AN INVESTIGATION TOWARDS DEVELOPING A MOLECULAR APPROACH TO IMPROVE THE EFFICIENCY OF COCONUT BREEDING BY RAPD-MARKER ASSISTED SELECTION

By

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ABSTRACT

Tall, dwarf green and San Ramon are three phenotypically distinct forms of coconut, currently used for production of hybrid seeds in Sri Lanka. Development of molecular markers for further improvement of important economic traits of these hybrids was thought important due to various constraints faced by conventional breeders. Sixty random primers (OPERON) were used for generation of RAPD profiles using DNA from 3 individuals of each form. All the sixty primers except one (OPC3) yielded RAPD-PCR products with coconut DNA. They generated a total of 326 consistently amplified bands of which 54 distinguished tall, dwarf green and San Ramon. Among RAPDs detected 16 and 8 were specific to tall and dwarf respectively, 18 distinguished dwarf and San Ramon from tall, 9 distinguished tall and San Ramon from dwarf and 3 distinguished tall and dwarf green from San Ramon. It is expected to study the segregation of these markers in F2 populations derived from tall x dwarf green and tall x San Ramon F1 palms.

INTRODUCTION

The genetic improvement of coconut by conventional methods has never reached the incomparable breeding achievements of rice, maize or wheat mainly due to constraints common to all perennial crops such as long juvenile phase and time and cost limitations, Coconut was further disadvantageous due to lack of a viable mechanism for vegetative propagation and the inherent heterozygosity associated with the commercially grown 'tall' coconut type, typica typica, (Liyanage, 1958)

Identification of genetically diverse parents for obtaining maximum heterosis is impracticable by screening coconut genotypes by morphological differences alone since in typica typica each individual is a unique heterozygous genotype and the phenotypic expression of most morphological traits is highly environmental-prone and often masks the true genetic variation. Therefore, it is advantageous to develop more direct biochemical methods to couple with the selection criteria used for determining parents for crosses aimed at improving important economic traits.

Molecular marker technology has gained immense popularity ever since the development of first DNA marker, Restriction Fragment Length Polymorphisms or RFLP (Botstein, *et al.*, 1980) and recognition of its utility by Beckmann and Soller (1993) for plant genome analysis. Advent of the polymerase chain reaction (PCR) by Mullis *et al*., (1986) resulted in development of a substantial array of marker-systems, such as Random Amplified Polymorphic DNA or RAPD (Williams, 1991), Simple Sequence Repeat Polymorphism or SSRP (Serikawa, *et al.*, 1992) and Amplified Fragment Length Polymorphisms or AFLP (Zabeau and Voss, 1993) for plant genome analysis.

RAPDs, being disadvantages by its dominant pattern of segregation and relatively poor reproducibility, it is the choice of many for studying 'new genomes' since other techniques require

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either previously cloned DNA fragments or DNA sequence information of the target genome or use of isotopes. In the past RAPD technique was rather prone to problems due to a lack of reproducibility of banding patterns between and within laboratories. To a great extent these problems have now been reduced by adherence to use of ultra purified and accurately quantified PCR reagents and protocols needed for reproducible results.

Coconut was not much benefited previously by DNA technology apart from a few studies. First published work was a less successful attempt made by Rhode *et al*., (1992) for detection of RFLPs by probing a repetitive EcoRI fragment of the coconut DNA which was characterized in the same study as a homology of a transposable element present in Copia and some other species. Subsequently, this information was utilized to develop a new system of markers called inverse sequence tagged repeats (ISTR) for characterization of coconut germplasm. Everard (1996a) constructed a library of coconut DNA by sub cloning Sau3AI fragments and had a meager success in detection of RFLPs on Southern blots of EcoRI, EcoRV and DraI digested fragments of tall and dwarf coconut DNA.

Everard *et al.,* (1996b) had better success with detection of polymorphisms among tall and dwarf coconut with RAPDs. They have detected 18 polymorphisms using 38 randomly generated 10-mer primers in RAPD-PCR. These primers segregated in a family of 18 F2 individuals in the Mendellian fashion assuring the potential of RAPDs for detection of genetic markers among major coconut varieties. Everard *et al.,* (1998) also demonstrated the ease of developing RAPD profiles for characterization of coconut germplasm.

In view of developing molecular markers to assist coconut breeding, detection of polymorphisms among coconut forms was considered as a priority. This paper describes early results of a long-term study aimed at detection of variety specific DNA polymorphisms among tall, dwarf green and San Ramon, the three varieties of coconut currently used in Sri Lanka for production of hybrid seeds for use in marker assisted selection after tagging to important traits.

