CHAPTER 4

Discussion

The coconut palm is an "orphan crop" according to Persley's (1990) definition, because it is primarily grown in developing countries. Breeding of this palm has not so far reached the expected goals for improvement of characters such as early flowering, yield, drought tolerance and pest and disease resistance. The commercial cultivars are inter or intra-varietal hybrids of outbreeding populations lacking homogeneity in the breeding material. The absence of methods for vegetative propagation and time and resource constraints created by long cycles of breeding have retarded the progress of breeding. Incorporation of suitable biotechnological approaches could be considered as a timely move in improving the new strategies of coconut breeding and conservation. Coconut palm has, so far, not benefited by the new tools of biotechnology. The only DNA-based study on coconut was a study which detected and sequenced *Eco*RI repetitive fragments in the coconut genome (Rohde *et al*., 1992). Therefore, the current study on random amplified polymorphic DNA (RAPDs) and restriction fragment length polymorphisms (RFLPs) in the coconut genome is important for coconut breeders and policy makers of coconut research.

4.1 Random Amplified Polymorphic Assay (RAPD) in the coconut palm

A number of different methods have been developed for using primers of arbitrary sequence in the polymerase chain reaction (PCR) with a view to analysing genetic variation. The random amplified polymorphic DNA (RAPD) method uses short primers (8 - 10 nucleotides) which anneal to genomic DNA at a low temperature, 36°C

(Williams *et al.*, 1990). Priming at pairs of closely spaced complementary sites allows the subsequent amplification of dispersed genomic sequences. The arbitrarily primed polymerase chain reaction (AP-PCR) described by Welsh *et al.*, (1991) used much longer primers (12 - 40 nucleotides) which anneal at higher temperatures (40 - 48 °C). The DNA amplified fingerprinting (DAF) approach uses primers as short as 5 nucleotides at lower annealing temperature conditions similar to RAPDs (Caetano-Anolles *et al.*, 1991).

At the time of writing, no previous reports had been found on the use of DNA-based assays in the coconut palm. RAPD was chosen for the assessment of genetic diversity in the coconut palm because it is most widely used of the three methods. This assay was in two parts. First, an attempt made was to analyse the segregation of RAPD markers in a small family comprising a tall and a dwarf parent and F_2 progeny of 18 individuals arising from the intercross of tall x dwarf hybrid (F_1). In the second part of the study the genetic diversity among 13 accessions from the coconut populations of Sri Lanka comprising the three varieties (*typica, nana* and *aurantiaca*) was assessed. These included four forms of the *typica* variety, (three replicas of the commercial tall form *typica typica, typica ran thembili, typica pora pol and typica gon thembili*) four local forms and two exotic forms of the *nana* variety; (Sri Lanka green dwarf, Sri Lanka yellow dwarf, Sri Lanka red dwarf and Sri Lanka brown dwarf, Cameroon red dwarf and Brazilian green dwarf), one *aurantiaca* variety (king coconut) and six germplasm accessions from the commercial form *typica typica* (*San Ramon*, Moorock, Pitiyakande, Debarayaya, Goyambokka and Akuressa).

The RAPD approach has gained wide acceptance over the past six years and has been applied in a wide range of organisms including bacteria (Welsh and McClelland, 1990), fungi (Varga and Croft, 1994) protozoa (Tibayrenc *et al.*, 1993), insects (Baruffi *et al.*, 1995), nematodes (Castagnone *et al.*, 1994), fish (Bardacki and Skibiski, 1994), mammals (Welsh and McClelland, 1990), grass (Stammers *et al.*, 1995), annual and semi-annual crops (Connolly *et al.*, 1994 Virk *et al.*, 1995), plantation crops (Shah *et al.*, 1994), fruit crops (Koller *et al.*, 1993) and forest trees (Chalmers *et al.*, 1994).

DNA templates for RAPD-PCR. Genomic DNA used for RAPD-PCR was isolated from young coconut leaves transported from Sri Lanka as fresh samples and subsequently stored at -70 °C. The modified method of Dellarporte (1983) used to isolate DNA from coconut samples gave a reasonably high yield of DNA with adequate quality for southern blot hybridisations and obviously for PCR studies. Although this technique proved successful for extraction of DNA from coconut leaves it is more laborious and time consuming than most other techniques developed for isolation of PCRs.

One of the advantages of PCR technology is it requires very low quantities of DNA for amplification reactions (25 - 50 ng per 25 µl reaction). The quality is also not a critical factor because only the sequence to be amplified has to be intact for PCR amplification. Therefore, there are now a number DNA protocols designed for rapid extraction of small quantities of DNA from plant material. For example the method developed by Edwards *et al.* (1991) to obtain DNA from a tissue sample of milligram amounts takes only 15 minutes to complete, allowing hundreds of individual samples to be processed in a single working day. The method consists of only three steps, maceration of the plant tissue in an eppendorf tube, centrifugation in an extraction buffer and isopropanol precipitation. The DNA extraction methods of Brunel (1992), Wang *et al.* (1993) and Guidet (1994) are more examples of rapid DNA isolation methods designed for PCR assays. Finding a suitable rapid DNA extraction protocol for isolation of coconut DNA is indeed a priority in view of the possible large scale application of RAPDs in the palm in the future.

The PCR conditions. The PCR conditions used in the production of RAPDs warrant a discussion. The reproducibility of RAPD fingerprints has been a major concern to many investigators. DNA amplifications in the RAPD-PCR uses very short primers which have less specificity than longer primers. Therefore, this method is highly sensitive to small variations in the temperature cycle, including the annealing temperature (Pammi *et al.*, 1994), annealing time (Virk *et al.*, 1995), number of cycles (Pammi *et al.*, 1994), DNA concentration (Virk *et al.*, 1995), primer concentration (Virk *et al.*, 1995), Mg^{2+}

concentration (Park and Kohel, 1994; Pamrai *et al.*, 1994) and utilisation of different thermal cyclers (Virk *et al.*, 1995). Further the purity of the DNA template, the source and manufacturer of Taq polymerase (Schierwater and Ender, 1993; Virk *et al.*, 1995; Loudan *et al.*, 1995) and manufacture of primer (Virk *et al.*, 1995) have also been shown to cause RADP-PCR variation. Williams *et al.*. (1990) the pioneers of the RAPD technique demonstrated the reproducibility of RAPD markers by mapping 11 polymorphisms in soybean using 60 F_2 segregating individuals. Virk *et al.* (1995) performed several experiments with rice DNA and confirmed the importance of the consistency of protocol to ensure the reproducibility of RAPD data throughout an experiment. Many laboratories have succeeded in optimising the reaction conditions with respect to their particular DNA samples, chemicals and equipment used. However, the comparability of diagnostic RAPD markers across laboratories still remains unknown.

PCR conditions used in the current study strictly followed the conditions described by Williams *et al.*, (1990) which is also laid down in the manufacturers instructions provided with the primers (OPERON Technologies Limited). The primer concentration 0.2 µM was doubled in the second assay because certain primers produced reliable banding patterns only when the primer was at higher concentrations than recommended in the standard protocol. This was also observed by Virk *et al.* (1995) with DNA from rice. The use of a single supplier of *Taq* (Bresatec), and the buffer and magnesium chloride supplied with the enzyme, helped to maintain these three critical constituents of the reaction constant across all samples throughout the experiments. Preparation of the reaction mix in bulk excluding only DNA made the PCR reaction constituents across the tubes identical for each primer. All these precautions were taken to ensure the reproducibility of the data at least within the laboratory throughout the assay. The repeatability of a high proportion of RAPD bands in repeat tests with DNA from tall and dwarf parents and the reasonably high proportion of monomorphic bands (69 out of 160 ie. approximately 43%) across 19 individuals indicated the consistency of the DNA bands produced in these assays.

Identification of PCR fragments: The accurate identification of amplified fragments is very important because the RAPD analysis is entirely based on scores of bands observed on a gel. A clear resolution of bands is needed to decide the presence or absence of a band. Fractionation of PCR products by 1.5% agarose gel electrophoresis followed by staining with ethidium bromide was adopted because it is the most common approach in RAPD assays which is the most common approach in RAPD assays (Jain *et al.*, 1994; Jermstad *et al.* 1994; Stiles *et al.*, 1993 etc). Since RAPD-PCR amplification products range from 100 - 3000 bp in size, a clear band resolution can be expected from 1.5% agarose. A great majority of bands resolved well enough to score without difficulty but in a number of instances deciding whether a band was present or absent became extremely difficult. Particularly with the bands that varied in their intensity across DNA samples. Uneven mobility of DNA across the gel lanes has also caused difficulties in scoring bands. Torres *et al.* (1993), preferred 2% agarose (1% agarose + 1% Nu-Sieve agarose) while Wilde *et al.* (1992) used 5% polyacrylamide (non denaturing) gels for resolving PCR bands. Pammi *et al.* (1994) used radiolabelled dCTP with ^{32}P in the PCR reaction and products were electrophoresed in 5% polyacrylamide (denaturing) followed by autoradiography. Caetano-Anolles *et al.*, (1991) resolved banding patterns using 5% poly-acrylamide gel electrophoresis followed by silver staining. Obviously a better resolution of bands could be expected from polyacrylamide gels but the use of radiolabelled primers can not be considered as a good alternative because one of the main reasons for the popularity of the RAPD technique is that it does not involve radioactive compounds. Silver staining is ideal but a long staining procedure follows polyacrylamide gel electrophoresis, making it more laborious than agarose gel electrophoresis. For automated RAPD typing a system is now commercially available which use fluoroscently labelled primers and an imaging device (Molecular Dynamics FluoroImager™ 575 and FragmeNT analysis software) to scan bands after polyacrylamide gel electrophoresis.

Reproducibility of RAPD fragments: Several authors have examined the reproducibilty of RAPD bands in experiments to measure genetic relationships between DNA samples. Keil and Griffin (1994) compared RAPD patterns generated from

combinations of 5 primers, 6 ramets and 2 clones of *Eucalyptus*. They observed very little variation within ramets with the same primer and the same clone and they claimed the quality of the DNA, free of single strand breaks as the essential factor for the reproducibility of the results. Kazan *et al.* (1993) studied the reproducibility of RAPD patterns in *Stylosanthus* species by replicating accessions with five individuals each and by repeating every PCR twice. They observed the same band patterns from individuals of the same accessions and also in repeated reactions. Kangfu and Pauls (1993) repeated all amplification reactions three to five times with two primers against alfalfa genomic DNA and found the amplifications produced consistent bands that could be scored over the repeated runs.

Mailer *et al.* (1994) studied genetic diversity among rapeseed cultivars using RAPDs and mentioned that clear, easily detectable and well resolved bands are highly reproducible over repeated runs with sufficient intensity to determine their presence or absence in samples with the same relative band intensity. The patterns comprising either faint or hard to detect bands were less reproducible. Koller *et al.* (1993) studied the RAPD patterns amplified from apple cultivars over a wide rage of DNA concentrations and found that even with more than 2000-fold change in DNA concentration the variation in the PCR amplifications were primarily quantitative and this is especially true for strongly amplified bands. Similarly Gonzalez and Ferrer (1993) observed 100% reproducibility with major bands and most of the minor bands in *Hordeum* species. Tinker *et al.* (1993), found that 19 out of 33 primers tested produced stable polymorphic products in spring barley in repeated runs.

RAPDs generated by one primer for a given DNA sample can be highly reproducible with clear polymorphic bands while another primer with the same DNA sample may produce weak bands, often arising from mismatched primer annealing which is sensitive to even very slight changes in the PCR condition. Therefore, the key factor is the choice of the correct primer to suit the DNA substrate. With the ready availability of 10-mer oligonucleotides of varying sequences, selection of a set of primers for any sample of

DNA is not a difficult task. Out of the 38 primers tested in the current work with coconut DNA, only 17 produced good scorable DNA bands. The other primers either did not produce any bands at all, did not produce bands across all the DNA samples or did not produce bands intense enough for accurate scoring.

Wolff *et al.* (1993) conducted a fractional factorial experiment and concluded by saying that optimal species/primer combination has to be found by trial and error. In many studies on RAPDs the best primer and DNA combinations were identified by initially screening a large number of primers. Mailer *et al.* (1993) initially tried 100 primers to select six primers capable of producing easily detectable, well resolved bands of 100% reproducibility to use in genetic assessment of *Brassica napus* cultivars. Similarly Jermstad *et al.* (1994), (Karzan *et al.* (1993), Torres *et al.* (1993) and Tinker *et al.* (1993) screened 142, 90, 53 and 33 primers respectively to select suitable primers for RAPD assays.

The presence of amplification fragments in the negative controls (PCR reactions without the template DNA) occurred rarely in the current study. In these few instance the artefacts appeared as very faint bands and bands of this size did not appear in samples where DNA were present. These artefacts are believed to occur as a consequence of self complementary ends. In OPB primers the self complementarity was avoided during primer design. Therefore the occasional artefacts may have formed as result of the ability of some primers to form complicated secondary structures (mentioned in the instructions booklet supplied with the primers, OPERON Technologies Limited). Torres *et al.* (1993) observed artefacts in about 10% of the control samples lacking DNA in their studies with *Vicia faba*. However, in no instances were these artefactual products observed in DNA supplemented samples. Kangfu and Pauls (1993) have also reported similar observations with RAPD studies carried out with alfalfa.

4.1.2 Segregation of RAPD markers in the F2 population

The first part of the study made on RAPDs was an assay of a pedigree of the coconut palm. This coconut pedigree come from a breeding experiment conducted at the Coconut Research Institute, Sri Lanka to evaluate F2 individuals originating from a cross between a *typica typica* individual and a *nana* green individual. The F_1 individuals of this pedigree were not available for the study. However, it was felt important to make use of this pedigree to study the inheritance pattern of the RAPD markers which distinguish the two parents. This assay was thought to be of paramount importance because the results would confirm the validity of the RAPD technique for detection of genetic variation in the coconut palm.

The results obtained by analysing 20 genotypes (the tall parent, the dwarf parent and 18 inbred, F_2 individuals) reflected quite positively on RAPDs as genetic markers. Seven out of the 38 primers tested against the tall and dwarf parents with RAPDs gave distinct and reproducible polymorphic bands. A total of 12 clearly scorable polymorphic bands were observed with these primers. These bands segregated among the F_2 individuals. Based on the assumption that each RAPD fragment is a unique locus and represents a dominant marker the bands exhibited Mendelian segregation. All the 12 bands initially selected by screening the tall and dwarf parent and the two bands subsequently selected, OPB7.2 and OPB11.4. which did not show polymorphism between the parents but segregated in the F2 population had acceptable goodness of fit ratios for simple principles of inheritance.

