Tropical Agricultural Research Vol. 8 1996, 124-135

Inheritance of RAPD Markers in the Coconut Palm *Cocos nucifera* L.

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ABSTRACT. *A RAPD assay was conducted on a coconut palm pedigree with the view of developing a molecular marker system to aid the coconut breeding strategy at the Coconut Research Institute of Sri Lanka (CRISL). Thirty eight 10 - 12mer primers were used in the single primer PCR to detect RAPDs among tall and dwarf parents and a F_2 progeny of 18 individuals. Seven primers detected 16 bands of which 12 of them distinguished parents. All except one RAPD followed Mendelian pattern of inheritance. This assay reflected positively on RAPDs as a genetic marker for use in the genetic assessment of the coconut palm.*

INTRODUCTION

Genetic improvement of the coconut palm by conventional methods is hindered by serious limitations such as lack of a viable cloning technique, long juvenile phase and risk of germplasm importation. Use of molecular-marker techniques such as Restriction Fragment Length Polymorphisms (RFLPs) (Botstein *et al.*, 1980) and Random Amplified Polymorphic DNAs (RAPDs) (Williams *et al.*, 1991) for genetic assessment of plant populations is well regarded for their high competence to detect naturally occurring polymorphisms in plant populations (Tanksley *et al.*, 1993). Both these techniques detect DNA polymorphisms and can be used to monitor segregation of DNA sequences among progeny of a genetic cross to construct a linkage map (Young, 1994). The RAPD approach in particular has gained wide acceptance among plantation crops such as tea (Wachira *et al.*, 1995), cocoa (Wilde *et al.*, 1992), rubber (Besse *et al.*, 1993) and oil palm (Shah *et al.*, 1994).

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Exploiting molecular markers in the coconut palm is important for directing the coconut breeding strategy for more efficient utilization of the palm's naturally occurring genetic variation. Coconut palm has so far, not much benefited by this new technology. The DNA-based studies on coconut include an investigation which detected and sequenced *EcoRI* repetitive fragments in the coconut genome (Rhode *et al.*, 1992) and subsequent use of these sequences for genome analysis by PCR amplification (Rhode *et al.*, 1995). The current authors have attempted to detect RFLPs in a pedigree of the coconut palm. This study failed to detect adequate RFLPs because the random coconut DNA clones (probes) that were used all contained copies of the same highly repetitive sequences (Everard, 1996). The work described here was conducted simultaneously for detection of RAPDs in the same family.

MATERIALS AND METHODS

The coconut palm family used in this study was grown in the Bandirippuwa Estate, Coconut Research Institute of Sri Lanka (CRISL). The family comprise parents; a tall *(typica typica)* x dwarf *(nana)* cross and 18 F_2 individuals. However, in most instances only 17 individuals were scored.

DNA extractions were performed by a modification of the procedure developed by Dellaporte *et al.*, (1988). Approximately 5 g of tender coconut leaves were cut into small pieces and ground in a mortar with a pestle in liquid nitrogen. The leaf powder was suspended in 30 ml of DNA extraction buffer 1 [200 mM tris-HCl (pH 8), 50 mM EDTA (pH 8), 250 mM sodium chloride, 2.5% PVP and 0.1% ß-Mercaptoethanol] and centrifuged for 10 min at 4,000 g in the Beckmann JA20 rotor.

The pellet was resuspended in 10 ml of DNA extraction buffer 2 [100 mM tris-HCl (pH 8), 50 mM EDTA (pH 8), 500 mM sodium chloride and 10 mM ß- Mercaptoethanol] and lysed overnight at 65°C with 1 ml of 20% SDS and 100 μl of Proteinase K (25 mg/ml). The lysate was chilled with 3 ml of cold 5 M potassium acetate (pH 4.8) for 30 minutes and centrifuged for 20 minutes at 25,000 g in the Beckmann JA20 rotor. Nucleic acids were separated from the aqueous supernatant by chilling for 30 minutes with 0.6 volumes of cold isopropanol followed by centrifugation for 15 minutes at 20,000 g in the Beckmann JA20 rotor.

