OPTIMISATION OF THE RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE FOR THE CHARACTERISATION OF SELECTED SOUTH AFRICAN MAIZE (*Zea mays* L.) BREEDING MATERIAL

by

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DECLARATION

I hereby declare that, unless specifically indicated,
this thesis is the result of my own investigation.

Nicola Rachel Edwards
December, 2000

PREFACE

The results presented in this thesis follow from a study which was carried out at the Department of Genetics, University of Natal, Pietermaritzburg, the Small Grain Institute, Agricultural Research Council, Bethlehem, and Mountain Home Laboratory, Mondi Forests, Hilton under the supervision of Dr Paul Shanahan.

The following oral paper and poster presentations have arisen from this study:-

1. Utilisation of Random Amplified Polymorphic DNA (RAPD) to characterise selected South African maize (*Zea mays L.*) genotypes. (Oral paper presented at the Eleventh South African Maize Breeding Symposium 15-17/3/94, Cedara) Warburton, N.R. and Shanahan, P.E.

2. RAPD identification of a potential marker for maize (*Zea mays* L.) leaf blight (*Helminthosporium* spp.) resistance. (Poster presented at the South African Plant Breeding Association Symposium 19-21/3/96, Potchefstroom) Warburton, N.R. and Shanahan, P. E

3. RAPD characterisation of seven selected South African maize (*Zea mays* L.) genotypes. (Poster presented at the Biotech SA'97 conference 21-24/1/97, Grahamstown) Edwards, N.R. and Shanahan, P.E.

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"I keep honest serving men
they taught me all I know
their names are What and
Why, and When and How,
and Where and Who" Rudyard Kipling

For my daughter Hannah Caroline Rachel Edwards

LIST OF ABBREVIATIONS AND DEFINITIONS OF SCIENTIFIC TERMS

A - absorbance reading (nm)

AFLP - amplified fragment length polymorphism

AP-PCR - arbitrarily primed PCR

a.i. - active ingredient

BAP - N6-benzylamino-purine

Callus - disorganised meristematic or tumor-like mass of plant cells formed under *in vitro* conditions

CTAB - cetyltrimethylammmonium bromide

DAF - DNA amplification fingerprinting

DNA - deoxyribonucleic acid

dNTP - deoxynucleotide triphosphate

EDTA - disodium ethylenediaminetetraacetic acid

Epi-genetic - non-genetic factors

EtBr - ethidium bromide

Explant - any portion of a plant which is placed *in vitro* on a nutrient medium

GLS - grey leaf spot

Haploid - having a single set of chromosomes/ monoploid

HTN - *Helminthosporium spp.* resistance gene

In vitro - culture of living material literally "in glass" i.e. on an artificial medium under aseptic conditions

KM - Kao and Michayluk

Leaf base - the lower third of the leaf blade and the leaf sheath

MS - Murashige and Skoog (1962) medium

MWM - molecular weight marker

MWM II - lambda DNA digested with *Hind*III (23130bp, 9416bp, 6682bp, 4361bp, 2322bp, 2027bp and 564bp)

MWM III - lambda DNA digested with *Hind*III and *Eco*R1 (21226bp, 5148bp, 4973bp, 4268bp, 3530bp, 2027bp, 1904bp, 1584bp, 1375bp, 947bp, 831bp and 564bp)

MWM V - pBR322 digested with *Hae*III (587bp, 540bp, 504bp, 458bp, 434bp, 267bp, 234bp, 213bp, 192bp, 184bp and 124bp)

m/v - mass per volume

NaCl - sodium chloride

nm - nanometers

NIL - near-isogenic line

OD - optical density

PAGE - polyacrylamide gel electrophoresis

PCR - polymerase chain reaction

PEG - polyethylene glycol

Plantlet - an apparently functioning connected shoot and root which develop either by the germination of a somatic embryo or by two separate events, shoot formation and subsequent root initiation

RAPD - Random amplified polymorphic DNA

RFLP - restriction fragment length polymorphism

rpm - revolutions per minute

SASEX - South African Sugar Association Experiment Station (Mt Edgecombe)

SDS - sodium dodecyl sulphate

SSR - simple sequence repeats/ microsatellite

Taq - *Thermus aquaticus* DNA polymerase enzyme

TEMED - N, N, N', N'-tetramethylethylenediamine

Tissue culture - cellular mass grown *in vitro* on solid media or supported with liquid media.

