DNA RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN THE IDENTIFICATION OF CLONAL VARIANTS OF *EUCALYPTUS*

Mornay Coulson

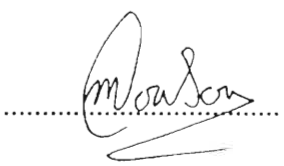
Submitted in partial fulfilment of the
requirements for the degree of
Master of Science,
in the
Department of Biology,
University of Natal,
Durban
1993

Durban
1993

PREFACE

The experimental procedures, described in this thesis, were undertaken in the Photosynthetic Nitrogen Metabolism Research Unit of the Biology Department, University of Natal, Durban. Studies were conducted from January 1991, to December 1993, under the supervision of Drs B.I. Huckett and M.P. Watt.

The findings reported represent original work by the author and have not been submitted in any form to another University. Where use was make of the work of others, they are duly acknowledged in the text.



M. Coulson

ACKNOWLEDGEMENTS

I would like say a sincere "thank you" to the supervisors of this thesis, Drs Barbara Huckett and Paula Watt. Barbara and Paula, over the past three years, your approachability, and willingness to provide expert scientific advise and personal encouragement have been very much appreciated. Working with you has been a valuable learning experience that I will never forget.

To my family, Mom and my husband, David. Thank you both for your love and support. I could not have done this without you.

Thanks are expressed to Dr. Barry Herman and Dave McKellar from Mondi Forests, for providing the plant material and much of the information required for the completion of this thesis.

The Foundation for Research and Development is acknowledged for financial support.

To my Father,
Ronald Aubrey Coulson

"THE ROSE STILL BLOOMS"

A rose once grew where all could see,
sheltered beside a garden wall,
And, as the days passed swiftly by,
it spread its branches, straight and tall.

One day, a beam of light shone through
a crevice that had opened wide-
The rose bent gently toward its warmth
then passed beyond to the other side

Now, you who deeply feel its loss
be comforted - the rose blooms there,
Its beauty even greater now,
nurtured by GOD's own loving care

Author unknown

ABSTRACT

The technique of restriction fragment length polymorphism (RFLP) analysis, of chloroplastic and genomic DNA, was investigated as a means of identifying eucalypt species and cultivars which are morphologically indistinguishable from one another. In order to resolve chloroplast DNA (cpDNA) RFLPs, a method was developed to extract high yields of intact chloroplasts from *Eucalyptus grandis* S/N M6. Starch contamination was reduced by incubation of saplings in the dark for 48 h prior to extraction and watering with a solution containing 370 mM Na-phosphate and 296 mM KNO_3. Optimal chloroplast yields (25 μg chlorophyll/g fresh mass) were obtained by chopping leaf material, using a vertical homogenizer, in a buffer containing 350 mM sorbitol, 50 mM tris-HCL and 5 mM EDTA, 0.1 % (w/v) bovine serum albumin, 0.15 % (w/v) 2-mercaptoethanol, 2 mM L-ascorbic acid and 1 mM $MgCl_2$, followed by washing of leaf pieces in a buffer containing only sorbitol, tris-HCL and EDTA. When these chloroplasts were used in an "in-organelle" DNA digestion procedure, polymorphisms were observed between the cpDNA profiles resolved for *E. grandis* S/N M6 and that of an outgroup species (spinach). However, the developed chloroplast extraction technique could not be used to obtain chloroplasts from various other eucalypt species, probably as a result of variability in the material at an ultrastructural or biochemical level.

For the analysis of genomic DNA RFLPs, a DNA extraction procedure was optimized for use with various eucalypt species and cultivars. This included the development of a purifcation technique during which DNA was ammonium acetate-ethanol precipitated and subjected to mini-dialysis. Following Dra I restriction of DNA, the extract was electrophoresed and Southern blotted onto both nylon and

nitrocellulose membranes. These were probed with a Hind-III resricted sample of the multilocus plasmid probe pV47-2. This probe was labelled using ^{32}P as well as a non-radioactive labelling substance digoxygenin (DIG). Hybridization conditions, including the composition of the hybridization buffer, were optimized for use with these labels, and DNA RFLPs (fingerprints) were resolved for the eucalypt species *E. grandis* and *E. macarthurii* and cultivars of *E. grandis* (S/N M6, TAG 5 and TAG 14). An average of 8.5 bands were detected with ^{32}P and 5.0 fragments with DIG. All the species and cultivars fingerprinted with the ^{32}P-label could be distinguished from one another. However, as a result of the reduced sensitivity of the DIG system, two of the *E. grandis* cultivars, S/N M6 and TAG 5, could not be differentiated. It is concluded that the latter system would be most suitable for incorporation into a routine eucalypt screening programme, although it is suggested that the colourimetric detection assay, used in this study to resolve DNA bands, be replaced by a more sensitive one.

TABLE OF CONTENTS

	Page number
Preface	i
Acknowledgements	ii
Devotion	iii
Abstract	iv
Table of contents	vi
List of Tables	xi
List of Figures	xii
List of Plates	xiv
List of Appendices	xv
Abbreviations and symbols	xvi
CHAPTER 1 GENERAL INTRODUCTION	1
1.1 The eucalypts	1
1.2 The eucalypt forestry industry in South Africa	1
1.2.1 Clonal forestry programmes	1
1.2.2 Problems encountered in the eucalypt breeding programme	2
1.3 Identification of plant cultivars	3
1.3.1 Classical methodologies	3
1.3.2 Molecular characterization of plant materials.	3
1.4 Aims of this study	5
CHAPTER 2 CHLOROPLAST DNA RFLP ANALYSIS	6
2.1 LITERATURE REVIEW AND RATIONALE	6
2.1.1 The chloroplast genome	6
2.1.1.1 Conformation and size of the genome	6
2.1.1.2 The chloroplast gene complement	8
2.1.1.3 Evolution of the chloroplast genome	9
2.1.1.4 The eucalypt chloroplast genome	10
2.1.1.5 Plastid inheritance	10
2.1.1.6 Inheritance of the chloroplast in the eucalypts	12

2.1.2	Systems for the analysis of cpDNA RFLP	12
2.1.3	Applications of chloroplast DNA analysis	13
	2.1.3.1 Taxonomic studies	13
	Population level	13
	Species level	14
	Genus level	14
	2.1.3.2 Plant breeding studies	15
	Genetic characterization	15
	Identification of hybrid material	15
	Managing of seed orchards	16
2.1.4	Rationale and introductory comments	16
2.2	MATERIALS AND METHODS	18
2.2.1	Plant material and growth conditions	18
2.2.2	Chloroplast isolation and purification	18
2.2.3	"In-organelle" DNA digestion and restriction fragment purification	19
2.2.4	Agarose gel electrophoresis of DNA fragments	19
2.2.5	Photography	20
2.2.6	Statistical analysis	20
2.3	RESULTS AND DISCUSSION	21
2.3.1	Chloroplast isolation and purification	21
	2.3.1.1 Pre-conditioning of leaf material	21
	2.3.1.2 Optimization of the chloroplast isolation protocol	23
2.3.2	Chloroplast DNA analysis	27
2.3.3	Applicability of the developed protocols for genetic identification in eucalypts	29
2.4	Concluding remarks	32

	CHAPTER 3 FINGERPRINTING OF GENOMIC DNA	34
3.1	LITERATURE REVIEW AND RATIONALE	34
3.1.1	General Introduction	34
3.1.2	Systems for the analysis of DNA	36
	3.1.2.1 Restriction enzymes for DNA cleavage	36
	3.1.2.2 Source and selection of probes	38
	Single-locus probes	38
	Multi-locus probes	38
	Oligonucleotide probes	39
	The M13 repeat probe	41
	The plasmid probe pV47-2	43
3.1.3	Applications of DNA polymorphism analysis	45
	3.1.3.1 Genome studies	45
	Construction of linkage maps	45
	Mapping of quantitative trait loci (QTLs)	48
	Phylogenetic analyses	48
	Development of conservation strategies	49
	Characterization of cultured material	49
	3.1.3.2 Identification of biological material	50
	Forensic applications	50
	Animal studies	50
	Plant breeding studies	51
3.1.4	Rationale and Introductory comments	53
3.2	MATERIALS AND METHODS	54
3.2.1	Plant material and growth conditions	54
3.2.2	Growth and maintenance of bacterial strains	54
3.2.3	Transformation of *E. coli*	54
3.2.4	DNA extraction and purification	55
	3.2.4.1 Plasmid DNA	55
	3.2.4.2 Plant genomic DNA	56
3.2.5	DNA assessment	57

3.2.6	Restriction enzyme digestion	58
3.2.7	Separation of fragments by agarose gel electrophoresis	58
3.2.8	Southern blotting	58
3.2.9	Random primer probe labelling and purification	60
	3.2.9.1 Labelling with ^{32}P	60
	3.2.9.2 Labelling with digoxygenin (DIG)-dUTP	60
3.2.10	Hybridization of labelled probe to membranes	61
3.2.11	Detection of bound probe	62
	3.2.11.1 ^{32}P detection	62
	3.2.11.2 DIG-detection	62
3.2.12	Photography	63
3.3	RESULTS	64
3.3.1	Eucalypt DNA isolation and purification	64
	3.3.1.1 Apparent yield and purity of the DNA obtained	64
	3.3.1.2 Effect of purification protocols on DNA yield determination	66
	Ammonium acetate-ethanol preciptation	66
	Spun column purification	67
	Dialysis	68
	Conclusions regarding the purification techniques	71
3.3.2	Probe preparation and labelling	72
	3.3.2.1 Plasmid extraction, purification and confirmation of identity	72
	3.3.2.2 Labelling of pV47-2	74
	^{32}P labelling	74
	DIG-labelling	75
3.3.3	Optimization of conditions for probe hybridization and detection	77
	3.3.3.1 Hybridization of probe to genomic DNA	77
	3.3.3.2 Detection of bound probe	80
3.3.4	Use of the developed procedures for the generation of eucalypt DNA fingerprints	84

3.3.4.1 Characterization of eucalypt species and cultivars 86

3.4 DISCUSSION 90

3.4.1 **Applications of the developed fingerprinting procedure** 90
3.4.1.1 Identification of eucalypt cultivars 90
3.4.1.2 Determination of degree of relatedness and genetic variation in eucalypt populations 92
3.4.1.3 Further applications of the eucalypt fingerprinting technology 93

3.4.2 **The feasibility of incorporating the developed protocol into a routine screening procedure for eucalypts** 94
3.4.2.1 Suitability of the DNA extraction procedure 94
3.4.2.2 Advantages of using the probe pV47-2 96
3.4.2.3 Comparison of the ^{32}P and DIG probe labelling and detection systems 97

3.4.3 **The use of RAPDS in the identification of plant cultivars** 100

CHAPTER 4 GENERAL CONCLUSIONS 102

REFERENCES 104

APPENDICES 128

LIST OF TABLES

Page number

Table 2.1 Effect of extraction buffer formulations on the yield of chloroplasts obtained from *Eucalyptus grandis* S/N M6. 24

Table 3.1 Examples of restriction enzymes used in studies of DNA polymorphisms. 37

Table 3.2 Probes used for DNA fingerprinting in plant genomes. 39

Table 3.3 Plants for which RFLP linkage maps have been constructed. 47

Table 3.4 Yields and purities of DNA samples extracted from three *Eucalyptus* species using the unmodified "CTAB" DNA isolation and purification protocol of Doyle and Doyle (1990). 65

Table 3.5 Effect of purification procedures on the apparent yield and purity of DNA samples obtained from *Eucalyptus grandis* S/N M6 and a *Eucalyptus grandis* x *Eucalyptus nitens* hybrid NG 1026. 69

Table 3.6 Scoring of bands detected on autoradiograph (Figure 3.12) and DIG blot (Figure 3.13) after hybridization of labelled probe to Dra I digested eucalypt DNA samples. 86

Table 3.7 "D" values calculated from the autoradiograph (Figure 3.12) (Table 3.5) and DIG blot (Figure 3.13) (Table 3.5) for all possible pairwise comparisons between eucalypt individuals. 87

Table 3.8 Calculated probabilities that the DNA fingerprints observed on the autoradiograph (Figure 3.12) and the DIG blot (Figure 3.13) (Table 3.6) would be identical for the eucalypt species *E. macarthurii* and *E.grandis* and the *E. grandis* cultivars TAG 14, TAG 5 and S/N M6. 88

LIST OF FIGURES

Page number

Figure 2.1 Gene map of the tobacco chloroplast genome (Shinozaki *et al.*, 1986). IR, inverted repeat; LSC, large single copy region; SSC, small single copy region. **7**

Figure 2.2 Effect of physical extraction procedures on the yield of chloroplasts obtained from *Eucalyptus grandis* S/N M6, using the optimized extraction buffer (Table 1). **26**

Figure 2.3 DNA restriction fragments, generated by "in-organelle" digestion of chloroplasts isolated from *Eucalyptus grandis* S/N M6 and separated by agarose gel electrophoresis. **28**

Figure 2.4 Comparison of the chloroplast DNA fragment profile of *E.grandis* with that of an outgroup. **28**

Figure 2.5 Chloroplast yields obtained from various eucalypt sources, using the protocol optimized for *Eucalyptus grandis* S/N M6. **29**

Figure 2.6 Comparison of chloroplast bands formed at the 58 % (w/v) to 30 % (w/v) sucrose interface, after centrifugation at 90 000 *g* for 30 min at 4 °C. **30**

Figure 2.7 Gross morphology of the second terminal leaf of A, *E.grandis*; B, *E. nitens* and C, *E. macarthurii*. **31**

Figure 3.1 Diagrammatic representation of the procedures involved in the production of restriction fragment profiles or DNA fingerprints".**35**

Figure 3.2 Restriction map of the M13 bacteriophage vector. **43**

Figure 3.3 Diagrammatic representation of the plasmid pV47-2 constructed by Longmire *et al.* (1990). **44**

Figure 3.4 Diagrammatic representation of the crossing schemes used in segregation analysis for the production of RFLP linkage maps. **46**

Figure 3.5 Side view of the capillary blot apparatus set up for the transfer of DNA from agarose gels to nylon or nitrocellulose membranes. **59**

LIST OF FIGURES cont.

Page number

Figure 3.6 Agarose gel electrophoresis of DNA samples extracted using the "CTAB" procedure and purified in various ways. 70

Figure 3.7 Agarose gel electrophoresis of the purified plasmid. 73

Figure 3.8 Dot blot of DIG labelled DNA. 76

Figure 3.9 Effect of hybridization buffer formulations on the background signal obtained after simple incubation of membranes in DIG-labelled pV47-2. 78

Figure 3.10 Autoradiograph of calf thymus and *Eucalyptus grandis* S/N M6 DNA fragments hybridized to ^{32}P labelled probe. 81

Figure 3.11 DIG blot of calf thymus and *Eucalyptus grandis* S/N M6 DNA fragments hybridized to DIG labelled probe. 82

Figure 3.12 Autoradiograph of eucalypt DNA fingerprints. 84

Figure 3.13 DIG blot of eucalypt DNA fingerprints. 85

LIST OF PLATES

Page number

Plate 3.1 Banding of pV47-2 and *E.coli* chromosomal DNA on a caesium chloride-ethidium bromide density gradient following centrifugation at 100 000 rpm at 18 °C for 4 h. **72**

LIST OF APPENDICES

Page number

Appendix 1. Flow diagram representing the protocol developed for the extraction of chloroplasts from *E. grandis* S/N M6. **128**

Appendix 2. Flow chart of the "in-organelle" digestion procedure used to resolve cpDNA RFLPs. **129**

Appendix 3. Diagram to represent the DNA extraction and purification procedure optimized for use with the euaclypt species and cultivars. **130**

Appendix 4. Diagrammatic representation of the procedures used to generate genomic DNA fingerprints for the eucalypts. **131**

ABBREVIATIONS

bp	basepair
BCIP	5-bromo-4-chloro-3-indolyl phosphate toluidinium salt
BSA	bovine serum albumin
^{o}C	degrees centigrade
cm	centimeter
cpDNA	chloroplast DNA
cpm	counts per minute
CsCl	caesium chloride
CTAB	hexadecyltrimethylammonium bromide
DPP	(3-(4-methoxyspiro[1,2-di-oxetane-3,2tricyclo[3.3.1.1$^{3.7}$]decan]-4-yl)phenyl phosphate)
DIG	digoxygenin
g	gram
h	hour
kb	kilobase
μCi	microcurie
μg	microgram
μl	microliter
M	molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
NBT	nitro blue tetrazolium salt
ng	nanogram
nm	nanometer
pers comm	personal commmunication
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
spp	species
U	enzyme units
V/cm	volts per centimeter
UV	ultra violet
v/v	volume by volume
w/v	weight by volume

CHAPTER 1
GENERAL INTRODUCTION

1.1 THE EUCALYPTS

Eucalyptus is a genus of mostly trees and some shrub species belonging to the family Myrtaceae (Pryor and Johnson, 1971). Also known as "gums", these trees are characterized by the exudation of resins from their barks and a characteristic menthol aroma (Phillips *et al.*, 1988).

Eucalypts are described as "multi-purpose" trees, capable of providing a great range of products from fixed nitrogen, animal fodder, shelter and edible fruits to sawn timber, mining timber, pulp, poles, firewood, charcoal, essential oils, honey and tannins (Turnbull, 1991). In 1991 there were eight millions hectares of eucalypts under plantation worldwide (Turnbull, 1991).

1.2 THE EUCALYPT FORESTRY INDUSTRY IN SOUTH AFRICA

The forestry industry is one of the most rapidly growing sectors of the South African economy with a total capital investment of R6 800 million in 1991 (Anonymous, 1991). Of the total 1 241 299 ha under tree plantation, a large proportion of the area, namely, 513 220 ha, is occupied by eucalypt species (Anonymous, 1991).

1.2.1 Clonal forestry programmes

Much of South Africa is dry and only a small proportion of the country is characterized by good rainfall, fertile soils and climates suitable for tree growth (Denison and Kietzka, 1993b). However, as those areas are scarce, they are highly in demand for the planting of agricultural crops and breeding of livestock. As a result, eucalypts are grown largely on marginal land sites in the southern Cape, Natal Midlands, Zululand, eastern and north-eastern Transvaal (Denison and Kietzka, 1993b). The eucalypt species selected for growth in these areas need to be well adapted to the environmental and climatic conditions of the site, show good growth performance, be tolerant to diseases and pests, and produce wood of high yield and quality for processing (Denison and Kietzka, 1993a). Such species have been obtained through clonal breeding programmes.

In a recent review published by Denison and Kietzka (1993a) those authors point out that the "clonal forestry" idea captured the imagination of South Africans in 1982/1983, around the time when there was a worldwide expansion in the mining and forestry industries. Since that time, the development of clonal tree breeding programmes has resulted in large -scale growth of the eucalypt forestry industry in South Africa.

According to the above authors, clonal forestry programmes involve selection of particular eucalypt species or hybrids which fulfil all the criteria described above. These are then mass propagated through vegetative cuttings or micropropagation of seedlings and planted out at appropriate sites. This ensures uniformity in the wood properties of all the trees found growing in a particular area and optimal wood production under a tested set of field conditions. Thus, the potential exists to establish thousands of hectares of clones producing wood and pulp of high quality.

1.2.2 Problems encountered in the eucalypt breeding programmes

The production of clonal plants is a costly and labour-intensive process, requiring a great capital input for the construction of glasshouse facilities and constant supervision in terms of the conditions required for plantlet propagation. The effort which goes into the production of healthy clonal plants is often lost through ignorance and negligence when the plants are handed over to the forester for planting in the field (Denison and Kietzka, 1993a). For example, many of the eucalypt cultivars which are multiplied as clonal plantlets, are phenotypically indistinguishable from one another. That is, they cannot be identified on the basis of morphological characteristics such a leaf shape or bark texture (B. Herman, pers comm). It is often the case that these clones are mis-identified by foresters once they are planted out in the field, and the implications of such a mistake are serious (B. Herman, pers comm). For example, a eucalypt clone A, which had been incorrectly identified as clone B, could be brought in from the field and found to have excellent wood properties. This would result in mass planting of clone B in an area, although this clone, unlike clone A, may not be suited for growth under those environmental conditions. Such a mistake could lead to a radical reduction in the productivity of eucalypt trees. Therefore, the need exists to develop a methodology which could be used to positively identify eucalypt cultivars and clones. In order to be useful in large-scale screening, the protocol developed would need to be reasonably simple to carry out and cost-effective.

1.3 IDENTIFICATION OF PLANT CULTIVARS

1.3.1 Classical methodologies

Traditionally, plants species and cultivars have been identified on the basis of their appearance or morphology. For example, individual plants were recognized on the basis of floral bract shape and colour, colour of the leaves, angle between lateral branches and the bole of the tree, colour and shape of flowers, plant height, to mention only a few, commonly scored, characteristics (Kemperman and Barnes, 1976; Mitton and Grant, 1980; Weier *et al.*, 1982). However, this method of cultivar identification requires extensive observation of plants in many different environmental conditions, as the phenotypic traits measured are often a product of environmental influences (Wrigley *et al.*, 1987). In addition, this methodology lacks definition and objectivity on the part of the persons characterizing the plant material (Wrigley *et al.*, 1987). Apart from the difficulties encountered when applying this method for plant identification, further problems have arisen when the material to be classified represents different species or cultivars, but, these are morphologically indistinguishable from one another. This has been found in the classification of certain *Cornus florida* (dogwood) (Santamour and McArdle, 1985) and *Populus tremuloides* (quaking aspen) cultivars (Rogstad *et al.*, 1991). Within these genera, trees belonging to various cultivars are so phenotypically similar, that they cannot be identified on the basis of their morphologies (Santamour and McArdle, 1985; Rogstad *et al.*, 1991). As mentioned previously (refer section 1.2.2), similar problems have been encountered in the identification of eucalypt cultivars.

1.3.2 Molecular characterization of plant materials

Recently, the ease and rapidity of generating data using molecular markers have made these the favoured traits for identifying plant materials (Prince *et al.*, 1992). These markers include protein profiles, HPLC chromatograms of secondary compounds, isozymes and DNA restriction fragment length polymorphisms (RFLPs) (Prince *et al.*, 1992).

Analysis of plant proteins requires electrophoretic separation of total soluble proteins, including isozymes, on starch or polyacrylamide gels, where molecules are separated on the basis of their molecular weight and charge density (McDonald, 1991). The characteristic banding patterns of protein molecules, produced after

separation using these systems, have allowed the distinction of cultivars and hybrids of *Zea mays* L. (maize) (Stuber *et al.*, 1988), *Avena sativa* L. (oat) (McDonald, 1991) and *Lens* (lentil) (de la Rosa and Jouve, 1992). However, protein analysis has failed to distinguish certain *Malus* (apple) varieties (Weeden and Lamb, 1985; Bournival and Korban, 1987) and *Rubus* (raspberry and blackberry) cultivars (Cousineau and Donnelly, 1989). In addition, as proteins are the products of gene expression, their presence may vary in tissues at different developmental stages and across variable environments (Beckman and Soller, 1983).

Analysis of DNA polymorphisms, using RFLPs, has become the most favoured molecular technique for identifying plant species and cultivars. Samples of genomic DNA, extracted from the tissue of interest, are digested with restriction enzymes and the resulting mixture of DNA fragments separated on an agarose gel (Botstein *et al.*, 1980). These are then Southern blotted onto membranes and probed with cloned DNA sequences which have been labelled in some way, usually using a radioisotope (Helentjaris *et al.*, 1986). Unlike the approach which relies on the scoring of morphological traits (refer section 3.1), this methodology distinguishes material at the level of the genome (Dowling and Brown, 1989). In addition, RFLP analysis offers many advantages over other molecular systems which have been used thus far to identify plant materials. For example: (1), the number of DNA polymorphisms which may be scored greatly exceeds the number of isozyme and protein loci available for assay; (2), these polymorphisms may occur in both the coding and non-coding regions of the genome; that is, analysis does not require the expression of genes before gene products can be assayed; (3), polymorphisms can be detected in most tissues, irrespective of their state of development; (4), DNA polymorphisms are usually co-dominant markers and can be resolved at various intensities to indicate both the heterozygous and homozygous condition; and (5), these markers are unlikely to have pleiotropic effects on other scorable characteristics (Neale and Williams, 1991; Dowling and Brown, 1989).

RFLP analysis has been carried out with both the chloroplastic and nuclear genomes. As the chloroplast genome has been reasonably conserved over evolutionary time, certain workers suggested that limited chloroplast DNA (cpDNA) polymorphisms would exist at a cultivar level (Banks and Birky, 1985). However, subsequent to this work, cpDNA polymorphisms were detected between accessions of *Dioscorea bulbifera* L (yam) (Terauchi *et al.*, 1991). Therefore, the potential exists for using this methodology in the identification of plant cultivars. In

fact, cpDNA analysis has been carried out with the eucalypts (Steane *et al.*, 1991), but, those authors used cpDNA polymorphisms to examine the difference in cpDNA organization between six species of *Eucalyptus*. Steane *et al.* (1991) were addressing phylogenetic questions and did not consider the issues which would be important in the optimization of a cpDNA analysis methodology for routine eucalypt cultivar identification.

Nuclear DNA polymorphisms have been utilized far more extensively than cpDNA analysis in the identification of materials from the level of the species to the individual. In particular, genomic DNA RFLP analysis has allowed workers to distinguish various varieties and cultivars belonging to the woody *Malus* (apple), *Rubus* (blackberry and raspberry), *Prunus* (prune) (Nybom *et al.*, 1989; Nybom, 1990a; 1990b; Nybom *et al.*, 1990; Nybom and Schaal, 1990a; 1990b; Nybom and Hall, 1991) and *Vitis* genera (grape) (Bowers *et al.*, 1993). However, no previous reports of nuclear DNA analysis in the genus *Eucalyptus* have been published.

1.4 AIMS OF THIS STUDY

The main aim of this study was to develop a protocol for RFLP analysis of eucalypt DNA which could be incorporated into a tree-breeding programme as a routine screening procedure for the identification of eucalypt species and cultivars. As discussed above, chloroplastic and genomic DNA RFLPs have been used by numerous authors to distinguish a variety of plant materials. Therefore, systems for the analysis of eucalypt DNA, obtained from both chloroplasts and nuclei, were investigated. This required optimization of protocols for extraction of the DNA, its restriction, visualization of the restriction fragments, followed by analysis of the polymorphisms.

CHAPTER 2
CHLOROPLAST DNA RFLP ANALYSIS

2.1 LITERATURE REVIEW AND RATIONALE

2.1.1 THE CHLOROPLAST GENOME

2.1.1.1 Conformation and size of the genome

Early reports of non-Mendelian inheritance and unequal transmission of maternal and paternal phenotypic characteristics suggested that certain genetic information was inherited through the cytoplasm of a cell rather than via the nucleus (Baur, 1909; Correns, 1909). Discovery of extra-chromosomal DNA in the chloroplast (Chun *et al.*, 1963; Sager and Ishida, 1963) and those other cytoplasmic organelles, mitochondria (Nass and Nass, 1963), confirmed these suspicions.

The presence of chloroplastic DNA was demonstrated first by characteristic DNA banding on caesium chloride gradients as a result of the unique bouyant density of these molecules (Chun *et al.*, 1963; Sager and Ishida, 1963). Those findings sparked extensive investigations into chloroplast DNA (cpDNA) structure and organization. Manning *et al.* (1971), for example, were the first workers to observe the circular nature of the cpDNA molecule while studying lysates of *Euglena* chloroplasts with the aid of the electron microscope. This circular structure has been confirmed by restriction analysis of cpDNA from *Zea mays* (maize) (Bedbrook and Bogorad, 1976), *Marchantia polymorpha* (liverwort) (Ohyama *et al.*, 1986), *Nicotiana tabacum* (tobacco) (Shinozaki *et al.*, 1986), *Oryza sativa* (rice) (Hiratsuka *et al.*, 1989) and *Pinus sylvestris* (pine) (Karpinska and Karpinski, 1993) and has resulted in the publication of circular restriction maps for these species. An example of such a gene map, constructed for tobacco, is given in Figure 2.1.

Twenty to 200 copies of the cpDNA molecule are found in each chloroplast, with genes being clustered into nucleoids which are scattered throughout the stroma (Palmer, 1987). Replication of this DNA is semi-conservative and occurs within the organelle independently of nuclear DNA replication (Chiang and Sueoka, 1967; Tewari and Wildman, 1967; Scott *et al*, 1968).

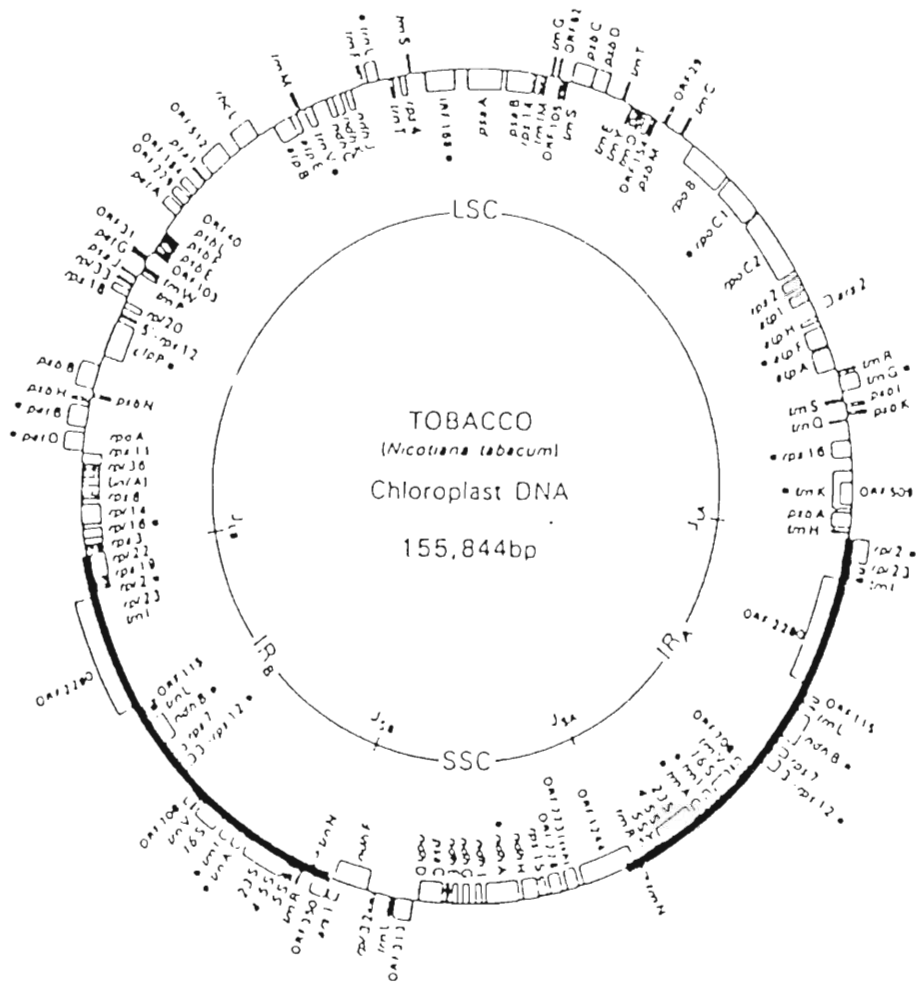


Figure 2.1 Gene map of the tobacco chloroplast genome (Shinozaki *et al.*, 1986). IR, inverted repeat; LSC, large single copy region; SSC, small single copy region. Reproduced from Sugiura (1992).

Within the plant kingdom chloroplast genome size varies from 2 000 kb in the alga *Acetabularia* (Green *et al.*, 1977), to 85 kb in another alga *Conidium fragile* (Hedberg *et al.*, 1981). However, these algae are exceptional as shown by comparison of this genome size with that of the liverwort (121 kb) (Ohyama *et al.*, 1986) and a range of angiosperm chloroplast genomes, where size is reasonably conserved (120 - 160 kb)

(Palmer, 1985). *Pelargonium hortorum* (geranium) is one of a few angiosperm exceptions, with cpDNA molecules measuring 217 kb (Palmer, 1985). Such size variation results from shrinking or spreading of an inverted repeat region which, in geranium, has increased in size from an average 22-26 kb (Bohnert *et al.*, 1982) to 76 kb (Palmer, 1985). There are two identical such inverted repeats in the chloroplast genome which subdivide the DNA molecule into a small and large single repeat sequence (Palmer, 1985) (Figure 2.1). Certain legumes such as *Pisum sativum* (pea) and *Vicia faba* (broad bean) (Koller and Delius, 1980) and a wide range of conifers (Palmer, 1985; Lidholm *et al.*, 1988) lack the inverted repeat region, which has been lost over evolutionary time (Palmer, 1985).

2.1.1.2 The chloroplast gene complement

Restriction analysis of the chloroplast genome in maize resulted in the publication of the first physical cpDNA map (Bedbrook and Bogorad, 1976). Today, the entire nucleotide sequence of this genome is known for liverwort (Ohyama *et al.*, 1986), tobacco (Shinozaki *et al.*, 1986) (Figure 2.1) and rice (Hiratsuka *et al.*, 1989).

Chloroplastic genes encode 3 - 5 rRNA, 30 - 31 tRNA and approximately 100 polypeptide species (Sugiura, 1992). Gene transcription and translation products include: tRNA molecules for chloroplastic protein synthesis (Haff and Bogorad, 1976); rRNAs and proteins (Bowman and Dyer, 1979; Eneas-Filho *et al.*, 1981); RNA polymerase subunits (Watson and Surzycki, 1983); components of the photosynthetic apparatus and associated enzymes (Reith and Cattolico, 1986; Fish and Bogorad, 1986); cytochrome b/f complexes (Heinemeyer *et al.*, 1984); ATP synthase (Krebbers *et al.*, 1982) and respiratory-chain elements (Ohyama *et al.*, 1986).

The chloroplast gene most widely sequenced is that encoding the large ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) subunit, *rbc* L (Sugiura, 1989). Complete sequence data for *rbc* L exists for a number of species such as *Spinacea oleracea* (spinach) (Zurawski *et al.*, 1981), maize (Krebbers *et al.*, 1982), tobacco (Shinozaki and Sugiura, 1982) and *Hordeum vulgare* (barley) (Zurawski *et al.*, 1984) and has been used to infer phylogenetic relationships between species (Zurawski and Clegg, 1987).

2.1.1.3 Evolution of the chloroplast genome

The idea that chloroplasts evolved from ancestral blue-green prokaryotes (cyanobacteria), which were engulfed by single-celled protists giving rise to early eukaryotic cells, was introduced by Schimper as early as 1893 (Keller, 1986). The discovery of chloroplastic DNA (Chun *et al.*, 1963; Sager and Ishida, 1963) provided strong evidence for this "endosymbiont" theory and led to speculations that chloroplast nucleotide sequences were remnants of an early prokaryotic genome (Margulis, 1971). In fact, studies at the molecular level have revealed greater nucleotide sequence homologies between chloroplasts and present day cyanobacteria than between these organelles and the cells in which they reside (Kite, 1986). In addition, chloroplast DNA is surprisingly prokaryotic in nature: the molecule is naked and circular, the 16S ribosomal RNA gene in chloroplasts is identical to that in cyanobacteria, regulatory gene sequences are similar to those in eubacteria, and the chloroplastic RNA polymerase enzyme is susceptible to antibiotics, as are bacterial enzymes (Taylor, 1974; Zablen *et al.*, 1975; Palmer, 1987).

The extant photosynthetic prokaryote *Prochloron*, which lives in symbiotic association with ascidians, has been named as the modern day counterpart of the organism that developed into the chloroplast of green plants (Stanier and Cohen-Bazire, 1977). In this alga the thylakoids are not enclosed within a membrane (Hoober, 1984) and the chloroplast genome is 20-30 times larger than most of the angiosperm genomes today (Palmer, 1985). According to Palmer (1985) this suggests that the reduction in cpDNA size occured during a short evolutionary period following endosymbiosis and the larger genome size of *Acetabularia* could probably be attributed to secondary increases in gene content. However, the highly uniform size of angiosperm cpDNA molecules suggests that selection maintains a restricted genome size within this group of plants (Palmer, 1985).

Apart from changes to the inverted repeat region, most of the variation in cpDNA sequence complexity may be attributed to small length mutations, 1 - 10 bp in size (Crouse *et al.*, 1984; Curtis and Clegg, 1984), caused by slippage and mispairing during DNA replication and repair (Zurawski *et al.*, 1984; Zurawski and Clegg, 1987). Another class of mutation involves 50 - 1 200 bp changes, but occurs less frequently (Fluhr and Edelman, 1981; Gordon *et al.*, 1982; Bowman *et al.*, 1983). Nucleotide substitutions in cpDNA are rare and occur mostly as silent changes in the third position of codons (Zurawski and Clegg, 1987), although missense substitutions clustered at the

ends of genes have been detected (Palmer, 1987). Where gene rearrangements have occurred, they generally involve simple inversions (Jansen and Palmer, 1987) but, as discussed previously (refer section 2.1.1.1), may include spreading or loss of the inverted repeat (Palmer *et al.*, 1987). The chloroplast genome of conifers, for example, differs from that of the angiosperms in having lost the large inverted repeat (Lidholm *et al.*, 1988; Strauss *et al.*, 1988). Extensive gene rearrangements have occurred in these conifers and there is more repetitive DNA, which may have accelerated the rate of evolutionary change (Strauss *et al.*, 1989).

Within the cpDNA of any individual, the evolutionary changes described above occur in a random fashion except in the inverted repeat regions, which evolve in perfect concert with one another. These repeats are always identical, possibly due to the operation of a gene-conversion or copy-correction mechanism (Palmer, 1985).

2.1.1.4 The eucalypt chloroplast genome

Chloroplast studies within the angiosperms have focused almost exclusively on herbaceous crop plants. Despite the known economic importance of certain woody species, little is known about their cpDNA structure (Strauss *et al.*, 1989). For example, the chloroplast genome size of various eucalypt species (135-150 kb) was determined only recently (Steane *et al.*, 1991). When cpDNA RFLPs of these species were analyzed, 50-200 bp differences in fragment lengths were observed, but Steane *et al.* (1991) were unable to determine if these resulted from point or length mutations. Estimated rates of nucleotide substitution in the eucalypt chloroplast genome range from 0.1-0.16 % per million years (Parks and Wendel 1990; Riesberg *et al.*, 1991) suggesting that these woody perennials have a slower rate of evolution than herbaceous annuals (Sytsma *et al.*, 1991). However, more extensive research into the basic structure and organization of this genome and that of other woody angiosperms is required before such complex evolutionary questions can be answered.

