Identification of RAPD markers linked to high yielding plants of *Hevea brasiliensis*

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Abstract

Since the upper limit of productivity of any trait of a living organism is determined by the genetic makeup, manipulation of the genetic makeup is important to achieve a high productive unit. Plant breeding is a proven technique used to modify the genetic makeup of a particular species. Therefore, genetically improved *Hevea* clones are produced by conventional breeding methods. Due to perennial nature of the crop, selection the elite genotypes from the breeding population is a serious problem in the current breeding programme. The exploration of genetic markers provides a good solution in breaking up of this limitation. Present study was carried out to find out the possibility to separate potentially high yielders from poor yielders, using the Randomly Amplified Polymorphic DNA (RAPD) technique for the establishment of a genetic marker for rubber breeders.
Due to the highly cross-pollinated nature of *Hevea*, 168 F_1 individuals from a cross between PB-235 and IAN-45/710 were used as the segregation population for the study. According to the yield performances, total population was aligned and 10 individuals from each extreme were subjected to Randomly Amplified Polymorphic DNA (RAPD) technique.
The bulked segregant analysis facilitates easy selection of potentially polymorphic primers for the two groups. Initially 17 primers showed variation in their banding pattern for the pooled DNA. When the individual plant selection, there were only 8 primers from the above 17 were given a polymorphism for the two phenotypic groups. Totally 59 bands were generated by those 8 Operon primers.
There are four bands, which were star graded as significant bands for the separation of two phenotypic groups in the RAPDistance computer programme. The 7^{th} band of OPE-14, the 2^{nd} band of OPE-07, the 5^{th} band of OPS-20, and the 1^{st} band of OPB-14 were the most significant bands for the separation of those two groups. These bands clearly differentiated the two groups. The tree diagram, which was generated by the total band scores for all primers, indicated three clusters; two high yielders and one all the poor yielders excluding one out of nine. These results showed that this technique could be successfully used in germplasm identification and genetic diversity analysis of *Hevea bracilliensis.*

Keywords: RAPD (Randomly Amplified Polymorphic DNA), Primers, Breeding population, Bulked segregant analysis.

Introduction

Sri Lanka is the sixth largest producer of natural rubber (NR) in the world and rubber is a one of the large-scale plantation crops in this country. The contribution of the rubber sector for the total Gross Domestic Production (GDP) is about 0.46%. Since 1996, total rubber production has declined gradually by 23% and the yield per hectare dropped by 6% to 712kg in 2000. The drop in production is due to both the lower extent under cultivation and lower yield per unit area. (Central Bank Report 2000).
According to the Rubber Research Institute (RRI) of Sri Lanka, the natural rubber industry basically depends upon two rubber clones, RRIC -100 and PB- 86. In the event of pest and diseases out break on these two rubber clones, the entire rubber industry could be in danger. Therefore, to minimize these risks, a clone diversity programme needs to be implemented.
The demand for NR in the world increases at the rate of 30000 MT yr^{-1} and there is a 0.8 MT deficiency between the NR production and the demand (Thilakarathne *et al.* 1997). Therefore it has been planned to increase the rubber production by introducing new technologies.
In Sri Lanka, there are no extra lands for the expansion of rubber cultivation. Hence, increasing the productivity of rubber lands is the only remedy to increase the NR production. Presently NR industry is facing a competitive situation with Synthetic Rubber (SR) in the world rubber market. Therefore to

increase the profitability of NR industry, cost of production should be reduced and the technical properties of NR should be improved.

The easiest and cheapest way to achieve these objectives is, by the use of genetically improved *Hevea* clones as planting material and replacing low yielding rubber clones by promising the high yielding and disease resistant clones.

Genetic improvement of *Hevea* is a long, time consuming and expensive procedure. It could take approximately 22 years for a new clone to be recommended for planting even on a limited scale. During this procedure, identification of clones at nursery level is necessary for the early selection of particular type of plants (i.e. disease resistant, high yielding etc).

