23	24	Degree of Association.
A18	9	+	-	-	+	+	-	-	+	+	-	+	-	50%
C16	18	+	-	-	-	-	-	-	-	-	+	-	-	30%
	25	-	+	+	-	-	-	-	-	+	-	-	-	60%
A10	26	-	+	-	+	+	+	-	-	+	+	-	-	50%
	28	+	-	-	-	+	+	+	-	-	-	-	-	70%
	29	-	+	-	+	-	+	-	-	+	+	-	-	60%

+ *Bands present ;* - *Bands absent*

and the resistant clone RRIC 100 in a single cluster shows the possible use of the selected markers in differentiating the germplasm related to RRIC 100 and RRIC 103. This has an important implication, because the genotypes that will fall in the RRIC100 cluster may contain the CLF disease resistant germplasm related to RRIC 100 origin.

Table 4 and 6 depict that, C16-18 band is the most suitable band for categorizing the nineteen genotypes into two groups of RRIC 100 clone and seven genotypes of its selfed progeny, and RRIC 103 clone and ten genotypes of its selfed progeny.

Other than the three main primers used, two of the most reliable primers A20 and A6 were also checked with few numbers of susceptible and resistant genotypes.

Prominent bands that segregate between two groups could be observed using these primers (Figure 3), which are suitable for future studies

In this study, even though the same DNA extraction procedure and PCR program were practiced for all the individuals, some genotypes were consistently difficult to be amplified. Further improvements of the PCR conditions have to be considered in the future experiments.

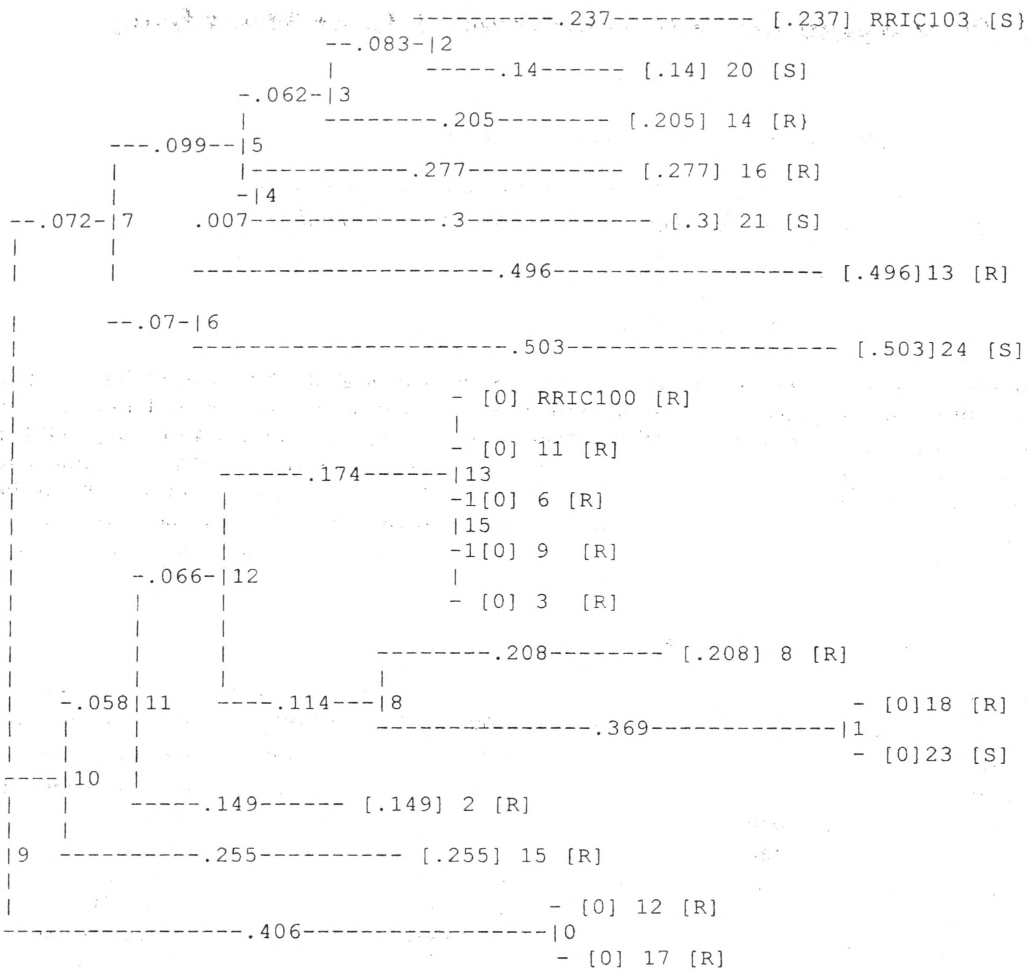

Fig. 2. Tree diagram for the nineteen genotypes.

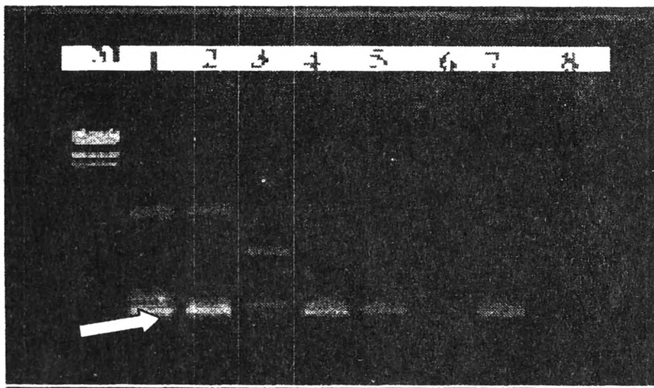

Fig. 3. Banding pattern obtained for four of the resistant genotypes and four of susceptible genotypes with the primer A20. Line M; ***Hind*** III digested λDNA. *Lane 1 and 2 resistant genotypes of 2 and 3 in RRIC 100 selfed progeny. Lane 3 and 4 represent the resistant genotypes of 13 and 14 in RRIC 103 selfed progeny. Lane 5-8; susceptible* genotypes of RRIC *103 selfed progeny 20, 22, 23 and 24. Arrow indicates the desirable band that differentiates the CLF disease resistant and CLF disease susceptible genotypes.*

CONCLUSIONS

Six-selected marker loci adequately differentiated the CLF disease resistant and susceptible genotypes by cluster analysis. They also grouped the RRIC100 and its related individuals separately from RRIC103 and its related individuals. This has an important implication in identifying RRIC100 related genotypes, which contain CLF disease resistant germplasm evolved in the breeding program.

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