Molecular Characterization of Recommended Rubber (*Hevea brasiliensis*) Clones of RRISL200 and RRISL2000 Series by using Random Amplified Polymorphic DNA (RAPD) Markers.

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ABSTRACT

Hevea brasiliensis **is commercially grown for natural rubber. Genetically mixed stands of rubber raised from impure nurseries are one of the reasons contributing to the low productivity and management difficulties in plantations. Presently, rubber cultivars are distinguished by means of their phenotypic traits. By this approach, it is difficult to identify the present generation of rubber clones, especially at the nursery stage. Therefore, a more objective method for clone identification is essential.**

Molecular markers are important tools to reveal the genetic differences between individuals avoiding any of the environmental influences. Therefore, the Random Amplified Polymorphic DNA (RAPD) marker technique was used for molecular characterization of recommended RRISL200 and RRISL2000 series clones.

The DNA samples from the two series of clones were subjected to Polymerase Chain Reaction (PCR) with three selected primers *viz*, OPA10, OPA12 and OPA20. Even with the most polymorphic primers selected, the genetic distances obtained proved close relatedness among the two series of clones. The two series of clones were not clearly separated into two groups. The clone RRISL2000 was classified separately from the other clones of this series. A key to identify eleven clones was developed based on the RAPD profiles of two primers OPA20 and OPA12. Seven clones were uniquely identified by the OPA20 primer. The remaining four clones were differentiated by the primer OPA12.

KEYWORDS: Rubbers, *Hevea brasiliensis*, RAPD Markers, Clone Identification, RRISL200 Series, RRISL2000 Series.

INTRODUCTION

Rubber tree (*Hevea brasiliensis*), is commercially grown for natural rubber. In extent wise rubber is the third largest plantation crop next to tea and coconut in Sri Lanka. It covers a land area of 114,000 ha and represents 7.5 percent of the cultivated land area. The total rubber production in year 2004 was 98.4mnkg (Anon, 2004). Natural rubber industry highly contributes to the economy of Sri Lanka, by earning 3718 million rupees as foreign exchange by exporting raw rubber and different rubber products, and providing employment to about 500,000 people both directly and indirectly.

The main criteria which determine the share of natural rubber in the world rubber market is the price, which in turn, determined by the cost of production. If natural rubber could be supplied at a lower price than that of the synthetic rubber, it will create an advantageous position for natural rubber in the global rubber market. The cheapest and convenient way to reduce the cost of production is, by increasing the productivity of natural rubber plantations, which can be achieved using genetically improved, high yielding planting materials. It takes approximately about 22 years to recommend an improved planting material as a clone.

There are 46 clones in the current clone recommendation of Rubber Research Institute of Sri Lanka (RRISL) (Attanayaka *et al.*, 2001). These clones have potential to provide dry rubber yield up to 2500kgha^{-1}yr^{-1}.

Since the genetic variability of present breeding population has been narrowed down due to long term directional selection, most of the newly recommended clones are phenotypically more alike. Therefore, visual identification of different genotypes is more difficult and not much accurate. However, at present, rubber cultivars are distinguished visually by their phenotypic trait. This traditional approach of clone identification poses several limitations to the rubber breeders and growers. This is because the phenotypic traits are influenced by the environmental conditions and age of the tree. Scarcity of trained people for clone identification is also a limitation. Therefore, more reliable and objective method for clone identification is necessary.

Mixed stands of rubber in most of rubber plantations give several management problems such as high variation in yield, girth and on the tapping system. Very commonly, majority of the mature and immature stands are impure, especially with the large number of new clones recommended. This has mainly arisen from the poorly maintained nurseries, where the genetic authenticity of the clones in many nurseries is questionable. Therefore, there is an urgent need to develop an accurate clone differentiation method.

Application of molecular biological strategies in genotype identification is now a common practice. Molecular markers play an important role in this respect and it can clearly differentiate the genetic material of two individuals, avoiding any of the environmental influences which cause variations in gene expression.

The Random Amplified Polymorphic DNA (RAPD) marker technique originally described by Williams *et.al.* (1990) and Welsh and McClelland (1990), have provided new opportunities for evaluating genetic variability in many crops.

The RAPD technique is relatively inexpensive, fast, reliable and very appropriate to use in an average plant breeder's laboratory. The usefulness of RAPD technique for the genetic differentiation of rubber clone has been reported by Attanayaka *et.al.*(2000), from their study on the genetic differentiation of RRIC 100 series clones and two germplasm selected clones.