Traditional approach for early selection of rubber clones is, by the use of physiological, anatomical and morphological characters for identification. Among anatomical characters, relationship between girth and yield, numbers of latex vessel rings and rubber production, and bark thickness and yield have been studied.

These traditional approaches possess several limitations. The most significant among them are, dependency of these traits on the environment conditions, difficulties of using these traits for selection at nursery level and lack of persons having a trained eye to distinguish cultivars by means of their morphological traits. Further, long-term directional selection has narrowed down the genetic variability of rubber and as a result present generations of clones are morphologically similar in appearance. Therefore visual identification of different genotypes is more difficult. This limitation is marked with the young bud wood nurseries, where plants do not completely express their distinguishable characteristics. This situation demands the development of more objective methods in genotype differentiation.

In plant breeding, the generation of improved genotypes is dependant upon the genetic recombination and segregation of traits among the progenies of a genetic cross between two parents. The identification of the desired recombinant trait among the segregating population can be an arduous task for the breeder.

Through DNA marker technology, a particular locus on a chromosomal segment, which is linked to a desirable trait, can be identified with a selectable DNA marker and the segregation of that particular segment (desirable trait) can be monitored in subsequent crossing. As tools to assist the plant breeder, DNA markers are more useful and powerful than morphological markers for genetic analysis of perennial crops. Since DNA markers are direct characters of the genotype and are independent of the environment and phenotype for their evaluation, they provide more accurate identification at a much earlier age.

Materials ans Methods

Basic Method: Bulked segregant analysis involves screening of the differences between two pooled DNA samples derived from a segregating population that originated from a single cross. Each pool, or bulk, contains individuals selected to have identical genotypes for a particular genomic region or a trait. Each pool is tested with RAPD and the selected polymorphic primers are then used to test individual plants.

Plant material

PB-235 and IAN-45-710 parents were crossed in 1999 and seedlings derived from them were grown at the multiplication nursery of the Department of Genetics and Plant breeding, RRI substation, Mathugama. Using the early performances (i.e. volume of latex, dry rubber weight, bark thickness and girth) of the progeny two sets of plants were selected from the two extremes of the population distribution. Mother plants, PB-235 and IAN-45-710 were collected from the department premises and Dartonfield estate respectively.

The immature leaf materials from the parents and the selected plants of the progeny were used for extraction of genomic DNA.

DNA extraction

Miniprep procedure for DNA extraction, which was first introduced by Dellaporta *et al.* 1983 was used for the isolation of genomic DNA.

Approximately 1 g of leaf materials was quickly frozen in liquid nitrogen and ground to a fine powder using a motar and pestle. Then the powder was quickly transferred to an orkridge tube and 15 ml of the extraction buffer (final concentration 100 mM Tris, 50 mM EDTA, 500 mM NaCl) was poured over the powder in the tube. Then 10μl of mercaptoethanol and 2ml of 10% SDS were added and the contents were mixed gently by inversion. As the next step the tube was incubated at 65 ^{0}C for 10 minutes. After the addition of 5 ml of 5M potassium acetate, the mixture was well mixed and incubated on ice for 20 minutes.

After about 20 minutes on ice, the contents were centrifuged at 14,500 rpm in a Beckmann J2-21 centrifuge for 20 minutes and the supernatant was taken in to a clean orkridge tube. Then 10 ml of isopropanol was added to the supernatant and mixed them gently and the mixture was incubated under – 20 °C for about 30 minutes minimum. The DNA was pelleted at 13,000 rpm for 15 minutes. DNA was allowed to dry by inverting the tube on a paper towel. The fully dried pellets were then re-dissolved in 0.7 ml of 50 mM Tris-EDTA (pH 8)and transferred to an eppendorf tube.
The eppendorf tube with DNA solution was spun for 10 minutes to remove any insoluble debris. The supernatant was decanted to a new microfuge tube and 2 μl of RNAse were added and incubate at 37 °C for 10 minutes to remove RNAs from the DNA matrix. Finally, 500 μl of isopropanol was added to the incubated Eppendorf and the tube was spun for 10 minutes to pellet the DNA. The DNA pellet was washed twice with 70% ethanol and allowed to dry. Then the dried pellet was re-dissolved in 35 μl of deionised distilled water.