The nature of the fragments from arbitrary amplified primers has not yet been adequately investigated to determine whether each fragment visualised in a gel can be considered as an independent character. However, based on experimental evidence most scorable RAPD amplification fragments segregate in the Mendelian fashion (Rieter *et al.*, 1992; Tinker *et al.*, 1993; Gonzalez and Ferrer, 1993; Heun and Helintjaris, 1993; Torres *et al.*, 1993; Jermstad *et al.*, 1994). Stammers *et al.* (1995) tested a set of six bands by

excising bands from the gel and using each as a hybridisation probe in Southern blot analysis of RAPD products. They found that the majority of fragments showed sequence homology to only a single band. Therefore, the general consensus is that each RAPD amplification fragment corresponds to an independent genetic locus and it has been found true especially when screening individuals within a species (Hallden *et al.*, 1994). Further there is evidence of RAPD and RFLP markers appearing synonymously in mapping positions (Jermstad *et al.*, 1994).

The RAPD markers tend to be inherited as dominant markers because they appear in similar fashion in both homozygous individuals and heterozygous individuals. Only the homozygous recessives can be definitely distinguished. Therefore this technique does not allow heterozygous and homozygous dominant individuals to be identified without subsequent crosses. In this study two RAPD loci OPB7.2 and OPB11.4 were inferred to be in the heterozygous state in the parents based on segregation of the amplified fragments in the F_2 progeny. The two RAPD fragments show clear variation in the band intensity across individuals. The segregation data suggested that OPB 7.2 was in the heterozygous state in both parents, appeared in similar intensity in the tall and dwarf amplified DNA profiles whilst showing a marked variation in the 13 F_2 individuals (10 strong bands versus 3 weak bands). However, the intensity of bands in the parents was the same as that of 10 strong bands in DNA samples from the F2 progeny. In the case of OPB11.4 a definite difference in the intensity of the two bands from the parent DNA is visible and complies well with the segregation that suggests heterozygosity in one parent. However, the OPB 11.4 band in F_2 individuals appear to show continuous variation rather than two discrete classes. Therefore, it is unlikely that heterozygotes can be scored reliably by quantitative differences in the band intensity observed in the gel. Even if such an attempt were to be made, the consistency of quantitative differences over repeated runs would have to be proven. Heun and Helintjaris (1993) studied the inheritance of RAPDs in F_1 hybrids of corn and observed situations where intensity differences were reproducible and followed the co-dominance pattern. Similarly Tinker *et al.* (1993) found one co-dominant marker from a sample of 31 RAPD polymorphisms in barley.

Another observation in the present study that warrants a discussion is the occurrence of bands in the amplified DNA samples of progeny that were not present in the parent samples. Such occurrences can be explained by the presence of organelle DNA (mitochondrial or plastid DNA) in the total DNA used as template for the PCR. Contamination of samples by foreign DNA (most likely microbial in origin) during the process of extraction, or mismatch annealing of primers are two other possibilities of such spurious bands. The coconut leaves sometimes can be seen to be infected by fungi when they are freshly transported.

Misidentification of parents can also be possibility because the parental crosses were performed long ago. This could easily happen in coconut through illegitimate pollinations, careless handling of pollen or the presence of insects inside pollination bag. These bags are put around the inflorescence to shield receptive flowers from alien pollen that could come by wind or insects. It should be noted that this pollination was actually performed in 1956. There is a reported instance of such "spurious" bands that behaved in the Mendelian manner in a phylogenetic analysis of Apple rootstocks (Landry *et al.*, 1994) and eventually lead to the discovery of an anomaly in parentage of these rootstocks.. However, this 'illegitimate' bands can not be regarded as a serious problem because they occur rarely and very often they are not completely reproducible. They can also be detected by their non - Mendelian inheritance (Heun and Helentjaris, 1993).

The Mendelian inheritance of all unambiguously polymorphic RAPD markers (distinguished by presence and absence of amplification products) in the coconut F_2 population confirms findings of many other authors. Tinker *et al.* (1993) tested 14 RAPD markers and found 13 out of them segregated in a Mendelian fashion in 27 inbred lines of barley. Heun and Helentjaris (1993) studied the inheritance patterns of RAPDs in corn and reported that 90 per cent of the unambiguously polymorphic markers were inherited as expected in the F_1 hybrids and concluded that the most predictable types of RAPD fragments were those of the greatest intensity in any one reaction while minor fragments

possesses a great propensity for irreproducibility. Jermstad *et al.* (1994) demonstrated Mendelian inheritance in 29 out of 30 RAPD loci based on single locus segregation in a sample of F_2 progeny in coastal Douglas-fir.. Lee *et al.* (1995) in a F_2 population of Cucurbita and Lu *et al.* (1995) in a F_1 population of Pinus have also detected Mendelian inheritance in all unambiguously polymorphic RAPD markers.

The purpose of this study on inheritance of RAPDs in a coconut F_2 family is to investigate the potential of RAPD markers as tools in mapping important traits of coconut in the future. It is evident from this study that detection of RAPD polymorphic markers is a relatively easy task. The procedures used in this study were relatively simple and the polymorphisms detected were repeatable and show Mendelian segregation. Most of the limitations discussed with the RAPD approach can be overcome by selection of primers to suit the DNA substrate from coconut and by standardising the RAPD-PCR by optimum concentrations of DNA, primer and other constituents of the PCR. The availability of random primers from commercial sources offers a great choice of primers and allows detection of a sufficient reproducible RAPDs in a short period of time. Therefore, generation of a linkage map for the coconut genome is possible provided the segregating populations arising from intercross and backcross populations of tall x dwarf F_1 hybrids are available.

The major limitation in using RAPDs to generate a linkage map for the coconut palm is the lack of clearly defined segregating populations in the coconut palm. The coconut breeders in the past have concentrated more on inter- and intra-varietal hybridisations rather than concentrating on long term evaluations of genetic crosses. Therefore, performing conventional genetic crosses such as those geared at constructing single seed descent lines has now become a pressing need because of the tremendous potential of RAPDs as genetic markers in the coconut palm. The other alternative at this stage is to resort to the bulk segregation technique assuming *typica* forms and *nana* forms as separate breeding lines. This possibility is further discussed in this chapter.

4.1.3 RAPD polymorphisms in assessment of genetic diversity in coconut populations

The genetic assessment of varieties, forms and germplasm accessions within the coconut species by the use of RAPDs revealed that RAPD technology is an effective method to study genetic diversity in the coconut palm. The RAPD based taxonomy of coconut accessions is in good overall agreement with classical taxonomy, although it contains a number of novel insights. The applicability of RAPDs for assessment of genetic diversity has also been well demonstrated in a number of tree crops and forest trees (Shah *et al.*, 1994, in oil palm; Wilde *et al.*, 1992, in cocoa; Castillo *et al.*, 1994, in coffee, Koller *et al.*, 1993 in apple; Chalmers *et al.*, 1994, in mahogany; Orozco- Daniel *et al.*, 1994, in *Populus* spp; Keil and Griffin, 1994 in eucalyptus etc.).

The coconut populations assayed : The 19 accessions of coconut palm assayed comprise three varieties with a number of forms and germplasm accessions within them. According to the classical classification (Liynage *et al.* 1958) *typica* (Tall), *nana* (Dwarf) and *aurantiaca* (King Coconut) are the three varieties of the palm in Sri Lanka.

Accessions of the variety *typica* : Within the *typica* variety *typica typica* (Commercial tall), *typica pora pol*, *typica ran thembili* and *typica gon thembili* are four forms. The three latter forms are each distinct from the *typica typica* by a single discrete character and quantitative differences in the fruit components. The characteristic of *typica pora pol* is the presence of thick shell, while *typica gon thembili* is characterised by the orange coloured nut. The characteristic of *typica ran thembili* is the pink colouration in the area underneath the carpels of the young nut. T_1, T_2 and T_3 are three sample populations of the *typica typica* descending from a single population of pooled parents.

The germplasm accessions Moorock, Pitiyakande, Akuressa, Goyambokka and Debarayaya are five populations of the form *typica typica* (Commercial tall) named according to the site of collection. These are believed to be isolated populations because

they were inbred for two or three generations while undergoing domestication[1]. No distinct morphological differences have been observed among these germplasm accessions or between them and the *typica typica* (Commercial tall). Quantitative differences are under investigation at the Coconut Research Institute (CRI), Sri Lanka.

San Ramon is an exotic tall-like form brought to the island from the Philippines by a coconut land owner. This variety is also classified as a form of *typica* by Philippine Coconut Authority (PCA) taxonomists (Carpio, 1982).

Accessions of the variety *nana* Within the *nana* (*javanica* according to PCA) variety, dwarf green, dwarf yellow, dwarf red and dwarf brown are four forms varying in nut colour. A *typica* dwarf type appears as a short stuttered palm with a trunk lacking the root bole. In a *typica typica* the bottom of the trunk has an inverted funnel shape because of the large root bole. The dwarf yellow form exhibits a tremendous variation within the form, some individuals even having well developed root boles and the appearance of the *typica* form gon thembili. Cameroon red dwarf and Brazilian green dwarf[2] are typical dwarf types and look almost identical to their Sri Lankan counterparts.

Accessions of the variety *aurantiaca*. *Aurantiaca* (King coconut) was classified as another variety by Liyanage (1959) because of its sweet nut water and intermediate stature. However, the general appearance of this palm is closer to the *nana* forms.

RAPD based diversity of the coconut palm accessions The RAPD technique has demonstrated its ability to discriminate plant populations at taxonomic levels from generic to clonal levels. Stammer *et al.* (1995) investigated genetic relationships between and within species of genera *Lolium* and *Festusa* and found good overall agreement with

---

[1] Growing coconut at a commercial level in tropical countries began in the nineteenth century to satisfy the need for coconut oil for industrial use. The use of coconut oil for soap manufacturing in developed countries began in 1841 (Benabadis, 1992).

[2] Imported from germplasm accessions at the Ivory Coast by CRI for breeding purposes in 1969 (Manthriratne, 1970)

classical taxonomy. Gonzalez and Ferrer (1993) accurately discriminated between 19 *Hordeum* species and subspecies by application of RAPDs. Jain *et al.* (1994) demonstrated the potential of RAPDs in identifying inherent variation and genetic relationships among genotypes in the mustard species, *Brassica juncea*.. Shah *et al.* (1994) distinguished different accessions of oil palm germplasm from Africa using RAPDs. Koller *et al.* (1993) demonstrated the capability of RAPDs to distinguish apple cultivars. Wilde *et al.* (1992) characterised cocoa clones by the RAPD application. RAPD primers are however, claimed to be more accurate in investigating relationships among populations of a single species or very closely related species than between less related species (Gonzalez and Ferrer, 1993; Hallden *et al.*, 1994).

In the present study on coconut palm populations in Sri Lanka, RAPD primers generated adequate polymorphism to distinguish the 19 intraspecific accessions screened. Of the 38 primers tested, 17 generated at least one polymorphic band. The sequences amplified by each primer varied in number (4 - 14) as well as in intensity, with a range of amplification products from 200 bp to 2200 bp. A total of 160 fragments was observed of which 91 exhibited polymorphism (57%) varying from 1 - 13 per primer with a mean of 5.3 per primer. The genetic distances based on Nei and Li's coefficient of similarity had the most distant accessions 0.524 apart indicating a substantial genetic diversity within the species.

The genetic diversity seen in populations of outcrossing plant species complicates the analysis of variation due to their inherent within population variation as no two genotypes of any accession are identical. Therefore, four individuals were sampled for each accession when leaves were harvested for DNA extraction. The estimation of genetic relatedness by random amplification of bulked genomic samples has been successful in heterogeneous populations of alfalfa (Kangfu and Pauls, 1993). According to Kangfu and Pauls (1993) the most important advantage of the DNA bulking procedure is the increase in efficiency by cutting down the number of reactions. The RAPD patterns obtained from bulked DNA samples generally consisted of superpositions of the patterns

seen for individuals with the exception that some faint bands that were present in the patterns from individual DNA samples were absent from the bulk DNA samples. Thus, the PCR patterns obtained from the bulked DNA samples represent a consensus pattern for the common markers in the sampled population. The number of plants that should be bulked for sampling the variabilities in a population has been discussed at length by Yang and Quiros (1993) in a similar study carried out with bulk DNA from celery cultivars. They have scored bulks made up of 5 - 50 individuals with primers showing polymorphic markers and found bulk of five individuals showed the same number of bands as those of 10, 20, 30, 40 and 50 plants. Virk *et al.* (1995) bulked leaf samples from 10 individuals per extract in wild rice populations to incorporate variation within accessions.

The optimum number of primers required for a genetic diversity assessment is dependent on many factors. If the assessment is between distant groups such as genera and species obviously more polymorphisms per primer could be expected. When assaying more closely related populations within a species, such as cultivars and clones, more primers would be required to obtain adequate polymorphisms to discriminate among them. However, it has been shown that by selection of primers yielding polymorphic bands across populations, a precise assessment of genetic diversity could be achieved with a very small number of primers. Rus-Kortekas *et al.* (1993) distinguished 11 of 15 tomato cultivars with four selected primers Similarly, Hu and Quiros (1991) discriminated between 14 broccoli and 12 cauliflower cultivars using as few as four primers. Tinker *et al.* (1993) distinguished 27 inbred lines of barley with seven primers.

The level of genetic polymorphisms detected within the coconut species is high, with 5.3 polymorphic fragments per primer among 19 accessions of the palm analysed by RAPD. This compares favourably with other intraspecies assessments: e.g. 4.5 polymorphic fragments per primer among germplasm accessions of oil palm from Cameroon, Tanzania, Nigeria and Zaire (Shah *et al.*, 1994), 4.0 polymorphic fragments per primer among 25 apple rootstocks (Landry *et al.*, 1994), 3.8 per primer among 23 cultivars of rapeseed from several breeding programmes (Mailer *et al.*, 1994), 3.7 per

primer among six cultivars of sweet potatoes (Connolly *et al.*, 1994), 2.7 per primer in rice germplasm collections obtained from a major world collection (Virk *et al.*, 1995). However, the polymorphisms per primer ratio is very high only if preselected primers were considered. The maximum dissimilarity, 0.524 (genetic distance between the most distant accessions T_3 and Brazilian green dwarf) is also an indication of the broad genetic base within the domesticated coconut palm population in Sri Lanka. This level of variation suggests that there is great potential for genetic improvement of the coconut palm.

The dendrogram based on genetic distances calculated by Nei and Li's coefficient for 91 RAPD fragments reflected the accepted separation of Liyanage *et al.* (1959) and later additions to the taxonomy (Wickramaratne, 1989; Perera and Peries, 1994) although the classification of some accessions were not placed exactly.