The pellet was suspended in 1 ml of DNA extraction buffer 3 [50 mM tris-HCl (pH 8), 20 mM EDTA (pH 8) and purified by extracting twice with phenol, chloroform and isoamyl alcohol (25:24:1 v/v/v)] and twice more with

chloroform and isoamyl alcohol (24:1, v/v). The nucleic acids were precipitated by adding a 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of cold isopropanol. The pellet was suspended in 200 μl TE buffer [10 mM tris-HCl (pH 8) and 1 mM EDTA (pH 8)]. RNAse (50 μg per ml) treatment was followed and quality and the quantity of the DNA were assessed by UV spectrophotometry and agarose gel electrophoresis.

DNA from individual leaf samples were used in single-primed polymerase chain reaction (PCR) to generate random amplified DNA profiles. Initially a total of thirty eight oligonucleotide primers (10-12 mer) were used. The PCR procedure was based on Williams *et al.* (1991) and the amplification reactions were performed in a thermocycler in a volume of 25 μl containing 1 x *Taq* reaction buffer, 2 mM Magnesium Chloride, 100 μM each of dATP, dCTP, dGTP and dTTP, 0.2 μM 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 minute at 94°C), annealing of primers (1 minute at 36°C) and extension of primers (2 minutes at 72°C).

The amplification products were made visible by 1.5% agarose gel electrophoresis. The primers were initially tested with DNA from parent palms to detect polymorphisms. The reactions were performed three times to confirm the reproducibility of the polymorphic fragments and the successful primers were used to assay F_2 individuals. Polymorphic fragments were scored for their presence and absence in the respective individuals.

Single locus segregation analysis was performed to determine the segregation of RAPDs detected by each primer. The RAPD markers were considered as dominant and the zygosities of the tall and the dwarf parents were scored on that basis for all possible genotypes. Independent chi squared values were obtained for each RAPD to test the goodness of fit of the expected ratio to the Mendelian ratio.

RESULTS AND DISCUSSION

The primers OPB1, 5, 7, 11, 18 and 12 mers 12mer2 and Check6 detected a total of 16 RAPDs among the two parent palms and the F_2 progeny. The amplification of these polymorphic DNA fragments was consistent over repeated runs. Inconsistent amplification products and faint bands were excluded from scoring. There were a few fragments observed only with DNA samples from F_2 individuals. These were also excluded from the analysis.

Bands were numbered from top to bottom as visible in the gels for each primer and their respective sizes were measured by comparing to the standard, *Hind*III lambda DNA.

Plates 1 and 2 serve as examples of two DNA profiles generated from RAPD-PCRs with primers OPB1 and OPB7, respectively. The DNA profile generated by primer OPB1 was simple and consisted of 9 distinct PCR fragments ranging from 200 bp to 1000 bp. Among these only five were clear and repeatable. The RAPD profile generated by the primer OPB7 is more complex and consisted of 15 amplified fragments ranging from 500 bp to 1800 bp of which only eight were clear and repeatable.