MATERIALS AND METHODS

Morphological description of the three coconut types, tall, dwarf green and San Ramon assayed is given in Table 1. Tender coconut leaves from three individuals each of these coconut varieties were used for isolation of DNA. Several methods have been used successfully for isolation of DNA from tender coconut leaves. Everard (1996) used a slightly modified mini prep protocol of Dellaporta *et al.,* (1983). Rhode *et al.,* (1995) and Ashburner *et al.,* (1997) used modifications of Murray and Thompson's (1980) CTAB method by Doyle and Doyle's (1990) and Roggers and Bendich's (1985) respectively. In the current study the method derived by Wiesing and Karl (1997) form Doyle and Doyle's (1990) was used for isolation of DNA from tender coconut leaves.

Table 1. Taxonomy, distinctive morphological and reproductive features and distribution of the 3 coconut varieties used for detection of RAPDs.

Accession	Taxonomy	Specific morphological and reproductive features	Source or origin
Tall (TT)	var. Typica form Typica (tall)	Tall stature, allogamous, heterogeneous, bear in 6 -7 years, medium sized nuts, 60 - 80 nuts per palm per year	Common Tall
San Ramon (SR)	var. Typica form San Ramon (tall)	Tall stature, allogamous, heterogeneous, bear in 6 -7 years, medium - large sized nuts, round shaped nuts, high copra content, about than 60 - 80 nuts per palm per year	Philippines
Dwarf green (DG)	var. Nana form green dwarf	Dwarf stature, autogamous, homogeneous, bear in 3 - 4 years, small sized green coloured nuts, low copra content, 80 - 120 nuts per palm per year	Common green dwarf

Procedure:

Young coconut leaves, harvested a day prior to extraction were washed in distilled water, wiped, deribbed and cut into small pieces. Approximately 3 g were ground with mortar and pestle to a fine powder in the presence of liquid nitrogen. The powder was dispersed in 15 ml of pre-warmed (60 ^{0}C) isolation buffer [2% CTAB (Sigma H6269), 1.4M NaCl, 20 Mm EDTA (pH 8.0), 100 mM Tris-HCl (pH 8.0) and 0.2% b-mercaptoethanol added just prior to dispersing the sample] in a capped polypropylene tube and incubated for 60 minutes at 60 ^{0}C.

The incubated suspension was extracted with 15 ml of chloroform: isoamyl alcohol (24:1) by mixing gently (inverting and back) for 10 minutes and centrifuged in Beckmann (J2-21), J17 rotor for 10 minutes and 6,000 rpm (5,000 g) at room temperature (30 ^{0}C). The aqueous phase was re-extracted with fresh chloroform-isoamyl alcohol and added RNAse A [50 mg/ml RNAse A (Sigma R6513) in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl)] to a final concentration of 100 mg/ml, and incubated at 37 ^{0}C for 30 minutes.

Cold isopropanol (0.6 volumes) was added to the RNAse A treated mixture and kept for 5 minutes at room temperature. DNA, appeared as a white fibrous tangle, was picked up with the aid of a bent Pasteur pipette and washed in 20 ml of washing solution (76% ethanol and 10 mM ammonium acetate) by gently agitating for about 20 minutes before spinning for 10 min. and 6000 rpm (5000 g) at 4 ^{0}C. Drained the supernatant and pellet was allowed to suspend in 2 ml of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] overnight without agitation at 4 ^{0}C.

The DNA suspension was treated with 1 ml (0.5 volumes) of 7.5M Ammonium acetate, chilled on ice for 15 minutes and centrifuged for 30 min. and 11,000 rpm (10,000 g) at 4 ^{0}C. Transferred the supernatant to a new tube, 2 volumes of 96% ethanol was added and kept at -20 ^{0}C for 60 minutes. The white fibrous tangle was picked up by a bent Pasteur pipette and washed on to a 1.5 ml eppendorf with 1 ml of 70% ethanol. Centrifuged in a microfuge for 10 min. and top speed (13,500 rpm). The final pellet was suspended in 500 ml of TE. The purity and quantity of the DNA were assessed by UV spectroscopy using DNA Calculator (Gene-Quant Pharamacia) subjecting a 50-fold dilution of the DNA suspension. The integrity and fidelity of the DNA isolated were checked by electrophoresing uncut DNA, restriction digested DNA (EcoRI, HindIII, PstI, AluI, Sau3AI, HaeIII) and PCR amplified (using primer OPA5) DNA on a 1% agarose gel.