This study investigated the possibility of genetic differentiation of the two recommended series of RRISL clones *viz*, RRISL200 and RRISL2000 using RAPD technique. It is also envisaged to develop a reliable molecular method to identify the clones recommended under RRISL200 and RRISL2000 series.

MATERIALS AND METHODS

This study was carried out at the laboratory of the Department of Genetics and Plant Breeding (DGPB), RRISL, Sub-station Niwithigalakale, Matugama, from December, 2004 to July, 2005.

Plant Materials

Leaves of the apple green stage were collected from all the recommended RRISL 200 and RRISL 2000 series clones from the bud wood nurseries maintained at the DGPB, RRISL, Niwithigalakale. Twenty six clones were used for this study (Table 1).

DNA Extraction

DNA was extracted from the leaves according to the method described by Withanage and Attanayaka (2004), (in press).

Thin slices of plant material were ground quickly with 400 µl extraction buffer (100 mM Tris HCl(pH 8.0), 50 mM NaCl, 1%SDS), in a motor. After the addition of another 400 µl of extraction buffer, the liquid phase was transferred to a 2 ml eppendorf tube which has been placed on 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 4.30 minutes in a microcentrifuge. Supernatant was transferred to another fresh tube and 1µl of RNAase was added. After mixing the content it was kept at room temperature for 10-15 minutes. 600-700 µl of 100% ethanol was then added, mixed well and spun at 7500 rpm for 4.30 minutes. The DNA pellet was washed twice with 70% ethanol for 1 minute at 1000 rpm and dried at room temperature. The DNA pellet was resuspended in 50µl of autoclaved ultra purified water and stored at 4 ^{0}C for further use.

Purity Analysis of Extracted DNA samples.

A DNA sample of two micro liters (2 µl) was mixed with one micro liter (1µl) of gel loading buffer and seven micro liters (7µl) of ultra purified water. The mixture was loaded on a 0.8% agarose gel for electrophoresis using 0.5 X TBE buffer. The DNA was visualized by ethidium bromide staining on a UV transilluminator.

Polymerase Chain Reaction (PCR)

Initial amplification was carried out using bulked DNA samples from the two series of clones separately. The concentrations of each of the RRISL 200 and RRISL2000 pools were estimated and were diluted to give concentration as 50 - 100 ng per 2 µl.

Table 1- The rubber clones used for the study.

RRISL 200 series clones		RRISL 2000 series clones
1). RRISL 201	11). RRISL 217	1). RRISL 2000
2). RRISL 203	12). RRISL 218	2). RRISL 2001
3). RRISL 204	13). RRISL 219	3). RRISL 2002
4). RRISL 205	14). RRISL 220	4). RRISL 2003
5). RRISL 206	15). RRISL 221	5). RRISL 2004
6). RRISL 208	16). RRISL 222	6). RRISL 2005
7). RRISL 210	17). RRISL 223	7). RRISL 2006
8). RRISL 211	18). RRISL 225	
9). RRISL 215	19). RRISL 226	
10). RRISL 216		

Polymerase Chain Reactions (PCR) were performed using 50-100 ng template DNA in a 20 µl reaction volumes comprising 1X (Mg^{2+} free) PCR buffer: (10 mM Tris HCl, pH 8.0, 50 mM KCl, 0.1%Triton-X100), 2.5mM $MgCl_2$, 2mM each of all four dNTPs and two unit of Taq polymerase (promega) and 16.5 ng primer covered with a drop of Mineral oil (Sigma). Amplification was performed in an Amplitron Π thermal cycler for 45 cycles. Each cycle had three steps as 94^{0}C for one minute, 36 ^{0}C for one minute and 72^{0} C for two minutes.

Primers

Amplification was carried out using decamer primers of the series OPA, OPB, OPC, OPE, OPY and OPS (Operon Technologies, Almenda, USA) .The most suitable primers were selected for the detailed studies based on the polymorphic and well resolved amplification profiles obtained from the bulked sample PCR. The reproducibility of these results was tested by repeating the experiment twice using the same DNA samples.

Amplified Products Resolving and Visualization

The amplified products were resolved by electrophoresis at 45 V in 1% agarose gel in 0.5X TBE buffer and visualized by ethidium bromide staining on a UV transilluminator. The bacterial Phage Lambda DNA of 100 ng, digested with Hind III restriction enzyme was used as the DNA size marker. The photographs of gels were taken by a Polaroid camera.