T_M - melting temperature

Tris-HCl - 2-amino-2-hydroxy-methyl-1,3propanediol

Tween - polyoxyethylene sorbitan monolaurate

U - unit

UV - ultraviolet

V - volts

v/v - volume per volume

w/v weight per volume

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Maize (*Zea mays* L.) is an important agronomic crop with the maize industry forming an important component of the South African economy. Considerable effort has been directed towards the genetic improvement of maize through both conventional breeding and biotechnology. Genotype identification by DNA fingerprinting is becoming an important activity in plant breeding. A widely used molecular based and relatively inexpensive method for DNA fingerprinting is the randomly amplified polymorphic DNA (RAPD) technique. The RAPD technique was tested in this study for its potential use in maize breeding programmes. Initial results using the technique showed a low degree of reproducibility, therefore both the DNA isolation and RAPD protocols were extensively optimised. DNA quality and quantity, and choice of *Taq* polymerase buffer were three of the variables found to be influential in ensuring reproducibility. The ability of the RAPD technique to characterise seven maize genotypes was evaluated. Sixty random oligonucleotide primers were screened. Forty two primers scored a total of 233 fragments (an average of 5.5 per primer), but not all primers gave reproducible profiles. Eighteen primers scored a total of 110 loci for the presence (1) and absence (0) of DNA fragments. RAPD markers were able to distinguish between all seven genotypes with five primers producing specific fragments for four genotypes. Genetic similarity matrices were calculated using two software programmes i.e. Genstat 5™ release 4.1 (1993) and PAUP (Phylogenetic Analysis Using Parsimony) 4.0 beta version (Swafford, 1998). Cluster analysis was used to generate dendrograms to visualise the genetic relationships of the seven maize genotypes (only minor differences were observed between the Genstat or PAUP method of analysis). Genetic diversity ranged from 0.62 to 0.96. The estimation of genetic relationship was in accordance with the presumed pedigree of the genotypes showing that the RAPD technique demonstrates potential for genome analysis of maize. The applicability of the technique for marker assisted selection was also evaluated. Near-isogenic lines (NILs) for leaf blight (*Helminthosporium* spp.) were screened for polymorphisms using a total of 120 primers. Ten primers identified polymorphisms between the NILs. Four primers produced five polymorphic fragments present in the resistant inbred K0315Y and absent in the susceptible inbred D0940Y. A small F_2 population of 14 individuals was produced by selfing the F_1 of a cross between K0315Y and D0940Y. To speed up the

generation time, the F_1 and F_2 plants were cultured by embryo rescue from 18d old harvested seed. One fragment of 627 base pairs produced by primer OPB-01 (5' GTTTCGCTCC 3') showed a 3:1 segregation in the small F_2 population and was considered putatively linked to the *HtN* gene for leaf blight resistance. This study shows that the RAPD technique does have application in maize breeding programmes.

Phenotypic identification of breeding lines and cultivars is a critical activity for plant breeding companies and seed laboratories. It allows them not only to control the propagation and marketing of their novel germplasm, but also to perform quality control on their products. Generally, phenotypic identification is based on morphological traits recorded in the field. These traits, however, seldom serve as unambiguous descriptors and may be challenged in parentage disputes. Additionally, phenotypic identification involves field trial evaluation of breeding material that is costly, labour intensive and subject to environmental influences (Hu and Quiros, 1991)

As an alternative to morphological markers, a number of laboratory methods including isozyme protein electrophoresis and high performance liquid chromatography of seed storage proteins have been successfully developed in the past two decades. However, a drawback of these techniques is the limited amount of polymorphisms they are able to detect among closely related genotypes (polymorphisms being differences between individuals at a molecular level). With the advent of molecular techniques, procedures based on structural differences in DNA have been proposed for cultivar identification. These include restriction fragment length polymorphisms (RFLPs) (Botstein, White, Skolnick and Davies, 1980; Beckman and Soller, 1983) and DNA fingerprinting involving hypervariable regions (Jeffreys, Wilson and Thein, 1985). Both of these techniques are able to detect numerous polymorphisms but are time consuming, costly and labour intensive procedures which are not suitable for high throughput applications (Hu and Quiros, 1991). Alternatively, genetic tests based on the Polymerase Chain Reaction (PCR) method (Mullis and Faloona, 1987; Saiki, Gelfand, Stoffel, Sharf, Higuchi, Horn, Mullis and Erlich, 1988a) are simple to perform but sequence information of the targeted DNA is required in order to design specific primers (Anderson and Fairbanks, 1990).