DNA obtained from the 9 individual coconut palms, 3 each of tall, 3 dwarf and 3 San Ramon were subjected to single-primed polymerase chain reaction (RAPD-PCR) to generate random amplified DNA (RAPD) profiles. A total of 60, oligonucleotide primers (10- mers), labeled OPA1 - OPA20, OPB1 - OPB20 and OPC1- OPC20 were used. These primers were obtained from OPERON Technologies, INC. The sequences of these primers were selected randomly keeping the requirements, G + C content between 60 - 70 % and avoiding self-complementarity at ends. The sequences of the 60 primers are given in Table 2.

The PCR procedure used was based on Williams *et al.* (1991). The amplification reactions were performed in a thermocycler (Perkin Elmer) in a volume of 25 ml containing 1 x Taq reaction buffer (10 mM Tris.HCl pH 8.3, 50 mM Potassium Chloride, 0.001% gelatin) supplied with the enzyme, 2mM Magnesium Chloride, 100 mM each of dATP, dCTP, dGTP and dTTP, 0.2 mM of a single 10 mer primer, 50 - 100 ng of genomic DNA and 0.5 units of Taq DNA polymerase. The reaction was allowed to progress in 45 repeating cycles of three phases, denaturing of the genomic DNA (1 minutes at 94 ^{0}C), annealing of primers (1 minute at 36^{0}C) and extension of primers (2 minutes at 72 ^{0} C). The amplification products were analyzed on 1.5-% agarose gels, stained in 0.5-mg/ml ethidium bromide for 20 min and exposing to UV for photographing with Polaroid 667 films. The amplified fragments (bands) were scored by visual inspection of the gel photograph and polymorphisms were scored as presence or absence of homologous bands. To check the consistency of polymorphisms detected each PCR was repeated for three times.

Table 2. Sequences of the 10-mer primers used to generate RAPD-PCR profiles with DNA from 3 individuals each of coconut varieties, tall, dwarf and San Ramon

Primer	Sequence	Primer	Sequence	Primer	Sequence
OPA1	CAGGCCCTTC	OPB1	GTTTCGCTCC	OPC1	TTCGAGCCAG
OPA2	TGCCGAGCTG	OPB2	TGATCCCTGG	OPC2	GTGAGGCGTC
OPA3	AGTCAGCCAC	OPB3	CATCCCCCTG	OPC3	GGGGGTCTTT
OPA4	AATCGGGCTG	OPB4	GGACTGGAGT	OPC4	CCGCATCTAC
OPA5	AGGGGTCTTG	OPB5	TGCGCCCTTC	OPC5	GATGACCGCC
OPA6	GGTCCCTGAC	OPB6	TGCTCTGCCC	OPC6	GAACGGACTC
OPA7	GAAACGGGTG	OPB7	GGTGACGCAG	OPC7	GTCCCGACGA
OPA8	GTGACGTAGG	OPB8	GTCCACACGG	OPC8	TGGACCGGTG
OPA9	GGGTAACGCC	OPB9	TGGGGGACTC	OPC9	CTCACCGTCC
OPA10	GTGATCGCAG	OPB10	CTGCTGGGAC	OPC 10	TGTCTGGGTG
OPA11	CAATCGCCGT	OPB11	GTAGACCCGT	OPC11	AAAGCTGCGG
OPA12	TCGGCGATAG	OPB12	CCTTGACGCA	OPC12	TGTCATCCCC
OPA13	CAGCACCCAC	OPB13	TTCCCCCGCT	OPC13	AAGCCTCGTC
OPA14	TCTGTGCTGG	OPB14	TCCGCTCTGG	OPC14	TGCGTGCTTG
OPA15	TTCCGAACCC	OPB15	GGAGGGTGTT	OPC15	GACGGATCAG
OPA16	AGCCAGCGAA	OPB16	TTTGCCCGGA	OPC16	CACACTCCAG
OPA17	GACCGCTTGT	OPB17	AGGGAACGAG	OPC17	TTCCCCCCAG
OPA18	AGGTGACCGT	OPB18	CCACAGCAGT	OPC18	TGAGTGGGTG
OPA19	CAAACGTCGG	OPB19	ACCCCCGAAG	OPC19	GTTGCCAGCC
OPA20	GTTGCGATCC	OPB20	GGACCCTTAC	OPC20	ACTTCGCCAC

RESULTS

The method of Weising and Karl (1997) for isolation of DNA from young coconut leaves was satisfactory. Sufficient quantities (67 mg/g of leaf tissue) of DNA were yielded with adequate quality for use in the RAPD-PCR. DNA was free of detectable amounts of RNA and accessible to restriction enzymes and Taq polymerase in the PCR.