2.1.1.5 Plastid inheritance

The demonstration of maternal inheritance of chlorophyll deficiency in *Mirabilis jalapa* (Correns, 1909) and bi-parental non-Mendelian inheritance of a pigmentation trait in *Pelargonium* x *zonale* (Baur, 1909) stimulated an interest in plastid and plastid DNA transmission. Patterns of cpDNA inheritance were traced originally using white and green plastids as parental markers (Tilney-Bassett, 1978), whereas today more

sophisticated techniques are employed. These include microscopic methods such as the staining of pollen with a fluorochrome dye to reveal the presence of proplastids (epifluorescence microscopy) (Corriveau *et al*, 1990), and ultrastructural analysis of plastids within gametophytes (Sears, 1980). Chloroplast DNA RFLP analysis of F1 progeny, produced during inter- and intraspecific crosses, has been used also to trace chloroplast inheritance (Hatfield *et al.*, 1985).

Within the angiosperms, chloroplasts are inherited either maternally, as in *Capsicum annum* (green pepper), *Nicotiana tabacum* (Corriveau and Coleman, 1988), *Lycopersicon esculentum* (tomato), *Petunia* x *hybrida* (Corriveau and Coleman, 1988; Hageman and Schroder, 1989) or bi-parentally, as shown for *Secale cereale* (rye) (Sears, 1980), *Oenothera organensis* (evening primrose) (Stubbe, 1984) and *Magnoliaceae* (Magnolias) (Sewell *et al.*, 1993). Certain species such as *Solanum tuberosum* (potato) show predominant maternal, as well as occasional biparental chloroplast inheritance (Tilney-Bassett, 1978). During such bi-parental plastid transmission the relative contributions of the maternal and paternal parents need not be equal, with certain crosses favouring a maternal bias (Tilney-Bassett and Almouslem, 1989). Plants containing two populations of parental chloroplasts may be chimeras, with different genotypes being expressed in different tissues of the same plant. This has been reported for *Oryza sativa* (Moon *et al.*, 1987) and for individuals in a *Pinus banksiana* - *Pinus contorta* (pine) sympatric region, where cpDNA variation within single branches was reported (Govindaraju *et al.*, 1988).

In contrast to conifers, where paternal chloroplast transmission is common, for example, in *Larix* spp (larches) (Szmidt *et al.*, 1987), *Picea* spp (spruces) (Neale *et al.*, 1986; Szmidt *et al.*, 1988) and *Pinus* spp (pines) (Wagner *et al.*, 1987), only 3 angiosperm species, namely, *Nicotiana plumbaginfolia* (Medygesy *et al.*, 1986), *Medicago sativa* (Schumann and Hancock, 1989) and a *Daucus* (carrot) subspecies (Boblenz *et al.*, 1990) demonstrate paternal chloroplast inheritance.

The parental source of plastids affects the dynamics of organelle genes in a population. (Harris and Ingram, 1991). Therefore, the mode of plastid transmission should be known before attempting to reconstruct phylogenies or infer genetic relationships using cpDNA data (Harris and Ingram, 1991).

2.1.1.6 Inheritance of chloroplasts in the eucalypts

It has been shown that the chloroplasts are inherited maternally in certain eucalypt species (Byrne *et al.*, 1993).

2.1.2 SYSTEMS FOR THE ANALYSIS OF cpDNA RFLPS

There are numerous practical considerations which favour the study of the chloroplastic genome over the nuclear genome. As mentioned previously (section 2.1.1), cpDNA molecules are abundant in chloroplasts and relatively easily extracted (Palmer, 1987). In addition, the genome is small with a low proportion of repetitive DNA which limits nucleotide substitution and other mutational events. Most of the 200 angiosperm chloroplast genomes examined thus far are similar in size, conformation, repeat structure, gene content and gene arrangement (Palmer, 1985). Therefore, the cpDNA molecule is thought to evolve at a conservative rate (1.5×10^{-9} substitutions per site per year) (Zurawski and Clegg, 1987) making it an ideal tool for phylogenetic and other investigations.

The simplest method of cpDNA analysis involves comparison of restriction fragment length polymorphisms. Single base substitutions or length mutations within the chloroplast genome often result in the loss or gain of restriction endonuclease cleavage sites (Palmer, 1987). Therefore, enzyme digestion of cpDNA produces polymorphic populations of fragments which differ in their molecular weights and copy number. After electrophoretic separation, these fragments may be visualized by ethidium bromide staining (Palmer and Zamir, 1982; Banks and Birky, 1985; Szmidt *et al.*, 1988; Ecke and Michaelis, 1990) or by hybridization to labelled cpDNA probes (Palmer, 1985; Steane *et al.*, 1991; Nissen *et al.*, 1992).

Isolated chloroplasts, separated from cell debris and associated contaminants, are an ideal source of chloroplastic DNA (Steane *et al.*, 1991), which may be further purified by caesium chloride equilibrium density gradient centrifugation (Szmidt *et al.*, 1988). Restriction fragment profiles produced on cleavage of this pure DNA may be resolved by ethidium bromide staining. However, there are numerous drawbacks to this procedure. These include requirements for large samples of leaf material and the economic cost of certain reagents. In addition, the period from organelle isolation to fragment detection is lengthy and complicated procedures increase the risk of introducing random breaks into the cpDNA molecule.

Another procedure for cpDNA analysis involves the isolation of total genomic DNA, restriction digestion, fragment separation and hybridization to cpDNA probes (Govindaraju *et al.*, 1988; Gawel and Jarret, 1991; Nissen *et al.*, 1992). This would involve the optimization of conditions for probe labelling, hybridization and RFLP detection. Availability of suitable cpDNA probes would be an additional limiting factor in such a study.

A further protocol for comparing cpDNA restriction profiles was described by Atchison *et al.* in 1976. Those workers presented a technique for "in-organelle" digestion, where restriction enzymes are added to a preparation of intact chloroplasts allowing DNA cleavage to proceed prior to release of fragments from the organelle. This procedure may be carried out with intact chloroplasts which need not be physiologically active.

2.1.3 APPLICATIONS OF CHLOROPLAST DNA ANALYSIS

2.1.3.1 Taxonomic studies

Population level

Low rates of evolutionary change within the chloroplast genome suggest that cpDNA analysis at the population level would be of limited value in assessing taxonomic relationships. This viewpoint has been supported by the findings of a comprehensive study by Banks and Birky (1985), who compared the cpDNA profiles of 100 individuals of *Lupinus texensis* from 21 different populations using 7 restriction enzymes. Those authors noted that within this large sample group 88 plants had identical chloroplast genomes (Banks and Birky, 1985). However, data have begun to accumulate which suggest that a lack of intraspecific cpDNA variation may not always be the rule. Lavin *et al.*, (1990) for example, have observed cpDNA variation in populations of *Gliricidia sepium* and *Astragalus molydenus*, as has Terauchi *et al.*, (1991) in accessions of *Dioscorea bulbifera* L. (yam). Such intrapopulational variability might be useful in studying evolutionary events such as autoploid versus allopolyploid speciation (Soltis and Soltis, 1989).

Species level

At the species level, cpDNA variation is sufficient to provide good phylogenetic resolution, but simple enough to be assessed by RFLP analysis (Palmer, 1987a). As a result, cpDNA studies have been used extensively in determining the degree of genetic variability and level of taxonomic affinity between species of the *Beta* (sugar beet) (Mikami *et al.*, 1984), *Picea* (Szmidt *et al.*, 1988), *Glycine* (soybean) (Doyle and Doyle, 1990a), *Pyrrhopappus* (Turner and Kim, 1990), *Rubus* (raspberry and blackberry) (Waugh *et al.*, 1990), *Musa* (banana and plantains) (Gawel and Jarret, 1991), *Ranunculaceae* (Johansson and Jansen, 1991), *Solanum* (eggplant) (Sakata *et al.*, 1991), and *Plantago* genera (Hooglander *et al.*, 1993). In most cases the RFLP data were used to assemble phylogentic trees which for *Nicotiana* (Rhodes *et al.*, 1981), *Rubus* (Waugh *et al.*, 1990) and *Solanum* (Sakata *et al.*, 1991) were in agreement with previously reported phylogenies constructed on the basis of morphological characteristics. However, cpDNA RFLP studies have an added advantage in that they provide quantitative estimates of species relationships which are difficult to obtain using morphological data (Waugh *et al.*, 1990). As a result, assessments of cpDNA variability have challenged existing phylogenetic hypotheses. For example, the morphologies and geographic ranges of two *Clarkia* species (*C. rostrata* and *C. epilobioides*) have been shown to differ greatly from one another and yet their cpDNA restriction fragment profiles are virtually indistinguishable (Sytsma and Gottlieb, 1986). This suggests that these plants belong to the same species, despite previous classifications to the contrary. In fact, RFLP studies have been used directly in assessing the taxonomic positions of newly described species in the genus *Glycine* (Doyle and Doyle, 1990a).

Genus level

Limited use has been made of cpDNA for inferring phylogenetic relationships at the level of genus and above (Palmer, 1987). Mutational events occur more frequently at higher taxonomic levels resulting in the production of extremely complex cpDNA RFLPs (Palmer, 1987). Therefore, intergeneric cpDNA comparisons are made by restriction endonuclease mapping of the chloroplast genome as Jansen *et al.* (1990) have achieved for 57 genera of *Asteraceae* and Soreng *et al.* (1990) for 34 genera from the family *Poaceae*.

2.1.3.2 Plant breeding studies

Genetic characterization

Successful plant breeding requires the development of techniques which allow identification of potentially useful, or harmful, genes within a population and which promote an understanding of genetic relationships within that population ensuring effective utilization of these genes (Clegg *et al.*, 1984; Waugh *et al.*, 1990). In the hope of developing such techniques many studies have been carried out to investigate the potential of cpDNA polymorphism analysis as a measure of genetic variability within and between populations (Waugh *et al.*, 1990; Gawel and Jarret, 1991; Neale and Williams, 1991; Nissen *et al.*, 1992). Those studies have shown that cpDNA profiles provide a measure of genetic variation within individuals, for example, of *Pinus monticola* (White, 1990), within species, as for *Hordeum* (barley) (Clegg *et al.*, 1984) and *Dioscorea bulbifera* L. (Terauchi *et al.*, 1991) and between species belonging to the *Glycine* (Doyle and Doyle, 1990), *Rubus* (Waugh *et al.*, 1990), *Musa* (Gawel and Jarret, 1991), *Ranunculaceae* (Johansson and Jansen, 1991) and *Solanum* genera (Sakata *et al.*, 1991). In particular, comparison of cpDNA profiles from cultivated and wild lines of barley has revealed a reduction in cpDNA variability within commercial species suggesting that levels of cytoplasmic diversity were restricted during domestication (Clegg *et al.*, 1984). Similarly, in yam varieties the chloroplast genome has been conserved, despite extreme morphological polymorphisms (Terauchi *et al.*, 1991). Studies such as those suggest that human intervention during the breeding of barley and yam crops results in introgression and a loss of cpDNA diversity within species (Clegg *et al.*, 1984; Terauchi *et al.*, 1991). Such findings may be crucial to plant breeders who hope to increase the genetic base of their crop species with the aim of producing new varieties.

Identification of hybrid material

Chloroplast DNA studies have proven useful in the classification of hybrid plant materials which are often difficult to identify on the basis of morphology, (Doyle and Doyle, 1988) and, their parentage is often unknown making it impossible to trace forward to the progeny (Hilu, 1988; Yatskievych *et al.*, 1988). For example, putative hybrid seedlots obtained from a zone of introgression in spruce were classified on the basis of their cpDNA profiles (Szmidt *et al.*, 1988). Hybrid plantlets of the sitka and white spruce species, obtained from that zone, were shown to be unique with regard to

eight chloroplast restriction fragments and these allowed the hybrids to be distinguished from pure sitka or white spruce individuals (Szmidt *et al.*, 1988). Those findings are particularly important as previous studies had shown that morphological characteristics cannot be used as genotype markers in spruce as these are determined by environmental influences (Falkenhagen and Nash, 1978). Recurrent hybrid formation within a *Polystichum* (fern) population has been detected also using cpDNA restriction profiles which have demonstrated the presence of two chloroplast genomes in hybrid individuals (Stein and Barrington, 1990). Therefore, the ability to monitor cpDNA variation has enabled workers also to determine the cytoplasmic constitution of hybrids.

Managing of seed orchards

In addition to being used as a marker of genetic variability, cpDNA analysis has been used to follow plastid inheritance (Frankel *et al.*, 1979; Stein and Barrington, 1990; White, 1990). This application may have great potential for use in plant breeding programmes, for example, in conifer populations, where chloroplasts are largely transmitted in the paternal gamete (Szmidt *et al.*, 1987; Neale *et al.*, 1986). Here, cpDNA analysis might allow workers to track pollen and detect pollen contamination in seed orchards (Straus *et al.*, 1989).

2.1.4 RATIONALE AND INTRODUCTORY COMMENTS

As mentioned in the introductory section, the main aim of the present study was to develop a molecular screening technique for the routine identification of eucalypts at the level of species and below. Chloroplast DNA RFLP analysis can be carried out using a reasonably simple protocol in which DNA is digested with a restriction enzyme, the extract subjected to electrophoresis and the fragments visualized by ethidium bromide staining. In addition, as previously described, cpDNA RFLPs may be used to distinguish individuals in a population and may have additional applications within breeding programmes. Consequently, the first approach in this study was to develop a methodology for cpDNA analysis in the eucalypts. This involved the choice, establishment and optimization of protocols for DNA extraction, purification and fragment separation followed by RFLP detection.

The choice of a procedure to be used routinely in any breeding programme requires consideration of financial and practical aspects. The cost of equipment, chemicals and

sample materials needed must be balanced against the time and labour required to run a series of analyses. Of the reported procedures for cpDNA analysis, that of "in-organelle" cpDNA digestion seemed most suited for incorporation into a routine screening program (refer section 2.1.3). The only pre-requisite for this protocol was a suspension of whole, purified chloroplasts. As no methods for chloroplast isolation from eucalypts had been published at the start of this study, initial experiments focused on the development of a rapid and cost effective mechanical organelle extraction procedure. It is envisaged that chloroplast preparations obtained, using the developed protocol, would be suitable for "in-organelle" DNA digestion and result in the production of cpDNA restriction fragment profiles which could be used for routine identification of eucalypts.

2.2 MATERIALS AND METHODS

2.2.1 PLANT MATERIAL AND GROWTH CONDITIONS

Most of the work was undertaken using *Eucalyptus grandis* S/N M6. In addition, *E. grandis* TAG 5 and TAG 14, *E. nitens*, *E.macarthurii* and *Spinacea oleracea* were used in selected experiments. Saplings of *Eucalyptus* species (Mondi Forests, SA) were grown potted, in soil, under 70 % shade conditions. Forty-eight hours prior to each chloroplast extraction, the plants were watered with 296 mM KNO_3 and 370 mM Na-phosphate and placed in the dark. Spinach leaves were purchased from a greengrocer.

2.2.1 CHLOROPLAST ISOLATION AND PURIFICATION

Young, fully expanded leaves (second to fifth terminal pairs) were used as the source of chloroplasts following surface sterilization in ice cold 1 % (w/v) sodium hypochlorite (20 min). Leaf samples (3-6 g) were ground, using a pestle and mortar, in a basic extraction buffer containing 350 mM sorbitol, 50 mM tris-HCl and 5 mM EDTA, pH 8.0. Alterations to this solution involved the addition of various combinations of 0.1 % (w/v) bovine serum albumin (BSA), 0.15 % (v/v) 2-mercaptoethanol, 2 mM L-ascorbic acid and 1 mM $MgCl_2$. The extraction buffers were used as a semi-frozen slush in a ratio of 6:1 (buffer:tissue). After grinding, the chloroplast brei was squeezed through two layers of muslin cloth, which had been pre-soaked in cold extraction buffer, and then the filtrate was passed through two layers of miracloth. For certain experiments tissue disruption was achieved using a vertical blender (Moulinex, blade length 15 mm) in 10 to 15 short bursts lasting 5 seconds each.

The chloroplast suspension was centrifuged at 2 200 rpm for 15 min at 4 °C and the pellet taken up in 1.7 ml of a resuspension buffer (350 mM sorbitol, 50 mM tris-HCl, 0.25 mM EDTA, pH 8.0) (Palmer, 1986). The entire volume of chloroplasts was loaded onto a discontinuous sucrose gradient (2.58 ml 58 % (w/v) sucrose, 1.0 ml 30 % (w/v) sucrose) and centrifuged at 50 000 rpm for 30 min at 4 °C. After removal from the gradient interface, chloroplasts were washed in 4 ml of resuspension buffer and pelleted by centrifugation at 2 200 rpm for 15 min at 4 °C.

Mitochondrial contamination was estimated using the succinate: cytochrome *c* oxido-reductase assay (Jackson 1985). Chlorophyll determinations were undertaken in 80 % (v/v) acetone, according to Arnon (1949).

2.2.3 "IN-ORGANELLE" DNA DIGESTION AND RESTRICTION FRAG MENT PURIFICATION

The method used was essentially that of Kut and Flick (1986). The chloroplast pellets obtained from 24 g of leaf material were resuspended in 200 μl of swelling buffer (200 mM NaCl, 20 mM $MgCl_2$, 0.01 % (w/v) BSA, 10 mM tris-HCl, pH 7.8) and incubated at 37 °C for 15 min. Restriction enzyme (30-50 U, Eco RI, Hind III, Xho I or Bgl II) (Boehringer Mannhein, Germany) was added and digestion allowed to proceed at 37 °C for various incubation periods (4-20 h). Following lysis of chloroplasts in the presence of 2 % (w/v) SDS, crystalline CsCl was added (final concentration 1.09 g/ml). Samples were then heated to 50 °C for 5 min and centrifuged (11 000 rpm for 5 min at 4 °C) to differentially separate lipid-based, carbohydrate and precipitated protein contaminants from soluble DNA. The clear DNA solution below the green pellicle was removed and diluted with 2 volumes of sterile distilled water. After a second centrifugation step to remove residual carbohydrates (11 000 rpm for 5 min at 4 °C), DNA was precipitated from the supernatant by the addition of 1 volume of ice cold 96 % (v/v) ethanol followed by storage at -20 °C overnight. DNA was recovered by centrifugation (11 000 rpm for 15 min at 4 °C), the pellet washed with 0.5 ml cold 80 % ethanol and air dried. The DNA was then resuspended in 10 μl of TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 7.5) and left at 4 °C for several hours to ensure complete solubilisation. RNA was removed by RNAse A digestion (0.05 μg/μl) at 25 °C for 10 min. Final DNA solutions were stored at - 20 °C.

2.2.4 AGAROSE GEL ELECTROPHORESIS OF DNA FRAGMENTS

Mini gels were run in TBE buffer (45 mM tris-borate, 1 mM EDTA, pH 8.0) on a Hoeffer-HE horizontal gel apparatus at 5.6 V/cm. Immediately prior to loading onto a 1 % (w/v) DNA-grade agarose gel (BioRad, USA), DNA samples were heat-treated at 65 °C for 10 min, ice-cooled, and mixed with 3 μl of dye loading buffer (50 % (w/v) sucrose, 4 M urea, 0.1 % (w/v) bromophenol blue, 1 mM EDTA, pH 7.6). Gels were stained with 0.5 μg/ml ethidium bromide (Sigma, USA) for 20 min and destained for an equivalent period with distilled water before being viewed by UV transillumination at 300nm.

2.2.5 PHOTOGRAPHY

Gel photographs were taken on black and white Kodak T Max film (ASA 400) using a red filter. Negatives were developed for 13 min at 20°C using Aculux developer (1 in 9 dilution) (Paterson Products, England). The developer was discarded, negatives rinsed with tap water and fixed (1 in 4 dilution) (Ilford, England) for 3-6 min at room temperature. Fixer was reusable so it was decanted back into a storage bottle. Negatives were then washed under running tap water and hung up to dry.

Black and white photographs were printed onto glossy Multigrade III photographic paper (MG Deluxe) (Ilford, England) using Multigrade filters (3-4) (Ilford, England).

2.2.6 STATISTICAL ANALYSIS

Chloroplast yields of three replicate samples from each treatment were determmined. These were analysed for mean and standard deviation. The significance of differences observed between treatments was assessed using Duncan's Multiple Range Test.

NOTE

Detailed diagrams of the methodologies developed for chloroplast isolation (Appendix 1) and "in-organelle" DNA digestion (Appendix 2) are given in the Appendices.

2.3 RESULTS AND DISCUSSION

2.3.1 CHLOROPLAST ISOLATION AND PURIFICATION

Having made the decision to analyze eucalypt chloroplast DNA using the "in-organelle" digestion procedure, the first step was the development of a protocol for the isolation of intact chloroplasts from this material. The choice of a chloroplast isolation procedure should be based on considerations of the source of plant material (Halliwell, 1981; Leegood and Walker, 1983; Walker, 1987) and the simplicity and speed of isolation, balanced against the functional integrity and purity of chloroplasts required (Berkowitz and Gibbs, 1985). Chloroplasts may be obtained directly by mechanical leaf disruption (Mills and Joy, 1980; Leegood and Walker, 1985; Kut and Flick, 1986; Palmer, 1986), or indirectly via the isolation of protoplasts which can be lysed to release organelles (Somerville *et al.*, 1981; Kobza *et al.*, 1989).

2.3.1.1 Pre-conditioning of leaf material

Excess starch, accumulated in chloroplastic granules, greatly reduces chloroplast yield during mechanical isolation During this procedure a rapid centrifugation step is required to separate chloroplasts from the cell fraction and during this process starch grains are dragged through the chloroplast membrane rupturing organelles (Halliwell, 1981; Leegood and Walker, 1983; Walker, 1987). An alternate route for organelle extraction involves prior protoplast isolation, followed by gentle rupture, which has made possible the isolation of intact chloroplasts with high starch content (Somerville *et al.*, 1981; Kobza *et al.*, 1989). However, this is a time-consuming and costly route of organelle extraction which makes it unsuitable for incorporation into a protocol for routine cpDNA diagnosis of eucalypt cultivars.

During early attempts to isolate chloroplasts from *E. grandis* leaves in this study, a white ring was observed around the crude chloroplast pellet (obtained after the first centrifugation step). According to Leegood and Walker (1983) this ring is indicative of high levels of starch and/or calcium oxalate contamination. To improve chloroplast yields it was essential, therefore, to devise a scheme to reduce starch levels before mechanical leaf grinding. This included watering with a phosphate (370 mM Na-phosphate) and nitrate (296 mM KNO_3) containing solution, followed by dark incubation of saplings for 48 h prior to organelle extraction.

The role of phosphate, at concentrations as low as 1.0 - 5.0 mM, in the inhibition of starch synthesis (Heldt *et al.*, 1977) and stimulation of its mobilization from the chloroplast (Steup *et al.*, 1976), has been well documented. Those effects have been attributed to phosphate partitioning between sinks (Steup *et al.*, 1976; Heldt *et al.*, 1977). During CO_2 assimilation, 3-phosphoglycerate (PGA) is exported from the chloroplast stroma into the cytoplasm. Each PGA molecule is counter-exchanged for a molecule of inorganic phosphate across the "phosphate translator" in the outer chloroplast envelope (Mathews and van Holde, 1991). In the cytosol PGA is converted to sucrose with the concomitant release of phosphate, which enters the chloroplast once again (Heldt *et al.*, 1977). Increased levels of inorganic phosphate would stimulate rates of this exchange, presumably resulting in a drain of triose-phosphate or starch precursor molecules from the chloroplast, where they would become limiting to starch production. Furthermore, phosphate has been shown to inhibit the enzyme ADP-glucose pyrophosphorylase, which catalyzes the conversion of glucose-1-phosphate to glucose (Heldt *et al.*, 1977). It is probable that starch formation would decline on depletion of this monosaccharides.

Nitrate is another substance which inhibits starch deposition (Klepper *et al.*, 1971; Ariovich and Cresswell, 1983). Sugars released from triose-phosphate breakdown in the cytoplasm are the prime energy source for NADH production which is essential to nitrate reductase activity (Klepper *et al.*, 1971). This enzyme is induced in the presence of its substrate and it is suggested that the requirement for reductant depletes simple sugar reserves in the cytoplasm, which are then replenished by import from the chloroplast stroma where these building blocks are no longer available for incorporation into starch (Klepper *et al.*, 1971; Ariovich and Cresswell, 1983).

The breakdown of starch in the absence of light is a well known phenomenon, with authors recommending that plant material be kept in the dark for periods ranging from 24 (Halliwell, 1981) to 72 h (Mills and Joy, 1980), before organelle isolation. In the present study, a 48 h dark incubation, together with the watering regime described previously, was effective in reducing starch contamination' as shown by the ringless appearance of the chloroplast pellet. Light microscopic observations of the purified chloroplast extract revealed minimal contamination by cell debris and a high percentage of apparently intact, type A chloroplasts (Lilley *et al*, 1975).

Therefore, this simple regime to reduce starch levels in eucalypt leaves was adopted for all future experiments.

2.3.1.2 Optimization of the chloroplast isolation protocol

During early experiments, leaves from *Eucalyptus grandis* S/N M6 were ground using a mortar and pestle in a basic buffer containing 350 mM sorbitol, 50 mM tris-HCl and 5 mM EDTA (Table 2.1). Aside from an osmoticum (sorbitol), to prevent shrinking or swelling of the organelles, and a buffering agent (tris), to maintain pH, EDTA was included also in the basic buffer as certain authors report a reduced chloroplast intactness in the absence of this compound (Somerville *et al.*, 1981; Kobza *et al.*, 1989). Under these conditions, the chloroplast yields obtained were low (1.72 μg chlorophyll/g fresh mass) when compared to yields obtained by Walker (1976) for spinach (50 - 70 μg chlorophyll/g fresh mass).

Soft-tissued materials such as spinach and pea are described as "ideal" for chloroplast extraction because they are easily homogenized and contain limited amounts of contaminating metabolites (Mills and Joy, 1980; Leegood and Walker, 1983; Walker, 1987). Woody plants such as the eucalypts, however, generally contain high levels of phenolics and oxidizing enzymes such as diphenol oxidase (DPO) (Palmer, 1987). Upon mechanical disruption, vacuolar contents are released into the cytoplasm, where they are oxidized to *o*-quinones which polymerize with themselves and cellular proteins, greatly reducing chloroplast yield (Berkowitz and Gibbs, 1985). In such instances, it is essential that anti-oxidants and protective agents be incorporated into the grinding medium.

The protein bovine serum albumin (BSA) is thought to bind quinones and phenolics (Berkowitz and Gibbs, 1985). Furthermore, BSA reportedly prevents organelle clumping (Mills and Joy, 1980), possibly due to charge stabilization on the chloroplast envelope (Walker, 1987). For these reasons, BSA (0.1 % (w/v)) was incorporated into the extraction buffer and retained, despite the fact that it did not significantly increase chloroplast yield (Table 2.1).

2-Mercaptoethanol is an inhibitor of DPO (Walker, 1980), and resulted in an 8.5 times increase in chloroplast yield when added to the basic buffer, probably due to its known role in reducing oxidation and maintaining protein integrity (Table 2.1). Ascorbic acid is another antioxidant frequently added to isolation media and is

reported to counteract the effects of DPO by reducing quinones back to diphenols (Walker, 1980). However, when ascorbic acid was added to the basic extraction buffer together with BSA and mercaptoethanol, significantly lower chloroplast yields were obtained (Table 2.1).

Cations Mg^{2+} and Mn^{2+}, used at low concentrations (0.2 mM), have been shown to enhance the separation of intact and broken chloroplasts (Nakatini and Barber, 1977). Other authors have reported similar beneficial effects of these cations, even in the presence of the chelator EDTA, although their specific action is unknown (Leegood and Walker, 1985). As shown in Table 2.1, chloroplast yield from *E.grandis* was not significantly altered by the addition of 1 mM $MgCl_2$.

The highest chloroplast yields (15.96 and 14.71 μg chlorophyll/g fresh mass respectively) were obtained by grinding in a modified buffer containing 350 mM sorbitol, 50 mM tris-HCL (pH 8.0), 5 mM EDTA, 0.1 % (w/v) BSA and 0.15 % (w/v) 2-mercaptoethanol, in the presence or absence of 2 mM L-ascorbic acid and 1 mM $MgCl_2$ (Table 2.1). As absolute yields were highest when using the more complex formulation, and because of the possible beneficial effects of the added components, this buffer was the one used in further chloroplast extractions from *E.grandis*.

Table 2.1 Effect of extraction buffer formulations on the yield of chloroplasts obtained from *Eucalyptus grandis* S/N M6. A basic buffer containing 350 mM sorbitol, 50 mM tris-HCl and 5 mM EDTA (pH 8.0) was supplemented with the various components shown. Leaf material was ground using a pestle and mortar. n = 3; A to D assigned according to Duncan's Multiple Range Test; values sharing the same letter are not significantly different.

Buffer number	Supplementary Components				Chloroplast yield (μg chorophyll/ g fresh mass)
	Bovine serum albumin 0.1% (w/v)	2-mercapto-ethanol 0.15% (w/v)	L-ascorbic acid 2 mM	$MgCl_2$ 1 mM	
1	-	-	-	-	1.72 D
2	+	-	-	-	1.04 D
3	+	+	-	-	14.71 AB
4	+	+	+	-	8.92 C
5	+	+	+	+	15.96 A

Although improved, the chloroplast yield did not match the yields of 300 μg chlorophyll per sample recommended for use with the "in-organelle" digestion procedure (Atchison *et al.*, 1976). Therefore, the mechanical procedures for tissue disruption were investigated with a view to further improving the yield of chloroplasts released from a limited source of leaf material.

Most authors recommend short bursts of motor-driven blending to release intact chloroplasts (Given, 1981; Kut and Flick, 1986; Palmer, 1986). Chopping of *E. grandis* leaf material using a chilled vertical homogenizer increased chloroplast yields from 15.96 (mortar and pestle) to 20.44 μg chlorophyll/g fresh mass (Figure 2.2). It is probable that this significant improvement was due to the speed of homogenization and separation of chloroplasts from the cell fraction. Limited exposure to phenolics and other harmful lytic substances may have minimized the risks of chloroplast rupture, resulting in increased chloroplast yields (Berkowitz and Gibbs, 1985).

Other manipulations were devised to further increase the efficiency of chloroplast extraction (Figure 2.2). Leaf material was frozen in liquid nitrogen prior to shattering with the blender. This technique is employed commonly in the isolation of whole cell DNA, from which cpDNA is purified (Dally and Second, 1989; Gawel and Jarret, 1991), but evidently proved too harsh for the isolation of organelles. Chloroplast integrity was destroyed, resulting in organelle yields of 0.876 μg chlorophyll/g fresh mass (Figure 2.2). In addition, attempts were made to salvage further chloroplasts from chopped leaf pieces. Gentle agitation of residual leaf material in a buffer, followed by filtration, centrifugation, resuspension and pooling of chloroplasts with those obtained in the primary pellet, improved chloroplast yield. This increase was significant when washing was carried out in the chloroplast resuspension buffer (Figure 2.2).

In conclusion, chloroplast yields of 25.11 μg chlorophyll/g fresh mass from *E. grandis* S/N M6 were obtained by chopping leaf material with the electrical blender in the buffer formulation previously found to be optimal, salvage of additional chloroplasts by washing of residual leaf pieces, and organelle purification on sucrose density gradients.

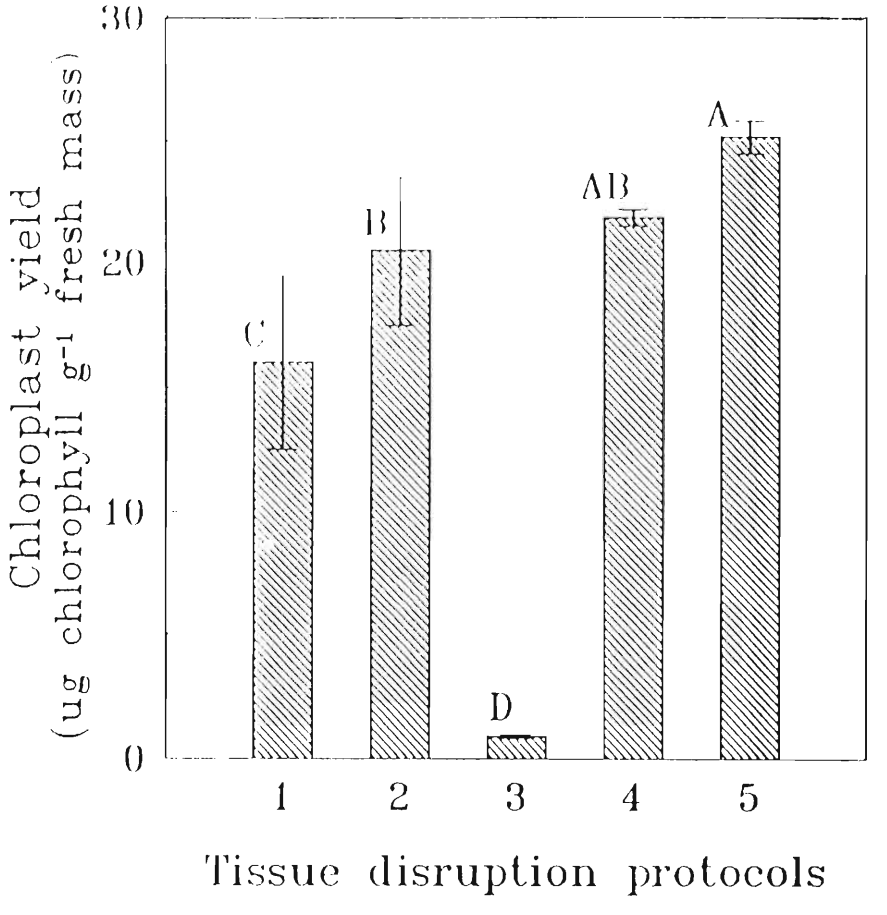


Figure 2.2 Effect of physical extraction procedures on the yield of chloroplasts obtained from *Eucalyptus grandis* S/N M6, using the optimized extraction buffer (Table 1). 1, grinding using a chilled pestle and mortar; 2, chopping with vertical blender for 10 to 15 5-second bursts; 3, freezing of leaves in liquid nitrogen followed by shattering using the blender; 4, chopping as in 2 followed by washing of residual leaf pieces in extraction buffer; 5, chopping as in 2 followed by washing of residual leaf pieces in resuspension buffer. n=3; mean ± SD; A to D assigned according to Duncan's Multiple Range Test; values sharing same letter are not significantly different.

The entire procedure, from tissue disruption to resuspension of the pure chloroplast pellet, could be carried out within a 2 h period. In addition, there was no requirement for costly reagents such as the enzyme extracts Cellulase, Pectinase or Hemicellulase used in protoplast isolation (Somerville *et al.*, 1981) or Percoll for density gradient purification of the organelles (Mills and Joy, 1981). The the major disadvantage of using sucrose as a gradient material is that it results in hypertonic

dehydration of chloroplasts and a subsequent loss of photosynthetic activity (Plaut, 1971; Kaiser *et al.*, 1981). However, as pointed out earlier, "in-organelle" digestion can be carried out using intact chloroplasts which need not be photosynthetically active. For routine chloroplast purification from various eucalypt cultivars, it would be economically practicable to use the cheaper gradient material, namely sucrose.

The chloroplast extract was shown also to be relatively pure and free of cellular debris, as demonstrated by microscopic observation. No mitochondrial contamination (0 %) was detected using the succinate: cytochrome *c* oxido-reductase marker enzyme assay.

2.3.2 CHLOROPLAST DNA ANALYSIS

Having developed and refined a simple and inexpensive methodology for the isolation of chloroplasts from a eucalypt cultivar, it was essential to establish the conditions for "in-organelle" DNA digestion, fragment separation and visualization of cpDNA restriction profiles.

A selection of four restriction enzymes, Eco RI, Hind III, Xho I and Bgl II (Kut and Flick, 1986) were incubated separately with chloroplasts for a 4 h period. Restriction fragment profiles were obtained for all of these endonucleases, although individual DNA bands were resolved most clearly on digestion with Bgl II (Figure 2.3A). An increased digestion period up to 8 h had no effect on these results as found by Atchison *et al.* (1976). In fact, in this study, digestion periods for longer than this (up to 20 h), resulted in a brown discolouration of the digestion mixture and degradation of cpDNA, as shown by the absence of DNA fragments on an agarose gel (Figure 2.3B).

In order to determine whether "in-organelle" digestion with Bgl II could be used to resolve cpDNA band differences between *E. grandis* and an outgroup, the entire procedure, from chloroplast isolation to visualization of cpDNA fragments, was carried out with *Spinacea oleracea* L. (spinach). Spinach was chosen as an outgroup based on its status as an "ideal" source material for the extraction of chloroplasts (refer section 2.3.2) (Walker, 1987). Using the methodology developed for *E. grandis*, spinach chloroplast yields of 112 μg chlorophyll/g fresh mass were achieved, which exceeded the values of 75 μg chlorophyll/g fresh mass obtained by Walker (1976). A clearly resolved cpDNA restriction profile also demonstrated differences in both the number and size of fragments compared with *E. grandis* (Figure 2.4).

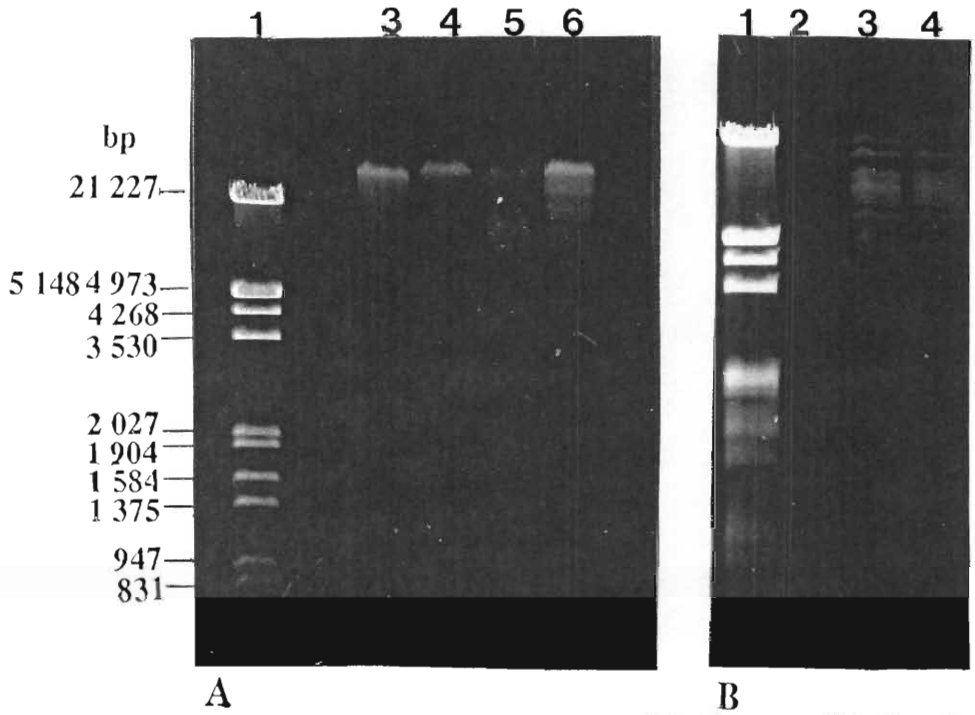


Figure 2.3 DNA restriction fragments, generated by "in-organelle" digestion of chloroplasts isolated from *Eucalyptus grandis* S/N M6 and separated by agarose gel electrophoresis. (A) Fragment profiles resulting from 4 h digestion with Eco RI (lane 3), Hind III (lane 4), Bgl II (lane 5), and Xho I (lane 6). (B) Fragment profiles resulting from Bgl II digestion for 4 h (lane 4), 8 h (lane 3) and 20 h (lane 2). For both (A) and (B) Lambda-Eco RI/Hind III DNA markers are shown in lane 1.

