CHAPTER 3

Results

3.1. Random Amplified DNA Polymorphisms in the Coconut Palm

Genomic DNA samples extracted from young leaves of individual palms were used as templates for PCR using single arbitrary primers. The amplification products generated by RAPD-PCR are strictly dependent on concentrations of the reaction constituents. Therefore, in order to assure the reproducibility of the RAPD-PCR fragments all possible measures were taken to maintain uniformity across reactions in all experiments. The concentrations of DNA samples were measured accurately to ensure that the amount of DNA amplified per reaction was within 50 - 100 ng in every reaction performed. The reaction constituents excluding the enzyme, primer and DNA template were made in bulk and stored at 4 °C The RAPD profiles generated by PCRs performed under experimental conditions laid down by Williams et al. (1990) as recommended in the instructions manual supplied with oligonucleotides from OPERON Technologies Limited (see chapter two, section 2.3.2) yielded reasonably intense, reproducible amplification products with a great majority of primers tested.

The random oligonucleotide primers OPB1 - 20, 12mer1 - 6 and Check1 - 12 were initially tested with DNA from three different varieties of coconut palm, *typica typica* (commercial tall form), *nana* green (dwarf form) and king coconut *aurantiaca* (king coconut form) to assess their ability to produce amplified DNA profiles. The primers that failed to produce clear, easily detectable bands over repeated runs were excluded. The primers that gave easily detectable well resolved bands were chosen for screening DNA samples from a number of sources of coconut. Most of the well resolved bands were

reproducible over repeated runs and had sufficient intensity to detect their presence or absence in samples with the same relative band intensity. RAPD profiles obtained with individual palm DNAs comprised 5 - 10 main bands ranging from 50 - 2000 bp in size. The number of amplified bands per profile per primer increased in reactions where the primer concentration was increased, in the experiments to assess genetic diversity in different coconut accessions (section 3.2).

The first RAPD experiment in coconut palm was designed to detect RAPD markers in a pedigree of a tall and dwarf cross existing in Sri Lanka and to assess the segregation of such RAPD markers in the inbred F_2 progeny of the dwarf x tall hybrid (F_1). The second experiment was designed to detect RAPD markers in 19 accessions of the coconut palm in Sri Lanka in order to assess the potential of RAPDs as a molecular marker to study genetic diversity.

3.1 Segregation of RAPD markers in an inbred (F_2) population of the coconut palm in Sri Lanka

The random oligonucleotides OPB1 - 20, 12mer1 - 6 and Check1 - 12 were initially used in single primer PCR (RAPD-PCR) to amplify DNA from the two parent palms and identify primers that consistently produce polymorphic DNA fragments in those palms. The reactions were performed three times to confirm the reproducibility of the polymorphic fragments. The primers, OPB1, OPB5, OPB7, OPB11, OPB18, 12mer2 and Check6 revealed genetic differences in the two parent palms and they were subsequently used for assaying individuals in the inbred progeny (F_2) of the tall and dwarf hybrid. The inbred progeny comprised 18 individuals but in most instances only 17 were recorded because the DNA from the individual F_2-18 did not readily amplify.

The amplified DNA profiles generated by these primers were size fractionated by 1.5% agarose gel electrophoresis and photographed for visual observation. The bands were scored for each primer across parents and F_2 individuals. Plates 3.1.1 - 3.1.2 serve

as examples of two photographs of DNA profiles after agarose gel electrophoresis. These gels display DNA amplification products obtained with primers OPB1 and OPB7 respectively with DNA from two parent palms and F_2 individuals. Two figures below each plate explain the insides in the plates (figures 3.1 and 3.2). These schematic diagrams also gives an insight of how the bands were scored from photographs of gels.

The RAPD profile generated by primer OPB1 comprised nine distinct PCR fragments ranging from approximately 200 bp - 1000 bp in size. Fragment one (1000 bp) was clear and monomorphic while fragment two (950 bp) was clearly amplified in only one individual (F_2-7) and appeared as faint bands in the dwarf parent and five other individuals of the F_2 progeny (F_2-8 F_2-9 F_2-10 F_2-13 and F_2-14). This fragment was not considered for scoring beacuse it was not consistently produced in previous PCRs. Fragment three (900 bp) was polymorphic and strongly amplified in the tall parent and in all the F_2 progeny except four individuals (F_2-2 F_2-4, F_2-8 and F_2-15). Fragment four (850 bp) was a strong band appearing only in the tall parent. This was not considered for scoring because it was not consistantly produced in previous PCRs. Fragment five (550 bp) was strongly amplified in only two F2 individuals and not seen in any of the two parents. Presence of similar unexpected amplification products (spurious bands) has also been observed by others (Heun and Helintjaris, 1993). Fragment six (300 bp) was another monomorphic fragment similar to fragment one and was well amplified in all the individuals posing no difficulty in scoring. Fragment seven (275 bp) was another spurious fragment like fragment five which was only seen in four individuals of the F_2 progeny (F_2-3, F_2-4, F_2-7 and F_2-13). This was also excluded from scoring because of the poor resolution of the bands. Fragment eight (250 bp) was polymorphic and strongly amplified in the dwarf parent and in all the F_2 progeny except three individuals (F_2-4 F_2-7, and F_2-13). Fragment nine (200 bp) was only seen in tall parent and this too did not appear consistently in previous PCRs.

Plate 3.1.1 Agarose gel (1.5%) containing size fractionated DNA amplified by RAPD-PCR using the primer **OPB1** (see figure 3.1.1 below for details of size fractionated DNA)

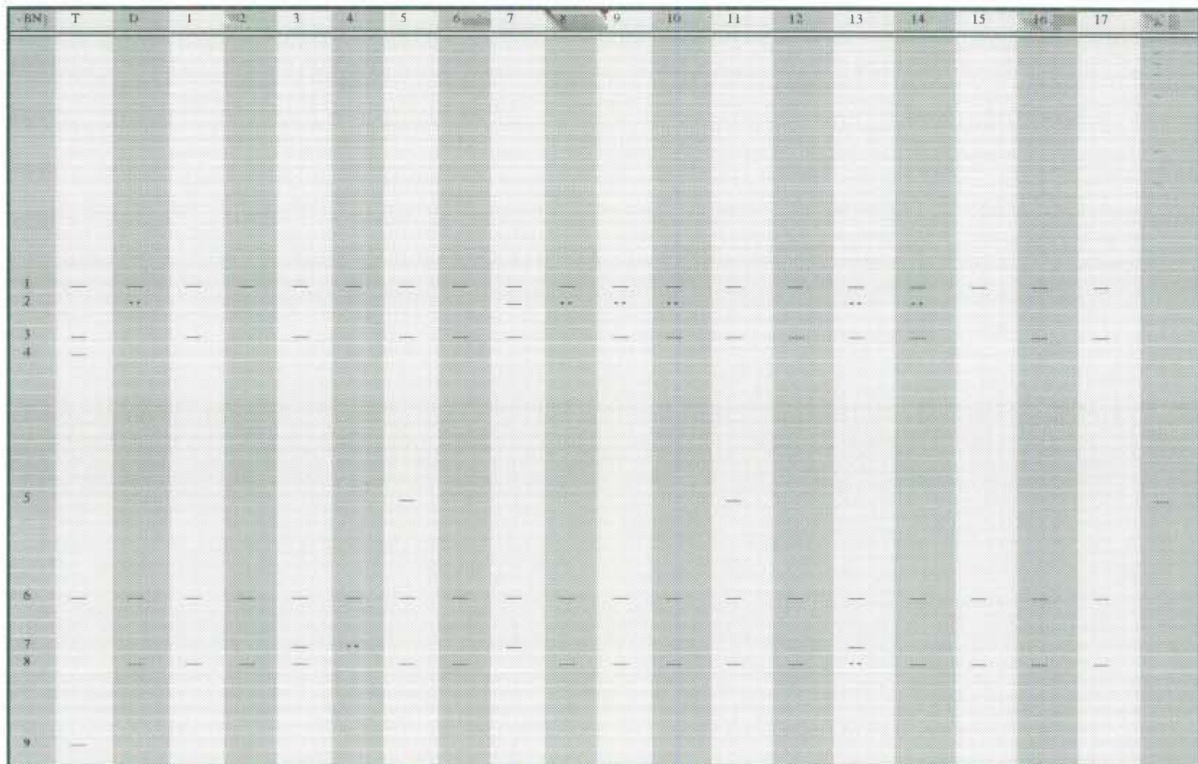

Figure 3.1.1 Schematic drawing of the agarose gel (1.5%) containing size fractionated DNA amplified by RAPD-PCR using the primer **OPB1**. BN, Band number. T, 'tall' parent (P1); D. 'dwarf' parent (P2) and 1 - 17, individuals of the f2 family are the sources of DNA for the RAPD-PCR. HindIII digested λ DNA is the standard DNA marker (the sizes of band 1, 2, 3, 4, 5, 6 and 7 from the most cathodal end are 23.1kb, 9.4kb 6.6kb, 4.4kb, 2.3 kb, 2.0 kb and 0.56 kb respectively). Bands appeared in broken lines were faint and not considered for scoring.

Plate 3.1.2 Agarose gel (1.5%) containing size fractionated DNA amplified by RAPD-PCR using the primer **OPB7** (see figure 3.1.2 below for details of size fractionated DNA)

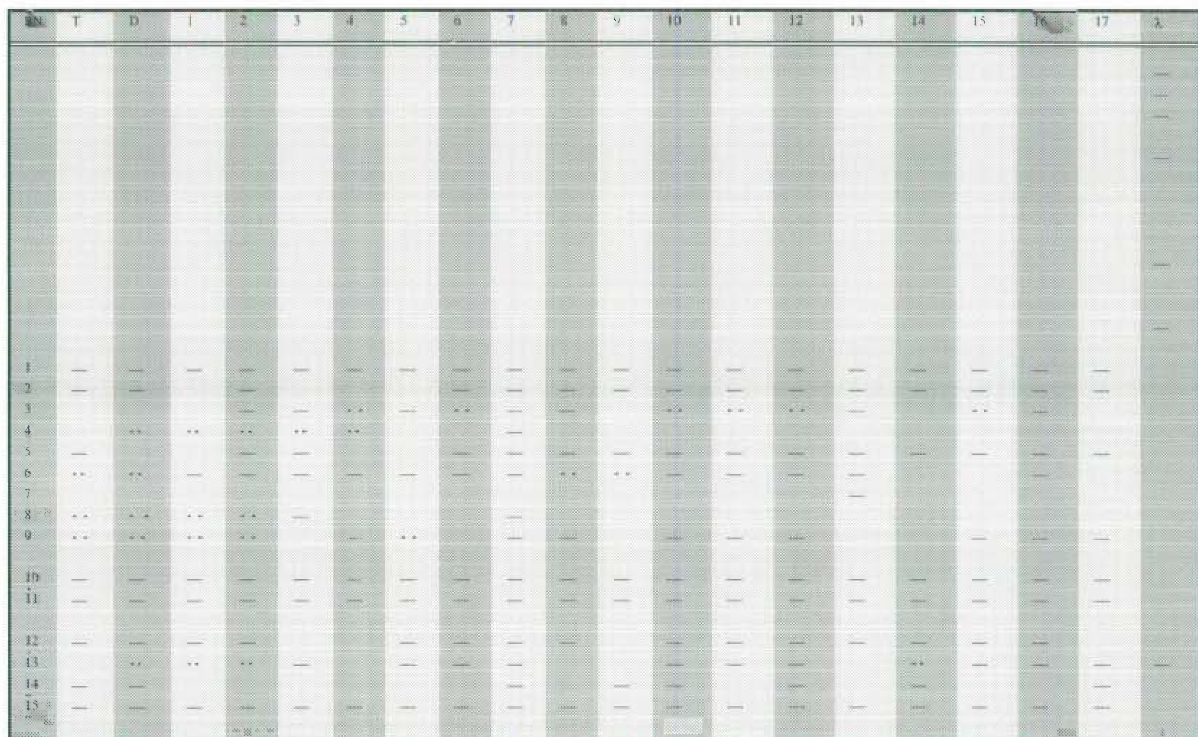

Figure 3.1.2 Schematic drawing of the agarose gel (1.5%) containing size fractionated DNA amplified by RAPD-PCR using the primer **OPB7**. BN, Band number. T, 'tall' parent (P1); D. 'dwarf' parent (P2) and 1 - 17, individuals of the f2 family are the sources of DNA for the RAPD-PCR. HindIII digested λ DNA is the standard DNA marker (the sizes of band 1, 2, 3, 4, 5, 6 and 7 from the most cathodal end are 23.1kb, 9.4kb 6.6kb, 4.4kb, 2.3 kb, 2.0 kb and 0.56 kb respectively). Bands appeared in broken lines were faint and not considered for scoring.

The amplified DNA profiles generated by the primer OPB7 provided an example of a more complex situation where the number of amplified fragments was high as 15 and the mobility of lower molecular weight DNA fragments was slightly uneven making it somewhat difficult to score shared bands. However, the intensity and resolution of fragments, 1, 2, 5, 10, 11, 12, 14 and 15 were strong and adequately resolved to score across all individuals. Fragment one (1,800 bp) was monomorphic and strongly amplified in all the individuals. Fragment two (1,700 bp) and fragment five 1,500 bp) were polymorphic. The former was common to both parents and was absent in four F_2 individuals (F_2-1, F_2-3, F_2-4 and F_2-5) and the latter was absent in the dwarf parent and three F_2 individuals (F_2-1, F_2-4 and F_2-5). Fragments 10 and 11 (725 bp and 700 bp respectively) were monomorphic. Fragments 11 and 12 (600 bp and 525 bp respectively) were polymorphic and both were present in the parents. Fragment 11 was absent in four F2 individuals whilst fragment 12 was absent in 11 F_2 individuals. Fragment 15 (500 bp) was also monomorphic and strongly amplified in all the individuals. Fragments 3, 4, 6 - 9 and 13 were not scored because they were either not consistent in the earlier assays or not strong and adequately resolved to score across all individuals with a reasonable accuracy.

The main amplification fragments primed by OPB1, OPB5, OPB7, OPB11, OPB18, 12mer2 and Check2 were scored from the gel photographsas described above for OPB1 and OPB7. The fragments were scored as '1' for presence and '0' for absence and are shown in table 3.1.1. The fragments scored against OPB1 and OPB7 were as explained earlier except for the changes in band numbers because only the fragments scored were numbered sequentially in this table. For example band OPB1.3 is OPB1.2 in this table because band OPB1.2 in p ate was not scored as a main amplification fragment.

The RAPD profile generated by primer OPB5 comprised sic clearly distinct fragments ranging from 550 - 1800 bp in size. Fragments one (1,800 bp) and two (1600 bp appeared strong and clear and exhibited olymorphism in the parents and individuals

of the F_2 progeny. The rest of the fragments wer monomorphic. Fragment one is absent in dwarf parent and in five F_2 individuals (F_2- 3, 4, 5, 7 and 8). Fragment two was absent in the tall parent and five F_2 individuals (F_2- 6, 9, 10, 13 and 14).

The RAPD profile generated by the primer OPB11 comprised seven main fragments ranging from 500 - 2000 bp in size, three of which are polymorphic. Fragments two (1,000 bp) and three (950 bp) exhibit polymorphism in the parents and individuals of the F_2 progeny. These two fragments segregated together as completely linked loci, absent in tall parent and seven F_2 individuals (F_2- 6, 7, 8, 10, 11, 14 and 15). Fragment four (700 bp) exhibited polymorphism only among the F_2 individuals as it was present in both parents and absent in five F_2 individuals (F_2- 10, 11, 15, 16 and 17).

Table 3.1.1 Main RAPD-PCR fragments scored for seven primers across *typica* (tall) and *nana* (dwarf green) parents and 17 F_2 individuals from F_1 (tall x dwarf) intercross population at the Coconut Research Institute, Sri Lanka. The presence of a band is denoted with '1' and its absence is denoted with '0'. 65

	OPB1					OPB5						OPB7							
RAPD band number Coconut palm	1	2[1]	3	4	5[1]	1[1]	2[1]	3	4	5	6	1	2[1]	3[1]	4	5	6[1]	7[1]	8
Typica parent (P_1)	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
Nana parent (P_2)	1	0	0	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1
F_2-1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1
F_2-2	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
F_2-3	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	0	0	1
F_2-4	1	0	0	1	0	0	1	1	1	1	1	1	0	0	1	1	0	0	1
F_2-5	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	0	1
F_2-6	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	1
F_2-7	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
F_2-8	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1
F_2-9	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1
F_2-10	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
F_2-11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
F_2-12	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
F_2-13	1	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1
F_2-14	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
F_2-15	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
F_2-16	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
F_2-17	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1

[1] Fragments exhibiting polymorphism

Table 3.1.1 continued on page 67

Table 3.1.1 continued from page 66

	OPB11						OPB18							12mer2									Check6			
RAPD band number / Coconut palm	1	2[1]	3[1]	4[1]	5	6	1	2	3[1]	4	5	6[1]	7	1	2	3	4	5	6	7	8	9[1]	1[1]	2[1]	3	4
Typica parent (P_1)	1	0	0	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1
Nana parent (P_2)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
F_2-1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1
F_2-2	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1
F_2-3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
F_2-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
F_2-5	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
F_2-6	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
F_2-7	1	0	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
F_2-8	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
F_2-9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
F_2-10	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1
F_2-11	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
F_2-12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
F_2-13	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
F_2-14	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
F_2-15	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1
F_2-16	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
F_2-17	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
F_2-18														1	1	1	1	1	1	1	1	1	1	0	1	1

[1] Clear discrete bands exhibiting polymorphism

The RAPD profiles generated by primer OPB18 comprised 7 main fragments ranging from 200 - 1500 bp in size, two of which were polymorphic. Fragments three (800 bp) and six (525 bp) were the two polymorphic fragments, the former was absent in tall parent and three F_2 individuals (F_2- 1, 5 and 7) and the latter was absent in tall parent and two F_2 individuals (F_2- 1 and 2).