All the Sixty primers except one (OPC3) yielded RAPD-PCR products with coconut DNA. They generated a total of 448 bands averaging 7 - 8 bands per primer. On repetitive runs, 326 (73%) bands appeared consistently and 138 (42%) exhibited polymorphism of which 54 distinguished tall, dwarf green and San Ramon, as specific to any one or two of the three genotypes. RAPDs detected by each primer, size of the amplified fragment and coconut forms characterized by each fragment are given in Table 2. Among 54 RAPDs detected 16 and 8 were specific to tall and dwarf respectively and 18 distinguished dwarf and San Ramon from tall, 9 distinguished tall and San Ramon from dwarf and 3 distinguished tall and dwarf green from San Ramon. Figures 1 and 2 illustrate RAPD profiles generated by primers, OPA10 and OPA11 with DNA obtained from 3 individuals each of the coconut forms, tall, dwarf and San Ramon.

DISCUSSION

The method for isolation of DNA by Wiesing and Karl (1997) derived from Doyle and Doyle (1990) was observed as appropriate for coconut. The method is simple, cheap and fast, the basic four steps; homogenization, lysis, precipitation and purification require minimum of time and working capacity while giving good yields of DNA. Preclusion of performing expensive, ultra-centrifugation-required CsCl gradients and handling hazardous phenol is noteworthy.

RAPD technique is very useful as it allows testing of large number of primer combinations within a very short period of time. Detection of 54 RAPDs to distinguish coconut forms tall, dwarf and San Ramon by merely testing 60 primers is remarkable in comparison to RFLP and SSRP techniques. The actual number of polymorphisms detected was very much high (> 100) but nearly 50%, lacked consistency over repeated runs of PCR.

The three forms of coconut tall, dwarf green and San Ramon are contrasting phenotypes for useful traits, precocity, nuts per palm, nuts per bunch, nut size and shape, copra per nut, copra per palm, tolerance to moisture stress etc., (Liyanage, 1958, Liyanage *et al.,* 1988, Fernando, 1987). Presence of these contrasting characters suggests these three forms as good candidates for analysis of markers for tagging to useful traits. Establishment of segregating populations by self pollination of F1 hybrids, tall x San Ramon and tall x dwarf is now in progress at the CRISL and breeders will soon have access to populations for analysis of the segregation of these markers. DNA analysis of bulk segregants to locate QTL by genotyping individuals divided into pools of contrasting phenotypes (Michelmore et al., 1991) is a promising alternative for finding linkage between molecular markers with useful traits in the coconut palm. Screening of more primers in similar manner is appreciable in parallel with the establishment of time consuming detection procedures required for RFLP and SSRP screening.

High level of genetic diversity between tall, dwarf green and San Ramon was also revealed by a molecular assay based on RAPDs (Everard *et al*., 1998). Tall and San Ramon in particular are very much diverse due to their heterozygous genomes associated with allogamy and their parallel evolution believed to have taken place in African and Asia Pacific regions respectively after dissemination of coconut from the center of origin, probably South East Asia (Harries, 1978, Ashburner, *et al*., 1997 Rhode, *et al.,* 1995). Dwarf being autogamous evolved at a less pace and resemble more of the form San Ramon. One third (18) of the RAPDs detected in this study separated tall from dwarf green and San Ramon in comparison to one sixth (9) of RAPDs that distinguished dwarf from tall and San Ramon agree with above observations.

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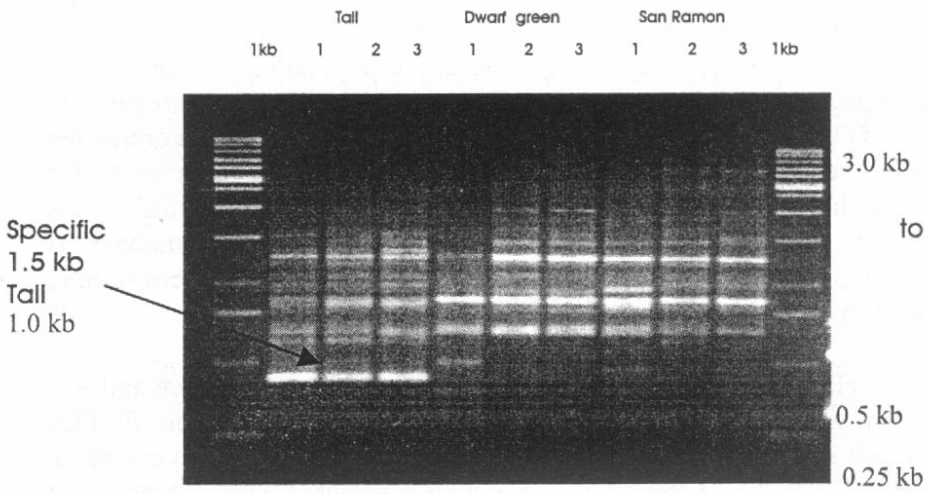

Figure 1. RAPD pattern of tall, dwarf and san ramon (3 individuals per variety) with primer OPA10.