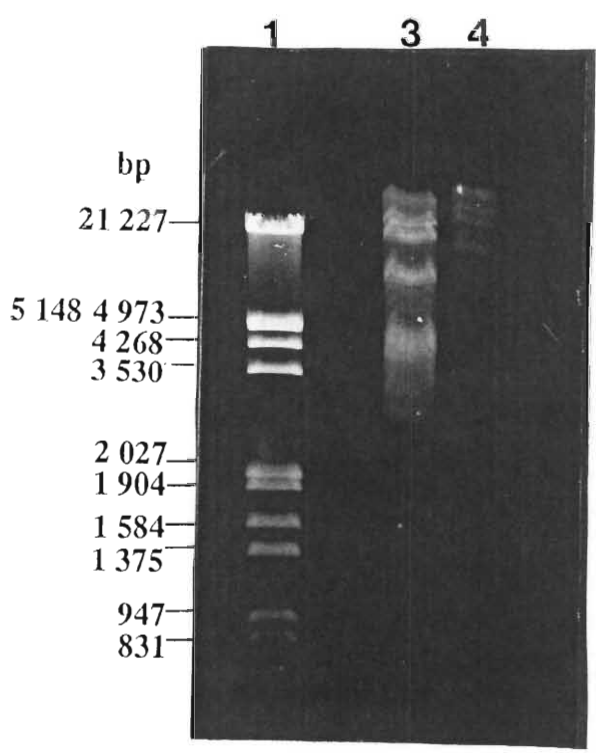


Figure 2.4 Comparison of the chloroplast DNA fragment profile of *E.grandis* with that of an outgroup. "In-organelle" DNA digestion (Bgl II, 4 h) was carried out using isolated chloroplasts of *Spinacea oleracea* (lane 3) and *Eucalyptus grandis* S/N M6 (lane 4). Lambda markers are shown in lane 1.

2.3.3 APPLICABILITY OF THE DEVELOPED PROTOCOLS FOR GENETIC IDENTIFICATION IN EUCALYPTS

Following the demonstration of cpDNA polymorphisms between *Eucalyptus grandis* S/N M6 and an outgroup (spinach), it was necessary to evaluate the developed methodology in terms of its usefulness as an identification tool for distinguishing eucalypt species and cultivars.

When the chloroplast isolation protocol developed for *E. grandis* S/N M6 was applied to other *Eucalyptus* species (*E. nitens* and *E. macarthurii*) and *E. grandis* cultivars (TAG 5 and TAG 14), significantly reduced chloroplast yields were obtained (Figure 2.5). Cultivars of *E. grandis* TAG 5 and TAG 14 yielded between 68 and 75 % fewer chloroplasts, *E. nitens* showed an 80 % reduction in yield and no intact chloroplasts were obtained from *E. macarthurii* (Figure 2.5). As it can be seen from photographs of the sucrose gradients, (Figure 2.6), there was a clear decline in the intensity of chloroplast bands at the gradient interface. To extract chloroplasts of sufficient yield for cpDNA analysis, it would be neccessary, therefore, to optimize the chloroplast isolation protocol for each sample material.

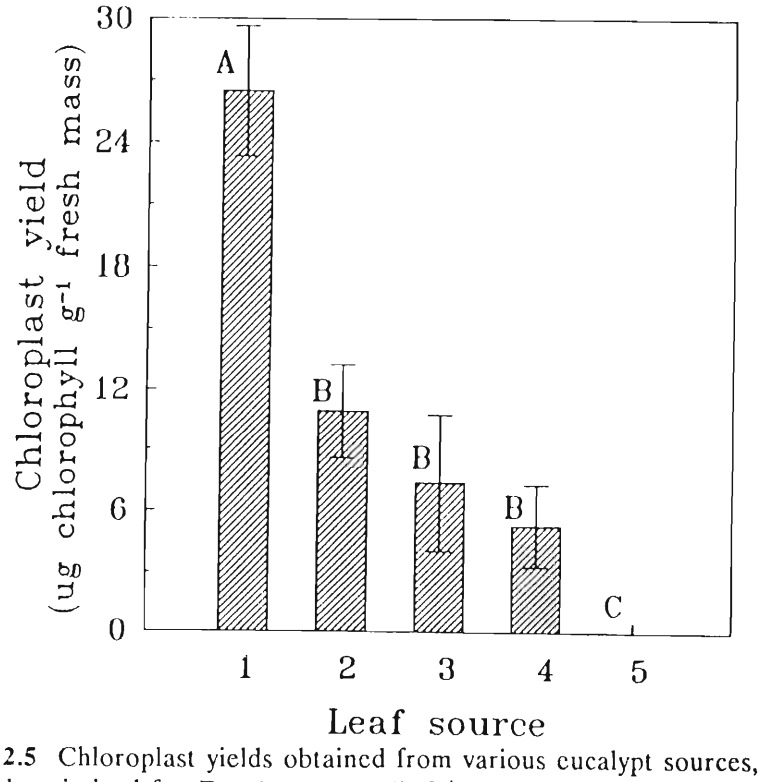


Figure 2.5 Chloroplast yields obtained from various eucalypt sources, using the protocol optimized for *Eucalyptus grandis* S/N M6. 1, *E. grandis* S/N M6; 2, *E. grandis* TAG 5; 3, *E. grandis* TAG 14; 4, *E. nitens*; 5, *E. macarthurii*. n=3; mean ± SD; A to D assigned according to Duncan's Multiple Range Test; values sharing same letter are not significantly different.

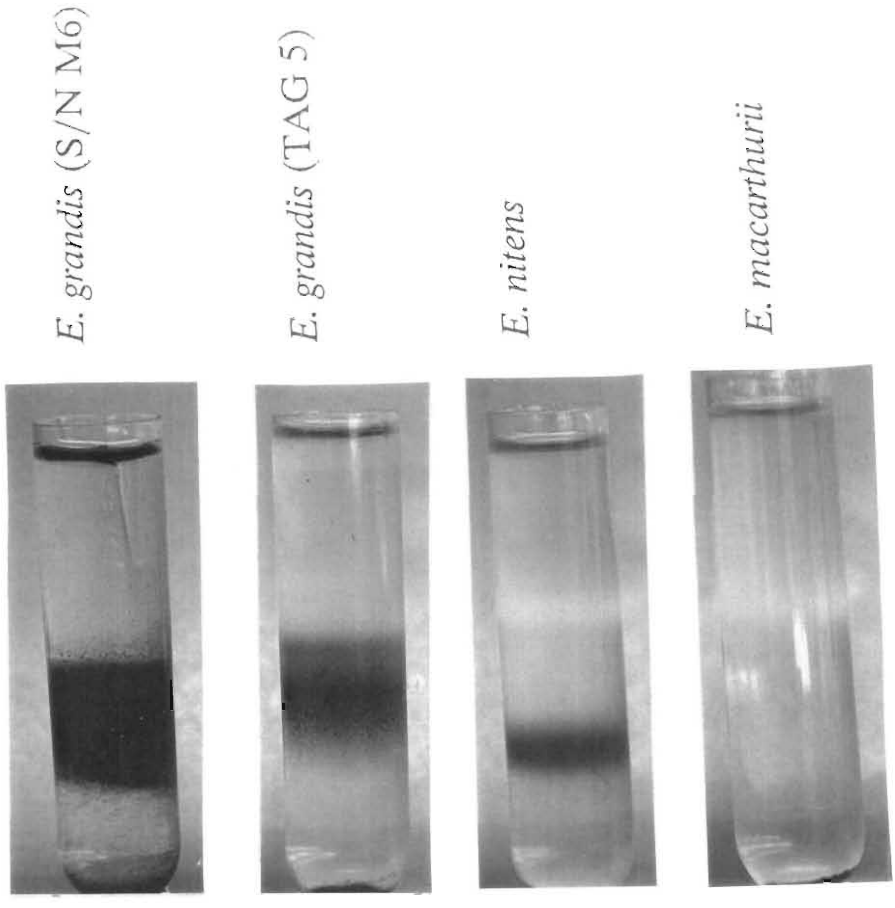


Figure 2.6 Comparison of chloroplast bands formed at the 58 % (w/v) to 30 % (w/v) sucrose interface, after centrifugation at 90 000 *g* for 30 min at 4 ° C.

In relation to the variable chloroplast yields obtained, it was interesting to note that the eucalypt species *E. grandis*, *E. nitens* and *E. macarthurii* have very different gross morphologies. Leaves sampled from these species vary in size, shape, texture (Figure 2.7) and colour. In addition, chloroplast extracts obtained from each of the species and cultivars have their own characteristic smell and shade of green (results not shown). These observations suggest differences at an ultrastructural or biochemical level, for example, in the levels of starch, phenolics and other secondary metabolites present in the leaf.

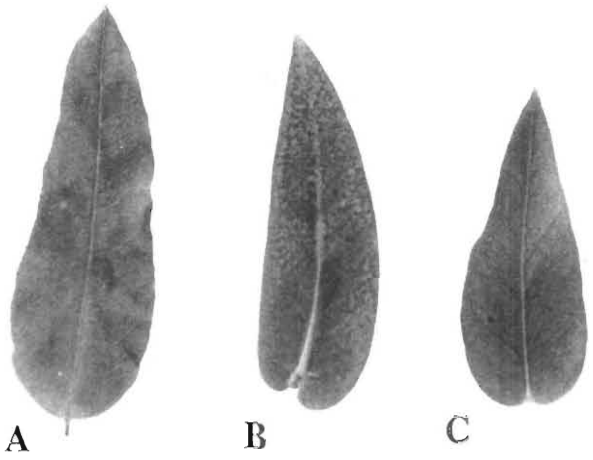


Figure 2.7 Gross morphology of the second terminal leaf of A, *E. grandis*; B, *E. nitens* and C, *E. macarthurii*.

Steane *et al.*, (1991), who used cpDNA analysis as a means of investigating phylogenetic relationships between eucalypt subgenera, similarly reported problems associated with "the reliability, variability in yield and speed of the chloroplast extraction procedure" from eucalypts. Those authors obtained chloroplasts by mechanical extraction using the protocol of Palmer (1986). Their only modification of that procedure was the inclusion of an overnight dialysis of chloroplasts, subsequent to density gradient purification (Steane *et al.*, 1991). According to Steane *et al.* (1991), this step removed residual contaminants such as tannins and phenolics, which may have degraded cpDNA. Although the purity of the chloroplast extract obtained was improved by dialysis, chloroplast yield was not considered to be important by those workers as they were using chloroplasts in a phylogenetic study, rather than developing a protocol for routine organelle isolation.

In the present study, requirements for a rapid and inexpensive procedure which would not waste sample material lead to the choice of "in-organelle" digestion as a protocol for cpDNA analysis (refer section 2.1.3). Preliminary results suggest that this approach would offer a valuable contribution to cpDNA restriction and analysis in soft-tissued plant materials such as spinach. However, problems arise when dealing with material recalcitrant to chloroplast isolation. Use of "in-organelle" restriction for cpDNA analysis in *Eucalyptus*, for example, would require continual modification of the chloroplast isolation protocol for each of the samples analyzed, as presented here for *E. grandis* S/N M6.

2.4 CONCLUDING REMARKS

Although Steane *et al.* (1991), using mechanical organelle extraction procedures, were able to generate and compare cpDNA restriction fragment profiles from various *Eucalyptus* species, they point out the limitations of the methodology used. The mechanical isolation protocol developed in this study for chloroplast extraction from *E. grandis* S/N M6 has advantages over the method used by Steane *et al.* (1991) and results in good yields of intact chloroplast from limited amounts of leaf material. In addition, the chloroplast extract is reasonably pure and free of mitochondrial contamination making chloroplasts ideal for use in other studies. However, problems of variable chloroplast yield from different eucalypt species and cultivars persist.

Steane *et al.* (1991) recommend an alternative procedure for cpDNA analysis in eucalypts, namely, hybridization of chloroplast probes to extracts of total genomic DNA. This approach, first described by Clark and Hansen (1983), has been used increasingly in phylogenetic studies of the chloroplast genome (refer section 2.1.2.2). However, this procedure requires purification of probes and optimization of protocols for probe labelling and detection, procedures which are both time-consuming and costly. Despite these disadvantages, investigations into the use of Southern hybridization techniques for routine screening and identification of eucalypts would be the only logical means of progressing in this study.

Failure to demonstrate species-specific restriction fragments within the euaclypt sub-genus *Monocalyptus* (Steane *et al.*, 1991) suggests that cpDNA at this taxonomic level may not be sufficiently variable to allow distinction of eucalypt species and cultivars. Therefore, it was decided to optimize condition for Southern

hybridization with a view to probing total genomic DNA for mini-satellite repeat regions. This would allow greater resolution of genetic variation at the cultivar and perhaps individual level.

CHAPTER 3
FINGERPRINTING OF GENOMIC DNA

3.1 LITERATURE REVIEW AND RATIONALE

3.1.1 GENERAL INTRODUCTION

At the level of the genome, mutations occur during which the DNA undergoes stable structural change (Mathews and von Holde, 1991). This may involve insertion or deletion of single nucleotide bases or insertion, deletion, and/or inversion of entire segments of DNA (Kirby, 1990). When such mutations occur within the non-coding regions of DNA they are "neutral", that is, they have no phenotypic effect and cannot be detected by changes in the appearance, or morphology, of the organism (Kirby, 1990). However, such changes may be detected by studying the restriction fragment profile of an individual, which is the pattern of DNA fragments produced on cleavage of DNA with bacterial restriction enzymes (Mathews and von Holde, 1991). These endonucleases cut eukaryotic DNA at 4-8 base pair recognition sequences and mutational events, such as those described above, occur often within these sequences resulting in either the loss or gain of enzyme recognition sites (Kirby, 1990). If a particular cleavage site is present, the enzyme recognizing it will cut the DNA into fragments of specific base pair length (Kirby, 1990). However, if the site is absent a population of different sized fragments will be produced (Figure 3.1).

Since mutational events are independent and separate, restriction sites are often polymorphic between individuals, and are either present or absent as mentioned above, or may be recognized by different restriction enzymes (Kirby, 1990). Sequence variation in DNA detected as a result of these differences are referred to as restriction fragment length polymorphisms or RFLPs (Botstein *et al.*, 1980). In addition, restriction sites often occur at positions in the DNA which flank regions of tandem repeat sequences, where the number of repeat units differs between individuals (Jeffreys, 1985a). As a result of the phenomena described above, cleavage of DNA from an individual using either a single or set of restriction enzymes, produces a unique population of restriction fragments. These fragments may be separated by electrophoresis and either visualized directly, for example, by ethidium bromide staining, or hybridized to single stranded DNA probes which are

labelled with a radioisotope (Maniatis *et al.*, 1982) or with a non-radioactive substance (Höltke *et al.*, 1992). Labelled probe, bound to complementary genomic DNA, is then detected by autoradiography or by a chemical assay to reveal an individual-specific restriction fragment pattern or DNA "fingerprint" (Figure 3.1).

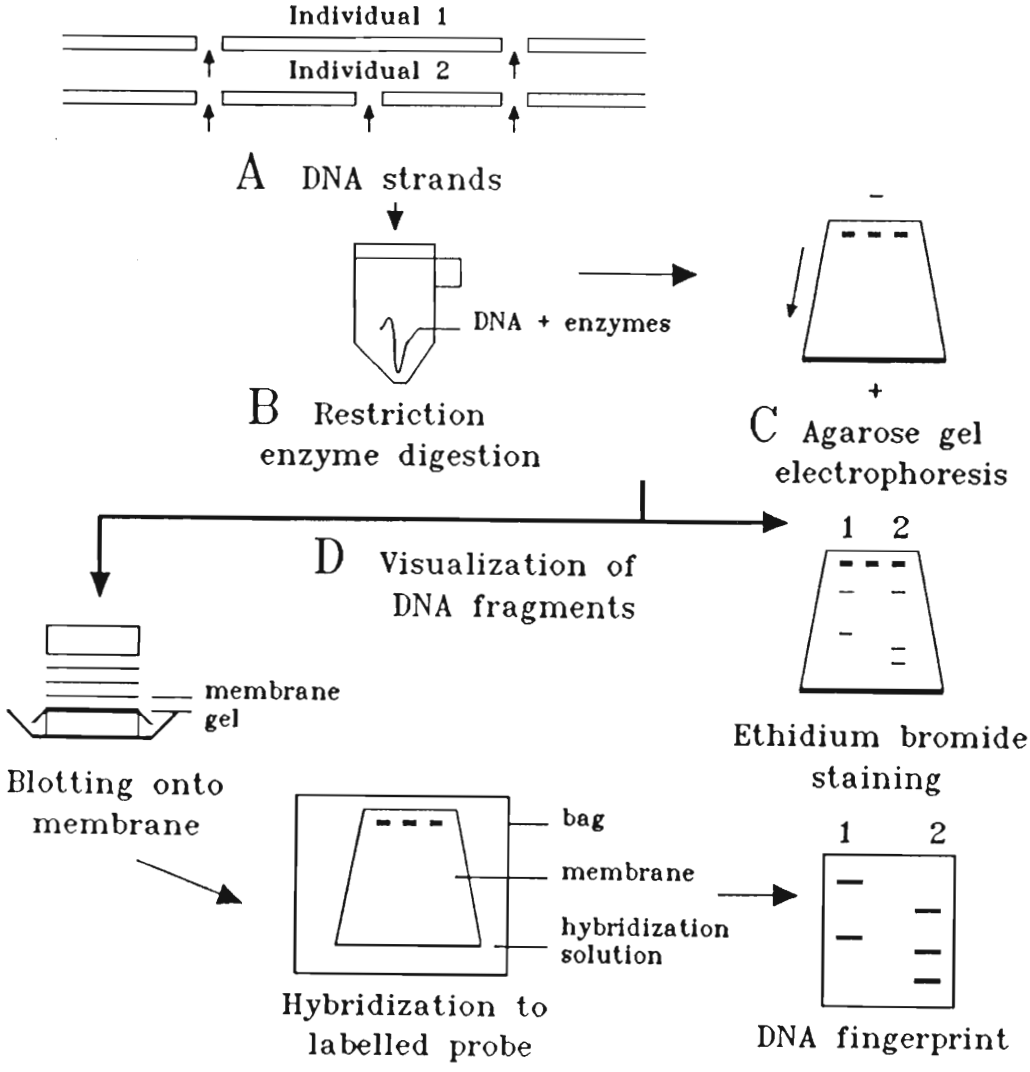


Figure 3.1 Diagrammatic representation of the procedures involved in the production of restriction fragment profiles or DNA "fingerprints". **A**, as a result of a mutational event individual number 1. has lost a restriction enzyme recognition site. **B**, restriction enzyme cleavage of DNA from this individual, **C**, followed by electrophoresis and, **D**, visualization of the DNA fragments, therefore, reveals a restriction fragment pattern or DNA "fingerprint" different from individual number 2.

3.1.2 SYSTEMS FOR THE ANALYSIS OF DNA

3.1.2.1 Restriction enzymes for DNA cleavage

The first known restriction enzyme, Hind III, was identified by Smith in 1970 and since then over 400 restriction enzymes have been isolated from approximately 200 bacterial strains (Kirby, 1990). The main function of these endonucleases is to protect bacteria from foreign DNA, for example, the DNA introduced into bacterial cells during viral infection (Dowling and Brown, 1989). As mentioned previously (refer section 3.1.1), these enzymes recognize 4-8 base pair sites on the unmethylated viral DNA and cleave it into many fragments, while the bacterial DNA itself is methylated and, therefore, protected against the action of these restriction enzymes (Lewin, 1987).

A number of factors need to be taken into account when restriction enzymes are selected for use in the study of DNA polymorphisms, namely the cost of the enzyme, its ability to digest DNA to completion and, most importantly, the potential of that particular enzyme to detect polymorphisms in the DNA of interest (Helentjaris, 1985; Miller and Tanksley, 1990).

In studies on mammalian, avian and human DNA, the enzymes Hinf I and Hae III have been used most extensively (Jeffreys *et al.*, 1985a; 1985b; Burke and Bruford, 1987; Wetton, *et al.*, 1987; Kirby, 1990) (Table 3.1). In human forensic studies, DNA cleavage with Hinf I has produced the largest number of resolvable DNA fragments (Jeffreys *et al.*, 1985a; 1985b, Kirby, 1990). Hae III has proved to be popular also in those studies as this enzyme is insensitive to methylation, ensuring that DNA is digested to completion, and because it has a 4 base pair recognition sequence which occurs frequently and results in the production of small DNA fragments easily resolved by gel electrophoresis (Kirby, 1990).

Restriction enzymes used most frequently in the study of plant DNA polymorphisms include Hae III (Rogstad *et al.*, 1988; Nybom, 1990a; Nybom, 1990b; Nybom *et al.*, 1990; Rogstad *et al.*, 1991) and Hinf I (Nybom, 1990a; Nybom *et al.*, 1990; Tzuri *et al.*, 1991; Weising *et al.*, 1991) as well as Dra I (Miller and Tanksley, 1990; Nybom *et al.*, 1990; Tzuri *et al.*, 1991) (Table 3.1). These enzymes digest DNA to completion and produce a spread of DNA fragments from which polymorphisms may be detected (Rogstad *et al.*, 1991; Weising *et al.*, 1991; Tzuri *et al.*, 1991).

As mentioned above, and as shown in studies of *Lycopersicon* spp (tomato), *Oryza sativa* (rice) and *Phaseolus vulgaris* (common bean) cultivars, the extent of DNA polymorphism visualized is determined by the size of the genomic DNA fragments generated by a particular restriction enzyme, which in turn is determined by the length of the enzyme recognition sequence (McCouch *et al*, 1988; Miller and Tanksley, 1990; Nodari *et al.*, 1992). For example, the enzyme Hinf I has a five base pair recognition sequence which occurs more frequently in the genome than the six base cutting site of Dra I (Table 3.1). As a result, Hinf I cleaves DNA more often than Dra I resulting in the production of relatively smaller fragments with Hinf I (2-5 kb) compared to those generated with Dra I (2-23 kb) (Tzuri *et al.*, 1991). As shown by Tzuri *et al.* (1991) the latter enzyme produces a more polymorphic and, therefore, informative DNA restriction pattern which those authors attribute to the generation of larger DNA fragments which are more likely to encompass insertions, deletions and rearrangements of DNA.

Table 3.1 Examples of restriction enzymes used in studies of DNA polymorphisms. Nucleotide bases, making up the enzyme recognition sequence, are represented by the letters A, adenine, C, cytosine, G, guanine and T, thymidine. Cleavage sites are shown by the arrows (↓ ↑).

Enzyme (bacterial source)	Recognition sequence 5' → 3'	References
Dra I (*Deinococcus radiophilus*)	↓ - TTT AAA - ↑	Miller and Tanksley, 1990; Nybom *et al.*, 1990; Tzuri *et al.*, 1991
Hae III (*Haemophillus aegyptius*)	↓ - GG CC - ↑	Jeffreys *et al.*, 1985a; Wetton *et al.*, 1987; Rogstad *et al.*, 1988; Kirby, 1990; Nybom, 1990b; Nybom *et al.*, 1990; Rogstad *et al.*, 1991
Hinf I (*Haemophilus influenzae Rf*)	↓ - G ANT C - ↑	Jeffreys *et al.*, 1985a; 1985b; Burke and Bruford, 1987; Kirby, 1990; Nybom, 1990b; Nybom *et al.*, 1990; Tzuri *et al.*, 1991; Weising *et al* 1991

3.1.2.2 Source and selection of probes

Probes are usually single stranded labelled DNA fragments which hybridize to complementary sequences in genomic DNA and are then detected using radioactive or non-radioactive assays. In the analysis of DNA polymorphisms, two types of probe are utilized routinely, namely single locus probes, which detect only one or a few loci, and multi-locus probes which bind to numerous loci (Kirby, 1990).

Single-locus probes

Single-locus probes are highly specific and bind complementary DNA sequences under high stringency conditions resulting in the resolution of one or two DNA bands (Kirby, 1990). These probes are usually prepared by enzymic digestion of a sub-sample of the genomic DNA to be analyzed (McCouch *et al.*, 1988; Gebhardt *et al.*, 1989; Miller and Tanksley, 1990). The fragments obtained during this digestion are separated by electrophoresis, a proportion of which (0.5-4.0 kb) are then isolated and used either directly as anonymous DNA probes (McCouch *et al.*, 1988; Gebhardt *et al.*, 1989; Miller and Tanksley, 1990), or ligated into plasmids which are maintained as probe libraries (Nodari *et al.*, 1992; Webb *et al.*, 1992). To ensure that the probes obtained in this way are enriched for single or low copy sequences, the enzyme Pst I is used often to generate genomic DNA fragments (Landry *et al.*, 1987; Burr *et al.*, 1988; Nodari *et al.*, 1992, Webb, 1992). This restriction endonuclease is methylation sensitive and, therefore, cleaves only within hypomethylated single-copy DNA regions generating 0.5-3.0 kb fragments which are 50-90 % enriched for low-copy-number sequences (Burr *et al.*, 1988).

Multi-locus probes

Multi-locus probes hybridize under low stringency conditions to many DNA loci resulting in the simultaneous resolution of numerous DNA fragments (Kirby, 1990). The first multilocus probes were developed by Jeffreys *et al.* (1985a). During the course of studies on the human myoglobin gene those authors had discovered a 33 base pair (bp) sequence repeated in tandem within the intron of the gene and the characteristic number of those repeat units was found to vary both within and between chromosomal loci (Jeffreys *et al.*, 1985a). Jeffreys and his co-workers realized that such hypervariable regions in the genome, which later became known as DNA "minisatellites", could act as probes for multiallelic variation between

individuals. Consequently, those workers purified the 33 bp sequence and ligated it into a pUC-13 vector to produce the plasmid pAV33.7 (Jeffreys *et al.*, 1985a). Cleavage of this plasmid with the restriction enzymes Bam HI and Eco RI yielded a 767 bp fragment composed almost entirely of 33 bp repeats and, when this sequence was used to probe a human genomic DNA library, approximately 40 hybridizing plaques were identified (Jeffreys *et al.*, 1985a). From those plaques eight probes were isolated and two, labelled 33.15 and 33.6, have been used extensively in the production of human and avian DNA fingerprints (Jeffreys *et al.*, 1985a; 1985b; Burke and Bruford, 1987; Wetton *et al.*, 1987; Kirby, 1990). Both the 33.6 and 33.15 probes have been hybridized also to plant DNAs producing fingerprints for Asian and African *Oryza* (rice) cultivars and *Dianthus* (carnation) varieties (Dallas, 1988; Tzuri *et al.*, 1991) (Table 3.2).

Oligonucleotide probes

Oligonucleotide probes are di-, tri- or tetra-nucleotide fragments which, under specific conditions, hybridize to simple repetitive genomic DNA sequences if these are perfectly complimentary (Ali *et al.*, 1986; Schafer *et al.*, 1988; 1988b; Kaemmer *et al*, 1992). This results in the production of DNA fingerprints of "microsatellite" repeat regions, as opposed to fingerprints obtained from the minisatellite regions described before. Microsatellite fingerprinting has been applied successfully to humans and numerous other animal species (Ali *et al.*, 1986; Epplen *et al.*, 1991), as well as to plant DNAs (Tzuri *et al.*, 1991; Weising *et al.*, 1991; 1992, Table 3.2).

Table 3.2 Probes used for DNA fingerprinting in plant genomes.

Plant material	Probe	Reference
Oryza sativa *O. glaberrima*	33.6, 33.15	Dallas, 1988
Rosa hybrida *Gerbera gamsoniii* *Dianthus* (species and cultivars)	33.6, 33.15	Tzuri *et al.*, 1991
Dianthus (species and cultivars)	R18.1	Tzuri *et al.*, 1991

Table 3.2 continued Probes used for DNA fingerprinting in plant genomes.

Plant material	Probe	Reference
Pinus torreyana *Asiminina triloba* *Polyalthia glauca* *Populus tremuloides* *Populus deltoides* *Lycopersicon esculentum*	M13 repeat	Rogstad *et al.*, 1988
Hordeum vulgare *Glycine* species	M13 repeat	Ryskov *et al.*, 1988
Rubus (species and cultivars)	M13 repeat	Nybom *et al.*, 1989; Nybom and Schaal, 1990b; Nybom *et al.*, 1990, Nybom and Hall, 1991
Malus (species and cultivars)	M13 repeat	Nybom, 1990a; Nybom *et al.*, 1990
Malus x *domestica*	M13 repeat	Nybom, 1990b
Acer negundo	M13 repeat	Nybom and Rogstad, 1990
Prunus species	M13 repeat	Nybom *et al.*, 1990
Cornus species	M13 repeat	Culpepper *et al.*, 1991
Picea abies (L.)	M13 repeat	Kvarnheden and Engström, 1991
Populus tremuloides	M13 repeat	Rogstad *et al.*, 1991
Equisetum arvense *Asparagus densiflorus* *Ficus benjamina* *Lens culinaris*	$(GATA)_4$	Weising *et al.*, 1991*
Brassica napus *Camelia sinensis* *Solanum tuberosum* *Nicotiana tabacum* *Lactuca sativa*		
Cicer arietinum (species and cultivars)		Weising *et al*, 1991, Weising *et al.*, 1992

Table 3.2 continued Probes used for DNA fingerprinting in plant genomes.

Plant material	Probe	Reference
Nicotiana (species and cultivars)	$(GTG)_5$	Weising *et al.*, 1991*
Dianthus (species and cultivars)		Tzuri *et al.*, 1991
Lens culinaris (species and cultivars)	$(GACA)_4$	Weising *et al.*, 1991*

* Note Weising *et al.* (1991) give extensive examples of material fingerprinted using oligonucleotide probes, from fungi to lower plants to monocotyledonous and dicotyledonous angiosperms. Only a selection of these examples are presented in this table.

The M13 repeat probe

M13 phage DNA has been used as a multi-locus tandem repeat probe to detect hypervariable minisatellite regions in genomic DNA (Table 3.2). The repeat sequence itself was discovered by Vassart *et al.* (1987) during a search for RFLPs associated with the human thyroglobin gene. Those authors observed that a set of thyroglobin probes, subcloned into the classical M13 bacteriophage vector, gave variable results when hybridized to human genomic DNA under different hybridisation conditions, in particular, when the composition of the hybridisation buffer was varied (Vassart *et al.*, 1987). Hybridisation solutions usually contain buffering reagents and labelled probe DNA and are the media in which the probe becomes bound to single-stranded genomic DNA fragments (Sambrook *et al.*, 1989). These fragments are usually immobilized on a nylon or nitrocellulose membrane and it is this membrane which is incubated in the hybridisation buffer at a set temperature for a period of time during which probe annealing takes place (Sambrook *et al.*, 1989). Also included in the hybridisation buffers are so-called "blocking reagents" which prevent non-specific binding of labelled probe DNA to the membrane background (Sambrook *et al.*, 1989). In their studies, Vassart *et al.*

(1987) found that the compounds used as blocking reagents directly determined the pattern of restriction fragments observed. For example, when those authors used herring sperm DNA to block non-specific binding of the probe to the membrane, no RFLPs were detected. However, when the herring sperm DNA was replaced with a milk based blocking compound, a complicated and highly polymorphic pattern of DNA bands was resolved (Vassart *et al.*, 1987). Similar results were obtained when wild-type M13 DNA, that is, M13 DNA which contained no human thyroglobin insert, was used to probe human genomic DNA (Vassart *et al.*, 1987). Therefore, Vassart *et al.* (1987) concluded that the pattern of DNA bands observed was unrelated to the nature of the probe itself, but resulted from hybridization of a portion of the M13 vector to the blocking reagents. In fact, those same authors went on to discover a 15 bp motif repeated in tandem at two places in the M13 protein III gene (Figure 3.2) and suggested that this repeat sequence had hybridized to minisatellite regions in the herring sperm DNA, but not to the milk based blocking reagent, accounting for the variation observed in the restriction fragment patterns (Vassart *et al.*, 1987). Vassart *et al.* (1987) then went on to isolate a 280 bp fragment from the M13 repeat DNA and when this sequence was used to probe a Hae III digest of human DNA, a polymorphic DNA restriction fragment pattern, or DNA fingerprint, was produced.

Subsequent to the work of Vassart *et al.* (1987), it has been found that M13 phage DNA can be used universally as a multi-locus probe in the fingerprinting of animals, plants and microorganisms (Ryskov *et al.*, 1988). In particular, a Cla I/Bsm I 780 bp M13 repeat fragment has been isolated (Rogstad *et al.*, 1988) which has been used extensively in the characterization of plant tissues (Ryskov *et al.*, 1988; Nybom *et al.*, 1989; Nybom, 1990a; 1990b; Nybom and Rogstad, 1990; Nybom and Schaal, 1990a; 1990b; Nybom *et al.*, 1990; Culpepper *et al.*, 1991; Kvarnheden and Engström , 1991; Nybom and Hall, 1991; Rogstad *et al.*, 1991; Tzuri *et al.*, 1991) (Table 3.2).

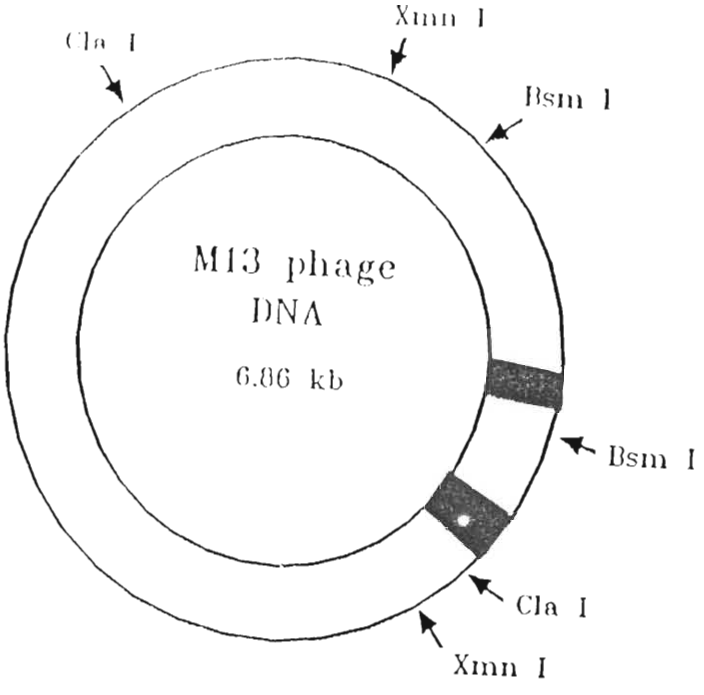


Figure 3.2 Restriction map of the M13 bacteriophage vector. The open boxes show the position of the tandem repeat sequences. Modified from Vassart *et al.* (1987).

The plasmid probe pV47-2

This plasmid is another multi-locus probe which was developed when Longmire *et al.* (1990) screened a Charon 40 human chromosome-16 library with the M13 repeat sequence isolated by Vassart *et al.* (1987). Several clones hybridizing to this repeat were isolated although one in particular, labelled clone 47, was free of other human minisatellite repeats such as the alu repeat sequence (Longmire *et al.*, 1990). Therefore, the insert of this clone was digested with the restriction enzyme Hind III and the 3.5 kb fragment produced subcloned into the Hind III site of a 2.8 kb pUC-8 plasmid to produce pV47-2 (J.L. Longmire, pers comm) (Figure 3.3). This plasmid was used then to probe Hae III digests of human DNA and was found to detect all fragments resolved using the M13 repeat (Vassart *et al.*, 1987) plus an average of 33 % more alleles (Longmire *et al.*, 1990).

Longmire *et al.* (1990) demonstrated also that the minisatellite regions detected with pV47-2 are inherited in a Mendelian fashion. When those authors hybridized

nick translated plasmid to DNA extracted from the members of a family group and from various unrelated males and females most of the DNA bands detected in the siblings were present in one or both of the parents, whereas the restriction fragment profiles of the unrelated individuals were very different (Longmire *et al.*, 1990).
In addition to its usefulness as a human DNA probe, pV47-2 has been hybridized also to DNA from a cross section of wildlife species and various domestic animals such as cattle (J.L. Longmire, pers comm; Dolf *et al.*, 1992).

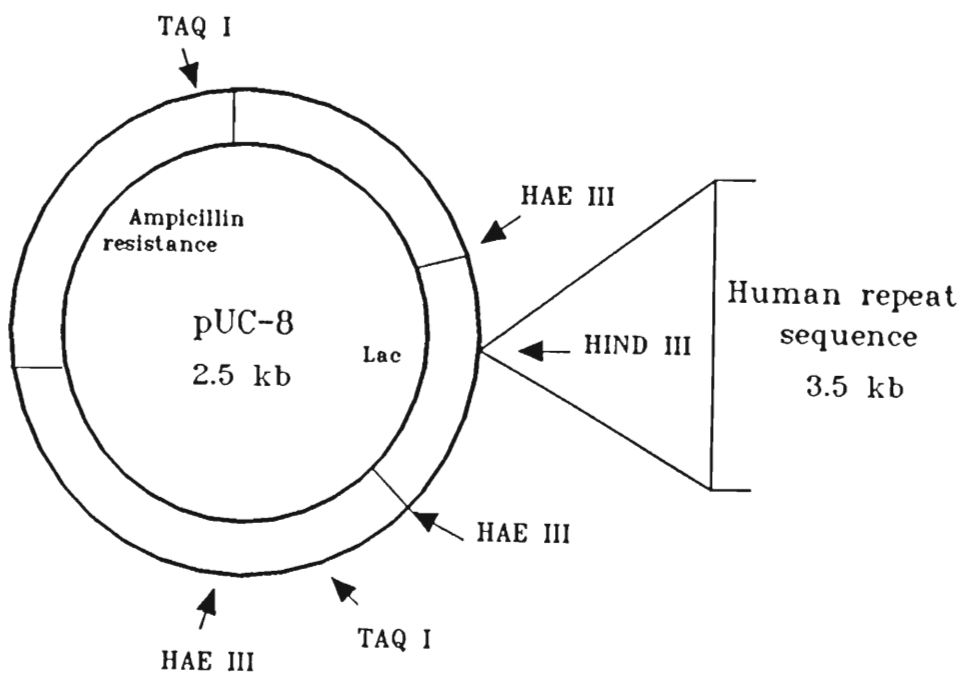


Figure 3.3 Diagrammatic representation of the plasmid pV47-2 constructed by Longmire *et al.* (1990). The position of the human DNA minisatellite repeat sequence is shown.