The RAPD profiles generated by the 12-mer primer, 12mer2 comprised 9 main fragments ranging from 200 - 1800 bp in size. The only fragment to exhibit polymorphism was fragment nine (200 bp). This was absent in the male parent and in four F_2 individuals (F_2- 1, 2, 10 and 14). The RAPD profile generated by the other 12 mer primer, Check 6 comprised 4 main fragments ranging from 300 - 1000 bp in size. The first two fragments exhibit polymorphism in both parents and among individuals in the F_2 progeny. Fragment one (1000 bp) was absent in the male parent and in seven F_2 individuals (F_2- 5, 9, 10, 11, 12, 15 and 16) and fragment two (950 bp) was absent in the dwarf parent and nine F_2 individuals (F_2- 3, 5, 6, 7, 8, 11, 15, 17 and 18).

Single locus segregation analysis was performed to determine whether the 16 polymorphic fragments scored against OPB1, OPB5, OPB7, OPB11, OPB18, 12mer2 and Check6 these RAPD markers segregated in the expected Mendelian ratio in the F_2 progeny. The RAPD makers were considered as dominant and the zygosities of the tall and the dwarf parents were scored on that basis for all possible genotypes. Independent χ^2 (chi square) values were obtained for each and every polymorphism to test the goodness of fit of the expected ratio to observed ratio in the in the F_2 progeny. The results of these χ^2 tests were given in Table3.1.2 .

The single locus segregation analysis shows that all but one polymorphism under investgation (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, OPB1.5, OPB5.1, OPB5.2, OPB7.3, OPB11.2, OPB11.3, OPB18.3, OPB18.6, 12mer2.9 and Check6.1 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, OPB11.3 and check6.1 also consistent with the intercross configuration (F1, 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 alleles at the RAPD loci Check6.2 was consistent only with the intercross configuration (F_1, Aa and aa) with one of the parents (P1 or P2) being heterozygous and the other being homozygous recessive. The data for the three loci OPB7.2, OPB7.6 and OPB11.4, where both parents (P_1 or P_2) were positive for the dominent allele fits well with the intercross configuration F_1, AA x 2Aa, x aa, with both parents (P_1 or P_2) being heterozygous. The data for alleles in the locus OPB7.7 can not be explained with any of the probable intercross configurations.

Further analysis of the genetic indenpendece of these segregating polymorphisms (linkage analysis) and mapping of the RAPD markers into linkage groups were not possible due to a number of limitations in the material. The data available at present was only from a single family of 18 individuals. The phenotypic characters of the parents and F2 individuals were not scored at the same environment and at the same age of the palms and the F1 population was not available for analysis. Therefore, separation of the 16 RAPD markers into linkage groups and correlation with phenotypic characters could not be undertaken with this limited information.

The results of this segregation analysis clearly indicate the potential of RAPDs as a molecular marker for generating a linkage map in the coconut palm. By careful selection of primers capable of producing well resolved, strongly amplified, reproducible polymorphic fragments among morphologically distinct parental types such as *typica typica*(commercial tall), *nana* (dwarf green form and dwarf yellow form) and typica *San Ramon* there is a strong likelihood of finding correlations between RAPDs and phenotypic characteristics. Some of the on-going progeny trials at the CRI were aimed at evaluating various combinations of inter and intravarietal hybrids. These trials comprise well characterised parent and F_1 material allowing the possibility for the establishment of an

adequate number of F_2 (intercross and backcross) families for detection of RAPD markers for segregation and linkage group analyses.

Table 3.1.2 Single locus segregation analysis of 14 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			
OPB1.2	900	+	-	++	--	3:1	13:4	0.02
				+ -	--	7:9		7.40*
OPB1.5	250	-	+	-	++	3:1	14:3	0.49
				-	+ -	7:9		10.29*
OPB5.1	1,800	+	-	++	--	3:1	12:5	0.18
				+ -	--	7:9		4.94*
OPB5.2	1,600	-	+	-	++	3:1	12:5	0.18
				-	+ -	7:9		4.94*
OPB7.2	1,700	+	+	++	+ -	15:1	13:4	8.67*
				+ -	++	15:1		8.67*
				+ -	+-	3:1		0.02
OPB7.3	1,500	+	-	++	--	3:1	14:3	0.49
				+ -	--	7:9		10.29*
OPB7.6	600	+	+	++	+ -	15:1	13:4	8.67*
				+ -	++	15:1		8.67*
				+ -	+-	3:1		0.02
OPB7.7	525	+	+	++	+ -	15:1	6:11	99.14*
				+ -	++	15:1		99.14*
				+ -	+-	3:1		14.29*
OPB11.2	1,000	-	+	-	++	3:1	10:7	2.37
				-	+ -	7:9		1.56
OPB11.3	950	-	+	-	++	3:1	10:7	2.37
				-	+ -	7:9		1.56
OPB11.4	700	+	+	++	+ -	15:1	12:5	15.56*
				+ -	++	15:1		15.56*
				+ -	+ -	3:1		0.17
OPB18.3	800	-	+	-	++	3:1	14:3	0.49
				-	+ -	7:9		10.29*
OPB18.6	525	-	+	-	++	3:1	15:2	1.59
				-	+ -	7:9		13.67*
12mer2.9	200	-	+	-	++	3:1	14:4	0.07
				-	+ -	7:9		8.46*
Check6.1	1,000	-	+	-	++	3:1	11:7	1.85
				-	+ -	7:9		2.20
Check6.2	950	+	-	++	--	3:1	9:9	6.00*
				+ -	--	7:9		0.28

* Statistically significant at $P = 0.05$

3.2 Assessment of Genetic Diversity in the Coconut Palm by RAPD Polymorphisms

Polymorphic RAPD amplification products are used to assess genetic relatedness between plant species and accessions within them. In these assays the presence and absence of shared DNA fragments amplified by RAPD-PCR are numerically analysed to estimate pair-wise genetic distances and clustering of samples. I have assessed the genetic diversity of 19 accessions of the coconut palm from Sri Lanka by using RAPD polymorphisms generated by 17 random primers. The morphologically based genetic diversity and the RAPD markers of these 19 accessions will be discussed in this section.

The coconut accessions used in the study were selected to represent the entire population of the coconut palm in Sri Lanka in terms of varietal classification, geographical distribution and sources of origin. The taxonomy, specific morphological features, and the origin of the 19 coconut accessions are summarised in table 3.2.1. The 19 accessions of the coconut palm selected for the assay comprised 12 tall (*typica*) types, 6 dwarf (*nana*) types and the aurantiaca variety, the indigenous *king coconut* of Sri Lanka. The first three accessions of the tall type (T_1, T_2 and T_3) were replicates of the commercial tall cultivar CRIC60. The next three accessions, gon thembili, pora pol and ran thembili are three other forms of the tall, rarely occuring in wild populations. Moorock, Pitiyakande, Goyambokka, Akuressa and Debarayaya are sub populations of the tall variety (common tall) regarded as genetically isolated because they were maintained for several generations by breeding within themselves in the locations from which they were collected.

Table 3.2.1 Taxonomy, distinctive morphological and reproductive features and distribution of the 19 coconut accessions of the coconut palm population in Sri Lanka used for the RAPD assay.

Accession	Taxonomy	Specific morphological and reproductive features	Collection site	Source or origin
ISG F4.1 (T_1)	var. *Typica* form *Typica* (tall)	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium sized nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Isolated Seed Garden, Chillaw	Tall x Tall (selected SLT)
ISG F4.2 (T_2)	var. *Typica* form *Typica* (tall) var.	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium sized nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Isolated Seed Garden, Chillaw	Tall x Tall (selected SLT)
ISG F4.3 (T_3)	var. *Typica* form *Typica* (tall) var.)	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium sized nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Isolated Seed Garden, Chillaw	Tall x Tall (selected SLT)
Gon Thembili (GN)	var. *Typica* form Gon Thembili	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium sized nuts, orange coloured nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Crop museum, CRI Lunuwila	Domesticated from wild palms
Pora Pol (PORA)	var. *Typica* form Pora Pol	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium - small sized nuts, thick shelled nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Crop museum, CRI Lunuwila	Domesticated from wild palms
Ran Thembili (RAN)	var. *Typica* form Ran Thembili	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium sized nuts, pink colouration in the mesocarp of the fruit, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Crop museum, CRI Lunuwila	Domesticated from wild palms
Moorock (Mk)	var. *Typica* form *Typica* (tall)	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, slightly bigger nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Germplasm repository, CRI	Moorock estate, Mawathagama
Pitiyakanda (PITY)	var. *Typica* form *Typica* (tall) var.	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium sized nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Germplasm repository, CRI	Pitiyakanda estate, Mawathagama
Goyambokka (Gb)	var. *Typica* form *Typica* (tall)	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium sized nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Germplasm repository, CRI	Goyambokka estate, Tangalle
Akuressa (Ak)	var. *Typica* form *Typica* (tall) var.	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium sized nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Germplasm repository, CRI	Razina estate, Akuressa
Debarayaya (DEB)	var. *Typica* form *Typica* (tall)	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium sized nuts, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Germplasm repository, CRI	Debarayaya estate, Debarayaya
San Ramon (SAN)	var. *Typica* form San Ramon (tall)	Tall stature, allogamous, heterogeneous, fruits in 6 -7 years, medium - large sized nuts, round shaped nuts, high copra content, 20 - 25 nuts per bunch, 60 - 80 nuts per palm per year	Germplasm repository, CRI	Imported from Philippines

Table 3.2.1 continued on page 73

Table 3.2.1 continued from page 72

Accession	Taxonomy	Distinctive morphological and reproductive features	Collection site	Source or origin
King Coconut (KING)	var. *Aurantiaca* form King coconut	Intermediate stature, autogamous, homogeneous, fruits in 6 -7 years, seasonal flower production, slightly small sized orange coloured nuts with sweet nut water, 25 - 50 nuts per bunch, 80 - 120 nuts per palm per year	Crop museum, CRI Lunuwila	Domesticated from wild palms
Dwarf Green (DG)	var. *Nana* form green dwarf	Dwarf stature, autogamous, homogeneous, fruits in 3 - 4 years, small sized green coloured nuts, low copra content, 25 - 50 nuts per bunch, 80 - 120 nuts per palm per year	Crop museum, CRI Lunuwila	Domesticated from wild palms
Dwarf Yellow (DY)	var. *Nana* form yellow dwarf	Dwarf stature, autogamous, homogeneous, fruits in 3 - 4 years, small sized green coloured nuts, low copra content, 25 - 50 nuts per bunch, 80 - 120 nuts per palm per year	Crop museum, CRI Lunuwila	Domesticated from wild palms
Dwarf Red (DR)	var. *Nana* form red dwarf	Dwarf stature, autogamous, homogeneous, fruits in 3 - 4 years, small sized red coloured nuts, low copra content, 25 - 50 nuts per bunch, 80 - 120 nuts per palm per year	Crop museum, CRI Lunuwila	Domesticated from wild palms
Dwarf Brown (DB)	var. *Nana* form brown dwarf	Dwarf stature, autogamous, homogeneous, fruits in 3 - 4 years, small sized brown coloured nuts, low copra content, 25 - 50 nuts per bunch, 80 - 120 nuts per palm per year	Germplasm repository, CRI	Domesticated from wild palms
Cameroon Red Dwarf (CRD)	var. *Nana* form red dwarf	Dwarf stature, autogamous, homogeneous, fruits in 3 - 4 years, small sized red coloured nuts, low copra content, 25 - 50 nuts per bunch, 80 - 120 nuts per palm per year	Germplasm repository, CRI	Imported from Cameroon
Brazilian Green Dwarf (BGD)	var. *Nana* form green dwarf	Dwarf stature, autogamous, homogeneous, fruits in 3 - 4 years, small sized green coloured nuts, low copra content, 25 - 50 nuts per bunch, 80 - 120 nuts per palm per year	Germplasm repository, CRI	Imported from Brazil

San Ramon is an exotic *typica* accession imported from the Philippines by a private coconut plantation owner. When this accession was imported is not known exactly but the CRI conservationists believe this form was introduced in the latter half of the last 19th century. This form is different from the local *typica* forms in many aspects. A 50-year old *San Ramon* palm attains an average height of about 22 meters which is about 2 meters more than the local form. In the *San Ramon* form the nuts are very large and they yield more copra than any other form of coconut.

Aurantiaca form *king coconut* is indigenous and characterised by the orange coloured leaf petioles and nuts. It differs from *typica* forms by its intermediate stature, autogamous nature and fruit characteristics (sweet nut water and low copra yield). King coconut differs from *Nana* (dwarf) forms by its longer juvenile phase.

The *Nana* (dwarf) forms differ from one another only by the colour of the fruit. The *Nana* yellow variety however, has individuals that appear more like the *Typica* form *gon thembili*. The last two *Nana* forms *Cameroon red dwarf* and *Brazilian green dwarf* were imported from a germplasm collection in the Ivory Coast in 1969 (Perera *et al.*, 1992).

DNA samples from the coconut accessions listed in table 3.2.1 were prepared in the manner described in section 2.2.1 for use in the RAPD-PCR. Young leaves picked from four individuals per accession were mixed and used for DNA extraction. This was thought necessary because one would expect to have a reasonable diversity between individuals of an accession due to the high heterogeneity of the *Tall* variety. Use of bulk DNA samples has been proved successful in estimating genetic relatedness in heterogeneous populations of alfalfa (Kangfu and Pauls, 1993).

Several precautions were taken to optimise the RAPD-PCR protocol to achieve successful amplifications from all the DNA samples at every single effort. The DNA concentrations of the DNA samples were measured accurately and all were diluted to the

same final concentration of 12.5 ng / µl to keep the volume of DNA added per tube identical (4 µl per 25 µl reaction) with all the DNA samples throughout the assay. *Taq* reaction buffer, magnesium chloride, dNTPs and water were mixed in bulk and aliquots were stored at 4 °C. Immediately before setting up the PCRs appropriate volumes of the above mixture, enzyme and primer were mixed thoroughly. DNA templates were then added to aliquots of the mixture. This procedure ensured uniformity in the concentrations of the reaction constituents across all the tubes.

In the earlier study (section 3.1) out of 38 primers tested 31 failed to produce polymorphisms with DNA from tall and dwarf individuals. Most of the unsuccessful primers did not even produce clear monomorphic bands. However, at a later stage it became evident that some of the unsuccessful primers generated strong RAPD band profiles when the concentration of the primer was doubled (increased to 10 picomoles). Therefore, in order to increase the number of RAPD-PCR fragments, for better assessment of the genetic diversity of the 19 accessions, primer concentration was increased in all the reactions. This has resulted not only in an increase in the number of primers useable in the RAPD-PCR analysis but also an increase in the number of scorable bands per primer.

The names of the primers and their sequences are indicated in chapter two (Table 2.3). All of the 38 primers were used to generate RAPD-PCR profiles with DNA from 19 coconut accessions. Out of the 38 primers tested, 18 effectively primed the amplification of coconut genomic DNA. The G+C content of the OPB primers were designed to be over 60 per cent and in 12-mer and Check primers they varied between 42 - 75 percent (see table 2.3). The failure of certain primers to generate RAPD profiles or the reduced number of fragments produced by some primers cannot be strictly attributed to the G + C content of the primer in this assay because some of the primers even with G + C contents as high as 70 - 75 percent (12-mer1, 12-mer4, OPB3, OPB9, OPB10, OPB16 and OPB19) failed to generate good DNA profiles. It is more likely that the combination of

primer sequence and DNA template is more decisive in generating a good reproducible DNA profile RAPD-PCR..

The amplification reactions with all the primers using all the DNA samples were not repeated in this assay due to time and resource constraints. Moreover, most of these primers gave consistent reproducible results between replicated amplifications with respect to scorable bands that were scored with DNA from tall and dwarf palms (Section 2.1). Amplifications with primers were repeated only when obvious problems with the PCR procedures occurred. When the PCR reactions were performed, two tubes in every experiment were allowed to go through the thermocycles with out adding the DNA template, to serve as negative controls. Occasionally amplification fragments appeared in these negative control reactions but they did not correspond to the bands in the test reaction.