PCR procedure

Since RAPD is a PCR based molecular marker assay, several numbers of PCRs were practiced.

PCR reaction mixture (20 μl)

- One unit of Taq polymerase.
- 50 mM KCl.
- 10 mM $MgCl_2$
- 10 mM Tris HCl (pH 8.3)
- 0.01% (w/v) Gelatin.
- 0.2 mM each dCTP, dATP, dTTP, dGTP.
- 20 pM at each primer.
- Genomic DNA. (~ 100ng)
- Distilled water up to 20μl.

Commercially PCR kits, which contain Taq polymerase, $MgCl_2$ and PCR buffer are available. Therefore, such PCR kits were used for this experiment.

PCR procedure

According to the number of reactions, the contents of cocktail were calculated and they were taken into a pre-sterilized Eppendorf tube. All the preparations were carried out on ice. 17.5 μl of the cocktail was first added to each pre-sterilized PCR tube and 0.5 μl of random primers and 2 μl of DNA were added to each PCR tube. Then the total volume of reaction mixture was 20μl. Each reaction mixture was topped with 30 μl mineral oil and the PCR tubes were placed on the wells of automated thermocycler to proceed the PCR programme. The PCR programme contains following parameters;

Initial denaturation temperature	96 °C	-4 minute
Denaturing temperature	94 °C	-1 minute.
Annealing temperature	36 °C	-1 minute
Primer extension temperature	72 °C	-2 minute.
Soaking temperature	4 °C.	
Total number of cycles	45.	

Selection of polymorphic primers

The bulked segregant analysis method was used to select primers, which has a high potential to give polymorphism between two groups.
Four plants from each category (i.e. from high yielding group and low yielding group) were randomly selected and 10μl DNA from each sample was pooled to form two pooled DNA samples.
Then these pooled DNA was used in PCR procedure. The amplified products were analysed on 1.2% agarose gels. The presence of RAPD bands was scored by visual inspection of the gel photograph and the presence or absence of band was scored as 1 or 0 respectively. Thirty random primers were subjected to initial PCRs of bulked DNA to select possible polymorphic primers and 10 random primers (out of 30) were selected for PCRs of individual plant DNA.

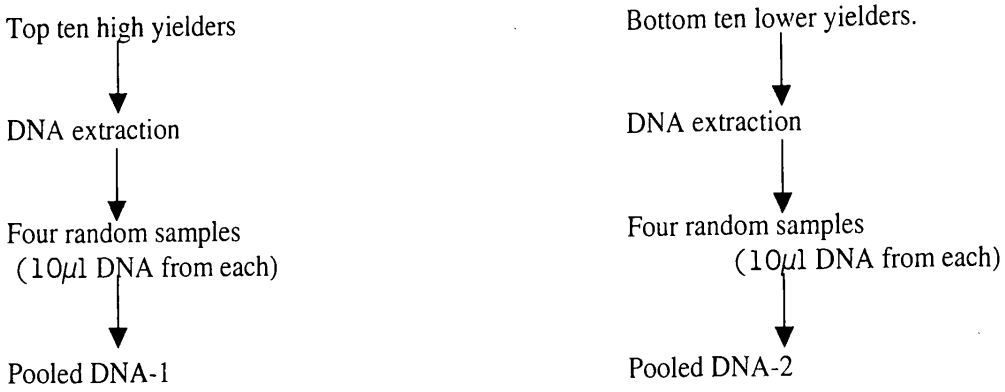

Figure 2. Preparation procedure of the pooled DNA.

Results and Discussion

Selection of plants from the progeny

According to the yield performances (i.e. volume of the tapped latex and the dry weight) 26 individuals were equally selected from the two extremes of the progeny for DNA extraction; 13 individuals were high yielders and the other 13 were the low yielders. From these, the top nine individuals and bottom nine individuals were used to cluster programmes.