3.1.3 APPLICATIONS OF DNA POLYMORPHISM ANALYSIS

3.1.3.1 Genome studies

DNA polymorphisms offer the opportunity to observe plant, animal and human genomes at the sequence level and have proved to be of great value in the study of genome organization and genome evolution, as well as in investigations into genetic diversity and the genetic relationships between organisms (Chang and Meyerowitz, 1991).

Construction of linkage maps

Genetic linkage occurs when two genes are closely spaced or linked on a chromosome and cosegregate during meiosis resulting in their being inherited together (Mathews and von Holde, 1991). The inheritance of certain of these genes may be followed, for example, by monitoring the presence or absence of their expression products and, therefore, these genes may act as genetic markers for the inheritance of other important, closely linked genes (Havey and Muehlbauer, 1989).

The potential of RFLPs as genetic markers became apparent during studies on the human globin gene, when Kan and Dozy (1978) observed a direct correlation between the sickle cell mutation carried by this gene and the presence of certain RFLP fragments. Thus, with the discovery of RFLPs a whole new group of genetic markers, apart from the isozyme and cytogenetic markers used previously, became available for genome analysis (Kan and Dozy, 1978). As mentioned above, by following the inheritance of such RFLP markers, the inheritance of tightly linked genes cosegregating with these markers could be traced. This has facilitated the construction of physical linkage maps which have allowed workers to link the pattern of restriction fragments observed in an individual with the presence or absence of genes of interest, for example, disease-causing genes in humans (Botstein *et al.*, 1980) or genes encoding important agronomic traits in plant species (for detailed references refer to Table 3.3).

Figure 3.4 gives a detailed account of how an RFLP linkage map would be constructed for self-compatible, inbreeding plant species such as *Zea mays* (maize) (Helentjaris *et al.*, 1986).

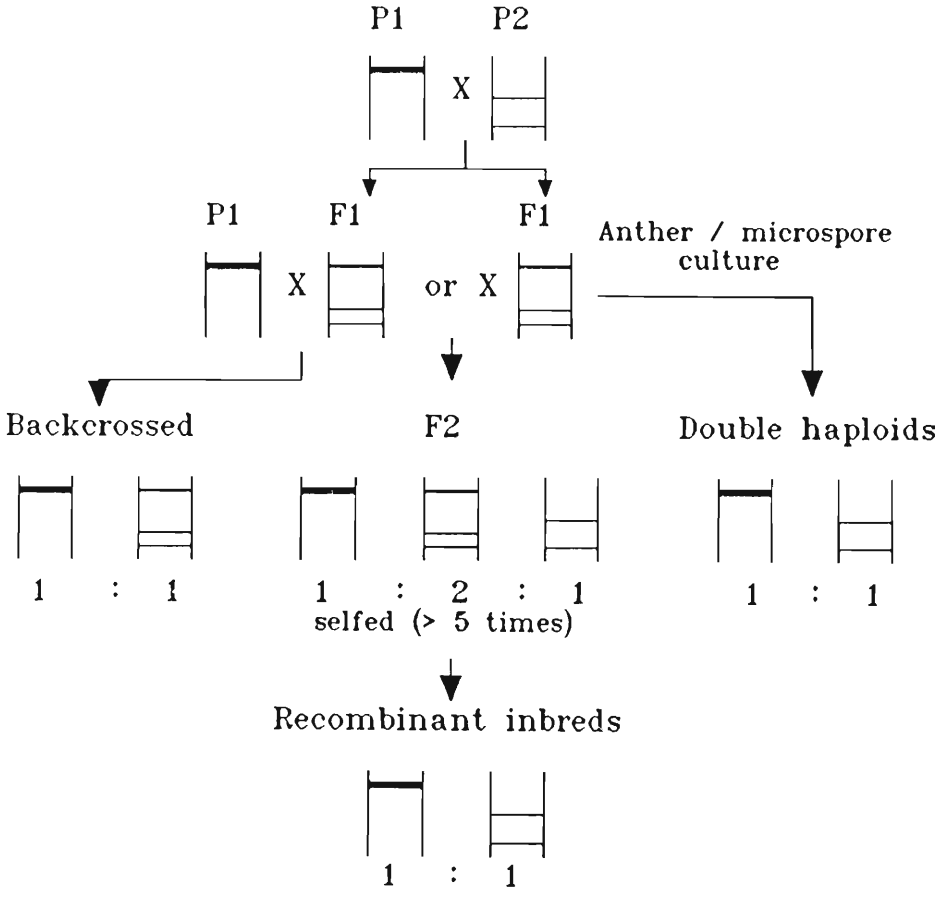


Figure 3.4 Diagrammatic representation of the crossing schemes used in segregation analysis for the production of RFLP linkage maps. The parallel lines (| |) represent double-stranded DNA and the bands between them (▬) chromosomal alleles. **A**, parents (P1 and P2) showing maximum polymorphic loci are crossed to produce the F1 population. In populations showing limited intraspecific variability, such as *Lycopersicon* (tomato), crosses may be made between different species to ensure mapping of as many markers as possible. **B**, the F1 population is selfed or **C**, F1 are backcrossed, to produce the F2 generation after which the inheritance of RFLP markers and the traits of interest are traced. **D**, RFLP maps may also be constructed by following segregating populations of double haploid lines. Modified from Gebhardt and Salamini (1992).

Table 3.3 Plants for which RFLP linkage maps have been constructed.

Species	Number of loci mapped	Reference
Lycopersicon esculentum (tomato)	>350	Helentjaris *et al.*, 1986; Bernatzky and Tanksley, 1986; Tanksley and Mutschler, 1990
Glycine species (soybean)	281	Kiem *et al.*, 1990 Lark *et al.*, 1993
Lens species (lentil)	20	Havey and Muel bauer, 1989
Zea mays (maize)	334-338	Helentjaris *et al.*, 1986; Burr *et al.*, 1988; Gardiner *et al.*, 1993
Lactuca sativa (lettuce)	46	Landry *et al.*, 1987
Oryza sativa (rice)	123	McCouch *et al.*, 1988*
Solanum tuberosum (potato)	122-141	Bonierbale *et al.*, 1988; Gebhardt *et al.*, 1989
Capsicum species (pepper)	80	Tanksley *et al.*, 1988
Brassica species (broccoli/ cabbage)	258	Slocum *et al.*, 1990 Chyi *et al.*, 1992
Arabidopsis thalaina	90	Chang and Meyerowitz, 1993
Avena species (oats)	-	Odonoughue *et al.*, 1992
Saccharum spontaneum	308	Dasilva *et al.*, 1993

Note: * Morphological and isozyme data were used to coordinate the cytogenetic and RFLP maps.
Morphological data was used here also.

Mapping of quantitative trait loci (QTLs)

Several complex traits are quantitative, that is, they show continual phenotypic variation due to the expression of independently assorted genes which act in combination with environmental factors (Chang and Meyerowitz, 1991). Such quantitative traits may be mapped by linking them, using statistical methods, to the segregation and inheritance of RFLP markers (Chang and Meyerowitz, 1991; Mansur *et al.*, 1993).

Quantitative trait mapping of an entire genome using RFLPs was first demonstrated in tomato, where multigenic traits for fruit mass, pH and the concentration of soluble solids, were mapped to several chromosome regions (Paterson *et al.*, 1988). Similarly, mapping of 72 RFLPs in the genome of *Glycine* (soybean) has lead to the identification of five genomic DNA regions associated with seed coat permeability (Kiem *et al.*, 1990b). During the domestication of this crop plant, the characteristic seed coat impermeability was lost. This trait would be of value to breeders of soybean today in improving seed viability and reducing fungal infection during seed storage (Chang and Meyerowitz, 1991). As mentioned above, RFLP analysis has played an important role in studies done thus far on the soybean genome and, in particular, has facilitated the identification of genomic regions which affect seed coat impermeability (Kiem *et al.*, 1990b). It is possible that in the future this information may be used to manipulate and possibly re-introduce this trait into cultivated soybean varieties.

Phylogenetic analyses

RFLP analysis is done at the level of the genome, allowing the detection of heritable changes in the nucleotide sequence of DNA (Wang *et al.*, 1992). These changes result largely from mutational events which occur over evolutionary time (refer section 3.1.1) and may be detected by comparison of shared and polymorphic restriction fragments between individuals (Chang and Meyerowitz, 1991). Such comparison of RFLP patterns has allowed workers to infer phylogenies for various species of organism and these are generally in agreement with classifications based on morphological and other characteristics (Chang and Meyerowitz, 1991, Takumi *et al.*, 1993). However, many more RFLP markers than other molecular markers are available enabling a number of RFLP loci to be studied simultaneously (McCough *et al.*, 1988), which suggests that the RFLP system would be most sensitive in detecting phylogenetic relationships between closely related individuals.

Song *et al.* (1990) investigated the phylogenetic relationships within *Brassica* (cabbage family) and between *Brassica* and its wild relatives using RFLPs. DNA samples, isolated from thirty eight *Brassica* cultivars (Chinese cabbage, turnip, brocolli, cauliflower and various others), were examined and on the basis of the restriction fragment patterns observed, these cultivars could be separated into two distinct groups, suggesting that they had evolved by different evolutionary pathways (Song *et al.*, 1990). Wang *et al.* (1992) similarly used RFLP analysis to demonstrate that the two major sub-species of cultivated *Oryza sativa* (rice), namely, indica and japonica, had arisen through separate domestication events.

Development of conservation strategies

As mentioned previously, RFLPs provide a means of measuring genetic diversity within the genome and, therefore, within populations of organisms. Hence, RFLP analysis has lead to the identification of species of birds and mammals which may be endangered (Kirby, 1990). For example, DNA fingerprinting of the peregrine falcon, Mauritius kestrel and whooping crane has shown the presence of limited gene pools and these findings have stimulated concern over the genetic fitness of these bird species (Longmire, 1989). Similarly, RFLP studies have provided a measure of the genetic diversity between endangered whale species and this information has proven invaluable in the development of conservation strategies for these animals (Hoelzel and Amos, 1988).

Characterization of cultured material

RFLPs have been used to characterize the genomes of hybrid and cybrid plant cell populations produced during *in vitro* culture (Imamura *et al.*, 1987; Piastuch and Bates, 1990). For example, Imamura *et al.* (1987) used repetitive, species-specific probes to estimate the extent of genetic diversity in somatic hybrids of *Hyoscyamus muticus* and *Nicotiana tabacum* (tobacco). Piastuch and Bates (1990) used this approach also to examine cybrids of *N. tabacum* and *N. plumbaginfolia*.

RFLPs have proved to be valuable also in the characterization of material produced in other culture systems. Rivard *et al.* (1989), for example, used RFLPs to distinguish true homozygous diploids of *Solanum chacoense* from plants produced through *in vitro* anther culture. RFLP probes were used also to show that *Juglans redia* (walnut) somatic embryos originated from zygotic as opposed to maternal tissue cultured in an *in vitro* system (Aly *et al.*, 1992).

3.1.3.2 Identification of biological material

As a result of the unique nature of DNA restriction fragment patterns (DNA fingerprints) (refer section 3.1.1 and section 3.1.2.2), technologies for the analysis of DNA polymorphisms have revolutionized methodologies for the identification of human, animal and plant materials.

Forensic applications

The earliest application of the DNA fingerprinting technology was in human pedigree analysis and the positive identification of individuals (Jeffreys *et al.*, 1985a; 1985b). Jeffreys *et al.* (1985b), for example, used a series of DNA fingerprints to confirm the relationship between a young Ghanian boy wanting to immigrate to the United Kingdom and his family, in particular his mother, already living there. Evidence provided by those workers allowed the boy to be re-united with his family and granted permanent residence in that country (Jeffreys *et al.*, 1985b). Many similar disputes regarding biological parentage have been resolved by comparing the DNA fingerprints of children with those of their putative parents (Odelberg *et al.*, 1988). DNA fingerprints have been used also as forensic evidence facilitating the positive identification of both the victims and suspects in crimes involving homicide, rape and other violent acts, and have been applied in accident cases and cases of missing persons (Anderson, 1989; King, 1989; Norman, 1989).

Animal studies

In animal species DNA fingerprinting has been used in pedigree analysis of dogs and cats, where proof of paternity is often necessary before an animal can be registered officially (Jeffreys *et al.*, 1987). DNA fingerprinting has been used also in a case of disputed paternity in a population of captive whales (Hewlett *et al.*, 1989). A whale cow named Bjossa had given birth to a calf and analysis of the DNA fingerprints of this calf and two whale bulls identified the bull named Hyak as the sire (Hewlett *et al.*, 1989). Another important application of DNA fingerprinting in animal studies has been in the identification of meat, furs and other materials obtained illegally by poaching of big game (Kirby, 1990). Here, DNA obtained from these tissues may be directly matched to samples obtained from the road-side remains of an animal and may lead to the conviction of wildlife poachers (Thommasen *et al.*, 1989).

Plant breeding studies

Technologies for the detection of DNA polymorphisms using mainly DNA fingerprinting have been used extensively in the identification of plant material in breeding programs. These polymorphisms have revealed genotypic diversity at various levels, that is, between individuals, for example, of *Acer negundo* (box elder) (Nybom and Rogstad, 1990), *Picea abies* (L.) (Norway spruce) (Kvarnheden and Engström, 1991) and *Populus tremuloides* (quaking aspen) (Rogstad *et al.*, 1991), between varieties and cultivars, as for *Oryza* spp (rice) (Dallas, 1988; Wang and Tanksley, 1992), *Rubus* spp (blackberry and raspberry) (Nybom *et al.*, 1989; 1990; Nybom and Schaal, 1990; Nybom and Hall, 1991), *Malus* spp (apple) (Nybom, 1990a; 1990b; Nybom *et al.*, 1990), *Prunus* spp (prune) (Nybom *et al.*, 1990), *Cornus* spp (dogwood) (Culpepper *et al.*, 1991), *Dianthus* spp (carnation) (Tzuri *et al.*, 1991), *Solanum* spp (potato) (Powell *et al.*, 1991; Schweizer *et al.*, 1993) and *Vitis* spp (grape) (Bowers *et al.*, 1993), and between species, for example, of *Beta* (sugar beet) (Nagamine *et al.*, 1989) and rice (Wang and Tanksley, 1992).

At the species level Nagamine *et al.* (1989) were able to detect restriction fragment polymorphisms between the cultivated sugar beet *Beta vulgaris* and a wild species *Beta nana*. According to those authors such RFLP analysis may aid the process of selection in plant breeding programs (Nagamine *et al.*, 1989). For example, RFLPs may allow workers to select individuals, produced from controlled cross experiments between cultivated and wild-type *Beta* species, which have favourable genes introgressed into their genomes (Nagamine *et al.*, 1989).

Another group of workers have used DNA fingerprinting in conjunction with the M13 repeat probe of Vassart *et al.* (1987) (refer section 3.1.2.2) to distinguish various varieties and cultivars of the woody *Malus*, *Rubus* and *Prunus* genera (Nybom *et al.*, 1989; Nybom, 1990a; 1990b; Nybom *et al.*, 1990; Nybom and Schaal, 1990a; 1990b; Nybom and Hall, 1991). The genus *Rubus*, for example, includes the raspberry cultivars which show considerable phenotypic plasticity and can not be distinguished on the basis of morphological characteristics (Nybom and Schaal, 1990b). In addition, isozyme analysis carried out on *Rubus* have not been able to distinguish red and purple raspberry varieties (Nybom and Schaal, 1990b). Therefore, within this genus, RFLP analysis has been particularly useful as means of identifying raspberry cultivars (Nybom *et al.*, 1989; 1990; Nybom and Schaal, 1990; Nybom and Hall, 1991). Nybom and Hall (1991), for example, demonstrated that

DNA fingerprints could be used to identify 13 different *Rubus* cultivars. In addition, minisatellite DNA analysis has revealed extensive inter- as well as intra-specific variation in some American *Rubus* species (Nybom, 1990b; Nybom and Schaal, 1990b).

The cultivated apple has been studied also by Nybom and co-workers (Nybom, 1990a; 1990b; Nybom and Schaal, 1990a). In the past ten years various cultivated apple varieties have been characterized using isozyme analysis, although closely related cultivars could not be distinguished unless several enzyme systems were analyzed simultaneously (Weeden and Lamb, 1985; Bournival and Korban, 1987). In subsequent studies on the apple genome, Nybom (1990a; 1990b) has shown that DNA minisatellite analysis has great potential as a technique for the identification, not only of apple cultivars, but, of individual apple genotypes (Nybom, 1990b).

In addition to the applications described above, where DNA fingerprinting has been used directly to identify plant material, this technology has other uses in plant breeding programmes. For example, fingerprints of box elder have been used to study genetic flow within insect pollinated elder species (Nybom and Rogstad, 1990). Similarly, Kvarnheden and Engström (1991) have investigated genetic diversity within a Norway spruce community using DNA fingerprinting and those authors suggest that this technology may be useful in the analysis of population structure and dynamics in conifer species. Another potential application of the fingerprinting technology, as described previously for humans and animal, is in paternity determination and studies of genetic relatedness between individuals. Nybom (1990b), for example, used fingerprints to determine paternity in apple cultivars. This application would be particularly useful when pollination occurs in seed orchards, where promising unidentified parental stock could be traced back from successful progeny using DNA fingerprints. In fact, numerous authors have used RFLPs to study the genetic efficiency of seed orchards (Szmidt, 1987; El-Kassaby *et al.*, 1988). In addition to seed production in these orchards, which occurs as a result of sexual fertilization, seeds and offspring may be produced apomictically, that is, without fertilization (Einset, 1951). This occurs with a few raspberry cultivars and Nybom *et al.* (1990) have suggested that minisatellite DNA analysis could be used to distinguish these apomictically derived offspring from those obtained during sexual crosses.

3.1.4 RATIONALE AND INTRODUCTORY COMMENTS

Chapter 2 described the development of a model methodology for chloroplast isolation from the eucalypt *Eucalyptus grandis* S/N M6, followed by successful use of the organelles in an "in-organelle" digestion procedure for the analysis of chloroplastic RFLPs. However, difficulties were encountered when attempts were made to extract chloroplasts from various other eucalypt species using the developed chloroplast isolation protocol. An alternative route for cpDNA analysis involves extraction of total genomic DNA, blotting of restricted DNA onto a membrane and visualization of the polymorphic fragments by hyridization of a labelled cpDNA probe. Parallel procedures are used in genomic fingerprinting, a technique which has allowed workers to differentiate material at wide ranging levels from the genus to the individual. Consequently, the second approach adopted in this study was to optimize protocols for genomic fingerprinting of the eucalypts.

The first requirement for fingerprinting is a supply of pure, intact genomic DNA which can be digested with various restriction enzymes. Therefore, it was neccessary to optimize a DNA extraction procedure which could be used to obtain reproducibly good yields of such DNA from a range of eucalypt materials. It was then essential to prepare and purify a suitable probe. The M13-derived plasmid pV47-2 was the probe of choice as this probe has been shown to detect numerous polymorphic loci (33 % more than the M13 repeat probe itself) (refer section 3.1.2.2). Following probe preparation it was neccessary to optimize conditions for probe labelling and hybridization of labelled probe to eucalypt genomic DNA. Systems are availabled which allow this to be carried out using radioisotopes as probe labels, but, more recently, non-radioisotopes have been used. It was decided to optimize probe labelling and detection systems using both the isotope ^{32}P and the non-isotope label digoxygenin (DIG), so that the results obtained with these sytems could be compared and contrasted. This would allow selection of a suitable protocol for incorporation into a eucalypt screening programme.

3.2 MATERIALS AND METHODS

3.2.1 PLANT MATERIAL AND GROWTH CONDITIONS

Potted saplings of *Eucalyptus grandis* S/N M6, *E. grandis* TAG 5, *E. grandis* TAG 14, *E. nitens*, *E. macarthurii* and an *E. grandis* x *E. nitens* hybrid NG 1026 were grown under 30 % full sunlight. These were placed in the dark 24 h prior to DNA extraction.

3.2.2 GROWTH AND MAINTENANCE OF BACTERIAL STRAINS

Colonies of of *E. coli* HB1O1 (ampicillin-sensitive) (Bolivar and Backman, 1979) and *E. coli* HB1O1/pV47-2 transformants were stab inoculated onto tryptose blood agar base (TAB) medium (33 g/l) (Difco, USA) with or without 50 μg/ml ampicillin. These stab cultures were incubated overnight at 37 °C and maintained in the dark, tightly sealed, at room temperature as long term stocks (up to 12 months). Short term bacterial stocks on streak plates were kept at 4 °C for 2 week periods.

Growth of bacteria was initiated by the transfer of streak plate colonies to 5 ml of Luria broth (LB) medium (10 g/l bacto-tryptone, 5 g/l bacto yeast extract, 10 g/l NaCl, pH 7.5, with or without 50 μg/ml ampicillin) (Maniatis *et al.*, 1982). Following overnight incubation at 37 °C bacteria were either used immediately or subcultured to generate larger volumes of culture.

3.2.3 TRANSFORMATION OF *E. coli*

The plasmid construct pV47-2 (Figure 3.1) (Longmire *et al.*, 1990) was obtained from Dr. Moira van Staden (The Biological Laboratories, Harvard University) and used with the permission of J.L. Longmire.

Bacterial transformation of *E. coli* HB1O1 with pV47-2 was carried out using a modified procedure of Cohen *et al.* (1972) (in Maniatis *et al.*, 1982). A single bacterial colony from a fresh streak plate was inoculated into a 1 l flask containing 100 ml of SOB medium (20 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 0.5 g/l NaCl, pH 7.0) (Maniatis *et al.*, 1982) and incubated for 3 h at 37 °C with shaking. Bacterial suspensions were transferred to sterile centrifuge tubes and kept on ice for

10 minutes before cells were pelleted (4 000 rpm for 15 min at 4 °C). The supernatant was discarded and the pellet resuspended in 10 ml ice-cold 0.1 M $CaCl_2$. Cells were then recovered by centrifugation (4 000 rpm for 15 min at 4 °C), resuspended once again (ice-cold 0.1 M $CaCl_2$, 0.04 ml/ml original culture) and stored overnight at 4 °C. Aliquots of the competent bacterial suspension were transferred to sterile tubes before plasmid DNA (0.25 ng/µl) was added, the suspension mixed and stored on ice for 30 min. Tubes were then incubated at 42 °C for 90 sec, before being chilled on ice for 1-2 min. SOC medium (SOB plus 20 mM glucose) (Maniatis *et al.*, 1982) was added (4 µl/µl bacterial suspension) and cultures incubated at 37 °C for 45 min. A volume of this culture (200 µl/90 mm plate) was spread over the surface of a SOB plate containing 20 mM $MgCl_2$ and 50 µg/ml ampicillin. Transformants were selected by overnight growth at 37 °C on this antibiotic medium.

3.2.4 DNA EXTRACTION AND PURIFICATION

3.2.4.1 Plasmid DNA

A modified procedure of Birnboim and Doly (1979) (in Draper, 1988) was followed for large scale extraction of pV47-2 from transformed *E. coli*. Aliquots of bacterial suspension, taken from overnight broth cultures, were subcultured into 2 l flasks (50 µl/flask) containing 500 ml each of LB plus 50 µg/ml ampicillin and incubated with vigorous shaking at 37 °C overnight. Bacterial cells were collected by centrifugation (8 000 rpm for 5 min at 4 °C), the supernatant discarded and the pellet resuspended in lysis solution (1 mg/ml lysozyme, 0.5 M glucose, 10 mM EDTA, 25 mM tris-HCl, pH 8.0) (0.08 ml/ml bacterial culture). Samples were incubated on ice for 10 min before the addition of alkaline sodium dodecyl sulphate (SDS) (0.2 M NaOH, 0.7 % (w/v) SDS final concentration). After mixing and a further 4 min incubation on ice, potassium acetate was added (1 M final concentration, pH 5.2). The suspension was mixed well, centrifuged (8 000 rpm for 10 min at 4 °C) and the supernatant filtered through a glass wool plug into a clean tube. Pre-chilled propane-2-ol (0.6 ml/ml sample) was added, followed by mixing and centrifugation at 8 000 rpm for 10 min at 4 °C. The DNA pellet was rinsed with a few drops of 70 % (v/v) ethanol and then ether (evaporated off in a fume cupboard) before being dissolved in TE buffer (1 mM EDTA, 10 mM tris-HCl, pH 7.4) (Maniatis *et al.*, 1982) (0.02 ml/ml original bacterial culture). Caesium chloride (1.31 g/ml) (Sigma, USA) and ethidium bromide (1.1 mg/ml) (Sigma, USA) were added to give the solution a final

density of 1.55 g/ml. Samples were centrifuged at 3 500 rpm at room temperature for 30 min. Ultracentrifuge tubes were filled with the supernatant, heat sealed and centrifuged at 100 000 rpm and 18 °C for 4 h. The lower diffuse plasmid band was visualized by UV light, drawn off with a hypodermic syringe and transferred to a sterile bottle.

Ethidium bromide was removed by partitioning with an equal volume of saturated butanol water. This was carried out several times before the sample was transferred to a length of dialysis tubing (prepared according to Draper *et al.*, 1988). Dialysis was carried out against a total volume of 2.5 l of TE buffer at 4 °C on a magnetic stirring pan. The buffer was changed every few hours and dialysis allowed to proceed for ± 2 days. The final solution of plasmid DNA was stored at -20 °C until further required.

The plasmid concentration was 1.122 mg/ml, total yield 2.24 mg, and A_{260}/A_{280} was 1.88.

3.2.4.2 Plant genomic DNA

Leaf tissue, pooled from at least three saplings (0.5-1.0 g), was frozen in liquid nitrogen before being ground using a mortar and pestle. The resulting powder was incubated in 5-7.5 ml of a hexadecyltrimethylammonium bromide (CTAB) (Sigma, USA) isolation buffer (2 % (w/v) CTAB, 1.4 M NaCl, 0.2 % (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM tris-HCl, pH 8.0), for 30 min at 60 °C (Doyle and Doyle, 1990b). The sample was extracted once with chloroform-isoamyl alcohol (24:1, v:v) and centrifuged (2 500 rpm for 10 min at 25 °C) to separate the phases. Following transfer of the aqueous phase to a clean centrifuge tube, two thirds the volume of cold isopropanol were added to precipitate the DNA. DNA was pelleted by centrifugation at 2 500 rpm for 10 min at room temperature and the pellet washed for 20 min in 10 ml wash buffer (76 % (v/v) ethanol, 10 mM ammonium acetate) (Doyle and Doyle, 1990b). After pelleting (2 500 rpm for 10 min at 25 °C), the DNA was air dried and resuspended in 1 ml TE buffer. RNA was removed by addition of preboiled RNAse A (final concentration 10 µg/ml) and incubation at 37 °C for 30 min. The sample was diluted with 2 volumes of TE buffer before DNA was precipitated by the addition of 2.5 volumes of cold 96 % (v/v) ethanol and stored at -20 °C overnight. The final DNA pellet, obtained by centrifugation at 3 500 rpm for 30 min at 4 °C, was solubilized in 200 µl TE by incubation at 4 °C overnight. DNA samples were stored at -20 °C until needed.

Various protocols for DNA purification were employed. These included: 1, Precipitation of DNA with ammonium acetate and ethanol (Doyle and Doyle, 1990b); 2, spun-column purification (Maniatis *et al*, 1982) and 3, dialysis against TE buffer (S. McRae, pers comm).

For the first procedure, ammonium acetate (pH 7.7) was added (final concentration 2.5 M) together with 96 % (v/v) ethanol and DNA precipitation allowed to proceed at -20 °C overnight (Doyle and Doyle, 1990b).

The second procedure involved removal of short contaminating DNA fragments by spun-column purification of the genomic DNA extract (Maniatis *et al.*, 1982). Syringes (1 ml) were plugged at the base with glass wool and filled with Sephadex G50 beads (Sigma, USA) which had been pre-swollen by incubation in STE buffer (0.1 M NaCl, 10 mM tris-HCl, 1 mM EDTA, pH 8.0) (Maniatis *et al.*, 1982) at 4 °C overnight. These were then suspended in test-tubes over eppendorf tubes (1.5 ml) and the columns packed by centrifugation at 3 000 rpm for 5 min. After equilibration of the columns with STE, the DNA sample was loaded and eluted during a final centrifugation step (3 000 rpm for 5 min at room temperature).

The third purification protocol was initiated by loading of 25-50 μl aliquots of genomic DNA onto 0.22 μm cellulose acetate filters (25 mm diameter) (Millipore, USA). These were floated in petri dishes containing 20 ml of TE buffer for ± 2 h (S. McRae, pers comm).

3.2.5 DNA ASSESSMENT

The concentration and purity of plasmid and genomic DNA samples were determined spectrophotometrically. Absorbance readings at 260 nm were used to calculate DNA concentration (A_{260} x 50 x dilution factor = concentration of sample in μg/ml) (Sambrook *et al.*, 1989) and the ratio of readings at 260 and 280 nm was used as a measure of sample purity (Sambrook *et al.*, 1989).

In addition to spectrophotometric readings, DNA was assessed visually by ethidium bromide staining of DNA fragments after electrophoretic separation on agarose gels (Sambrook *et al.*, 1989).

3.2.6 RESTRICTION ENZYME DIGESTION

Digestion of genomic, plasmid and lambda DNA was carried out with the appropriate restriction enzyme (5 U/μg DNA) (Boehringer Mannheim, Germany) using the Boehringer Mannheim buffer set for 1½-3 h at 37°C. In all cases the enzyme reaction was stopped by rapid cooling of the sample on ice followed by storage at -20°C.

3.2.7 SEPARATION OF FRAGMENTS BY AGAROSE GEL ELECTROPHORESIS

Mini gels were run in TBE buffer (45 mM tris-borate, 1 mM EDTA, pH 8.0) (Maniatis *et al.*, 1982) on a Hoeffer-HE horizontal gel apparatus at 5.6 V/cm. Immediately prior to loading onto a 1 % (w/v) DNA-grade agarose gel (BioRad, USA), DNA samples were heat-treated at 65°C for 10 min, ice-cooled, and mixed with 3 μl of dye loading buffer (50 % (w/v) sucrose, 4 M urea, 0.1 % (w/v) bromophenol blue, 1 mM EDTA, pH 7.6) (Maniatis *et al.*, 1982). Gels were stained with 0.5 μg/ml ethidium bromide for 20 min and destained for an equivalent period with distilled water before being viewed by UV transillumination at 300 nm.

Electrophoretic separation of DNA fragments for Southern analysis was carried out on 12 x 15 cm, 0.8 % (w/v) DNA-grade agarose gels (Biorad, USA). These were run on a Biorad horizontal gel apparatus at 20 V for 18 h, with continuous circulation of TAE buffer (40 mM tris-acetate, 1 mM EDTA, pH 8.0) (Maniatis *et al.*, 1982).

3.2.8 SOUTHERN BLOTTING

Following electrophoresis, DNA fragments were either neutral blotted (Maniatis *et al.*, 1982) onto Hybond-C-extra nitrocellulose membranes (Amersham, UK) or alkali blotted (Amersham protocol) onto Hybond-N^+ nylon membranes (Amersham, UK). In both cases, prior to blotting, DNA was depurinated by soaking the gel in 0.25 M HCl for 15 min. After washing with distilled water, the gel was submerged in a volume of denaturation buffer (1.5 M NaCl and 0.5 M NaOH) and left for 30-45 min.

When blotting onto nitrocellulose, the gel was rinsed and neutralized by incubation for 30 min in 1 M tris-HCl (pH 7.4), 1.5 M NaCl and 0.001 M EDTA. A capillary blot was set up (Figure 3.3) using 20 x SSC (3 M NaCl, 0.3 M Na citrate) (Maniatis *et al.*, 1982) as a transfer buffer. For alkali blotting onto nylon, the gel was removed from the depurination buffer, rinsed, equilibrated in alkali transfer buffer (0.25 M NaOH, 1.5 M NaCl) and a capillary blot set up using as shown in Figure 3.5. DNA transfer was allowed to proceed overnight.

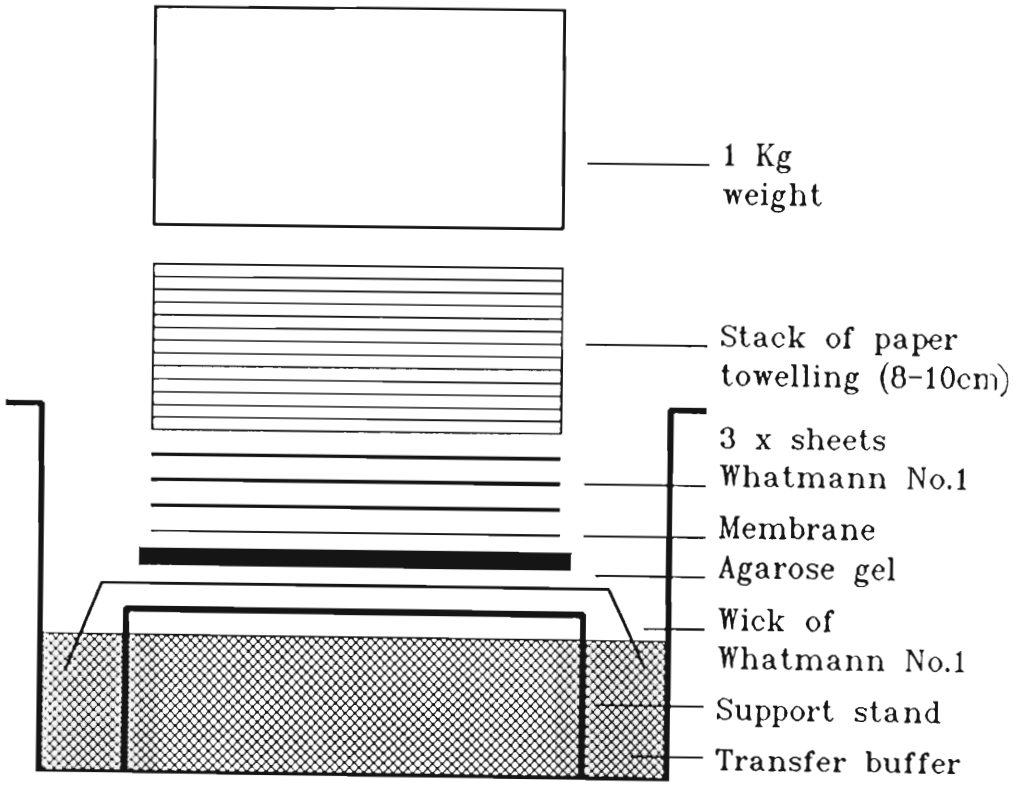


Figure 3.5 Side view of the capillary blot apparatus set up for the transfer of DNA from agarose gels to nylon or nitrocellulose membranes.

Nitrocellulose filters were washed in 6 x SSC and nylon membranes in 2 x SSC for 5 min. Membranes were air dried and DNA permanently fixed by baking at 80°C for 2 h. Blots were either probed immediately or stored with silica gel in an airtight container at room temperature.

3.2.9 RANDOM PRIMER PROBE LABELLING AND PURIFICATION

3.2.9.1 Labelling with ^{32}P

[α-^{32}P]dTTP was incorporated into Hind III restricted pV47-2 fragments according to the "oligolabelling" technique of Feinberg and Vogelstein (1983). The reaction mixture contained 0.4 mg/ml bovine serum albumin (BSA), 44.8 mM tris-HCl (pH 8.0), 4.84 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 19.36 μM each of dATP, dCTP, dGTP, 200 mM Hepes (pH 6.6), 5.4 OD units/ml random hexanucleotide primers, 100 ng denatured probe (100°C, 10 min), 4 U Klenow enzyme and 250 μCi [$\alpha^{32}P$]dTTP in a final volume of 100 μl. Label incorporation was allowed to proceed at room temperature overnight and the reaction terminated by the addition of EDTA (final concentration 7.3 mM). Unincorporated nucleotides were removed by spun-column purification of the labelled probe (Maniatis *et al.*, 1982).

To measure [$\alpha^{32}P$]dTTP incorporation, labelled probe was precipitated with trichloroacetic acid (TCA) (final concentration 5 % (w/v)) in the presence of carrier DNA (30 μg calf thymus DNA/μl probe) (Maniatis *et al.*, 1982). After a 15 min incubation on ice, samples were suction-filtered using a Millipore Micro Filter apparatus (Millipore, USA) through GF/C glass filters (25 mM diameter) (Whatmann, UK) and washed (5 x 5 ml cold 5 % (w/v) TCA and 3 x 5 ml cold 96 % (v/v) ethanol). Filters were submerged in Beckman "Ready Value" scintillation cocktail (5 ml) and counted in a Beckman LS 7500 Liquid Scintillation Counter. Specific activities varied slightly, with a typical value being 9.5 x 10^9 cpm/μg probe.

3.2.9.2 Labelling with digoxygenin (DIG)-dUTP

Hind III cleaved denatured plasmid was labelled with DIG-dUTP according to manufacturer's instructions (Boehringer Mannhein, Germany). The 20 μl reaction volume contained a hexanucleotide mixture, buffering agents, 100 μM each of dATP, dCTP and dGTP, 350 μM of DIG-dUTP, 100-300 ng denatured probe and 2

U Klenow enzyme. Labelling was carried out for 20 h at 37 °C and stopped by the addition of EDTA (20 mM final concentration, pH 8.0). Labelled probe was precipitated with 500 mM LiCl and pre-chilled 96 % (v/v) ethanol (3.75 µl/µl labelling solution). Samples were incubated at -80 °C for at least 1 h before the DNA was pelleted (11 000 rpm for 15 min at 4 °C). After washing of the pellet with cold 70 % (v/v) ethanol it was dried under vacuum and dissolved in 50 µl TE. DIG-labelled probe was stored at -20 °C until required for hybridization experiments.

3.2.10 HYBRIDIZATION OF LABELLED PROBE TO MEMBRANES

Three hybridization protocols were used, namely (A), a procedure modified from Sambrook *et al.* (1989), (B), the DIG-hybridization protocol (Boehringer Mannheim, Germany) and (C), a protocol for "fingerprinting" with M13 based probes (Westneat *et al.*, 1988).