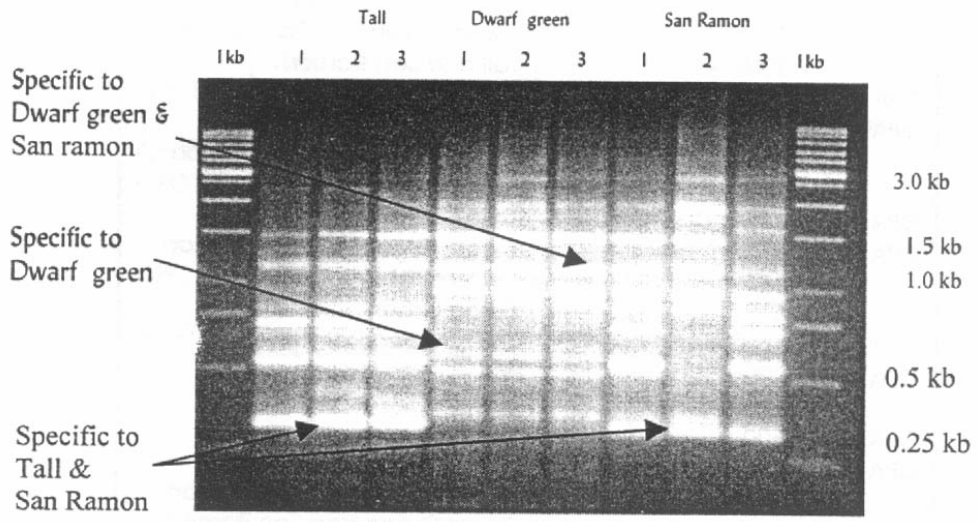

Figure 2. RAPD pattern of tall, dwarf and san ramon (3 individuals per variety) with primer OPA11.

Table 3. RAPDs detected among coconut forms, tall, dwarf green and san ramon

Primer	Fragment size (base pairs)	Sources of DNA positive for amplification
OPA 1	1,900	Tall and San Ramon
OPA 4	725	Dwarf Green and San Ramon
	300	Tall and San Ramon
OPA 5	1,500	Tall
OPA9	750	Tall
	725	Dwarf Green and San Ramon
	280	Tall and San Ramon
OPA 10	475	Tall
OPA 11	975	Dwarf Green and San Ramon
	525	Dwarf Green
	500	Dwarf Green
	300	Tall and San Ramon
OPA12	800	Tall
	1,200	Tall and Dwarf Green
OPA14	600	Dwarf Green
OPA 15	950	Tall
	450	Dwarf Green and San Ramon
OPA 16	250	Dwarf Green and San Ramon
OPA 18	800	Tall
OPA 19	2,250	Dwarf Green
	850	Tall and San Ramon
OPA 20	1,500	Dwarf Green and San Ramon
	700	Tall
OPB 1	1,000	Dwarf Green and San Ramon
OPB 2	550	Dwarf Green
	500	Tall and San Ramon
OPB 3	500	Dwarf Green and San Ramon
OPB 5	1,450	Tall
OPB 6	500	Tall and Dwarf Green
OPB 8	1,000	Dwarf Green and San Ramon
OPB 11	500	Tall
	475	Dwarf Green and San Ramon
	300	Tall
OPB 13	450	Dwarf Green and San Ramon
OPB 15	800	Dwarf Green and San Ramon
OPB 16	700	Tall and San Ramon
	500	Dwarf Green and San Ramon
	100	Dwarf Green and San Ramon
OPB 17	1,500	Tall
OPC4	1,200	Tall
	900	Tall
OPC 6	1,500	Dwarf Green
	1,300	Dwarf Green
	400	Dwarf Green and San Ramon
OPC 7	1,000	Tall and San Ramon
OPC10	600	Tall and San Ramon
	300	Dwarf Green and San Ramon
OPC13	800	Tall
OPC14	800	Tall
	475	Dwarf Green
	450	Tall and Dwarf Green
OPC 16	750	Dwarf Green and San Ramon
	700	Tall
OPC 18	250	Dwarf Green and San Ramon

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