Photographs were taken after size fractionation of the amplified DNA on 1.5% agarose gels. *Hind*III digested λ DNA was used as a size marker to estimate molecular weights of the amplified DNA fragments. The same pattern was followed in assigning lanes in the gel for loading DNA samples. Plates 3.2.1 and 3.2.2 serves as examples of two photographs of gels. Figures 3.2.1 and 3.2.2 set out underneath the two plates respectively describe the results obtained from gels.

The amplified fragments were scored by visual observation. The different amplification fragments visible as products of different molecular weights did vary in their intensity and resolution. Therefore, the bands were scored for the presence versus absence of products rather than grading quantitatively on the level of intensity. Only clear discrete bands were scored and in cases of doubt the negative of the photographs were examined because the band resolution is relatively better in the transparent negative image. The presence of a smear was always considered as absence of a band. Bands were scored as "1" for presence and "0" for absence and were initially recorded on paper as a matrix

with each sample forming one column of the matrix and each band one row. The bands scored against each primer across the sources of DNA are presented in Table 3.2.2

Out of the 38 primers tested, 17 primers generated profiles with clear, discrete amplified fragments of DNA with varying molecular weights. Ten of the primers failed to reveal polymorphisms in the tall and the dwarf palm in the earlier assay. All of these 17 primers produced amplified polymorphisms with DNA from the 19 coconut accessions. The DNA profiles generated by these primers in this assay yielded more bands of scorable intensity than those obtained with the same primers with the DNA from tall and dwarf parents and the F_2 progeny.

The total number of amplification products and number of polymorphic DNA fragments amplified among the 19 accessions of the coconut palm by each primer are given in Table 3.2.2. The codes numbers of random primers, their sequences, the total numer of bands scored for each primer and the size ranges in the molecular weights of the amplified bands for each primer are also included in this tabulation. The 17 primers produced a total of 160 amplified DNA fragments across the 19 accessions of the coconut palm. Of the 160 fragments, 91 (56.8%) exhibited polymorphisms between the accessions. The mean number of amplification products per primer is 9.4 $\pm$ 0.6 and the mean number of polymorphic amplification products per primer is 5.4 $\pm$ 0.7. The sizes of amplified fragments approximately ranged between 200 - 2200 bp in size. The primer OPB12 gave 14 amplified fragments across the 19 accessions which is the highest for a primer and primers OPB1, OPB18 and Check7 yielded more than 10 bands per primer. OPB6 gave the lowest yield of four amplified bands. Check 7 yielded the highest number of polymorphic bands across the 19 coconut accessions whilst OPB1 and OPB18 has also yielded 11 and 9 polymorphic bands respectively

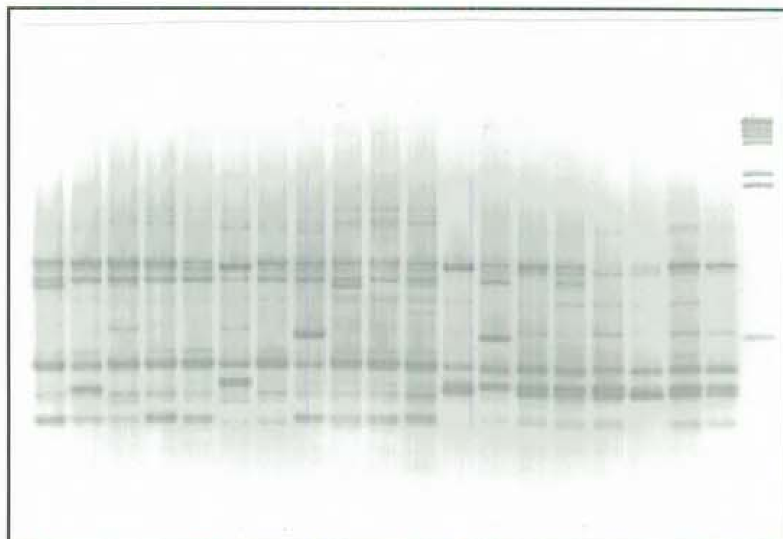

Plate 3.2.1 Agarose gel (1.5%) containing size fractionated DNA amplified by RAPD-PCR using the primer **OPB1** (see figure 3.2.1 below for details of size fractionated DNA)

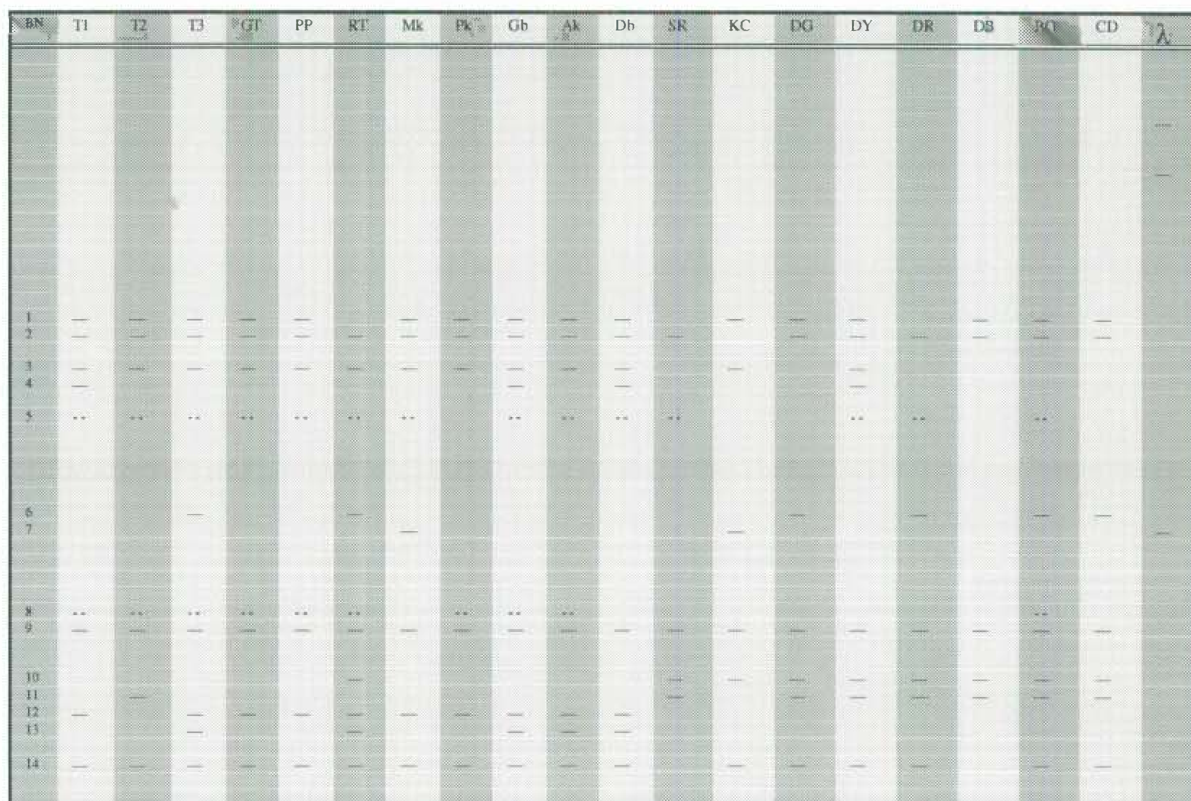

Figure 3.2.1 Schematic drawing of plate 3.2.1, the agarose gel (1.5%) containing size fractionated DNA amplified by the RAPD-PCR using the primer **OPB1** BN, Band number. T1, T2 and T3 , *typica typica* populations; GT, *typica gon thembili*; PP, *typica pora pol*; RT, *typica ran thembili*; Mk, *typica typica* Moorock; Pk, *typica typic*a Pitiyakanda; Gb, *typica typic*a Goyambokka; Ak, *typica typic*a Akuressa; Db, *typica typic*a Debarayaya; SR, *typica San Ramon*; KC, *Aurantiaca king coconut*; DG, DY, DR and DB, forms of the Sri Lanka *nana* (dwarf), green, yellow, red and brown; CD, Cameroon *nana* form red; Brazilian Cameroon *nana* form green. λ, standard DNA marker, HindIII digested λ DNA (the sizes of band 5, 6 and 7 from the most cathodal end are 2.3 kb, 2.0 kb and 0.56 kb respectively). Bands shown in broken lines were faint and not considered for scoring.

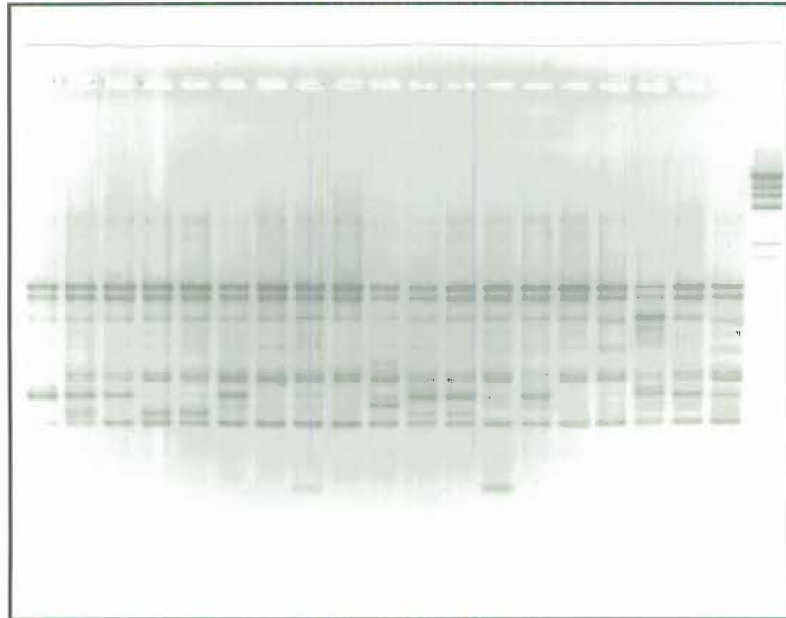

Plate 3.2.1 Agarose gel (1.5%) containing size fractionated DNA amplified by the RAPD-PCR using the primer **OPB7** (see figure 3.2.1 below for details of size fractionated DNA)

Figure 3.2.2 Schematic drawing of plate 3.2.1, the agarose gel (1.5%) containing size fractionated DNA amplified by the RAPD-PCR using the primer **OPB7.** BN, Band number. T1, T2 and T3 , *typica typica* populations; GT, *typica gon thembili*; PP, *typica pora pol*; RT, *typica ran thembili*; Mk, *typica typica* Moorock; Pk, *typica typic*a Pitiyakanda; Gb, *typica typic*a Goyambokka; Ak, *typica typic*a Akuressa; Db, *typica typic*a Debarayaya; SR, *typica San Ramon*; KC, *Aurantiaca king coconut*; DG, DY, DR and DB, forms of the Sri Lanka *nana* (dwarf), green, yellow, red and brown; CD, Cameroon *nana* form red; Brazilian Cameroon *nana* form green. λ, standard DNA marker, HindIII digested λ DNA (the sizes of band 1, 2, 3, 4, 5, 6 and 7 from the most cathodal end are 23.1kb, 9.4kb 6.6kb, 4.4kb, 2.3 kb, 2.0 kb and 0.56 kb respectively). Bands shown as broken lines were faint and not considered for scoring.

Table 3.2.2 RAPD-PCR bands scored for 17 primers across 19 accessions of the coconut palm population in Sri Lanka. The presence of a band is denoted with a "1" and its absence is denoted with "0" . The bottom line gives the number assigned to polymorphic bands in the computer aided RAPD distance analysis.

	Primer OPB1												Primer OPB4										OPB5					
RAPD band number Coconut accession	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
T1	1	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	0	0	1	1	0	1	1	1	0	1	1	1
T2	1	1	1	0	0	0	1	0	1	0	0	1	1	1	1	0	0	1	1	1	0	1	1	1	0	1	1	1
T3	1	1	1	0	1	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	0	1	1	1
Gon thembili	1	1	1	0	0	0	1	0	0	1	0	1	1	1	1	0	0	0	1	1	0	1	1	1	0	1	1	1
Pora pol	1	1	1	0	0	0	1	0	0	1	0	1	1	1	1	0	0	1	1	1	0	1	1	1	0	1	1	1
Ran thembili	0	1	1	0	1	0	1	1	0	1	1	1	1	1	1	0	0	0	1	1	0	1	1	1	0	1	1	1
Moorock	1	1	1	0	0	0	1	0	0	1	0	1	1	1	1	0	0	0	1	1	0	1	1	1	0	1	1	1
Pitiyakanda	1	1	1	0	0	1	1	0	0	1	0	1	1	1	1	0	0	0	1	1	0	1	1	1	0	1	1	1
Goyambokka	1	1	1	1	0	0	1	0	0	1	1	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	1	1
Akuressa	1	1	1	0	0	0	1	0	0	1	1	1	1	0	1	0	0	0	1	1	0	1	1	1	0	1	1	1
Deharayaya	1	1	1	1	0	0	1	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1	1	1	0	1	1	1
San Ramon	0	1	0	0	0	0	1	1	1	0	0	0	1	0	1	0	0	0	1	1	0	1	1	1	1	1	1	1
King coconut	1	0	1	0	0	1	1	1	0	0	0	1	1	0	0	0	1	1	1	1	0	1	1	0	1	1	1	1
Dwarf green	1	1	0	0	1	0	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Dwarf yellow	1	1	1	1	0	0	1	1	1	0	0	1	1	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1
Dwarf red	0	1	0	0	1	0	1	1	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
Dwarf brown	1	1	0	0	0	0	1	1	1	0	0	0	1	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1
Cameroon red dwarf	1	1	0	0	1	0	1	1	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
Brazilian green dwarf	1	1	0	0	1	0	1	1	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1
RAPD polymorphic loci number	1	2	3	4	5	6		7	8	9	10	11	-	12	13	14	15	16	-	-	17	-		18	19			

Table 3.2.2 continued on page 81

Table 3.2.2 continued from page 80

	OPB5			OPB6				OPB7										OPB8							OPB11			
RAPD band number / Coconut accession	29	30	3	32	33	34	35	30	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
T1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	0	1	0	0	1
T2	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	0	1	1	0	1	0	0	1
T3	1	1	1	0	0	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	0	1	0	0	1
Gon thembili	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	1	0	1	1	0	1	0	0	1
Pora pol	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	0	1	1	1	1	1	0	0	1
Ran thembili	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	0	1	1	1	1	1	0	0	1
Moorock	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	1	0	0	1	1	1	1	0	1	1
Pitiyakanda	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	0	1	0	0	1	1	1	1	0	0	1
Goyambokka	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	1	0	0	1	1	1	1	0	1	1
Akuressa	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	0	1	0	0	1	1	1	1	1	0	1
Debarayaya	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	0	0	1	1	0	1	0	0	1
San Ramon	1	0	1	0	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	0	1	1	0	0	0	1	1
King coconut	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	1	1	1	1	0	0	1
Dwarf green	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	0	1
Dwarf yellow	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	0	1	0	0	1	1	1	1	1	0	1
Dwarf red	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	1	0	1	1	1	0	1	1	1	1	1	0	1
Dwarf brown	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	1
Cameroon red dwarf	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	0	1	1	1	1	1	0	1
Brazilian green dwarf	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1
RAPD polymorphic loci number		20		21	22								23	24	25		26	27		28	29			30	31	32	33	

Table 3.2.2 continued on page 82

Table 3.2.2 continued from 81

	OPB11				OPB12													OPB13								OPB15		
RAPD band number / **Coconut accession**	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84
T1	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	0	1	0	1	1	1	0	1	0	1	1	1	1
T2	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	0	1	0	1	1	1	0	1	0	1	1	1	1
T3	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	1
Gon thembili	1	0	0	1	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	0	1	1	1	0	1	1	1	1
Pora pol	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	0	1	0	1	1	1	0	1	1	1	1	1	1
Ran thembili	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	0	1	0	1	1	1	0	1	0	1	1	1	1
Moorock	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	0	0	1	0	1	1	1	1
Pitiyakanda	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	0	0	1	0	1	1	1	1
Goyambokka	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	0	0	1	0	1	1	1	1
Akuressa	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	0	0	1	0	1	0	1	1
Debarayaya	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1
San Ramon	1	0	0	1	1	1	1	1	0	0	1	1	0	0	1	0	1	0	1	0	0	0	1	0	1	1	1	1
King coconut	1	0	1	1	1	1	1	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1	1	1	0	1	1
Dwarf green	1	0	0	1	1	1	1	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1	1	1	1	1	1
Dwarf yellow	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	0	1	1	1	0	0	0	1	1	1	0	1	1
Dwarf red	1	0	0	1	1	1	1	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1	0	1	0	1	1
Dwarf brown	1	0	0	1	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1
Cameroon red dwarf	1	0	0	1	1	1	1	1	0	0	0	1	0	0	1	0	1	1	1	0	0	0	1	1	1	1	1	1
Brazilian green dwarf	1	0	0	1	1	1	1	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1	0	1	0	1	1
RAPD polymorphic loci number		34	35						36	37	38		39	40		41		42		43	44	45		46		47		