Table 2. Tree numbers of selected individuals

High yielders	Low yielders
42	70
73	75
159	124
61	111
72	113
67	117
106	164
55	150
81	126

Selection of polymorphic primers

The bulked DNA from each extreme was subjected to RAPDs. 30 primers were tested and 17 primers out of them showed polymorphism. (eg: Plate 2)

Individual assessment of Randomly Amplified Polymorphic DNA

Highly polymorphic primers, which were revealed from bulked segregant assay, were used for individual assessment. Eight such primers were subjected to PCRs of individual plant DNA.

Table 3. Nucleotide sequence of the primers used for the individual assessment and their banding pattern.

Primer	Nucleotide sequence 5'-3'	# of amplified bands	# of polymorphic bands
OPE-18	GGACTGCAGA	10	10
OPE-03	CCAGATGCAC	6	2
OPE-14	TGCGGCTGAG	7	6
OPE-07	AGATGCAGCC	8	5
OPC-01	TTCGAGCCAG	9	6
OPC-16	CACACTCCAG	7	5
OPS-20	TCTGGACGGA	8	8
OPB-14	TCCGCTCTGG	4	3

Distance matrix and dendogram of the progeny

The "RAPDistance" computer programme proposed by Armstrong *et al* in 1995 was used for the detection of genetic distance between individuals of the progeny and for the cluster analysis. The pair wise comparison between individuals was applied for the detection of genetic distance. As shown in table 4 the genetic distance between the samples ranged from 0.103448 to 0.423729. This programme calculated pair wise distance between the DNA samples using the band data, which were fed in to data file. Similarities between individuals were calculated by using "Dise formula" .

Dise formula,

2*n11/((2*n11)+n01+n10)

n11- number of common bands

n01- number of specific bands (x =1, y = 0)

n10- number of specific bands (x =0, y = 1)