The discovery that single primers of arbitrary nucleotide sequence (Williams, Kubelik, Livak, Rafalski and Tingey, 1990; Welsh and McClelland, 1990) will amplify a specific set of arbitrary distributed loci in any genome (Williams *et al.*, 1990), laid the foundation for high

output of genetic markers that can be used for a variety of purposes. This recently developed and novel technique, termed Arbitrary-Primed PCR (AP-PCR) (Welsh and McClelland, 1990) or Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990), is based on the amplification of random DNA sequences in the genome produced by PCR, with oligonucleotide primers of arbitrary sequence. This method does not depend on DNA sequence information. The specific amplified products are inherited as dominant markers and segregate in Mendelian fashion, and these products can therefore effectively be used as genetic markers (Williams *et al.*, 1990). The advantages of this technique are: (i) the ability to detect extensive polymorphisms in both single copy and repetitive DNA; (ii) simplicity in that the analysis of RAPD markers requires no DNA cloning, Southern blotting or hybridisations with labelled radioactive probes, with markers being simply scored from an ethidium bromide stained agarose gel following electrophoresis (Echt, Erdahl and McCoy, 1992); and (iii) only small amounts of DNA are required which allows the analysis of DNA extracted from single seeds and young seedlings (Hu and Quiros, 1991). RAPD markers therefore, provide the geneticist with a tool for applications in gene mapping, population genetics, molecular systematics and marker assisted selection in plant and animal breeding. Data can be generated faster and with less labour than other methods. The process can be set up in a small laboratory and there is no need for radioactive isotopes (Williams *et al.*, 1990).

In the field of plant breeding, RAPD markers are being used extensively for the identification and characterisation of cultivars and clones (Hu and Quiros, 1991; Demeke, Adams and Chibber, 1992; Wu, Krutovskii and Straus, 1999), for the detection and analysis of genetic diversity (Haley, Afanador, Miklas, Stavely and Kelly, 1994; Marmey, Beeching, Harmon and Charrier, 1994; Harvey and Botha, 1996; Dvorak, Jordan, Hodge and Romero, 2000), and for the estimation of outcross rates (Fritsch and Reisberg, 1992). They are also proving useful for high density genetic mapping (Reiter, Williams, Feldman, Rafalski, Tingey and Scolnik, 1992). To a lesser extent the RAPD technique has been applied to problems of phylogeny and pedigree inheritance in F1 hybrids of maize (*Zea mays* L.) (Heun and Helentjaris, 1993), detection of interspecific gene introgression (Orozco-Castillo, Chalmers, Waugh and Powell,

1994), characterisation of somatic hybrids in potato (*Solanum tuberosum*) (Baird, Cooper-Bland, Waugh, De Main and Powell, 1992), the determination of parentage in maize (Welsh, Honeycutt, McClelland and Sobral, 1991) and apple (*Malus domestica*) (Harada, Matsukswa, Sato, Ishikawa, Niizeki and Saito, 1993), and pedigree analysis in sugarcane (*Saccharum spp.*) (Huckett and Botha, 1996).

Another major challenge for plant breeders is the identification of suitable sources of disease resistance genes, and their incorporation into adapted germplasm. Assays for disease resistance are often based on qualitative responses in the field and fail to identify specific resistance genes without the use of race testing (Barua, Chalmers, Hackett, Thomas, Powell and Waugh, 1993). DNA genetic markers form the basis of most current strategies for genome analysis, gene mapping and germplasm identification. In addition, it has been argued that genetic markers could be useful in breeding programmes since they would facilitate the accurate, rapid and early screening of progeny independent of environmental and ontogenic factors. A number of markers linked to dominant genes in important crops have been characterised (Martin, Williams and Tanksley, 1991). The linkage of polymorphisms to genetic regions of interest has been determined most successfully using pairs of backcross derived near-isogenic lines (NILs) (Paran, Kesseli and Michelmore, 1991). The principle being the identification of markers located in the linkage block surrounding the introgressed gene (Melchinger, Lee, Lamkey and Woodman, 1990).