Table 3.2.2 continued on page 83

Table 3.2.2 to continued from 82

	OPB15				OPB17										OPB18											
RAPD band number Coconut accession	85	86	87	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111
T1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1
T2	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1
T3	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1
Gon thembili	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1
Pora pol	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
Ran thembili	1	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1
Moorock	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1
Pitiyakanda	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1
Goyambokka	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
[illegible]	1	1	1	1	1	0	1	1	1	1	1	1	1	1	?	?	?	?	1	?	?	?	1	?	?	1
Debarayaya	1	1	1	1	1	0	1	1	0	1	1	1	0	1	0	0	0	1	1	0	1	1	1	1	1	1
San Ramon	1	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	1	1	1
King coconut	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	0	0	1	0	0	1
Dwarf green	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	0	1	1	1	0	1	1	1	1	1
Dwarf yellow	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	1	1	1
Dwarf red	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	1	1	1
Dwarf brown	1	1	1	1	0	0	1	0	1	1	1	1	0	1	0	0	0	1	1	1	0	1	1	1	1	1
Cameroon red dwarf	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	1	1	1
Brazilian green dwarf	1	1	1	1	1	1	1	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	1	1	1	1
RAPD polymorphic loci number				48	49	50		51	52				53		54	55	56	57		58	59	60		61	62	

Table 3.2.2 to continued on page 84

Table 3.2.2 continued from page 83

	12MER2									CHECK2										CHECK6							
RAPD band number / **Coconut accession**	112	13	14	15	16	17	18	19	120	121	22	23	24	25	26	27	28	29	130	131	32	33	34	35	36	37	138
T1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	0	1	1	0	0	1	1
T2	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	0	1	1	0	0	1	1
T3	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	0	1	1	0	0	1	1
Gon thembili	1	1	1	1	1	1	1	1	0	1	0	0	1	0	1	0	1	1	0	1	0	0	0	1	1	1	1
Pora pol	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	0	1	0	0	1	0
Ran thembili	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	0	1	1	0	1	0	0	1	0
Moorock	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	0	0	0	0	1	1	1
Pitiyakanda	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1	1
Goyambokka	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	0	1	1	1
Akuressa	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	0	1	1	1	1	0	1	1	0	0	1	1
Debarayaya	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	?	?	?	?	?	?	1	?
San Ramon	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	0	1	1	0	1	1
King coconut	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1	1	1	1	1	0	1	0	0	1	1
Dwarf green	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1	1	0	1	0	0	1	1
Dwarf yellow	1	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	1	1	0	1	1	0	1	1
Dwarf red	1	1	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	0	1	1	0	1	1	0	1	1
Dwarf brown	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1
Cameroon red dwarf	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1
Brazilian green dwarf	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	0	1	1
RAPD polymorphic loci number									63	64	65	66	67	68		69			70	71	72	73	74	75	76		77

Table 3.2.2 continued on page 85

Table 3.2.2 continued from page 84

	CHECK7													CHECK 8							
RAPD band number / Coconut accession	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159
T1	0	1	0	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	0	1	1
T2	0	1	0	1	0	1	0	0	1	0	1	1	0	1	1	1	1	1	0	1	1
T3	0	0	0	1	1	0	0	0	1	0	1	1	0	1	1	1	1	1	0	1	1
Gon thembili	0	1	0	1	1	0	0	0	1	0	1	1	0	1	1	1	1	1	0	1	1
Pora pol	0	1	0	1	1	0	0	0	1	0	0	1	1	1	1	1	1	1	0	1	1
Ran thembili	0	1	1	1	1	0	0	0	1	0	0	1	1	1	1	1	1	1	0	1	1
Moorock	1	1	0	0	1	0	0	0	0	1	1	0	0	1	1	1	1	1	1	1	1
Pitiyakanda	1	1	0	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	0	1	1
Goyambokka	1	1	0	1	1	0	1	1	0	0	1	1	0	1	1	1	1	1	0	1	1
Akuressa	1	1	0	1	1	0	0	0	0	0	1	1	0	1	1	1	1	1	1	1	1
Debarayaya	1	1	0	1	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	1	1
San Ramon	0	1	1	0	1	1	0	1	0	0	1	1	1	1	1	1	1	1	0	1	1
King coconut	0	1	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	0	1	1
Dwarf green	0	1	0	0	0	0	0	1	0	0	0	1	1	1	1	1	1	1	0	1	1
Dwarf yellow	0	1	1	1	0	1	0	1	0	0	0	1	1	1	1	1	1	1	0	1	1
Dwarf red	0	1	0	0	0	0	0	1	0	0	1	1	1	1	1	1	1	1	0	1	1
Dwarf brown	0	1	0	0	0	1	1	1	0	0	0	1	1	1	1	1	1	1	0	1	1
Cameroon red dwarf	1	1	1	0	0	1	1	1	0	1	0	1	0	1	1	1	1	1	0	1	1
Brazilian green dwarf	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1
RAPD polymorphic loci number	78	79	80	81	82	83	84	85	86	87	88	89	90						91		

The 91 polymorphic bands were numbered sequentially to identify them as RAPD loci and the approximate size of each band was measured by comparing their electrophoretic mobility with standard DNA fragments of the HindIII digested lambda DNA. The number assigned, the primer responsible and the approximate size in base pairs of each amplified fragment are tabulated in table 3.2.4.

Table 3.2.3 Code names of the primers, their sequences, total bands and number of polymorphic bands scored against each primer across 19 accessions of the coconut palm

Code number of the primer	Primer sequence	No. of amplified fragments	No. of polymorphic fragments	Range of molecular weights (bp)
OPB1	GTTTCGCTCC	12	11	200-1000
OPB4	GGACTGGAGT	10	6	250-1800
OPB5	TGCGCCCTTC	9	3	250-1800
OPB6	TGCTCTGCCC	4	2	800-1600
OPB7	GGTGACGCAG	10	4	300-1800
OPB8	GTCCACACGG	8	4	300-1600
OPB11	GTAGACCCGT	7	5	300-2000
OPB12	CCTTGACGCA	14	6	200-1800
OPB13	TTCCCCCGCT	8	5	650-2000
OPB15	GGAGGGTGTT	8	2	350-1200
OPB17	AGGGAACGAG	10	5	300-2000
OPB18	CCACAGCAGT	12	9	200-2200
12Mer2	TCATCCGCTTCC	9	1	200-1800
Check2	AATCGCTTATGA	10	7	550-1800
Check6	GTCTGAATGACC	8	7	300-1600
Check7	GGCAACGCGGT C	13	13	300-2000
Check8	ACCGCGATTGCC	8	1	200-1100

Table 3.2.4 Sizes of polymorphic bands amplified by 17 arbitrary primers in RAPD-PCRs among 19 accessions of the coconut palm

Primer	Polymorphic band number	Approximate base pair length of the band (bp)	Primer	Polymorphic band number	Approximate base pair length of the band (bp)
OPB1	1	1000	**OPB17**	47	1200
	2	975		48	350
	3	900		49	2000
	4	875		50	1800
	5	580		51	1775
	6	565		52	1675
	7	400		53	375
	8	375	**OPB18**	54	2200
	9	300		55	2000
	10	275		56	1800
	11	200		57	1700
OPB4	12	1525	**OPB6**	58	650
	13	1500		59	625
	14	1300		60	600
	15	1250	**OPB7**	61	550
	16	1200		62	250
	17	650	**12MER2**	63	200
OPB5	18	1600	**CHECK2**	64	1800
	19	1450		65	1400
	20	275		66	1350
OPB6	21	1600		67	1300
	22	1400		68	1100
OPB7	23	560		69	800
	24	525		70	525
	25	500	**CHECK6**	71	1600
	26	300		72	1200
OPB8	27	1600		73	800
	28	1300		74	600
	29	900		75	450
	30	300		76	425
OPB11	31	2000		77	300
	32	1300	**CHECK7**	78	2000
	33	950		79	1800
	34	325		80	1725
	35	300		81	1600
OPB12	36	525		82	1300
	37	500		83	800
	38	475		84	700
	39	425		85	450
	40	375		86	400
	41	250		87	375
OPB13	42	2000		88	350
	43	1700		89	325
	44	1675		90	300
	45	1300	**CHECK8**	91	350
	46	700			

The genetic relatedness of the DNA samples was numerically assessed by calculating pair-wise distances based on shared amplification products and by construction of a dendrogram. Genetic distance (F) is an estimate of similarity between two samples calculated on the basis of the probability that an amplified fragment from one plant will also be found in another. The two most common estimates for genetic distance are Nei and Li's (1979) matching coefficient and Jaccards coefficient (Virk *et al.*, 1995).

Nei and Li's coefficient $F' = 2 \times N_{AB1} \div (N_A + N_B)$

Jaccard's coefficient $F'' = N_{AB1} \div (N_T - N_{AB0})$

(N_A, number of bands in accession A,
N_B, number of bands in accession B,
N_{AB1}, number of bands common in both accessions
N_{AB0}, number of bands present in others but not in A and B)

Nei and Li's coefficient was used to calculate the pair-wise genetic distances (1 - F') between all the accessions using the microcomputer software package, RAPDISTANCE (version 1.02) developed by John Armstrong, Adrain Gibbs, Rod Peakall and George Weiller of the Australian National University, Canberra, Australia.

Table 3.2.5 presents the genetic distance matrix based on Nei and Li's coefficient for 19 accessions of the coconut palm revealed by the 17 random oligonucleoide primers. The matrix highlights the distinctions between and within the varieties, form and population of the coconut palm. The genetic distances in the matrix were used to find mean distances between and within tall and dwarf accessions and between San Ramon and king coconut accessions because these four were the most morphologically separable groups. The results are given in table 3.2.6.

The mean genetic distance within the entire population (19 accessions), 0.33, is an indication of the existing genetic variability within the accessions assayed (33% genetic dissimilarity in the genomic DNA). The most genetically distant pairs of accessions are T3 and Brazilian green dwarf, king coconut and gon thembili, T3 and king coconut, T3 and brown dwarf, T3 and Cameroon red dwarf, all of these recording more than 50% dissimilarity. The closest pair is Brazilian green dwarf and Cameroon red dwarf separated only by a distance of 0.096. T1 and T2 also share a greater proportion of polymorphic RAPDs resulting in a low distance of 0.115.

The genetic variation within the tall accessions is relatively higher than that in the dwarf accessions. This is an expected result because of the distinct morphological differences between different tall forms and geographical isolation of tall germplasm accessions. Dwarfs other than yellow dwarf differed morphologically from each other only in the colour of their fruit.

Table 3.2.5 Matrix showing values of genetic distance (d) between 19 accessions of the coconut palm.

	T1	T2	T3	GT	PP	RT	MK	PT	GB	AK	DE	KC	SR	DG	DY	DR	DB	CD	BD
T1	0.0																		
T2	0.115	0.0																	
T3	0.173	0.187	0.0																
GT	0.214	0.292	0.252	0.0															
PP	0.122	0.207	0.245	0.229	0.0														
RT	0.247	0.306	0.346	0.288	0.135	0.0													
MK	0.200	0.257	0.298	0.280	0.233	0.352	0.0												
PT	0.180	0.237	0.298	0.260	0.214	0.274	0.142	0.0											
GY	0.214	0.269	0.288	0.252	0.210	0.321	0.142	0.200	0.0										
AK	0.169	0.224	0.244	0.247	0.203	0.320	0.151	0.151	0.132	0.0									
DE	0.247	0.290	0.333	0.291	0.262	0.367	0.234	0.255	0.188	0.157	0.0								
SR	0.393	0.428	0.516	0.468	0.445	0.437	0.413	0.434	0.414	0.440	0.477	0.0							
KC	0.389	0.402	0.517	0.511	0.340	0.413	0.431	0.431	0.452	0.415	0.428	0.487	0.0						
DG	0.377	0.408	0.448	0.485	0.333	0.378	0.414	0.434	0.415	0.400	0.431	0.290	0.303	0.0					
DY	0.346	0.333	0.458	0.434	0.339	0.386	0.381	0.319	0.365	0.346	0.354	0.362	0.287	0.285	0.0				
DR	0.420	0.391	0.478	0.452	0.411	0.402	0.419	0.376	0.420	0.404	0.460	0.287	0.301	0.170	0.195	0.0			
DB	0.384	0.395	0.500	0.494	0.377	0.405	0.422	0.443	0.403	0.448	0.462	0.274	0.310	0.163	0.270	0.195	0.0		
CD	0.420	0.434	0.515	0.450	0.394	0.384	0.420	0.420	0.420	0.425	0.458	0.297	0.355	0.168	0.232	0.157	0.171	0.0	
BD	0.412	0.445	0.524	0.461	0.387	0.377	0.411	0.392	0.412	0.398	0.469	0.270	0.369	0.165	0.287	0.134	0.207	0.096	0.0

Table 3.2.6 Mean genetic distances between and within varieties of the coconut palm

Sources of diversity	Mean genetic distance	Standard deviation of the mean genetic distance	Range of the mean genetic distance
Within all the 19 accessions	0.33	0.11	0.10 - 0.52
Within tall accessions	0.24	0.06	0.12 - 0.52
Within dwarf accessions	0.19	0.05	0.10 - 0.37
Between tall and dwarf accessions	0.41	0.04	0.32 - 0.29
Between tall accessions and the San Ramon accession	0.44	0.03	0.39 - 0.52
Between dwarf accessions and the San Ramon accession	0.29	0.03	0.27 - 0.36
Between tall accessions and the king coconut accession	0.43	.054	0.34 - 0.52
Between dwarf accessions and the king coconut accession	0.32	0.03	0.29 - 0.37
Between San Ramon accession and the king coconut accession	0.49	-	-

The average genetic distance between tall and dwarf accessions (0.41) is well above the average genetic distances within the *typica* (0.24) and *nana* (0.19) indicating a clear separation of the two groups. The average genetic distance between tall accessions and the San Ramon accession (0.44) is higher than the genetic distance between San Ramon and dwarf accessions (0.29). This is an unexpected result according to the taxonomy of the San Ramon form. San Ramon was classified as a Tall form by the Philippine Coconut Authority. Morphologically it is very different from the Sri Lankan tall palms and also from the dwarf types. It also differs from the indigenous *aurantiaca* variety, king coconut by a genetic distance of 0.49. Average genetic distances between tall accessions and the king coconut palm (0.43) and dwarf accessions and the king coconut palm (0.32) indicate that the intermediate *aurantiaca* variety share a higher proportion of genomic DNA with dwarfs than talls. Morphologically, too, king coconuts look more like dwarfs.

The data matrix of genetic distances was used to generate the best tree for these data using the option NJTREE (Neighbour joining tree) in the RAPDISTANCE package. This option uses a method of clustering accessions by utilising the unweighted pair group method analysis described by Sneath and Sokal (1973). This package also has another option to test whether the tree calculated from the set of distances reflects a tree-like signal in the data, or is merely an artefact that could arise from a random set of data. This test permutation tail probability analysis (PTP), as it is called, is based on the concept that a tree calculated from random distances will have a larger total branch length than a tree with structure. Thus the test compares the tree calculated with the original data with 20 trees calculated from the same data but dispersed randomly across the samples.

The neighbour joining tree of the 19 accessions of the coconut palm is shown in figure 3.2.3. The mean length of the random trees 2.81 which is significantly higher than the length of the original tree which is 2.10. This original tree deviates from a random structure because the 171 pair-wise distance values did not show a normal-like distribution as expected from a random set of data. The distance values are distributed in two peaks: one around 0.25 - 0.30 and the other around 0.35 - 0.40. Whereas the randomised pair-wise distance values showed a normal distribution with a single peak at around 0.30 - 0.35 (see table 3.2.7). All this information supports the validity of the dendrogram presented in Figure 3.2.1.