At the first hierarchical level two major groups separated, with all the tall accessions except *San Ramon* forming one cluster and the rest in the second cluster. The overall genetic similarity within the tall cluster (76%) was less than that in the dwarf cluster (81%). This is an expected result due to the heterogeneous nature of the tall type resulting from its predominantly outbreeding reproductive behaviour. Furthermore, the dwarf types have been selected and maintained in cultivation (Benabadis, 1992) thus conserving less diversity. The accessions in the tall cluster further fell into two groups with sub clusters of T_1, T_2 and T_3, germplasm accessions of the *typica typica* form and one *typica* form, *typica gon thembili* in one cluster and the other two forms of *typica*, *typica ran thembili*, and *typica pora pol* grouped in the other cluster. This separation are in line with the classical taxonomy, except for grouping of *typica gon thembili* within the *typica typica* group rather than separating it as a different form.

The palms of the form *typica gon thembili* differ morphologically from the palms of the form *typica typica* only by its orange coloured nuts. The quantitative differences in husked nut weight, a character estimated to have a heritability value of 0.95, is zero. The other fruit characteristics do vary but not as much as *typica pora pol and typica ran*

thembili (Liyanage *et al.*, 1988). Therefore, classifying *typica gon thembili* within the of *typica typica*, rather than including it in a different taxa, could be worth considering. A better analysis of this classification of *typica* could be obtained by screening all the forms of *typica*, including *typica bodiri* and *typica kamandala* which show marked differences in the fruit characteristics, by RAPD markers. The former, *typica bodiri* produces very small nuts averaging about 100 nuts per bunch and requiring approximately 20,000 nuts to produce a ton of copra. In comparison, *typica kamandala* produces very large nuts with just two or three nuts per bunch and requiring approximately 2,700 nuts to produce a ton of copra. The commercial form *typica typica* produces average size nuts with 20 -25 per bunch, requiring about 4,750 nuts to produce a ton of copra (Liyanage, 1958).

Clustering of the germplasm accessions of the form *typica typica* in a separate branch from the commercial T_1, T_2 and T_3 is a clear indication of the diversity in these heterogeneous populations, which the conservationists expected. These populations believed to have been independently domesticated and kept apart from the commercial cultivars of the *typica typica*. The coconut germplasm collections at the CRI were basically biased collections because the conservationists (Rathnasiri and Wickremaratne, 1988; Perera and Peries, 1993 and 1994) were keener on establishing gene banks of 'wild' populations (domestic populations maintained for several generations by open pollination or forced pollination within the populations) rather than random collections because of the logistical constraints (space and cost in particular) in establishing large gene banks. The conservationists preferred coconut populations with favourable characters such as nut size, copra yield, ability to withstand drought etc., for conservation in gene banks.

Further grouping of germplasm accessions Moorcok and Pitiyakande together and Goyambokka, Akurassa and Debarayaya together fits well with their geographical distributions. The former two were collected from an area called Mawathagama in the north central province towards the border of the central province whilst the other three were collected from the southern part of the country. The sites of collection of these germplasm accessions are far apart and chances of cross pollinating between any of the

populations are meagre. Moorock and Pitiyakande share a similar agroclimatic condition in the intermediate wet zone while Goyambokka and Debarayaya share a similar agroclimatic condition in the intermediate dry zone more towards the coast. Akuressa is in the intermediate dry zone away from the coast.

Ecotypic adaptation as a possible reason for separation of coconut populations was suggested by Harries (1978) based on data collected from geographically diverse coconut populations. Natural selection pressure exerted by frequent dry spells in the intermediate dry zone is likely to have an impact on existing 'wild' populations in south Sri Lanka. But without a clear understanding of the history of these palm populations it is difficult to make a valid judgement. A more detailed RAPD analysis using germplasm accessions representing more finely defined agroclimatic zones over the entire coconut growing area in the country would allow this complex situation to be addressed with greater accuracy.

The accessions in the predominantly *nana* (dwarf) cluster had a *nana* form *dwarf yellow* and the only *aurantiaca* form used in the study, *king coconut* separating from the rest of the dwarf forms and the *typica* form *San Ramon*. *Dwarf yellow* and *king coconut* differ from *typica* dwarf palm by their stature. Dwarf yellow vary greatly with its stature ranging from a typical *typica* type (tall large trunk with a root bole) to a typical dwarf type (short small trunk without a root bole). King coconut has an intermediate stature and flowers late which is uncharacteristic of the variety *nana*. Therefore, the separation of these two types at this level of hierarchy reflects positively on the ability of RAPDs to identify genetic differences in the coconut palm.

In the second sub cluster of the *nana* forms, the tall form from the Philippines, *San ramon* is included with the typica dwarf forms, *dwarf green, dwarf brown, dwarf red, Cameroon red dwarf and Brazilian green dwarf*. The clustering of the latter forms is in agreement with the morphologically based taxonomy because they all are typical dwarf types varying only in nut colour irrespective of their origin. The close relatedness between

Sri Lanka dwarf forms green, red and brown and exotic forms Cameroon red and Brazilian green indicates the narrow genetic base shared by these types and agrees well with their autogamous nature and selection for domestication.

The *typica* form, *San ramon* : The clustering of the *typica* form, *San ramon* with the *nana* forms is unexpected but is an extremely exciting result which warrants a lengthy discussion. San Ramon palms are majestic trees, very tall and erect with stout trunks and massive boles. The nuts are large and round with a thick kernel which constitutes about 50% of the nut. *San Ramon* kernel is 1.4 cm thick (1.35 cm in commercial typica) with a copra content of 350 - 400 g per nut (210 - 225 g per nut in commercial tall; Fernando, 1988). A special feature of this palm is its ability to withstand long spells of drought. It is allogamous as characteristic of *typica*. It does not in any respect resemble a *nana* form.

The placement of the form *San ramon* in a cluster with typical *nana* forms separate from *typica* forms although unexpected, confirms with Carpio's (1982) observations on diversity of pollen proteins. The electrophoresis of pollen protein extracts from morphologically distinct populations of the coconut palm in the Philippines have also demonstrated a grouping of San Ramon with the nana type.

Pollen proteins from eleven morphologically distinct coconut populations in the Philippines, extracted with 1% Sodium chloride (pH 6.7) without urea and stained with Coomassie blue, provided some discrimination (Carpio, 1982). The populations screened comprised seven *typica* forms (Laguna, LAG var. *typica*; Baybay, BAY var. *typica*; Tag*nana*n, TAG var. *typica*; *San Ramon*, SRM var. *typica*; Bago-Oshiro, BAO var. *typica*; Zamboanga, ZAM var. *typica*; Rennel, RNL var. *typica*;) and four *nana* forms (Tacunan, TAC var. *javanica, green dwarf* form; Catigan, CAT var. *javanica, green dwarf* form; Yellow Dwarf, YD var. *javanica, yellow dwarf* form; Orange Dwarf, var. *javanica, orange dwarf* fotm). The eleven forms were separated into six major groups. *San Ramon* clustered with orange dwarf and yellow dwarf while two green dwarfs

separated to another cluster. All the ot ıer *typica* types clustered in pairs to form the other four groups (see Figure 4.1).

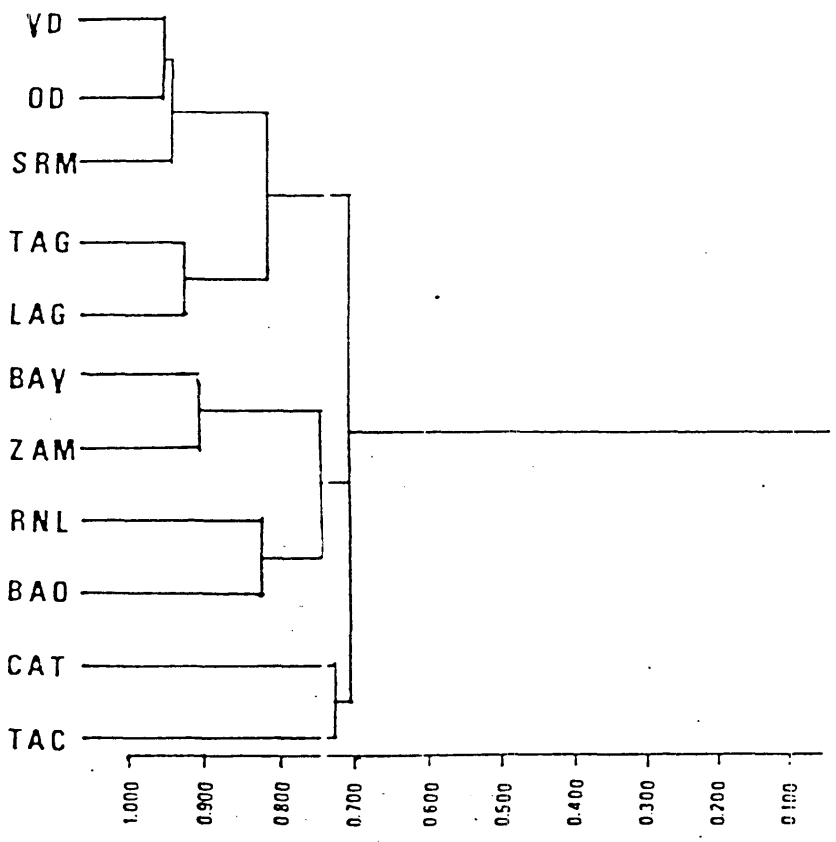

Figure 4.1 Dendrogram obtained usir g Jaccard's coefficient of similarity and clustering based on electrophoretic separation o ' pollen proteins (reproduced from Carpio, 1982). YD, *nana* yellow, OD, *nana* Orange, SRM, *typica* San Ramon, TAG, *typica* Tagnanan, LAG, *typica* Laguna, BAY, *typica* Bay bay, ZAM, *typica* Zamboanga, RNL *typica* Rennel, BAO, *typica* Bago-Oshiro, BAO, *typic a* Catigan, TAC,, *nana* Tacunan.

Clustering of 'tall coconut' *San Ramon* with 'dwarf coconut' types as revealed by RAPDs and pollen protein polymorphisms indicate a unique genetic make up in the *San Ramon* form. It is 0.44 and 0.29 apart from *typica* accessions and *nana* accessions respectively and suggests that it would worthwhile testing for probable crosses between *typica* and *nana* forms. It is interesting to note that such crosses are already under evaluation at the CRI. The relatively large nut with higher copra content and its ability to tolerate prolonged drought conditions (Fernando, 1989) led CRI breeders to establish multi-locational trials to evaluate *San Ramon* x *typica typica* and *San Ramon* x *nana* forms, *dwarf green* and *dwarf yellow* (Fernando *et al.*, 1986, 1987; Rathnasiri *et al.*, 1987, 1988 and 1989). These trials are now in progress and analysis of results on early vegetative growth and precocity indicated *San Ramon* x Sri Lanka Tall hybrids exhibit heterosis in a number of locations (Everard and Peries, 1990, 1991, 1992, 1993 and 1994). The palms are now in bearing age in most of these trials but have not yet reached the accepted maturity age (approximately 9 years of age) to consider analysis of fruit components and recording of yield data.

4.2 Restriction fragment length polymorphisms in the coconut palm

The Restriction fragment length polymorphism (RFLP) technique is a powerful tool in biotechnology that can potentially increase the effectiveness and efficiency of plant breeding. Besides direct sequence comparisons RFLPs are the most sensitive tool for the detection of DNA differences within or between species. The molecular basis of RFLPs is the loss or gain of a restriction site due to a point mutation within the enzyme's recognition sequence, or a molecular event leading to insertion, deletion or inversion of DNA sequences. Both situations result in a length difference of genomic restriction fragments detectable on Southern blots. RFLPs exist in potentially unlimited numbers and their detection is only limited by the molecular differences existing between available genotypes and the effort applied to detect them.

Tagging of genes of interest with tightly linked RFLP loci, analysis of complex polygenic characters (QTL mapping), assessment of genetic diversity and phylogenetic relationships and identification of genotypes (DNA fingerprinting) are major areas of RFLP-application that have been discussed (Young *et al.*, 1990). RFLPs have aided for improvement in many crops such as maize, wheat, tomato, potato and soybean (Helentjaris, 1987; Sharp *et al.*, 1989 Tanksley *et al.*, 1988; Bonierbale *et al.* 1988; and Keim *et al.*, 1990). Although RFLPs have been generated for many crops that are important to industrialised nations, there has been little effort made on crops grown primarily in developing countries (Persley, 1990).

An attempt was made in the present study to detect RFLPs in the coconut palm population of Sri Lanka by using cloned coconut DNA sequences and minisatellite (M13) and microsatellite sequences as probes. Although the study did not reveal an adequate number of RFLPs the exercise was a worthwhile investigation because it yielded some useful information for planning further studies on developing RFLPs in the coconut palm. In this regard, issues that warrant a discussion include isolation of genomic DNA from fresh young leaves of the coconut palm with adequate quality for restriction digestion and Southern blotting, factors involved in construction of a library construction of a genomic DNA library and repetitive sequences in the coconut DNA.

DNA extraction for RFLP studies: The protocol developed by Dellaporte *et al.* (1983) for extraction of DNA from fresh (frozen) young coconut leaves yielded an average of 40 ng of DNA per milligram of leaf tissue. This miniprep protocol for isolation of total plant DNA developed is simple and inexpesnive and yielded DNA with reasonable purity. The DNA digested well with a range of restriction enzymes and smeared uniformly when digested and electrophoresed on agarose. Most methods of DNA extraction and purification of plant DNA use caesium chloride density gradients to eliminate enzyme-inhibiting polysaccharides (Murray and Thompson, 1980; Taylor and Powell, 1982, Ausubel *et al.*, 1987). These procedures are expensive and time consuming since ultra-centrifugation is required.

The miniprep protocol for isolation of total plant DNA developed by Dellaporte *et al.* (1983) has an initial homogenisation step and uses phenol:chloroform:isoamyl alcohol instead of caesium chloride gradient purification. Modifications made to this protocol were pounding of leaves in liquid nitrogen, suspending the leaf material in a homogenisation buffer containing PVP and mercaptoethanol (adopted from Thomas *et al.* 1993), and removal of intact starch grains and chloroplasts by differential centrifugation (adopted from Kirkpatrick *et al.* 1987). Coconut leaves are waxy and difficult to grind in a Waring blender, but they powdered well with liquid nitrogen. Coconut leaves are also rich in polyphenolic compounds and in early assays the DNA pellets appeared brown due to presence of these compounds as impurities. The inclusion of PVP in the homogenisation buffer inhibited the browning of the extract. Similar effects of PVP have also been reported with DNA extractions of *Vicia faba* (Rogers and Bendish, 1985).

The DNA yield (40 ng per milligram of leaf tissue) compares well with DNA yields obtained by Rogers and Bendish (1985) from a variety of leaves using the CTAB (cetyltrimethylammonium bromide) nucleic acid extraction procedure of Murray and Thompson (1980). The average DNA yields recorded for *Nicotiana tabacum*, *Petunia hybrida*, *Triticum aestivum* and *Vicia faba* were 38, 14, 52 and 40 ng per milligram leaf tissue respectively.