Screening of Whole DNA Samples

Highly polymorphic primers between two pools were used to screen individual genotypes of the two series of clones.

Data Analysis

The data were analyzed using "RAPDistance", RAPD data analyzing computer base software package.

RESULTS AND DISCUSSION

Selection of Primers

Initially 47 primers from OPA, OPB, OPC, OPE, OPS and OPY primer series were screened using two bulked DNA samples made from RRISL200 and RRISL2000 series of clones. Among the 47 primers tested, 10 primers showed polymorphism between two pools of RRISL200 and RRISL2000 series. Three polymorphic primers namely OPA20, OPA12 and OPA10 were selected to screen all individual genotypes of the two series of clones (Fig. 1).

Screening of the Genotypes

Out of 26 clones tested, only eleven clones showed amplification products with the three primers used (Table 2). Therefore, only the RAPD data of these eleven clones were used to observe the genetic relatedness. The selected eleven clones were RRISL 210, 216, 218, 220, 221, 223, 2000, 2002, 2003, 2004 and 2006. These three primers produced a total of 160 bands, out of which 116 were polymorphic. All the bands produced by the primer OPA20 and OPA12 were polymorphic while OPA10 produced 60% polymorphic bands (Table 2).

Table 2-Details of the RAPD profiles obtained by selected three primers with the eleven genotypes.

Primer	Sequence	Number of amplified bands	Number of polymorphic bands.
1) OPA20	GTTGCGATCC	10	10
2) OPA12	TCGGCGATAG	12	12
3) OPA10	GTGATCGCAG	10	6

A pair wise comparison of RAPD profiles between clones was used for the detection of genetic distance between eleven *Hevea* clones tested. The cluster diagram (Fig. 2) showed no clear separation of the two series of clones RRISL200 and RRISL2000. The RRISL216 and RRISL2000, having an average genetic distances of 0.612 and 0.602 respectively, proved to be the most distinct clones from the rest of the clones (Table 3). The two most genetically related clones were RRISL2003 and RRISL2006 with the genetic distance value of 0.333. All the RRISL2000 series clones were clustered together except RRISL2000 which was clustered with RRISL216 and RRISL218. This indicates the genetic distinctness of the RRISL2000 from the other RRISL2000 series clones. *Hevea* breeding follows a cyclic breeding pattern in which the superior clones of one generation becomes the parents for the next generation. Hence, close genetic relatedness of the clones of these two series can be expected.

In this study, the initial selection of primers using the pooled DNA samples of each of the two series of clones had not resulted clear genetic differentiation of the two series. This is because in most of the selected primers, the polymorphism had been resulted due to a presence of a single positive genotype in one of the bulked sample (Table 4). Therefore, though these positive bands were able to differentiate between two bulked samples, the absence of the same band reflects the genetic similarity between the individuals of the two samples.

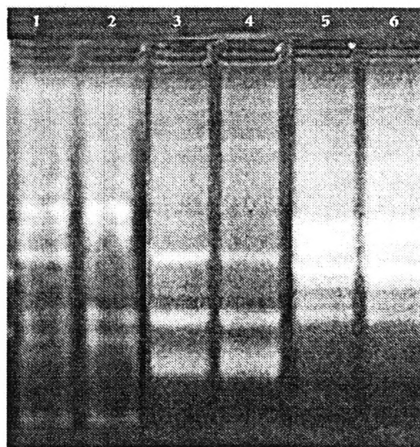

Fig. 1- The polymorphism shown by three of primers, OPA20, OPA12, and OPA10 between two bulked DNA samples of RRISL200 and RRISL2000.

Lane 1: RRISL200 series bulked DNA and primer OPA20 ,
Lane 2: RRISL2000 series bulked DNA and primer OPA20 ,
Lane 3: RRISL200 series bulked DNA and primer OPA12 ,
Lane 4: RRISL2000 series bulked DNA and primer OPA12,
Lane 5: RRISL200 series bulked DNA and primer OPA10,
Lane 6: RRISL2000 series bulked DNA and primer OPA10.

Clone Identification.

An effort was made to develop a RAPD based clone identification key (Fig. 3) to identify the eleven RRISL200 and RRISL2000 series clones using minimum number of primers.

Out of the eleven clones used in this study seven clones could be uniquely identified by the RAPD banding pattern produced by OPA20.