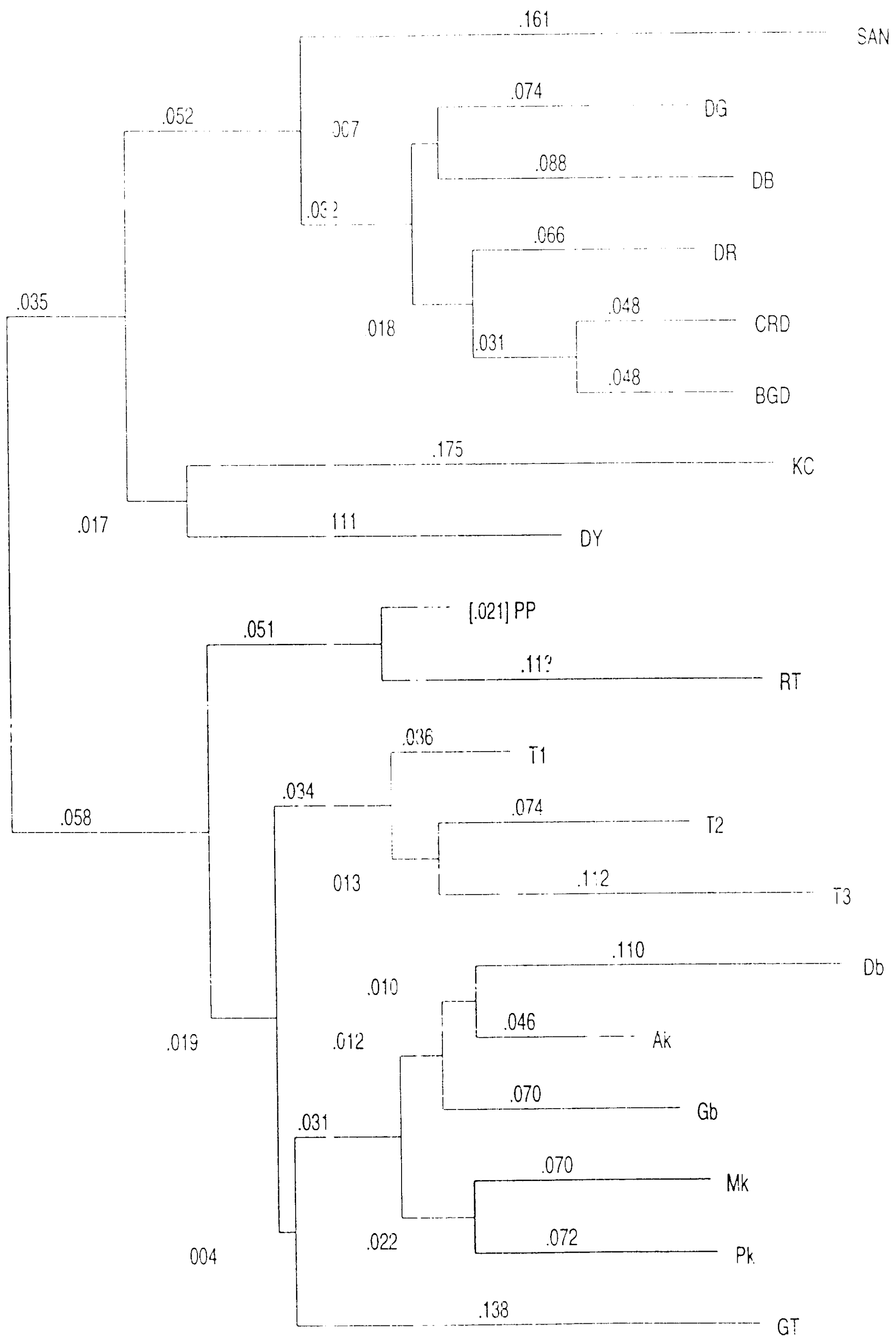

Figure 3.2.3. Dendrogram of coconut accessions determined by clustering pair-wise genetic distances estimated by Nei and Li's coefficient.

Table 3.2.7 The frequency of pair-wise d stance values at 0.05 intervals of the original distance data matrix and the randomised data matrix as re vealed by the permutation tail probability analysis.

Range of distance values	Frequency of pairs in the original tree	Mean frequency of pairs in the random trees	Range in frequencies of pairs in the random trees	Standard deviation of frequencies of pairs in random trees
0.00-0.05	0	0.00	0 - 0	0.00
0.05-0.10	1	0.00	0 - 0	0.00
0.10-0.15	7	0.00	0 - 0	0.00
0.15-0.20	16	0.20	0 - 1	0.41
0.20-0.25	20	5.65	3 - 11	2.13
0.25-0.30	25	35.35	27 - 47	5.41
0.30-0.35	15	71.05	55 - 82	7.98
0.35-0.40	24	44.80	34 - 52	5.20
0.40-0.45	42	12.15	9 - 15	2.16
0.45-0.50	15	1.60	0 - 7	1.70
0.50-0.55	6	0.20	0 - 1	0.41
0.55-0.60	0	0.00	0 - 0	0.00

In general the dendrogram reflects the separation of the accessions based on our understanding of morphological characters and reproductive behaviour. The hierarchical distribution of the 19 coconut accessions in the tree indicate a ready separation of the *typica* (tall) accessions from the dwarf accessions in spite of the unexpected inclusion of the Philippine *typica* form San Ramon in the dwarf cluster. The *aurantiaca* form, king coconut although classically considered as a unique variety shares more dwarf-like characters.

Clustering within the *typica* accessions at subsequent hierarchical levels grouped the *typica typica*, “commercial talls” (T1, T2 and T3), *typica typica* germplasm accessions, Moorock (Mk), Pitiyakanda (Pk), Goyambokka (Gb), Akuressa (Ak) and Debarayaya (Db) and as other *typica* form, *typica gon thembili* together is a separate branch from the two other typica forms, *typica ran thembili* and *typica pora pol.* The three replicates of the commercial tall cultivar (T1, T2 and T3) are more closely related compared to others in that cluster. In the adjoining cluster the germplasm accessions grouped together at a much greater level of genetic diversity. The accessions from same

geographical origin joined together, Moorock (Mk) and Pitiyakanda (Pk) from Mawathagama in the Kurunegala district of the north central province and Goyambokka (Gb) and Debarayaya (Db) and Akuressa (Ak) from neighbouring villages in the southern province of Sri Lanka.

The second big cluster comprises all *nana* forms, dwarf green, dwarf yellow, dwarf red, dwarf brown (Sri Lankan varieties), dwarf green from Brazil and dwarf red from Cameroon, the *aurantiaca* form king coconut and typica form, *typica San Ramon* from the Philippines. Classically the most dwarf-like accessions (palms of short stature without the root bole) Sri Lankan green, red, and brown dwarf forms, Cameroon red dwarf form, and Brazilian green dwarf form grouped well within the main cluster. Sri Lankan dwarf yellow show greater morphological variation within the form and is often misclassified as gon thembili, the tall form in nature. Therefore, the separation of Sri Lankan dwarf yellow and the more intermediate looking variety king coconut form the main dwarf cluster is a good indication of the ability of RAPD markers to detect genetic diversity at a great depth within varieties of the coconut palm.

The clustering of the coconut accessions based on RAPD polymorphisms gave an excellent correlation with the classical classifications. The grouping of accessions at all levels reflected the best fits that could be expected in terms of shared morphology, reproductive behaviour and geographical isolation. This general good agreement found between classical classifications and those produced using RAPDs strongly favours the applicability of RAPDs assay for detection of genetic diversity in the coconut germplasm.

3. 3 Restriction Fragment Length Polymorphisms in the Coconut Palm

Restriction fragment length polymorphisms (RFLPs) were studied using genomic DNA extracts from immature leaves of the forms of the coconut palm, *typica typica, typica san ramon* and *nana* green and nana red. A random genomic DNA library was constructed of high copy and low copy sequences from coconut DNA. These clones were labelled with α^{32}P-dATP and hybridised to digested DNA on Southern blots to detect RFLPs. The protocols for isolation of DNA, restriction enzyme digestion, agarose gel electrophoresis, alkaline Southern blotting to nylon membranes and hybridisation methods are all described in chapter two, section 2.4.

3.3.1 Isolation of DNA

The method developed from Dellaporte *et al.* (1988) for extraction of DNA (see section 2.2.1) from frozen fresh young coconut leaves gave adequate quantities of DNA with reasonably good quality for use in restriction endonuclease digestions. The optimum number of samples that could be handled at a time was eight with 5 g of leaf material per sample for isolation of DNA. The powdering of fresh leaves in liquid Nitrogen in the mortar and pestle was the most labor ous step because of the waxy nature of the coconut leaves. Usually it took about 20 minutes to powder a leaf sample of 5 g. The early steps, homogenisation, lysis, protein precipitation and DNA precipitation from the supernatant were all performed in 40 ml centrifuge tubes. The rest of the steps, phenol, chloroform and iso-amyl alcohol (PCA) extraction, RNAse treatment and final DNA precipitation were carried out in microfuge tubes. The first PCA extraction was always time consuming because separating the top layer without disturbing the inter-phase between the top DNA and bottom PCA layer was somewhat difficult. The whole DNA isolation procedure including quantitative (UV spectrum analysis) and qualitative (agarose gel electrophoresis) assessment of DNA, required about two days.

The average yield of DNA obtained over a series of extractions was 40 ng per milligram of leaf tissue. The UV absorption spectra of the DNA samples gave clear peaks at 256 nm wavelength. The agarose gel electrophoresis of DNA samples revealed high molecular weight DNA with marginal degradation (see plate 3.3.1). Immediately after each extraction the DNA isolates were diluted and divided into aliquots of 100 μl (approximately 100 ng /μl) each and were stored at -20°C for use in restriction digestions.

Plate 3.3.1 DNA isolated from six different forms of the coconut palm. Lanes 1 -7, *typica typica*, *typica gon thembili*, *typica san ramon*, *nana green*, *nana green* (Brazilian), *nana red* (Cameroon) and *aurantiaca* king coconut (200 ng per lane) respectively and lane 8, *Hin*dIII digested λ DNA (400 ng). The sizes of λ DNA bands are 23.13 kb, 9.42 kb, 6.56 kb, 4.36 kb, 2.32 kb, 2.03 kb and 0.56 kb respectively.

3.3.2 Selection of RFLP probes

As a source of potential RFLP probes, a DNA library was constructed of sequences from coconut DNA. To construct this library, coconut DNA *Sau*3AI fragments of approximately 500 bp - 2000 bp were ligated into *Bam*HI digested Bluescribe vector, pTZ18. Plasmids were transformed into bacterial strain *E. coli* PMC112 by electroporation. Clones containing recombinant plasmids were selected using IPTG/Xgal screening and maintained as glycerol stocks at - 70°C . These colonies were pre-screened for high and low copy sequences by hybridisation with labelled total genomic DNA. A total of 98 colonies were picked for use as probes for RFLP screening (details of the experimental procedures are found in section 2.4.1. The nomenclature for these clones is AE1, AE2, ...AE98.

In the first series of experiments on constructing the library, the stock of *E. coli* PMC 112 competent cells were transformed readily by recombinant plasmids. In two electroporations the petri-dishes plated with competent cells gave more than 1,000 transformed colonies with a ratio of 1:16, white to blue colonies. The first four electroporations yielded 127 white clones. These colonies were innoculated to 2 ml of SOB medium (Table 2.1.2) supplemented with 100 µg / ml ampicillin, grown overnight and then spotted onto LB plates containing 100 µg / ml ampicillin by pipetting out 1 µl of each culture. This procedure was adopted in order to maintain uniformity in the size of colonies for accurate assessment of the intensity of the binding signal following hybridisation with labelled total genomic DNA. This is the criterion for deciding whether a colony is low or high copy. Each plate was made in duplicate for further confirmation of the results. Plate 3.3.2 show hybridisation intensity 50 colonies in two colony lift replicas. After careful examination, 44 high copy clones were selected and labelled as AE1 - AE44. The colonies that did not hybridise could be either low copy clones or clones containing no coconut inserts. The high copy clones AE1 - AE44 were maintained as glycerol stocks at - 70°C as potential RFLP probes.

In the second series of experiments a further 54 recombinant colonies were selected by pre-screening another 129 transformed colonies. Upon pre-screening these colonies 27 high copy clones were selected as in the previously study. A further 27 potential low copy colonies were picked at random from those that did not hybridise. The presence of inserts in these low copy colonies were confirmed by observing digested plasmids. These 54 colonies were also maintained as glycerol stocks at - 70°C as potential RFLP probes.

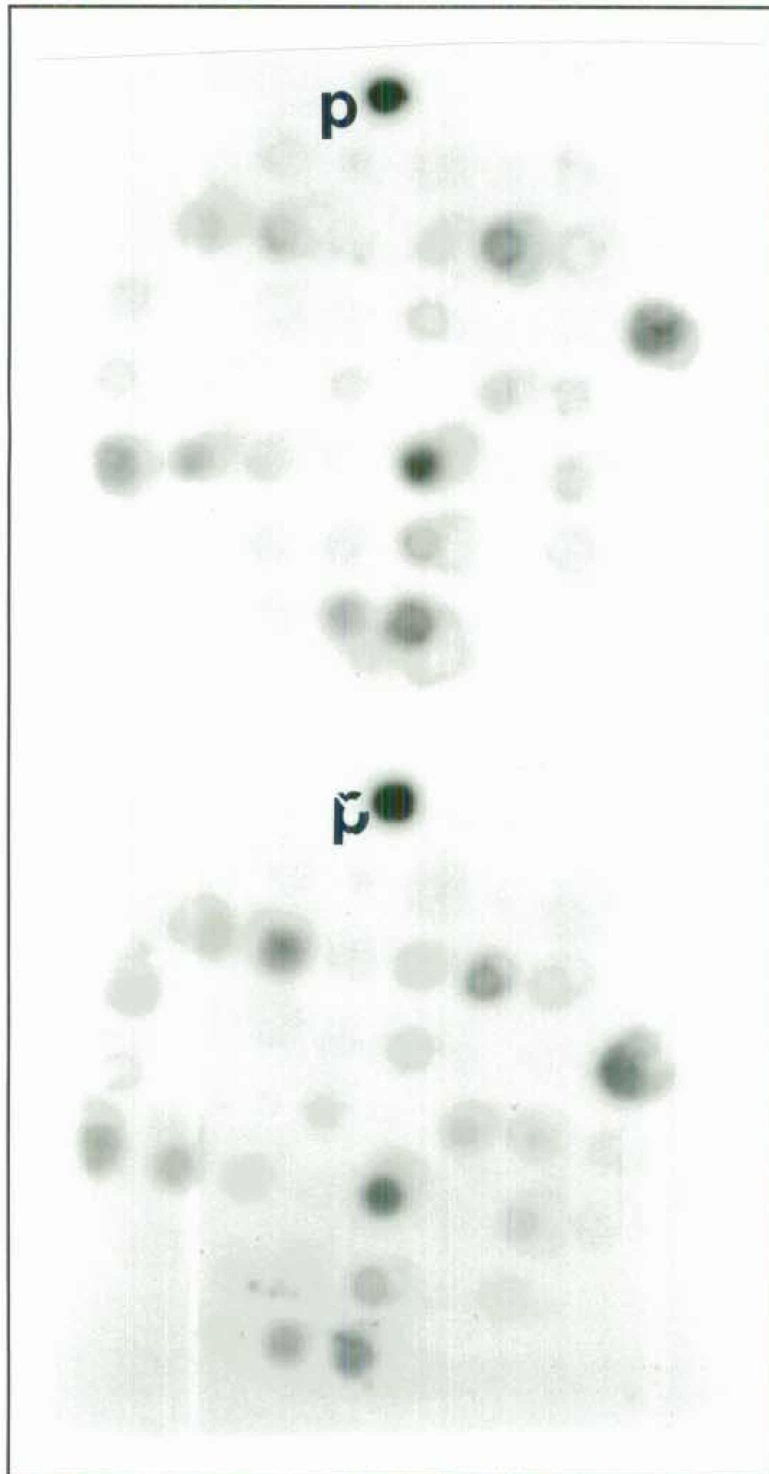

Plate 3.3.2 Autoradiograph of colony lifts after hybridisation with radiolabelled genomic coconut DNA. Filters labelled as 1.1 and 1.2 are duplicate lifts blotted separately from two plates containing colonies, 1 - 50. P, denatured genomic DNA (positive control).

3.3.3 Digestion of coconut genomic DNA by restriction endonucleases

Initially six restriction enzymes, *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Pst*I were used for digestion of coconut DNA (see section 2.4.2 for details of the digestion procedure). The Southern blot hybridisation studies revealed the enzymes, *Dra*I, *Eco*RI and *Eco*RV readily cleave coconut DNA. Restriction digestions were performed in 50 µl reactions using approximately 5 µg of genomic DNA per reaction. DNAs from *typica typica, typica san ramon, nana* green, nana red (Cameroon), nana green (Brazilian) and *aurantiaca* king coconut were digested with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Pst*I in bulk in this manner and stored at 4 °C for transfer to Southern blots.

The DNA digests when electrophoresed on a 1% agarose gel appeared as uniform smears. *Dra*I, *Eco*RI and *Eco*RV digests produce unique band patterns on ethidium bromide stained gels after electrophoresis, showing the presence of sequence repeats of varying sizes. Plate 3.3.3 A, displays DNA from *typica typica* and *nana* green after digesting with *Dra*I, *Eco*RI and *Eco*RV. *Dra*I-cleaved coconut DNA shows a prominent band of approximately 1150 bp in size. *Eco*RI-digested coconut DNA displays two prominent bands of approximately 1400 bp and 1300 bp in size. Further a minor band of 3700 bp can also be seen. *Eco*RV-cleaved coconut DNA revealed a ladder like band pattern extending from approximately 2700 bp to 300 bp comprising at least 10 identifiable bands of approximate lengths, 2700 bp, 2500 bp, 2100 bp, 1800 bp, 1700 bp, 1000 bp, 800 bp, 600 bp, 400 bp and 300 bp.

