CHAPTER 1

Introduction

The coconut palm *Cocos nucifera* is an important plantation crop in developing countries throughout the tropics. Coconut is an outbreeding perennial crop with no methods available for vegetative propagation. Hence optimum utilisation of the natural variability for genetic improvement of the coconut palm by traditional breeding methods is restrained by serious limitations. Exploiting molecular biological techniques, especially the use of molecular markers, to enhance the coconut breeding strategies appears a timely move in future breeding programmes. This thesis is the result of a study made to investigate the possibilities of using molecular markers in coconut breeding.

1.1 The coconut palm

Cocos nucifera is a member of the monocot family Arecaceae (Palmaceae) and is the only species of the genus. Based on morphological characters and breeding systems, the species was divided into several sub divisions such as varieties, forms and populations most often named according to their origin (Ohler, 1984). For instance three varieties and 15 forms have been distinguished in the coconut palm population in Sri Lanka (Liyanage, 1958, Wickremaratne *et al*, 1986, Perera *et al.*, 1992). Names and specific morphological features and reproductive differences of the types are indicated in Table 1.1. All these varieties are diploid and have a chromosome number 2n = 32. No incompatibility mechanisms have been found between or within these varieties.

Coconut is believed to have originated in South East Asia and spread to other areas by natural dispersal or sea currents. Colonial governments further disseminated

coconut palm by transporting them to Africa and Latin America and developed plantations throughout the tropics by the end of the 19th century. The major coconut growing countries, Philippines, Indonesia, India, Malaysia, Vietnam, Sri Lanka and Thailand are in Asia. Countries such as Papua New Guinea and Vanuatu in the South Pacific region, Ivory Coast, Mozambique and Tanzania in the African region and Mexico, Brazil and Jamaica also noteworthy as coconut growing countries (Benabadis, 1992).

Most productive coconut plantations are commonly found in coastal areas. The palms are adapted to agro-climatic zones in lower altitudes comprising sandy or sandy loam soils of high moisture retention capacity with a reasonably good drainage. Coconut also prefers an evenly distributed rainfall of around 1000 mm a year (Mahindapala and Pinto 1991).

Currently the total area under coconut cultivation is approximately 11 million ha and is distributed in around 80 countries. It is mainly grown in countries such as Philippines, Indonesia, India, Papua New Guinea, Mexico, Malaysia, Vietnam, Sri Lanka, Mozambique, Tanzania and Ivory Coast. In Sri Lanka coconut is the most widely grown plantation crop and occupies an area of 416,000 ha accounting for approximately 21% of the total land area under agriculture in the island (Fernando et al., 1995). Coconut is predominantly a smallholder's crop.

Coconut was the major source of vegetable oil in world trade during the early parts of 20th century before it was surpassed by oil palm (*Elaeis guineensis*), soy bean (*Glycine max*), sunflower (*Helianthus annuus*) and rapeseed (*Brassica napus*). Today it accounts for only 5% of the world vegetable oil supply. However, coconut oil has a comparative advantage over most other vegetable oils because of its 48% lauric acid content. Lauric acid has a wide range of both edible and industrial uses, such as cooking oil, detergents, cosmetics, lubricants and pharmaceutical's (Manicad, 1995). Despite the setback in the world oil trade, coconut still remains the most important crop in countries like Sri Lanka and Philippines. For many small islands coconut is the main earner

of foreign exchange (Benabadis, 1992). The export earnings from coconut in Sri Lanka in 1993 were approximately A$100 million (Fernando *et al.*, 1995).

Table 1.1 : Varieties, forms and taxonomic characteristics of the coconut palm in Sri Lanka

Variety / **Form**	**Specific morphological feature (s)**	**Reproductive behaviour**
Typica (Tall)	Tall stature, late flowering (6 years after planting), continuous flower production, hardy palms tolerating a wide rage of environments	Outbreeding
Typica	Commercially grown type	
Navasi	Soft fibred husk	
Gonthembili	Orange coloured Nuts	
Ranthembili	Pink colouration in the mesocarp of the young fruit	
Porapol	Thick shell	
Bodiri	Small and numerous nuts	
Kamandala	Few large nuts	
Dikiri	Jelly -like kernel	
Nana (Dwarf)	Dwarf stature, early flowering (4 years after planting), seasonal flower production, less hardy, palms suffers adversely from drought, pests and diseases	Inbreeding
Green	Green coloured nuts	
Yellow	Yellow coloured nuts	
Red	Red coloured nuts	
Brown	Brown coloured nuts	
Aurantiaca (Thembili)	Intermediate stature, late flowering, seasonal flower production, leaf petioles and nuts are orange coloured, nut water sweet, suffers adversely from drought	Inbreeding
King coconut	Common type	
Navasi thembili	Soft fibred husk	
Rathran thembili	Pink colouration in the mesocarp of the young fruit	

In Sri Lanka coconut is grown chiefly for domestic use. The domestic uses of coconut are many. Almost every part of the palm has a use. The trunk provides wood for building rafters and furniture. Woven leaves are used to thatch roofs while ribs of the leaflets forms the base of brooms and baskets. The sugary sap of the inflorescence serves as the base for palm wine (toddy), alcoholic spirits (arrack), vinegar and jaggery (sugar cake). The parts of the nut (fruit) are the most valuable, the husk providing fiber (coir) for mats and ropes, the shell providing charcoal and the kernel providing copra and oil (for manufacturing soap, margarine and cosmetics), desiccated coconut (for confectioneries) and milk (cooking ingredient). In Sri Lanka the per capita consumption of coconuts is about 2 nuts per day per family. It provides approximately 25% of the caloric requirement of the average Sri Lankan diet and around 80% of the estimated total production is consumed domestically (Fernando, et al. 1995).

1.2 Genetic diversity

The coconut palm has been characterised on the basis of the mode of pollination, morphological differences and geographical distribution. Attempts have also been made to characterise coconut ecotypes using protein and polyphenol polymorphisms.

Tall and dwarf types of the coconut palm differ significantly from each other, mainly due to mode of pollination In all varieties staminate and pistillate phases do not overlap and hence they are cross pollinated in nature (Liyanage, 1958). In contrast, dwarf palms have overlapping phases facilitating self-pollination in nature. However, no incompatibility mechanisms have been observed in the coconut palm (Sangare et al. 1978).

Morphological differences that are attributed to genetic diversity in the coconut palm are differences in the stature, colour of leaf petioles, colour of nuts, qualitative and quantitative differences in the fruit components (Liyanage, 1958). Tables 1.1 and 1.2 illustrate the use of such morphological differences to characterise genotypes of the coconut palm in Sri Lanka.

Coconut genotypes have also been characterised according to their geographic origin (Bretting and Goodman, 1989). Germplasm conservationists in particular collect and conserve coconut accessions from different geographic locations as there is a likelihood that those not modified by man-made dissemination could have evolved as nearly pure or homozygous lines.

The studies based on enzyme, polyphenol and carotenoid differences, although not extensive can be considered as more appropriate for characterisation of the coconut palm because these are not affected by the environment. However, the initial attempts made to compare enzyme polymorphisms did not reveal adequate diversity within the groups tested (Carpio, 1982). The studies based on polyphenol polymorphisms demonstrated clustering between and within the populations studied indicating a significant difference between tall and dwarf types and within dwarf types of different origin (Jay *et al.* 1989). Studies on carotenoids in tall, dwarf and tall x dwarf hybrids have shown that genotypes within the tall variety share the same carotenoids while differences in carotenoids were observed between tall and dwarfs genotypes. Carotenoid characteristics of dwarfs dominated in the hybrids (Jayasekera, 1979).

Table 1.2. Some quantitative characters of different varieties and forms of the coconut palm in Sri Lanka

Variety / Form	Husked nut weight (g)	Shell weight (g)	Copra / nut (g)	Nuts/ton of Copra
Typica (Tall)				
Typica	692	157	211	4,750
Navasi	606	169	178	5.675
Gonthembili	692	197	229	4,400
Ranthembili	827	197	219	4,600
Porapol	533	225	141	7,150
Bodiri	194	51	51	19,900
Kamandala	1600	320	375	2,700
Nana (Dwarf)				
Green	285	70	112	8,950
Yellow	429	87	111	9,050
Red	276	56	86	11,675
Aurantiaca				
King coconut	398	168	141	7,150
Navasi thembili	461	98	139	7,250

1.3 Coconut breeding in Sri Lanka

The major progress in coconut breeding came after attempts were made in Fiji in 1928 and in India in 1932 to hybridise dwarf x dwarf and dwarf x tall (intra-varietal hybrids) palms from geographically different populations (Benabadis, 1992). Subsequently, other countries, too, attempted to develop hybrids because heterosis has been demonstrated in a wide range of inter-origin crosses (de Nuce de Lamothe and Wuidart, 1981). PB121 (Malayan Yellow Dwarf x West African Tall), MAWA (Malayan Red Dwarf x West African Tall) and CRIC60 (Sri Lanka Tall x Sri Lanka Tall) are some hybrids of the coconut palm which are widely used in commercial plantations.

Coconut is predominantly a smallholder's crop. Hence coconut research is undertaken by state funded research institutions. In Sri Lanka the Coconut Research Institute (CRI) was formed as a statutory body in 1930 to deal with research problems of the crop. Since the inception of CRI breeding was a set priority. The coconut breeding research activities at the CRI during the 60-year period from 1930 - 1980 has been reviewed by Liyanage *et al.* in 1988. The current breeding programme was formulated in 1985 and progress of the ongoing experiments is periodically reported in the annual reports and other publications of the CRI.

The Sri Lanka Tall (SLT) palm has been the mainstay of the coconut industry in Sri Lanka because of its ability to tolerate a wide range of environments. It attains a height of about 20 meters approximately 50 years after planting. The palm comes into flower about six years after planting and begins to bear steadily after 10 - 12 years and remains productive for over 60 years. The average yield of SLT palm varies between 40 - 60 nuts per palm per year or 7,500 - 10,000 nuts per ha per year (Liyanage *et al.*, 1988).

The early studies on breeding were first aimed at developing a criterion for selection of seed parents. In the beginning it was hypothesised that a seed palm should have characters such as short straight stem, closely set leaf scars, short well oriented

fronds, short bunch stalks, reasonably good number of female flowers, inflorescences spread around the crown, a large number of nuts and a high husked nut weight to qualify as a seed palm. Progeny trials were established to test the hypothesis and it became evident that weight per husked nut has a very high heritability value (0.95) and was named the top indicator in selection of seed parents (Liyanage, 1967 and 1980). The other characters which proved useful were short internode length, high setting of female flowers into fruits, high kernel weight and high yield of copra per palm per year. Based on this information a palm selection programme was initiated and a massive pool of palms were selected from coconut plantations for use as seed parents. These palms were named as plus palm talls (PPT).

The results of correlations obtained between some important characters in the palm are also noteworthy. It was found that the nuts that germinate early give rise to palms that flower early and consequently lead to a higher production of nuts and copra in the adult palm. This eventually led to a very important decision that culling of seedlings arising from late-germinating seeds to be used as a measure to ensure better adult palms with uniformity in plantations.

In the late 1940's techniques were developed for the collection and processing of pollen for artificial pollinations. This made way for hybridisation trials and as a result two intra and inter varietal hybrids, CRIC60 and CRIC65 were developed. CRIC60, derived by crossing selected SLT x SLT palms is capable of producing over 110 nuts per palm per year averaging 225 g of copra per nut under rainfed conditions with minimal management. This hybrid can tolerate a wide range of environments and hence was recommended for planting in all the coconut growing areas in the country. CRIC65 derived by crossing Green Dwarf (SLGD) x SLT comes into flower about 2 years earlier than CRIC60 and is capable of producing over 120 nuts per palm per year with about 210 g of copra per nut in a favourable environment. This hybrid cannot tolerate drought stress and pests and diseases and hence was recommended for planting in home gardens or under irrigated

conditions. Three seed gardens have been established and maintained by the CRI to mass produce these varieties (Liyanage *et al.*, 1988)..

The national planting programme is an activity geared to maintain the population of coconut palms in Sri Lanka. Based on the age, mortality and area under cultivation of the coconut palm in the country it was estimated that at least one million seedlings should be planted every year to maintain the balance of the coconut population (Liyanage, 1985). With a view to carrying out replacements with genetically superior material, CRI was entrusted to supply seed nuts for the national planting programme. Currently approximately 20% of the seed nuts come from seed gardens and the rest come from the plus palms. The seed nuts are raised in nurseries and seedlings are culled on the basis of late germination and poor seedling characters and selected seedlings are issued for planting. The expected per palm increase in the yield by replanting with these elite material is about 50%, that is an increase from existing yield of 40 - 60 nuts per palm per year up to 60 - 80 nuts per palm per year with n a period of approximately 25 years.

The objectives of the current breeding programme at the CRI are to develop cultivars, for high yield under high input for large scale plantations, and optimum yield under low input for smallholder plantations. Besides high or optimum yield, tolerance to moisture stress and pest and diseases are other important characters in evaluating the cultivars. Conservation of germplasm is another important function of the breeders at the CRI.