The maximum number of samples that a person can handle in this method is eight at a time and a minimum of two days is required to obtain DNA from eight samples. This is however, essential because of the need for large amounts of DNA for RFLP studies. With the CTAB procedure which was designed to obtain DNA from milligram amounts of fresh material 24 - 36 samples can be processed in one day by a person using only microfuge tubes (Rogers and Bendish, 1985). This latter technique is preferred for DNA extraction for use in procedures involving PCR, where only nanogram amounts of DNA is required. Rhode *et al.* (1992) who cloned and sequenced an EcoRI restricted coconut DNA fragment used the CTAB extraction procedure to isolate DNA from coconut leaves.

Harrison *et al.*, (1992) who extracted coconut DNA for detection of myco-plasma-like organisms associated with lethal ye lowing disease in the coconut palm used palm heart tissue instead of leaf tissue to iso ate DNA. Their method was also a modification of that by Dellaporte *et al.* (1983).

Random coconut genomic DNA library: Cloned DNA sequences that are randomly distributed throughout tl e entire genome are used to screen RFLPs. These sequences may be cloned from random genomic sequences or may be cDNA sequences derived from purified messenger RNA. It is not necessary for the clones to come from a library of known genes or sequences that code for proteins. For RFLP mapping, clones bearing non-coding sequences are sometimes considered better than cDNA clones (Young *et al.*, 1990). Not much difference has been reported in the efficiency of detection of RFLPs by random clones and cDNA clones in *Eucalyptus* (Byrne *et al.*, 1994). However, Havey and Muehlbauer (989) observed an increased efficiency of detection of polymorphism using cDNA libraries in lentil and lettuce and suggested that this may be related to the large genome size of hese plants.

A library of coconut DNA sequences was constructed by digesting total genomic DNA with the restriction endonuclease *Sau*3AI, recovering 0.5 kb - 2 kb fragments from an agarose gel, cloning these fragments into the plasmid vector pTZ18 and transforming the recombinants into *E. coli* strain PMC112. Recombinant clones were screened by colony hybridization and a total of 71 (27%) high-copy clones were detected from 264 recombinant clones. A further 27 colonies were retained as low-copy clones.

The general consensus in constructing a genomic plant DNA library is to clone single and low-copy DNA sequences. Therefore, the methylation sensitive enzyme *Pst*I is used to digest the DNA to obtain inserts as there is a tendency in plants for repetitive sequences to be methylated (Miller and Tanksley, 1990). This was also shown for other species such as maize (Burr *et al.*, 988), peanut (Kochert *et al.*, 1991) and *Hevea* (Besse *et al.*, 1994). However, in rice the DNA appears to be less methylated than other species

(McCouch *et al.*, 1988). The percentage of high-copy clones (27%) in the coconut library is higher comparing to 4% of *Hevea* (Besse *et al.*, 1994), 10% of Peanut (Kochert *et al.*, 1991) and 21% of *Eucalyptus* (Byrne *et al.*, 1994) libraries originated from PstI digested DNA inserts. The restriction enzyme Sau3AI is not methylation sensitive and this may account for the higher percentage of high-copy clones in the coconut library.

Digestion of coconut genomic DNA by restriction endonucleases: The coconut DNA samples digested with the restriction enzymes, *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Pst*I, appeared as uniform smears when electrophoresed on agarose gels indicating complete or near complete digestion. The presence of intensely stained bands in *Dra*I, *Eco*RI and *Eco*RV digested DNA indicated the presence of repetitive sequences in the coconut genome. Rohde *et al* (1992) detected highly repetitive sequences in the coconut genome by analysing EcoRI restricted genomic DNA. They have cloned and sequenced the 1.3 kb and 1.4 kb EcoRI fragments and showed that these fragments are members of a single family of repetitive sequences with sequence variation due to point mutations, insertions and deletions. The EcoRI fragment of 1.3 kb was the same as the 1.4 kb fragment except for a 84 bp deletion at its 5' end.

Southern blot hybridisations with high-copy and low-copy clones: Initial probing of 18 Southern blots containing DNA from six forms of coconut, using three pairs of high-copy clones, revealed bands only when the coconut DNA was digested by *Dra*I, *Eco*RI and *Eco*RV. The blots containing *Bam*HI, *Hind*III and *Pst*I digests resulted only in hybridising signals in the top molecular weight region (approximately 23 kb) without resolving any sharp bands. The failure of high-copy clones to bind with discrete bands with methylation sensitive enzyme PstI digested coconut DNA might be an indication of the methylation of repeat sequences in the coconut genome. However, these three enzymes were excluded from further studies.

The Southern blot screening of *Dra*I, *Eco*RI and *Eco*RV digested DNA from *typica typica* and *nana* green, upon probing by high-copy clones, resolved in a majority of

probes detecting fragments of same size with each enzyme. In blots containing *Dra*I digested DNA; the 1150 bp fragment, *Eco*RI digested DNA; fragments of 2700 bp, 2500 bp, 1400 bp and 1300 bp and *Eco*RV digested DNA; fragments of 1800 bp, 600 bp and 400 bp were the major bands detected by most clones, regions detected by most clones. This raised the question of whether these bands represented the same fragment or different fragments of the same size?

The *Eco*RI repetitive fragments of approximately 1,300 bp and 1400 bp sequenced by Rhode *et al.*, (1992) provide clear evidence for the presence of a repetitive sequence family in the coconut genome. Detection of *Eco*RI digested fragments of 1,300 bp by 16 pairs of high-copy clones and 1,400 bp by 16 pairs of high-copy clones favours the idea that these probes hybridise fragments in the same family or repetitive sequences. Further, hybridization intensity was observed to be very strong with these bands. Therefore, it is reasonable to infer that these fragments of same size represent of the same sequence of DNA.

It can be conclusively stated that the 1150 bp *Dra*I fragment, detected by 15 pairs of high-copy clones, *Eco*RI fragments, 2700 bp, 2500 bp, 1400 bp and 1300 bp detected respectively by 7, 9, 16 and 16 pairs of high-copy clones respectively and *Eco*RV fragments 2700 bp, 2100 bp, 1800 bp, 1000 bp, 600 bp, 400 bp and 300 bp detected by 7, 9, 18, 7, 27, 22 and 7 pairs of high-copy clones respectively are all sharing repetitive sequences belonging to a single element family. Similar observation has been reported in *Eucalyptus* (Byrne *et al.*, 1994) where the majority of high-copy clones detected only one locus.

Based on the assumption that bands of similar size hybridise to high-copy clones are homologous the actual number of fragments detected by these clones comes down to 30 (9, 7 and 14 with *Dra*I, *Eco*RI and *Eco*RV digests respectively). Out of these 30 fragments only one, the 300 bp fragment of *Eco*RV digested DNA, exhibited polymorphism between *typica typica* and *nana* green. It appears that the use of low-copy

clones has reduced the efficiency of probes from genomic libraries in the detection of polymorphism in the palm. A similar comment was made by Byrne *et al.*, (1994) who studied RFLPs in *Eucalyptus* by probing with high-copy clones from a random DNA library and with cDNA clones.

The 300 bp *Eco*RV fragment which demonstrated polymorphism between the parents *typica typica* (male) and *nana* green (female) did not segregate in the F_2 family and showed a maternal inheritance pattern. None of the F_2 progeny had the 300 bp *Eco*RV fragment detected by probe AE25 in the *typica typica* parent. This raises the possibility that the high copy clones hybridised to this fragment might be originating from mitochondrial (mtDNA) or chloroplast DNA (cpDNA). The current DNA extraction procedure allows very little opportunity for inclusion of organelle DNA in the genomic DNA extracts. The first centrifugation step, i.e. 4,000 g for 10 minutes minimises the release of such DNA and hence it is very unlikely to assertion that all these hybridisations have resulted from organelle DNA.

Further these probes appear to have consistently hybridised to 1.3 kb and 1.4 kb *Eco*RI fragments in same blots. Rohde *et al.* (1992) have cloned and sequenced *Eco*RI repeat fragments of similar size (1.3 kb and 1.4 kb) in the coconut genome and described these fragments as a family of highly repetitive sequences in the coconut genome which show a remarkable sequence homology to the *copia*-like group of retroposons. It is very likely that the sequences detected by above probes and the sequences described by Rohde *et al.* (1992) are homologous. Therefore, it is reasonable to assume that the high copy clones used in the current RFLP assay shared sequences in the above repetitive element and have originated from genomic DNA. Although there was enough evidence to ascertain the genomic origin of the cloned sequences in the current assay this situation clearly highlights the need for prescreening colonies with organelle DNA before probing for RFLPs and in particular when high copy clones are used as DNA probes.

Another possibility for this non-Mendelian behavior of the F_2 individuals could be that the two palms assayed as grand parents of the F_2 family may not be their actual grand-parents as indicated in records. These crosses were performed in 1960's and it is not righteous to rule out any possible illegitimate pollinations as collection, processing of pollen and pollination has to be carried out with great care in the coconut palm to avoid contamination. These F_2 individuals however, behaved as a true progeny derived from above grand parents when RAPDs were

assayed. For markers that distinguisl varieties, *typica* from *nana* this segregation is possible irrespective of the individuality of parents as long as the F_1 individual is a true hybrid of *typica* and *nana* . Therefore, the possibility of parental error still remain as a possible explanation for the non-Mendellian inheritance of the above 300 bp RFLP.

The results of the last 18 blots appears to have been affected by the poor quality of the Southern blots. The auotoradiographs appeared cloudy and none of the bands resolved sharply. Therefore, the polymorphic *Eco*RV fragments, 2100 bp and 1800 bp detected by high copy clones and 2600 bp, 2400 bp, 2200 bp and 1,100 bp detected by low copy clones (last nine blots) need to checked for reproducibility. However, in the last nine blots the bands produced were unique for each combination of primers. Therefore, the chances of detecting non-homologous RFLPs will be higher when probing with low copy clones because larger the number of fragments detected, greater the probability that polymorphisms will be detected.

According to Young *et al.*, (990) highly repetitive sequences are unsuitable for RFLP assays because they yield banding patterns that are too complex to interpret. However, the high copy coconut DNA clones did not yield complex bands. The highest number of bands yielded by a pair of clones was eight (AE17 + AE24) and those were *Dra*I fragments. This pair of probes have also detected seven *Eco*RV fragments and none of these patterns were complex in spite of the very high hybridisation intensity of the probes which did not resolve bands properly in the dark background. None of these bands, however, are polymorphic.

Most breeders construct single and low-copy libraries by using one of two methods, either cloning cDNA sequences derived from purified messenger RNA or cloning random genomic DNA sequences enriched for single and low-copy DNA sequences. The latter is the most common and in this case DNA sequences for cloning is often obtained by cleaving genomic DNA by the methylation sensitive restriction enzyme, *Pst*I. It is documented that single and low-copy sequences in plants tend to be under-methylated (Tanksley *et al.*, 1989) and hence use of *Pst*I is advantages in obtaining a DNA fraction highly enriched in single and low-copy DNA sequences.

It should be noted however, that Byrne *et al.*, (1994) have been able detect RELPs in *Eucalyptus* using high-copy clones. This may be a consequence of compact genome with a relatively low abundance of repeated DNA sequences in *Eucalyptus*. This also agrees well with the observation that there is not much difference in the efficiency of detection of RFLPs by random high-copy clones and cDNA clones in *Eucalyptus* (Byrne *et al.*, 1994). The *Populus* genome is also believed to be compact with fewer repetitive sequences because no differences were observed in the proportion of single clones from methylation sensitive *Pst*I fragments and methylation non-sensitive *Xba*I fragments which detected RFLPs (Bradshaw *et al.*, 1994).

The limited success achieved with the present RFLP assay therefore, is likely to be associated with the high-copy number of the clones chosen for assaying. The presence of the repetitive sequence family (Rohde *et al.*, 1992) in the coconut genome and the selection of high-copy clones detected by strong signals have combined to affect the efficiency of the detection of RFLPs in the current assay.

In conclusion it can be stated that use of low-copy clones from methylation sensitive *Pst*I fragments would have been more desirable for construction of RFLP probes for screening coconut genotypes. RFLP assays based on screening by low-copy clones have shown successful results in a range of plants. Gentzbittel *et al.* (1994) used 181 low-copy clones to probe DNA digested by four restriction enzymes (331 probe/enzyme combinations) and found 73 probes detected polymorphisms (40%) among inbred lines of the cultivated sunflower, *Helianthus annuus*. Besse *et al.*, (1994) obtained 25 clones that detected polymorphism among wild and cultivated populations of *Hevea brasiliensis*. Xu *et al.* (1994) checked 413 single or low-copy number sequences by probing DNA from parental lines digested with five enzymes, and found 210 (51%) detected polymorphisms in the parental lines with at least one of the restriction enzymes. Use of low-copy clones also has the additional advantage that there is no necessity to pre-screen plasmid clones for the presence of organelle DNA.

Southern blot hybridisation with mini satellite and microsatellite probes : Micro-satellites and mini-satellites are abundant, multiallelic, co-dominant markers uniformly distributed in the eukaryotic genome (Litt and Luty, 1989; Vassart *et al.*, 1987; Jeffereys, 1985). These are simple tandem repeats (variable number tandem repeats) revealing a high degree of variability and thus called DNA fingerprints (Jeffereys *et al.*, 1985). These fingerprints allow

identification of individuals in man, other mammals such as swine, birds, fungi and protozoa (Ryshkov *et al.* 1988; Rohrer *et al.*, 1994; Epplen, 1988; Rogstad *et al.*, 1988; Tautz, 1989). The linkage map of the human genome developed on the basis of segregation of the dinucleotide repeat, $(CA)_n$, is remarkable. This map comprises 813 markers in 23 linkage groups corresponding to 22 autosomes and the X chromosome (Weissenbach *et al.*, 1992).

The ability of these markers to identify individuals at the DNA level makes them useful for breeders to identify genotypes, varieties and cultivars and to determine genetic variability in populations. The presence of repetitive sequences in plant genomes and variability of sequences has been demonstrated in plants including rice (Dallas, 1988), *triticeae* species (Vershinin *et al.*, 1994), *Brassica* (Poulson *et al.*, 1993); turnip (Rogstad, 1994), tomato (Vosman *et al.*, 1992) and a range of plants, *Triticum aestivum, Secale cereale, Daucus carota saitvas, Brassica oleracea, Petunia hybrida, Nicotina tabacum, Horedeum vulgare* and *Beta vulgaris* (Beyermann *et al.*, 1992).

The results obtained from Southern blot hybridisation of coconut DNA with M13 (minisatellite), and oligonucleotide probes, $(CA)_9$, $(GAA)_6$, $(GAT)_8$, $(TAA)_6$, $(GTT)_8$ and $(GATA)_4$ were unsuccessful. The blots containing genomic DNA digested by restriction enzymes, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III and *Rsa*I failed to resolve bands when hybridised with M13. The presence of continuous smears along DNA tracts, except for a single band resolved in *Dra*I digests and another in EcoRI digests, were the only evidence for hybridisation to discrete fragments. .