Using procedure A, membranes were pre-hybridized overnight at 42 °C in a solution (0.2 ml/cm^2 membrane) containing 6 x SSPE (0.9 M NaCl, 6 mM EDTA, 60 mM NaH_2PO_4, pH 7.7), 5 x Denhardt's solution (0.1 % (w/v) Ficoll, 0.1 % (w/v) polyvinylpyrrolidone, 0.1 % (w/v) BSA), 50 % (v/v) formamide, 0.1 % (w/v) SDS and 0.1 mg/ml denatured, sheared salmon sperm DNA. Once the membrane had been transferred to hybridization solution (as for pre-hybridization plus 10 % (w/v) dextran sulphate) the radiolabelled probe was denatured (100 °C for 10 min), added and hybridization carried out for 24 h at 42 °C. Membranes were then passed through five wash steps to remove unbound probe. Filters were agitated: 1, in 2 x SSC and 0.5 % (w/v) SDS for 5 min at room temperature; 2, in 2 x SSC and 0.1 % (w/v) SDS for 15 min at room temperature; 3, in 0.1 x SSC and 0.5 % (w/v) SDS for 30 min at 37 °C; 4, in 0.1 x SSC and 0.5 % (w/v) SDS for 30 min at 68 °C and; 5, in 0.1 x SSC for a few min (Maniatis *et al.*, 1982).

Hybridization by procedure B was carried out using a DIG kit according to the manufacturers instructions (Boehringer Mannheim, Germany). Membranes were incubated in the pre-hybridization solution (5 x SSC, 1 % (w/v) blocking reagent, 0.1 % (w/v) N-lauroylsarcosine, 0.02 % (w/v) SDS, pH 7.0) (20 ml/cm^2 membrane) for 4 h at 68 °C. Hybridization using 25 µl of solution per cm^2 of filter (pre-hybridization solution plus 5 ng/µl labelled, denatured probe) was carried out overnight at 68 °C. Filters were then washed twice for 5 min at room temperature using 2 x SSC and 0.1 % (w/v) SDS, and twice again for 15 min at 68 °C with 0.1 x SSC and 0.1 % (w/v) SDS.

The Westneat *et al.* (1988) hybridization protocol was procedure C. Blots were pre-hybridized by incubation in a buffer (7 % (w/v) SDS, 1 % (w/v) BSA, 1 mM EDTA (pH 8.0) and 0.263 M Na_2HPO_4, pH 7.2) (25 μl buffer/cm^2 membrane) at 65 °C overnight. Labelled probe was denatured by boiling for 10 min and then ice-cooled before being added to the pre-hybridization solution (25 ng α^{32}P-labelled probe/ml or 20-40 ng DIG-labelled probe/ml). Excess DIG-labelled pV47-2 was stored at -20 °C until needed again. Following hybridization (65 °C for 48 h), the membrane was washed to remove any unbound DNA. Washes comprised three 15 min washes with 2 x SSC and 0.1 % (w/v) BSA, the first two at room temperature and the third at 65 °C. Finally, the membrane was rinsed briefly at room temperature with 1 x SSC.

3.2.11 DETECTION OF BOUND PROBE

3.2.11.1 ^{32}P detection

Blots were wrapped in cling film and subjected to autoradiography in the presence of intensifying screens (-80 °C for 16 h). X-Ray film (Hyperfilm-MP) (Amersham, UK) was developed in AGFA-GEVAERT developer (1 in 10 dilution) (Amersham, UK) for 1 min at room temperature and fixed .

3.2.11.2 DIG-detection

Digoxygenin labelled DNA bound to the membrane was detected by a colour reaction, according to the manufacturers instructions (Boehringer Mannheim, Germany). Membranes were washed for 1 min in buffer 1 (150 mM NaCl, 100 mM tris-HCl, pH 7.5), before being incubated for 30 min in buffer 2 (buffer 1 plus 1 % (w/v) blocking reagent). The antibody conjugate was diluted (1 in 5 000) in buffer 2 and the membrane agitated in this solution for 30 min. After two 15 min washes in buffer 1, the membrane was equilibrated in buffer 3 (100 mM NaCl, 50 mM $MgCl_2$, 100 mM tris-HCl, pH 9.5) for 2 min. The colour solution was made up with nitro blue tetrazolium salt (NBT) (337.5 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (BCIP) (175 μg/ml) dissolved in buffer 3. The membrane was covered with this solution and kept in a black bag in a dark cupboard for ± 16 h. Colour development was terminated by washing of the membrane for 5 min in 1 mM EDTA and 10 mM tris-HCl (pH 8.0) and the membrane stored in this solution.

3.2.12 PHOTOGRAPHY

Black and white photographs were taken using Kodak T Max film (ASA 400). A red filter was used for gel photographs taken under UV light (300 nm). T Max film were developed for 13 min at 20°C using Aculux developer (1 in 10 dilution) (Paterson Products, England). The developer was discarded, negatives rinsed with tap water and fixed (1 in 4 dilution) (Ilford, England) for 3-6 min at room temperature. Fixer was reusable so it was decanted back into a storage bottle. Negatives were then washed under running tap water for 30 min and hung up to dry. Black and white photographs were printed onto glossy Multigrade III photographic paper (MG Deluxe) (Ilford, England) using Multigrade filters (3-4) (Ilford, England).

Colour photographs were taken on Kodak colour print film (ASA 100).

NOTE

Diagrammatic representations of the methodologies developed for DNA extraction (Appendix 3) and production of eucalypt fingerprints (Appendix 4) are given in the Appendices.

3.3 RESULTS

3.3.1 EUCALYPT DNA ISOLATION AND PURIFICATION

The first step towards the generation of DNA fingerprints is the isolation of genomic DNA from the tissue to be analyzed. Therefore, establishment of a procedure for DNA extraction from *Eucalyptus* trees is essential if DNA restriction profiles are to be used for cultivar identification. To obtain fingerprints of individual eucalypt saplings, sufficiently high yields of DNA need to be extracted from small amounts of tissue and DNA must be sufficiently pure to facilitate restriction enzyme digestion. However, there are often problems associated with the extraction of genomic DNA from woody tissues, generally due to co-isolation of phenolic and polysaccharide contaminants which render the DNA inaccessible to restriction enzymes and result in low fragment yields (Doyle and Doyle 1990b; Culpepper *et al.*, 1991; Kvarnheden and Engström, 1991; Weising *et al.*, 1991). As the "CTAB" procedure of Doyle and Doyle (1990b) has been used successfully in the isolation of DNA from woody oaks and walnut, it was decided to attempt DNA extraction from various eucalypt saplings using this protocol.

3.3.1.1 Apparent yield and purity of the DNA obtained

During earlier investigations (Chapter 2), attempts to isolate high yields of intact chloroplasts from a range of eucalypt saplings using a single organelle isolation protocol had failed, possibly due to inherent variabilities in the material at a structural and/ or biochemical level (Figure 2.7). In the light of those findings, DNA extraction using the CTAB procedure was attempted from three eucalypt species, namely *E. grandis*, *E. nitens* and *E. macarthurii*, to ascertain whether the Doyle and Doyle protocol could be used successfully in the isolation of genomic DNA from a spectrum of eucalypt materials. Spectrophotometric measurements of DNA absorbance at 260 and 280 nm were used to determine the yields and purities of the DNA samples obtained (Table 3.4).

Table 3.4 Yields and purities of DNA samples extracted from three *Eucalyptus* species using the unmodified "CTAB" DNA isolation and purification protocol of Doyle and Doyle (1990b).

Species	DNA yield (μg/g fresh mass)	Purity of sample (A_{260}/A_{280})
E. grandis	87.5	1.86
E. nitens	87.2	1.73
E. macarthurii	201.0	1.88

As can be seen from Table 3.4, DNA yields ranged from 87.2 μg/g fresh mass obtained for *E. nitens* to 201.0 μg/g fresh mass extracted from *E. macarthurii*. These values seem to be acceptable when compared to DNA yields of 100-350 μg/g fresh mass isolated from Norway spruce by Kvarnheden and Engström (1991). DNA extracts also had A_{260}/A_{280} ratios between 1.73 and 1.88 (Table 3.4), indicating that the samples were reasonably pure, that is, mostly free of contaminants such as protein and RNA.

For visual confirmation of the integrity and purity of these DNA extracts, samples were run on an agarose gel. As DNA loads of 0.5 μg per well are required on a mini-gel for clear visualization of DNA fragments after ethidium bromide staining (Maniatis *et al.*, 1982), 0.5-1μg DNA samples from each of the eucalypt species were subjected to electrophoresis. However, no DNA bands were detected after incubation of the gel in ethidium bromide, indicating that there was insufficient DNA present for visualization by this staining technique. Therefore, DNA yield, determined spectrophotometrically, was not an accurate reflection of the true yield of DNA in each sample. This discrepancy between apparent and true yield could not have resulted from DNA degradation during long term storage of samples as DNA was subjected to electrophoresis almost immediately following CTAB extraction. In fact, Doyle and Doyle (1990b), who devised the protocol used here for DNA extraction in the presence of CTAB, also experienced difficulties when attempting to quantify DNA yields using absorbance readings at 260 nm. Despite the fact that those authors include a purification step in their extraction procedure, in which DNA is selectively precipitated with 2.5 M ammonium acetate, they stress that residual CTAB molecules are found in the samples which interfere with spectrophotometric DNA measurements. It is probable that these molecules absorb at 260 nm and together with small fragments of sheared genomic DNA result in an

overestimation of the yield of high molecular weight DNA in each sample and subsequent underloading on an agarose gel.

3.3.1.2 Effect of purification protocols on DNA yield determination

In order to accurately quantify DNA using spectrophotometric techniques, it was essential to develop an improved procedure to purify the DNA extract of CTAB, short nucleotides and other contaminating molecules. Three different purification protocols were compared, namely, DNA precipitation by ammonium acetate and ethanol as used in the previous experiment (Doyle and Doyle 1990b), spun-column purification (Maniatis *et al.*, 1982) and mini-dialysis (S. McRae, pers comm). To determine the efficacy of these procedures, DNA yields and A_{260}/A_{280} ratios of purified samples were compared to those obtained for a "crude" DNA extract, where DNA had simply been ethanol precipitated and not subjected to any further purification (Table 3.5). These apparent DNA yields, determined spectrophotometrically, were compared then to the yields observed after electrophoretic separation and staining of undigested DNA on an agarose gel (Figure 3.6A). Restrictability of each of the DNA extracts was determined also by digesting samples with an excess of the restriction enzyme Hinf I over a period of hours (Figure 3.6B).

In this set of experiments DNA samples were extracted and purified from both *E. grandis* and an *E. grandis* x *E. nitens* hybrid, once again to investigate the potential application of these purification protocols to a range of eucalypt materials.

Ammonium acetate-ethanol precipitation

Ammonium acetate treatment of DNA extracts has been shown to result in selective precipitation of RNA (Weising, 1991). However, where crude DNA was obtained using the Doyle and Doyle (1990b) extraction protocol, RNA was removed by RNAse A digestion. This was followed by dilution of the DNA extract and addition of ammonium acetate in conjunction with ethanol (Doyle and Doyle 1990b). It is probable that ammonium acetate added at this stage in the extraction procedure would have an alternative function, that is, to increase the amount of DNA which could be precipitated from the supernatant (Doyle and Doyle 1990b).

In this study, precipitation of *E. grandis* DNA in the presence of 96 % (v/v) ethanol

and 2.5 M ammonium acetate (treatment B) more than doubled apparent yields from 115 μg/g fresh mass for the crude extract which had simply been ethanol precipitated (treatment A) to 239.0 μg/g fresh mass (treatment B) (Table 3.5). However, this increase in DNA yield (treatment B, Table 3.5) was somewhat exaggerated, as shown by agarose gel electrophoresis of undigested crude (lane 2), and ammonium acetate precipitated samples (lane 3) (Figure 3.6A). Ethidium bromide staining of this gel revealed bands of similar intensity, suggesting that the yields of DNA obtained from these two treatments (A and B) were similar (lanes 2 and 3, Figure 3.6A). Although ammonium acetate precipitation was intended as a purification step, the apparent purity of *E. grandis* DNA obtained after use of this treatment decreased, probably insignificantly, from 1.71 (treatment A) to 1.68 (treatment B) (Table 3.5). This had no effect on the restrictability of the DNA, which could be digested to completion with Hinf I during a 2 h incubation period (lane 3, Figure 3.6B).

For the *E. grandis* x *E. nitens* hybrid, ammonium acetate-ethanol treatment resulted in an apparent 6 fold increase in DNA yield, from 86.7 μg/g fresh mass (treatment A) to 505.0 μg/g fresh mass (treatment B) (Table 3.5). This result was confirmed by agarose gel electrophoresis, where no DNA was visible for the crude extract (lane 6), but an intense band was detected after DNA had been precipitated with ammonium acetate (lane 5) (Figure 3.6A).

The apparent purity of the DNA sample extracted from the *E. grandis* x *E.nitens* hybrid increased after ammonium acetate-ethanol precipitation from 1.55 (treatment A) to 1.77 (treatment B) (Table 3.5). Both the purified (lane 5) and crude (lane 6) DNAs were accessible to the restriction enzyme Hinf I resulting in complete digestion, as shown by the smear of DNA fragments visualized after agarose gel electrophoresis of the samples (Figure 3.6A).

Spun column purification

Centrifugation of a sample through a spun column results in small molecules being retained within the beads of the column, while larger particles are eluted in the void volume (Maniatis *et al.*, 1982). Hence, Sephadex G50 spun-column purification of DNA was attempted with the expectation that short nucleotides and other small contaminants would be trapped in the mini-column allowing large genomic DNA strands to pass through in the void volume.

Spun column treatment of *E. grandis* DNA resulted in an apparent 80 % loss of DNA yield, with only 23 μg DNA/g fresh mass being obtained after purification using this procedure (treatment C), compared to 115.6 μg/g fresh mass extracted for the crude sample (treatment A) (Table 3.5). This reduction in yield was confirmed by agarose gel electrophoresis of the spun column purified sample, where no DNA could be detected after ethidium bromide staining (lane 4, Figure 3.6A).

The purity of *E. grandis* DNA appeared to decrease after spun column purification from 1.71 (treatment A) to 1.67 (treatment C) (Table 3.5). Furthermore, only partial DNA digestion occurred on incubation of an aliquot of the purified sample with Hinf I, as shown by the slightly diffuse appearance of the DNA band detected after agarose gel electrophoresis (lane 4, Figure 3.6B). DNA yields obtained for the *E. grandis* x *E. nitens* hybrid showed an apparent 75 % decrease from 86.7 μg/g fresh mass (treatment A) to 21.5 μg/g fresh mass after spun column purification (treatment C) (Table 3.5). An unexpectedly intense DNA band was detected after agarose gel electrophoresis of this purified DNA (lane 8), despite the apparently low yield (treatment C) (Table 3.5).

Spun column purification of *E. grandis* x *E. nitens* DNA increased the apparent purity of the sample from 1.55 (treatment A) to 1.73 (treatment C) (Table 3.5). However, this treatment did not render the DNA more accessible to the restriction enzyme Hinf I, rather it hindered digestion as shown by agarose gel electrophoresis of the sample (lane 8, Figure 3.6B). After a period of enzymic digestion only partial degradation of DNA had occurred, resulting in detection on the gel of a short smear of fragments below the main DNA band (lane 8, Figure 3.6B).

Dialysis

Mini-dialysis of DNA samples is a simple protocol which has proven effective in the purification of small volumes of crude DNA (S. McRae, pers comm). Aliquots of DNA are applied to the surface of membrane filters and these filters are floated in a buffer, allowing small contaminants to diffuse from the sample into the dialysis buffer. In this study, a modified mini-dialysis procedure was used in which samples of DNA were dialyzed against TE buffer for 2 h (S. McRae, pers comm).

Mini-dialysis increased the apparent yield of DNA obtained from the *E. grandis* x *E. nitens* hybrid from 86.7 μg/g fresh mass (treatment A) to 116.0 μg/g fresh mass (treatment D) (Table 3.5). This result was confirmed by agarose gel electrophoresis of DNA samples, where no band was visible for the crude extract (lane 6), but a diffuse band was observed after DNA had been subjected to dialysis (lane 7) (Figure 3.6A).

The apparent purity of the DNA extract obtained from the *E. grandis* x *E. nitens* hybrid improved after dialysis from 1.55 for the crude DNA (treatment A) to 1.76 (treatment D) (Table 3.5). The DNA extract was pure enough to allow complete digestion with the restriction enzyme Hinf I (lane 7, Figure 3.6B).

Table 3.5 Effect of purification procedures on the apparent yield and purity of DNA samples obtained from *Eucalyptus grandis* S/N M6 and a *Eucalyptus grandis* x *Eucalyptus nitens* hybrid NG 1026. DNA was extracted using the "CTAB" protocol (Doyle and Doyle 1990b) and the yields of crude DNA (A) compared to those obtained after purification by precipitation with 2.5 M ammonium acetate (B), spun-column filtration (C) and dialysis against TE (D).

Plant Source	Treatment	DNA yield (μg/g fresh mass)	DNA purity (A_{260}/A_{280})
Eucalyptus grandis	A	115.6	1.71
	B	239.0	1.68
	C	23.0	1.67
	D	-	-
E.grandis/ E. nitens	A	86.7	1.55
	B	505.0	1.77
	C	21.5	1.73
	D	116.0	1.76

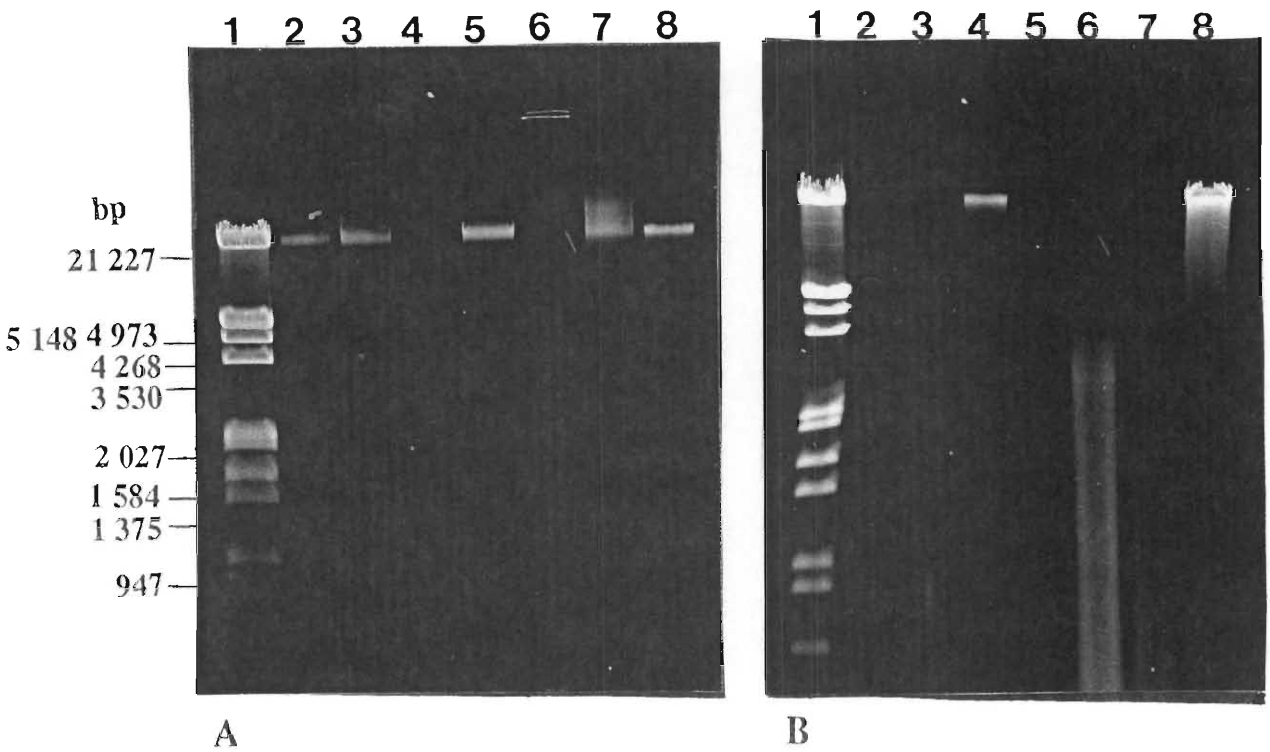


Figure 3.6 Agarose gel electrophoresis of DNA samples extracted using the "CTAB" procedure and purified in various ways. Total genomic DNA, undigested (A) and Hinf I digested (B), from *Eucalyptus grandis* S/N M6 (lanes 2-4) and a *Eucalyptus grandis* x *Eucalyptus nitens* hybrid NG 1026 (lanes 5-8) were purified by precipitation with 2.5 M ammonium acetate, or centrifugation through Sephadex G50 spun columns, or dialysis against TE buffer.

Lane 1: Lambda-EcoR I/ Hind III markers
Lane 2: *E. grandis* - crude
Lane 3: *E. grandis* - ammonium acetate precipitated
Lane 4: *E. grandis* - spun column filtered
Lane 5: *E.grandis* x *E.nitens* - ammonium acetate precipitated
Lane 6: *E.grandis* x *E.nitens* - crude
Lane 7: *E.grandis* x *E.nitens* - dialysed
Lane 8: *E.grandis* x *E.nitens* - spun column filtered

Conclusions regarding the purification techniques

Certain unexpected results were obtained during the course of this series of experiments. For example, the protocols used to "purify" *E. grandis* DNA apparently reduced the purity of certain DNA samples (treatment B and C) compared to the crude extract (treatment A) (Table 3.5). As this was noted only with *E. grandis* DNA samples, it is suggested that there is greater structural and/or biochemical variability within individual leaves of this species, compared to leaves of the *E. grandis* x *E. nitens* hybrid, which results in particularly inconsistent yields when crude DNA is extracted from different batches of leaf material. In fact, when crude *E. grandis* DNA was extracted using the Doyle and Doyle (1990b) protocol on two separate occasions during the course of these experiments, yields varied from 87.5 μg/g fresh mass (Table 3.4) to 239.0 μg/g fresh mass (treatment B, Table 3.5). Similarly DNA purity ranged from 1.86 (Table 3.4) to 1.68 (treatment B, Table 3.5). Therefore it is possible that the use of separately extracted crude *E. grandis* DNA samples in the three purification protocols had made it difficult to compare the efficacy of these procedures. Variable DNA yields may have resulted also from inconsistent handling of leaf material during the course of the extraction procedure.

Despite the shortcomings described above, trends were evident on examination of the results presented in Table 3.5 and those shown on the agarose gels, Figure 3.6A and Figure 3.6B. Ammonium acetate-ethanol treatment increased the yield of DNA which could be extracted from the *E. grandis* x *E. nitens* hybrid (treatment B, Table 3.5 (lane 5, Figure 3.6A). This DNA, and that precipitated for the *E. grandis* sapling, could be digested to completion with Hinf I during a 2 h incubation period (lanes 3 and 5, Figure 3.6B). However, spun column filtration of DNA samples reduced both the yield of DNA obtained from *E. grandis* leaf material (treatment C, Table 3.4) (lane 4, Figure 3.6A) and the restrictability of the DNA samples purified from both saplings (lanes 4 and 8, Figure 3.6B). Dialysis of *E. grandis* x *E. nitens* DNA against TE buffer, once again, produced a sufficiently pure sample to allow complete DNA cleavage with the Hinf I restriction enzyme (Lane 7, Figure 3.6B). On the basis of the findings described above, it was decided to abandon spun column purification, but combine the ammonium acetate and dialysis treatments for the purification of high molecular weight DNA to be used in all subsequent experiments.

After DNA samples had been purified using each of the purification protocols, it remained difficult to correlate spectrophotometric determinations of DNA yield and purity (Table 3.5) with results obtained after agarose gel electrophoresis of the purified fractions (Figure 3.6A). Therefore, it was decided that samples from all DNA extracts should be run on an agarose gel prior to their being restricted and blotted onto membranes. This would provide a more accurate measure of the quantity and quality of the DNA samples available for DNA fingerprinting.

3.3.2 PROBE PREPARATION AND LABELLING

Having refined a protocol for the extraction and purification of DNA from *Eucalyptus* saplings, the next step was the establishment and optimization of procedures for the preparation and labelling of the M13 derived probe encoded in pV47-2 (Figure 3.3).

3.3.2.1 Plasmid extraction, purification and confirmation of identity

To facilitate labelling of the plasmid probe, it was essential that a pure preparation of pV47-2 be obtained. After transformation of ampicillin-sensitive *E. coli* HB101 with this plasmid, selection of transformed bacteria and their growth in a nutrient medium containing ampicillin, pV47-2 was isolated using a large scale preparation procedure modified from Birnboim and Doly (1979) by Draper (1988). Crude plasmid extracts were purified by caesium chloride-ethidium bromide equilibrium density gradient centrifugation (Plate 3.1). Once the plasmid DNA had been drawn off from these gradients it was further purified by ethidium bromide removal and dialysis against TE buffer.

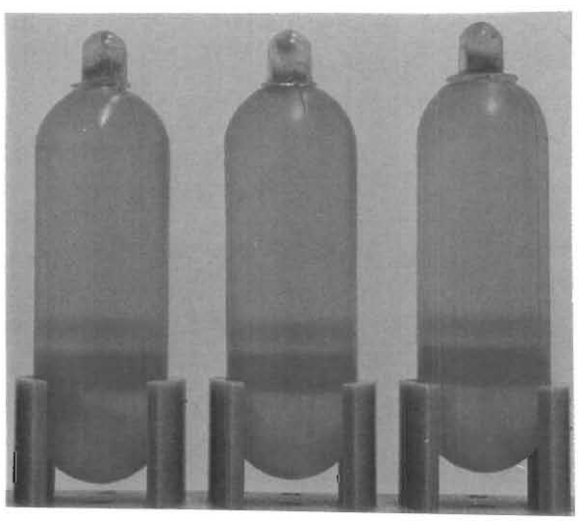


Plate 3.1 Banding of pV47-2 and *E.coli* chromosomal DNA on a caesium chloride-ethidium bromide density gradient following centrifugation at 100 000 rpm at 18 °C for 4 h.

The observed width of the pV47-2 band on the caesium chloride gradient (Plate 3.1) suggested that a high yield of plasmid DNA had been obtained. Spectrophotometric assessment of DNA revealed a good plasmid yield of 1.22 mg/ml. The plasmid DNA was also pure and free of contaminants, as shown by the A_{260}/A_{280} ratio of 1.88.

For visual confirmation of plasmid size and purity of the extract, an undigested sample was run on an agarose gel (lane 3, Figure 3.7). An aliquot of Hind III digested plasmid was run on the same gel to confirm its identity (lane 1, Figure 3.7).

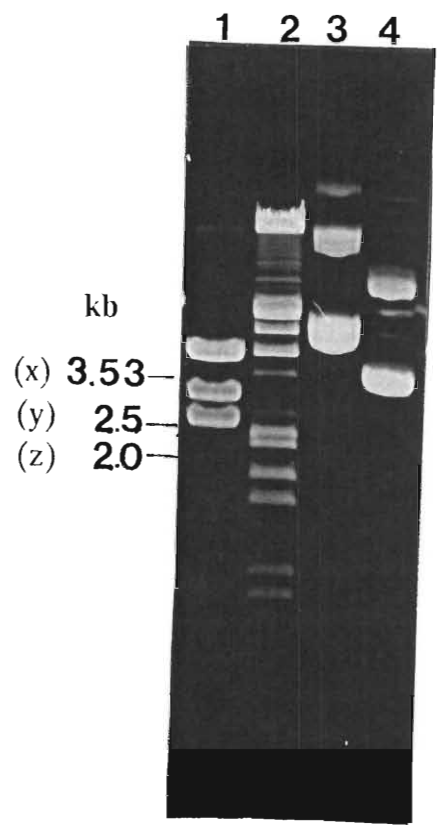


Figure 3.7 Agarose gel electrophoresis of the purified plasmid. Samples (0.5μg) of undigested (lane 3) and Hind III restricted plasmid (lane 1) were run against undigested pBR322 (Boehringer Mannheim, Germany) (lane 4) and Lambda EcoR I/Hind III markers (lane 2).

As can be seen from Figure 3.7, no chromosomal DNA or RNA contamination was present in the plasmid preparation. Three bands were detected after electrophoresis of the purified plasmid (lane 3, Figure 3.7), indicating that it contained three populations of plasmid molecules. It is probable that the bands visualized represent covalently closed circular plasmid (fastest moving), nicked circular plasmid and concatenated plasmid (slowest moving) (lane 3, Figure 3.7). These bands occurred closer to the well of the agarose gel than those visualized for unrestricted pBR322 (Boehringer Mannheim, Germany) (lane 4, Figure 3.7), demonstrating that the purified plasmid was of higher molecular weight than pBR322. The plasmid pV47-2 is larger, with a molecular weight of 6.5 kb (J.L. Longmire, pers comm) compared to 4.36 kb for pBR322 (Sutcliffe, 1979).

Hind III cleavage of plasmid DNA produced fragments of approximately 3.53 kb (x), 2.5 kb (y) and 2.0 kb (z) (lane 1, Figure 3.7). It is probable that two of these fragments (y and z), comprised the linearized 2.8 kb pUC-8 plasmid (the reason why two fragments were observed, as opposed to one, is unknown), while the third fragment (x) was the 3.5 kb tandem repeat insert (J.L. Longmire, pers comm) (Figure 3.3).

For final confirmation of the identity of the purified plasmid and to check that it contained the human repeat probe insert, a Hind III digested sample was separated by electrophoresis, along with a purified fraction of the insert (obtained from Dr. Moira van Staden along with the sample of the entire plasmid) (refer section 3.3.3). The position of the 3.5 kb fragment coincided precisely with that of the insert (results not shown).

It was concluded therefore, that the plasmid purified in this series of experiments was in fact pV47-2, and that it contained the tandem repeat insert to be hybridized to the eucalypt genomic DNA.

3.3.2.2 Labelling of pV47-2

P^{32}-labelling

Traditionally, ^{32}P has been incorporated into strands of probe DNA by "nick translation" (Rigby, 1977) or more recently, using the "random primer" labelling technique of Feinberg and Vogelstein (1983). The latter approach was used to label

Hind III restricted, denatured pV47-2. Once the labelling reaction had been terminated, the probe extract was purified of unincorporated nucleotides by centrifugation through Sephadex G50 spun columns (Maniatis *et al.*, 1982).

Liquid scintillation counting revealed that the purified probe DNA (100 ng) was labelled to a high specific activity (1.5×10^9 to 9.5×10^9 cpm/μg of probe).

DIG-labelling

Various techniques for non-radioactive probe labelling have been developed over the past several years, for example, incorporation of digoxygenin (DIG)-dUTP into probe DNA (Mühlegger *et al.*, 1988; Höltke *et al.*, 1992). DIG is an artificial hapten which is bound by a spacer arm to uridine nucleotides which in turn are incorporated into probes by random priming (Feinberg and Vogelstein, 1983). DIG particles are detected using polyclonal anti-DIG FAB fragments which are conjugated to alkaline phosphatase and may be visualized by enzymic reactions with colour or chemiluminescent substrates (Mühlegger *et al.*, 1988; Höltke *et al.*, 1992).

In this study, an NBT/BCIP substrate system was used (Boehringer Mannheim, Germany), which allowed detection of DIG-labelled DNA by the development of a purple-black precipitate on the blot membrane. This colour reaction was used as a visual measure of the efficiency of DIG-dUTP incorporation into pV47-2. After 200-300 ng of this plasmid had been incubated in the DIG labelling mixture and the resulting DIG-labelled pV47-2 purified by LiCl precipitation, a dilution series was blotted onto a nylon membrane alongside known concentrations of labelled control DNA (DIG kit, Boehringer Mannheim, Germany) (Figure 3.8). To check the efficiency of the labelling reaction as carried out in this laboratory, an aliquot of a second purified plasmid pBR328 (DIG Kit, Boehringer Mannheim, Germany) was labelled also and applied to the same membrane (Figure 3.8). Following the DIG detection reaction, the relative intensities of colour development of the experimentally labelled and control DNAs were compared (Figure 3.8).

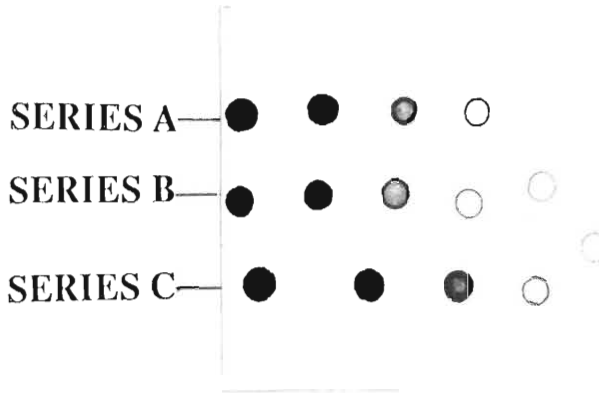


Figure 3.8 Dot blot of DIG labelled DNA. A dilution series (1 μl samples) of labelled control DNA (Boehringer Mannheim, Germany) (series A), DIG labelled pBR328 (series B) and DIG labelled pV47-2 (series C) were blotted onto a nylon membrane and the intensity of the colour precipitate obtained with the DIG detection reaction compared between samples.

As can be seen from the dot blot, DIG-dUTP was incorporated into both pBR328 (series B) and pV47-2 (series C) (Figure 3.8), indicating that the labelling reaction was carried out successfully. The intensity of colour development for DIG labelled pV47-2 (series C) appeared equivalent to that obtained for the labelled control DNA (series A) (Figure 3.8). As the concentration of DIG-labelled DNA in the control sample was known and this could be extrapolated to the pV47-2 sample, it was estimated that 0.13 ng of DIG labelled pV47-2 had been produced per hour, per 0.5 ng of DNA template originally added to the labelling mixture. However, this estimate of the efficiency of the DIG labelling procedure was based on subjective comparisons of blot intensities, unlike the precise measurement of label incorporation obtained after using ^{32}P.

3.3.3 OPTIMIZATION OF CONDITIONS FOR PROBE HYBRIDIZATION AND DETECTION

In order to generate eucalypt DNA fingerprints, labelled probe DNA needed to be hybridized to genomic DNA and the bound probe detected to reveal DNA polymorphisms.

3.3.3.1 Hybridization of probe to genomic DNA

Early attempts to hybridize ^{32}P-labelled probe to Hinf I restricted eucalypt genomic DNA, blotted onto nitrocellulose and nylon membranes, were carried out using a standard procedure described by Sambrook *et al.* (1989). However, genomic DNA RFLPs could not be detected as a result of background smearing and a low signal to noise ratio. Similar problems of background discolouration were encountered after probe DNA had been DIG-labelled and hybridized to eucalypt DNA using the DIG protocol. It is essential to include reagents in the hybridization buffer which block such non-specific binding of probe DNA to the membrane. The blocking agents used in the hybridization buffer of Sambrook *et al.* (1989), were Denhardt's reagent (Denhardt, 1966) and sheared denatured salmon sperm DNA, while in the DIG buffer a blocking reagent of unknown composition was used (DIG Kit, Boehringer Mannheim, Germany). However, it was suspected that these compounds were not suitable for use with the human tandem repeat region of pV47-2, used here as a probe. This region shows a certain homology with the repeat sequence from the M13 bacteriophage (Longmire *et al.*, 1990), which binds non-specifically to herring sperm DNA (Vassart *et al.*, 1987) and DNA extracted from various other sources (Ryskov *et al.*, 1988). Therefore, it was probable that the human repeat insert had bound the salmon sperm carrier DNA in the Sambrook *et al.* (1989) buffer, resulting in the discolouration of the membrane background observed in earlier experiments. As background smearing was observed also after use of the DIG hybridization protocol, it was suspected that the blocking reagent used in the DIG hybridization buffer contained some form of DNA, which was bound also by the pV47-2 DNA.

To test the hypotheses presented above and to establish a hybridization protocol which could be used successfully with the human pV47-2 probe, nitrocellulose and nylon membranes free of any bound DNA were incubated in various hybridization buffers in the presence of Hind-III restricted, DIG- labelled, pV47-2 (Figure 3.9). The solutions tested included: (1), DIG buffer (5 x SSC, 1 % (w/v) N-

lauroylsarcosine Na-salt, 0.1 % (w/v) SDS, 0.02 % (w/v) blocking reagent, pH 7.0) (DIG Kit, Boehringer Mannheim, Germany) (series A); (2), DIG buffer plus salmon sperm DNA (composition as given above, but with blocking reagent replaced with 0.1 mg/ml sheared denatured salmon sperm DNA) (series B); and (3), the buffer of Westneat *et al.* (1988) (0.263 M NA_2HPO_4, 1 % (w/v) BSA, 7 % (w/v) SDS, 1 mM EDTA, pH 8.0) (series C) (Figure 3.9). The buffer formulation of Westneat *et al.* (1988) was included as it has been used successfully for hybridisation of DNA from various sources to M13 based probes (Nybom and Rogstad, 1990; Nybom and Schaal, 1990a; 1990b; Nybom *et al.*, 1990; Nybom and Hall, 1991; Rogstad *et al.*, 1991).