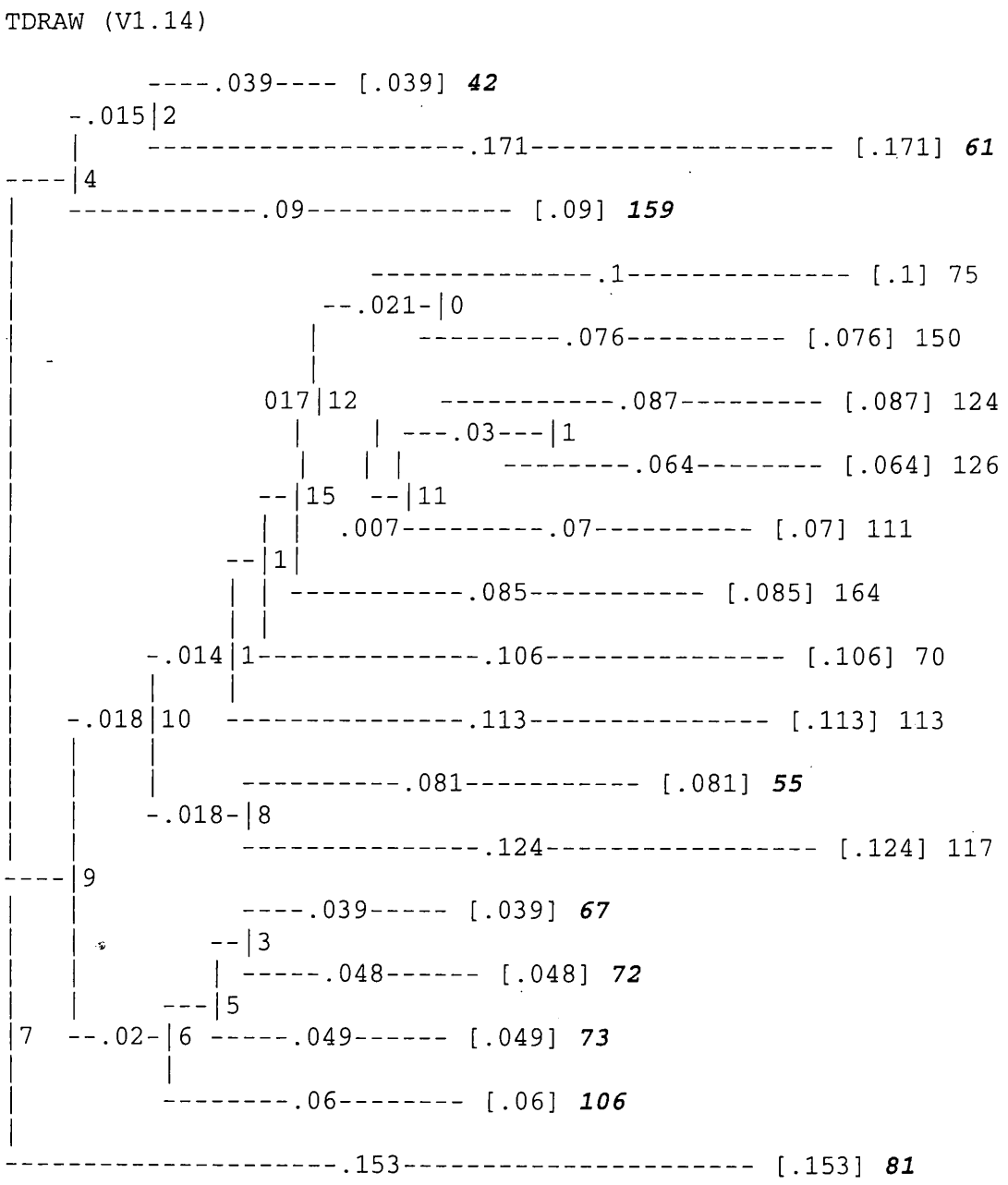

Figure 3. Tree diagram (Dendogram) for the selected individuals of the progeny.

When the banding scores of total polymorphic primers were fed in to the programme, the above tree diagram was obtained. The most significant primers for the separation of these two groups were also given. In the score sheet those primers were star graded (Tables 5 and 6).

Table 5. RAPDCORL BANDS output with their significance for clustering

Band No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	significance
1	0	0	1	0	1	0	1	1	1	1	1	1	1	0	1	0	0	1	
2	0	0	1	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1	
3	0	0	1	0	1	1	1	1	0	1	1	0	1	0	0	0	0	0	
4	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	
5	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	
6	1	0	1	0	1	1	1	1	1	1	1	0	0	0	0	1	0	1	
7	0	0	1	0	0	0	0	1	1	0	1	0	0	0	1	1	0	0	
8	1	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	0	
9	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
10	1	1	1	0	0	1	1	0	1	0	0	0	1	0	0	0	1	0	
11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
13	1	1	1	1	0	1	0	0	0	1	1	1	1	0	1	0	1	0	
14	1	1	0	1	1	0	0	0	1	1	1	0	1	1	1	0	1	1	
15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
17	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
18	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
19	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	
20	0	0	0	1	1	0	1	0	0	1	0	1	1	0	0	0	0	1	
21	1	0	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	0	
22	1	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	
23	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	
24	1	0	1	1	1	0	0	1	1	0	1	1	0	1	0	1	1	0	
25	1	0	1	1	1	1	0	1	1	0	1	1	0	1	1	1	1	1	
26	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	
27	1	0	1	1	0	1	1	0	1	1	0	1	1	0	0	0	0	0	
28	0	1	0	1	0	1	1	1	1	0	0	0	0	0	0	0	0	0	
29	1	0	1	1	0	1	1	1	1	1	0	1	0	1	1	1	1	0	
30	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
31	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
32	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	***
33	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	
34	1	1	1	1	0	1	0	1	1	0	0	0	1	1	1	1	1	0	
35	0	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	
36	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	0	1	
37	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	***
38	0	0	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	
39	1	0	1	0	0	1	1	1	1	1	0	0	0	0	0	1	1	0	
40	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	
41	1	1	1	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	
42	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	****
43	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	
44	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
45	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
46	1	1	0	1	1	1	0	1	1	1	1	0	1	0	1	1	1	0	
47	1	1	0	1	0	0	1	1	0	0	1	0	0	1	0	1	0	1	
48	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	
49	1	1	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	0	
50	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	1	0	
51	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
52	1	1	1	1	1	1	1	0	1	0	0	1	0	0	0	0	0	0	
53	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
54	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
55	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	
56	**1**	**1**	**1**	**1**	**1**	**1**	**0**	**1**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	***
57	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
58	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	
59	1	0	0	1	1	0	1	0	0	1	0	1	0	1	1	1	0	0	