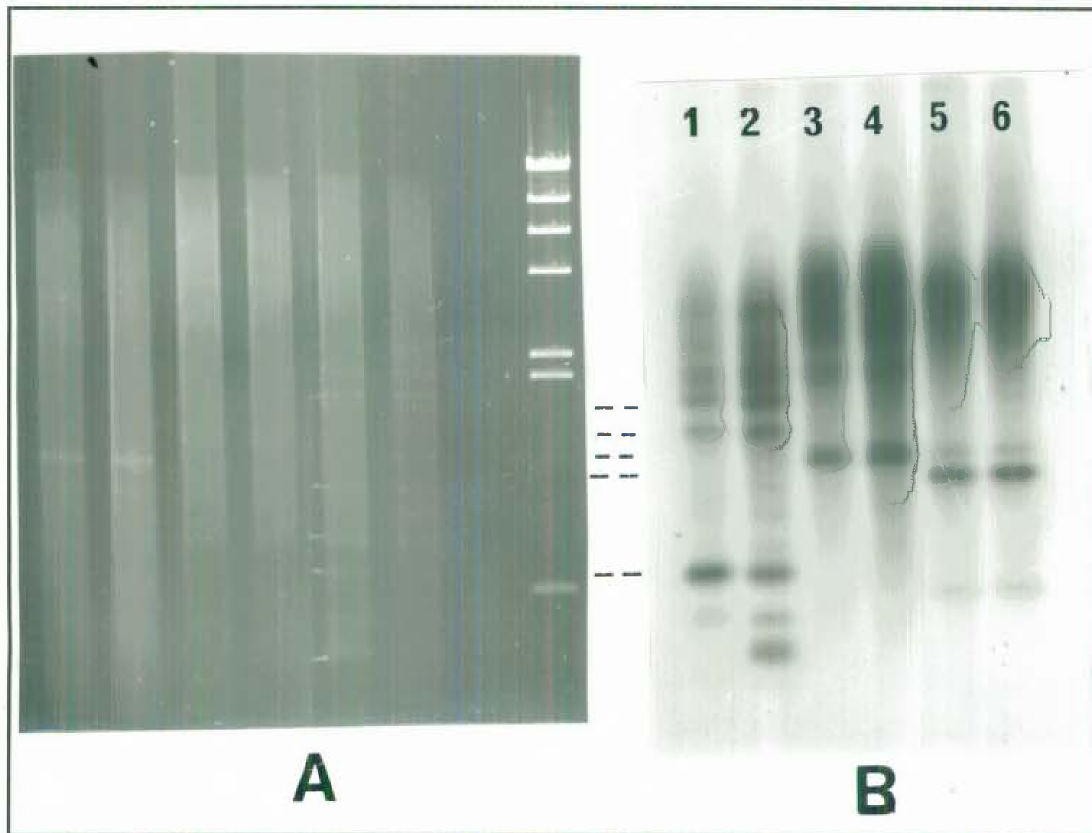

Plate 3.3.3 **A** . *Dra*I, *Eco*RI and *Eco*RV digested coconut DNA (gel photograph). Lanes 1, 3 and 5, *typica typica* DNA digested by *Dra*I, *Eco*RI and *Eco*RV respectively; lanes 2, 4 and 6, *nana* green DNA digested by *Dra*I, *Eco*RI and *Eco*RV respectively; lane 8, *Hin*dIII digested λ DNA (size markers 23.13 kb, 9.42 kb, 6.56 kb, 4.36 kb, 2.32 kb, 2.03 kb and 0.56 kb). **B.** Autoradiograph of a *Dra*I, *Eco*RI and *Eco*RV digested coconut DNA Southern blot hybridised by high copy clone, AE24. Lanes 1, 3 and 5, *nana* green DNA digested by *Eco*RV, *Eco*RI and *Dra*I respectively; lanes 2, 4 and 6, *typica typica* digested by *Eco*RV, *Eco*RI and *Dra*I respectively. The lines between the plates indicate the repetitive sequences in ethidium bromide stained gels which are detected in the Southern blots.

3.3.4 Southern blot hybridisations with potential RFLP clones

Southern blots of digested DNAs were prepared for hybridisation with potential RFLP clones (details described in section 2.4.2). For the first series of Southern blot hybridisations DNA isolated from six forms of coconut, *typica typica*, *typica san ramon*, *nana green*, *nana red* (Cameroon red dwarf), *nana green* (Brazilian green dwarf) and *aurantiaca* king coconut were digested separately by six restriction enzymes, *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Pst*I. 2.5μg samples of digested DNA were separated on 1% agarose gels. The first six lanes of the gel were respectively loaded with restriction digested DNA from *typica typica*, *nana green*, *aurantiaca* king coconut, *typica san ramon*, *nana red* (Cameroon) and *nana green* (Brazilian). The eighth lane was loaded with *Hin*dIII digested λ DNA size marker. A total of 18 blots, 3 per enzyme were prepared for hybridisation with potential RFLP clones AE1 to AE6.

Probes were labelled with α^{32}P-dATP using a random primed labelling kit and unincorporated nucleotides were removed from probes using a Sephadex G50 column. Denatured labelled probes were paired, AE1+AE2, AE3+AE4 and AE5+AE6 to use two at a time. Pre-hybridisation and hybridisation were performed at 42°C in 50% formamide and blots were washed with a final stringency of 1 x SSC, 0.1% SDS at 65°C. Blots were autoradiograhped at - 70 °C for one to seven days (details given in section 2.2.3). Band positions were determined by superimposing the negatives of actual-size gel photographs of the electrophoresed gels. Binding to standard DNA marker bands 23.4 kb and 4.4 kb by plasmid DNA used further to confirm the estimation of band sizes. Only well resolved bands were scored. High molecular bands, smears and bands that were not clearly visualised in the hybridisation background of the DNA track were excluded from scoring. The characteristics of probes, AE1 to AE6 in relation to coconut DNA digested by restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Pst*I are summarised in Table 3.3.1.

The autoradiographs showed that 10 out of 18 blots probed by AE1+AE2, AE3+AE4 and AE5+AE6 (three blots per enzyme) produced a total of 18 bands while the other 8 blots produced only high molecular weight bands. The band patterns are simple and the maximum number of bands found in a blot was three (see plate 3.3.4). This is somewhat uncharacteristic for high copy clones which usually yield complex banding patterns. AE1+AE2, yielded only one band in each blot behaving like single low copy clones. Probes AE3+AE4 and AE5+AE6 respectively yielded more bands per with *Dra*I, *Eco*RI and *Eco*RV digested coconut DNA. *Bam*HI and *Pst*I digested coconut DNA did not bind with any of the above six probes to produce bands other than the high molecular band. *Hind*III digested coconut DNA annealed only with probe AE3+AE4 and resulted in a single band (see plate 3.3.5).

The Southern blots containing *Dra*I digested DNA showed two bands, 1800 bp and 1150 bp. Pairs of RFLP probes, AE3+AE4 and AE5+AE6 both hybridised to the 1150 bp band while only AE3+AE4 hybridised to the 1800 bp band. *Eco*RI digested blots yielded three bands 1900 bp, 1400 bp and 1300 bp. The latter was detected by all the three pairs of RFLP probes while 1400 bp fragment was detected by two pairs of probes AE3+AE4 and AE5+AE6. The 1900 bp site was detected only by AE3+AE4. *Eco*RV digested blots revealed four bands. An 1800 bp fragment and 1000 bp fragment were shared by AE3+AE4 and AE5+AE6 while the 800 bp and 600 bp appeared in blots probed with AE1+AE2 and AE5 + AE6 respectively.

All of the above 18 bands were monomorphic across the six coconut forms screened. Close observation of these banding patterns suggest that these three probes share common binding sites. The 18 fragments actually recognise only nine fragments bands, one from *Hind*III (900 bp), two from *Dra*I cleavage sites (1800 bp and 1150 bp), three from *Eco*RI cleavage sites (1900 bp, 1400 bp and 1300 bp) and four from *Eco*RV cleavage sites (1800 bp, 1000 bp, 800 bp and 600 bp). It is noteworthy that these shared bands coincide with repetitive sequence sites visualised by ethidium bromide in gels of *Dra*I, *Eco*RI and *Eco*RV digested DNA (Plate 3.3.3 A and B).

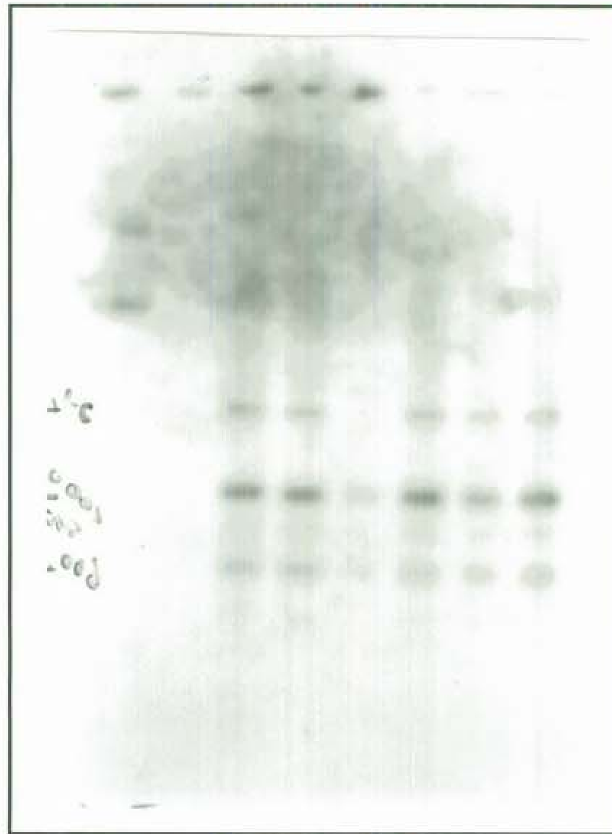

Plate 3.3.4. Autoradiograph of an *Eco*RV digested coconut DNA Southern blot hybridised with a radiolabelled probe consisting of two high copy clones AE3+AE4. Lane 1, *Hin*dIII digested λ DNA fragments 23.4 kb and 4.4 kb indicating hybridisation with the probe, lanes 3 - 8, *nana green* (Brazilian), *nana red* (Cameroon) and *typica san ramon*, *aurantiaca* king coconut, *nana green, typica typica* respectively.

Plate 3.3.5. Autoradiograph of an *Pst*I digested coconut DNA Southern blot hybridised with a radiolabelled probe consisting of two high copy clones AE3+AE4. Lane 1, *Hind*III digested λ DNA fragments 23.4 kb and 4.4 kb indicating hybridisation with the probe, lanes 3 - 8, *nana green* (Brazilian), *nana red* (Cameroon) and *typica san ramon*, *aurantiaca* king coconut, *nana green, typica typica* (200 ng per lane) respectively.

Table 3.3.1 Southern blot results of *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Pst*III digested genomic DNA of six coconut forms, *typica typica, nana* green, *aurantiaca, typica san ramon, nana* red Cameroon, *nana* green Brazilian hybridised to probes AE1 to AE6 from a random coconut DNA library.

RFLP Probe	Restriction enzyme	No. of bands	App. band size (bp)	Source of DNA and presence ('1') or absence of ('0') band					
				typica typica	*nana* green	*auranti-aca king coconut*	*typica san ramon*	*nana red Ca-meroon*	*nana* green Brazil
AE1+AE2	BamHI	HMB	–	–	–	–	–	–	–
	DraI	1	1150	1	1	1	1	1	1
	EcoRI	1	1300	1	1	1	1	1	1
	EcoRV	1	600	1	1	1	1	1	1
	PstI	HMB	–	–	–	–	–	–	–
	HindIII	HMB	–	–	–	–	–	–	–
AE3+AE4	BamHI	HMB	–	–	–	–	–	–	–
	DraI	2	1800 1150	1 1	1 1	1 1	1 1	1 1	1 1
	EcoRI	3	1900 1400 1300	1 1 1	1 1 1	1 1 1	1 1 1	1 1 1	1 1 1
	EcoRV	3	1800 1000 600	1 1 1	1 1 1	1 1 1	1 1 1	1 1 1	1 1 1
	PstI	HMB	–	–	–	–	–	–	–
	HindIII	1	900	1	1	1	1	1	1
AE5+AE6	BamHI	HMB	–	–	–	–	–	–	–
	DraI	1	1150	1	1	1	1	1	1
	EcoRI	2	1400 1300	1 1	1 1	1 1	1 1	1 1	1 1
	EcoRV	3	1800 1000 800	1 1 1	1 1 1	1 1 1	1 1 1	1 1 1	1 1 1
	PstI	HMB	–	–	–	–	–	–	–
	HindIII	HMB	–	–	–	–	–	–	–

For the second series of Southern blot hybridisations the number of coconut forms used were restricted to *typica typica*, *typica san ramon* and *nana green* in the first six blots and only to *typica typica* and *nana green* in the subsequent blots. The restriction enzymes *Bam*HI, *Hind*III and *Pst*I were not considered for further assays because they did not prove promising with AE1 - AE6. The rest of the procedure was the same and clones AE7 to AE44 were used with two radiolabelled probes per blot for hybridisation. The characteristics of probes, AE7 to AE44 in relation to coconut DNA digested by restriction enzymes *Dra*I. *Eco*RI and *Eco*RV are summarised in Table 3.3.2.

The banding patterns observed with 19 pairs of probes (AE7 - AE44) did not appear as complex profiles in most instances although some *Dra*I and *Eco*RV digested DNA yielded high multiple band profiles. The most complex banding patterns came from blots hybridised with two pairs of probes AE17+AE24 and AE18+AE19 yielding eight and seven bands respectively with *Dra*I digested coconut DNA. Similarly the blots having *Eco*RV digested DNA gave eight bands with the probe AE28+AE33 and seven bands with the probe AE17+AE24. Here again most pairs of probes shared common banding positions and more often on sites where repetitive sequences were visible on ethidium bromide stained gels.

A total of 33 bands were scored with 19 pairs of probes (AE7 - AE44) in *Dra*I digested *typica typica* and *nana* green DNA blots. Out of the 19 pairs of clones seven did not produce any bands except for high molecular weight bands. In the rest the number of bands per blot varied from one to eight. The most common band, 1150 bp, was shared by 12 pairs of probes. The band, 1400 bp, was shared by six pairs of probes while bands, 800 bp and 560 bp were each shared by three pairs of probes. Bands, 2700 bp, 2100 bp and 1000 bp were each shared by two pairs of probes while bands 4400 bp and 1000 bp were unshared fragments. Therefore, the actual number of fragments detected by these 19 probes (38 clones) with *Dra*I digested coconut DNA is nine (4400 bp, 2700 bp, 2100 bp, 1800 bp, 1400 bp, 1150 bp, 1000 bp, 800 bp and 560 bp). None of these 9 fragments exhibited polymorphism between *typica typica and nana green* coconut types.

Table 3.3.2 Southern blot results of *Dra*I, *Eco*RI and *Eco*RV digested genomic DNA of three coconut forms, *typica typica*, *nana* green, and *typica san ramon* hybridised to probes AE7 to AE44 from a random coconut DNA library.