Considerable effort has been directed towards the genetic improvement of maize through both conventional breeding and biotechnology (Shillito, Carswell, Johnson, Di Maio and Harms, 1989). Cultivated maize is a member of the grasses and cereals family *Gramineae*, tribe *Maydeae*, genera *Zea*, species *mays* L. Of all the domesticated plants, maize has been the most extensively studied genetically and cytogenetically. Maize is a monocotyledonous, cross-pollinating annual plant of tropical origin which is highly heterogenous (Neuffer, 1994). The maize plant is one of the most versatile of the domesticated plants worldwide. In general maize grain on a dry mass basis is composed of 77% starch, 2% sugar, 9% protein, 5% oil,

5% pentosan and 2% ash. It provides more animal feed in both grain and forage form and due to the chemical composition of the grain lends itself to more industrial uses than any other crop. The industrial uses of maize are animal feed; dry milling to produce cornflour, breakfast cereals, grits and oil; wet milling to produce starch, dextrose, syrup and oil; and distilling and fermentation to produce alcohols, organic acid and amino acids (Bansal, 1992).

Maize is one of the most widely grown agronomic crops and is of considerable significance to South African as well as world agriculture. Maize ranks as the worlds third most important grain crop after wheat (*Triticum aestivum*) and rice (*Oryza sativa*) (Shillito *et al.*, 1989). The maize industry in South Africa is a very important component of this country's economy, and in addition maize is the staple diet of many rural South Africans. Hence, commercial maize farmers and developing small farmers require robust good quality cultivars that are easy to grow and have drought and insect tolerance as well as disease resistance. This necessitates the breeding and release of high yielding genotypes that are well adapted to the South African environment. Significant increases in yield have been made in South Africa due to the persistant efforts of plant breeders in producing better cultivars with disease resistance. The major diseases that affect South African maize are leaf blight (*Helminthosporium turcicum*); grey leaf spot (*Cercospora zeae-maydis*); common rust (*Puccinia sorghi*); maize streak virus; root, stalk and cob rots (*Diplodia maydis, Gibberella zeae* and *Fusarium moniliforme*); tassel smut (*Sphacelotheca reiliana*); and boil smut (*Ustilago maydis*) (Barrow and Bell, 1988). Plant breeders want to be able to protect their germplasm as well as screen for the necessary disease resistance and other advantageous characteristics in their segregating populations once crosses are made using superior germplasm.

Grey leaf spot (GLS) is currently the most important leaf disease of maize in South Africa (Smit and Flett, 2000). The disease is caused by the fungus *Cercospora zeae-maydis* and was first identified in the Greytown area of KwaZulu Natal in the early 1990s (Ward, 1992). It has subsequently spread along the mist belt to neighbouring areas on the South African Highveld. The epidemiology of the disease under South African conditions is being studied and under

optimal environmental conditions yield losses of up to 60% can be experienced (Ward, Laing and Rijkenberg, 1997). This holds grave economic implications for the maize breeding industry in terms of yield reductions particularly in Kwazulu Natal where the disease is most prevalent. A technique that could easily identify the genotypic presence or absence of resistance to GLS (and other diseases) before planting or in young seedlings would be advantageous.

Host plant genetic resistance has the potential to be the most effective and economical method of preventing losses due to GLS. Resistant or tolerant maize hybrids have been reported in the United States but few if any can be classified as highly resistant (Thompson, Bergquist, Payne, Bowman and Goodman, 1987). Identification of additional GLS resistant inbreds and more information on inheritance patterns will help facilitate the development of elite resistant hybrids in South Africa. Resistance to GLS is a highly heritable, quantitative trait with genes that act primarily in an additive manner, with these results being complicated by genotype-environment interactions (Saghai-Maroof, Van Scoyoc, Yu and Stromberg, 1993).

It has been proposed that RAPD markers may be useful for the characterisation of introgressed single chromosome segments (Devos and Gale, 1992; Schachermayr, Siedler, Gale, Winzeler, Winzeler and Keller, 1994). In maize, many resistance genes have been located to specific chromosomes or chromosome arms by means of traditional breeding methods, but less have been localised at the molecular level and only a very small number of these have been tagged using RAPD markers (Simcox and Bennetzen, 1993). DNA RAPD analysis would provide a potentially powerful tool for identifying markers linked to loci responsible for controlling resistance to maize diseases. As this work has not been done with South African genotypes, an easily scored marker linked to the gene(s) conferring a particular resistant phenotype would therefore represent an important tool for plant breeders.