Two bands produced by this primer, A20-3 and A20-4 were specific for two clones RRISL216 and RRISL2000, respectively. Absence of the band number A20-6 was characteristic to the clone RRISL 223. The clone RRISL2003 produced only a single band A20-6 with this primer. The two clones RRISL218 and RRISL220 could also be identified by the polymorphism showed by two bands A20-8 and A20-9. The rest of the four clones could be categorized into two groups based on the similarity of the banding patterns produced by this primer. One group of clones consisted of RRISL210 and RRISL2002, while the other group comprised RRISL221 and RRISL2004. The individual differentiation of these four clones was possible using the primer OPA12 in the second step of the procedure. The presence of the bands A12-2, A12-3, A12-7 and A12-10 in RRISL2002 differed from the RRISL210 and the band number A12-4 was present in RRISL210. The bands A12-1, A12-5, A12-10 and A12-12 were present in RRISL2004. These bands were absent in RRISL 221. The band A12-6 was present in RRISL 221 and it was not present in RRISL 2004. Using these DNA profiles, these clones could be differentiated from the second step of the key.

Table 3- The average genetic distances between eleven clones

Clone	210	216	218	220	221	223	2000	2002	2003	2004	2006
210	0.0										
216	0.636	0.0									
218	0.577	0.590	0.0								
220	0.577	0.589	0.447	0.0							
221	0.542	0.604	0.387	0.505	0.0						
223	0.661	0.620	0.458	0.468	0.408	0.0					
2000	0.612	0.577	0.561	0.643	0.623	0.594	0.0				
2002	0.389	0.591	0.531	0.487	0.493	0.609	0.609	0.0			
2003	0.538	0.648	0.493	0.447	0.447	0.460	0.577	0.485	0.0		
2004	0.507	0.690	0.517	0.426	0.423	0.545	0.677	0.397	0.408	0.0	
2006	0.507	0.577	0.517	0.426	0.480	0.493	0.545	0.458	0.333	0.500	0.0
Averages	0.555	0.612	0.508	0.512	0.491	0.532	0.602	0.505	0.484	0.529	0.484

Table 4- Details of the polymorphic bands between two pools of DNA samples

Band number	RRISL 200 DNA pool	RRISL 2000 DNA pool	Number of genotypes giving the positive band.	
			RRISL 200 series clones	RRISL 2000 series clones
1).A20-3	+	-	1	0
2).A20-4	-	+	0	1
3).A20-7	+	-	2	0
4).A20-8	+	-	3	0
5).A12-9	+	-	1	0
6).A12-12	-	+	0	1
7).A10-5	-	+	0	1
8).A10-7	+	-	1	0
9).A10-10	+	-	1	0