In the score sheet,

Sample Number 1-9 ⟶ High yielding category.

Sample Number 10- 18 ⟶ low yielding category.

Table 6. Primers, number of their bands and significance

Primer	Band range	Number of bands	Significant band
OPE-18	1-10	10	No
OPE-03	11-16	6	No
OPE-01	17-25	9	No
OPE-14	26-32	7	7^{th} (***)
OPS-20	33-40	8	5^{th} (***)
OPE-07	41-48	8	2^{nd} (****)
OPC-16	49-55	7	No
OPB-14	56-59	4	2^{nd} (***)

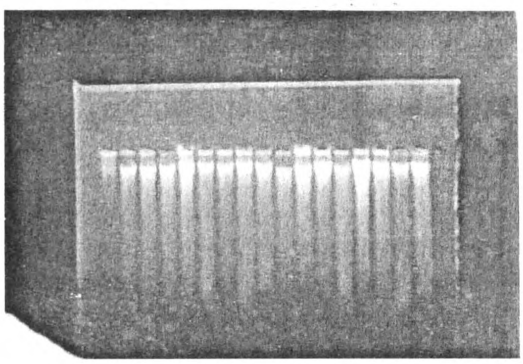

Plate 1: Initial concentration of extracted DNA. First lane contains 250 ng of • DNA.

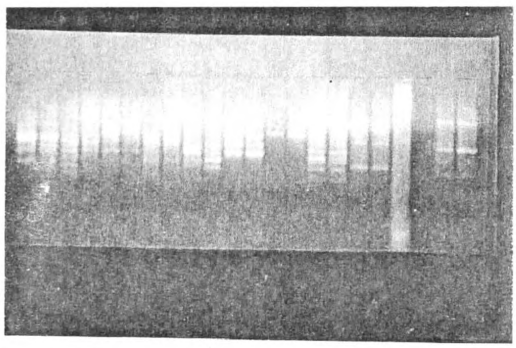

Plate 2. The bands given for the pooled • DNA

Every odd number lane contains pooled DNA from randomly selected high yielding individuals.
Every even number lanes contain pooled DNA from randomly selected lower yielding individuals.

Lane 1-2: OPE-13 Lane 9-10: OPY-05 Lane 3-4: OPE-14 (POLYMORPHIC)
Lane 5-6: OPE-15 Lane 7-8: OPE-18 (POLYMORPHIC) Lane 11-12: OPY-11
Lane 13-14: OPY-17 Lane 15-16: OPS-20