A number of multi-locational trials have been established to evaluate improved cultivars and progenies arising from varying combinations of parents selected on the basis of high yield potential, consistency in the bearing pattern and better performance under drought situations. More than 50 populations of coconut palms in the island have been established in the research station for conservation of the coconut gene pool in the country. These germplasm accessions are also under evaluation for utilisation in the future breeding programmes.

1.4 Constraints for conventional breeding

The optimum utilisation of natural genetic variability for improvement of the coconut palm by conventional breeding methods is restrained by several limitations. Conventional breeding methods in plants are generally based on accurate estimation of genetic parameters by careful assessment of F_2 or back-cross populations and cross hybridisations between inbred lines.

In the coconut palm careful assessment of F_2 populations is restricted by the lack of vegetative propagation mechanisms in the crop. A proper assessment of a population can never be made without true replicas because most of the economic traits show polygenic inheritance and are affected by the environment. Lack of vegetative propagation also limits the possibility of producing clones from elite individuals.

Development of inbred lines from a heterozygous plant usually requires about six breeding cycles of inbreeding. Hence to obtain pure lines from heterozygous tall coconuts would require at least 60 years as the breeding cycle of the coconut palm is around 10 years. The cost and land requirement for a such an exercise can not be justified in terms of the anticipated benefits in 100 years time.

The risk involved in importation of germplasm is another constraint for attempting genetic improvement by hybridisation between ecotypes. Coconuts have deadly diseases in some countries (eg. Cadang Cadang in Philippines, Lethal Yellowing in India) which are caused by mycoplasma-like organisms. Very little is known about the pathogenecity of these diseases and therefore, importing coconut in any form to a country free of such diseases would endanger the industry.

1.5 Prospects of biotechnology for breeding of coconuts

The prospects of biotechnolc gy to improve the coconut palm is dependent on the improvement of tissue culture proce dures a nd development of isoenzyme or molecular marker systems. Introduction of nev genetic information into coconut palm by DNA transformation is still technically re mote. Lack of tissue culture systems and lack of knowledge of the genetics of the fac tors that control or modify important traits such as precocity, yield, quality, tolerance to noisture stress or pest or disease resistance is also a limit for extensive application of reco mbinant DNA technology (Jones, 1989).

1.5.1 *In vitro* culture techniques

Research work is under w ay at several laboratories including the CRI for development of methods for vegetative propagation of the coconut palm. These studies have been progressing for nearly 20 years but the advances made so far have not yet reached the expectations. Tissue culture in the coconut palm has been reviewed by Jones (1989) and Benabadis (1992).

Somatic embryogenesis is b elieved to be the only possible way of vegetatively propagating the coconut palm. This has been induced from callus derived from young leaves, rachillae, seedling leaves and zygotic embryos. Although there were signs of early development in the culture process further multiplication proceeds in an unbalanced way and leads to incomplete plantlets (Karunaratne *et al.*, 1991a., Jones, 1989., and Benabadis, 1992). Karunaratne *et al.*, (1991a) showed that the most embryogenic material was explanted from 10 to 20 cm long l eaves of 12 to 24 months old plants and that the morphogenic capacity was highest a t the leaf base. This situation not only limits the availability of leaf material for rese arch but prevents results of previous work being reproduced if the same developmenta stage is not selected for explanting.

Experiments are in progress at the CRI to increase the somatic embryo formation by using different combinations of auxins and cytokinin in the culture media and to improve the shoot formation capacity of the germinating embryos with the use of organic nitrogen sources. The ideal levels of auxins and cytokinin in the regeneration medium have not yet been ascertained. It is believed that a gradual shift in the concentration of both auxins and cytokinin during the regeneration phase is imperative. Thus studies concerning the availability, uptake and metabolism of synthetic auxins and cytokinin is seen as an essential component of research towards the success of tissue culture of the coconut palm by the researchers at the CRI (Fernando *et al.*, 1995)

However, a method for *in vitro* germination of excised embryos has been developed and improved (Del Rosario and de Gusman, 1976). The embryo culture technique was developed with the intention of using as an *in-vitro* based technique for collecting and transporting germplasm from distant populations and as an embryo rescue method for propagating the indigenous tall form "dikiri pol". Development of an *in vitro* assay for drought tolerant coconut germplasm is also another useful practical possibility of the embryo culture technique (Karunaratne *et al.*, 1991b).

Two methods have been developed for *in-vitro* based germplasm collection using the embryo culture technique. First is the slow growth method where embryos are dissected and cultured in a special agar-based medium which suppresses further growth and development of embryos without affecting their viability. The second is the field explanting method where similar culture techniques are used but a compact inflatable glove box is used for aseptic explanting and culturing at the field site. This development paved the way for probable conservation of germplasm *in vitro* and reduction in the bulk of transport in germplasm exchange programmes.

The embryo culture method was successfully exploited to rescue the embryo of "Dikiri pol", the makapuno type coconut form in Sri Lanka and obtained planting material for propagation. These coconuts are characterised by a soft jelly-like endosperm which is

highly valued in the confectionary industry. The natural "dikiri pol" palms yield about 2 - 21% "dikiri" type nuts while the rest are normal tall type nuts. Manifestation of the "dikiri" character is believed to be due to homozygosity for a single recessive allele in the triploid endosperm of the nut (Zuniga, 1953). These "dikiri" nuts cannot germinate in nature and the embryo culture technique has been successfully used to propagate "dikiri" coconut palms from "dikiri" nuts. The additional advantage of establishing seedlings propagated by *in-vitro* culture of "dikiri" embryos is that they are capable of producing 100% "dikiri" nuts when self-pollinated (Fernando *et al.*, 1995).

An attempt has been made to develop a screening method for drought tolerance by using *in-vitro* cultured embryos. Water stress conditions were simulated during *in-vitro* germination and subsequent plant development was accomplished in the presence of sodium chloride. In sodium chloride stress media approximately 78% of embryos survived 170 mM sodium chloride and only 12% were able to tolerate a concentration of 320 mM sodium chloride when embryos from common Sri Lanka Tall were used. A comparative assessment of embryos derived from two drought susceptible cultivars of coconut CRIC65 and Dwarf green and two putative drought tolerant cultivars Ambakelle special and San Ramon, showed that at 170 mM salt, 71% and 29% respectively of the former two survived while all embryos of the latter two survived the stress. At a salt concentration of 230 mM all failed to survive in the former two and 18% survived in the latter two (Karunaratne, *et al.*, 1991). These results clearly indicate the possibility of applying this technique for screening coconut genotypes for drought tolerance which is very important in view of the yield variation in coconut due to weather fluctuations and changes in the global weather patterns.

Anther culture is an *in vitro* technique which could be used to develop homozygous plants. The technique involves embryoid development of pollen grains and doubling chromosome number to produce dihaploids. These will eventually become fully homozygous fertile diploids which can be perpetuated by self pollination or used as

parents in hybridisation programmes. Work on anther culture of coconut is in progress in the Philippines (Thanh-Tuyen and de Guzman, 1983 as quoted by Jones, 1989).

1.5.2 Isozyme technique

Isozymes are multiple forms of an enzyme which have the same catalytic specificity but can be physically separated due to differences in molecular weight, charge or both. These proteins are thus, phenotypic characters which reflect the genetic composition of their source and by which estimates of genetic variability, by rates of polymorphism and heterozygosity, can be determined. The role of isozyme analysis in the study of plant population structure and genetic conservation has been extensively reviewed by Brown and Weir (1983). Although isozymes are limited in the number of markers available, they are relatively cheap, easy to assay, and satisfy most of the requirements that an ideal genetic descriptor should possess. They are co-dominant and not altered by the environment.

Several attempts have already been made to study the isozyme patterns in coconut. Benoit (1979) reported isozymic variation between and within populations of the coconut palm in the Philippines using nine isozymes and using embryo and haustorium as sources. Carpio (1982) have studied isozyme patterns in 12 morphologically distinct coconut populations in the Philippines using four isozymes and pollen and meat protein extracts. The study has not revealed significant differences between populations except for the peroxidase isozyme which showed some variation in pollen proteins from different sources. More recent studies conducted at the CRI indicated young actively growing leaf tissues obtained from the most recently opened fronds as a better source for isozyme analysis. Among three isozymes tested, esterase (EST), glutamate oxaloacetate trasaminase (GOT) and peroxidase (PER), the esterase system has shown a very high level of polymorphism with a maximum of five different alleles within individuals of the tall form (Fernando, 1995).

1.5.3 Molecular marker techniques

Molecular markers are DNA fragments either detected by hybridisation with specific sequences of DNA or amplified by short oligonucleotide primers in the polymerase chain reaction. Molecular marker systems possess a great potential in enhancing genetic improvement programmes in plantation crops. They are developmentally stable and exist in potentially unlimited numbers. The expression of these markers is not influenced by the environment. They behave as either dominant or codominant Mendelian markers, free of pleiotropic effects on economically important characters. Moreover, molecular marker analysis is based on the use of purified DNA, which can be extracted from almost any type of tissue and hence plants need not be grown in a particular environment to obtain material for assays.

The most important application of molecular markers for crop improvement is in selecting individuals that carry genes of economic importance. When a gene of interest can be tagged with a tightly linked molecular marker, selection can be based on scoring for the molecular marker rather than for the gene itself. In polygenic characters molecular markers can identify the regions in the genome containing loci for such traits and facilitate mapping of quantitative trait loci which in turn provide a direct method for selecting individuals carrying desirable combinations of genes.

The potential of molecular markers for genetic improvement of coconut needs to be discussed in the light of the existing knowledge of the genetics of the crop. The chances of developing quantitative trait loci maps based on molecular markers in coconut is again a long term objective. However, use of molecular markers could reduce the breeding cycle for attaining homozygosity or near homozygosity for traits of importance. The number of plants required for field trials which at present are large and expensive could be reduced. There is the possibility of using existing field trials for tagging molecular markers with phenotypic characters because it is likely that ancestral palms may be found, as coconut has a long life span. Molecular markers could be used to identify the levels of genetic

diversity between and within natural populations and hence can be applied for screening and utilisation of the germplasm.

Restriction fragment length polymorphisms (henceforth, RFLPs) was the first type of DNA molecular marker used to detect genetic polymorphisms in crop plants. RFLPs became popular since their inception in early 1980's and now is are being used in almost every crop. The Random Amplified Polymorphic DNA technique (henceforth, RAPDs) was developed more recent. RAPDs came into use in early 1990's and became even more popular especially among those who deal with perennial crops. DNA polymorphisms based on the simple sequence repeats distributed in the genome (micro and mini satellite variations) is another technique developed for DNA profiling studies (DNA fingerprinting) in plants.

1.5.3.1 Restriction Fragment Length Polymorphisms

RFLPs are DNA polymorphisms revealed by the differences in lengths of the restriction endonuclease digested DNA fragments of eukaryotic genomes detected by hybridising with labelled cloned DNA sequences homologous to a particular DNA fragment. Restriction endonucleases recognise a specific base sequence and cleave DNA at a particular point in or adjacent to the site. Two individuals may not always have DNA molecules cut at the same position(s) by a particular enzyme because the recognisable sites in the genome can be abolished or new sites can be created by base changes. Shifting of restriction sites by transposition of large elements can also cause changes in the size of restriction fragments (Beckmann and Soller, 1983).

Experimentally, RFLP assaying is based on hybridising cloned DNA sequences with blots that contain restriction digested DNA from the individuals concerned, such as parents, F1 and F2 or backcross populations, germplasm accessions etc. For each cloned DNA sequence, the pattern of DNA bands for each individual can be used to assess the genetic relationships. Thus the two major requirements for RFLP analysis are purified

DNA from individuals and a library of cloned DNA sequences (putative RFLP clones) derived from the organism of interest (Tanksley *et al.* 1989., Young *et al.*, 1991a). Simple methods have been developed for obtaining large amounts of DNA from plant material for blots (Dellaporte *et al*, 1983., Murray and Thompson, 1980., Rogers and Bendich, 1985) and for construction of DNA libraries by cloning cDNA sequences derived from purified mRNA or random sequences from cleaved genomic DNA (Sambrook *et al*, 1989., Asubel, *et al.*, 1989).

The detection and potential uses of RFLPs as genetic markers in crop improvement has been well considered by Beckmann and Soller (1983) and Soller and Beckmann (1983). RFLPs are expected to be numerous, developmentally stable and act as co-dominant Mendelian markers free of pleiotropic effects on economically important traits. The areas emphasised as potential uses of RFLPs in crop improvement were varietal and parentage identification, identification of genetic loci affecting quantitative economic traits and genetic improvement programmes, including screening and evaluation of germplasm resources, marker assisted introgression, improvement of commercial hybrids and within population selection. The methodologies involved in RFLP detection and costs of applying RFLPs to genome mapping and other breeding applications have also been discussed and concluded that in most cases anticipated costs appear to be commensurate with the scientific and economic value of the application.