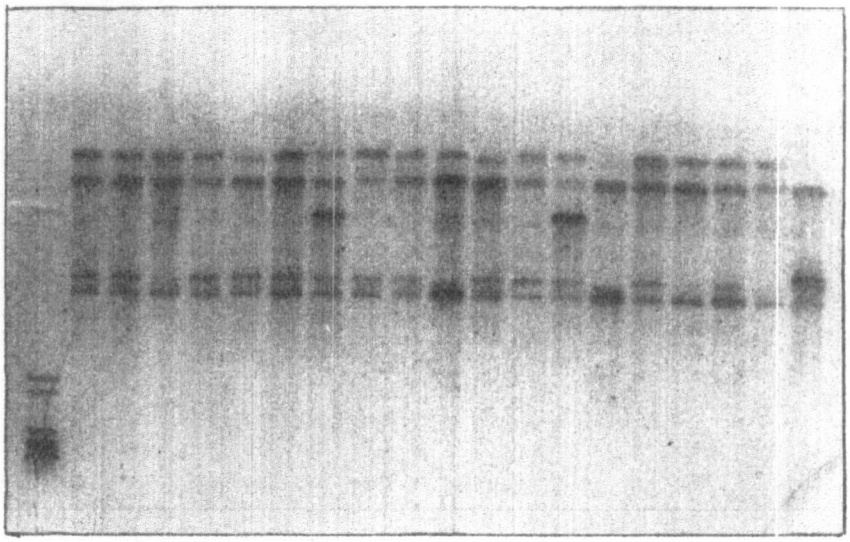

Plate 1. **Agarose gel (1.5%) containing size fractionated DNA amplified by RAPD-PCR using the primer OPB1. Lane 1, tall parent; land 2, dwarf parent and lanes 1-17, individuals of the F_2 family are the sources of DNA for the RAPD-PCR. HindIII digested lambda DNA is the standard DNA marker (the sizes of band 1, 2, 3, 4, 5, 6 and 7 from the most cathodal end are 23.1 kb, 9.4 kb, 6.6 kb, 4.4 kb, 2.3 kb, 2.0 kb and 0.56 kb respectively).**

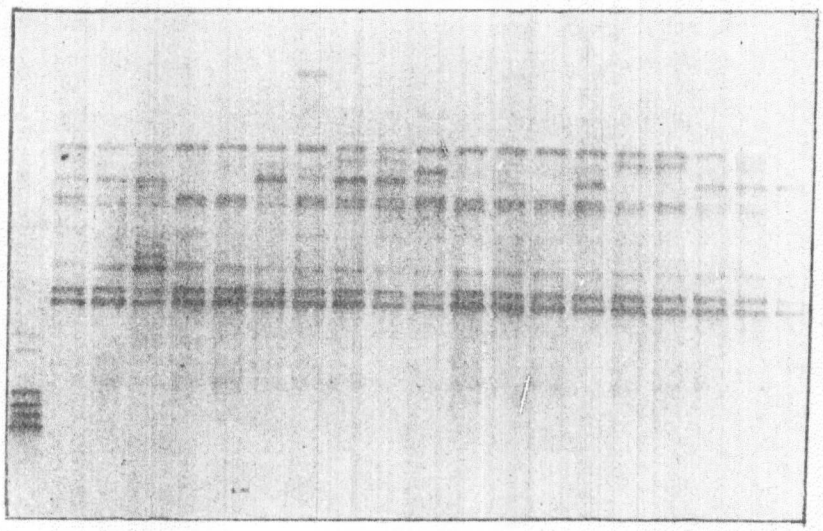

Plate 2. **Agarose gel (1.5%) containing size fractionated DNA amplified by RAPD-PCR using the primer OPB7. Lane 1, tall parent; lane 2, dwarf parent and lanes 1-17, individuals of the F_2 family are the sources of DNA for the RAPD-PCR. HindIII digested lambda DNA is the standard DNA marker (the sizes of band 1, 2, 3, 4, 5, 6 and 7 from the most cathodal end are 23.1 kb, 9.4 kb, 6.6 kb, 4.4 kb, 2.3 kb, 2.0 kb and 0.56 kb respectively).**

The number of unambiguous polymorphic bands produced by the seven primers among 'tall' and 'dwarf' parents and F_2 individuals is given in Table 1. The seven primers have produced a total of 45 clear repeatable fragments averaging 6 - 7 bands per primer. The size of bands ranged from 200 bp - 2,000 bp. Among these bands 16 exhibited polymorphisms averaging 2-3 polymorphic bands per primer.