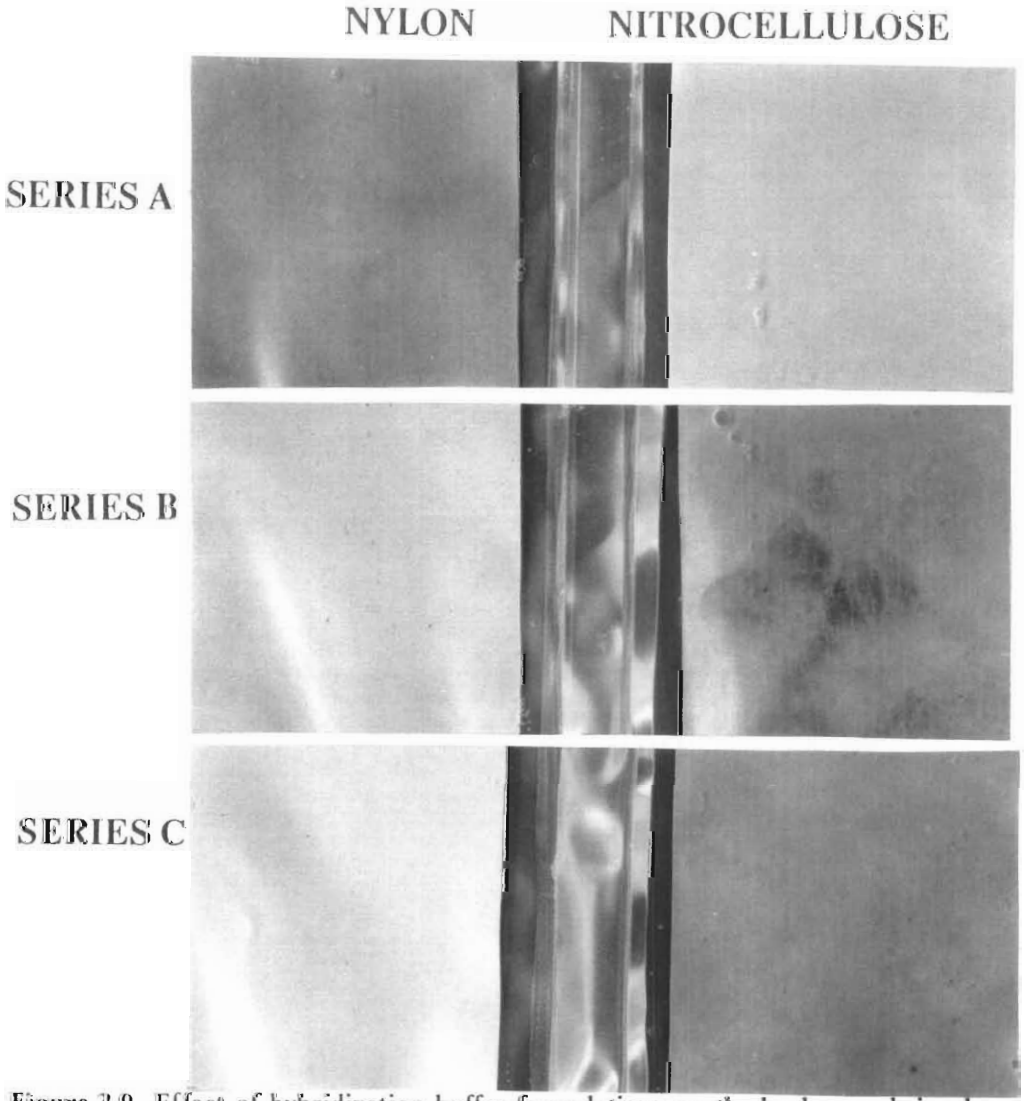


Figure 3.9 Effect of hybridization buffer formulations on the background signal obtained after simple incubation of membranes in DIG labelled pV47-2. Nitrocellulose and nylon membranes were incubated for 48 h at 65 °C in DIG buffer containing 1 % blocking reagent (Boehringer Mannheim, Germany) (series A), DIG buffer with 0.1 mg/ml sheared denatured salmon sperm (series B) and the buffer of Westneat *et al.* (1988) containing 7 % SDS and 1 % BSA (series C).

Incubation of a nitrocellulose membrane in the DIG hybridization solution containing 1 % blocking reagent (Boehringer Mannheim, Germany), resulted in a reasonably clear background although a slight yellow discolouration of the membrane was noted (series A, Figure 3.9). However, after a nylon membrane had been incubated in the same solution, it was covered with a brown precipitate (series A, Figure 3.9).

When nitrocellulose was incubated in the basic DIG buffer in which the blocking reagent had been replaced with 0.1 mg/ml sheared denatured salmon sperm, blotches of red-brown precipitate were observed on the membrane (series B, Figure 3.9). A light coating of this precipitate was evident also on a nylon membrane after it had been incubated in the same hybridization solution (series B, Figure 3.9).

Incubation of nitrocellulose in the hybridization buffer of Westneat *et al.* (1988) resulted in the formation of a dark brown precipitate on the membrane (series C, Figure 3.9). However, when a nylon membrane was incubated in this buffer the background was absolutely clear, there was no evidence of any discolouration (series C, Figure 3.9).

Results shown in Figure 3.8 thus confirmed suspicions that the labelled pV47-2 probe fragments had bound both the DIG blocking reagent (series A) and the sheared denatured salmon sperm (series B), resulting in discolouration of both the nitrocellulose and nylon membranes to a lesser or greater degree. Therefore, the hybridization buffers containing these blocking reagents could not be used in subsequent experiments. However, non-specific binding of probe DNA to a nylon membrane was prevented when it was incubated in the hybridization buffer of Westneat *et al.* (1988) (series C, Figure 3.9). Since this buffer was formulated specifically for use with M13 based probes it contains no carrier DNA, but instead includes SDS and BSA as blocking reagents (Westneat *et al.*, 1988). These compounds evidently attach to the nylon membrane preventing the labelled probe from binding and resulting in a clear background (series C, Figure 3.9). Therefore, it was decided that eucalypt DNA would be blotted onto nylon membranes in all subsequent experiments and that labelled probe would be hybridized to these membranes using the protocol of Westneat (1988).

From Figure 3.9 it is evident that the extent and nature of background discolouration observed on nitrocellulose and nylon membranes differed after these had been incubated in the same hybridization solution. This was expected as these membranes bind compounds, such as DNA, by different mechanisms, namely, by hydrophobic interactions on the nitrocellulose and/or covalent bonding on the nylon (Sambrook *et al.*, 1989). Therefore, nitrocellulose and nylon membranes would have interacted differently with the DIG blocking reagent, salmon sperm DNA, BSA, SDS and labelled probe DNA, accounting for the variable background discolouration observed (Figure 3.9). However, the exact nature of these interactions is not known.

3.3.3.2 Detection of bound probe

Two DNA detection systems, namely, the ^{32}P and DIG systems, were compared to determine which would best resolve eucalypt DNA fingerprints. For test blots, samples of calf thymus and *E. grandis* S/N M6 DNA were fingerprinted. Calf thymus mammalian DNA was selected for comparison to the eucalypt plant DNA as these DNAs would be vastly different allowing the detection of restriction fragment polymorphisms.

Aliquots of calf thymus and *E. grandis* DNA were digested with Dra I after which 10 and 15 μg samples of each were run in duplicate on an agarose gel before being blotted onto a nylon membrane. One half of the blot was hybridized to ^{32}P labelled probe using the Westneat protocol (Westneat *et al.*, 1988) and the bound probe detected by autoradiography (Figure 3.10). The other half was hybridized to DIG labelled probe using the same procedure, but bands were visualized by the formation of an insoluble, precipitate on the blot membrane (Figure 3.11).

DNA fragments visualised using both the detection systems ranged in size from 21 to 0.83 kb, with a clustering of bands between approximately 5 and 1.38 kB (Figure 3.10 and Figure 3.11). These bands were visible in lanes where 10 μg of genomic DNA had been loaded (lanes 1 and 3) as in lanes which contained 15 μg of DNA (lanes 2 and 4) (Figure 3.10 and 3.11).

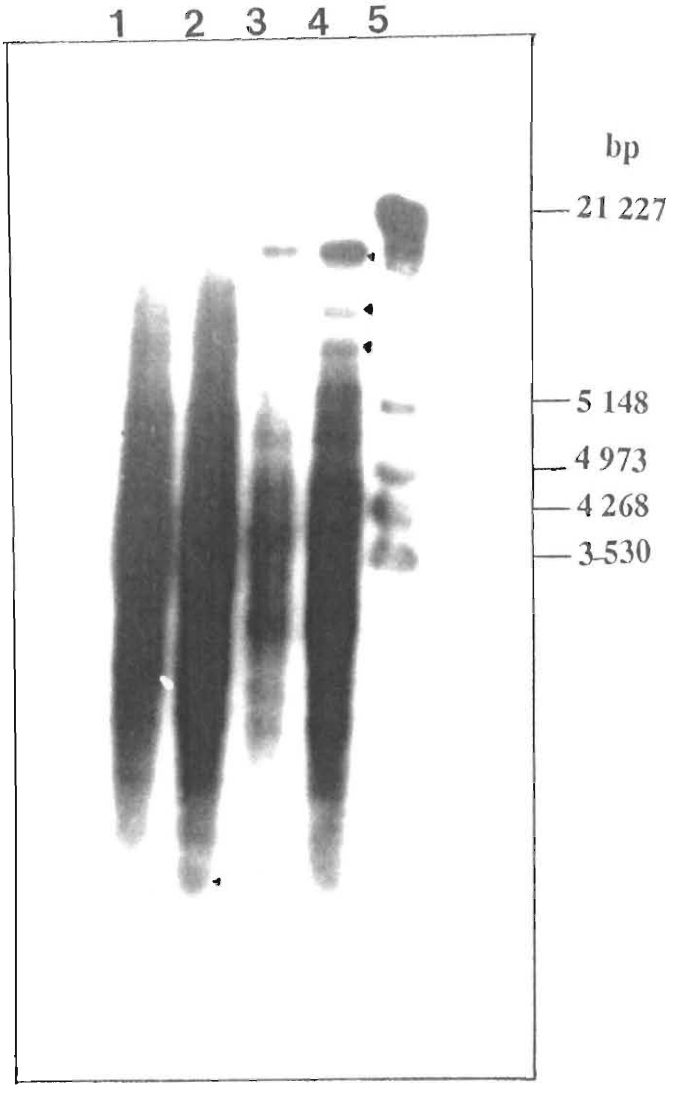


Figure 3.10 Autoradiograph of calf thymus and *Eucalyptus grandis* S/N M6 DNA fragments hybridized to ^{32}P labelled probe. Calf thymus and eucalypt DNA samples were Dra I digested and subjected to electrophoresis (25 V, 18 h) before being blotted onto a nylon membrane. After hybridization of 10 μg calf thymus DNA (lane 1), 15 μg calf thymus DNA (lane 2), 10 μg eucalypt DNA (lane 3), 15 μg eucalypt DNA (lane 4) and Lambda Eco RI/Hind III markers (lane 5), to Hind III restricted, ^{32}P labelled pV47-2, bands were detected on an autoradiograph. Autoradiographs were developed at -80 ° C for 16 h in the presence of intensifying screens. Differences between the fragment profiles of calf thymus and eucalypt DNA are indicated by the arrows.

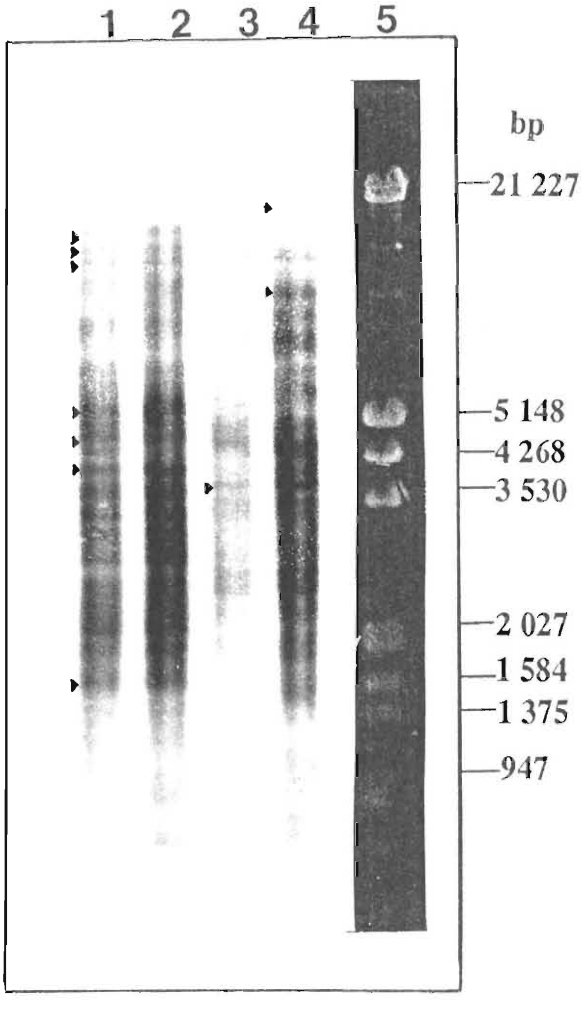


Figure 3.11 DIG blot of calf thymus and *Eucalyptus grandis* S/N M6 DNA fragments hybridized to DIG labelled probe. Calf thymus and eucalypt DNA samples were Dra I digested and subjected to electrophoresis (25 V, 18 h) before being blotted onto a nylon membrane. After hybridization of 10 μg calf thymus DNA (lane 1), 15 μg calf thymus DNA (lane 2), 10 μg eucalypt DNA (lane 3), 15 μg eucalypt DNA (lane 4) and Lambda Eco RI/Hind III markers (lane 5), to Hind III digested, DIG labelled pV47-2, bands were visualized using the DIG detection reaction. This was carried out in the dark at room temperature for 16 h. Differences between the fragment profiles of calf thymus and eucalypt DNA are indicated by the arrows.

Although Lambda DNA molecular weight markers were present on the membranes hybridised to both DIG- and ^{32}P-labelled probe, Lambda fragments were detected only on the autoradiograph (Figure 3.10). An additional high molecular weight eucalypt DNA band (21 kb) was visualized also using ^{32}P (lanes 3 and 4, Figure 3.10), which was absent on the DIG blot (lanes 3 and 4, Figure 3.11). However, these clearly distinguishable bands were the exception, with the most of the eucalypt (lanes 3 and 4) and calf thymus DNA (lanes 1 and 2) appearing as a smear after development of the autoradiograph (Figure 3.10). This inability to resolve individual bands made it difficult to identify differences in the calf thymus and eucalypt DNA restriction fragment profiles (Figure 3.10). However, using the DIG protocol, it was possible to differentiate bands (Figure 3.11). As can be seen from Figure 3.11, immediate differences were evident in the molecular weights and number of fragments detected for calf thymus (lanes 1 and 2) and eucalypt DNA (lanes 3 and 4) (Figure 3.11).

Detection of labelled probe, bound to genomic DNA fragments on the membrane, using the ^{32}P and DIG systems, occurs by very different mechanisms. For example, during the development of an autoradiograph emissions from the ^{32}P-labelled probe result in the appearance of diffuse black bands on the X-Ray film (Stryer, 1981), whereas DIG-labelled probe is usually detected by a colourimetric reaction which results in the formation of a purple-brown precipitate on the blot membrane (Boehringer Mannheim, Germany). In this particular case, the bands on the autoradiograph (Figure 3.10) have merged to produce a smeared signal, while distinct bands are discernable on the DIG blot (Figure 3.11). This difference in results may be attributed to the nature of the detection systems, as described above. The bands produced on the autoradiograph are diffuse and if closely spaced, these bands may have merged to produce the smear seen in Figure 3.10. However, this smearing and the fact that additional bands were detected on the autoradiograph (Figure 3.10) which were not present on the DIG blot (Figure 3.11) suggest also that the ^{32}P system is more sensitive than the DIG system with a greater number of fragments being detected.

In conclusion, the DIG protocol revealed the greater number of distinct fragment bands (Figure 3.10), whereas a smear of DNA was visualized on the ^{32}P autoradiograph (Figure 3.11). However, as it was suspected that the ^{32}P system was more sensitive, it was decided to use both protocols for fingerprinting. As bands could be detected in both cases with the lower DNA load of 10 μg per well, this amount of sample was used in all subsequent experiments.

3.3.4 USE OF THE DEVELOPED PROCEDURES FOR THE GENERATION OF EUCALYPT DNA FINGERPRINTS

Having established procedures for eucalypt DNA extraction, restriction and blotting onto nylon membranes, followed by probe hybridization and detection, the next step was the generation of fingerprints for various eucalypt species and cultivars.

Samples of Dra I digested DNA (10 μg) from *Eucalyptus grandis* S/N M6, *E. grandis* cultivars TAG 5 and TAG 14 and *E. macarthurii* were blotted onto nylon membranes. One of these membranes was hybridized to ^{32}P-labelled probe and DNA fragments detected by autoradiography (Figure 3.12), while the other was incubated with DIG labelled probe and the eucalypt fragments visualized by the formation of a colour precipitate on the membrane (Figure 3.13).

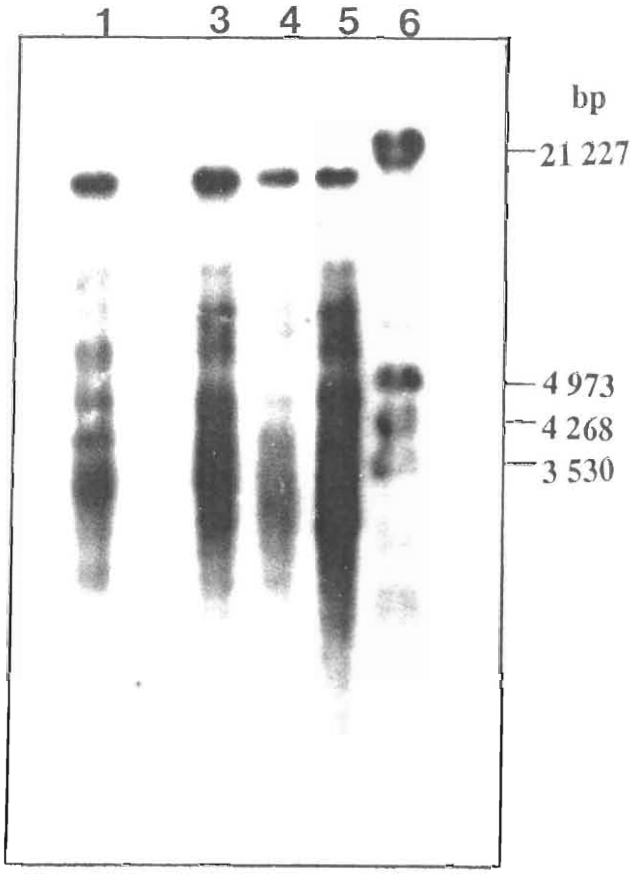


Figure 3.12 Autoradiograph of eucalypt DNA fingerprints. DNA extracted from various eucalypt species and cultivars was Dra I digested, samples (10 μg) subjected to electrophoresis (24 V, 18 h) and fragments blotted onto a nylon membrane. Following hybridization of ^{32}P-labelled pV47-2 fragments to DNA from *Eucalyptus macarthurii* (lane 1), the *Eucalyptus grandis* cultivar TAG 14 (lane 3), the *Eucalyptus grandis* cultivar TAG 5 (lane 4), *Eucalyptus grandis* S/N M6 (lane 5) and Lambda Eco RI/Hind III markers (lane 6), bands were detected by autoradiography at -80 ° C for 16 h in the presence of intensifying screens. The mean number of DNA fragments detected per sample was 8.5.

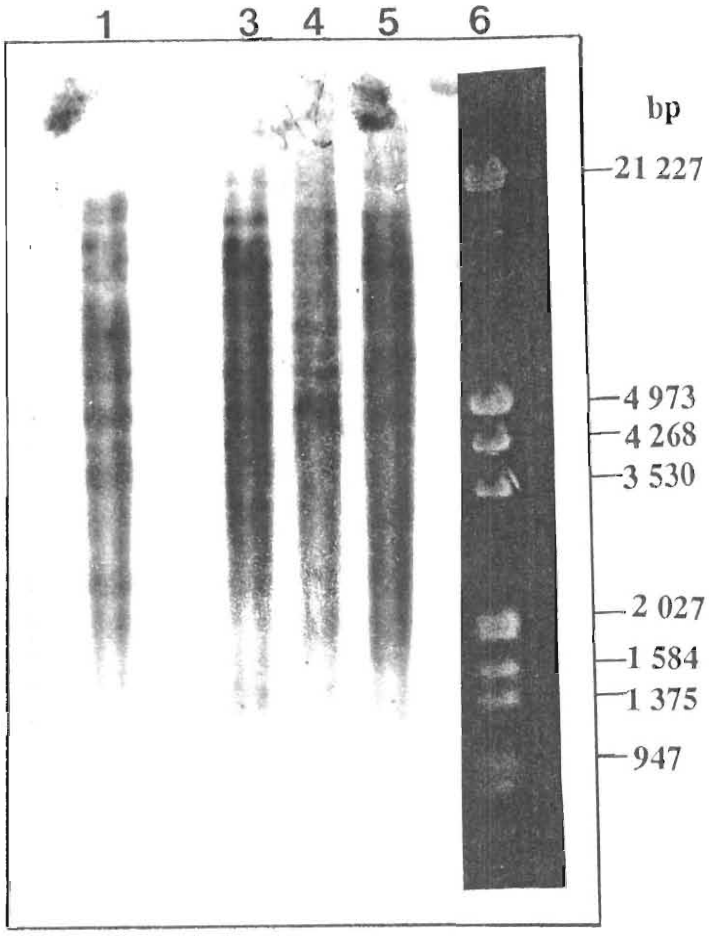


Figure 3.13 DIG blot of eucalypt DNA fingerprints. DNA extracted from various eucalypt species and cultivars was Dra I digested, samples (10 μg) subjected to electrophoresis (24 V, 18 h) and fragments blotted onto a nylon membrane. Following hybridization of DIG-labelled pV47-2 fragments to DNA from *Eucalyptus macarthurii* (lane 1), the *Eucalyptus grandis* cultivar TAG 14 (lane 3), the *Eucalyptus grandis* cultivar TAG 5 (lane 4), *Eucalyptus grandis* S/N M6 (lane 5) and Lambda Eco RI/Hind III markers (lane 6), bands were visualized by the DIG detection reaction which was carried out in the dark at room temperature for 16. The mean number of fragments detected per sample was 5.0.

3.3.4.1 Characterization of eucalypt species and cultivars

To compare the DNA fingerprints of the various eucalypt species and cultivars, the autoradiograph (Figure 3.12) and DIG blot (Figure 3.13) were analyzed according to a procedure described by Jeffreys *et al.* (1985a). Discernible DNA bands, occurring at a fixed distance from the origin (the well of the agarose gel which was marked on the nylon membrane) were assigned a number and scored for presence in the other eucalypt samples (Table 3.6).

Table 3.6 Scoring of bands detected on autoradiograph (Figure 3.12) and DIG blot (Figure 3.13) after hybridization of labelled probe to Dra I digested eucalypt DNA samples. Individuals possessing a particular fragment are indicated with a "+".

Probe label	Band number	Plant material			
		E.macarthurii	*E.grandis* TAG 14	*E.grandis* TAG 5	*E.grandis* S/N M6
^{32}P	1	+	+	+	+
	2		+	+	+
	3	+	+	+	+
	4		+	+	+
	5	+	+		+
	6	+	+	+	+
	7	+			
	8	+	+	+	+
	9		+	+	+
	10	+	+	+	
	11	+	+		
DIG	1	+	+	+	+
	2	+	+		
	3	+	+	+	+
	5	+	+	+	+
	6	+	+	+	+
	7	+			
	8	+			

DNA fingerprints produced using the ^{32}P detection system were species and cultivar-specific, that is, neither the two eucalypt species (*E. grandis* and *E. macarthurii*) (lane 1 and lane 5) nor the *E. grandis* cultivars sampled (TAG 14, TAG 5 and S/N M6) (lane 3, lane 4 and lane 5) had identical restriction fragments

(Figure 3.12) (Table 3.6). However, fingerprints resolved on the DIG blot (Figure 3.13) showed the banding patterns of *E. grandis* S/N M6 (lane 5) and that of a second cultivar of this species, TAG 5 (lane 4), to be identical implying that the two individual trees, from which DNA was sampled for these fingerprints, belong to the same cultivar. In addition, the restriction fragment profiles of these two saplings were almost indistinguishable from that of the third *E. grandis* cultivar, TAG 14 (lane 3, Figure 3.13), apart from one additional band scored for TAG 14 (Table 3.6).

To determine whether similarities and differences noted in the DNA fingerprints could be used as a measure of the genetic relationship between eucalypt saplings, the bands from each sample, observed on the autoradiograph (Figure 3.12) (Table 3.6) and the DIG blot (Figure 3.13) (Table 3.6), were compared using the similarity index "D" described by Wetton *et al.* (1987) (Table 3.7). Here the probability of band sharing between individuals A and B, that is, D_{AB} was equal to $2N_{AB}/N_A + N_B$, where N_{AB} was the number of fragments shared by A and B and N_A and N_B the total number of bands detected in individuals A and B respectively; "D" values may range from 0 when there are no bands in common to 1 when fragment patterns are identical (Wetton *et al.* 1987).

Table 3.7 "D" values calculated from the autoradiograph (Figure 3.12) (Table 3.5) and DIG blot (Figure 3.13) (Table 3.5) for all possible pairwise comparisons between eucalypt individuals.

Individuals compared	"D" value obtained ^{32}P	D I G
E. macarthurii/*E. grandis* S/N M6	0.500	0.727
E. macarthurii/*E. grandis* TAG 5	0.625	0.727
E. macarthurii/*E. grandis* TAG 14	0.778	0.833
E. grandis S/N M6/*E. grandis* TAG 5	0.875	1.000
E. grandis S/N M6/*E. grandis* TAG 14	0.889	0.889
E. grandis TAG 5/*E. grandis* TAG 14	0.889	0.889

Of the "D" values calculated from the autoradiograph (Figure 3.12) the lowest (0.500) was obtained when comparing the taxonomically distant *Eucalyptus* species of *E. macarthurii* and *E. grandis* S/N M6 (Table 3.7). However, "D" values increased to 0.625 and 0.778 when *E. macarthurii* was compared to the other *E. grandis* cultivars TAG 5 and TAG 14 respectively (Table 3.7). "D" values increased even further when pairwise comparisons were carried out between *E. grandis* S/N M6/*E.*

grandis TAG 5, *E. grandis* S/N M6/*E. grandis* TAG 14 and *E. grandis* TAG 5/*E. grandis* TAG 14, ranging from 0.875 to 0.889 (Table 3.7). These high "D" values were expected as these are all cultivars of *E. grandis* and, therefore, genetically similar to one another.

"D" values obtained from the DIG blot (Figure 3.13) revealed similar trends to those observed on the autoradiograph (Figure 3.11). As was expected, comparison of the *Eucalyptus* species *E. macarthurii* and *E. grandis* S/N M6 (Figure 3.13) once again gave the lowest "D" value (0.727) (Table 3.7). It is interesting to note that this value was the same as that obtained when *E. macarthurii* was compared to the *E. grandis* cultivar TAG 5 (Table 3.7). In addition, when the fingerprints of the *E. grandis* S/N M6 and the TAG 5 saplings were compared directly, a "D" value of 1.000 was obtained which according to Wetton *et al.* (1987) indicates that these trees belong to the same cultivar, although it was not possible to determine conclusively whether the trees were both *E. grandis* S/N M6 or *E. grandis* TAG 5. This suspicion was further supported when the *E. grandis* S/N M6 and *E. grandis* TAG 5 cultivars were compared seperately to the third *E. grandis* cultivar TAG 14, with both comparisons yielding a "D" value of 0.889 (Table 3.7).

In order to determine the probability that two species or cultivars would exhibit identical fingerprints, the average "D" value for the species/cultivars being compared was raised to the mean number of fragments recorded for those species/cultivars (Jeffreys *et al.*, 1985b). For example, the probablity that cultivars A, B and C would have identical fingerprints would be equal to D_{ABC} (mean number of fragments for A,B and C), where D_{ABC} is the average D value calculated for all the pairwise comparisons between these three cultivars (Jeffreys *et al.*, 1985b).

Table 3.8 Calulated probabilities that the DNA fingerprints observed on the autoradiograph (Figure 3.11) and the DIG blot (Figure 3.12) (Table 3.6) would be identical for the eucalypt species *E. macarthurii* and *E.grandis* (all cultivars) and the *E. grandis* cultivars TAG 14, TAG 5 and S/N M6.

Material compared	Probability	
	^{32}P	DIG
Eucalyptus species	3.9×10^{-3}	1.73×10^{-1}
Eucalyptus cultivars	3.43×10^{-1}	7.17×10^{-1}

The calculated probability of band sharing between the various eucalypt species was approximately 100 fold less when fragments were resolved using the ^{32}P labelling and detection system (Figure 3.12) compared to the DIG protocol (Figure 3.13) (Table 3.8). This difference reflects the enhanced sensitivity of the ^{32}P system.

The probabilities that the fingerprints of the eucalypt cultivars would be identical was very much increased when compared to the probability values calculated for the species (Table 3.8). This trend was evident for both the ^{32}P and DIG systems (Table 3.8).

In conclusion, preliminary results suggest that genomic DNA fingerprints may be used to identify both *Eucalyptus* species and cultivars (Figure 3.12, Figure 3.13, Table 3.6 and Table 3.8). In addition, simple "D" values calculated from such fingerprints may provide an idea of the level of genetic relatedness within the eucalypts (Table 3.7).

3.4 DISCUSSION

In this study, a successful methodology was developed for the production of DNA fingerprints for *Eucalyptus* species and cultivars. Restriction fragment patterns were resolved for *Eucalyptus macarthurii* and the *Eucalyptus grandis* cultivars TAG 14, TAG 5 and S/N M6, after Dra I digests of genomic DNA were probed with the multi-locus probe pV47-2 of Longmire *et al* (1990) (Figure 3.12 and Figure 3.13). Fingerprints were produced using both the ^{32}P (Figure 3.12) and DIG-labelling and detection systems (Figure 3.13).

3.4.1 APPLICATIONS OF THE DEVELOPED FINGERPRINTING PROCEDURE

3.4.1.1 Identification of eucalypt cultivars

An important pre-requisite for any commercial plant breeding programme is an understanding of the available genetic base from which improved varieties or cultivars may be selected or generated (Denison and Quaile, 1987). In certain breeding programmes, as has occurred with *Eucalyptus*, the accumulation of such information is particularly difficult as problems are encountered in the identification of cultivars currently grown commercially (B. Herman, pers comm).

Traditionally, plant cultivars have been classified or identified on the basis of morphological characteristics (refer section 1.3.1). However, as mentioned previously, these phenotypic traits are often unreliable and result in confusion between the persons "growing, breeding, marketing" and generally wanting to identify these plants. Similar difficulties have been encountered in the breeding of eucalypts, where cultivars of economically important species such as *Eucalyptus grandis* are often phenotypically indistinguishable from one another.

In the present study, the technique of DNA fingerprinting was investigated as an alternative method for distinguishing eucalypts from one another. The decision was made to develop a fingerprinting methodology as this procedure has facilitated the identification of a variety of agronomically important woody species and cultivars from the *Malus* (apple), *Rubus* (blackberry and raspberry) and *Prunus* (black cherry) genera (Nybom *et al.*, 1989; Nybom, 1990a; 1990b; Nybom *et al.*, 1990; Nybom and

Schaal, 1990a; 1990b; Nybom and Hall, 1991; Parent and Page, 1992), and *Vitis* (grape) genus (Bowers *et al.*, 1993).

Comparison of the fingerprint profiles of the *Eucalyptus* species *E. macarthurii* and *E. grandis* revealed species-specific banding patterns when the DNA blots were probed with both ^{32}P and DIG-labelled probe (Figure 3.12 and Figure 3.13). However, the average "D" value calculated from the pairwise comparison between these species (Table 3.7), when raised to the mean number of fragments resolved for the species (refer section 3.3.4.1), yielded probability values of 3.9×10^{-3} for the autoradiograph (Figure 3.12) and 1.73×10^{-1} for the DIG blot (Figure 3.13) (Table 3.8). This rating was devised by Jeffreys *et al.* (1985b) and according to those authors, values such as those obtained for the eucalypt species (Table 3.8) indicate a high probability that *E. macarthurii* and *E. grandis* would exhibit identical DNA fingerprints. When this calculation was carried out between the three *E. grandis* cultivars, the probability of complete band sharing increased even further to 3.43×10^{-1} and 7.17×10^{-1} for the autoradiograph and DIG blot respectively (Figure 3.12 and Figure 3.13) (Table 3.8). This result was not unexpected, as few fragment differences were detected between the fingerprints of these cultivars generated with the ^{32}P system (Figure 3.12) (Table 3.7), and no polymorphisms were noted between the *E. grandis* cultivars of TAG 5 and S/N M6 using the DIG protocol (Figure 3.13) (Table 3.7).

The probability of observing complete concordance in the banding patterns of the eucalypt cultivars (Table 3.8) are orders of magnitude greater than the 3×10^{-11} calculated after fingerprinting random samples of human DNA (Jeffreys *et al.*, 1985b), or values of 3.166×10^{-4} found in quaking aspen (Rogstad *et al.*, 1991), or ratings of 4.34×10^{-4} calculated in raspberry cultivars (Nybom and Hall, 1991). This apparent lack of significant polymorphism between the eucalypt cultivars may have resulted from the high incidence of band smearing observed on the autoradiograph (Figure 3.12) and, to a certain extent, on the DIG blot (Figure 3.13). This smearing made it extremely difficult to identify polymorphic fragments clearly and resulted in a low mean number of bands being scored for individual samples, namely an average of 8.5 bands from the ^{32}P autoradiograph (Figure 3.12) and 5.0 on the DIG blot (Figure 3.13), compared to the 11.4 fragments scorable for certain *Rubus* cultivars (Nybom *et al.*, 1990). An alternative, although less likely, explanation for the high probabilities values shown in Table 3.8 could be that the eucalypt saplings were mis-identified by the forestry breeders themselves, prior to these plants being

sampled for DNA extraction. As mentioned previously, eucalypts are difficult to identify on the basis of morphological characteristics and it is possible that the saplings labelled *E. grandis* TAG 5 and *E. grandis* S/N M6 belonged to the same cultivar, although this would be impossible to establish unequivocally.

In this study, preliminary investigations into the use of DNA fingerprints as a tool for identifying eucalypt cultivars have shown that polymorphisms may be observed using both the ^{32}P and DIG systems (Figure 3.12 and Figure 3.13). However, the extent of these polymorphisms and their degree of usefulness to the eucalypt forestry industry would need to be established more fully. Therefore, it is suggested that the next step in this series of investigations would be to fingerprint at least three replicate saplings belonging to each of the *E. grandis* cultivars. The restriction fragment profiles resolved from these samples would indicate whether the eucalypt fingerprints are reproducible within a cultivar or whether they are individual-specific. If this were the case it might be necessary to bulk the DNA extracted from three or more replicate saplings before DNA restriction and blotting onto a membrane (Arnheim *et al.*, 1985; Giovannoni *et al.*, 1991). This would ensure that the resulting DNA fingerprints would give a more accurate reflection of inter-cultivar differences.

3.4.1.2 Determination of the degree of relatedness and genetic variation in eucalypt populations

In this series of investigations, genetic relatedness between eucalypt species and cultivars was calculated using the "D" value developed by Wetton *et al.* (1987) (refer section 3.3.4.1) (Table 3.7). "D" values were lowest when the fingerprints of the eucalypt species *E. macarthurii* and *E. grandis* were compared (0.500 and 0.727) and increased when comparisons were made between the *E. grandis* cultivars (0.875 to 1.000) (Table 3.7). At this point it is important to note that these figures were calculated from the fingerprints resolved on the autoradiograph (Figure 3.12) and DIG blot (Figure 3.13), respectively, and although the values were not identical, similar trends were revealed using both these detection systems. The range in calculated "D" values, from low (inter-specific comparisons) to high (intra-specific) was expected, as Wetton *et al.* (1987) state that "D" values may range from 0 when there is no band sharing between individuals, that is, when they are genetically unrelated, to 1, when two individuals are genetically identical to one another and have all their DNA bands in common. Therefore, it is clear that these "D" values

may give an idea of the level genetic relatedness between the eucalypts, as has been achieved with various *Rubus* (Nybom and Schaal 1990b; Nybom *et al.*, 1990), *Malus* (Nybom *et al.*, 1990), *Prunus* (Nybom *et al.*, 1990) and *Acer* (box elder) (Nybom and Rogstad, 1990) cultivars. However, the degree of informativeness of this measure is limited as several factors may interfere with the calculation of "D" values from DNA fingerprints, such as: comigration of non-allelic markers; linkage disequilibria between loci; and an inability to detect and score DNA bands in the lower molecular weight range (Lynch, 1988). In addition, these values are determined by the restriction enzymes used to digest DNA prior to its being blotted onto a membrane, as well as the minisatellite probe used to resolve the fingerprints (Weising *et al.*, 1989; Nybom *et al.*, 1990). Therefore, "D" values may be used a measure of genetic relatedness only within a particular series of experiments, with comparisons across different studies yielding only tentative results (Nybom, 1990b). On the basis of these observations it is suggested that the calculation of "D" values from eucalypt fingerprints may or may not prove to be of value as part of a routine screening programme.

3.4.1.3 Further applications of the eucalypt fingerprinting technology

Apart from the applications of the developed methodology described thus far (refer sections 3.4.1.1 and 3.4.1.2), it is probable that the eucalypt fingerprinting procedure will have further uses in future eucalypt breeding and research programmes. For example, the fingerprinting methodology might prove useful as a tool for determining the paternity of promising eucalypt cultivars. This has been achieved in the *Malus* genus, where parental seed of certain apple cultivars has been identified using DNA fingerprints (Nybom and Schaal, 1990a). Similarly, in a eucalypt breeding program, DNA fingerprints could be used to point out promising parental stock to be used in conventional breeding crosses which may the lead to the selection of improved eucalypt cultivars.

The DNA fingerprinting technique might also have potential application in other aspects of eucalypt research. For example, this technology may provide a means of resolving the controversy which rages regarding the phylogenetic origins of a number of eucalypt species. Authors have had diverging opinions regarding the evolutionary history of these species, with certain groups suggesting that they have a monophyletic origin and others, a polyphyletic origin (Pryor and Johnson, 1971). Fingerprinting data could provide a means of answering such biosystematic

questions. DNA fingerprinting procedures could be applied also in the development of conservation strategies for the maintenance of a broad genetic base within the eucalypts. This would be important, as concern exists over the level of genetic variation within individual eucalypt species and the distribution of genome diversity within and amongst eucalypt populations (Pryor and Johnson, 1971).

One of the most important applications of the RFLP technology would be in the mapping of the eucalypt genome. Certain eucalypt traits have been identified as having high priority, such as the requirement for frost tolerance (Raymond *et al.*, 1992) and an ability to survive in arid environments (Aradhya and Phillips, 1993). It is probable that a eucalypt RFLP map would allow identification of genetic markers linked to such important phenotypic characteristics.

3.4.2 THE FEASIBILITY OF INCORPORATING THE DEVELOPED PROTOCOL INTO A ROUTINE SCREENING PROCEDURE FOR EUCALYPTS

Before any diagnostic procedure can be incorporated into a program for the routine screening of plant materials, it is important that an evaluation be carried out of the "costs" involved of including such a step. This would include assessment of the capital input needed, as well as consideration of additional "costs" in terms of the time and labour required for the running of the procedure.

3.4.2.1 Suitability of the DNA extraction protocol

A technique for DNA extraction, developed by Doyle and Doyle (1990b), was optimized for use with the eucalypts. This included the development of a process to purify the DNA extract of unwanted contaminants and short oligonucleotide sequences by ammonium acetate-ethanol precipitation (Weising *et al.*, 1991), followed by mini-dialysis of samples (S. McRae, pers comm) (Table 3.5 and Figure 3.6A). These procedures, used in tandem, produced DNA yields ranging from 96.25 to 1116.5 μg/ g fresh mass (results not shown). Such yields compare favourably to values of 100 to 300 μg/g fresh mass obtained by Kvarnheden and Engström (1991) who extracted DNA from woody species of Norway spruce. In addition, sufficient DNA for RFLP fingerprinting was extracted from small amounts of tissue (0.5-1.0 g) which is important, as only limited amounts of leaf material could be sampled from young eucalypt saplings.