RFLP studies on plants to date have been numerous. These cover almost the entire range of commercially important plants including a few forest trees. To document a list of more recent studies; rice (*Oryza sativa*; Yu *et al.* 1995), wheat (*Triticum aestivum*; Siedler *et al.* 1994), maize (*Zea mays*; McMullen, *et al.* 1994), sweet corn (*Zea mays*; Gerdes and Tracy 1994), sorghum (*Sorghum bicolor*; Cui *et al.* 1995), oat (*Avena steralis*; Rooney *et al.* 1994), rye (*Secale cereale*; Svitashev *et al.* 1995), barley (*Hordeum vulgare*; Svitashev *et al.* 1995), pigeon pea (*Cajanus cajan*; Nadimpalli *et al.* 1993), lentils (*Lens culinaris*; Havey and Muelhlbauer 1989), mung bean (*Vigna radiata*; Young *et al.* 1993), peanut (*Arachis hypogea*; Kochert *et al.* 1991), soybean (*Glycine*

max; Landau and Gresshoff *et al.* 1994), bean (*Phaseolus vulgaris*; Adam-Blondon *et al.* 1994), carrot (*Dacus carota*; Schulz *et al.* 1994), cabbage (*Bassica* spp; Dos-Santos *et al.* 1994), beet (*Beta vulgaris*; Salentijn *et al.* 1995), Lettuce (*Lactucae* spp; Witsenboer *et al.* 1995), cucumber (*Cucumis sativus*; Kennard *et al.* 1994), alfalfa (*Mediago sativa*; Echt, *et al.* 1994), tomato (*Lycopersicon esculentum*; Lindhout *et al.* 1994), citrus (Fatta-Del-Bosco, 1993), potatoes (*Solanum tuberosum*; Gebhardt *et al.* 1994), sweet potatoes (*Ipomoea batatas*; Jarret and Austin *et al.* 1994), casava (*Manihot esculenta*; Marmey *et al.* 1995), date palm (*Phoenix dactylifera*; Corniquel and Mercier *et al.* 1994), oil palm (*Elaeis guineensis*; Jack, 1995), rapeseed (*Brassica napus*; Van-Deynze *et al.* 1995), sunflower (*Helianthus annuus*; Gentzbittel *et al.* 1995), grape (*Vitis vinifera*; Bourquin *et al.* 1993), apple (*Malus* spp; Gardiner *et al.* 1994), pepper (*Capsicum* spp; Prince *et al.* 1995), sugar cane (*Saccharum* spp; Lu *et al.* 1994), tobacco (*Nicotina tobacum*; Tivang *et al.* 1994), cocoa (*Theobroma cacao*; N'Goran *et al.* 1994), cotton (*Gossypium hirsutum*; Brubaker and Wendel, 1994), rubber (*Hevea brasiliensis*; Besse *et al.* 1994), bamboo (*Phyllostachys* spp; Friar and Kochert, 1994), pine (Pinus spp; Groover *et al.* 1994), eucalyptus (*Eucalyptus nitens*: Byrne *et al.* 1994) and poplar (*Populus* spp; Tivang *et al.* 1994), have all been subjected for RFLP assays.

The above studies were basically aimed at identifying genetic diversity within or between groups at various levels such as species, sub species, germplasm accessions comprising ecogeograhical groups, domesticated and wild cultivars and inbred and outbred lines, etc. RFLP analyses of the species of *Brassica* (cabbage, broccoli and cauliflower), *Vitis* (grape) and *Capsicum* (pepper) serve as good examples of the application of RFLPs to examine interspecific variation (Dos-Santos *et al.* 1994, Bourquin *et al.* 1993 and Prince *et al.* 1995). In these studies phylogenetic trees were constructed based on genetic distances measured by RFLPs to determine relationships between the species.

Examples of RFLP based genetic assessment studies at the intraspecific level include the work of Siedler *et al.* (1994) who characterised the genetic diversity within

European winter wheat (*Triticum aestivum*) germplasm and observed a high degree of variation. Havey and Muelhlbauer (1989) screened RFLPs in thirty accessions of wild and domesticated lentils (*Lens culinaris*) and revealed high variability between accessions. Kochert *et al.* (1991) assessed the genetic variability in peanut (*Arachis hypogaea*) cultivars and found that they share a narrow germplasm base. Gentzbittel *et al*, observed a similar low genetic variability in cultivated sunflower (*Helianthus anus*). Both these studies suggested the need for creation of new source populations by crossing with wild populations for further genetic improvement of the crops.

Laurent *et al.* (1994) assessed the RFLP variability in 203 clones of the cocoa (*Theobroma cacao*) genome and detected a high level of polymorphism that distinguish populations from different sources. RFLP-assessed genetic diversity in wild and cultivated populations of rubber (*Hevea brasiliensis*) proved that even the inbred cultivated clones have conserved a relatively high level of genetic diversity. Poplars (*Populas* spp) and Eucalyptus (*Eucalyptus nitens*) are examples of two forest trees which have been subjected to RFLP analysis (Liu *et al.*, 1993, Byrne, *et al.*, 1994).

RFLPs have been used to generate linkage maps in several agricultural crops. RFLP linkage maps are generated by hybridising F2 or back cross (segregating) populations from parents with differing genomes using single copy DNA sequences cloned randomly from the genome. The greatest advantage of RFLPs is that are scored as codominant alleles facilitating the phenotypic detection of heterozygotes. Many traits of economic importance are under the control of multiple genes that work in harmony to produce a genotype. In mapping quantitative trait loci (QTL) parents that differ significantly from the trait of interest are analysed with RFLPs. Each marker is then tested for the likelihood that it is linked to a QTL with a significant effect on the trait of interest. Linkage-1 and Mapmaker are two software packages widely developed for mapping RFLP loci. Linkage-1 carries out chi squared analysis of single locus and two-locus data to give goodness-of-fit estimates of linkage while mapmaker uses maximum likelihood

algorithms to produce linkage data from variety of segregating populations (King *et al.* 1991).

In recent years RFLP linkage maps have been developed for a large number of economically important plants. These include rice (McCouch *et al.* 1988), maize (Helentjaris *et al.* 1987), sorghum, (Xu *et al.* 1994), barley (Shin, *et al.* 1990), rye (Korzun, 1994), potatoes (Gebhardt, 1989), soybean (Keim *et al.* 1990), faba bean (Torres *et al.* 1993), tomato (Helentjaris *et al.* 1987), citrus (Cai *et al.* 1994), crucifers (Change *et al.* 1988), carrot (Schulz, *et al,* 1993), beet (Pillen, *et al.* 1993), cucumber (Kennard, *et al.* 1994), lettuce (Landry, *et al.* 1987), alfalfa (Echt *et al.* 1994), rapeseed (Fereira *et al.* 1994), sunflower (Gentzbittel *et al.* 1994), cotton (Reinisch *et al.* 1994), pine (Devey *et al.* 1991), poplar (Bradshaw, 1994) and the model plant system *Arabodopsis thalina* (Chang *et al.* 1988).

Disease resistance genes were the first category to be mapped extensively using RFLPs because they are mostly controlled by alleles at a single locus and governed by dominant genes. Genes for resistance to gall midge in rice (Mohan *et al.,* 1994), dwarf mosaic virus in maize (McMullen and Louie, 1989), downy mildew in lettuce (Landry *et al.,* 1987) tobacco mosaic virus, root-knot nematode, yellow leaf curl virus, *fusarum, verticillium* and *pseudomonas* in tomato (Young *et al.* 1988, Michelson *et al.* 1994, Tanksley, *et al.* 1989), nematode *Globodera rostochiensis* in potato (Barone, *et al.* 1990) and resistance to small animals due to the presence of limarase in Trifolium repens (Hughes, *et al.,* 1990) are examples of such major genes tagged with RFLP markers.

Unlike resistance to diseases most other economic traits are quantitative characters and are of polygenic inheritance. In order to map these quantitative trait loci (QTLs), a population derived from parents that differ significantly for the trait of interest is analysed with RFLPs. Each marker can then be tested for the likelihood that it is linked to a QTL with a significant effect on the trait of interest. For example in a backcross the population can be separated for heterozygous and homozygous groups for

the RFLP allele of the recurrent parent. The phenotypic values of these sub groups can then be compared by analysis of variance (ANOVA). If there is a significant difference between the sub-groups the RFLP may be linked to a QTL. By repeating this analysis for RFLPs distributed over the entire genome a map could be generated with RFLPs tagged with economic traits such as precocity, overall yield, stress tolerance, nutritional quality etc.

Mapping QTLs with RFLPs has been carried out in tomatoes for a number of economic traits including time of flowering, fruit set, time of ripening (Lindhout *et al.* 1994), soluble solid content in the fruit (Patterson *et al.*, 1988), water use efficiency (Martin, *et al.* 1989), insecticide resistance (Nienhuis *et al.* 1987) and root knot nematode resistance (Klein-Lankhorst, 1991). For each of these characters several RFLP markers on different linkage groups have been identified. Similarly, in maize plant, height and days to flowering (Koester *et al.*, 1993), grain yield and grain yield components (Veldboom and Lee, 1994), protein and starch concentration (Goldman *et al.*, 1993), pollen germination and growth of pollen tube (Sari-Gorla *et al.*, 1994), resistance to wheat streak mosaic virus (McMullen *et al.* 1994), resistance to leaf blight (Simcox and Bennetzen, 1993), stalk and ear rot resistance (Pe *et al.*, 1993) and rust resistance (Sanz-Alferez *et al.*, 1995) are important characters tagged with RFLP loci on different RFLP linkage groups. Wood specific gravity in pine (Groover *et al.*, 1994), cold and salt tolerance in citrus (Cai *et al.*, 1994), tuber shape in potato (Van-Eck *et al.*, 1994), semidwarf character in wheat (Storlie and Tabert, 1993), panicle characteristics and seed weight (Pereira *et al.*, 1995), brown pericarp in rice (Yu *et al.*, 1995) and seed colour in rapeseed (Van Deynze *et al.*, 1995) are also some useful traits linked with RFLP markers.

Tagging of economic traits with RFLPs is useful for marker-assisted selection in crop improvement programmes. When a gene is mapped with RFLPs, selection for that gene can be based on tightly linked RFLPs rather than phenotypic scoring. This can be extremely valuable if a trait is recessive, difficult to assay, or obscured by the expression of other traits. This also helps to score a trait at a very early stage and for characters such as

flowering time and yield even before they are expressed in the plant. RFLPs can also be used to select against unwanted DNA from one of the parents.

Theoretical considerations and cost effectiveness of using RFLPs in varietal identification and genetic improvement has been well reviewed (Soller and Beckmann, 1983., Beckmann and Soller, 1983., Tanksley, 1983). In terms of cost per breeding application the cost for evaluation and mapping of quantitative loci was found to be higher for segregating populations than inbred populations. Yet it was argued to be economical when populations were at a selection plateau and the traits under improvement were of great economical value (Beckmann and Soller, 1983).

1.5.3.2 Random Amplified Polymorphic DNA

Random amplified polymorphic DNA (henceforth RAPDs) is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence using the polymerase chain reaction (Williams *et al.*, 1991., Welsh and McClelland, 1991). Primers identify specific base sequences in the genome and anneal to these sites at both strands of the denatured DNA template. If two priming sites on opposite strands lie within an amplifiable distance from each other (< 5000 bp) and are appropriately oriented, a discrete DNA product is produced through thermocyclic amplification. The presence of each amplification product identifies complete or partial homology between the genomic DNA and the oligonucleotide primer at each end of the amplified product (Tingey and Tufo, 1993).

In most experiments each primer will direct the amplification of several discrete loci in the genome, making the assay an efficient way to screen for nucleotide sequence polymorphisms between individuals. The number of different amplification products for each reaction depends upon the primer sequence, the genomic sequence and size of the genome. Williams *et al.*, (1991) showed the frequency of polymorphism detection in

Neurospora crassa as 2.5 per primer after testing 88 primers. Similar frequencies were obtained for *Arabidopsis* (0.3) corn (1.0) and soybean (0.5).

RAPD assaying therefore, is based on random amplification of DNA fragments by the single random primer polymerase chain reaction using DNA from the individuals concerned whether parents, inbred or backcross populations, isogenic lines, germplasm accessions etc. Therefore, the requirements for a RAPD assay are a small amount of DNA and a set of random primers. The amount of DNA required for a reaction is approximately 25 - 100 ng which is a much less than the 1 - 10 µg, required for a Southern blot in a RFLP assay. Several rapid DNA extraction methods have now been developed for use in RAPD assays (Edwards *et al.* 1991.. Brunel, 1992 and Wang *et al.* 1993). The primers used for these reactions are usually of 10-base length with randomly chosen sequences with the requirement that (G + C) content is 60 - 70 % and that they have no self complementary ends.

Application of the RAPD assay is now more widespread than the application of the RFLP assay. The RAPD technology has quickly gained widespread acceptance because it is quick and the chances of detecting polymorphisms are high. Assessment of genetic diversity, characterisation of germplasm and study of introgression are domains of the RAPD markers that have not previously benefited from the use of molecular markers. RAPDs have also generated linkage maps in a large number of plants that had not been mapped by either RFLPs or isozymes. They have further extended isozyme or RFLP based linkage maps by adding RAPDs into such maps.