Results of the single locus segregation analysis for the 16 polymorphic fragments is given in Table 2. The analysis shows that all but one polymorphism (OPB7.7) segregated in the Mendelian fashion in the F_2 progeny at a significance level of $P < 0.05$. The RAPD loci. OPB1.2, 1.5, 5.1, 5.2, 7.3, 11.2, 11.3, 18.3, 18.6 and 12 mer 2.9 and Check 6.1 were all in the intercross configuration (F_1 Aa) with one of the parents (P_1 or P_2) being homozygous dominant and the other being homozygous recessive. The segregation pattern for RAPD loci OPB11.2 and 11.3 and Check 6.1 is also consistent with the

intercross configuration (F_1 Aa and aa) with one of the parents (P_1 or P_2) being heterozygous and the other being homozygous recessive. The data for the alleles at the RAPD loci Check 6.2 was consistent only with the intercross

Table 1. Sequence of oligonucleotide primers, number of scorable and repeatable bands produced by two 12 mer and five 10-mer primers in the RAPD-PCR with DNA from 'tall' and 'dwarf' parents and 18 F_2 individuals.

Primer	Sequence 5' to 3'	Number of scorable and repeatable bands	Range in size (bp)	Number of polymorphic band
OPB1	GTTTCGCTCC	5	200 - 1000	2
OPB1	TGCGCCCTTC	6	550 - 1800	2
OPB1	GGTGACGCAG	8	500 - 1800	4
OPB1	GTAGACCCGT	6	500 - 2000	3
OPB1	CCACAGCAGT	7	200 - 1500	2
12mer2	TCATCCGCTTCC	9	200 - 1800	1
Check6	GTCTGAATGACC	4	300 - 1000	2

configuration (F_1 Aa and aa) with one of the parent (P_1 or P_2) being heterozygous and the other being homozygous recessive. The data for the three loci OPB7.2, 7.6 and 11.4, where both parents (P_1 or P_2) were positive for the dominant allele fits well with the intercross configuration F_1, AA x 2Aa, x aa with both parents (P_1 and P_2) being heterozygous. The data for alleles in the locus OPB7.7 can not be explained with any probable intercross configurations. One possibility for such an observation is the presence of two different bands of the same size in the parents.

The inheritance of RAPD markers in the coconut F_2 population confirms findings of many other authors (Tinker *et al.*, 1993; Heun and Helentjaris, 1993; Jermstad *et al.*, 1994; Lee and Jeon, 1995). All these studies suggest that most predictable types of RAPD fragments are those of the greatest intensity in any one reaction while minor fragments possess a great propensity.

Table 2. Single locus segregation analysis of 16 RAPD loci in F_2 progeny of the coconut palm.

RAPD locus	Approximate size of the fragment (bp)	Parent phenotype		Possible parent genotype		Expected ratio in F_2 (+ : -)	Observed ratio in F_2 (+ : -)	Chi square
		Tall	Dwarf	Tall	Dwarf			
OPB 1.2	900	+	-	++	--	3 : 1	13 : 4	0.02
				+-	--	7 : 9		7.40
OPB 1.2	250	-	+	--	++	3 : 1	14 : 3	0.49
						7 : 0		10.20
OPB 5.1	1800	+	-	++	--	3 : 1	12 : 5	0.18
				+-	--	7 : 9		4.94
OPB 5.2	1600	-	+	--	++	7 : 1	12 : 5	0.18
						7 : 9		4.94
OPB 7.2	1700	+	+	++	+-	15 : 1	13 : 4	8.67
				+-	++	15 : 1		8.67
				+-	+-	3 : 1		0.02
OPB 7.3	1500	+	+	++	--	3 : 1	14 : 3	0.49
				+-	--	7 : 9		10.29
OPB 7.6	600	+	+	++	+-	15 : 1	13 : 4	8.67
				+-	++	15 : 1		8.67
				+-	+-	3 : 1		0.02
OPB 7.7	525	+	+	++	+-	15 : 1	6 : 11	99.14
				+-	++	15 : 1		99.14
				+-	+-	3 : 1		14.29

Cont'd.....