Taking the above into consideration, the aim of this study was to assess the utility of the RAPD protocol (using DNA isolated from selected maize genotypes) in terms of reproducibility (Chapter 3). In addition, using a total of 11 South African developed maize

genotypes, two further studies were undertaken to test the potential of the RAPD technique in a maize breeding program. Firstly, the level of polymorphism revealed, and inheritance of marker bands were used to determine whether seven selected maize genotypes could be identified using the Random Amplified Polymorphic DNA (RAPD) technique (Chapter 4), and secondly two different pairs of genotypes were used to investigate whether molecular markers linked to resistance genes could be identified using the RAPD technique (Chapter 5). The term genotype is used in the broadest sense throughout this thesis to encompass the maize inbreds, single crosses, backcross and F_2 populations characterised in this study.

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

A DNA fingerprint is the display of a set of DNA fragments from a specific DNA sample. When DNA fingerprints of related samples are compared, common bands as well as different bands will be observed. When these differences are observed in an otherwise identical fingerprint, such differences are referred to as DNA polymorphisms. DNA fingerprinting can therefore be used to visualise DNA polymorphisms between samples. These fingerprints may be used as a tool for determining the identity of specific DNA samples or to assess the relatedness between samples. Fingerprints are also used as the source for molecular markers to generate linkage maps or to identify molecular markers linked to phenotypic traits and or genetic loci (Lee, 1995).

Many DNA fingerprinting techniques have been developed in the past few years and are generally based on one of two strategies: (i) classical, hybridisation-based fingerprinting e.g. Restriction Fragment Length Polymorphisms (RFLPs), which involves the cutting of genomic DNA with restriction endonucleases followed by electrophoretic separation of the DNA fragments that are detected by Southern Hybridisation with probes targeted to hypervariable regions of DNA (Jeffreys *et al.*, 1985). This restriction cleavage allows the detection of polymorphisms in DNA fingerprints, which can result from alterations in the DNA sequence including mutations abolishing or creating a restriction site, and insertions, deletions or inversions between two restriction sites; and (ii) PCR-based fingerprinting. This involves the *in vitro* amplification of particular DNA sequences using specific or arbitrary primers and a thermostable polymerase. Amplification products are separated by electrophoresis and detected by staining or use of labelled primers. Techniques in this category included Random Amplified Polymorphic DNA (RAPD), DNA Amplification Fingerprinting (DAF) and Arbitrary Primer PCR (AP-PCR) (Welsh and McClelland, 1990; Williams *et al.*, 1990; Caetano-Anollés, Bassan and Gresshoff, 1991).

1.2 Molecular marker systems

The association between markers and traits was first reported by Sax (1923). Since then much attention has been given to the potential uses of markers in practical plant breeding programmes (Smith, Smith and Wall, 1991b). Traditionally, markers based on morphological differences between individuals are used, but not all types of markers are suitable for breeding applications. Morphological and cytological markers are typically associated with deleterious effects or are difficult to observe in breeding populations and are thus of little use (Tanksley, Young, Paterson and Bonierbale, 1989). Molecular markers in contrast are free from such associations i.e. genetic markers are not influenced by the environment and may be more precisely linked to desirable traits and monitored in successive generations (Lee, 1995).

1.2.1 Isozyme analysis

The development and extensive use of isozyme and other biochemical markers represent a significant improvement on morphological markers as they characterise greater genetic diversity (Doebley, Goodman and Stuber, 1981; Tanksley *et al.*, 1989). Isozyme analysis is one of the methods of generating biochemical markers. It has been used extensively for characterisation of genetic resources in several plant species (Smith, Ordman and Wall, 1991a). Isozyme analysis results from translation events and relies on the detection of polymorphisms among isolated enzymes that differ in their electrophoretic mobility (Helentjaris, Slocum, Wright, Schaefer and Niehuis, 1986; Tanksley *et al.*, 1989). It has the advantage of being relatively rapid and inexpensive compared to other methods, but its usefulness in breeding programmes is limited by the lack of polymorphism, the relatively small number of loci and alleles required for analysis, and the need to use a variety of methods for marker detection (Andersen and Fairbanks, 1990).

1.2.2 Restriction fragment length polymorphisms (RFLPs)

Compared with morphological characteristics and biochemical (non-DNA based) polymorphisms, the DNA genome provides a significantly more powerful source of genetic polymorphism. The

development of markers based on DNA probes has introduced a new dimension to the characterisation of genetic resources. Restriction fragment length polymorphism (RFLP) overcomes some of the problems of isozyme analysis. The RFLP procedure relies on the detection of genetic polymorphism in DNA fragments and provides substantially more polymorphism than isozyme or other protein markers. The number of polymorphisms that can be generated is practically unlimited and the technique may be standardised regardless of marker and species (Botstein *et al.*, 1980).