A wide range of plants including grasses, cereals, vegetables and plantation crops and forest trees have been subjected to RAPD assays. The majority of these studies were for assessment of genetic diversity at inter or intraspecific levels. Discrimination of the species of crucifers (*Brassica* spp; Thorman *et al.* 1994), lotus (*Lotus* spp Campos *et al.* 1994), coffee (*Coffea* spp Orozco *et al.* 1994), raspberry (*Rosaceae* spp; Parent *et al.* 1993), blueberry (*Vaccinium* spp Rowland and Levi, 1994), and garlic (*Allium sativum*;

Mass and Klass 1995); sub species of *Eucalyptus globulous* (Nesbitt *et al.* 1995), cultivars of *rice (Oryza sativa*; Virk *et al.* 1994), *Theobroma cacao* (Ronning *et al.* 1994), date palm (*Phoenix dactylifera*; Corniquel and Mercier *et al.* 1994),), rye (*Secale cereale*; Iqbal and Rayburn *et al.* 1994); varieties and clones of tea (*Camellia sinensis*; Wachira *et al.* 1995) and germplasm accessions of fig (*Ficus carica*; Khadari *et al.* 1995), soybean (*Glycine max*; Abdelnoor *et al.* 1995), cocoa (*Theobroma cacao*; Ronning *et al.* 1995), egg plant (*Solanum melongena*; Karihaloo *et al.* 1994), banana (*Musa accuminata*; Baht and Jarret, 1995), sweet potatoes (*Ipomoea batatas*; Jarret and Austin, 1994), casava (*Manihot esculenta*; Marmey *et al.* 1994), Lettuce (*Lactucae* spp; Waycot and Fort, 1995), oil palm (*Elais guineensis*: Shah *et al.*, 1994), trembling aspen (*Populus tremuloides*; Daniel, 1994) and mahoganies (*Meliaceae* spp; Griffin, 1994) serves as noteworthy examples of such applicat ons.

Besides the demonstration of the ability of RAPDs to reveal genetic diversity between closely related genotypes several other observations on the use of RAPDs were also reported from the above studies Virk *et al.*, 1994 who assessed rice (*Oryza sativa*) germplasm collections at IRRI (International Rice Research Institute) commended the unprecedented speed, efficiency and reliability of the RAPD technique while mentioning that the sources and concentrations of primer can affect the amplification pattern. Heun and Helentjaris (1993) observed inheritance of RAPDs in segregating populations of corn (*Zea mays*) and revealed that the most predictable types of RAPD fragments were those of the greatest intensity in any one reaction and also that once the Mendelian inheritance of a RAPD fragment has been verified within an F_2, its use is quite reproducible. They have observed few RAPD fragments showing aberrant inheritance patterns and attributed that to uniparental inheritance of fragments amplified from organellar DNA. The RAPD analysis of oat (*Avena steralis*) accessions resulted in more definitive separation of clusters of accessions in comparison to previous separations based on isozymes (1994). Studies on barley indicated RAPDs detected an adequate number of markers to distinguish inbred lines with less effort than RFLPs (Tinker *et al.*, 1993).

RAPD technique has been able to detect enough polymorphisms to distinguish celery cultivars which were believed to be low in their level of polymorphism (Yang and Quiros, 1993). However, a high percentage (23%) of spurious bands have been observed indicating the importance of multiple runs and strict control of the experimental conditions. Much higher levels of polymorphism were detected in *Brassica* when broccoli and cauliflower cultivars were screened than previous attempts with RFLPs (Hu and Quiros, 1991). Varieties and cultivars of Oilseeds, *Brassica napus* (rapeseed) and *Brassica juncea* (Indian mustard) have also been distinguished by RAPDs without much effort (Jain *et al.*, 1994, Mailer *et al.*, 1994). It is interesting to note that polymorphic bands used to assess polymorphisms in rapeseed cultivars were found to be stable after automating the reactions in four thermocyclers.

In *Stylosanthus* (legume) the RAPD technique generated enough polymorphisms to obtain phylogenetic distinctions among accessions (Kazan *et al.*, 1993). Studies on alfalfa (*Medicago sativa*) which emphasised using bulk DNA for RAPD screening of heterogeneous crops has also produced promising results. The RAPD technique also generated adequate polymorphisms to distinguish ten cultivars of papaya (*Carica papaya*) and the authors quoted "RAPD technology is a rapid, precise and sensitive technique for genomic analysis" (Stiles *et al.*, 1993). RAPD studies on sweet potato (*Ipomoea batatas*) produced good results in estimating genetic distances between cultivars. Further it was emphasised that RAPDs have a great potential for tagging genes for weevil resistance and culinary/nutritional quality (Connolly, 1994).

RAPD markers have been extensively studied in apple (*Malus domestica*). A cladogram has been constructed to determine the phylogenetic relationships among a number of apple cultivars. Further a DNA fingerprinting system based on 13 RAPD loci amplified by five primers has been developed for rapid identification of apple rootstock (Landry *et al.*, 1994). In a parallel study another 11 cultivars were differentiated by RAPDs. A key based on consistent RAPD markers was proposed to differentiate apple cultivars (Koller *et al.*, 1993).

RAPD markers are now progressively being used for creation of high density maps. Due to the speed and efficiency of the RAPD system it has been possible to construct maps for plants of large genomes within a reasonably short period of time (Tingey and Tufo, 1993). However, inability to detect codominant markers is a disadvantage in the RAPDs. Most of the recent genome mapping projects have involved isozymes and RFLPs in combination with RAPDs to generate linkage maps.

The RAPD-marker map constructed for the model plant genome *Arabidopsis thalina* is a noteworthy experience. Reiter *et al.* 1992 has been able to construct a 320-marker-loci map for *Arabidopsis thalina* in just four months with two full time workers by screening 1200 primers. By comparison the time taken to construct an RFLP map in soybean of 600 loci was two years for five full time workers. Another RAPD map developed with an unprecedented time was that of loblolly pine which contained 191 loci and took only 6 person months (Chaparr *et al.*, 1992 as quoted by Tingey and Tufo, 1993). More examples of RAPD linkage maps of economically important plants that have been developed recently are; cucurbita hybrid (Le *et al.* 1995), pine (*Pinus* spp; Kubisak *et al.* 1995), sugi (*Criptomeria japonica*; Mukai *et al.* 1995), banana (*Musa accuminata*; Faure *et al.* 1995), barley (*Hordeum vulgare*; Guise *et al.* 1994), carrot (*Dacus carota*; Schulz *et al.* 1993), poplar (*Populus* spp; Bradshaw *et al.* 1995) and blueberry (*Vaccinium* spp Rowland and Levi, 1994).

RAPD marker tagging of major genes or QTLs have also been established for many disease resistance and other economic traits in plants has been successful. Tomato mosaic virus resistance (Ohmori *et al.* 1995), powdery mildew resistance in pea (*Pisum sativum*; Hart *et al.* 1995) and low Cadmium uptake in wheat (Penner *et al.* 1995) are traits governed by major genes and tight linkage with RAPD fragments have been established. Several other examples of RAPD marker-linked disease resistance traits include powdery mildew resistance (Li *et al.* 1995) and leaf rust resistance (Procunier *et al* 1995) in wheat, gall midge resistance (Nair *et al.* 1995) in rice, downy mildew resistance and turnip mosaic virus resistance in lettuce (Witsenbeer *et al.* 1995), black leaf spot

resistance in Chinese elm (*Ulmus parvifolia*; Bennet *et al.* 1995), beet cyst nematode resistance (Salentjin *et al.* 1995), bean rust resistance (Johnson *et al.* 1995), scab resistance in apple (Young and Kruger, 1994), head smut resistance in sorghum (Skorupska *et al.* 1995), tobacco mosaic virus in tomato (Dax *et al.* 1994), crown rust resistance in oats (Penner *et al.* 1994), resistance to anthracnose in bean (Mahe *et al.* 1995), stalk and ear rot resistance in maize (Pe at al. 1993).

As with RFLPs mapping of QTLs have also been carried out for several economic traits. Fresh weight of roots, sprouting ability and rooting ability of *Eucalyptus grandis* (Grattopglia *et al* 1995), linoleic acid concentration in rape seed (Tnhuanpaa *et al.* 1995), milling energy requirement of barley (Chalmers *et al.* 1993) serves as examples of QTLs mapped with RAPDs.

Tagging of economic traits with RAPDs is also useful in marker-assisted selection in crop improvement programmes. When a gene is mapped with RFLPs, selection for that gene can be based on tightly linked RFLPs rather than phenotypic scoring. This can be extremely valuable if a trait is recessive, difficult to assay or obscured by the expression of other traits. This also helps to score a trait at a very early stage and for characters such as flowering time and yield even before they are expressed in the plant. RFLPs can also be used to select against unwanted DNA from one of the parents.

There were several studies which were undertaken to compare RFLPs and RAPDs in revealing genetic variation in plants. Liu and Firnier (1993) carried out a study to compare isozyme, RFLP and RAPD markers in revealing genetic variability in two species of *Populus,* trembling aspen (*P. tremuloides*) and big tooth aspen (*P. grandidentata*). The study concluded that RFLPs and RAPDs have a high potential as markers to assist in breeding of aspens. RAPDs consistently revealed greater variation than RFLPs and allozymes. However, the capacity to detect genetic variability by RAPDs was questioned mainly for two reasons: the dominant nature of the marker and the

assumption that each amplified fragment represents a RAPD locus with two alleles. The latter is not always the case since deletion and insertion events that occur in the region between the primers will result in an amplified fragment migrating to a different position. Despite these arguments the patterns of variation observed by the two types of markers were found to be consistent.

Another comparative assay made on *Brassica napus* revealed consistency among data from RFLPs and RAPDs (Hallden, 1994). The RFLP and RAPD assays detected highly similar relationships between genotypes of *B. napus* which were also consistent with known pedigree data. This study estimated the optimum number of probes or primers sufficient to demonstrate genetic relat onships in the *B. napus* genome as 30.

Polymorphisms revealed by sequence-tagged sites (STS) detected by combination of PCR and RFLP analysis has also been used in combination with RFLPs and RAPDs in genome mapping of *populus* (Olson *et al.* 1989, Bradshaw *et al.* 1994). The STS approach is the use of PCR primers designed from terminal sequences of RFLP probes of low-copy-number sequences to amplify DNA fragments. This was considered a better alternative to RAPDs in analysing the wheat genome (Talbert *et al.* 1994).

Besides the ability of molecular markers to detect genetic variation cost is another factor which has been locked into by the investigators. The study on the evaluation of relative merits of RFLPs and RAPDs using maize (*Zea mays*) produced some useful results (Ragot and Hoisington, 1993). In terms of genotyping costs none had advantage over the other in spite of the fact that RAPDs were more economical for small scale projects. Cost for probe development in RFLPs was not included in this study because they have already been developed for maize. For a crop which had not been studied the cost for developing RFLPs, therefore would definitely be greater than for that of RAPDs.

1.5.3.3 Variable Number Tandem Repeat Polymorphisms (VNTRs)

DNA polymorphisms revealed by short tandemly repeated nucleotide motifs in the eukaryotic genome is also a technique currently applied to the identification of genetic diversity in plants and animals. Unlike in the prokaryote, the eukaryotic genome is large and possesses a large proportion of repeated DNA scattered throughout the genome (Britton and Kohne, 1968). These tandem-repetitive sequences are termed satellite DNA and are contained in loci exhibiting moderate to high levels of allelic variation among individuals, providing a useful tool for DNA fingerprinting or distinguishing individuals by DNA profiling (Jeffereys *et al* , 1985 Nakamura, *et al*, 1992). Allelic variation in satellite DNA containing loci are believed to be due to the differences in the number of the repeat unit or the 'core' sequence (Jeffereys *et al* , 1985) and thus have been termed as variable number tandem repeat loci (hence for h VNTRs).

VNTR sequences are generally classified according to the size of their monomeric unit. The length of the core sequence can vary from 2-4 nucleotides as in simple sequence repeats or microsatellites to 5 - 100 nucleotides as in minisatellites (Tautz, 1989, Jeffereys *et al* , 1985a and b) or even greater (more than 100 nucleotides) as in macrosatellites (Jabs *et al.* 1986). The small repeat units of microsatellites may occur in any part of the genome and usually with an overall length of less than 100 nucleotides.

The evolutionary development of satellite DNA is not yet fully understood. Several hypotheses were put forward and the basic consensus of opinion is a two stage process: repetition of short sequences and amplification of the repeats on a large scale (Maio *et al.*, 1977). Initially it was believed that unequal crossing over between homologous chromosomes was the cause for initial creation of repeats. After sequencing minisatellite regions detected by human myoglobin 33-repeat probe Jeffereys *et al.* (1991) suggested recombination events that occur between sister chromatids (replication slippage) after DNA replication as the cause. The differences in these satellites, said to

occur due to point mutations, deletions and insertions, preferentially occurred at one end of the tandem array (Jeffereys *et al.*., 1991).

The function of satellite DNA is believed to have no direct impact on phenotypes (Dover, 1980). The simple sequence nature, hypervariability and lack of transcription makes it difficult to assign a function (Bell *et al.* 1982). Therefore, VNTR's are not often used to tag phenotypic characters.