The blots containing coconut DNA digested by *Dra*I, *Eco*RI and *Eco*RV, and hybridised with $(CA)_9$, $(GAA)_6$, $(GAT)_8$, $(TAA)_6$, $(GTT)_8$ and $(GATA)_4$, probes also failed to produce the expected DNA fingerprints. Changes made to the hybridisation conditions did not give any improvement or signs of resolving bands. However, with both the minisatellite, M13 and microsatellite, high molecular weight fragments of λ DNA hybridised well with probes indicating that the Southern transfers had been successful.

The conditions of hybridisation and stringency wash used in this study followed standard conditions used by most of the above authors who had successful results. Vosman *et al.* (1992) used different conditions of hybridisation and stringency wash and obtained well resolved bands after probing with $(GATA)_4$ in tomato DNA. The micro-satellite probe $(GATA)_4$ has detected microsatellite variations in a number of plants (*Brassica napps*, Poulson *et al.*, 1993; *Triticum aestivum*, *Secale cereale, Daucus carota sativas, Brasica oleracea, Petunia hybrida, Nicotina tabacum, Hordeum vulgare* and *Beta vulgaris*, Beyermann *et al.*, 1992). However, the $(GATA)_4$ probe failed to produce bands with coconut DNA under standard hybridisation conditions and also under the conditions used by Vosman *et al.* (1992).

These disappointing results parallel somewhat the observations of Thomas *et al.* (1993) on *Vitus Vinifera* who failed to detect microsatellites with GA, GT, CAC repeats and M13 with Southern blot technology. However, they detected bands with GATA repeats in the same study with the same experimental conditions. It appears that the application of Southern blot technique for detection of minisatellite and microsatellite polymorphisms for each genome has to be developed individually by varying conditions and restriction enzymes. On the other hand, cloning of sequences to identify adjacent sequences which can be used as primers to determine microsatellite variations by the PCR technique appears a better alternative because once the primers are made DNAs can be routinely screened at a rapid pace with little effort.

4.3 Relative merits of RFLPs and RAPDs as probable candidates for coconut DNA analysis

The present DNA assay, which was aimed towards detecting molecular markers for use in coconut breeding, proceed over a period of two years. RFLP and RAPD assays progressed simultaneously after the DNA isolation procedure was developed. The time spent on laboratory studies was about 16 months. The actual cost involved in the

study could not be estimated as budgeting of articles was not made separately for my study.

The RFLP study consisted of more than 60 Southern blot hybridisations. The procedures for these studies included; isolation of µg amounts of DNA from leaf samples, constructing DNA` libraries, restriction digestions, size fractionation of restriction fragments by agarose gel electrophoresis, Southern transfers, preparation of plasmids for probing, oligolabelling of probes, hybridisations, stringency washes and autoradiographing. The RFLP assaying progressed throughout the entire 16-month duration of the study. The RAPD study consisted of more than 150 PCR experiments, each involving a PCR amplification (automated) and detection of amplified products by agarose gel electrophoresis. The RAPD studies progressed over a period of nine months. In terms of time and simplicity of application, RAPD technique is definitely more appropriate than the RFLP technique for an "orphan" crop (Persley, 1990) like coconut which is grown primarily in the developing world. However, the relative merits of the two marker types will have to be judged by the actual ability of each marker to detect genetic variation.

The most desirable genetic markers are codominant, multiallelic, abundant in the genome of the chosen organism and applicable across reasonably wide phylogenetic distances. Ideally such markers are also easily, rapidly and inexpensively assayed. None of the above marker types meets all these criteria. RAPDs are undoubtedly the simplest and least expensive method for tracking DNA based markers but are dominant markers and hence relatively uninformative compared to RFLPs which are codominant and multiallelic markers.

RFLP and RAPD methods have different molecular properties. Each DNA band detected by Southern blot analysis using a restriction enzyme with a six base pair recognition sequence evaluates the template DNA for differences at 12 base pairs. On the other hand, each DNA band amplified using a single decamer primer is a result of an

interaction between primer and temp ate DNA at up to 20 base pairs (Bradshaw *et al.*, 1994). Heun and Helentjaris (1993) postulated the apparent variation resulting from RAPD markers exhibit is not due to point mutations in the recognition sites but rather to competition between PCR products. This was argued because a number of mapping studies have shown that many RAPD markers deviate from their expected patterns of inheritance. However, Thormann and Osborn (1992) interpreted this result as being due to non-homology of identically sized RAPD fragments, because RFLP and RAPD assays detected highly similar genetic distances among a set of Brassica napus individuals but gave different results when comparing among different Brassica species.

In addition several factors must be considered in using RAPD markers for assessing genetic variation in diploid outcrossing organisms. The first factor is the dominant nature of the RAPD markers. If a locus is polymorphic, with the recessive allele present at low frequency, almost all copies of that allele will be carried in heterozygotes and will go undetected by the RAPD assay, resulting in that locus being scored as monomorphic. This will lead to under estimation of true genetic variation. The second factor is the assumption that each fragment represents one RAPD locus with only two alleles, which corresponds to the presence and absence of the fragment observed on the gel. This is not always the case as deletion and insertion events that occur in the region flanked by the primers will result in an amplified fragment migrating into a different position, causing it to be scored as a separate locus rather than an additional allele at the first locus. This will lead to an over estimation of the true genetic variation

However, there are a number of comparative studies on RAPDs and RFLPs which favour the use of either technique or both in combination as suitable for genotyping of plants. Liu and Furnier (1993) examined the genetic variation between and within two species of *Populus*, *P. tremuloides* trembling aspen) and *P. grandidentata* (big tooth aspen) by RFLP and RAPD assay procedures. They used 138 random *Populus* genomic probes predominantly of low-copy number sequences and four decamer primers to assay RFLPs and RAPDs in 235 individuals. They have detected a considerable amount of

genetic variation between and within the two species by both types of markers, although RAPDs revealed greater variation than RFLPs with less effort per polymorphic locus detected.

Bradshaw *et al.* (1994) evaluated RFLPs and RAPDs for linkage map construction in the *Populus* genome using an inter-specific F_1 hybrid and F_2 populations. They have 232 RFLP probes (majority low-copy) and 180 decamer primers to detect RFLPs and RAPDs respectively. The results of this study concluded that a mixed marker map composed of a large number of RAPD markers and a smaller number of RFLP markers could take advantage of the attributes of the two marker types, simplicity, rapidity and low cost of RAPDs and the co-dominance of RFLPs.

Hallden *et al.* (1994) evaluated RFLP and RAPD markers in a comparison of *Bassica napus* breeding lines showing varying degrees of genetic relatedness. The RFLP and RAPD assays detected highly similar relationships between the genotypes screened. Ragot and Hoisington (1993) compared costs involved in genotyping by RFLPs and RAPDs. The results revealed RAPDs as most economical for small and medium size projects and RFLPs for more extensive genotyping projects.

4.4 Conclusions

The result of the present study provide clear evidence of the potential of RAPDs as useful molecular markers in coconut breeding and conservation strategies. Without any prior knowledge of the coconut genome a reasonably good number of RAPD markers were obtained allowing the study of genetic relationships in 19 coconut accessions, within a period of about six months. The results were consistent with morphological based classifications.

RFLP technique is much more difficult to develop within a short period of time because it requires prior knowledge of the genome and more expertise in molecular

biological techniques. The repetitive sequences in the coconut genome hindered the detection of useful RFLP markers and thus it is suggested that a DNA library consisting of low-copy clones would be a better choice for screening RFLPs.

The RAPD technique demonstrated its potential as a valuable marker system for tagging useful traits and as a tool to discriminate between groups within the coconut species. The limited assessment of varieties, forms and germplasm accessions exhibited that exploitable genetic variation within the coconut population in Sri Lanka. Confirmation of merging of the *San Ramon* form with *Nana* forms by two different biochemical markers is also a noteworthy discovery.

The usefulness of RAPDs in generating genetic maps was demonstrated when Reiter *et al* (1992) placed over 250 RAPD markers on a recombinant inbred population of *Arabidopsis thalina* in four person months. However, the dominant inheritance of RAPD markers poses a problem with plants that lack inbred populations in genetic mapping or back cross populations (Tingey and del Tufo, 1993). Plants that have the ability to produce dihaploids can overcome this problem without going through a rigorous series of selfing or back crossing to produce homozygous lines (Powell, 1990). For the coconut palm neither of these techniques are immediately available except for the existence of F_1 populations from carefully set out crosses. Establishment of sufficient F_2 intercross and backcross populations would allow a possibility of attempt at generating RAPD linkage map.

There is still an immediate possibility for tagging of simple genetic traits by application of the bulk segregant analysis. This method analyses two bulked DNA samples gathered from individuals segregating in a single population by RAPD markers. Each bulk is composed of individuals that differ for a specific phenotype or individuals at either extreme of a segregating population (Michelmore *et al.*, 1991). Therefore, characters such as resistance to diseases (eg. lethal yellowing, cadang cadang) can be targeted by RAPD markers.

One of the key findings of this study is the demonstration that RAPDs can be used to genetically fingerprint coconut populations. This will have a major impact on the management of coconut genetic resources. The coconut germplasm maintained in gene banks can be characterised rapidly by RAPD markers. The conventional characterisation by scoring the coconut genetic descriptors laid down by the International Board for Plant Genetic Resources (IBPGR) is a long procedure taking about 10 years. Further development of RAPD makers would also assist in the elimination of duplicates, allow rationalisation of existing gene banks and allow future germplasm expeditions to be targetted toward collection of material possessing maximum levels of genetic diversity.

References

Abdelnoor, R.V., Barros, E. G. D. and Moreiar, M. A. (1995). Determination of genetic diversity within Brazilian soybean germplasm using RAPD technique and comparative analysis with pedigree data. *Revista Brasilaria de Genetica* **18** : 265 - 273

Adam-Blondon, A. F., Sevignac, M., Bannerot, H. and Dron, M. (1994). SCAR, RAPD and RFLP markers linked to a dominant gene (are) conferring resistance to anthracnose in common bean. *Theoretical and Applied Genetics* **88** : 865 - 870

Arens, P., Odinot, P., van Heusden, A. W. Lindhout, P. and Vosman, B. (1995). GATA- and GAGC- repeats are not evenly distributed through the tomato genome. *Genome* **38** : 84 - 90

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1989). Current protocols in molecular biology. Vol. 1. John Wiley and Sons New York

Baht and Jarret (1995). Random Amplified Polymorphic DNA genetic diversity in Indian Musa germplasm. *Genetic Resources and Crop Evolution* **42** : 107 - 118

Bardakci, F. and Skibinski, D. O. F. (1994). Application of the RAPD technique in *Tilapia* fish : species and subspecies identification. *Heredity* **73** : 117 - 123

Baruffi, L., Damiani, G., Guglielmino, Bandis, C., Malacrida, A. R. and Gasperi, G. (1995). Polymorphism within and between populations of *Ceratitis capitata*: comparison between RAPD and multilocus enzyme electrophoresis data. *Heredity* **74** : 425 - 437

Beckmann , J. S. and Soller, M. (1983). Restriction fragment length polymorphisms in genetic improvement: methodologies, mapping and costs. *Theoretical and Applied Genetics* **67** : 35 - 43

Bell, G.I., Selby, N.J. and Rutter, W.J. (1982). The highly polymorphic region near the human insulin gene is composed of simple tandemly repeating sequences. *Nature* **295**: 33-35

Benabadis, B. K. (1992). Coconut and date palm *in* Biotechnology of perennial fruit crops ed. Hammersdilag and Litz, K. E. C. A. B. International pp 383 - 400

Benet, H., Guries, R. P. Boury, S. and Smally, E. B. (1995). Identification of RAPD markers linked to a black leaf spot resistance gene in Chinese elm RAPD. *Theoretical and Applied Genetics* **90** : 1068 - 1073

Benoit, H. (1979). Isozymic variation between within and between coconut populations. Paper presented during the 5th session of the FAO Technical Working Party on Coconut Production, Protection and Processing, Manila, December 3 - 8, 1979.

Besse, P., Lebrun, P., Seguin, M. and Lanaud, C. (1993). DNA fingerprints in *Hevea brasiliensis* (rubber tree) using human minisatellite probes. *Heredity* **70** : 237 - 244

Besse, P., Seguin, M., Lebrun, P., Chevallier, M. H., Nicolas, D. and Lanaud, C. (1994). Genetic diversity among wild and cultivated populations of *Hevea brasiliensis* assessed by nuclear RFLP analysis. *Theoretical and Applied Genetics* **88** : 199 - 207

Beyermann, B., Nurnberg, P., Weihe, A., Meixner, M., Epplen, J. T. and Borner, T. (1992). Fingerprinting plant genomes with oligonucleotide probes specific for simple repetitive DNA sequences. *Theoretical and Applied Genetics* **83** : 619 - 694

Bonierbale, M. W., Plaisted, R. L. and Tanksley, S. D. (1988). RFLP maps based on common set of clones reveal modes of chromosomal evolution in potato and tomato. *Genetics* 120 : 1095 - 1103

Bourquin J. C., Sonko, A., Otten., L. and Walter, B. (1993). Restriction fragment length polymorphism and molecular taxonomy in *Vitis vinifera* L. *Theoretical and Applied Genetics* **87** : 431 - 438

Bradshaw Jr, H. D., Villar, M., Watson, B. D., Otto, K. G., Stewart, S. and Stettler, R. F. (1994). Molecular genetics of growth and development in *Populus*. III. A genetic linkage map of a hybrid poplar composed of RFLP, STS and RAPD markers. *Theoretical and Applied Genetics* **89** : 167 - 178

Bradshaw Jr, H. D. and Stettler, R. F. (1995). Molecular genetics of growth and development in *Populus*. IV. Mapping QTLs with large effects on growth, form and phenology traits in a forest tree. *Genetics* **139** : 963 - 973

Britton, R. J. and Khone, D. E. (1963). Repeated sequences in DNA. *Science* **161** : 529 - 540

Brown, A. H. D. and Wier, B. S. (1983). Measuring genetic variability in plant populations: *in* Isozymes in plants genetics and breeding, Part A. S. D. Tanksley and T. J. Orton eds. pp 219 - 239. Elsvier Amsterdam.