RFLP Probe	Restriction enzyme	No. of bands	App. band size (bp)	Source of DNA and presence ('1') or absence of ('0') band		
				typica typica	*nana* green	*typica san ramon*
AE7+AE8	DraI	HMB	–	–	–	–
	EcoRI	2	1400 1300	1 1	1 1	1 1
	EcoRV	1	600	1	1	1
AE9+AE10	DraI	HMB	–	–	–	–
	EcoRI	3	2400 1400 1300	1 1 1	1 1 1	1 1 1
	EcoRV	5	2400 2300 1700 600 400	1 1 1 1 1	1 1 1 1 1	1 1 1 1 1
AE11+AE12	DraI	HMB	–	–	–	–
	EcoRI	2	1400 1300	1 1	1 1	1 1
	EcoRV	3	2400 1700 600	1 1 1	1 1 1	1 1 1
AE13+AE43	DraI	HMB	0	0	0	
	EcoRI	3	2400 1400 1300	1 1 1	1 1 1	
	EcoRV	4	2400 1800 600 400	1 1 1 1	1 1 1 1	
AE14+AE44	DraI	HMB	–	–	–	
	EcoRI	HMB	–	–	–	
	EcoRV	1	2500	1	1	
AE15+AE23	DraI	HMB	–	–	–	
	EcoRI	3	2400 1400 1300	1 1 1	1 1 1	
	EcoRV	5	2500 1800 600 400 300	1 1 1 1 1	1 1 1 1 0	
AE16+AE42	DraI	1	1150	1	1	
	EcoRI	3	2400 1400 1300	1 1 1	1 1 1	
	EcoRV	5	2500 1800 1000 600 400	1 1 1 1 1	1 1 1 1 1	

Table 3.3.2 continued on page 110

Table 3.3.2 continued from page 109

RFLP Probe	Restriction enzyme	No. of bands	App. band size (bp)	Source of DNA and presence ('1') or absence of ('0') band		
				typica typica	*nana* green	*typica san ramon*
AE17+AE24	DraI	8	4400	1	1	–
			2700	1	1	
			2100	1	1	
			1800	1	1	
			1400	1	1	
			1150	1	1	
			1000	1	1	
			800	1	1	
	EcoRI	3	3700	1	1	–
			2700	1	1	
			2400	1	1	
	EcoRV	7	2700	1	1	–
			2500	1	1	
			1800	1	1	
			1000	1	1	
			800	1	1	
			600	1	1	
			400	1	1	
AE18+AE19	DraI	7	2700	1	1	–
			2100	1	1	
			1800	1	1	
			1400	1	1	
			1150	1	1	
			1000	1	1	
			800	1	1	
	EcoRI	3	3700	1	1	–
			2700	1	1	
			2400	1	1	
	EcoRV	4	2700	1	1	–
			1800	1	1	
			600	1	1	
			400	1	1	
AE20+AE25	DraI	1	1150	1	1	–
	EcoRI	3	2700	1	1	–
			1400	1	1	–
			1300	1	1	
	EcoRV	5	2700	1	1	–
			1800	1	1	
			600	1	1	
			400	1	1	
			300	1	0	
AE21+AE41	DraI	3	1400	1	1	
			1150	1	1	
			800	1	1	
	EcoRI	2	1400	1	1	
			1300	1	1	
	EcoRV	4	2400	1	1	
			2100	1	1	
			600	1	1	
			400	1	1	

Table 3.3.2 continued on page 111

Table 3.3.2 continued from page 110

RFLP Probe	Restriction enzyme	No. of bands	App. band size (bp)	Source of DNA and presence ('1') or absence of ('0') band		
				typica typica	*nana* green	*typica san ramon*
AE22+AE40	DraI	1	1150	1	1	–
	EcoRI	2	1400	1	1	–
			1300	1	1	–
	EcoRV	4	1800	1	1	–
			600	1	1	
			400	1	1	
			300	1	0	
AE23+AE26	DraI	1	1150	1	1	
	EcoRI	3	2700	1	1	
			1400	1	1	
			1300	1	1	
	EcoRV	8	2700	1	1	
			1800	1	1	
			600	1	1	
			400	1	1	
			300	1	0	
AE27+AE39	DraI	3	1400	1	1	
			1150	1	1	
			560	1	1	
	EcoRI	1	3700	1	1	
	EcoRV	2	2100	1	1	
			1800	1	1	
AE28+AE33	DraI	3	1400	1	1	
			1150	1	1	
			560	1	1	
	EcoRI	4	2700	1	1	
			2400	1	1	
			1400	1	1	
			1300	1	1	
	EcoRV	8	4400	1	1	
			2700	1	1	
			2500	1	1	
			2100	1	1	
			1800	1	1	
			600	1	1	
			400	1	1	
			300	1	0	
AE29+AE38	DraI	1	1150	1	1	
	EcoRI	3	2700	1	1	
			1400	1	1	
			1300	1	1	
	EcoRV	6	2700	1	1	
			1800	1	1	
			1000	1	1	
			600	1	1	
			400	1	1	
			300	1	0	

Table 3.3.2 continued on page 112

Table 3.3.2 continued from page 111

RFLP Probe	Restriction enzyme	No. of bands	App. band size (bp)	Source of DNA and presence ('1') or absence of ('0') band		
				typica typica	*nana* green	*typica san ramon*
AE31+AE37	DraI	1	1150	1	1	
	EcoRI	4	2700	1	1	
			2400	1	1	
			1400	1	1	
			1300	1	1	
	EcoRV	6	2700	1	1	
			1800	1	1	
			1000	1	1	
			600	1	1	
			400	1	1	
			300	1	0	
AE32+AE36	DraI	HMB	–	–	–	–
	EcoRI	2	1400	1	1	
			1300	1	1	
	EcoRV	4	2100	1	1	
			1800	1	1	
			600	1	1	
			400	1	1	
AE34+AE35	DraI	3	1400	1	1	
			1150	1	1	
			560	1	1	
	EcoRI	1	1300	1	1	
	EcoRV	3	2300	1	1	
			1800	1	1	
			600	1	1	

The 19 pairs of probes yielded a total of 47 bands in *Eco*RI digested *typica typica* and *nana* green DNA blots. Only one pair failed to yield bands with these blots and all the others gave bands between one to four per blot. The bands, 1400 bp and 1300 bp were the most common and were annealed by 15 probes (30 clones) and 14 probes (28 clones) respectively. Bands, 2700 bp and 2400 bp were also shared by 7 pairs of probes each while band, 3700 bp was shared by three pairs of probes. Band 2500 bp was an unshared fragment. Therefore the actual number of fragments detected by the these 19 pairs of probes (38 probes), was only six fragments (3700, 2700, 2500, 2400, 1400 and 1300 bp). Here too, none of these fragments exhibited polymorphism between *typica typica* and *nana* green coconut types. The blot of *Eco*RI digested coconut DNA hybridised with probe AE28+AE33 is displayed in plate 3.3.3 -B.

All the 19 pairs of probes yielded bands with *Eco*RV digested *typica typica* and *nana* green DNA blots. A total of 82 bands were produced at an average of 4.3 bands per

blot per pair of probes. The probe, AE28+33 gave eight bands which is the highest in all blots studied. The size of bands ranged from 4400 bp to 300 bp. The 600 bp band was shared by 17 out of 19 pairs of probes tested. Bands of 1800 bp and 400 bp were each shared by 14 pairs of probes while bands, 2700 bp and 400 bp were each shared by seven pairs of probes. The other bands that were shared by more than one pair of primers are 2500 bp (five shared), 2400 bp (four shared), 2300 bp (two shared), 2100 bp (four shared), 1700 bp (two shared) and 1000 bp (four shared). Therefore, the actual number of fragments detected by the these 19 probes (38 clones), was thirteen, 4400 bp, 2700 bp, 2500 bp, 2400 bp, 2300 bp, 2100 bp, 1800 bp, 1700 bp, 1000 bp, 800 bp, 600 bp, 400 bp and 300 bp). Amongst these 13 fragments 12 were monomorphic. The only band that exhibited polymorphism between *typica typica* and *nana* green coconut types is a fragment of 300 bp. This polymorphism was detected by seven probes, AE15+AE23, AE20+AE25, AE22+AE40, AE28+AE33, AE23+AE26, AE29+AE28 and AE31+AE37. The RFLP fragments of *Eco*RV digested coconut DNA revealed by the probe AE28+AE33 are displayed in plate 3.2.3 -B.

For the third series of Southern blot hybridisations *Eco*RV digested DNA from coconut forms, *typica typica*, *typica san ramon* and *nana green* was probed with 54 clones (AE44 - AE98). These 54 clones comprise 27 high copy clones and 27 low copy clones and these were used separately with three per blot for screening of RFLPs. The characteristics of probes, AE45 to AE98 in terms of low and high copy and in relation to coconut DNA digested by the restriction enzyme *Eco*RV are summarised in Table 3.3.3.

Eight out of nine blots probed by the 27 high copy clones produced bands. These eight blots yielded a total of 29 bands of which bands 2100 bp, 1800 bp, 1400 bp, 800 bp, 600 bp and 400 bp were common to at least two blots. The first band, 2100 bp is a polymorphic band and was shared by five probes, AE45+AE46+AE47, AE51+AE52+AE53, AE63+AE64+AE65, AE78+AE79+AE80 and AE87+AE88+AE89. All of these detected the same polymorphism at this fragments distinguishing *typica typica* from *typica san ramon* and *nana green*. The second band, 1800 bp was shared by four

probes, AE45+AE46+AE47, AE78+AE79+AE80, AE87+AE88+AE89 and AE96+AE97+AE98 and these primers have also detected polymorphism distinguishing *typica typica* from *typica san ramon* and *nana green.* The rest of the four bands were monomorphic across *typica typica, typica san ramon* and *nana green.* Band three, 1400 bp was shared by only two probes (combinations), AE51+AE52+AE53 and AE63+AE64+AE65. The 800 bp band was not shared. The last two bands, 600 bp and 400 bp were shared by all the eight high copy clone combinations.

Only three out of nine blots probed by the 27 low copy clones produced bands. The other blots gave only high molecular weight bands. The banding patterns exhibited by low copy clones did not have bands shared by the different combinations of probes. The three blots yielded a total of six bands each identifying unique fragments, 2700 bp, 2400 bp, 2200 bp, 1100 bp, 750 bp and 700 bp. The first two bands, 2700 bp and 2400 bp detected by probe AE66+AE67+AE68, exhibit polymorphism at these fragments distinguishing *nana green* from *typica typica* from *typica san ramon.* Bands three and four, 2200 bp and 1100 bp detected by probe AE93+AE94+AE95 also exhibit polymorphism at these two fragments distinguishing *typica typica* from *typica san ramon* and *nana green.* Bands five and six, 750 and 700 detected respectively from probe combinations AE66+AE67+AE68 and AE60+AE61+AE62 are monomorphic. The 700 bp fragment hybridised with AE60+AE61+AE62 is shown in Plate 3.3.6. The autoradiograph obtained by probing *Eco*RV digested DNA from coconut forms *typica typica, nana green* and *typica san ramon* with AE66+AE67+AE68 and AE93+AE94+AE95 are displayed plate 3.3.3 - D and E.

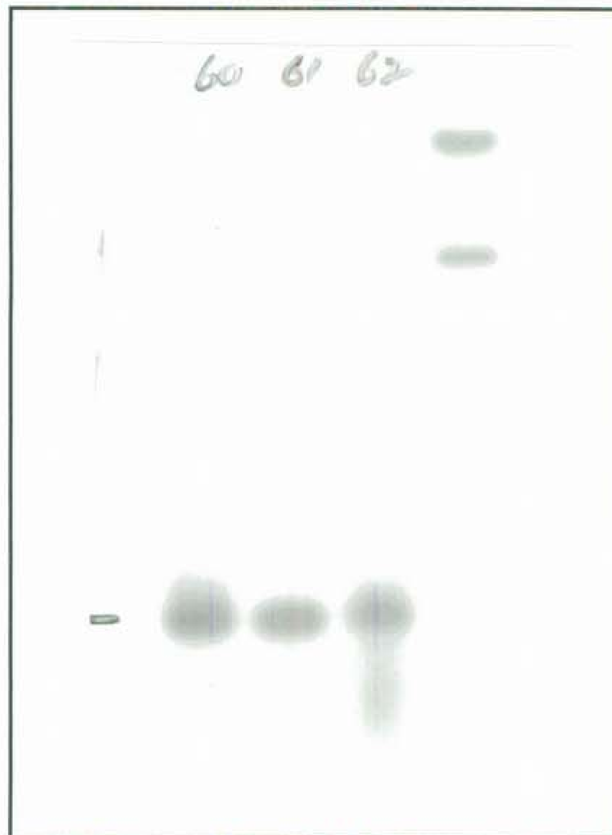

Plate 3.3.6 Autoradiograph of an *Eco*RV digested coconut DNA Southern blot hybridised with a radiolabelled probe consisting of three low copy clones AE60, AE61 and AE62. Lane 1, *typica typica*, lane 2, nana green and lane 3, *typica* san ramon. Lane 4 λ DNA fragments 23.4 kb and 4.4 kb indicating strong hybridisation with the probe.

In summary the overall results of the Southern blot hybridisation of coconut DNA indicated that the first 44 high copy clones (AE1 - 44) had detected a total of 180 DNA fragments on Southern blots comprising coconut DNA digested by *Dra*I (37 bands), *Eco*RI (53 bands) and *Eco*RV (90 bands). These different high copy clones however, appeared to detect bands of the same size where the DNA was digested with the same enzyme indicating that the actual number of different loci detected is very much less than

the number of fragments detected. The numbers of *Dra*I, *Eco*RI and *Eco*RV fragments detected by all probe combinations are 9, 7 and 14 respectively.

Only one out of these 30 fragments, exhibited polymorphism and this same polymorphism was detected by 7 pairs of clones. The lack of polymorphism in all shared bands except the 300 bp fragment (EcoRV digested DNA) and presence of the polymorphism in all blots that showed the 300 bp band, clearly supports the assumption that bands of similar size hybridised by these high copy clones are homologous. The subsequent assay of *Eco*RV digested DNA probed with a further 27 high copy clones also revealed similar results. The total of 29 bands observed probably represented only six fragments. The two polymorphic fragments detected, 2100 bp and 1800 bp were also shared.

The most common bands detected by the high copy clones, 1150 bp in *Dra*I digests (15 probes shared), 1400 bp (15 probes) and 1300 bp (16 probes) in *Eco*RI digests and 2700 bp (7 probes), 2100 bp (9 probes), 1800 bp (20 probes), 1000 bp (7 probes), 600 bp (25 probes), 400 bp (22 probes) and 300 bp (7 probes) in *Eco*RV digests also correspond to repeat sequences visible in the ethidium bromide stained gel containing *typica typica* and *nana* green DNA digested by the three enzymes (plate 3.3.3). In contrast, the bands hybridised by the low copy clones did not occur in more than one blot indicating that all these six fragments represents a unique locus. Therefore, it is quite evident that these high copy clones were derived from highly repetitive sequences in the coconut genome.

Further analysis of these RFLPs was not undertaken at this stage of the study due to logistical constrains. The project was funded only for two years and since all RFLPs except one (300 bp) were found at the final stage of the programme, detection of these polymorphisms by using single probes and studying their segregation in the F2 family was not performed. The RFLP (300 bp) was separately analysed and AE25 was found as a single clone capable of detecting the RFLP. Subsequently a blot containing

*Eco*RV digested DNA from a family of tall and dwarf parents and from 18 individuals of their F2 was probed with AE25. The RFLP however, did not segregate in the F_2 family. None of the F_2 individuals showed the 300 bp fragment indicating that the band shows maternal inheritance.

In summary 98 eight probes and six restriction enzymes were used in 124 probe-enzyme combinations and a total of 211 RFLP loci were detected across typica and nana coconut forms. The high copy RFLP clones did not yield complex banding patterns as expected. The hybridisation patterns were simple and a great majority of cloned sequences shared common binding sites in the Southern blots of genomic DNA. The most common binding sites were also observed on ethidium bromide stained gels containing DraI, EcoRI and EcoRV digested DNA. On the contrary RAPD technique was yielding far greater amounts of useable data, for the effort made. RFLPs although conceptually more sound than RAPDs, as a method is constrained by time and money.

Table 3.3.3 Southern blot results of *Eco*RV digested genomic DNA of three coconut forms, *typica typica*, *nana* green, and *typica san ramon* hybridised to probes AE45 to AE98 from a random coconut DNA library.

RFLP Probe	Restriction enzyme	No. of bands	App. band size (bp)	Source of DNA and presence ('1') or absence of ('0') band		
				typica typica	*nana* green	*typica san ramon*
AE45+AE46+AE47 (High copy)	EcoRV	4	2100 1800 600 400	0 0 1 1	1 1 1 1	1 1 1 1
AE51+AE52+AE53 (High copy)	EcoRV	4	2100 1400 600 400	0 1 1 1	1 1 1 1	1 1 1 1
AE54+AE55+AE56 (High copy)	EcoRV	HMB	–	–	–	–
AE57+AE58+AE59 (High copy)	EcoRV	2	600 400	1 1	1 1	1 1
AE63+AE64+AE65 (High copy)	EcoRV	5	2100 1400 1000 600 400	0 1 1 1 1	1 1 1 1 1	1 1 1 1 1
AE72+AE73+AE74 (High copy)	EcoRV	3	800 600 400	1 1 1	1 1 1	1 1 1
AE78+AE79+AE80 (High copy)	EcoRV	4	2100 1800 600 400	0 0 1 1	1 1 1 1	1 1 1 1
AE87+AE88+AE89 (High copy)	EcoRV	4	2100 1800 600 400	0 0 1 1	1 1 1 1	1 1 1 1
AE96+AE97+AE98 (High copy)	EcoRV	3	1800 600 400	0 1 1	1 1 1	1 1 1
AE48+AE49+AE50 (Low copy)	EcoRV	HMB	–	–	–	–
AE60+AE61+AE62 (Low copy)	EcoRV	1	700	1	1	1
AE66+AE67+AE68 (Low copy)	EcoRV	3	2700 2400 750	0 0 1	1 1 1	0 0 1
AE69+AE70+AE71 (Low copy)	EcoRV	HMB	–	–	–	–
AE75+AE76+AE77 (Low copy)	EcoRV	HMB	–	–	–	–
AE81+AE82+AE83 (Low copy)	EcoRV	HMB	–	–	–	–
AE84+AE85+AE86 (Low copy)	EcoRV	HMB	–	–	–	–
AE90+AE91+AE92 (Low copy)	EcoRV	HMB	–	–	–	–
AE93+AE94+AE95 (Low copy)	EcoRV	2	2200 1100	0 0	1 1	1 1

3.3.5 Southern blot hybridisations with the minisatellite probe M13

M13 phage DNA, as a probe, has produced hypervariable polymorphic patterns in Southern blots of genomic DNA in organisms belonging to different taxa including animals, plants and micro-organisms (Ryskov *et al.,* 1988). M13 was therefore, used to probe DNA samples digested with five restriction enzymes. The Southern blot hybridisations of coconut DNA digests however, failed to produce the expected complex fingerprint patterns. The details of the experiments are as follows:

Initially 10 µg of DNA from a *typica typica* palm and a *nana* green palm were digested separately with *Dra*I, *Eco*RI and *Eco*RV and electrophoresis was carried out on a 7% agarose, 20-cm long TBE gel. The gel was blotted onto a nylon membrane Hybond N (Amersham) by capillary blotting. The membrane was hybridised to single stranded M13 (Pharmacia), labelled with α-^{32}P-ATP by the random priming method (Bresatec) according to manufacturers recommendation. Pre-hybridisation was carried out at 60 °C for 2 hours and hybridisation was at the same temperature overnight in 10 ml of 0.263M Na-phosphate buffer with 7% SDS, 1mM EDTA (pH 8.0) and 1% BSA (Table 2.1.4). The membrane was washed with 0.263M Na-Phosphate buffer and 0.1% SDS for 20 minutes at 60 °C, then with 2 x SSC and 0.1% SDS twice for 20 minutes at 60 °C. The membrane was autoradiographed for 14 days at - 70 °C with an intensifying screen.