VNTRs are usually detected by either Southern hybridisation of restriction endonuclease digested genomic DNA with labelled mini or microsatellite probes or by amplifying the genomic DNA using the polymerase chain reaction primed by synthetic oligonucleotides complementary to sequences flanking the repeat region. Most commonly used minisatellite probes include human minisatellites of a 33 bp core sequence (Jeffreys *et al.* 1985a) and a sequence from bacteriophage M13 (Vasart *et al.*, 1987). The microsatellite probes comprise either di, tri or tetra nucleotide 'core' sequences. $(CA)_8$, $(CT)_8$, $(GT)_{12}$, $(GTG)_5$, $(GATA)_4$, $(GACA)_4$ and $(GACA)_4$ are some commonly used microsatellite probes in detecting VNTRs in plants (Sharon *et al.*, 1992., Poulson *et al.*, 1992., Vosman *et al.*, 1992., Arens *et al.*, 1995., Beyerman, *et al.*, 1995).

Human minisatellite probes were derived from a core sequence in the human myoglobin minisatellite. A pure repeat probe was prepared by purification of a single 33 bp repeat and recombinants were constructed by head to tail ligation and cloning of the repeat. Then a recombinant, pAV 33 7 was obtained which comprised 23 repeats of the 33bp core sequence. This clone was used to screen a human DNA library of inserts of 10 - 20 Kb to obtain more clones carrying repeats of the same core. These clones, pAV 33.1, 33.3, 33.4, 33.5, 33.6, 33.10, 33.11 and 33.15 were subsequently sequenced for in depth understanding of this minisatellite in the human genome. These pAV 33 clones derived from human genomic DNA were also found to produce fingerprints in plants such as rice, tomato and rubber (Dallas, 1988, Rogstad *et al.*, 1988 and Besse *et al.*, 1993).

A tandemly repeated 15 bp fragment in the protein III gene of the bacteriophage M13 has also proven its potential as a minisatellite to produce finger prints in human and bovine DNA (Vasart, *et al*, 1987). Subsequently the universality of M13 as a probe for detection of DNA fingerprints has been proven (Ryskov, 1988, Rogstad, 1988). A successful application of M13 in clarifying patterns of speciation and diversity in blackberries (*Rubus* spp; Nybom, 1995) has been reported.

The applicability of microsatellites to fingerprint plant genomes has been investigated by Beyermann (1992) for a range of plant species: *Triticum aestivum, Secale cerele, Horedum vulgare, Beta vulgaris, Petunia hybrida, Brassica oleracea* and *Nicotiana tabacum* and concluded that simple sequence repeat DNA fingerprinting is a powerful tool in breeding research enabling the identification of genotypes, cultivars and varieties. Other studies include DNA fingerprinting of tomato (*Lycopersicon esculentum*, Vosman *et al.*, 1992, Arens *et al.*, 1995), turnip (*Brassica rapa*, Rogstad, 1994), *Carica papaya* (Sharon *et al.*, 1992), rapeseed (*Brassica napus,* Poulson, *et al.*, 1993 and grapevine (*Vitis vinifera,* Thomas *et al*, 1993).

1.6 Aims and objectives of the present study

In order make a genuine genetic advance in the breeding of coconut palm by overcoming the serious limitations currently faced by coconut breeders, the present research study was planned to explore the possibility of using different molecular marker techniques for identification of genetic diversity in coconut palms from Sri Lanka. The material available was from tall *(typica)* and dwarf (*nana*) populations, an F2 family derived from a tall and dwarf cross and some accessions of the coconut population of tall and dwarf coconuts existing in Sri Lanka.

The objectives of the present investigation are,

1. Develop and optimise a technique for the isolation of DNA from coconut leaf material

2. Screen the test material for detection of Random Amplified DNA Polymorphisms (RAPDs):

3. Construct a random genomic library of coconut DNA and screen the test material for detection of Restriction Fragment Length Polymorphisms (RFLPs):

4. Screen the test material for detection of mini and micro satellite polymorphisms using M13 and microsatellite probes by Southern hybridisations and the PCR.

The only description in the literature of an attempt to develop molecular markers in the coconut palm was the detection and sequencing of highly repetitive sequences in the genome, revealed by the presence of bands following digestion of coconut genomic DNA with *Eco*RI. RFLP assays using these repetitive sequences as molecular probes has not detected any polymorphism among tall and dwarf coconut types originating from Papua New Guinea, Solomon Islands and Malaysia (Rohde, *et al.*, 1992). One other molecular biological technique applied in coconut was an attempt made by Harrison *et al.* (1992), to develop a probe to detect myco-plasma- like organisms causing lethal yellowing disease in the coconut palm.

CHAPTER 2

Materials and Methods

2.1.1 Materials and equipment

The general chemicals used were of analytical grade except for ethanol and phenol which were purchased as laboratory grade and redistilled before use. The materials, including common chemicals, unusual biochemical compounds and molecular biological kits, were purchased from the suppliers or manufacturers indicated in Table 2.1. The special equipment used along with the manufacturers are listed in Table 2.2

2.1.2 Solutions and bacterial growth media

Composition of commonly used solutions and bacterial growth media are detailed in Tables 2.3 and 2.4 respectively.

2.1.3 Coconut material used for DNA analysis

Tender coconut leaves were transported from Sri Lanka for isolation of DNA for molecular marker assay procedures. An F_2 family of 18 individuals descended from a tall and dwarf cross (grand parents) followed by the F_1 intercross, planted in the Bandirippuwa estate (BE), Lunuwila, was used to study the patterns of inheritance of the molecular markers.

Additional leaves were collected from different forms of *typica, nana* and *aurantiaca* in the Crop Museum, Bandirippuwa estate (BE), Lunuwila and germplasm accessions planted in the Germplasm Conservation Block at the Pottukulama Research

Station (PRS), Pallama for use in mo ecular marker characterisation studies. Here the leaf samples were taken from at least 4 ir dividuals per form or accession and pooled prior to isolation of DNA. The details of palm s used in the entire study are listed in Table 2.5.

Table2.1 Common c iemicals, biochemical compounds and molecular bi ological kits and their sources

Name of chemical / biochemical / kit	Supplier / Manufacturer
Acrylamide	BDH Laboratory Supplies
Agar	Difco laboratories
Agarose	Progen Industries Limited
Agarose LMP (low melting point)	Gibco, Life Technologies, Inc
Amberlite MB-1 deionisati n resin	Bio-Rad
Ampicillin	Boehringer Mannheim
Ammonium acetate	BDH Laboratory Supplies
Ammonium persulfate (AP S)	M & B, May and Baker Limited
Bactoagar	Difco laboratories
Boric acid	BDH Laboratory Supplies
bis-acrylamide (N,N' Met ylene-bis-acrylamide)	Sigma Chemical Company
Bovine Serum Albumin (B SA)	Sigma Chemical Company
Chloroform	Univar, Ajax Chemicals
Dextran sulfate	Sigma Chemical Company
Dithiothreitol (DTT)	Boehringer Mannheim
DNA polymerase I Klenow fragment	Bresatec
DNA T_4 ligase	Bresatec
DNA T_4 PNK (polynucleotide kinase)	Pharmacia
Deoxynucleside tri phosphates (DNTP's)	Boehringer Mannheim
Ethanol	CSR limited
Ethidium bromide	Sigma Chemical Company
Ethylenediamine tetra a etic ac d disodium salt (EDTA)	Univar, Ajax Chemicals
Formamide	BDH Laboratory Supplies
Bresa clean Kit	Bresatec
Gigaprime DNA labelling l it	Bresatec
Glucose	Univar, Ajax Chemicals
Glycerol	ICN biochemical compounds
Glycine	UNIVAR, Ajax Chemicals
Isopropyl-β-D-thiogalact opyranside (IPTG)	Promega Corporation
Herring Sperm DNA	Sigma Chemical Company
Hydrochloric Acid	BDH Laboratory Supplies

Table 2.1 continued on pa ge 34

Table 2.1 continued from page 33

Name of chemical/biochemical/kit	Supplier/Manufacturer
Isopropanol	BDH Laboratory Supplies
Lambda standard DNA	Sigma Chemical Company
Lysozyme	Boehringer Mannheim
M13mp18 phage DNA	Pharmacia
β-Mercaptoethanol	Sigma Chemical Company
Phenol	BDH Laboratory Supplies
Potassium acetate	BDH Laboratory Supplies
Potassium orthophosphates	Univar, Ajax Chemicals
Proteinase K	Boehringer Mannheim
Polyvinylpyrolidone (PVP)	Sigma Chemical Company
pTZ18 Plasmid DNA	Pharmacia
Restriction endonucleases	Pharmacia
Ribonuclease A	Boehringer Mannheim
α^{32}P Adenosine triphospha e	Dupont
γ^{32}P Deoxyadenosine triph(sphate	Dupont
Sephadex G-50	Pharmacia
Sodium azide	Sigma Chemical Company
Sodium acetate	BDH Laboratory Supplies
Sodium chloride	BDH Laboratory Supplies
Sodium citrate	BDH Laboratory Supplies
Sodium dodecyl sulfate	BDH Laboratory Supplies
Sodium hydroxide	BDH Laboratory Supplies
Sucrose	BDH
Super-base Sequencing Kit	Bresatec
Taq DNA polymerase with *Taq* buffers set	Bresatec
Tris base	Boehringer Mannheim
N,N,N',N'-Tetramethylene liamine (TEMED)	Sigma Chemical Company
Urea	Univar, Ajax Chemicals
5-bromo-4-chloro-3-indoly -β-galactoside (X-gal or BCIC)	Progen
Yeast extract	Difco

Table 2.2 Special equipment used and their manufacturers

Equipment	Manufacturer
pH meter Model 109pH/ mV Meter	Activon
Thermal cycler	Bartelt Instruments Ltd.
DU50 Spectrophotometer	Beckman
J2 - 21 Centrifuge	Beckmann
Microfuge (Bench top)	Beckman
Electrophoresis power supplies	Biorad
Sequencing gel apparatus	Biorad
Gel electrophoresis apparatus	Pharmacia
Laminar flow cabinet Model CF 43S.1	Gelmen Clemco Pty. Ltd
MP-4 Land Camera	Polaroid
Positive / negative 665 film	Polaroid
Medical x - ray films	Fuji photo Film Company
X - ray developer and fixer (1:4)	Ilford Pty. Ltd
Bench top orbitol shaking incubator	Paton Scientific
Hybridisation oven	Hybaid
Electroporator	Biorad
Whatman filter paper (Quality no. 1)	W and R Balston Ltd

Table 2.3 Composition of commonly used solutions

Name of solution	Composition of solution
Gel loading buffer	0.02% bromophenol blue 50% glycerol 5 mM tris.HCl pH 7.5 at room temperature, approx. 22 ^{0}C
TE buffer	10 mM tris.HCl 1 mM EDTA-di-sodium salt pH 8 at room temperature, approx. 22 ^{0}C
TBE electrophoresis buffer	130 mM tris base 45 mM boric acid 2.5 mM EDTA-di-sodium salt pH 8.8 at room temperature (approx. 22 ^{0}C) Prepared as 10 x concentrate
TAE electrophoresis buffer	40 mM tris base 20 mM sodium acetate 1 mM EDTA-di-sodium salt pH 8.2 at room temperature (approx. 22 ^{0}C) Prepared as 20 x concentrate

Table 2..3 continued on page 36

Table 2.3 continued from page 35

Name of solution	Composition of solution
Ligation buffer	60 mM tris.HCl 10 mM magnesium chloride 1 mM dithiothreitol (DTT) 5 µg / ml BSA pH 7.5 at room temperature (approx. 22 ^{0}C) Prepared as 10 x concentrate
SSC	0.15 M sodium chloride 0.015 M sodium citrate Prepared as 20 x concentrate
Denhardt's solution	0.02% polyvinylpyrolidone 0.02% Ficoll 0.02% BSA
Formamide pre-hybridisation medium	50% Formamide 5 x SSC 5 x Denhardt's solution 1% Glycine 50 mM potassium phosphate, pH 6.2 -6.5 200 µg/ml Herring sperm DNA, sonicated and heat denatured
Formamide hybridisation medium	50% Formamide 5 x SSC 1 x Denhardt's solution 10% Dextran Sulfate 20 mM potassium phosphate, pH 6.2 -6.5 100 µg/ml Herring sperm DNA, sonicated and heat denatured Made 90% of final volume
Non-formamide hybridisation medium	0.263 M sodium phosphate buffer, pH 7.2 7% SDS 1 mM EDTA pH 8.0 1% BSA
Spun column stop buffer	10 mM tris.HCl pH 7.5 1 mM EDTA pH 7.5 0.2 % sodium dodecyl sulfate
GET solution	50 mM glucose 25 mM tris-HCl pH 8.0 10 mM EDTA Prepared as 20 x concentrate
Alkaline SDS	0.2 M sodium hydroxide 1% SDS