Brubaker, C. L. and Wendel, J. F. (1994). Reevaluating the origin of domesticated cotton (*Gossypium hirsutum* : malvaseae) using nuclear restriction fragment length polymorphisms (RFLPs). *American Journal of Botany* 81 : 1309 - 1326

Brunel, D. (1992). An alternative, rapid method of plant DNA extraction for PCR analyses. *Nucleic Acids Research* **20** : 4676

Burr, B., Burr, R. A. and Thompson, K. H., Albertson,M.C. and Stuber, C.W. (1988) Gene mapping with recombinant inbreds in maize. *Genetics* **118**: 519 - 526

Byrne, M., Moran, G. F., Murrell J. C. and Tibbits, W. N. (1994). Detection and inheritance of RFLPs in *Eucalyptus nitens*. *Theoretical and Applied Genetics* **89** : 397 - 402

Caetano-Anolles, G., Bassam, B. J. and Gresshoff, M. (1991). DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Biotechnology* **19** : 553 - 557

Cai, Q., Guy, C. L. and Moore G. A. (1994). Extension of the linkage map in citrus using RAPD markers and RFLP mapping of cold acclimation responsive loci. *Theoretical and Applied Genetics* **89** : 606 - 614

Campus, L. P., Raelson, J. V. and Grant, W. F. (1994). Genome relationships among lotus species based on RAPD. *Theoretical and Applied Genetics* **88** : 417 - 422

Carpio, C. B. (1982). Biochemical studies of Cocos nucifera L. Kalikasan. Philippines Journal of Biology **11**: 319 - 338

Castagnone-Sereno, P., Vanlerberghe - Masutti, F. and Leroy, F. (1994). Genetic polymorphism between and within *Meloidogyne* species detected with RAPD markers. *Genome* **37** : 904 - 909

Chalmers, K. J., Newton, A. C., Waugh, R., Wilson, J. and Powell, W. (1994). Evaluation of the extent of genetic variation in mahoganies (Meliaceae) using RAPD markers. *Theoretical and Applied Genetics* **87** : 314 - 320

Chalmers, K. J., Barua, U. M. Hackett, C. A., Thomas, W. T. B. Waugh, R. and Powell (1993). Identification of RAPD markers linked to genetic factors controlling the milling energy requirement in barley. *Theoretical and Applied Genetics* **89** : 504 - 508

Chang, C., Bowman, A. W. DeJohn, Lader, L., and Meyerowitz, M. (1988). RFLP linkage map for *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, **85** : 6831 - 6835

Connaly, A. G., Goodwin, I.D. Cooper, M. and Delacy, I. H. Interpretation of RAPD marker data for fingerprinting sweet potato genotypes. *Theoretical and Applied Genetics* **88** : 332 - 336

Corniquel, B. and Mercier, L. (1994). Date palm (*Phonix dactylifera* L) cultivar identification by RFLP and RAPD. *Plant Science* **101** : 163 - 172

Cui, Y. X., Xu, G. W., Magill, C. W., Scherts, K. F. and Hrt, G. E. (1995). RFLP based assay of Sorghum bicolour (L) Moench genetic diversity. *Theoretical and Applied Genetics* **90** : 787 - 796

Dallas, J. F. (1988). Detection of DNA fingerprints of cultivated rice by hybridisation with a human minisatellite DNA probe. *Proceedings of the national Academy of Sciences USA* 85 : 6831 - 6835

Daniel, K., Chong, X., Yang, R. C. and Yeh, F. C. (1994). Nucleotide divergence between populations of trembling aspen (Populus tremuloides) estimated with RAPDs. *Current genetics* 0(: 374 - 376.

Dax, E., Livneh, O., Edelbaum, O., Kedar, N., Gavish, N., Karchi, H., Milo, J., Sela, I. and Rabonowitch, H. D. (1994). A RAPD molecular marker for the Tm2a gene in tobacco. *Euphytica* **74:** 159 - 163

de Nuce de Lamothe, M. and Wuicart, W. (1981). Les cocotiers grands a port Bouet (Cote d'Ivore) II. Grand rennell, Grand salomon, Grand thailande, Grand Nouvelles Hebrides. *Oleagineux* **36** : 353 - 365

del Rosario, A. G. and de Guzman, E. V. (1976). The growth of coconut 'Makapuno' embryos in vitro as effected by mineral composistion and sugar level of the medium during the liquid and solid culture. *Phillipine Journal of Science* **105** : 215 - 222

Dellaporte, S. L., Wood, J. and Hicks, J. B. (1983). A plant DNA minipreparation: version II. *Plant Molecular Biology Reporter* **1**: 19 - 21

Dos-Santos, J. B., Niehuis, J., Skorch, P., Tivang, J. and Slocum, M. K. (1994). Comparison of RAPD and RFLP genetic markers in determining genetic similarity among *Brassica oleracea* L. genotypes. *Theoretical and Applied Genetics* **87** : 909 - 915

Dover, G. (1980). Ignorant DNA? *Nature* **285**: 47 - 49 VNTRs

Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988). High efficient transformation of *E. coli* by high voltage electroporation *Nuceleic Acids Research* **16**: 6127-6145

Echt, C. T., Kidwell, K. K., Knapp, S. J., Osborn, T. C. and McCoy, T. J. (1994). Linkage mapping in diploid alfalfa. *Genome* **37**: 61 - 71

Edwards, K., Johnstone, C. and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* **19** : 1349

Epplen, J. T., Ammer, H., and Kammerbauer, C. (1991). Oligonucleotide fingerprinting using simple repeat motifs. T. Burke, G Dolf, A. J. Jefferies and R. Wolf (eds) In: DNA fingerprinting approaches and applications pp 50 - 69

Everard, J. M. D. T., Peries R. R. A., Fernando, W. B. S., Kularatne, H. S. G., Padmasiri, M. H. L. and Fernando, M. A. S. (1990) Pollination of selected palms at the isolated seed garden, Ambakelle, in different combinations and evaluation of the progeny. *Annual report of the Coconut Research Institute of Sri Lanka.* R. Mahindapala (ed.). pp 42 - 52

Everard, J. M. D. T., Peries R. R. A., Fernando, W. B. S., Kularatne, H. S. G., Padmasiri, M. H. L., Jayatilake, R. and Attanayake, R. B. (1991) Pollination of selected palms at the isolated seed garden, Ambakelle, in different combinations and evaluation of the progeny. *Annual report of the Coconut Research Institute of Sri Lanka.* R. Mahindapala (ed.). pp 49 - 61

Everard, J. M. D. T., Peries R. R. A., Fernando, Fernando, W. B. S., Padmasiri, M. H. L., Jayatilake, R., Abeysekera, J. D. J. and Attanayake, R. B. (1992, 1993) Pollination of selected palms at the isolated seed garden, Ambakelle, in different combinations and evaluation of the progeny. *Annual report of the Coconut Research Institute of Sri Lanka.* R. Mahindapala (ed.). unpublished

Fatta-Del-Bosco, S. (1993). Use of chemiluminescent system for non-radioactive RFLP analysis on Citrus. *Journal of Genetics and Breeding* **47** : 183 -185.

Faure, S., Noyer, J. L., Horry, J. P., Bakry, F., Lanaud, C. and Gonzales D. L. D. (1993). A molecular marker-based linkage map of diploid banana (*Musa accuminata*) *Theoretical and Applied Genetics* **87** : 517 - 526

Fereira M. E., Williams, P. H. and Osborn, T. C. (1994) RFLP mapping of *Brassica napus* using doubled haploid lines. *Theoretical and Applied Genetics* **89** : 615 - 621

Fernando, W. M. U. (1987). San Ramon big with promise. *Coconut bulletin* **4**: 1

Fernando, W. M. U., Kondasinghe, . J. S., Ratnasiri, W. G. A. and Padmasiri, M. H. L. (1989) Pollination of selected palms at the isolated seed garden, Ambakelle, in different combinations and evaluation of the progeny. *Annual report of the Coconut Research Institute of Sri Lanka.* R. Mahindapala (ed.). 50 - 53

Fernando, W. M. U., Everard, J. M D. T., Peries R. R. A., Fernando, Fernando, W. B. S., Padmasiri, M. H. L., Jayatilake, R., Abeysekera, J. D. J. and Attanayake, R. B. (1994) Pollination of selected palms at the isolated seed garden, Ambakelle, in different combinations and evaluation of the progeny. *Annual report of the Coconut Research Institute of Sri Lanka.* R. Mahindapala (ed.) Unpublished

Fernando, W. M. U., Fernando, S., Vidanaarachchi, V., Peries R. R. A., Everard, J. M. D. T., Periyapperuma, K and Karunaratne, S. (1995). Coconut biotechnology research with emphasis on tissue culture - The Sri Lankan experience. Unpublished report. Coconut Research Institute of Sri Lanka.

Friar and Kochert (1994). Phylogeny of wild and cultivated *Solanum* species based on nuclear restriction fragmen length polymorphisms (RFLPs). *Theoretical and Applied Genetics* **79** : 360 - 368

Gardiner, S. E., Shu, J. M., Whitehead, H. C. M. and Madie, C. (1994). The New Zealand apple genome mapping project. *Euphytica* **77**: 77 - 81

Gebhardt, C., Ritter, E., Debner, T., Schachtschabel, U., Walkemeier, B., Uhrig, H. and Salamani, F. and. (1989). RFLP analysis and linkage mapping in *Solanum tuberosum. Theoretical and Applied Genetics* **78** : 65 - 75

Gebhardt, C., RFLP mapping in potato of qualitative and quantitative genetic loci conferring resistance to potato pathogens. *American Potato Journal* **71** : 339 - 345

Gentzbittel, L., Zhang, Y. X., Vear, F., Griveau, B. and Nicolas, P. (1994). RFLP studies of genetic relationships among inbred lines of the cultivated sunflower, *Helianthus annuus* L, : evidence for distinct restorer and maintainer germplasm pools. *Theoretical and Applied Genetics* **89** : 419 - 425

Gentzbittel, L., Zhang, Y. X., Vear, F., Griveau, B. and Nicolas, P. (1994). Development of a consensus RFLP map of cultivated sunflower (*Helianthus annuus* L,). *Theoretical and Applied Genetics* **90** : 1079 - 1086

Gerdes, J. T. and Tracy, W. F. (1994) Diversity of historically important sweet corn inbreds as estimated by RFLPs, morphology, isozymes and pedigrees. *Crop Science* **34** : 26 - 33

Goldman, I. L., Rocheford, T. R. anc Dudley J. W. (1994). Molecular markers associated with maize kernel oil concentration in an Illinois high protein x Illinois low protein cross. *Crop Science* **34** : 908 - 915

Gonsales, J. M. and Ferrer, E. (1993). Random amplified polymorphic DNA analysis in *Horedeum* species. *Genome* **36** : 1029 - 1031

Grattapaglia, D and Sederoff (1995 . Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo test cross. *Genetics* **79** : 187 - 193

Griffin, A. R. and Keil, M. (1994). Use of RAPD in the discrimination and verification of genotypes in Mahogony. *Theoretical and Applied Genetics* **90** : 998 - 1006

Groover, A., Devey, M., Fiddler, T. (1994). Identification of quantitative trait loci influencing wood specific gravity in an outbred pedigree of loblolly pine. *Genetics* **138** ; 1297 - 1300

Guidet, F. (1990). Rapid method for plant DNA extraction. *Nucleic Acid Research* **18** : 4955

Guise, H., Jenson, H. A. G., Mathiassen, H (1994). Distribution of RAPDs on linkage map of barley. *Hereditas* **120** : 267 - 273

Hallden, C., Nilsson, N. O., Rading, I. M. and Sall, T. (1994). Evaluation of RFLP and RADP markers in a comparison of *Brasica napus* breeding lines. *Theoretical and Applied Genetics* **88** : 123 - 128

Harries, H. C. (1978). Evolution, dissemination and classification of *Cocos nucifera*. *Botanical review* **44** : 265 - 320

Harrison, N. A., Bourne, C. M., Cox, R. L., Tsai, J. H. and Richardson, P. A. (1992). DNA probes foe detection of mycoplasma-like organisms associated with lethal yellowing disease of palms in Florida. *Phytopathology* **82 :** 216 - 224

Hart, L., Weiss, H, Stephan, U., Zeller, F. J. and Jahoor, A. (1995). Molecular identification of powdery mildew resistance in common wheat. *Theoretical and Applied Genetics* **90** : 601 - 606

Havey, M. J. and Muehlbauer, F. J. (1989). Variability for restriction fragment lengths and phylogenies in lentil. *Theoretical and Applied Genetics* **77** : 839 - 843

Heun, M., and Helentjaris, T. D. (1993). Inheritance of RAPDs in F_1 hybrids of corn. *Theoretical and Applied Genetics* **85** : 961 - 968

Heun, M., Murphy, J. P. and Phillips, T. D. (1994). A comparison of RAPD and isozyme analyses for determining the genetic relationships among *Avena sterilis* L. accessions. *Theoretical and Applied Genetics* **87** : 689 - 696

Hu. J. and Quiros, C. F. (1991). Identification of broccoli and cauliflower culivars with RAPD markers. *Plat Cell Reporter* 10 : 505 - 511

Iqbal, M. J. and Rayburn , A. L. (1994). Stability of RAPD markers for determining cultivar specific DNA profiles in rye. *Euphytica* 75 : 215 - 220

Jack, P. L., Dimitrijevic, T. A. F. and Mayes, S. (1995). Assessment of nuclear, mitochondrial and chloroplast RFLP markers in oil palm (*Elaeis guineensis*). *Theoretical and Applied Genetics* **90** : 643 - 649

Jain, A., Bhatia, S., Banga, S. S., Prakash, S. and Laksmikumaran, M. (1994). Potential use of random amplified polymorphic DNA (RAPD) technique to study the genetic diversity in Indian mustard (*Brassica juncea*) and its relationship to heterosis. *Theoretical and Applied Genetics* **88** : 116 - 122

Jarret, R. L. and Austin, D. F. (1994). Genetic diversity and systematic relationships in sweet potato (*Ipomoea batatas*) and related species as revealed by RAPD analysis. *Genetic Resources and Crop Evolution* **41** : 165 - 173

Jay, P., Bourdex, R., Potier, F. and Sanlaville, C. (1989). Note on polymorphism of coconut leaf polyphenols. *Oleagineux* **44** : 151 - 161

Jayasekera, C. (1979). *Annual report of the Coconut Research Institute of Sri Lanka*. M. Pethiyagoda (ed), pp 38 - 39

Jeffreys, A. J., Wilson, V. and Thein, S. L. (1985a). Individual-specific 'fingerprints' of human DNA. *Nature* **316** : 76 - 79

Jeffreys, A. J., Brookfield, J. F. Y. and Semeonoff, R. (1985b). Positive identification of an immigration test-case using human DNA finger prints. DNA. *Nature* **317** : 818 - 819

Jefferys, A. J., Royle, N. J. , Patel, I (1991). Principles and recent advances in human DNA fingerprinting. In DNA fingerprinting approaches and applications. T. Burke, G. Dolf, A.J. Jefferies and R. Wolff (eds). pp 1 - 19

Jermstad, K. D., Reem, A. M., Henifin, J. R., Wheeler, N. C. and Neale, D. B. (1994). Inheritance of restriction fragment length polymorphisms and random amplified polymorphic DNAs in coastal Douglas-fir. *Theoretical and Applied Genetics* **89** : 758 - 766

Jones, L. H. (1989). Prospects of biotechnology in oil palm (*Elaeis* guineensis) and coconut (*Cocos nucifera*) improvement. *Biotechnology and genetic engineering reviews*. **7** : 281 - 296

Jones, L. H. (1991). Perennial vegetable oil crops *in* Persley, G. J. ed., Agricultural Biotechnology: Opportunities for international development. CAB International, Wallingford, pp. 213 - 224

Kangfu, Y and Pauls, K. P. (1993). Rapid estimation of genetic relatedness among heterogeneous populations of alfalfa by rapid amplification of bulked genomic DNA samples. *Theoretical and Applied Genetics* **86** : 788 - 794

Karunaratne, S., (1988). Short-term *in vitro* preservation of coconut seed material; A method to facilitate field collection and transport of coconut germplasm. *Cocos* **4** : 40 - 47

Karunaratne, S., Kurukularachchi, C and Gamage, C. (1985). A report on the culture of embryos of dwarf coconut. *Cocos* **3** : 1 - 8.