RAPD locus	Approximate size of the fragment (bp)	Parent phenotype		Possible parent genotype		Expected ratio in F_1 (+ : -)	Observed ratio in F_2 (+ : -)	Chi square
		Tall	Dwarf	Tall	Dwarf			
OPB 11.2	1000	-	+	- -	+ +	3 : 1	10 : 7	2.37
				- -	+ -	7 : 9		1.56
OPB 11.3	950	-	+	- -	+ +	3: 1	10 : 7	2.37
				- -	+ -	7 : 9		1.56
OPB 11.4	700	+	+	+ +	+ -	15 : 1	12 : 5	15.56
				+ -	+ +	15 : 1		15.56
				+ -	+ -	3 : 1		0.17
OPB 18.3	800	-	+	- -	+ +	3 : 1	14 : 3	0.49
				- -	+ -	7 : 9		10.29
OPB 18.6	525	-	+	- -	+ +	3 : 1	15 : 2	1.59
				- -	+ -	7 : 9		13.67
12 mer 2.9	200	-	+	- -	+ +	3 : 1	14 : 4	0.07
				- -	+ -	7 : 9		4.86
Check 6.1	1000	-	+	- -	+ +	3 : 1	11 : 7	1.85
				- -	+ -	7 : 9		2.20
Check 6.2	950	+	-	+ +	- -	3 : 1	9 : 9	6.00
				+ -	- -	7 : 9		0.28

* $p < 0.05$

for irreproducibility. RAPDs generated by one primer for a given DNA sample can be highly reproducible with clear bands while another primer with the same DNA sample may produce weak bands, often arising from mismatched primer annealing which is sensitive to even very slight changes in the PCR condition. The key factor here is the choice of the correct primer to suit the DNA substrate. The availability of random primers from commercial sources offers a great choice of primers and allows detection of sufficient reproducible RAPDs in a short period of time.

The nature of the fragments from arbitrary amplified primers has not yet been adequately investigated to determine whether each fragment visualized in a gel can be considered as an independent character. However, most scorable RAPD amplification fragments segregate in the Mendelian fashion and the general consensus is that each RAPD amplification fragment corresponds to an independent genetic locus and this is especially true when screening individuals within a species (Tinker *et al.*, 1993; Heun and Helentjaris, 1993; and Hallden *et al.*, 1994). Further there is evidence of RAPD and RFLP markers appearing synonymously in mapping positions (Jermstad *et al.*, 1994).

The RAPD markers tend to be inherited as dominant markers because they appear in similar fashion in both homozygous individuals and heterozygous individuals. Only the homozygous recessives can be definitely distinguished. Therefore, this technique does not allow heterozygous and homozygous dominant individuals to be identified without subsequent crosses. Heun and Helintjaris (1993) studied the inheritance of RAPDs in F_1 hybrids of corn and observed situations where intensity differences were reproducible and followed the co-dominance pattern. Similarly Tinker *et al.* (1993) found one co-dominant marker from a sample of 31 RAPD polymorphisms in barley. However, in the current study the two RAPD loci OPB7.2 and OPB11.4 which were inferred to be in the heterozygous state in the parents, did not confirm the above observations.

The usefulness of RAPDs in generating genetic maps was well demonstrated by Reiter *et al.* (1992). However, dominant inheritance of RAPD markers poses a problem with plants that lack inbred populations or back cross populations in genetic mapping (Tingey and del Tufo, 1993). Lack of sufficient F_2 intercross and backcross populations is a major limitation in using RAPDs for generating a linkage map for the coconut palm. Some of the on-going progeny trials at the CRISL comprise well characterized parent and F1 material for establishment of F_2 (intercross and backcross) families. Alternatively, bulk segregation technique (Michelmore *et al.*, 1991) can be

applied by assuming *typica* forms, *San Ramon* and *nana* forms as separate breeding lines.

CONCLUSIONS

The results of this segregation analysis clearly indicate the potential of RAPDs as a molecular marker for generating a linkage map for the coconut palm. By careful selection of primers which are capable of producing well resolved, strongly amplified, reproducible polymorphic fragments among morphologically distinct parental types such as *typica typica* (commercial tall), *nana* (dwarf green and dwarf yellow) and *typica San Ramon*, there is a strong likelihood of finding correlations between RAPDs and economic traits.

ACKNOWLEDGEMENTS

The authors are grateful to Agricultural Research Project of the Ministry of Agriculture, Sri Lanka for funding this research study and University of New England, Armidale, NSW, Australia for providing laboratory facilities.

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