Table 23 continued on page 37

Table 23 continued from page 36

Name of solution	Composition of solution
5M potassium acetate (for 1 litre)	294.42 g potassium acetate in sterile water made up to 600 ml 115 ml glacial acetic acid 285 ml sterile water
20 % acrylamide solution (for 1 litre) for sequencing gel	193 g acrylamide 6.7 g bis-acrylamide 467 g urea in sterile water made up to 1 litre Stir for 30 min and store in 20 - 30 g Amberlite MB-1 deionising resin
46 % urea solution for sequencing gel	460 g urea in sterile water made up to 1 litre Stir for 30 min and store in 20 - 30 g Amberlite MB-1 deionising resin
10 x TBE for sequencing gel electrophoresis (for 1 litre)	121 g tris base 7.4 g EDTA 53.4 g boric acid in sterile water made up to 1 litre pH 8.3
Coconut DNA extraction buffer 1	200 mM tris-HCl pH 8 50 mM EDTA pH 8 250 mM sodium chloride 2.5 % PVP 0.1 % β - mercaptoethanol
Coconut DNA extraction buffer 2	100 mM tris-HCl pH 8 50 mM EDTA pH 8 500 mM sodium chloride 10 mM β - mercaptoethanol
Coconut DNA extraction buffer 3	50 mM tris-HCl pH 8 20 mM EDTA pH 8

Table 2.4 Bacterial culture media

Bacterial culture media	**Composition of solution**
Luria-Bertini broth (L broth) + Ampicillin	50 mg / ml bacto-yeast extract 10 mg / ml bacto tryptone 85.6 mM sodium chloride 100 µg / ml Ampicillin
Luria-Bertini broth (L broth) + Ampicillin plates	50 mg / ml bacto-yeast extract 10 mg / ml bacto tryptone 85.6 mM sodium chloride 1.5% (w/v) bactoagar 100 µg / ml Ampicillin
SOC medium	50 mg / ml bacto-yeast extract 20 mg / ml bacto tryptone 5 mM sodium chloride 2.5 mM potassium chloride 0.5 mM magnesium chloride 0.5 mM magnesium sulfate 2 mM glucose
2YT + Ampicillin	16 mg / ml tryptone 10 mg / ml yeast extract 5 mg / ml sodium chloride 100 µg / ml Ampicillin
2YT + Ampicillin	16 mg / ml tryptone 10 mg / ml yeast extract 5 mg / ml sodium chloride 1.5% (w/v) bactoagar 100 µg / ml Ampicillin

Table 2.5 Details of coconut palms used for DNA assays. BE, Bandirppuwa estate, PRS, Pottukulama Research Station.

Palm variety	Palm form	Palm identification number / name of accession	Palm location
typica	form, *typica* (T)	Banded palm at BE	BE
nana	form, green (DG)	Banded palm at BE	BE
DG x T (inbred) 1		570	BE
DG x T (inbred) 2		635	BE
DG x T (inbred) 3		655	BE
DG x T (inbred) 4		694	BE
DG x T (inbred) 5		709	BE
DG x T (inbred) 6		837	BE
DG x T (inbred) 7		856	BE
DG x T (inbred) 8		865	BE
DG x T (inbred) 9		868	BE
DG x T (inbred) 10		903	BE
DG x T (inbred) 11		907	BE
DG x T (inbred) 12		911	BE
DG x T (inbred) 13		917	BE
DG x T (inbred) 14		935	BE
DG x T (inbred) 15		940	BE
DG x T (inbred) 16		945	BE
DG x T (inbred) 17		959	BE
DG x T (inbred) 18		967	BE
typica	form, *typica*	2-palm plot	BE (crop museum)
typica	form, gon-thembili	2-palm plot	BE (crop museum)
typica	form, ran-thembili	2-palm plot	BE (crop museum)
typica	form, pora pol	2-palm plot	BE (crop museum)
typica	*typica*	Moorock (20-palm plot)	PRS (germplasm block)
typica	*typica*	Pitiyakanda (20-palm plot)	PRS (germplasm block)
typica	*typica*	Akuressa (20-palm plot)	PRS (germplasm block)
typica	*typica*	Debarayaya (20-palm plot)	PRS (germplasm block)
typica	*typica*	Goyambokka (20-palm plot)	PRS (germplasm block)
typica	san ramon	Clovis (20-palm plot)	PRS (germplasm block)
aurantiaca	form, king coconut	2-palm plot	BE (crop museum)
nana	form green	Brazilin (20-palm plot)	PRS (germplasm block)
nana	form red	Malayan (20-palm plot)	PRS (germplasm block)
nana	form, green	2-palm plot	BE (crop museum)
nana	form, brown	20-palm plot	PRS (germplasm block)
nana	form, red	2-palm plot	BE (crop museum)
nana	form, green	2-palm plot	BE (crop museum)
nana	form, green	Brazil (20-palm plot)	BE (crop museum)
nana	form, red	Cameroon (20-palm plot)	BE (crop museum)

2.2 Methods

2.2.1 Isolation of DNA from coconut leaves

Coconut leaves (leaflets) were cut into small pieces measuring about 1 x 6 cm. These pieces were excised from tender leaves without the rib, sealed in plastic bags and airfreighted. They were stored immediately on arrival at -70°C. The time gap between leaf harvest and arrival was mostly about 5 days. Leaves turning brown (drying) or infected by fungi were discarded and only yellow healthy looking samples were selected for extraction of DNA. The young yellow leaflets were preferred as they have a low polysaccharide content.

The basic protocol suggested for preparation of genomic DNA from plant tissue consists of lysis (by a detergent sarkosyl), digestion (with Proteinase K) and purification (removing the insoluble debris from the lysate, precipitation and purification of the DNA on a caesium chloride gradient (Ausubel *et al.*, 1987). The method used in the present assay to isolate DNA from the coconut leaves is a modified version of the miniprep protocol for isolation of total plant DNA developed by Dellaporte *et al.* (1983). This method also follows the same procedure as the basic protocol except for an initial homogenisation step and use of phenol:chloroform:isoamyl alcohol instead of caesium chloride gradient purification.

The steps involved in the current extraction procedure are, homogenisation of frozen leaves, lysis, high salt precipitation of proteins and polysaccharides, extraction of the supernatant with an organic solvent, treatment with ribonuclease and precipitation of DNA from the aqueous phase with ethanol. After trying various buffers and reagents the following protocol was developed as it was found to be the most promising in terms of yield and quality of DNA. The significant improvements in the protocol include pounding of leaves in liquid nitrogen and suspending in a homogenisation buffer containing PVP and

mercaptoethanol (adopted from Thomas *et al.* 1993) and removal of intact starch and chloroplast by differential centrifugation (adopted Kirkpatrick *et al.* (1987).

The isolation of genomic DNA from coconut leaf samples were routinely performed in the following manner.

Homogenisation. Coconut leaves were first rinsed with distilled water and blotted dry with fine paper towelling. Then about 5 g were weighed and cut into small strips about 1 mm thick and pounded in a small mortar (3 cm diameter) with a pestle adding liquid Nitrogen several times to keep frozen while grinding. The leaf powder was then allowed to thaw for a while before suspending in 30 ml of DNA extraction buffer 1 (200 mM tris-HCl pH 8, 50 mM EDTA (pH 8, 250 mM sodium chloride, 2.5 % PVP and 0.1% β - Mercaptoethanol. See Table 2.3). The samples were then centrifuged for 10 min at 4,000 g in the Beckman JA20 rotor.

Lysis. The pellet was resuspended in 10 ml of DNA extraction buffer 2 (100 mM tris-HCl pH 8, 50 mM EDTA pH 8, 500 mM sodium chloride and 10 mM β - Mercaptoethanol. See Table 2.3). The suspension was then lysed overnight at 65^{0}C with 1 ml of 20% SDS and 100 µl of Proteinase K (25 mg / ml). The lysate was chilled with 3 ml of cold 5 M potassium acetate (pH 4.8) for about 30 minutes in an ice slurry before it was centrifuged for 20 minutes at 25,000 g in the Beckman JA20 rotor.

Precipitation of DNA. Nucleic acids were separated from the aqueous supernatant by chilling for 30 minutes with 0.6 volumes of cold Isopropanol followed by centrifugation for 15 minutes at 20,000 g in the Beckman JA20 rotor. The pellet was suspended in 1 ml of DNA extraction buffer 3 (50 mM tris-HCl pH 8, 20 mM EDTA pH 8. See Table 2.3) and divided into two 500 µl aliquot's in 1.5 ml eppendorf tubes for further purification.

Purification of DNA by phenol, chloroform and isoamyl alcohol. The aliquot's were first mixed thoroughly with 250 µl of tris-neutralised phenol (pH 8.3) by gently inverting and re-inverting the tubes for about two minutes. Then they were further mixed in the same fashion by adding 250 µl of chloroform and isoamyl alcohol (24:1, v/v). The tubes were then centrifuged for 5 min in a microfuge at top speed. The upper aqueous layer was carefully transferred to a new tube without disturbing the white interface and the step was repeated by adding 250 µ of phenol followed by 250 µl of chloroform and isoamyl alcohol. The upper aqueous phases were re-extracted twice with equal volumes chloroform and isoamyl alcohol (24:1, v/v).

Nucleic acids were precipitated again by adding a 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of cold isopropanol. The precipitates were collected by centrifugation for 15 minutes in the microfuge at top speed and resuspended in a volume of 200 µl TE buffer (10 mM tris-HCl, pH 8 and 1 mM EDTA, pH 8). The aliquot's were then consolidated and treated with RNAse (50 µg per ml) and incubated at 37^0C for 2 hours. Thereafter the DNA samples were aliquoted and stored at -20^0C for use in molecular marker assays. Prior to storage they were incubated at 65^0C for 10 minutes to inactivate any possible DNAse activity and prevent degradation of the samples. An aliquot of 5 µl was used for quantitative and qualitative analysis of the DNA (see section 2.6).

2.2.2 DNA analysis

DNA samples were analysed by spectrophotometry and agarose gel electrophoresis to determine size, purity and quantity of DNA isolated.

Spectrophotometry. DNA samples were diluted and subjected to spectrophotometric analysis to estimate the quantity and the purity of the DNA by UV absorptivity. Approximately 50 µl of diluted DNA (amount of dilution depended on the

concentration of DNA present in the sample) was placed into a clean cuvette and absorbence readings were taken at wave lengths between 230 nm and 330 nm. The reading at 260 nm enabled calculation of the concentration of DNA based on the observation that at 260 nm a double stranded DNA solution with an absorbence of 1 has 50 µg / ml DNA.

A characteristic DNA curve gives a peak at 260 nm. The ratio of the reading at 260 nm to the reading at 280 nm provides an estimate for the purity of the DNA. Pure preparations of DNA have values between 1.8 and 2.0. If the sample is contaminated with protein or phenol, the ratio will be significantly less than 1.8 and accurate quantitation of the DNA is not possible (Manak, 1993). In all the instances where the shape of the curve was not characteristic, the DNA samples were redigested with SDS and Proteinase K and re-extracted with phenol, chloroform and isoamyl alcohol.

Agarose gel electrophoresis. Electrophoresis through agarose gels is the standard method used to separate, identify, purify and transfer DNA to hybridising membranes. Analytical gel electrophoresis using 1% agarose in 1 x TBE buffer (Table 2.1.3) was routinely employed to confirm the presence of DNA in samples. Gels for purifying DNA fragments were prepared with low-melting-agarose (LMP agarose) in TAE buffer (Table 2.1.3). Other gels included 0.7% agarose in TAE (200 ml, 20cm x 10 cm long gels) for Southern blots and 1.4% agarose in TBE for resolving random amplified DNA fragments within a small size range (less than 5kb).

In each instance agarose was suspended in the appropriate volume; 20 ml, 40 ml, 80 ml or 200 ml with 1 x TBE or 1 x TAE and boiled carefully in a microwave oven until all the agarose particles were dissolved. When water was lost by evaporation, more water was added to adjust to the original volume. Then the solution was cooled to approximately 55 ^{0}C, poured into a gel tray, and allowed to set with combs in position for about 30 minutes. DNA samples were mixed with 0.1 volume of loading buffer (Table 2.3) and were loaded into wells after immersing the gel in a tank buffer of the same

composition as the gel buffer. The gel was then electrophoresed at a constant current of 80 mA for small gels and at a constant voltage of 40 V for larger (200 ml) gels. DNA was detected by staining the gel in ethidium bromide (1 µg / ml) for 20 minutes. DNA stained with ethidium bromide fluoresces orange under UV light, allowing it to be photographed with polaroid film. Hence the DNA was viewed using a UV transilluminator (peak wavelength 302 nm for analytical gels, or 366 nm for preparative gels) and photographed with the MP-4 Land camera. The sizes of the DNA fragments were determined by using lambda DNA *Hind*III size markers carrying fragments measuring 23.1, 9.42, 6.56, 4.36, 2.32, 2.03, 0.56 and 0.12 kilobases.

DNA extraction from Agarose gels. "Bresaclean" (Table 2.1) extraction was performed with DNA excised from low melting agarose (LMP) gels in 1 x TAE buffer for purifying DNA from the agarose. The procedure used was recommended by the manufacturer of the kit. Briefly, the gel slice was melted at 55^0C in 5.5 M sodium iodide, and then the DNA was bound to a silica matrix (glass milk). The impurities were removed by washing and DNA was eluted into TE buffer at 55^0C.