- (Negative sign)-absence of the band
+ (Positive sign)-presence of the band

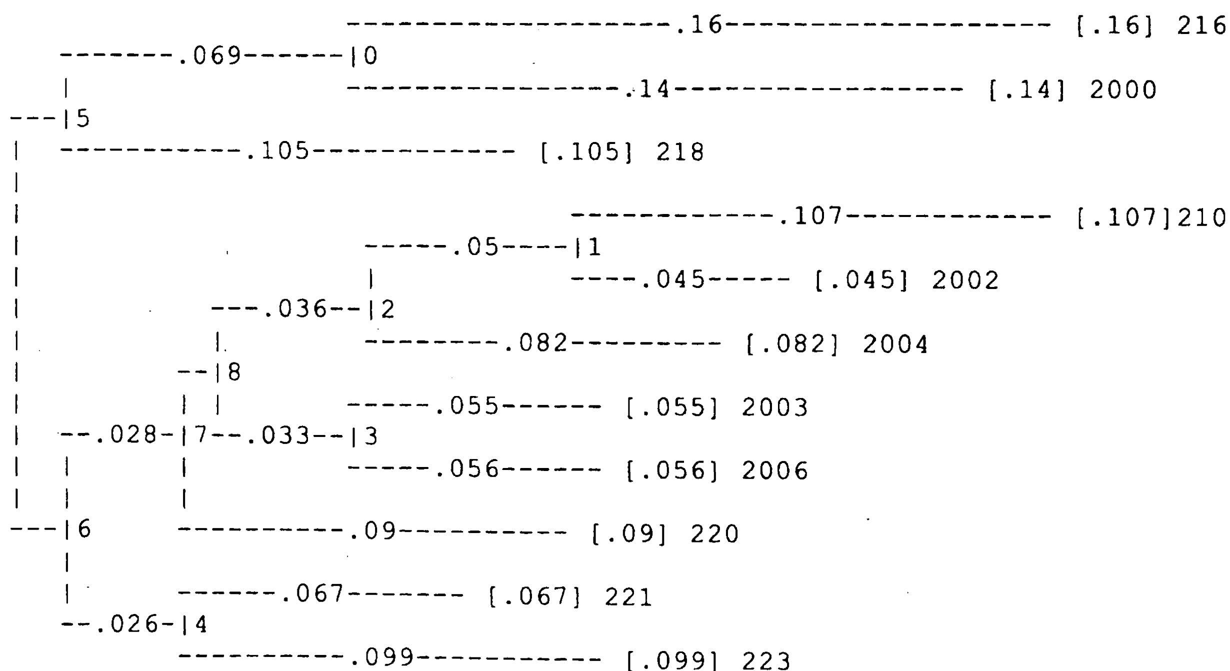

Fig. 2- The cluster diagram showing the genetic relatedness of the eleven RRISL 200 and RRISL 2000 clones.

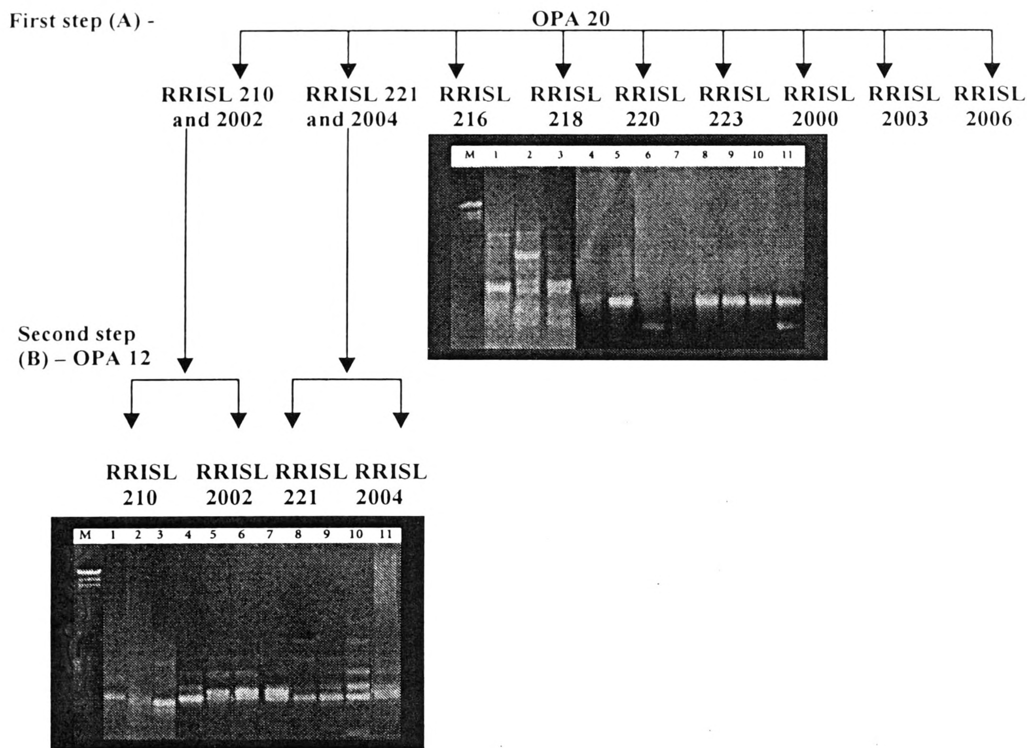

Fig. 3- The clone identification key.
M-DNA size marker (Hind III digested ,bacterial phage Lambda DNA)

Lane 1-RRISL 210,	Lane 4-RRISL 220,	Lane 7-RRISL 2000,	Lane 10-RRISL 2004,
Lane 2-RRISL 216,	Lane 5-RRISL 221,	Lane 8-RRISL 2002,	Lane 11-RRISL 2006.
Lane 3-RRISL 218,	Lane 6-RRISL 223,	Lane 9-RRISL 2003,	

CONCLUSIONS

The genetic differentiation of two RRISL200 and RRISL2000 series clones was not possible using RAPD screening. Initial primer selection using the two bulked samples *viz* RRISL200 and RRISL2000 was not successful in identifying useful polymorphic primers for genetic differentiation studies. The clone RRISL2000 was genetically distinct from the other clones in the RRISL2000 series. A molecular marker based identification key was developed to identify the eleven rubber (*Hevea brasiliensis*) clones of RRISL200 and RRISL2000 series.

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