Karunaratne, S. and Periyapperuma, K. (1989). Culture of immature embryos of coconut, Cocos nucifera L. : Callus proliferation and somatic embryogenesis. *Plant Science* **62** : 247 - 253

Karunaratne, S., Gamage, C. and Kovoor, A. (1991a). Leaf maturity a critical factor in embryogenesis. *J. Plant Physiology* **139** : 27 - 31

Karunaratne, S., Santha, S.S. and Kovoor, A. (1991b). An in vitro assay for drought tolerant coconut germplasm. *Euphytica* **53** : 25 - 30

Karihaloo, J. L., Brauner, S. and Gottlieb (1994). RAPD variation in the egg plant. *Theoretical and Applied Genetics* **90** : 767 - 770

Kazan, K., Manners, J. M. and Cameron, D. F. (1993). Genetic variation in agronomically important species of *Stylosanthus* determined using random amplified polymorphic DNA markers. *Theoretical and Applied Genetics* **85** : 882 - 888

Keil, M. and Grifin, A. R. (1994). Use of random amplified polymorphic DNA (RAPD) markers in the discrimination and verification of genotypes in *Eucalyptus*. *Theoretical and Applied Genetics* **89** : 442 - 450

Keim, P., Diers, B. W. and Shoemaker, R. C. (1990). Genetic analysis of soybean with molecular markers. *Theoretical and Applied Genetics* **79** : 116 - 122

Kennard, W.C., Potter, K., Dijkhu zen, A., Meglic, V., Staub, E. E. and Havey, M. J. (1994). Linkage among RFLP, RAPD, isozyme, disease resistance and morphological markers in narrow and wide crosses of cucumber. *Theoretical and Applied Genetics* **89** : 42 - 48

Khadari, B., Lashermes, P. and Kje lberg, F. (1995). RAPD fingerprints for identification and genetic characterisation of fig genotypes. *Journal of Genetic and Breeding* **49**:77 - 85

King, G., Alston, F. H., Battle, I., Chevreau, E., Gessler, C., Janes, J., Lindhout, Manganaris, A. G. (1991). The 'European Apple Genome Mapping Project' - developing strategy for mapping genes coding for agronomic characters in tree species. *Euphytica* **56** : 89 - 94

Kirkpatrick, B., Stenger, D.C., Morris, T. J. and Purcell, A. H. (1987). Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. *Science***238**:197 - 199

Klien-Lankhorst, R., Rietweld, P. and Machieels (1991). RFLP markers linked to the root knot nematode resistance gene in tomato. *Theoretical and Applied Genetics* **81**:667 - 667

Kochert, G., Halward, T. Branch, W. D. and Simpson, C. E. (1991). RFLP variability in peanut (*Arachis hypogaea* L) cultivars and wild species. *Theoretical and Applied Genetics* **81**:565 - 570

Koester, R. P., Sisco, P. H. and Stuber, C. W. (1993). Identification of QTLs controlling days to flowering and plant height in two near isogenic lines of maize. *Crop Science* **33**: 1209 - 1216

Koller, B., Lehmann, A., McDermott, J. M. and Gessler, C. (1993). Identification of apple cultivars using RAPD markers. *Theoretical and Applied Genetics* **85**:901-904

Korzum, V., Karte, N. Plaschke, J. and Boerner (1994). Construction and screening of a rye DNA library for RFLP mapping. *Cereal Research Communications* **22**:151-157

Kubisiak T. L. Nelson, C. D., Nance, W. L. and Stine, M. (1995). RAPD linkage mappingin a long leaf pine x slash pine. *Theoretical and Applied Genetics* **9** 1119-1127

Landry, B. S., Li, R. Q., Cheung, W. Y. and Granger, R. L. (1994). Phylogeny analysis of 25 apple root stocks using RAPD markers and tactical gene tagging. *Theoretical and Applied Genetics* **89** : 847 - 852

Landau Ellis, D., and Gresshoff, P. M. (1994). The RFLP molecular marker closely linked to the supernodulation locus of soybean contains three inserts. *Molecular Plant Microbe Interactions* 7 : 432 - 433

Laurent, V., Risterucci, A. M. and Lanaud, C. (1994). Genetic diversity in cocoa revealed by cDNA probes. *Theoretical and Applied Genetics* **88** : 193 - 198

Lee, Y. H., Jeon, H. J. (1995) Use of RAPDs for linkage group analysis in interspecific hybrid F-2 generation of Cucurbita. *Journal of Korean Society of Horticultural Science* **36** : 323 33

Lindhout, P., Van-Heusden, S., Pet, G., Van-Ooijen, J. W., Sandbrink, H., Verkerk, R. and Zabel, P. (1994). Perspectives of molecular marker assisted breeding of earliness in tomato. *Euphytica* **79** : 279 - 286

Liu, Z. and Furnier, G. R. (1993). Comparison of allozyme, RFLP and RAPD markers for revealing genetic variation within and between trembling aspen and big tooth aspen *Theoretical and Applied Genetics* **87** : 97 - 105

Liyanage, D. V. (1958). Varieties and forms of coconut palms grown in Ceylon. *Ceylon Coconut Quarterly* **9** : 1 - 10

Liyanage, D. V. and Abeywardene, V. (1958). Correlations between seed nut, seedling and adult palm characters in coconut. *Tropical Agriculture* **CXIII** : 1 - 16

Liyanage, D. V. (1985). Consultants report on coconut breeding strategies. Coconut Research Institute of Sri Lanka.

Liyanage, D. V. (1967). Identification of genotypes of coconut palms suitable for breeding. *Experimental Agriculture* **3** : 205 - 210

Liyanage, D. V., Wickramaratne, M. R. T. and Jayasekera, C. (1988). Coconut breeding in Sri Lanka : a review. *Cocos*, **6** : 1 - 26

Lu, Y. H., D'Hont, A., Paulet, F., Grivet, L., Arnaud., M. and Glaszmann, J. C. (1995). Molecular diversity and genome structure in modern sugarcane varieties. *Euphytica* 78 : 217 - 226

Mahe, A., Bannerot, H., and Grisvard, J. (1995) Construction of near isogenic lines to investigate the efficiency of different resistant genes to anthracnose. *Theoretical and Applied Genetics* **90** : 859 - 864

Mahindpala, R. and Pinto J. L. J. G. (1991). Coconut cultivation. Coconut Research Institute of Sri Lanka. 162 pp

Mailer, R. J., Scratch, R. and Frister sky, B. (1994). Discrimination among cultivars of rapeseed (*Brassica napus* L.) use DNA polymorphisms amplified by arbitrary primers. *Theoretical and Applied Genetics* **87**: 697 - 704

Manak, M. M. (1993). Sample preparation *in* DNA probes (ed. Keller, G. H. and Manak, M. M.) pp 27 - 65

Manak, M. M. (1993). Radioactive labelling procedures *in* DNA probes (ed. Keller, G. H. and Manak, M. M.) pp 137 - 170

Manak, M. M. (1993). Hybridisation formats and detection procedures *in* DNA probes (ed. Keller, G. H. and Manak, M. M.) pp 199 - 220

Manicad, G. (1995). Biotechnology and the Philippine coconut farmers. *Biotechnology and development monitor*, **23**: 7 - 10

Manthrirtane, M . Personal communication

Maio, J. J. Brown, F. L. and Musich, P. H. (1977). Sub unit structure of chromatin and the organisation of eukaryotic repetitive DNA. *Journal of Molecular Biology* : **117** : 637 - 655

Marmey, P. , Beeching, J. R., Hamon, S. and Charrior, A. (1994). Evaluation of cassava germplasm collections using RAP markers. *Euphytica* **74 :** 203 - 209 cassava RAPD general

Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). Molecular cloning: A Laboratory Manual. Cold Spring harbour Laboratory. USA

Martin, B., Nienhuis, J., King, G. and Schaefer, A. (1989). Restriction fragment length polymorphisms associated with water use efficiency in tomato. *Science* **243**: 1725 - 1728

Mass, H. I. and Class, M. (1995). Intraspecific differentiation of Garlic by isozyme and RAPD makers. *Theoretical and Applied Genetics* **91** : 89 - 97

McCouch, S.R., Kochert, G., Yu, H., Wang, Z.Y., Khush, G. S., Coffman, W. R. and Tanksley, S. D. (1988). Molecular mapping of rice chromosomes. *Theoretical and Applied Genetics* **76** : 815 - 829

McMullen, M. D., and Louie R. (1989). The linkage of molecular markers to a gene controlling the symptom response in maize to maize dwarf mosaic virus. *Molecular Plant Microbe Interactions* 2 : 309

McMullen, M. D., Jones, M. W., Simcox, K. D. and Louie R. (1994). Three genetic loci control resistance to wheat streak mosaic virus in maize inbred pa405. *Molecular Plant Microbe Interactions* 7 : 708 - 712

Michelmore, R. W. Paran, I. and Kesseli, R. V. (1991). Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the national Academy of Sciences of USA* **88** : 9828 - 9832.

Michelson, I., Zamir, D., and Czosnek, H. (1994). Accumulation and translocation of tomato yellow leaf curl virus in a Lycorpersicon breeding line containing the L chilense TYLCV tolerance gene Ty-1. *Phytpathology* **84** ; 928 - 933

Miller, J. C. and Tanksley, S. D. (1990). Effect of different restriction enzymes, probe, source and probe length on detecting restriction fragment length polymorphisms in tomato. *Theoretical and Applied Genetics* **80** : 385 - 389

Mohan, M., Nair, S., Bentur, J. S. Rao, U. P. and Bennet, J. (1994). RFLP and RAPD mapping of the rice Gm2 gene that confers resistance to biotype 1 of gall midge. *Theoretical and Applied Genetics* **87** : 782 - 788

Mukai Y., Suyama, Y., Tsumura, Y.. Kawahara and T. Murai, M. (1995). A linkage map for sugi based on RFLP and RAPD and isozyme loci. *Theoretical and Applied Genetics* **90** : 835 - 840

Murray, M. G. and Thompson, W. F. (1980). Rapid isolation of high molecular weight DNA. *Nucleic Acids Research* **8:** 4321 - 4325

N'Goran, J. A. K., Laurnet, V. and Risteerucci, A. M. and Lanaud, C. (1994). Comparative genetic diversity studies of *Theobroma cacao* L using RFLP and RAPD markers. *Heredity* **73** : 589 - 597

Nadimpillai, R. G., Jarret, R. L. and Kochert, G. (1993). Phylogenetic relationships of the pigeon pea (*Cajanus cajun* based on nuclear fragment length polymorphisms. *Genome* **36** : 216 - 223

Nair , S., Bentur, J. S., Rao, U. P. and Mohan, M. (1995) DNA markers tightly linked to Gall midge resistance gene are potentially useful for marker aided selection in rice breeding. *Theoretical and Applied Genetics* **91** : 68 - 73

Nakamura, Y., Leppert, M., O'Connell, P., Wolf, R., Holm,T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E. and White, R. (1992). VNTR markers for human gene mapping. *Science* **235** : 1616 - 1622

Nesbitt, K. A., Potts, B. M., Vaillancourt, R. E., West, A. K. and Reid J. B. (1995). Partitioning and distribution of RAPD variation in forest tree species, *Eucalyptus globulous*. *Heredity* **74** : 628 - 637

Nienhuis, J. T., Heletjaris, T., Solcum, M., Ruggero, B., and Scaefer, A. (1987). RFLP analysis of loci associated with insect resistance in tomatoe. *Crop Science* **27** : 297

Nybom, H. (1995). Evaluation of interspecific crossing experiments in facultatively apomictic blackberries (*Rubus* subgen. *Rubus*) using DNA fingerprinting. *Hereditas* **122** : 57 -65

Ohler, J. G. (1984). Coconut, tree of life. Plant production and protection paper 57. FAO. Rome.

Ohmori, T., Murata, M., and Motoyoshi (1995). RAPD markers linked to the tobacco mosaic virus gene, Tm-1, in omato. *Japanese Journal of Genetics* **70** : 179 -184

Orozco-Castillo, C., Chalmers, K. J., Waugh, R. and Powell, W. (1994). Detection of genetic diversity and selective gene introgression in coffee using RAPD markers. *Theoretical and Applied Genetics* **87** : 934 - 940

Pammi, S., Schertz, K., Xu, G., Hart, G. and Mullet, J. E. (1994). Random amplified polymorphic DNA markers n sorghum. *Theoretical and Applied Genetics* **89** : 80 - 88

Parent, J. and Page, D. (1992). Identification of Raspberry cultivars by nonradioactive DNA fingerprinting. *HortScience* **27** : 1108- 1110

Park, Y. H. and Kohel, R. J. (1994). Effect of concentration of $MgCl_2$ on RAPD. *Biotechniques* **16** : 652 - 654

Patterson, A. H., Lander, E. S., Hewitt, J. D. and Tanksley, S.D. (1988). Resolution of quantitative traits into Mendelian factor by using a linkage map of RFLPs. *Nature* **335** : 721 -726

Pe, M. E., Gianfranceschi, L., Taramino, G., Tarchini, R., Angelini, P., Dani, M., and Binelli, G. (1995) Mapping QTLs for resistance to *Gibberella zeae* infection in maize. *Molecular and General Genetics* **: 241** : 11 - 16

Penner, G. A., Chong, J., Wight, C. ². Molnar, S. J. and Fedak, G. (1993) Identification of an RAPD marker for the C rown rust resistance in oats. *Genome* **36**: 818 - 820

Penner G. A., Clarke, J., Bezte, and Leisle, D. (1995). Identification of RAPD markers linked to a gene governing C d uptake in durum wheat. *Genome* **38** : 543 -547.