Storage of DNA samples. All DNA samples were suspended in 1 x TE buffer and stored at 4^0C. In instances where large quantities of DNA were extracted the samples were aliquoted and stored at - 20 ^{0}C.

2.3 Detection of Random Amplified Polymorphic DNA in the coconut

DNA obtained from different coconut varieties, forms, accessions and parent and F2 population were used in single-primed polymerase chain reaction (PCR) to generate random amplified DNA (RAPD) profiles. The size and sequence of the primers used, PCR procedure and other details were as follows:

2.3.1 Size and sequence of the primers

Altogether, thirty eight oligonucleotide primers (10-12 mers) were used in the current RAPD assay. Primers labelled 12-mer 1 to 12-mer 6 and Check 1 to Check 12 were obtained from Dr. C. Beard of the Institute of Biotechnology, UNE. The rest, OPB1 to OPB20 were from the OPERON 10-mer kit (Kit B) manufactured by OPERON Technologies, INC. The sequences of these oligonucleotide primers are indicated in Table 2.6. According to manufacturers these sequences were selected randomly, with the requirement that their (G + C) is 60 - 70 % and that they have no self complementary ends.

Table 2.6 Sequences of oligonucleotide primers used to generate RAPD Profiles

Code	Sequence 5' to 3'	Code	Sequence 5' to 3'
12 mer 1	CTCCGGGGTGTG	OPB	GTTTCGCTCC
12 mer 2	TCATCCGCTTCC	OPB	TGATCCCTGG
12 mer 3	AACCCTTTTCA	OPB	CATCCCCCTG
12 mer 4	TGGCCGCCGTTG	OPB	GGACTGGAGT
12 mer 5	TGGCCACCGTTG	OPB	TGCGCCCTTC
12 mer 6	TAGCCGCCGTTG	OPB	TGCTCTGCCC
Check 1	TCATAAGGCGAT	OPB	GGTGACGCAG
Check 2	AATCGCTTATGA	OPB	GTCCACACGG
Check 3	GGGACTGGAAA	OPB	TGGGGGACTC
Check 4	TTTTCCCAGTCC	OPB 10	CTGCTGGGAC
Check 5	AGGTCATCAGAC	OPB 11	GTAGACCCGT
Check 6	GTCTGAATGACC	OPB 12	CCTTGACGCA
Check 7	GGCAACGCGGTC	OPB 13	TTCCCCCGCT
Check 8	ACCGCGATTGCC	OPB 14	TCCGCTCTGG
Check 9	AAGCTTCACTCC	OPB 15	GGAGGGTGTT
Check 10	GGGAGTAAGCTT	OPB 16	TTTGCCCGGA
Check 11	GACATACGCGAA	OPB 17	AGGGAACGAG
Check 12	TCGCCGTATGGC	OPB 18	CCACAGCAGT
		OPB 19	ACCCCCGAAG
		OPB 20	GGACCCTTAC

2.3.2 Polymerase chain reaction procedure

Experimentally the RAPD assay is carried out by polymerase chain reaction with a single 10 or 12-mer primer. The PCR procedures were based on Williams et al. (1991)

and were also discussed in documentation from the suppliers of *Taq* polymerase (Bresatec) and random primers (OPERON Technologies). The amplification reactions were performed in a thermocycler (Table 2.1.2) in a volume of 25 µl containing 1 x *Taq* reaction buffer (10 mM Tris.HCl pH 8.3, 50 mM Potassium Chloride, 0.001% gelatine) supplied with the enzyme, 2mM Magnesium Chloride, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM of a single 10 mer primer, 50 - 100 ng of genomic DNA and 0.5 units of *Taq* DNA polymerase. The reaction was allowed to progress in 45 repeating cycles of three phases, denaturing of the genomic DNA (1 minutes at 94°C), annealing of primers (1 minute at 36°C) and extension of primers (2 minutes at 72 °C. The amplification products were analysed on 1.5% agarose gels.

2.3.3 Analysis of RAPD profiles

The presence of RAPD bands were scored by visual inspection of the gel photograph and the presence or absence of band was scored as 0 or 1 respectively. In instances of doubt the negatives of the polaroid film were checked.

RAPD profiles obtained for tall and dwarf parents and F2 individuals were tested using the chi square test to determine whether the RAPDs detected were behaving in the Mendelian fashion. RAPD profiles obtained with different varieties, forms and accessions of coconut were analysed using the computer software RAPDistance package developed at the Plant Science Centre of the Australia National University. For this analysis the data were transformed into a 1 or 0 matrix over all genotypes and fragment positions scored.

2.4 Detection of Restriction Fragment Length Polymorphisms (RFLPs)

A random coconut DNA library was constructed by transforming *Escherichia coli* strain PMC 112 with recombinant plasmid pTZ18 . Clones containing high copy sequences were detected by hybridising recombinant colonies back to radiolabelled genomic DNA (Grunstein and Hogness, 1975). High copy and low copy clones were used to probe restriction endonuclease digested DNA from coconut forms to detect RFLPs. A total of 98 clones (71 high copy and 27 low copy) were obtained and were stored in 50% (v/v) glycerol. The details of the procedure were as follows:

2.4.1 Search for RFLP probes

Restriction digestion of insert and vector DNA. Restriction endonucleases cleave DNA at specific sites and the activity of these enzymes depends on the amount of DNA in the sample, the optimal salt concentration, and temperature for the activity of a given restriction enzyme. The restriction endonucleases were supplied with the 10 x incubation buffer (One-Phor-All, Pharmacia) with a table indicating the concentrations of buffer and the reaction temperatures to be used for each restriction enzyme.

Approximately 10 µg of coconut DNA were digested with *Sau*3AI and fragments of 1 -3 kb were size separated in a low melting agarose gel (insert DNA). Similarly about 1 µg of plasmid, pTZ18 was digested with *Bam*HI to linearise the plasmid. Both insert and vector DNA were purified by "Bresa clean" (Table 2.1.2). The recovery and relative concentrations of samples of the two preparations were checked by agarose gel electrophoresis. The respective recognition sequences of *Bam*HI and *Sau*3AI are G↓GATCC and ↓GATC . Therefore, following digestion the open (sticky) ends of the plasmid and cleaved sites of coconut DNA fragments result in the same over hanging sequence.

$^{5'}$NNN$^{3'}$ $^{5'}$GATCNNNN$^{3'}$

$_{3'}$NNNCTAG$_{5'}$ $_{3'}$NNNN$_{5'}$

allowing for insertion of coconut DNA restriction fragments into the plasmid.

Ligation. Ligation of cohesive ends of coconut DNA insert fragments and pTZ18 vector fragments, mixed at 3 : 1, was carried out in a 10 µl volume containing 1 x ligation buffer (Table 2.1.3), 0.5 mM ATP and 2 units of the enzyme, T_4 ligase. The reaction was incubated for 4 hours at 15 ^{0} C. The ligation products were checked by electrophoresing in agarose gel.

Transformation of *Escherichia coli* by electroporation: Electroporation technique was used to transform *E. coli* (strain PMC 112) with ligated plasmid. Competent *E. coli* stocks for use in electroporation were prepared according to the method of Dower, *et al.* (1988). The method involved several steps of washing to ensure low conductivity in the bacterial suspension.

Bacteria were grown to an optical density of 0.5 - 0.8 (at 550 nm), and then rapidly chilled to ice temperature. The culture was centrifuged at 2,500 g for 10 minutes and the cells resuspended in a volume of ice cold distilled water equal to the initial volume of the culture. The cells were agair centrifuged as above, and resuspended in ice cold distilled water to half the initial culture volume. After re-centrifugation as before, bacteria were washed in 0.02 volumes of 10% (vol/vol) glycerol to 0.2 - 0.3% of the original culture volume. Bacteria prepared in this way were stored in 40 µl aliquot's, frozen at - 70 ^{0}C until required.

Electroporation was performed in a 0.2 cm wide electroporation cuvette at 0 ^{0}C after thawing the competent cell suspension and adding 1 µl of the ligation mixture. A 2.5 kV pulse from a 25 µF capacitor (Bio-Rad Gene Pulser) was delivered to a cuvette.

Immediately after the pulse the contents were transferred to a bacterial growth medium (SOC) for incubation.

Detection of recombinant bacterial colonies: The vector pTZ18 allows cloning into the lacZ gene which encodes β galactosidase. When this gene is disrupted by the insertion of foreign DNA it is no longer capable of converting the substrate BCIG to a blue colour in the presence of the inducer IPTG (Manak, 1993). Hence if transformed colonies are grown in a medium containing IPTG and X-gal, recombinant colonies with coconut DNA would appear white while those transformed parental plasmids ligated back without inserts would appear blue. This phenomenon was applied in this study to detect *E. coli* PMC 112 bacteria transformed with coconut DNA inserted in the plasmid pTZ18. The procedure was as follows:

Immediately after elctroporation the bacteria were transferred into a 10 ml tube containing 2 ml of SOC (Table 2.4) supplemented with ampicillin to a final concentration of 100 µg per ml. This was allowed to incubate for one hour at 37 ^{0}C in a bench top orbital shaking incubator. Meanwhile L broth + ampicillin plates were prepared by plating each with 200 µl of a solution containing, 10 µl of X - gal (20 mg / ml in dimethyl formamide), 50 µl of IPTG (100 mM) and 140 µl of SOC. After a one hour incubation 200 µl from the culture were spread on one plate. The rest of the culture was centrifuged at 3000 g for five minutes and the pellet was plated on a second plate. Both inoculated plates were incubated overnight at 4) ^{0}C. The colonies were examined on the following morning and all the white colonies were picked.

Detection of clones homologous to high copy-number sequences : Grunstein colony hybridisation technique was employed to detect clones homologous to high copy-number sequences. The method as described by Maniatis *et al.* (1982) involves transfer of colonies to hybridisation membranes. The details of the procedure were as follows:

All the white colonies were cultured separately in 2 ml volumes of ampicillin supplemented SOB medium for approximately 12 hours and 2 ul of each culture were spotted on L-broth + ampicillin plates in a regular array and replicated on two plates. The colonies were allowed to grow overnight at 40^{0}C and were transferred by carefully laying Hybond-N (Amersham) nylon membranes over the plates. The nylon membranes were cut into the size slightly smaller than the culture plate and the orientation was labelled as N, E, S and W and were baked overnight. The plates were labelled in line with the orientation labels on the membranes before lifting up. The membranes were first treated by laying them colony-side up on blotting paper soaked with 10% SDS for five minutes. Thereafter, the membranes were treated with 1.5 M NaCl, 0.5 M NaOH, and then on blotting paper soaked with 1.5 M NaCl , 0.5 M Tris each for 5 minutes. Thereafter the cell debris in the membranes was washed off with 5 x SSC with a soft paint brush. Each membrane was spotted with approximately 100 ng of denatured chromosomal DNA (prepared by suspending in chromosomal DNA in 1.5 M NaCl and 0.5 M NaOH) as the positive, control before they were air dried and baked in a hot cupboard (90 ^{0}C) for 2 hours. The filters were stored for hybridisation with a labelled probe at a convenient time.

Prior to hybridisation approximately 100 ng of chromosomal DNA was labelled using the oligo-labelling or random priming system. Briefly in this method random primers are annealed to the denatured single strands of the template. Then the enzyme DNA polymerase I, Klenow fragment adds nucleotide residues to the 3'-OH termini of the random primers in the presence of three unlabelled dNTPs (deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate) and α-P^{32}-dATP (a radioisotope of deoxyadenosinecytidine triphosphate) which serve as precursor molecules for DNA synthesis. The labelling reaction was performed using the reaction components supplied with the Gigaprime DNA labelling kit (Table 2.1) and according to the instructions provided with the kit.

The unincorporated nucleotides were removed by spinning the products of the labelling reaction through a Sephadex column. The procedure in brief is as follows: A

column of 0.9 ml was prepared by trapping equilibrated Sephadex G50 with spun column stop buffer in a 1 ml syringe with a siliconised glass wool plug in the bottom. The labelling products, after incubation, were treated with spun column stop buffer (Table 2.3) to stop the reaction and were then spun through the column 1000 rpm for one minute in the bench centrifuge.

Hybridisation was performed in Stark pre-hybridisation and hybridisation media (50% formamide, Table 2.1.3) in the Hybaid mini hybridisation oven (Table 2.1.2). The nylon membranes placed between nylon mesh soaked in 2 x SSC were prehybridised in five ml of prehybridisation solution at 42 ^{0}C for not less than two hours. This was carried out in Hybaid glass tubes and care was taken to eliminate air bubbles. Freshly denatured, labelled and purified DNA was added to 3.6 ml of the hybridisation solution and the volume was made up 4 ml by adding distilled water. This solution replaced the prehybridisation solution and membranes were further incubated over 10 hours at the same temperature.

The probe was removed after the hybridisation and the filters were washed 5 times in 2 x SSC, 0.1% SDS at room temperature, followed by two 30-minute washes at 65 ^{0}C in the same solution. The washed filters were examined by the radiation monitor to detect the intensity of the radioactivity and were autoradiograph with X-ray film for as long as required at - 70 ^{0}C with an intensification screen. The X-ray films were processed by soaking in X - ray developer and in X - ray fixer for 3 minutes.