Additional advantages of the developed DNA extraction and purification technique were the speed with which it could be carried out, the economy of the procedure and its relative simplicity. A crude DNA pellet was obtained approximately 24 hours after grinding eucalypt leaf material and final purification of this DNA was achieved after a further 24 hours (refer section 3.3.4.2). Numerous samples could be processed simultaneously and it is estimated that this would allow DNA to be extracted from at least 20 batches of leaf material over a two day period. Such large scale DNA extraction would be particularly advantageous in a commercial screening programme, where a single batch of chemicals could be made up and used immediately to extract DNA from a number of samples over a short period of time. The chemicals required to make up the DNA extraction solutions are not highly specialized, with the most expensive component, the hexadecyltrimethylammonium bromide (CTAB), being relatively in-expensive ($ 20.35 per 100g) (Sigma, 1992) when compared to the caesium-chloride ($ 84.60 per 100g) (Sigma, 1992) and ethidium-bromide ($ 867.60 per 100g) (Sigma, 1992) required by most other DNA extraction protocols for the preparation of high molecular weight DNA (Gawel and Garret, 1991; Nissen *et al.*, 1992). In addition, as a result of the simplicity of the DNA extraction protocol, which includes only five basic steps namely, grinding of leaf material, incubation in a CTAB buffer, extraction of protein and other contaminants in a chloroform:isoamyl alcohol mixture, precipitation and resuspension of the DNA, and final nucleic acid purification, it could be carried out by almost any person with little laboratory experience. Such a worker could be trained, with a minimum amount of effort, to extract DNA from the eucalypts on a routine basis using this procedure.

The only difficulty encountered when using the DNA extraction procedure optimized for use with the eucalypts, was the inability to correlate spectrophotometric determinations of DNA concentration with the estimates of DNA yield obtained by comparing the banding intensities of markers and sample on an agarose gel (refer section 3.3.1.2) (Figure 3.6A and Table 3.5). Doyle and Doyle (1990b) reported that they encountered similar problems when they attempted to quantify DNA using spectrophotometric methods and attributed this to "interference of residual CTAB in the samples". In this study, DNA samples were subjected to ammonium acetate-ethanol precipitation and dialysis (refer section 3.3.1.2) in an attempt to remove these and other small molecular weight contaminants. However, it remained difficult to correlate spectrophotometric determinations of DNA yield with the results observed on an agarose gel (results

not shown). Therefore, in all subsequent experiments, DNA was quantified using a combination of these techniques. Running of the samples on an agarose gel served not only as a tool for the quantification of the DNA product, but also provided a visual check of the integrity and purity of the DNA sample (Figure 3.6A). Therefore, it would be advantageous to incorporate such a step into a routine screening programme.

3.4.2.2 Advantages of using the probe pV47-2

Use of the multi-locus probe pV47-2, in conjunction with the ^{32}P and DIG-labelling systems, facilitated the detection of DNA polymorphisms in eucalypt species and cultivars (Figure 3.12 and Figure 3.13). This probe was developed by Longmire *et al.* (1990) (Figure 3.3) and has been used to generate DNA fingerprints for a variety of mammalian species (Dolf *et al.*, 1992). From a review of the literature, it appears that the present study contains the first report on the use of pV47-2 in the probing and analysis of plant DNA polymorphisms.

As is shown in Table 3.2 (refer section 3.1.1.2) various DNA sequences have been used as probes in the fingerprinting of plant DNA. In the woody *Malus*, *Rubus* and *Prunus* genera, the DNA repeat region isolated from the M13 bacteriophage (Ryskov *et al.*, 1988), has been the probe used most extensively (Nybom *et al.*, 1989; Nybom, 1990a; 1990b; Nybom and Rogstad, 1990; Nybom and Schaal, 1990a; 1990b; Nybom *et al.*, 1990; Nybom and Hall, 1991). However, Longmire *et al.* (1990) have shown that the M13-derived pV47-2 repeat probe is able to detect an average of 33 % more alleles than those visualized using M13 DNA itself. On the basis of these results, and as a supply of pV47-2 was available, this plasmid was used to fingerprint the eucalypts.

As was suggested by Longmire *et al.* (1990) a greater number of DNA fragments were detected, in this study, with pV47-2. On one set of DNA fingerprints, generated using ^{32}P-labelled probe (Figure 3.12), 8.5 bands were detected per genotype which is more than the averages of 7.5 fragments scored in *Prunus* or 7.75 recorded in *Malus* after using the M13 probe (Nybom *et al.*, 1990). At this point it is important to note that the average number of bands detected per genotype on any fingerprint is determined also by the choice of restriction enzyme used to digest the genomic DNA prior to its being blotted onto a membrane (refer section 3.1.2.1) (McCouch, 1988; Miller and Tanksley, 1990; Nodari *et al.*, 1992). However, all the

fragment averages given above were obtained after restriction of the DNA with Dra I, so comparisons could be made between the eucalypt fingerprints (Figure 3.12) and those mentioned previously (Nybom *et al.*, 1990). From these comparisons various potential advantages of using the pV47-2 probe in a eucalypt breeding program were identified. For example, this probe could be used to screen closely related eucalypt individuals, and because of its sensitivity, it is probable that pV47-2 would resolve a greater number of polymorphic bands than those detected with other probes such as the M13 vector. An additional advantage of using pV47-2 as a probe would be the ease with which this plasmid can be prepared and purified (refer section 3.3.2.1). In this study, a single plasmid extraction step generated a sufficiently high yield of pV47-2 (1.22 mg/ml) to produce a series of eucalypt fingerprints. The plasmid was also pure, as shown by the A_{260}/A_{280} ratio of 1.88, and the appearance of the plasmid on an agarose gel (Figure 3.7). Although plasmid preparation and purification was carried out by caesium-chloride/ethidium-bromide equilibrium density gradient centrifugation (refer section 3.2.4.1), which is an extremely costly procedure, this was carried out only once and produced sufficient plasmid to last the entire duration of the fingerprinting experiments, with an additional plasmid stock to spare. Therefore, the use of pV47-2 in a commercial screening program would be an economically viable prospect.

3.4.2.3 Comparison of the ^{32}P and DIG probe labelling and detection systems

Eucalypt DNA fingerprints were resolved when both the ^{32}P and DIG protocols were used to label pV47-2 and then to detect where this labelled probe had bound eucalypt genomic DNA (Figure 3.12 and Figure 3.13).

The efficiency of probe labelling using the ^{32}P system was high, as shown by scintillation counts carried out on ^{32}P-labelled pV47-2 (between 1.5 and 9.5 x 10^9 cpm/μg of TCA-precipitated probe) (refer section 3.3.2.2). However, the efficiency of labelling using the DIG system could not be determined accurately, only an estimate of the rate of labelling could be calculated using a DIG blot onto which a dilution series of DIG-labelled pV47-2 had been blotted alongside various control samples (Figure 3.8) (0.13 ng of DIG labelled pV47-2 was produced per hour per 0.5 ng of DNA template incorporated into the labelling reaction). The ability or inability to quantify the amount of labelled probe would have important implications if either of these two labelling systems were to be incorporated into a commercial eucalypt screening programme. For example, if numerous eucalypt

DNA blots were to be probed using a supply of labelled pV47-2, it would be important to know exactly how much label was available, as this would give an indication of the number of fingerprints which could be generated before the supply of probe was exhausted. Therefore, use of the ^{32}P probe labelling system might facilitate more accurate short term planning. However, a major drawback to using any ^{32}P-labelled substance is the short half life of the isotope itself (14.28 days) (Mathews and von Holde, 1991). As a result of the instability of this isotope ^{32}P-labelled pV47-2 could be used to produce fingerprints for a period of less than 28 days after probe labelling afterwhich there would be no detectable signal. Any probe DNA labelled with ^{32}P would have to be used almost immediately and could not be stored away for use in future experiments. By contrast, the DIG-label is known for its stability, which allows DIG-labelled probe to be stored at -20°C for extended periods of time (Boehringer Mannheim). It is probable that such a stable substance would be favoured for use in a eucalypt fingerprinting program as any excess probe could be stored away and utilized in later fingerprinting attempts.

Apart from the instability of ^{32}P, additional disadvantages of using this isotope as a probe-label in a screening program could be identified. For example, ^{32}P is extremely dangerous and any person working with it would be exposed to a potential health risk (Mathews and von Holde, 1991). As such, this substance could be handled only by competent persons who were fully trained in the use of such hazardous radioisotopes. This requirement would need to be taken into consideration when setting up a fingerprinting programme for the eucalypts, in which ^{32}P was to be used as a probe-label. Persons with appropriate radioisotope work experience would have to be employed, or, unskilled individuals would have to be trained in the procedures followed when working with these substances. In addition, specialized laboratory facilities would have to be established. These laboratories would need to fulfill certain governmental requirements regarding the safety of persons handling the isotopes, and the disposal of radioactive waste (Keller and Manak, 1989; Pollard-Knight, 1990). Therefore, it is clear that the decision to include ^{32}P in a programme for the fingerprinting of eucalypts would be an extremely costly one, requiring a considerable financial outlay at the start, for the recruitment of skilled personnel and establishment of facilities in which the isotope work could be carried out, followed by continued, substantial, financial input at regular intervals for the purchase of the isotope itself.

A further disadvantage of using the ^{32}P protocol would be the time required to carry out the fingerprinting procedure. In this study, for example, a minimum of 5 days was required from the probe labelling step to the generation of eucalypt fingerprints on an autoradiograph (refer section 3.2.9, 3.2.10 and 3.2.11). However, using the DIG system, fingerprints were produced after a 2-3 day period (refer section 3.2.9, 3.2.10 and 3.2.11), which would be a far more convenient time period if fingerprints were to be generated on a routine basis.

A decision to use either the ^{32}P or DIG-labelling systems would have to be based on the considerations mentioned above but, more importantly, these systems should be evaluated on the basis of the polymorphisms revealed between eucalypt species and cultivars (Figure 3.12 and Figure 3.13). As reported previously, DNA polymorphisms were revealed between both eucalypt species and cultivars using the ^{32}P system (Figure 3.12), whereas two of the eucalypt cultivars appeared to be genetically identical after they were fingerprinted using the DIG system (Figure 3.13) (refer section 3.4.1). It is probable that the DNA polymorphisms which exist between the two *E. grandis* cultivars were revealed on the autoradiograph as a greater number of bands were detectable per genotype on these fingerprints (8.5) (Figure 3.12) compared to those revealed on the DIG blot (5.0) (Figure 3.13). As explained previously (refer section 3.3.4.1), it is suspected that additional bands could be scored using the ^{32}P system as the detection procedure, for the identification of eucalypt DNA fragments which have bound ^{32}P-labelled probe, is more sensitive than the colorimetric detection assay used to resolve DIG-bound DNA fragments. This is supported by the finding that DNA fragments from a phage Lambda Hind III/Eco RI digest, included on the eucalypt DNA blots as molecular weight markers (lane 6, Figure 3.12 and Figure 3.13), were resolved only on the autoradiograph and not on the DIG blot. It is possible that this was due also to the enhanced sensitivity of the ^{32}P detection system compared to the DIG protocol.

In conclusion, it is clear that the DIG protocol would be most suitable for incorporation into a eucalypt screening program, if factors such as the cost and ease of running the procedures are considered. However, the ^{32}P system produced the more informative DNA fingerprints which revealed eucalypt DNA polymorphisms at a cultivar level. Therefore, the "ideal" eucalypt fingerprinting procedure would need to combine the convenience of the DIG system with the sensitivity of the ^{32}P protocol. Recent advances in the development of alternative substrates for use in

the DIG procedure may provide the ultimate system for meeting these requirements. In this study, as mentioned previously, use was made of a colourimetric assay for the detection of DIG-labelled DNA fragments (refer section 3.2.11.1) (Boehringer Mannheim, Germany). This assay relies on the formation of a purple-brown precipitate on the blot membrane at the exact location where DIG-labelled probe has bound eucalypt genomic DNA (Boehringer Mannheim, Germany). Apart from the problems, described previously, with the sensitivity of this system, this precipitate is also insoluble and difficult to remove from the surface of the blot membrane, making it extremely difficult to reprobe (Düring, 1991; Höltke *et al.*, 1992). However, various new chemiluminescent substrates have been developed for use with the DIG protocol and amongst these is a substance called DPP (3-(4-methoxyspiro[1,2-di-oxetane-3,2-tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl phosphate) (Boehringer Mannheim, Indianapolis). Detection of DIG-labelled DNA using this substrate has been shown to have a sensitivity equivalent to that of the ^{32}P system and, as DPP does not create any colour precipitate, reprobing of blots is possible (Höltke *et al.*, 1992). It is suggested that this substrate be used to replace the colour substrate (NBT/BCIP) used previously in the DIG experiments. With an improved sensitivity in the system, it is expected that the DIG procedure would allow polymorphic bands to be resolved between eucalypt cultivars, while remaining sufficiently convenient and cost effective for incorporation into a routine screening programme for the identification of eucalypts.

3.4.3 THE USE OF RAPDS IN THE IDENTIFICATION OF PLANT CULTIVARS

After the present study had been initiated, two groups of workers, namely, Welsh and McClelland and Wiliiams *et al* (1990) developed a procedure now know as Random Amplification of Polymorphic DNAs, or, RAPDs. Using the polymerase chain reaction and random oligonucleotide probes, those authors amplified samples of genomic DNA and when these fractions were separated on an agarose gel and stained with ethidium bromide, discrete sets of fragments were resolved. Those banding patterns were used then to fingerprint genomes (Welsh and McClelland, 1990) and as genetic markers (Williams *et al.*, 1991). Subsequent to the work of those authors, RAPDs have been used in genetic linkage mapping (Quiros *et al.*, 1991; Echt *et al.*, 1992; Reiter *et al.*, 1992), as markers for disease resistance genes (Martin *et al.*, 1991; Miklas *et al.*, 1993; Penner *et al.*, 1993), and as a tool for the identification of plant cultivars (Demeke *et al.*, 1993; Torres *et al.*, 1993; Yang and

Quiros, 1993). There are a number of advantages to using the RAPD technique, including the requirement for small amounts of genomic DNA which can be screened with a universal set of primers in a short period of time (Haley *et al.*, 1993). As a result, many authors favour this methodology for cultivar identification over the apparently "laborious and costly" route of RFLP analysis (Penner *et al.*, 1993; Yang and Quiros, 1993). However, there are also certain disadvantages associated with the use of the RAPD methodology. For example, this Polymerase Chain Reaction (PCR)-based technique may be prone to artefacts caused by contamination of the RAPD reaction mixture (Newbury and Ford-Lloyd, 1993). In addition, there may be competition for primers between the regions of the genome being amplified, which may result in irreproducible results between amplification attempts (Newbury and Ford-Lloyd, 1993). Therefore, despite the growing popularity of this technique, many obstacles would need to be overcome in the development of a protocol for RAPD analysis in the eucalypts. In the light of the succesess obtained with the RFLP protocol developed in this study, the suggestion remains to further optimize this methodology. By using an alternative substrate in the DIG detection reaction it is probable that the developed procedures could be used to resolve DNA fingerprints of eucalypt cultivars on a routine basis.

CHAPTER 4
GENERAL CONCLUSIONS

A protocol for chloroplast isolation was developed which allowed the extraction of good yields of intact organelles (25 μg chlorophyll/g fresh mass) from the eucalypt *E. grandis* S/N M6. These chloroplasts were used in the "in-organelle" DNA digestion procedure, and differences were resolved in the cpDNA RFLP patterns of *E. grandis* S/N M6 and an outgroup species *S. oleracea* (spinach). However, use of the developed organelle extraction procedure with other eucalypts (*E. macarthurii*, *E. grandis* TAG 5 and TAG 14), resulted in low chloroplast yields (68-80 % lower than those obtained for *E. grandis* S/N M6). This was attributed to variability in the material at a biochemical and/or ultrastructural level. Therefore, it would be essential to optimize the developed chloroplast isolation protocol for use with each of the eucalypt species and cultivars to be analyzed. This would be an extremely costly and time-consuming process. It is concluded, therefore, that this protocol for cpDNA analysis would not be suitable for incorporation in a eucalypt screening programme.

Subsequently, methodologies for genomic DNA fingerprinting of the eucalypts were developed. These included protocols for extraction and purification of genomic DNA, and hybridization and detection of labelled probe bound to the DNA.

With the use of the developed DNA isolation and purification technique, DNA yields between 87.2 and 201.0 μg/g fresh mass were obtained, which compared favourably with yields achieved for other woody species (Kvarnheden and Engström, 1991). In addition, the DNA was reasonably pure and free of contaminants, as shown by A_{260}/A_{280} ratios between 1.73 and 1.88.

Systems for the hybridization and detection of labelled probe, bound to eucalypt genomic DNA, were optimized using ^{32}P and a non-radioactive labelling substance dioxygenin (DIG). High yields of the M13-derived plasmid probe pV47-2 were extracted and purified (1.22 mg/ml). Hind-III restricted fractions of pV47-2 were labelled to a high efficiency with the isotope and DIG. Hybridization of these labelled probes to membranes, onto which genomic DNA had been blotted, resulted in the resolution of DNA fingerprints for all the eucalypt species and cultivars tested (*E. macarthurii*, *E. grandis* TAG 5 and TAG 14). An average of 8.5 DNA bands were detected with ^{32}P, whereas only 5 fragments were resolved per sample on the

DIG blot. This was attributed to the enhanced sensitivity of the isotope detection system. However, it is concluded that this system would not be suitable for incorporation into a eucalypt screening programme as it is hazardous to carry out, time-consuming and costly. It is suggested that the DIG system be optimized further to include a chemiluminescant assay in the detection procedure. Certain authors claim that recently developed DIG assays provide a sensitivity equivalent to that of the radioisotope protocols (Höltke *et al.*, 1992).

REFERENCES

Ali, S., Müller, C.R. and Epplen, J.T. (1986). DNA fingerprinting by oligonucleotide probes specific for simple repeats. *Hum.Genet.* 74: 239-243.

Aly, M.A.M., Fjellstrom, R.G., McGranahan, G.H and Parfitt, D.E. (1992). Origin of walnut somatic embryos determined by RFLP and isozyme analysis. *HortSci.* 27(1): 61-63.

Anderson, A. (1989). DNA fingerprinting on trial. *Nature* 342: 844.

Anonymous. (1991). *South African forestry facts leaflet*. South Africa : Promotion Committee Forestry Council.

Arandhya, K.M. and Phillips, V.D. (1993). Genetic variability in elite *Eucalyptus* provenances and progenies selected for arid environments in Hawaii. *Bioresource Technol.* 45(2): 145-150.

Ariovich, D.I. and Cresswell, C.F. (1983). The effect of nitrogen and phosphate on starch accumulation in two varieties of *Panicum maximum* Jacq. *Plant, Cell Environ.* 6: 138-143.

Arnheim, N., Strange, C. and Erlich, H. (1985). Use of pooled DNA samples to detect linkage disequilibrium of polymorphic restriction fragments and human disease: studies of the HLA class II loci. *Proc.Natl.Acad.Sci. USA* 82: 6970-6974.

Arnon, D.I. (1949). Copper enzymes in chloroplasts. Polyphenoloxidases in *Beta vulgaris*. *Plant Physiol.* 24: 1-14.

Atchison, B.A., Whitfeld, P.R. and Bottomley, W. (1976). Comparison of chloroplast DNAs by specific fragmentation with EcoRI endonuclease. *Mol. Gen. Genet.* 148: 263-269.

Barry Herman, Mondi Forests, Hilton, Natal.

Banks, J.A. and Birky Jr., C.W. (1985). Chloroplast DNA diversity is low in a wild plant, *Lupinus texensis*. *Proc.Natl.Acad.Sci.USA.* 82: 6950-6954.

Baur, E. (1909). Das Wesen und die Erblichkeitsverhältnisse der "Varietates albomarginatae hort" von *Pelargonium zonale*. *Z. Indukt. Abstammungs-Vererbungsl.* 1: 330-351.

Beckmann, J.S. and Soller, M. (1983). Restriction fragment length polymorphism in genetic improvement: methodologies, mapping and cost. *Theor.Appl.Genet.* 67: 33-43.

Bedbrook, J.R. and Bogorad, L. (1976). Endonuclease recognition sites mapped on *Zea mays* chloroplast DNA. *Proc.Natl.Acad.Sci.USA* 73: 4309-4319.

Berkowitz, G.A. and Gibbs, M. (1985). Chloroplasts as a whole. In *Modern Methods of Plant Analysis, Volume I, Cell Components*, eds. Linskens, J.F. and Jackson, J.F. Heidelberg, New York, Toronto : Springer-Verlag. pp 152-181.

Bernatzky, R. and Tanksley, S.D. (1986). Toward a saturated linkage map in tomato based on isozymes and random cDNA sequences. *Genetics* 112: 887-898.

Boblenz, K., Nothnagel, T., and Metzlaff, M. (1990). Paternal inheritance of plastids in the genus *Daucus*. *Mol.Gen.Genet.* 220: 489-491.

Bohnert, H.J., Crouse, E.J. and Schmitt, J.M. (1982). Chloroplast genome organization and RNA synthesis. *Encycl.Plant Physiol.* 14B: 475-530.

Bolivar, F. and Backman, K. (1979). Plasmids of *Escherichia coli* as cloning vectors. *Methods Enzymol.* 68: 245.

Bonierbale, M.W., Giraudat, J., Nam H-G. and Goodman, H.M. (1988). RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. *Genetics* 120: 1095-1103.

Botstein, D., WHite, R.L., Skolnick, M. and Davis, R.W. (1980). Construction of genetic linkage map in man using restriction fragment length polymorphisms. *Amer.J.Human Genet.* 32: 314-331.

Bournival, B.L. and Korban, S.S. (1987). Electrophoretic analysis of genetic variability in the apple. *Sci.Hortic.* 31: 233-243.

Bowers, J.E., Bandman, E.B. and Meredith, C.P. (1993). DNA fingerprint characterization of some wine grape cultivars. *Amer.J.Enol.Vit.* 44(3): 266-274

Bowman, C.M., Bonnard, G. and Dyer, T.A. (1983). Chloroplast DNA variation between species of *Triticum* and *Aegilops*. Location of the variation on the chloroplast genome and its relevance to the inheritance and classification of the cytoplasm. *Theor.Appl.Genet.* 65: 247-262.

Bowman, C.M. and Dyer, T.A. (1979). 4.5S ribonucleic acid, a novel ribosome component in the chloroplasts of flowering plants. *Biochem.J.* 183: 605-613.

Burke, T. and Bruford, M.W. (1987). DNA fingerprinting in birds. *Nature* 327: 149.

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

Byrne, M., Moran, G.F. and Tibbits, W.N. (1993). Restriction map and maternal inheritance of chloroplast DNA in *Eucalyptus nitens*. *J.Hered.* 84(3): 218-220.

Chang, C. and Meyerowitz, E.M. (1991). Pant genome studies: restriction fragment length polymorphism and chromosome mapping information. *Curr.Op.Biotech* 1: 178-183.

Chiang, K.-S. and Sueoka, N. (1967). Replication of chloroplast DNA in *Chlamydomonas reinhardi* during vegetative cell cycle, its mode and regulation. *Proc.Natl.Acad.Sci.USA* 57: 1506-1513.

Chun, E.H.L., Vaugham, M.H. and Rich, A. (1963). The isolation and characterization of DNA associated with chloroplast preparations. *J.Mol.Biol.* 7: 130-141.

Chyi, Y.S., Hoenecke, M.E. and Sernyk, J.L. (1992). A genetic linkage map of restriction fragmnt length polymorphism loci for *Brassica rapa* (syn *Campestris*). *Genome* 35(5): 746-757.

Clark, E.M. and Hanson, M.R. (1983). Analysis of chloroplast genomes in small amounts of tissue. *PMB Rep.* 1: 77-79.

Clegg, M.T., Brown, A.H.D. and Whitfeld, P.R. (1984). Chloroplast DNA diversity in wild and cultivated barley: implications for genetic conservation. *Gene.Res.Camb.* 43: 339-343.

Correns, C. (1909). Vererbungsversuche mit blass(gelb)grünen und buntblättrigen Sippen bei *Mirabilis jalopa, Urtica pilulifera* und *Lunaria annua*. *Z.Indukt. Abstammungs-Vererbungsl.* 1: 291-329.

Corriveau, J.L., and Coleman, A.W. (1988). Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. *Amer.J.Bot.* 75: 1443-1458.

Corriveau, J.L., Goff, L.J. and Coleman, A.W. (1990). Plastid DNA is not detectable in the male gametes and pollen tubes of an Angiosperm (*Anthirrhinum majus*) that is maternal for plastid inheritance. *Curr.Genet.* 17: 327-330.

Cousineau, J.C. and Donnelly. (1989). Identification of raspberry cultivars in vivo and in vitro using isoenzyme analysis. *HortScience* 24: 490-492.

Crouse, E.J., Bohnert, H.J. and Schmidtt, J.M. (1984). Chloroplast RNA synthesis. In *Chloroplast Biogenesis, Seminar Series of the Society for Experimental Biology* ed. Ellis, R.J, Vol. 21. New York : Cambridge University Press. pp 83-136.

Culpepper, J.H., Sayavedra-Soto, L.A., Bassam, B.J. and Gresshoff, P.M. (1991). Characterization of *Cornus* (Dogwood) genotypes using DNA fingerprinting. *J.Amer.Soc.Hort.Sci.* 116(6): 1103-1107.

Curtis, S.E. and Clegg, M.T. (1984). Molecular evolution of chloroplast DNA sequences. *Mol.Bio.Evol.* 1: 291-301.

Dallas, J.F. (1988). Detection of DNA "fingerprints" of cultivated rice with a human minisatellite DNA probe. *Proc.Natl.Acad.Sci USA* 85: 6831-6835.

Dally, A.M. and Second, G. (1989). Chloroplast DNA isolation from higher plants: an improved non-aqueous method. *Plant Mol.Biol.Rep.* 7(2): 135-143.

Dasilva, J.A.G., Sorrells, M.E., Burnquist, W.L. and Tanksley, S.D. (1993). RFLP linkage map and genome analysis of *Saccharum spontaneum*. *Genome* 36(4): 782-791.

de la Rosa, L. and Juove, N. (1992). Genetic variation for isozymes and proteins in Spanish primitive cultivars and wild subspecies of *Lens*. *Euphytica* 59: 181-187.

Demeke, T., Kawchuk, L.M. and Lynch, D.R. (1993). Identification of potato cultivars and clonal variants by random amplified polymorphic DNA analysis. *American potato J.* 70(8): 561-570.

Denhardt, D.T. (1966). A membrane-filter technique for the detection of complementary DNA. *Biochem.Biophys.Res.Commun.* 23: 641.

Denison, N.P. and Kietzka, J.E. (1993a). The development and utilisation of vegetative propagation in Mondi for commercial afforestation programmes. *S.A.For.J.* 165: 47-54.

Denison, N.P. and Kietzka, J.E. (1993b). The use and importance of hybrid intensive forestry in South Africa. *S.A.For.J.* 165: 55-60.

Denison, N.P. and Quaile, D.R. (1987). The applied eucalypt programme in Mondi Forests. *S.A.For.J.* 142: 60-66.

Dolf, G., Glowatzki, M-L. and Gaillard, C. (1992). DNA fingerprinting in cattle using the probe pV47. *Anim.Genet.* 23: 63-69.

Dowling, T.E. and Brown, W.M. (1989). Allozymes, mitochondrial DNA and levels of phylogenetic resolution among four species of minnows (*Notropis: Yprinidae*). *Syst.Zool.* 38: 126-143.

Doyle, J.J. and Doyle, J.L. (1990a). A chloroplast DNA phylogeny of the wild perennial relatives of soybean (*Glycine* subgenus *Glycine*): congruence with morphological and crossing groups. *Evolution* 44(2): 371-389.

Doyle, J.J. and Doyle, J.L. (1990b). Isolation of plant DNA from fresh tissue. *Focus* 12(1): 13-15.

Draper, J., Scott, R., Armitage, P. and Walden, R. (1988). *Plant genetic transformation and gene expression*. Oxford, London, Edinburgh, Boston, Palto Alto, Melbourne : Blackwell Scientific Publications. pp 28-34.

Düring, K. (1991). Ultrasensitive chemiluminescent and colorigenic detection of DNA, RNA and proteins in plant molecular biology. *Ana.Biochem.* 196: 433-438.

Echt, C.S., Erdahl, L.A. and McCoy, T.J. (1992). Genetic segregation of random amplified polymorphic DNA in diploid cultivated alfalfa. *Genome* 35: 84-87.

Ecke, W. and Michaelis, G. (1990). Comparison of chloroplast and mitochondrial DNA from five morphologically distinct *Beta vulgaris*: sugar beet, fodder beet, beet root, foliage beet and Swiss chard. *Theor.Appl.Genet.* 79: 440-442.

El-Kassaby, Y.A., Ritland, K., Fashler, A.M.K. and Devitt, W.J.B. (1988). The role of reproductive phenology upon the mating structure of a Douglas-fir seed orchard. *Silvae Genet.* 37: 76-82.

Eneas-Filho, J., Hartley, M.R. and Mache, R. (1981). Pea chloroplast ribosomal proteins: characterization and site of synthesis. *Mol.Gen.Genet.* 184: 484-488.

Epplen, J.T., Ammer, H. and Epplen, C. (1991). Oligonucleotide fingerprinting using simple repeat motifs: a convenient, ubiquitously applicable method to detect hypervariability for multiple purposes. In *DNA fingerprinting: Approaches and applications* eds Burke, T., Dolf, G., Jeffreys, A.J. and Wolff, R. Basel : Birkhäuser. pp 50-69.

Falkenhagen, E.R. and Nash, S.W. (1978). Multivariate classification on provenance research. *Silvae Genet.* 27: 14-23.

Feinberg, A.P. and Vogelstein, B. (1983). A teechnique for radiolabelling DNA restriction fragments to a high specific activity. *Anal.Biochem.* 132: 6-13.

Fish, L.E. and Bogorad, L. (1986). Identification and analysis of maize P700 chlorophyll a proteins of PSI-A1 and PSI-A2 by high pressure liquid chromatography analysis and partial sequence determination. *J.Biol.Chem.* 260: 1413-1421.

Fluhr, R. and Edelman, M. (1981). Conservation of sequence arrangement among higher plant chloroplast DNAs: Molecular cross hybridization among the *Solanaceae* and between *Nicotiana* and *Spinacia*. *Nucl.Acids Res.* 9: 6841-6853.

Frankel, R., Scowcroft, W. R. and Whitfeld, P.R. (1979). Chloroplast DNA variation in isonuclear male-sterile lines of *Nicotiana*. *Mol.Gen.Genet.* 169: 129-135.

Gardiner, J.M., Coe, E.H., Meliahancock, S., Hoisington, D.A. and Chao, S. (1993). Development of a core RFLP map in maize using an immortalized-F2 population. *Genetics* 134(3): 917-930.

Gawel, N.J. and Jarret, R.L. (1991). Chloroplast DNA restriction fragment length polymorphisms (RFLPs) in *Musa* species. *Theor.Appl.Genet.* 81: 783-786.

Gebhardt, C., Ritter, E., Debener, T., Schachtschabel, V., Wakemeier, B., Uhrig, H. and Salamini, F. (1989). RFLP analysis and linkage mapping in *Solanum tuberosum*. *Theor.Appl.Genet.* 78: 65-75.

Gebhardt, C. and Salamini, F. (1992). Restriction fragment length polymorphism analysis of plant genomes and its application to plant breeding. *Int.Rev.Cytol.* 135: 201-237.

Giovannoni, J.J., Wing, R.A., Ganal, M.W. and Tanksley, S.D. (1991). Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. *Nuc.Acids Res.* 19: 6553-6558.

Given, C.V. (1981). Chloroplast purification: Is there a procedure of choice? *PMB Newsletter* 2: 28-36.

Gordon, K.H.J., Crouse, E.J., Bohnert, H.J. and Herrmann, R.G. (1982). Physical mapping of differences in chloroplast DNA of the five wild-type plastomes in *Oenothera* subsection *Euoenothera*. *Theor.Appl.Genet.* 61: 373-384.

Govindaraju, D.R., Wagner, D.B., Smith, G.P. and Dancik, B.P. (1988). Chloroplast DNA variation within individual trees of a *Pinus banksiana-P. contorta* sympatric region. *Canad.J.Forest.Res.* 18: 1347-1350.

Green, B.R., Muir, B.L and Padmanabhan, U. (1977). The *Acetabularia* chloroplast genome: small circles and large kinetic complexity. In *Progress in Acetabularia Research*, ed. Woodcock, C.F.L. New York : Academic. pp 107-122.

Haff, L.A. and Bogorad, L. (1976). Hybridization of maize chloroplast DNA with transfer nucleic acids. *Biochim* 15: 4105-4109.

Hageman, R. and Schroder, M. (1989). The cytological basis of the plastid inheritance in angiosperms. *Protoplasma* 152: 57-64.

Haley, S.D., Miklas, P.N., Stavely, J.R., Byrum, J. and Kelly, J.D. (1993). Identification of RAPD markers linked to a major rust resistance gene block in common bean. *Theor.Appl.Genet.* 86: 505-512.

Halliwell, B. (1981). *Chloroplast metabolism*. Oxford, New York: Clarendon Press. pp 13-25.

Harris, S.A. and Ingram, R. (1991). Chloroplast DNA and biosystematics: The effects of intraspecific diversity and plastid transmission. *Taxon* 40: 393-412.

Hatfield, P.M., Shoemaker, R.C. and Palmer, R.G. (1985). Maternal inheritance of chloroplast DNA within the genus *Glycine* subgenus *Soya*. *J.Heredity* 76: 373-374.

Havey, M.H. and Muehlbauer, F.J. (1989). Linkages between restriction fragment length , isozyme and morphological markers in lentil. *Theor.Appl.Genet.* 77: 395-401.

Hedberg, M.F., Huang, Y.S., and Homersand, M.H. (1981). Size of the chloroplast genome of *Conidium fragile*. *Science* 213: 445-447.

Heinemeyer, W., Alt, J. and Herrmann, R.G. (1984). Nucleotide sequence of the clustered genes for apocytochrome b6 and subunit 4 of the cytochrome *b/f* complex in the spinach plastid chromosome. *Curr.Genet.* 8: 543-549.

Heldt, H.W., Ja Chon, C., Maronde, D., Herold, A., Stankovic, Z.S., Walker, D.A., Kraminer, A., Kirk, M.R. and Heber, U. (1977). Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. *Plant Physiol.* 59: 1146-1155.

Helentjaris, T., King, G., Slocum, M., Siedenstrang, C. and Wegman, S. (1985). Restriction fragment length polymorphisms as probes for plant diversity and their development as tool for applied plant breeding. *Pl.Mol.Biol.* 5: 109-118.

Helentjaris, T., Slocum, M., Wright, S., Schaefer, A. and Nienhuis, J. (1986). Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. *Theor.Appl.Genet.* 72: 761-769.

Hewlett, K.G., Chamberlin-Lea, J., Goldie, P.L.,Duffield, D.A. and Stevens, T.A. (1989). Determination of paternity in killer whales (*Orcinus orca*) by chromosomes and DNA fingerprinting *Eigth Biennial Conference on the Biology of Marine Mammals.* Pacific Grove, CA : The Society for Marine Mammalogy.

Hilu, K.W. (1988). Identification of the "A"genome of finger millet using chloroplast DNA. *Genetics* 118: 163-167.

Hiratsuka, J., Shimada, H., Whittier, R.F., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C.R., Meng. B.Y., Li, Y., Kanno, A., Nishizawa, Y., Hirai, A., Shinozaki, K. and Sugiura, M. (1989). The complete sequence of the rice (*Oryza sativa*) chloroplast genome: intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. *Mol.Gen.Genet.* 217: 185-194.

Hoelzel, A.R. and Amos, W. (1988). DNA fingerprints and "scientific" whaling. *Nature* 333: 305.

Höltke, H.J., Sagner, G., Kessler, C. and Schmitz, G. (1992). Sensitive chemiluminescent detection of digoxygenin-labeled nucleic acids: a fast and simple protocol and its applications. *Biotechniques* 12(1): 104-112.

Hoober, J.K. (1984). *Chloroplasts*. New York : Plenum Press. pp 19-45.

Hooglander, N., Lumaret, R. and Bos, M. (1993). Inter-intraspecific variation of chloroplast DNA of European *Plantago* spp. *Heredity* 70(3): 322-334.

Imamura, J., Saul, M.W. and Potrykus, I. (1987). X-ray irradiation promoted asymmetric hybridisation and molecular analysis of the products. *Theor.Appl.Genet.* 74: 445-450.

Jackson, J.F. (1985). Mitochondria. In *Modern Methods of Plant Analysis, Volume 1, Cell Components*, eds. Linskens, H.F. and Jackson, J.F. Berlin, Heidelberg, New York and Toronto : Springer-Verlag. pp 296-303.

Jansen, R.K., Holsinger, K.E., Michaels, H.J. and Palmer, J.D. (1990). Phylogenetic analysis of chloroplast DNA restriction site data at higher taxonomic levels: and example from the *Asteracea*. *Evolution* 44(8): 2089-2105.

Jansen, R.K. and Palmer, J.D. (1987). A chloroplast inversion marks an ancient evolutionary split in the sunflower family (*Asteraceae*). *Proc.Natl.Acad.Sci.USA* 84

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

Jeffreys, A.J., Hillel, J., Hartley, N., Bulfield, G., Morton, D., Wilson, V., Wong, Z. and Harris,S. (1987). The implications of hypervariable DNA regions for animal identification. *Anim.Genet.* 18(1): 141-142.

Jeffreys, A.J., Wilson, V. and Thein, S-L. (1985a). Hypervariable "minisatellite" regions in human DNA. *Nature* 314: 67-73.

Johansson, J.T. and Jansen, R.K. (1991). Chloroplast variation among five species of *Ranunculaceae*: structure, sequence divergence, and phylogenetic relationships. *Pl.Syst.Evol.* 178: 9-25.

Jonothan Longmire, Life Sciences Division, Los Alamos Laboratory, USA.

Kaemmer, D., Afza, R., Weising, K., Kahl, G. and Novak, F.J. (1992). Oligonucleotide and amplification fingerprinting of wild species and cultivars of banana (*Musa* spp). *Biotechnology* 10(9): 1030-1035.

Kaiser, W.M., Kaiser, G., Schoner, S. and Niemanis, S. (1981). Photosynthesis under osmotic stress. Differential recovery of photosynthetic activities of stroma enzymes, intact chloroplasts, protoplasts, and leaf slices after exposure to high solute concentrations. *Plants* 153: 430-435.

Kan, Y.W. and Dozy, M. (1978). *Proc.Natl.Acad.Sci. USA* 75: 5631-5635.

Karpinska, B. and Karpinski S. (1993). The chloroplast genome of *Pinus sylvestris* - physical map and localization of chloroplast genes. *Can.J.For.Res.* 23(2): 234-238.