The autoradiograph appeared as dark smears along the lanes of DNA without resolving bands of scorable intensity. A faint band appeared in the region of 1000 bp in *Dra*I digested DNA of both *typica typica* and *nana* green while another faint band appeared in the region of 4.4 kb in the *Eco*RI digested DNA of *nana* green. Only a uniform dark smear was exhibited in the lane carrying *Eco*RV digested DNA. However, bands corresponding to *Hin*dIII digested λ DNA fragments of 23.1 kb, 9.4 kb, 6.6 kb and 4.4 kb were visible, indicating hybridisation of M13 with these high molecular weight fragments of λ DNA (see Plate 3.3.7).

The experiment was repeated using DNA from 12 different coconut palms digested separately by two restriction enzymes, the four-base cutters, *Hae*III and *Rsa*I. The ten palms included one individual from each coconut form, *typica typica*, *typica* gon thembili, *typica* pora pol, *typica* san ramon, *nana* green, *nana* yellow, *nana* red, *nana* red from Cameroon, *nana* green from Brazil, *aurantiaca* king coconut and two other individuals of *typica typica* and *aurantiaca* king coconut individuals. Here again the autoradiographs did not show DNA fingerprints with any of the enzymes. However, once again the bands, 23.1 kb, 9.4 kb, 6.6 kb and 4.4 kb fragments of *Hin*dIII digested λ DNA appeared in all the blots.

Finally two large blots one containing *Dra*I and the other containing *Eco*RI digested freshly prepared DNA of 14 palms, one each from *typica typica* Goyambokka, *typica typica* Akuressa, *typica typica* Deberayaya, *typica typica* gon thembili, *typica* pora pol, *typica* san ramon, *nana* green, *nana* yellow, *nana* red, *nana* red from Cameroon, *nana* green from Brazil, *aurantiaca* king coconut, were probed with labelled M13 to check the reproducibility of previously obtained faint bands. The autoradiograph of the blot containing *Dra*I digests once again showed a faint band (approximately 1000 bp) in six individuals while the band was not resolved in others. The autoradiograph of the blot containing *Eco*RI digests, showed the faint band (approximately 4.4 kb) earlier seen in DNA from two individuals, *nana* yellow and *typica* san ramon. No further experiments were attempted using M13 as a probe.

Plate 3.3.7. Autoradiograph of a *DraI*, *Eco*RI and *Eco*RV digested coconut DNA Southern blot hybridised with radiolabelled single stranded M13 bacteriophage DNA. Lanes 1, 3 and 5, *typica typica* digested by *DraI*, *Eco*RI and *Eco*RV respectively; lanes 2, 4 and 6 *nana* green DNA digested by *DraI*, *Eco*RI and *Eco*RV respectively. Lane 7, λ DNA fragments 23.13 kb, 9.42 kb, 6.56 kb, 4.36 indicating strong hybridisation with the probe.

3.3.5 Southern blot hybridisations with the microsatellite probes

To investigate whether different simple sequence motifs (microsatellites) are present in the coconut palm genome, *Dra*I, *Eco*RI and *Eco*RV digested DNA of two different individuals of *typica typica* and *nana green* were hybridised to six different probes on Southern blots. Similarly to the M13 probed blots, the autoradiographs appeared as dark smears on DNA lanes and no fingerprinting patterns were visualised. The details of the experiments are as follows:

Six Southern blots were made by using 10 µg DNA from a *typica typica* palm and a *nana* green palm each digested separately with *Dra*I, *Eco*RI and *Eco*RV and probed separately with six different oligonucleotides. The digests were electrophoresed on 7% agarose 20-cm long TBE gel and Southern blotted onto a nylon membrane Hybond N (Amersham). The oligonucleotide probes, $(CA)_9$, $(GAA)_6$, $(GAT)_8$, $(TAA)_6$, $(GTT)_8$, and $(GATA)_4$, were 5'-end labelled by T_4 kinase reaction using γ-^{32}P-ATP. The labelled oligonucleotides were cleaned by running through Bio Gel P30 spin columns. The membranes were consecutively hybridised with labelled probes.

The pre-hybridisation and hybridisation temperature was at 10 °C below the T_m value for each oligonucleotide. The T_m values for individual oligonucleotides were: $(CA)_9$: 60 °C, $(GAA)_6$: 56 °C, $(GAT)_8$: 67 °C, $(TAA)_6$: 47 °C, $(GTT)_8$: 67 °C and $(GATA)_4$: 50 °C. Pre-hybridisation (2 hours) and hybridisation (overnight) was carried out at temperatures determined for each probe in 10 ml of 0.263M Na-phosphate buffer with 7% SDS, 1mM EDTA (pH 8.0) and 1% BSA (Table 2.1.4). Membranes were washed for 15 minutes at room temperature in 0.9M sodium chloride and 6 x SSC and rinsed twice for one minute in 2 x SSC at the temperature used for hybridisation. Membranes were autoradiographed for 14 days at - 70 °C with an intensifying screen.

The autoradiograph of the Southern blot probed by $(CA)_9$ did not show any bands in all the six lanes containing *Dra*I, *Eco*RI and *Eco*RV digested *typica typica* and

nana green DNA. Apart from dark background smearing even a trace of banding was not visible. The first four high molecular weight fragments *Hin*dIII digested λ DNA (23.1 kb, 9.4 kb, 6.6 kb and 4.4 kb) hybridised to the oligonucleotide $(CA)_9$ and appeared as clear bands in the autoradiograph.

The autoradiograph of the Southern blot probed by $(GAA)_6$ also did not show fingerprints of multiple bands. However, a single band in the region of 2.5 kb was seen with *Eco*RI digested DNA of both *typica typica* and *nana* green DNA. This probe did not anneal to any of the fragments of *Hin*dIII digested λ DNA.

The autoradiograph of the Southern blot probed by $(GTT)_8$ showed a very strong background in all the six lanes containing *Dra*I, *Eco*RI and *Eco*RV digested *typica typica* and *nana* green DNA. A very faint band (4.5 kb) was present in *Eco*RI digested *nana* green DNA in the dark background. This probe annealed strongly to a 6.6 kb fragment of the *Hin*dIII digested λ DNA.

The autoradiographs of Southern blots probed with $(TAA)_6$, $(GAT)_8$, and $(GATA)_4$ (see Plate 3.2.8) also failed to show any bands other than dark smears in lanes containing DNA of *Dra*I, *Eco*RI and *Eco*RV digested *typica typica* and *nana* green. Apart from $(GAT)_8$ which annealed to 23.1 kb fragment of the *Hin*dIII digested λ DNA others did not anneal to any fragments of the *Hin*dIII digested λ DNA.

Because of the inability to detect simple sequence repeats, $(CA)_9$, $(GAA)_6$, $(GAT)_8$, $(TAA)_6$, $(GTT)_8$ and $(GATA)_4$ in the coconut genome by the hybridisation procedure used above a further attempt was made with a different hybridisation solution. The solution is a buffer consisting of 5 x SSC, 0.5% blocking reagent (Boehringer, Mannheim), 0.1% N-lauryl-sarcosine and 0.02% SDS. The blot was washed for 15 minutes in 6 x SSC at room temperature and once for 1 minute in 6 x SSC at the hybridisation temperature (Vosman, et al., 1992). A Southern blot consisting of *Eco*RI digested DNA from 14 palms, one individual each from coconut forms, *typica typica*,

typica gon thembili, *typica* pora pol, *typica* Ran thembili, *typica* san ramon, *typica typica* Akuressa, *typica typica* Goyambokka, *typica typica* Moorock, *nana* green, *nana* yellow, *nana* red, *nana* red from Cameroon, *nana* green from Brazil and *aurantiaca* king coconut, was hybridised with labelled oligonucleotide $(GATA)_4$. The autoradiograph of the Southern blot once again failed to reveal any discrete bands in spite of uniform dark smears along the lanes of DNA and the four clear bands at the positions of high molecular weight fragments of *Hind*III digested DNA, 23.1 kb, 9.4 kb, 6.6 kb and 4.4 kb in the blot.

3.3.6 Detection of microsatellite sequences using the polymerase chain reaction

A genomic library comprising small coconut DNA inserts (300 bp - 600 bp) in pTZ18 was constructed to isolate microsatellite sequences which could be used to develop primer sequences for polymerase chain reaction (PCR) analysis of microsatellite alleles, for amplification of microsatellite alleles. The procedure used for construction of the library was same as that used for production of clones for RFLP screening, except for the size of the inserts. Small inserts were used to facilitate sequencing.

One hundred white colonies were probed separately with γ^{32}P-dATP end-labelled oligonucleotides, $(CA)_9$, $(TAA)_8$ and $(GAA)_8$. These probes detected a total of 39 colonies by annealing to coconut DNA inserts in the plasmid DNA. The colony numbers and the nomenclature of the prospective colonies containing sequence repeats are shown in Table 3.3.4.

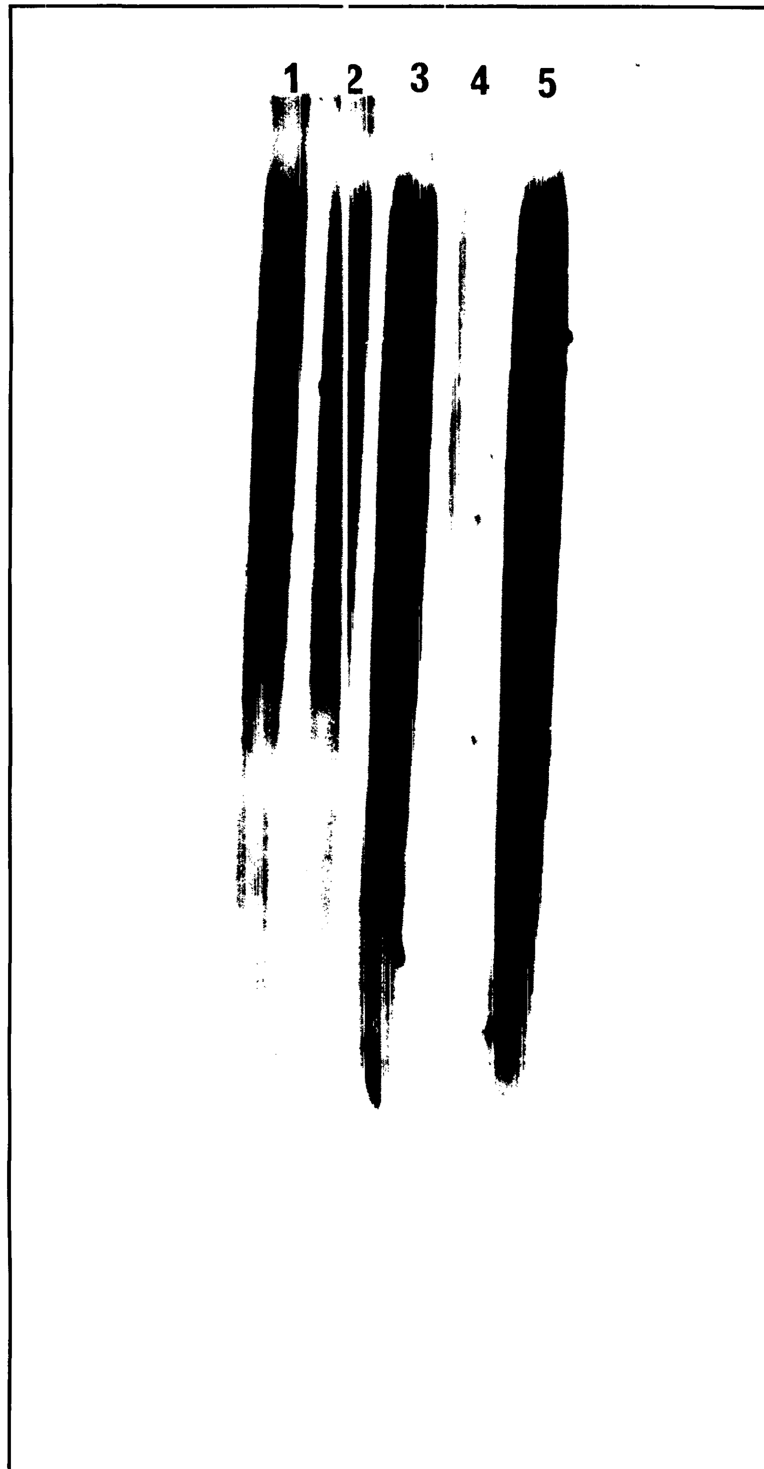

Plate 3.3.8. Autoradiograph of a *DraI* digested coconut DNA Southern blot hybridised with endlabelled simple sequence repeat $(GATA)_4$. Lanes 1 - 5, *DraI* digested DNA of *typica typica*, *nana* green, *nana* yellow, *aurantiaca* king coconut and *typica* san ramon respectively.

Table 3.3.4 The colony numbers and the nomenclature of the prospective colonies containing simple sequence repeats (SSR), $(CA)_9$, $(GAA)_8$ and $(TAA)_6$ in the coconut DNA inserted to *E. coli* PMC 112 via the blue scribe plasmid pTZ18.

Colony number	Simple sequence repeat (SSR)	Label of the SSR clone	Colony number	Simple sequence repeat (SSR)	Label of the SSR clone
1			51		
2	$(TAA)_8$	MS1	52		
3			53	$(CA)_9$	MS21
4			54	$(CA)_9$	MS22
5	$(CA)_9$, $(TAA)_8$	MS2	55	$(CA)_9$, $(TAA)_8$	MS23
6	$(CA)_9$, $(TAA)_8$	MS3	56		
7	$(CA)_9$	MS4	57	$(CA)_9$, $(GAA)_8$, $(TAA)_8$	MS24
8	$(TAA)_8$	MS5	58		
9	$(GAA)_8$	MS6	59	$(CA)_9$, $(GAA)_8$, $(TAA)_8$	MS25
10	$(TAA)_8$	MS7	60		
11	$(CA)_9$, $(GAA)_8$	MS8	61		
12	$(TAA)_8$	MS9	62		
13			63		
14	$(TAA)_8$	MS10	64		
15			65	$(TAA)_8$	MS26
16			66		
17			67		
18			68	$(CA)_9$, $(TAA)_8$	MS27
19	$(TAA)_8$	MS11	69		
20			70		
21			71		
22	$(TAA)_8$	MS12	72		
23	$(GAA)_8$, $(TAA)_8$	MS13	73		
24			74		
25	$(CA)_9$	MS14	75		
26			76	$(GAA)_8$, $(TAA)_8$	MS28
27			77	$(GAA)_8$	MS29
28			78	$(TAA)_8$	MS30
29	$(TAA)_8$	MS15	79		
30			80	$(TAA)_8$	MS31
31			81		
32	$(CA)_9$	MS15	82		
33	$(GAA)_8$, $(TAA)_8$	MS16	83		
34			84		
35	$(GAA)_8$	MS17	85		
36	$(TAA)_8$	MS18	86	$(CA)_9$	MS32
37			87	$(GAA)_8$, $(TAA)_8$	MS33
38			88	$(GAA)_8$, $(TAA)_8$	MS34
39			89		
40			90	$(CA)_9$, $(TAA)_8$	MS35
41			91	$(CA)_9$	MS36
42	$(GAA)_8$	MS19	92		
43			93		
44	$(TAA)_8$	MS20	94	$(TAA)_8$	MS37
45			95	$(CA)_9$, $(TAA)_8$	MS38
46			96	$(GAA)_8$	MS39
47			97		
48			98		
49			99		
50			100		

The inserts of the first six colonies were sequenced by a version of the dideoxy chain termination method of Sanger *et al.* (1977). These were performed according to the protocol provided with the Bresatec Super-Base sequencing kit using the T_7 promoter primer and the universal primer for forward and reverse reactions. Sequences of inserts have been obtained for two of the colonies MS5 and MS6. The sequences obtained from the two sequencing directions were identical, but did not carry the expected sequence in the insert. Therefore, it appeared that colonies selected on the basis of their ability to bind with end-labelled oligonucleotides did not contain necessarily the repeat sequences. The reason for this non-specific binding could be due to inadequate purification of the labelled probe away from unincorporated nucleotides by the Bio Gel P30 quick spin. Due to time constraints no further attempts were made to isolate micro-satellite sequences by this method.