Perera, A. A. L., Peries, R. R. A., Att anayake, R. B. and Fernando, W. B. S. (1992, 1993 and 1994). Annual Reports of the Coconut Research Institute of Sri Lanka. R. Mahindapala (ed). Unpublish ed

Pereira, M. G., Ahnert, D., Lee, M. and Klier, K. (1995).Genetic mapping of QTLs for panicle characteristics. *Revista Brasileira de Genetica* **18** : 249 - 257

Persley, G. J. (1990). Beyond Mendel's garden: Biotechnology in the service of world agriculture. CAB Internation al, Wallingford. U.K

Poulson, G. B., Kahl, G. and Weis ng, K. (1992). Abundance and polymorphism of simple repetitive DNA sequences in *Brassica napus* L. *Theoretical and Applied Genetics* **85** : 994 - 1000.

Prince, J. P., Lacney, V. K., Angeles C. Blauth, J. R., and Kyle M. M. (1995). A survey of DNA polymorphism within the genus Capsicum and the fingerprinting of Pepper cultivars. *Genome* **38** ; 224-231

Ratnasiri, W. G. A. and Kularatne, H S. G. (1989). Conservation of San Ramon. *Annual Report of the Coconut Research Institute* of Sri Lanka. R. Mahindapala (ed). p.64

Ratnasiri, W. G. A. and Kularatne, H. S. G. (1989). Establishment of "filed gene bank". *Annual Report of the Coconut Research Institute of Sri Lanka.* R. Mahindapala (ed). p.64

Rogers, S. O. and Bendich, A. J. (1985). Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology* **5** : 69-76

Rogstead, S. H. (1994). Inheritance in turnip of variable-number tandem-repeat genetic markers revealed with synthetic repetitive DNA probes. *Theoretical and Applied Genetics* **89**: 824 - 830

Rohde, W., Salamani, F., Ashburner, G. R. and Randles, J. W. (1992). An *Eco*RI repetitive sequence family of the coconut palm *Cocos nucifera* L. shows sequence homology to copia-like elements. *Journal of Genetics and Breeding* **46** : 391 - 393

Rohrer, G. A., Alexander, L. J., Keele, J. W., Smith, T. P. and Beatie, C. W. (1994). A microsatellite linkage map of the porcine genome. *Genetics* **136** : 231 - 245

Ronning, C. M. , Schnell, R. J. and Khun, D. N. (1995). Inheritance of RAPD markers in *Theobroma cacao. Journal of American Society of Horticultural Science* **120** : 681 - 686

Rooney, W. L. (1994). Identification of RFLP markers linked to crown rust resistance in oat. *Crop Science* : **34** : 940 - 944

Rowland, L. J. and Levi, A. (1994). RAPD based genetic linkage map of blueberry derived from a cross between diploid species. *Theoretical and Applied Genetics* **87** : 863 - 868

Reiter, R. S., Williams, J. G. K., Feldman, K. A., Rafalski, J. A., Tingey, S. V. and Scolnik, P. A. (1992). Global and local genome mapping an *Arabidopsis thalina* by using recombinant inbred lines and random amplified polymorphic DNAs. *Proceedings of the National Academy of Sciences, USA* **89** : 1477 - 1481

Rus-Kortecaas, W., Smulders, M. J. M., Arens, P. and Vosman, B. (1994). Direct comparison of levels of genetic variation in tomato detected by GACA - containing microsatellite probe and by random amplified polymorphic DNA. *Genome* **37** : 375 - 381

Ryskov, A., P. Jincharadze, A. G., Frosnyak, M. I. Ivanov, P. L. and Limborska, S. A. (1988). M13 phage DNA as universal marker for DNA fingerprinting of animals, plants and mocroorganisms. 233 : **388** - 392

Salentjin, E. M. J., Arens, M. J. B., Lange, W., De-Bock, T. S. M., Stiekema, W. J. and Klein-Lankhorst, R. M. (1995). Isolation and characterisation of RAPD based markers linked to the beet cyst nematode resistance on chromosome 1 of *B. patellaris.. Theoretical and Applied Genetics* **90** : 885 - 891

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). "Molecular cloning: A laboratory manual", Cold Spring harbour Laboratory Press

Sangare, A., Rognon, F. and de Nace de Lamothe, M. (1978). Les phases males et femelles de l,inflorescence du cocotier. Influence sur le mode de production. *Oleagineux* **32** : 609 - 617

Sanz-Alferez, S., Richter, T. E., Hulbert, S. H., and Bennetzen, J. L. (1995). The rp3 disease resistance gene of maize. *Theoretical and Applied Genetics* **91** : 25 - 32

Sari-Gorla, M., Pe, M. E. and Rossini L (1994). Detection of QTLs controlling pollen germination and growth in maize. *Heredity* **72** : 332 - 335

Schierwater, B. and Ender, A. (1993). Different thermostable DNA polymerase may amplify different RAPD products. *Nucleic Acids Research* **19 :** 4676

Schulz, B. Westpal, L and Wrickle, G. (1994). Linkage groups of isozymes, RFLP and RAPD markers in carrot (*Daucus carota*). *Euphytica* **74** : 67 -76

Shah, F. H., Rashid, O., Simons, A J. and Dunsdon, A. (1994). The utility of RAPD markers for the determination of genetic variation in oil palm (*Elaeis guineensis*). *Theoretical and Applied Genetics* **89** : 713 - 718

Sharon, D., Hillel, J. Vainstein, A. and Lavi, U. (1992). Application of DNA fingerprints for identification and genetic analysis of *Carica papaya* and other *Carica* species. *Euphytica* **62** : 119 - 126

Shin et al (1990) SST-PCR for barley genome mapping. *Theoretical and Applied Genetics* **84** : 1002 - 1008

Siedler, H., Messmer, M. M., Schachermayr, G. M. Winzler, H., Winzler, M. and Keller, B. (1994). Genetic diversity in European wheat and spelt breeding material based on RFLP data. *Theoretical and Applied Genetics* **88** : 994 - 1003

Simcox, K. D. and Bennetzen, J. L. (1993). The use of molecular markers to study *Setosphaeria turcia* resistance in maize. *Phtopathology* **83** : 1326 - 1330

Skorupska, H. T., Choi, I. S., Rao, A. and Bridges, W. C. (1994) Resistance to soybean cyst nematode and molecular polymorphisms in various sources of Peking soybean. *Euphytica* **75 :** 63 - 70

Soller, M. and Beckmann, J. S. (1983). Genetic polymorphisms in varietal identification and genetic improvement. *Theoretical and Applied Genetics* **67** : 25 - 33

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98** : 503 - 517

Stammers, M., Harris, J., Evans, G. M., Hayward, M. D. and Forester, J. W. (1995). Use of random PCR (RAPD) technology to analyse pylogenetic relationships in the Lolium / Festuca complex. *Heredity* **74** : 19 - 27

Stiles, J. I., Lemme, C. Sondur, S., Morshidi, M. B. and Manshardt, R. (1993). Using randomly amplified polymorphic DNA for evaluating genetic relationships among papaya cultivars. *Theoretical and Applied Genetics* **89** : 697 - 701

Storlie, E. W. and Talbert, L. E. (993). Cause of tall off type in a semidwarf spring wheat. *Crop Science* **33** : 6 131 - 1135

Svitashev, S. K., Vershinin, A. V., Trunova, S. A., Pershina, L. A. and Shumy, V. K. (1995). Molecular analysis of the genomes of wide hybrids in cereals. *Hereditas* **122** : 25 - 31

Talbert, L. E., Blake, N. K., Chee, P. W, Blake, T.K. (1994). Evaluation of SST PCR products as molecular markers in wheat. *Theoretical and Applied Genetics* **87** : 789 - 794

Tanhuanpaa, P., Vilkki, P., Vilkki, J. and Pulli, S. (1995). Genetic polymorphism at RAPD loci in spring turnip rape. *Agricultural Science in Finland* **2** : 303 -310

Tanksley S. D., (1983) Molecular markers in plant breeding. *Plant Molecular Biology Reporter* **1** : 3- 8

Tanksley, S. D., Young, A.H., Paterson, M. W. and Bonierbale M. W. (1989). RFLP mapping in plant breeding: New tools for an old science. *Biotechnology* **7**: 257 - 264

Tautz, D. (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nuecleic Acids Research* **17**: 6463 - 6472

Thomas, M. R., Matsumuto., Cain P. and Scott, N. S. (1993). Repetitive DNA of Grapevine: classes present and sequences suitable for cultivar identification. *Theoretical and Applied Genetics* **86** : 173 - 180

Thorman, C. E. (1994), Ferreira, M. E. Camargo, L. E. A. Tivang, J. G. and Osborn, T. C. (1994). Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. *Theoretical and Applied Genetics* **88** : 973 - 980

Tingey, S. V. and del Tufo, J. P. (1993). Genetic analysis with random amplified polymorphic DNA markers. *Plant Physiology* **101** : 349 - 352

Tinker, N. A., Mather, D. E. and Fortin, M. G. and (1994). Pooled DNA for linkage analysis ; Practical and statistical considerations. *Genome* **37** : 976 - 984

Tinker, N. A., Fortin, M. G. and Mather, D. E. (1993). Random amplified polymorphic DNA and pedigree relationships in spring barley. *Theoretical and Applied Genetics* **85** : 999 - 1004

Tivang, J. G., Nienhuis, J., Smith, O. S. (1994) . Estimation of sampling variance of molecular marker data using the boot strap procedure. *Theoretical and Applied Genetics* **89** : 259 - 264

Torres, A. M., Weeden, N. F. and Martin, A. (1993). Linkage among isozyme, RFLP and RAPD markers in *Vicia faba*. *Theoretical and Applied Genetics* **85** : 937 - 945

Van Deynze A. E. Landry, B. S. and Pauls, K. P. (1995). The identfication of RFLPs linked to seed colour gene in *Brassica napus*. *Genome* **38** : 534 - 542

Van-Eck, H. J., Jacobs, J. M. E., Stan, P., Ton, J., Jacobson, E. (1994). Multiple alleles for tuber shape in diploid potato detected by qualitative and quantitave genetic analysis using RFLPs. *Genetics* **137** : 303 - 309

Varga, J. and Croft, H. (1994). Assignment of RFLP, RAPD and isoenzyme markers to *Aspergillus nidulans* chromosomes, using chromosome-substituted segregants of a hybrid of *A. nidulans* and *A. quadrilinelatus*. *Current Genetics* **25** : 311 - 317

Vassart, G., Georges, M., Monsior, R., Brocas, H. A. S. and Christophe, D. (1987). A sequence in M13 phage detects hypervariable minisatelites in human and animal DNA. *Science* **235** : 683 - 684.

Vershinin, A., Sviashev, S., Gummesson, P. O., Salomon, B., von Bothmer, R. and Bryngelsson, T. (1994). Characterisation of a family of tandemly repeated DNA sequences in Triticeae. *Theoretical and Applied Genetics* **89** : 217 - 225

Veldboom , L. R., and Lee, M. (1994). Moleculare marker facilitated studies of morphological traits of in maize. II determination of QTLs for grain yield and yield components. *Theoretical and Applied Genetics* **89** : 451 - 458

Virk, P. S., Ford-Lloyd, B. V., Jackson, M. T. Newbury, H. J. (1994). Use of RAPD for the study of diversity within plant germplasm collections. *Heredity* **74** : 170 - 179

Vosman, B., Arens, P., Rus-Kortekaas and Smulders, M. J. M. (1992). Identification of highly polymorphic DNA regions in tomato. *Theoretical and Applied Genetics* **85** : 239 - 244

Wachira, F. M., Waugh, R. Hacket, C. A. and Powell, W. (1995). Detection of genetic diversity in tea using RAPD markers. *Genome* **38** : 201 - 210

Wang, H., Qi, M. and Cutler, A.J. (1993). A simple method of preparing plant samples for PCR. *Nucleic Acids Research* **21 :** 4152 - 4253

Wang, G. R. and Binding, H. (1993). Somatic hybrids and cybrids of *Senecio fuchsii* Gmel. (x) *jacobaea* L. *Theoretical and Applied Genetics* **87** : 561 - 567

Welsh, J., Peterson, C. and McClelland, M. (1991). Polymorphisms generated by arbitrarily primed PCR in the mouse : application to strain identification and genetic mapping. *Nucleic Acids Research* **19** : 303 - 306

Welsh, J., and McClelland, M. (1991). Genomic fingerprints produced by PCR with consensus tRNA gene primers. *Nucleic Acids Research* **19** : 861 - 865

Weissenbach, J., Gyapay, G., Dib, C. Vignal, A., Morissette, J., Millasseau, P., Vaysseix, G. and Lathrop, M. (1992). A second generation linkage map of the human genome. *Nature* **359** : 794 - 801

Wickremaratne, M. R. T. and Ratnasiri, W. G. A. (1986). New variety block of crop museum. *Annual Report of the Coconut Research Institute of Sri Lanka.* R. Mahindapala (ed). P. 56

Wilde, J., Waugh, R. and Powell, W. (1992). Genetic fingerprinting of Theobroma clones using randomly amplified polymorphic DNA markers. *Theoretical and Applied Genetics* **83** : 871 - 877

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. (1991). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18** : 6531 - 6535

Witsenboer, H., Kesseli, R. E. Fortin, M. G., Stangehellini, M., and Michelmore, W. (1995). Sources and Genetic structure of a cluster of genes for resistance to three pathogens in lettuce. *Theoretical and Applied Genetics* **91** : 178 - 188

Xu, G. W., Magill, C. W., Schertz, K. F. and Hart, G. E. (1994). A RFLP linkage map of *Sorghum bicolour* (L.) Moench. *Theoretical and Applied Genetics* **89** : 139 - 145

Yang, X. and Quiros, C. (1993). Identification and classification of celery cultivars with RAPD markers. *Theoretical and Applied Genetics* **86** : 205 - 212

Young, N. D., Menacio-Hautea, D , Fatokun., C. A. and Danesh, D. (1990). RFLP technology, crop improvement, and international agriculture. Contribution No 18603. Minnesota Agricultural Experiment Station. USA, p 221 - 229, p 237 - 245

Young, N. D., Danesh, D. and Menacio-Hautea, D. and Kumar L. (1993). Mapping oligogeneic resistance to powdery mildew in mung bean with RFLPs. *Theoretical and Applied Genetics* **87** : 243 - 249

Yu, Z. H., McCouch, S. R., Kinoshita, T., Sato, S. and Tanksley, S. D. (1995). Association of morphological and RFLP markers in rice (*Oryza sativa*, L). *Genome* **38** : 566 - 574

Yu, K. and Pauls, K. P. (1993). Rapid estimation of genetic relatedness among heterogeneous populations of alfalfa by random amplification of bulked genomic DNA samples. *Theoretical and Applied Genetics* **86** : 788 - 794

Zuniga L (1953). The probable inheritance of the Makapuno character in coconut. *Philippine Journal of Agriculture* **36** : 402 - 413