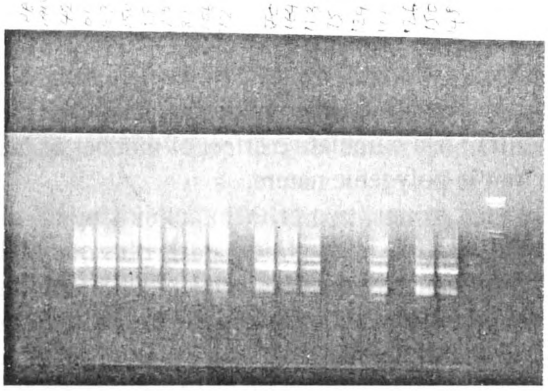

Plate 3. Bands for the primer OPC-16

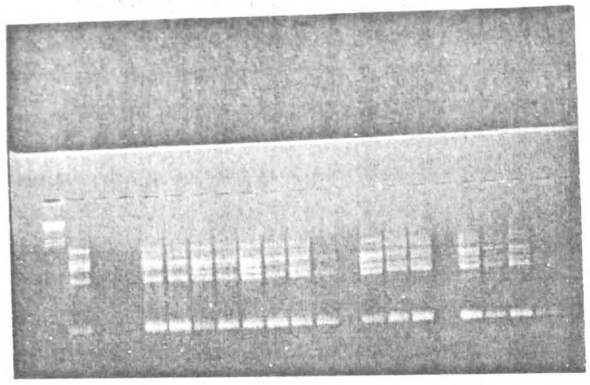

Plate 4: Bands for primer OPC-04

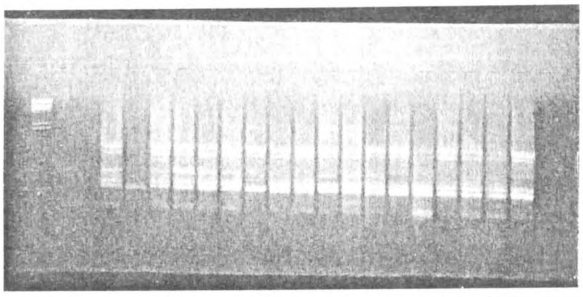

Plate 5. Bands for primer OPC-01

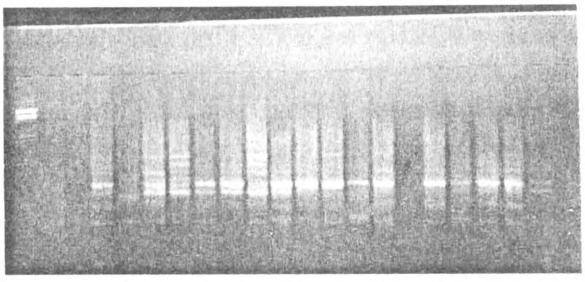

Plate 6. Bands for primer OPE-14

Conclusion

Bulked segregant analysis is a method developed for rapid identification of markers linked to any specific region of the genome. The underlying principle of bulked segregate analysis is the grouping of informative individuals together so that a particular genomic region can be studied against randomised genetic background of unlinked loci. In each bulk the individuals are identical for the trait or gene of interest. Although, bulked segregate analysis gives easy results for monogenic or qualitative traits, it could be extended to the analysis of genetically complex traits (Quantitative traits) by screening bulks of informative individuals. When the quantitative trait is controlled by a few major genes (QTLs), the comparison of bulks of extreme individuals could be easy by markers linked to QTL (Michelmore *et al.* 1991).

In this study the desired trait (i.e yield) was not governed by a single gene. Since the contribution of each gene for the trait varies in polygenic conditions, the phenotype cannot be clearly distinguished from the genetic level. Because the overall trait is a cumulative effect of number of genes, it is extremely difficult to find a molecular marker for a trait in polygenic nature.

In this study, there were three cluster groups, two of them comprising all the high yielding individuals while the other cluster composed of all low yielding individuals plus one high yielder (i.e. 55). The high yielding nature of this "55"can be a cumulative result of many of poor yielding genes.

The other important factor is that, further studies should be carried out to find whether the selected groups behave similarly at their economic lifetime. In order to confirm as a RAPD marker, it must be carried out the same experiment for several other high yielding individuals and low yielding individuals.

In this study, same DNA extraction procedure and same PCR programme was practised for all selected individuals of the progeny and both parents. However, IAN 45/710 (parent) was rarely amplified. In

DNA level, there was nc significant difference of IAN 45/710 with others. The general principle in RAPDs is that the amplification depends on the presence of inverted repeats in the genome at an amplifiable distance. But, it cannot be a reason for no amplification of IAN 45/710, because there were several numbers of primers tested on IAN 45/710. The purity of DNA is not much considered on RAPD amplification because RAPDs answers even for partially purified DNAs. Therefore another trials should be carried out to find the problem in amplification of in 45/710 because the parental variation must be studied in order to achieve a fair conclusion.

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