Keller, E.F. (1986). One woman and her theory. *New Scientist* 46-50.

Keller, G.H. and Manak, M.M. (1989). *DNA probes*. Basingstoke, UK : Macmillan Publishers Ltd.

Kemperman, J.A. and Barnes, B.V. (1976). Clone size in American aspens. *Canad.J.Bot.* 54: 2603-2607.

Kiem, P., Diers, B.W., Olson, T.C. and Schoemaker, T.C. (1990a). RFLP mapping in soybean: association between marker loci and variation in quantitative traits. *Genetics* 126: 735-742.

Kiem, P., Diers, B.W. and Schoemaker, R.C. (1990b). Genetic analysis of soybean hard seededness with molecular markers. *Theor.Appl.Genet.* 79: 465-469.

King, M-C. (1989). Genetic testing of identity and relationship. *Am.J.Hum.Genet.* 44: 179-181.

Kirby, L.T. (1990). *DNA fingerprinting: An introduction*. Canada : Stockton Press. pp 75-252.

Kite, G. (1986). Evolution by endosymbiosis: the inside story. *New Scientist* 50-52.

Klepper, L., Flescher, D. and Hageman, R.H. (1971). Generation of reduced nicotinamide adenine dinucleotide for nitrate reduction in green leaves. *Plant Physiol.* 48: 580-590.

Kobza, J.M., Brandon, M. and Seeman, J.R. (1989). Isolation of photosynthetically active protoplasts and intact chloroplasts from *Phaseolis vulgaris*. *Plant Sci.* 65: 77-182.

Koller, B. and Delius, H. (1980). *Vicia faba* chloroplast DNA has only one set of ribosomal RNA genes as shown by partial denaturation and R-loop analysis. *Mol.Gen.Genet.* 178: 261-269.

Krebbers, E.T., Larrinura, I.M., McIntosh, L. and Bogorad, L. (1982). The maize chloroplast genes for the β and ϵ subunits of the photosynthetic coupling factor CF1 are fused. *Nucl.Acids Res.* 10: 4985-5002.

Kut, S.A. and Flick, C.E. (1986). A miniprep system for analysis of chloroplast DNA restriction enzyme digest fragments in *Nicotiana*. *PMB Rep.* 4: 48-55.

Kvarnheden, A. and Engström, P. (1991). Genetically stable, individual-specific differences in hypervariable DNA in Norway spruce, detected by hybridization to a phage M13 probe. *Can.J.For.Res.* 22: 117-123.

Landry, B.S. Kesseli, R.V., Farrara, B. and Michelmore, R.W. (1987). A genetic map of lettuce (*Latuca sativa* L.) with restriction fragment length polymorphism, isozyme, disease resistance, and morphological markers. *Genetics* 116: 331-337.

Lark, K.G., Weisemann, J.M., Matthews, B.F., Palmer, R., Chase, K and Macalma, T. (1993). A genetic map of soybean (*Glycine max* L.) using an intraspecific cross of two cultivars-*Minosy* and *Noir*. *Theor.Appl.Genet.* 86(8): 901-906.

Lavin, M., Mathews, S., Hughes, C., Marriott, H. and Shelly, S. (1990). Intraspecific chloroplast DNA diversity is high in some wild species of *Leguminosae*. *Amer.J.Bot.* 77: Suppl. 144.

Leegood, R.C. and Walker, D.A. (1983). Chloroplasts. In *Isolation of Membranes and Organelles from Plant Cells. Biological Technical Series*, eds. Hall, J.L. and Moore, A.L. London : Academic Press. pp 185-210.

Leegood, R.C. and Walker, D.A. (1985). Chloroplasts and protoplasts. In *Techniques in Bioproductivity and Photosynthesis*, eds. Coombs, J., Hall, D.O., Long, S.P and Scurlock, J.M.O. Oxford, New York, Toronto, Sydney, Frankfurt : Pergamon Press. pp 118-132.

Lewin, B.M. (1987). *Genes, third edition*. New York : Wiley.

Lidholm, J. Szmidt, A.E., Hällgren, J.-E. and Gustafsson, P. (1988). The chloroplast genome of conifers lack one of the rRNA-encoding inverted repeats. *Mol.Gen.Genet.* 212: 6-10.

Lilley, R.McC., Fitzgerald, M.P., Rientis, K.G. and Walker, D.A. (1975). Criteria for intactness and the photosynthetic activity of spinach chloroplast preparations. *New Phytol.* 75: 1-10.

Longmire, J. (1989). Molecular genetic studies of endangered species. *Northwest Association of Forensic Scientists Spring 1989 Meeting Abstracts*. Ashland :National Fish and Wildlife Forensics Laboratory.

Longmire, J.L., Kraemer, P., Brown, N.C., Hardekopf, L.C. and Deaven, L.L. (1990). A new multi-locus fingerprinting probe: pV47-2. *Nuc.Acids Res.* 18(6): 1658.

Lynch, M. (1988). Estimation of relatedness by DNA fingerprinting. *Mol.Biol.Evol.* 5: 584-599.

Maniatis, T., Fritsch, F.F. and Sambrook, J. (1982). *Molecular cloning: A laboratory mannual*. Cold Spring Harbor New York : Cold Spring Harbor Press.

Manning, J.E., Wolstenholme, D.R., Ryan, R.S., Hunter, J.A. and Richards, O.C. (1971). Circular chloroplast DNA from *Euglena gracilis*. *Proc.Natl.Acad.Sci.USA* 68: 1169-1173.

Mansur, L.M., Lark., K.G., Kross, H.. and Oliveira, A. (1993). Interval mapping of quantitative trait loci for reproductive, morphological, and seed traits of soybean, (*Glycine max* L.). *Theor.Appl.Genet.* 86(8): 907-913.

Margulis, L. (1971). Symbiosis and evolution. *Scientific American* 225: 49-57.

Martin, G., Williams, J.G.K and Tanksley, S.D. (1991). Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. *Proc.Natl.Acad.Sci. USA* 88: 2336-2340.

Mathews, C.K. and van Holde, K.E. (1991). *Biochemistry*. Redwood City, CA : The Benjamin/Cummings Publishing Company.

McCouch, S., Kochert, G, Yu, Z.H., Wang, Z.Y., Kush, G.S., Coffman, W.R. and Tanksley, S.D. (1988). Molecular mapping of rice chromosomes. *Theor.Appl.Genet.* 76: 815-829.

McDonald, M.B. (1991). Blotting of seed proteins from isoelectrically focused gels for cultivar identification. *Seed Sci.Technol.* 19: 33-40.

Medgyesy, P., Pày, A. and Màrton, L. (1986). Transmission of paternal chloroplasts in *Nicotiana*. *Mol.Gen.Genet.* 204: 195-198.

Mikami, T., Kishima, Y., Sugiura, M. and Kinoshita, T. (1984). Chloroplast DNA diversity in the cytoplasms of sugar beet and its related species. *Plant Sci.Lett.* 36: 231-235.

Miklas, P.N., Stavely, J.R. and Kelly, J.D. (1993). Identification and potential use of a molecular marker for rust resistance in common bean. *Theor.Appl.Genet.* 85: 745-749.

Miller, J.C. and Tanksley, S.D. (1990). Effect of different restriction enzymes, probe source, and probe length on detecting restriction fragment length polymorphism in tomato. *Theor.Appl.Genet.* 80: 385-389.

Mills, W.R. and Joy, K.W. (1980). A rapid method for isolation of purified, physiologically active chloroplasts, used to study the amino acid distribution of amino acids in pea leaves. *Planta* 148: 75-83.

Mitton, J.B. and Grant, M.C. (1980). Observations on the ecology and evolution of quaking aspen, *Populus tremuloides*, in the Colorado front range. *Amer.J.Bot.* 67: 202-209.

Moon, E., Kao, T.-H. and Wu, R. (1987). Rice chloroplast DNA molecules are heterogenous as revealed by DNA sequences of cluster genes. *Nucl.Acid Res.* 15: 611-630.

Mühlegger, K., Batz, H.G., Böhm, S., Eltz, H., Höltke, H.J. and Kessler, C. (1988). Synthesis and use of new digoxygenin-labeled nucleotides in non-radioactive labelling and detection of nucleic acids. *Nucleosides Nucleotides* 8: 1161-1163.

Nagamine, T., Todd, G.A., McCann, K.P., Newbury, H.J. and Ford-Lloyd, B.V. (1989). Use of restriction fragment length polymorphism to fingerprint beet at the genotype and species level. *Theor.Appl.Genet.* 78: 847-851.

Nakatini, H.Y. and Barber, J. (1977). Am improved method for isolating chloroplasts retaining their outer membranes. *Biochem. Biophys. Acta* 461: 510-512.

Nass, M.M. and Nass, S. (1963). Intramitochondrial fibers with DNA characteristics. *J.Cell Biol.* 19: 593-611.

Neale, D.B., Wheeler, N.C. and Allard, R.W. (1986). Paternal inheritance of chloroplast DNA in Douglas-fir. *Can.J.For.Res.* 16: 1152-1154.

Neale, D.B. and Williams, C.G. (1991). Restriction fragment length polymorphism mapping in conifers and applications to forestry genetics and tree improvement. *Can.F.For.Res.* 21: 545-554

Newbury, H.J. and Ford-Lloyd, B.V. (1993). The use of RAPD for assessing variation in plants. *Pl.Growth.Reg.* 12: 43-51.

Nissen, S.J., Masters, R.A., Lee, D.J. and Rowe, M.L. (1992). Comparison of restriction fragment length polymorphisms in chloroplast DNA of five leafy spurge (*Euphorbia* spp.) accessions. *Weed Science* 40: 63-67.

Nodari, R.O., Koinange, E.M.K., Kelly, J.D. and Gepts, P. (1992). Towards an integrated linkage map of common bean. 1. Development of genomic DNA probes and levels of restriction fragment length polymorphism. *Theor.Appl.Genet.* 84: 186-192.

Norman, C. (1989). Caution urged on DNA fingerprinting. *Science* 245: 669.

Nybom, H. (1990a). DNA fingerprints in sports of "Red Delicious" apples. *HortScience* 1: 1641-1642.

Nybom, H. (1990b). Genetic variation in ornamental apple trees and their seedlings (*Malus,Rosaceae*) revealed by DNA "fingerprinting" with the M13 repeat probe. *Hereditas* 113: 17-28.

Nybom, H. and Hall, H.K. (1991). Minisatellite DNA "fingerprints" can distinguish *Rubus* cultivars and estimate their degree of relatedness. *Euphytica* 53: 107-114.

Nybom, H. and Rogstad, S.H. (1990). DNA "fingerprints" detect genetic variation in *Acer negundo* (*Aceraceae*). *Pl.Syst.Evol.* 173: 49-56.

Nybom, H., Rogstad, S.H. and Schaal, B.A. (1990). Genetic variation detected by use of the M13 "DNA fingerprint" probe in *Malus, Prunus* and *Rubus* (*Rosaceae*). *Theor.Appl.Genet.* 79: 153-156.

Nybom, H. and Schaal, B.A. (1990a). DNA "fingerprints" applied to paternity analysis in apples (*Malus x domestica*). *Theor.Appl.Genet.* 79: 763-768.

Nybom, H. and Schaal, B,A. (1990b). DNA "fingerprints" reveal genotypic distributions in natural populations of blackberries and raspberries. *Amer.J.Bot.* 77: 883-888.

Nybom, H., Schaal, B.A. and Rogstad, S.H. (1989). DNA "fingerprints" can distinguish cultivars of blackberries and raspberries. *Acta Horticulturae* 262: 305-308.

Odelberg, S.J., Demers, D.B., Westin, E.H. and Hossaini, A.A. (1988). Establishing paternity using minisatellite DNA probes when the putative father is unavailable for testing. *J.Forensic Sci.* 33: 921-928.

Odonoughue, L.S., Wang, Z., Roder, M., Kneen, B., Leggett, M., Sorrells, M.E. and Tanksley, S.D. (1992). An RFLP-based linkage map of oats based on a cross between two diploid taxa (*Avena atlantica x A. hirtula*). *Genome* 35(5): 765-771.

Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z.., Aota, S., Inokuchi, H. and Ozeki, H. (1986). Chloroplast gene organization deduced from the complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. *Nature* 322: 572-574.

Ozeki, H., Umesono, K., Inokuchi, H., Kohchi, T. and Ohyama, K. (1989). The chloroplast genome of plants: a unique origin. *Genome* 31: 169-174.

Palmer, J.D. (1985). Comparative organization of chloroplast genomes. *Ann.Rev.Genet.* 19: 325-354.

Palmer, J.D. (1986). Isolation and Structural Analysis of Chloroplast DNA. In *Methods in Enzymology, Volume 118, Plant Molecular Biology*, eds. Weissbach, A. and Weissbach, H. Orlando, San Diego, New York, Austin, London, Montreal, Sydney, Tokyo, Toronto : Academic Press Incorporated. pp 167-187.

Palmer, J.D. (1987). Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. *The American Naturalist* 130: S6-S29.

Palmer, J.D., Nugent, J.M. and Herbon, L.A. (1987). Unusual structure of geranium chloroplast DNA: a triple-sized inverted repeat, extensive gene duplications, multiple inversions and two repeat families. *Proc.Natl.Acad.Sci.USA* 84: 769-773.

Palmer, J.D., and Zamir, D. (1982). Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*. *Proc.Natl.Acad.Sci.USA.* 79: 5006-5010.

Parent, J.G. and Page, D. (1992). Identification of raspberry cultivars by nonradioactive DNA fingerprinting. *HortScience* 27(10): 1108-1110.

Parks, C.R. and Wendel, J.F. (1990). Molecular divergence between Asian and North American species of *Liriodendron* (Magnoliaceae) with implications for interpretation of fossil floras. *Amer.J.Bot.* 77: 1243-1256.

Paterson, A.H., Lander, E.S., Hewitt, J.D., Peterson, S., Lincoln, S.E. and Tanksley, S.D. (1988). Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. *Nature* 335: 721-726.

Penner, G.A., Chong, J., Levesque-Lemay, M., Molnar, S.J. and Fedak, G. (1993). Identification of a RAPD marker linked to the oat stem rust gene *Pg 3*. *Theor.Appl.Genet.* 85: 702-705.

Phillips, R.S., Bram, L.L. and Dickey, N.H. (1988). *Funk and Wagnalls New Encyclopedia*. United States : Funk and Wagnalls Incorporated. pp 393-394.

Piastuch, W.C. and Bates, G.W. (1990). Chromosomal analysis of *Nicotiana* asymmetric hybrids by dot blotting and in situ hybridization. *Mol.Gen.Genet.* 222: 97-103.

Plaut, Z. (1971). Inhibition of photosynthetic carbon dioxide fixation in isolated spinach chloroplasts exposed to reduced osmotic potentials. *Plant Physiol.* 48: 591-595.

Pollard-Knight, D. (1990). Current methods in non-radioactive nucleic acid labelling and detection. *Technique* 2: 113-132.

Powell, W., Phillips, M.S., McNicol, J.W. and Waugh, R. (1991). The use of DNA markers to estimate the extent and nature of genetic variability in *Solanum tuberosum* cultivars. *Ann.Appl.Biol.* 118: 423-432.

Prince, J.P., Loaiza-Figueroa, F. and Tanksley, S.D. (1992). Restriction fragment length polymorphism and genetic distance among Mexican accessions of *Capsicum*. *Genome* 35: 726-732.

Pryor, L.D. and Johnson, L.A.S. (1971). *A classification of the eucalypts*. Canberra : The Australian National University. pp 1-45.

Quiros, C.F., Hu, J., This, P., Chevre, A.M. and Delseny, M. (1991). Development and chromosomal localisation of genome-specific markers by polymerase chain reaction in *Brassica*. *Theor.Appl.Genet.* 82: 632-727.

Raymond, C.A., Owen, J.V. and Ravenwood, I.C. (1992). Genetic variation for frost tolerance in a breeding population of *Eucalyptus nitens*. *Silvae Genetica* 41(6): 355-362.

Reiter, R.S., Williams, J.G.L., Feldman, K.A., Rafalski, J.A., Tingey, S.V. and Scolnik, P.A. (1992). Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and random amplified polymorphic DNAs. *Proc.Natl.Acad.Sci. USA* 89: 1477-1481.

Reith, M. and Cattolico, R.A. (1986). Inverted repeat of *Olisthodiscus luteus* chloroplast DNA contains genes for both subunits of ribulose-1,5-bisphosphate carboxylase and the 32000-dalton Q_B protein: phylogenetic implications. *Proc.Natl.Acad.Sci.USA* 83: 8599-8603.

Rhodes, P.R., Zhu, Y.S. and Kung, S.D. (1981). *Nicotiana* chloroplast genome I. chloroplast DNA diversity. *Mol.Gen.Genet.* 182: 106-111.

Rieseberg, L.H., Beckstrom-Sternberg, S.M., Liston, A. and Arias D.M. (1991). Phylogenetic and systematic inferences from chloroplast DNA and isozyme variation in *Helianthus* sect *Helianthus* (Asteraceae). *Systematic Botany* 16: 50-76.

Rigby, P.W.J., Dieckmann, M., Rhodes, C and Berg, P. (1977). Labelling deoxyribonucleic acid to high specific activity in vitro by nicktranslation with DNA polymerase I. *J.Mol.Biol.* 113: 237-251.

Rivard, S.R., Cappadocia, M., Vincent, G., Brisson and Landry, B.S. (1989). Restriction fragment length polymorphism (RFLP) analyses of plants produced by in vitro anther culture of *Solanum chacoense* Bitt. *Theor.Appl.Genet.* 78: 49-56.

Rogstad, S.H., Nybom, H. and Schaal, B.A. (1991). The tetrapod "DNA fingerprinting" M13 repeat probe reveals genetic diversity and clonal growth in quacking (sic) aspen (*populus tremuloides, Salicaceae*). *Pl.Syst.Evol.* 175: 115-123.

Rogstad, S.H., Patton, J.C. and Schaal, B.A. (1988). M13 repeat probe detects DNA minisatellite-like sequences in gymnosperms and angiosperms. *Proc.Natl.Acad.Sci.USA* 85: 9176-9178.

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

Sakata, Y., Nishio, T. and Matthews, P.J. (1991). Chloroplast DNA analysis of eggplant (*Solanum melongena*) and related species for their taxonomic affinity. *Euphytica* 55: 21-26.

Sager, R. and Ishida, M.R. (1963). Chloroplast DNA in *Chlamydomonas*. *Proc.Natl.Acad.Sci.USA* 50: 725-730.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular cloning: A laboratory manual, second edition*. Cold Spring Harbor New York : Cold Spring Harbor Laboratory Press.

Santamour, F.S. and McArdle, A.J. (1985). Cultivar checklists of the large-bracted dogwoods: *Cornus florida*, *C. kousa*, and *C. nuttallii*. *J.Arborculture* 11: 29-36.

Schäfer, R., Zischler, H., Birsner, U., Becker, A., Epplen, J.T. (1988). Optimized oligonucleotide probes for DNA fingerprinting. *Electrophoresis* 9: 369-374.

Schumann, C.M. and Hancock, J.F. (1989). Paternal inheritance of plastids in *Medicago sativa*. *Theor.Appl.Genet.* 78: 863-866.

Schweizer, G.,Borisjuk, N., Borisjuk, L., Stadler, M., Stelzer, T., Schilde, L. and Hemleben, V. (1993). Molecular analysis of highly repeated genome fractions in *Solanum* and their use as markers for the characterization of species and cultivars. *Theor.Appl.Genet.* 85(6-7): 801-808.

Scott, N.S., Shah, V.C. and Smillie, R.M. (1968). Synthesis of chloroplast DNA in isolated chloroplasts. *J.Cell Biol.* 38: 151-157.

Sears, B.B. (1980). Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. *Plasmid* 4: 233-255.

Sewell, M.M., Qui, Y.L., Parks, C.R. and Chase, M.W. (1993). Genetic evidence for trace paternal transmission of plastids in *Liriodendron* and *Magnolia* (*Magnoliaceae*). *Amer.J.Bot* 80(7): 854-858.

Sharmaine McCrae, Forestek Division, CSIRO, Natal.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Shinozaki, K.Y., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H. and Sugiura, M. (1986). The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. *EMBO.J.* 5: 2043-2049.

Shinozaki, K. and Sugiura, M. (1982). The nucleotide sequence of the tobacco chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. *Gene* 20: 91-102.

Slocum, M.K., Figdore, S.S., Kennard, W.C., Suzuki, J.Y. and Osborn, T.C. (1990). Linkage arrangement of restriction fragment length polymorphism loci in *Brassica oleracea*. *Theor.Appl.Genet.* 80: 57-64.

Smith, H.O. and Wilcox, K.W. (1970). A restriction enzyme from *Hemophilus influenzae*, I: purification and general properties. *J.Mol.Biol.* 51: 393-409.

Soltis, D.E. and Soltis, P.S. (1989). Allopolyploid speciation in tragopogon: insights from chloroplast DNA. *Amer.J.Bot* 76(8): 1119-1124.

Somerville, C.R., Somerville, S.C. and Ogren, W.L. (1981). Isolation of photosynthetically active protoplasts and chloroplasts from *Arabidopsis thaliana*. *Plant Sci. Lett.* 21: 89-96.

Song, K., Osborn, T.C. and Williams, P.H. (1990). *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLps) 3. Genome relationships in *Brassica* and related genera and the origin of *B. oleracea* and *B. rapa* (syn. *campestris*). *Theor.Appl.Genet.* 79: 497-506.

Soreng, R.J., Davis, J.I. and Doyle, J.J. (1990). A phylogenetic analysis of chloroplast DNA restriction site variation in *Poaceae* subfam. *Pooideae*. *Pl.Syst.Evol.* 172: 83-97.

Smith, D.B. and Adams, W.T. (1983). Measuring pollen contamination in seed orchards with the aid of genetic markers. *Proc.17th South.For.Tree Improve.Conf.* pp 69-77.

Stanier, R.Y. and Cohen-Bazire, G. (1977). Phototropic prokaryotes: The cyanobacteria. *Ann.Rev.Micro.* 31: 225-274.

Steane, A., West, A.K., Potts, B.M., Ovenden, J.R., and Reid, J.B. (1991). Restriction fragment length polymorphisms in chloroplast DNA from six species of *Eucalyptus*. *Aust. J. Bot.* 39: 399-414.

Stein, D.B. and Barrington, D.S. (1990). Recurring hybrid formation in a population of *Polystichum x Potteri*: evidence from chloroplast DNA comparisons. *Ann.Missouri.Bot.Gard.* 77: 334-339.

Steup, M., Peavey, D.G., and Gibbs, M. (1976). The regulation of starch metabolism by inorganic phosphate. *Biochem. Biophys. Res. Comm.* 72: 1554-1561.

Strauss, S.H., Neale, D.B. and Wagner, D.B. (1989). Genetics of the chloroplast in conifers. Biotechnology research reveals some surprises. *Journal of Forestry* 87(8): 11-17.

Strauss, S.H., Palmer, J.D., Howe, G. and Doerksen, A.H. (1988). Chloroplast genomes of two conifers lack a large inverted repeat and are extensively rearranged. *Proc.Natl.Acad.Sci.USA* 85: 3898-3902.

Stubbe, W. (1984). Biparental plastid inheritance in *Oenothera organensis* Munz. *Acta.Bot.Neerl.* 33: 229.

Stuber, C.W., Wendel, J.F., Goodman, M.M. and Smith, J.S.C. (1988). Techniques and scoring procedures for starch gel electrophoresis of enzymes from maize (*Zea mays*). *N.C.Agr.Res.Serv., N.C. State Univ.Tech.Bull.* 286: 1-87.

Sugiura, M. (1989). The chloroplast chromosome in land plants. *Annu.Rev.Cell Biol.* 5: 51-70.

Sugiura, M. (1992). The chloroplast genome. *Plant Mol.Biol.* 19: 149-168.

Sutcliffe, J.G. (1979). Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. *Cold Spring Harbor Symp.Quant.Biol.* 43: 77.

Sytsma, K.J. and Gottlieb, L.D. (1986). Chloroplast DNA evolution and phylogenetic relationships in *Clarkia* sect. *Peripetasma* (*Onagraceae*). *Evolution* 40: 1248-1261.

Sytsma, K.J., Smith, J.F. and Berry, P.E. (1991). Biogeography and evolution of morphology, breeding systems, and flavanoids in the Old World *Fushia* (sic) sect *Skinnera* (Onagraceae): evidence from chloroplast DNA. *Amer.J.Bot.* (IN PRESS)

Szmidt, A.E., Alden, T. and Hällgren, J.E. (1987) Paternal inheritance of chloroplast DNA in *Larix*. *Plant Mol.Biol.* 9: 59-64.

Szmidt, A.E., Kassaby, Y.A., Sigurgeirsson, A., Alden, T., Lindgren, D. and Hällgren J.-E. (1988). Classifying seedlots of *Picea sitchensis* and *P. glauca* in zones of introgression using restriction analysis of chloroplast DNA. *Theor.Appl.Genet.* 76: 841-845.

Takumi, S., Nasuda, S., Liu, Y.G. and Tsunewaki, K. (1993). Wheat phylogeny determined by RFLP analysis of nuclear DNA. 1: Einkorn wheat. *Jap.J.Genet.* 68(1): 458-464.

Tanksley, S.D., Bernatsky, R., Lapitan, N.L. and Prince, J.P. (1988). Conservation and gene repertoire but not gene order in pepper and tomato. *Proc.Natl.Acad.Sci. USA* 85: 6419-6423.

Tanksley, S.D. and Mutschler, H.A. (1990). Linkage map of the tomato (*Lycopersicon esculentum*, ZN=24. In *Genetics Maps* ed, O'Brian, S. Cold Spring Harbor New York : Cold Spring Harbor Laboratory Press. pp 6.100-6.102.

Taylor, F.J.R. (1974). Implications and extensions of the serial endosymbiosis theory of the origin of eukaryotes. *Taxon* 23: 229-258.

Terauchi, R., Terachi, T. and Tsunewaki, K. (1991). Intraspecific variation of chloroplast DNA in *Dioscorea bulbifera* L. *Theor.Appl.Genet* 81: 461-470.

Tewari, K.K. and Wildman, S.G. (1967). DNA polymerase in isolated tobacco chloroplasts and nature of the polymerized product. *Proc.Natl.Acad.Sci.USA* 57: 689-696.

Thommasen, H.V., Thomson, M.J., Schutle, G.G. and Kirby, L.T. (1989). Development of DNA fingerprints for use in wildlife forensic science. *Wildl.Soc.Bull.* 17: 321-326.

Tilney-Bassett, R.A.E. (1978). The inheritance and genetic behaviour of plastids. In *The Plastids*, eds. Kirk, J.T.O. and Tilney-Bassett, R.A.E. Amsterdam : Elsevier. pp 251-524.

Tilney-Bassett, R.A.E. and Almouslem, A.B. (1989). Variation in plastids inheritance between *Pelargonium* cultivars and their hybrids. *Heredity* 63: 145-153.

Torres, A.M., Millan, T. and Cubero, J.I. (1993). Identifying rose cultivars using random amplified polymorphic DNA markers. *HortScience* 28(4): 333-334.

Turnbull, J.W. (1991). Future use of *Eucalyptus*: opportunities and problems. In *Proceedings of the symposium on intensive forestry: The role of the eucalypts*, ed. Schönau, A.P.G. pp 1-27.

Turner, B.L. and Kim, K-J. (1990). An overview of the genu *Pyrrhopappus* (*Asteraceae: Lactuceae*) with emphasis on chloroplast DNA restriction site data. *Amer.J.Bot.* 77(7): 845-850.

Tzuri, G., Hillel, J., Lavi, U., Haberfeld, A. and Vainstein, A. (1991). DNA fingerprint analysis of ornamental plants. *Pl.Sci.* 76: 91-97.

Vassart, G., Georges, M. and Monsieur, R. (1987). A sequence in M13 phage detects hypervariable minisatellites in human and animal DNA. *Science* 235: 683-684.

Wagner, D.B., Furnier, G.R., Saghai-Maroof, M.A., Williams, S.M., Dancik, B.P. and Allard, R.W. (1987). Chloroplast DNA polymorphism in lodgepole and jack pines and their hybrids. *Proc.Natl.Acad.Sci.USA* 84: 2097-2100.

Walker, D.A. (1976). CO_2 fixation by intact chloroplasts: photosynthetic induction and its relation to transport phenomena and control mechanisms. In *The Intact chloroplast*, ed. Barber, J. Amsterdam : Elsevier. pp 235-278.

Walker, D.A. (1987). *The Use of the Oxygen Electrode and Fluorescence Probes in Simple Measurements of Photosynthesis.* University of Sheffied : Oxgraphics Limited. pp 107-114.

Walker, J.R.L. (1980). Enzyme isolation from plant and the phenolic problem. *What's New in Plant Physiol.* 11: 33-36.

Wang, Z.Y. and Tanksley, S.D. (1992). Polymorphism and phylogenetic relationships among species in the genus *Oryza* as determined by analysis of nuclear RFLPs. *Theor.Appl.Genet.* 83: 565-581.

Watson J.C. and Surzycki, S.J. (1983). Both the chloroplast and nuclear genomes of *Chlamydomonas reinhardtii* share homolgy with *Escherichia coli* genes for transcriptional and translational components. *Curr.Genet.* 7: 201-210.

Waugh, R., van de Ven, W.T.G., Phillips, M.S. and Powell, W. (1990). Chloroplast DNA diversity in the genus *Rubus* (*Rosaceae*) revealed by Southern Hybridization. *Pl.Syst.Evol.* 172: 65-75.

Webb, D.M., Knapp, S.J. and Tagliani, L.A. (1992). Restriction fragment length polymorphism and allozyme linkage map of *Cuphea lanceolata*. *Theor.Appl.Genet.* 83: 528-532.

Weeden, N.F. and Lamb, R.C. (1985). Identification of apple cultivars by isozyme phenotypes. *J.Amer.Soc.Hortic.Sci.* 110: 509-515.

Welsh, J. and McClelland, M. (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nuc.Acids Res.* 18: 7213-7218.

Weier, T.E., Stocking, C.R., Barbour, M.G. and Rost, T.L. (1982). *Botany: and introduction to plant biology*. New York : John Wiley and Sons. pp 376.

Weising, K., Beyermann, B., Ramser, J. and Kahl, G. (1991). Plant DNA fingerprinting with radioactive and digoxigenated oligonucleotide probes complementary to simple repetative DNA sequences. *Electrophoresis* 12: 159-169.

Weising, K., Kaemmer, D., Weigand, F., Epplen, J.T. and Kahl, G. (1992). Oligonucleotide fingerprinting reveals various probe-dependent levels of informativeness in chickpea (*Cicer arietinum*). *Genome* 35: 436-442.

Westneat, D.F., Noon, W.A., Reeve, H.K. and Aquadro, C.F. (1988). Improved hybridization conditions for DNA "fingerprints" probed with M13. *Nuc.Acids Res.* 16(9): 4161.

Wetton, J.H., Carter, R.E., Parkin, D.T. and Walters, D. (1987). Demographic study of a wild house sparrow population by DNA fingerprinting. *Nature* 327: 147-149.

White, E.E. (1990). Chloroplast DNA in *Pinus monticola* 2. survey of within-species variability and detection of heteroplasmic individuals. *Theor.Appl.Genet.* 79: 251-255.

Williams, J.G.L., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. (1990). DNA polymorphisms amplified by arbitray primers as useful genetic markers. *Nuc.Acids Res.* 18: 6531-6535.

Wrigley, C.W., Batley, I.L. and Skerritt, J.H. (1987). Complementing traditional methods of identifying cereal varieties w th novel procedures. *Seed Sci. Technol.* 15: 679-688.

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

Yatskieych, G., Stein, D.B. and Gastony, G.J. (1988). Chloroplast DNA evolution and systematics of *Phanerophlebia* (*Dryopteridaceae*). *Proc.Natl.Acad.Sci. USA* 85: 2589-2593.

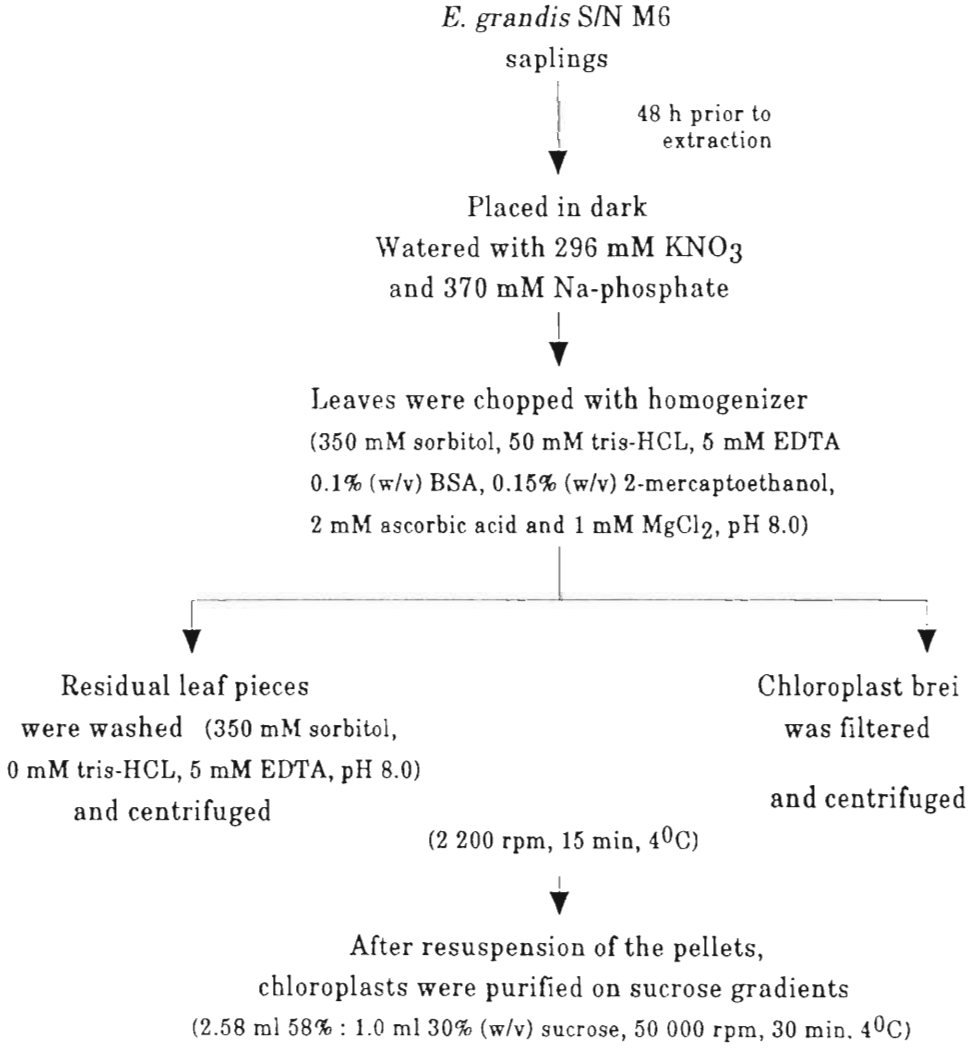
Zablen, L.B., Kissil, M.S., Woese, C.R. and Buetow, D.E. (1975). Phylogenetic origin of the chloroplast and prokaryotic nature of its ribosomal RNA. *Proc.Natl.Acad.Sci. USA* 72: 2418-2422.

Zurawski, G. and Clegg, M.T. (1987). Evolution of higher-plant chloroplast DNA-encoded genes: implications for structure-function and phylogenetic studies. *Ann.Rev.Pl.Physiol.* 38: 391-418.

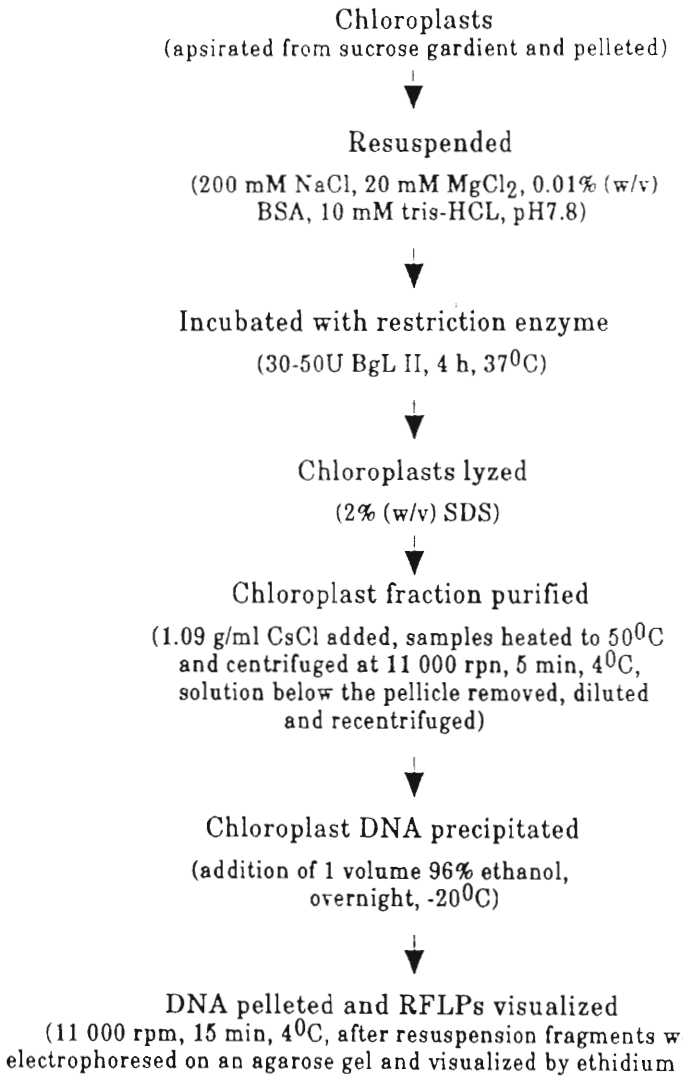
Zurawski, G., Clegg. M.T. and Brown., A.D.H. (1984). The nature of nucleotide sequence divergence between barley and maize chloroplast DNA. *Genetics* 106: 735-749.

Zurawski, G., Perrot, B., Bottomley, W. and Whitfeld, P.R. (1981). The structure of the gene for the large subuunit of ribulose 1,5-bisphosphate carboxylase from spinach chloroplast DNA. *Nuc.Acids Res.* 9: 3251-3269.

Appendix 1. Flow diagram representing the protocol developed for the extraction of chloroplasts from E.grandis S/N M6.



Appendix 2. Flow chart of the "in-organelle" digestion procedure used to resolve cpDNA RFLPs.



Appendix 3. Diagram to represent the DNA extraction and purification procedure optimized for use with the eucalypt species and cultivars.

Appendix 4. Diagrammatic representation of the procedures used to generate genomic DNA fingerprints for the eucalypts.