The colonies that hybridised strongly with the radiolabelled probe were selected as high copy colonies and others as low copy colonies. A total of 98 colonies were selected, 71 high copy and 27 low copy and re-cultured in SOB + ampicillin and stored in glycerol following the instructions in Maniatis *et al.*, (1982). These colonies were named as A1, A2, ... A98. The last twenty seven colonies were picked as low copy colonies

2.4.2 RFLP assay procedure

Chromosomal DNA isolated from tall and dwarf coconuts was digested with restriction endonucleases and subjected to agarose gel electrophoresis. Gels were Southern blotted on Hybond-N (Amersham) nylon membranes and hybridised with randomly-primed ^{32}P-labelled DNA probes from the coconut DNA library. Following hybridisation the membranes were exposed to X-ray films and were scored for the presence of RFLPs. The details of the procedure are as follows:

Digestion of coconut DNA: Most of the coconut DNA digests were performed in 50 µl volumes, containing approximately 10 µg DNA, appropriate concentration of the buffer and 10 units (1 µl) of the restriction enzyme incubated overnight (8 - 16 hours) at 37 ^{0} C. The restriction digests were concentrated by ethanol precipitation before checking by electrophoresis in agarose gels. Initially six restriction endonucleases namely, *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Pst*I and *Hind*III were used and in later assays only *Eco*RV was used.

Southern blotting: The technique of Southern blotting (Southern, 1975) was used to transfer DNA to nylon membranes. Briefly this technique involves the transfer of electrophoretically size fractionated, denatured DNA from an agarose gel matrix to a solid support such as nitrocellulose or nylon membranes by passive diffusion. Relative positions of the DNA fragments are preserved during transfer to the membrane. The basic steps involved in Southern transfer are agarose gel electrophoresis, depurination (nick DNA fragments greater than 10 kb to ease transfer), denaturation, neutralisation and transfer.

DNA digested from tall and dwarf coconuts was electrophoresed in 40 ml - 1% agarose gels in 1 x TBE with approximately 5 µg of digested DNA per well. The DNA was electrophoresed for about 100 minutes at a constant voltage of 100V. The gels were stained in ethidium bromide (1 µg / ml) and photographed the approximately life size. The

gels were then soaked in 0.25 M HCL for two seven-minute periods with gentle agitation for depurination. Then they were washed in distilled water and soaked in the denaturing solution, 0.5 NaOH for another two seven-minute washes. Finally the gels were washed again by soaked in a neutralisation solution, 1M ammonium acetate and 0.02M NaOH for two ten-minute washes.

Nylon membranes (Hybond N) cut into the size of gels were labeled according to the probe, the orientation was marked and the membranes were dry sterilized prior to the transfer. To do a transfer of DNA from gel to membrane a plastic container was filled to about 2 cm with 20 x SSC. A platform was made and on it were placed 3 x Whatman 3MM paper wicks soaked in 20 x SSC. Air bubbles were removed by rolling a clean glass rod across the wicks and the gel was placed upside down. The air trapped between the gel and paper wicks was removed and the nylon membrane was overlaid on top the gel. The rest of the area surrounding the gel was covered by a plastic film (glad wrap). Three sheets of Whatman 3MM paper were cut to the size of the gel, wetted with 20 x SSC and placed on top of the membrane, and the air bubbles were removed again by rolling a clean glass rod across the surface. A stack of absorbent paper towelling (approximately 10 cm high) was placed on top of the gel and covered with a glass plate with light weight on top. The transfer was allowed to proceed overnight. Following transfer the position and orientation of the wells were marked and the membrane was washed in 2 x SSC to remove any agarose sticking to the membrane. The membrane was then air dried at 37 C for 30 minutes and baked at 80 C for at least 2 hours to fix the DNA.

Preparation of Plasmid DNA for hybridisation : Plasmid DNA for probing RFLPs was prepared by the alkaline lysis method as described by Birnboim and Doly (1979) with few modifications. In his method DNA is prepared from plasmid-containing bacterial cultures by treating with sodium dodecyl sulfate and sodium hydroxide which denatures proteins and DNA. Renatured plasmid DNA is recovered by neutralising the medium with potassium acetate (pH 4.8), and the bacterial DNA and proteins are

precipitated. The miniprep procedure routinely performed for RFLP probing was as follows:

Two ml of SOB or L-broth culture medium (Table 2.1.4) supplemented with 100 µg/ml ampicillin (in a 10 ml disposable capped tube) was inoculated with a single colony grown on L-broth agar plates supplemented with 100 µg/ml ampicillin and allowed to incubate overnight at 37^{0}C in a shaker to grow to saturation. .5 ml of the culture was centrifuged in a microcentrifuge tube for 1 minute at 12,000 r. p. m.. and the liquid was removed leaving the bacterial pellet as dry as possible.

The pellet was resuspended in 100 µl of ice cold lysozyme solution (10 mM EDTA pH 8.0, 25 mM tris-HCl pH 8.0, 15% sucrose and 2 mg / ml lysozyme) and incubated at room temperature for 5 minutes. Then 300 µl of a solution of 0.2 N NaOH and 0.1% SDS was added, the contents mixed by inverting rapidly, and stored in an ice slurry for 5 minutes. Then 150 µl of ice cold 3 M potassium acetate (pH 4.8) was added, mixed well, stored in ice for 5 more minutes and centrifuged at 12,000 rpm for 5 minutes.

The supernatant was transferred into a fresh microcentrifuge tube and digested with 2.5 µl of RNAse A (10 mg/ml) at 37^{0}C for 30 minutes. Thereafter the contents were extracted once with equal volumes of phenol : chloroform (1 : 1) and once with diethyl ether. The plasmid DNA was precipitated by adding two volumes of ethanol, mixed by gentle inversion, storing in ice for 2 minutes and collected by centrifugation at 12,000 rpm for 5 minutes. The precipitate was washed with 1 ml of 70% ethanol, re-centrifuged for 5 minutes and dried briefly in a vacuum desiccator. Finally the pellet was suspended in 50 µl of TE buffer and about 5 µl was checked in a 1% agarose gel. Plasmid DNA was linearised by digesting with *Eco*RI before being labelled for use in Southern hybridisation.

Labelling of probes and hybridisation: Labelling of probes and hybridisation were carried out following the same procedures used to label coconut DNA and

hybridisation of filters in the colony hybridisation. The blots were washed after hybridisation in a similar manner and were exposed to X-ray films at - 70 ^{0}C.

2.4.3 Detection of polymorphic patterns using M13 phage as a probe

Radiolabelled M13 bacteriophage was used as a minisatellite probe to detect polymorphic patterns in Coconut DNA from different individuals. DNA from individuals from the parent palms and several F_2 individuals and DNA from individuals belonging to several tall populations and dwarf populations were used in this study. The procedure applied was similar to the RFLP procedure described above except that for 20 cm long gels were used for size fractionation of digested DNA and different hybridisation conditions and the stringency washes were used.

Coconut DNA from different individuals was digested with *Eco*RV or *Hae*III and electrophoresed in 200 ml, 20 x 15 cm 0.7% agarose gels. Each well was loaded with about 5 µg of digested DNA The gels were run for 20 hours at a constant voltage of 40 V. The DNA was Southern blotted to nylon membranes and prehybridised for one hour in the hybridisation solution (non-formamide hybridisation medium, Table 2.1.3) at 55 ^{0}C. Single stranded M13mp18 (Pharmacia) was radiolabelled with α^{32}P dATP using the random primer method and unincorporated nucleotides were removed by spin columns. The hybridisation was carried out overnight in a fresh volume of hybridisation solution containing the radiolabelled probe. The membranes were washed twice for 15 minutes with a low stringency wash solution (2 x SSC, 0.1% SDS) and once for 15 minutes in the same solution at 60 ^{0}C. The blots were exposed to X-ray films at -70 ^{0}C with an intensifying screen for a required period of time to observe DNA fingerprinting patterns.

2.4.4 Detection of microsatellite sequences using oligonucleotide probes

Six oligonucleotides carrying microsatellite (simple sequence repeats) sequences, $(CA)_9$, $(TAA)_9$, $(GAA)_9$, $(GTT)_9$, $(GAT)_9$ and $(GATA)_4$ were used as probes to detect DNA fingerprinting patterns in the coconut palm. These oligonucleotides were end-labelled with γ-^{32}P-ATP according to Fritsch *et al.* (1989). The labelled products were purified by running through a Bio Gel P30 spin column. Southern blots were prepared from 20-cm long gels as in the case with M13 probing.

The non-formamide hybridisation solution used with M13 probing was used with individually labelled probes at temperatures calculated for each oligonucleotide (T_m - 10°C). The blots after one-hour prehybridisation and overnight hybridisation were washed once in 2 x SSC, 0.1% SDS at 65 °C before exposing to x-ray film.

2.4.5 Detection of microsatellite sequences using the polymerase chain reaction

An attempt has been made to sequence small coconut DNA inserts presumed to have microsatellite sequences in order to construct primers for the polymerase chain reaction for amplification of microsatellite alleles. The detailed procedure is as follows:

Construction of the genomic library: A coconut genomic library was made using the same procedure described in section 2.4.1 but with inserts ranging from 300 - 600 bp to facilitate sequencing. These colonies were screened by probing with γ-^{32}P-ATP end-labelled oligonucleotides, $(CA)_9$, $(GA)_9$, $(ATT)_9$, $(GAA)_9$, $(GTT)_9$, $(GAT)_9$ and $(GATA)_4$. Five positive clones were identified for sequencing the inserted fragment.

Isolation of plasmid DNA for sequencing: In order to isolate plasmid DNA for the sequencing reaction a 10 ml overnight culture was made in 2 YT broth (Table 2.1.4)

supplemented with 100 µg / ml ampicillin after inoculating the medium from a single colony of recombinant bacteria grown in 2 YT ampicillin supplemented plates. The culture was centrifuged for 10 minutes at 1,500 g in the Beckman JA20 rotor. The bacterial cells were resuspended in 300 µl of lysozyme (50% (v/v) 2 x GET (Table 2.1.3), 2 mg / ml lysozyme, 50 µg / ml) / RNAse A (0.01% RNAse A, 10 mM tris.HCl pH 7.5, 0.015 M NaCl) and transferred to a microcentrifuge tube and allowed to lyse on ice for 30 minutes.

The lysate was treated with 600 µl of an alkaline SDS solution (0.2 M NaOH, 0.1% SDS) and incubated on ice for 5 minutes. The cell debris, protein and bacterial DNA were precipitated by adding 450 µl of cold 5M potassium acetate (pH 4.8) and by keeping on ice or another 5 minutes. The suspension was centrifuged at 12,000 g for 10 mins. The supernatant, after spinning again at the same speed for ten minutes, was separated into two aliquot's of equal volume and transferred into fresh tubes for ethanol precipitation.

The plasmid DNA was first obtained by adding 2 volumes of redistilled absolute ethanol, incubating 5 minutes at room temperature and centrifuging at 12,000 g for 15 minutes. The pellet was dried under vacuum, resuspended in 20 µl TE buffer and extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) twice and once with chloroform : isoamyl alcohol (24 : 1). The DNA was recovered again by adding 0.1 volume of 3M sodium acetate (pH 4.5) and 2.5 volumes of redistilled absolute ethanol. The tube was held at -20 ^{0}C for 20 minutes before centrifugation at 12,000 g for 10 minutes. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 20 µl of TE buffer. A 1 µl from the sample was electrophoresed on a 1% agarose gel.

DNA sequencing : Sequencing reactions were based on the dideoxy chain termination method of Sanger *et al.* (1977) and were performed according to the protocol provided with the Bresatec Super-Base sequencing kit. Approximately 5 µg of double

stranded DNA was sequenced using T7 promoter and universal primer. The reactions were labelled with labelled with α^{35}S dATP.

Polyacrylamide denaturing gel: DNA sequencing products were electrophoresed on 8% polyacrylamide gels. The gels were prepared by mixing 56 ml of 20% polyacrylamide solution (Table 2.1.3), 70 ml of 46% urea (Table 2.1.3), 14 ml of 10 x TBE and 160 µl of 1.1 M ammonium persulphate and 80 µl of 6.6 m TEMED as polymerising agents. The mixture was poured into a SEQI-GEN sequencing apparatus (Bio-RAD) and allowed to set overnight.

After assembling the gel apparatus with 1 x TBE tank buffer, the gel was pre-run until it reached 50 °C. Reaction products were denatured by heating to 70 °C for three minutes. Then, 2.5 µl of each reaction product was loaded onto the gel. The gel was run at a constant power of 130 W (2300 V, 250 mA). The gel was run for approximately 4 hours while maintaining the temperature at 50°C and paused for the second loading and run for a further 3 hours. The gel was then transferred to 3MM Whatman filter paper and dried on a Bio-Rad gel drier at 80°C for 2 hours.

Autoradiography : After drying the gel was exposed to X - ray film (Fuji Medical X - ray film) for 4 days. The autoradiographs were then developed and fixed.

Analysis of the sequences: The sequences were read to identify microsatellite repeat sequences in the insert fragments.