The RFLP procedure requires plant DNA extraction, DNA digestion with a restriction enzyme, separation of the fragments on an agarose gel, Southern blot transfer of DNA on to a membrane, hybridization of a probe to the membrane-bound DNA, and detection of the probe using autoradiography (Botstein *et al.*, 1980). An alternative to one of the disadvantages of RFLP markers, namely the use of radioactive probes, is provided by the availability of sensitive nonradioactive chemiluminescent detection systems (Ragot and Hoisington, 1993).

Differences in DNA between individuals may result from sequence differences of nucleotides in homologous regions. The term RFLP describes this variation (King, Figdore, Helentjaris, Neuhausen, Niehuis, Slocum, Suzuki and Wright, 1990). RFLPs are inherited in simple Mendelian fashion, are co-dominant in that heterozygotes can be distinguished from either homozygote (different bands are produced for the dominant and recessive characters) and provide complete genetic information at a single locus and therefore can function as genetic markers (Botstein *et al.*, 1980).

RFLPs have been used to generate extensive genetic maps of several important plant species including maize (Helentjaris *et al.*, 1986; Hoisington and Coe, 1990) within several months (as opposed to several years with morphological markers) and have been used to increase the efficiency of plant breeding by association of molecular markers with agronomic traits for marker assisted selection. New genetic markers can be identified from an existing RFLP map, but there is little information available on allele sizes and the distribution of markers in the germplasm.

The potential to generate a large number of RFLP markers has provided an efficient means for exploring the evolutionary relationship between species and populations (Helentjaris *et al.*, 1986; Bonierbale, Plaisted and Tanksley, 1988), for identifying cultivars and genotypes (Melchinger, Messmer, Lee, Woodman, Lamkey, 1991; Smith *et al.*, 1991a), for mapping genes that control qualitative (Niehuis, Helentjaris, Slocum, Reggero and Schaeffer, 1987; Young, Zamir, Ganal and Tanksley, 1988) as well as quantitative traits (Edwards, Helentjaris, Wright and Stuber, 1992), and as starting points for genome walking and jumping experiments designed to isolate specific loci based on map positions (Rommens, Iamuzzi, Keram, Drumm, Melmer, Dean, Rozmahel, Cole, Kennedy, Hidakain, Zsiga, Buchwood, Tsui and Collins, 1989).

1.2.3 Polymerase chain reaction-based genetic markers

The development of polymerase chain reaction (PCR) technology (Saiki *et al.*, 1988a) has provided new tools to detect polymorphism. The PCR is an *in vitro* DNA amplification procedure, which involves two oligonucleotide primers that flank the DNA segment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences and extension of the annealed primers with DNA polymerase (Saiki *et al.*, 1988a). These primers are designed to hybridise to opposite strands of the target sequence and are oriented so DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of that DNA segment. Since the extension products are also complementary to and capable of binding primers, successive cycles of amplification continue to double the amount of DNA synthesised in the previous cycle. This results in an exponential accumulation of the specific target fragment, approximately 2n, where n is the number of cycles of amplification performed. The discovery of a thermostable DNA polymerase isolated from the bacterium *Thermus aquaticus (Taq)* substantially improved the performance of this procedure (Saiki, Gyllensten and Erlich, 1988b).

PCR-based genetic markers are generated from specific primers determined from known DNA sequences. This includes allele specific primers and primers flanking minisatellites and microsatellites (Paran and Michelmore, 1993). These powerful technologies allows the

assessment of genetic variability, determining varietal purity and assessing germplasm (Weeden, Timmerman, Hemmat, Kneen and Lodhi, 1992). Weining and Langridge (1991) used PCR with primers (15-25 bp) based on the nucleotide sequence of a known alpha-amylase gene sequence for the identification and mapping of polymorphism in wheat cv. Chinese Spring lines. The widespread use of these types of markers is, however, limited as they rely on predetermined variation or on the genomic distribution and organisation of tandem repeats. Many minisatellites alleles are too large to be amplified, and microsatellites are only sparsely distributed in plant genomes (Weber, 1991).

1.2.3.1 Random amplified polymorphic DNA markers

A novel procedure for the detection of DNA polymorphisms which overcomes these difficulties was developed more recently. The arbitrary-primer polymerase chain reaction (AP-PCR) of Welsh and McClelland (1990) (discussed in Section 1.2.3.4) and the random amplified polymorphic DNA (RAPD) procedure of Williams *et al.* (1990) are modifications of the basic PCR technique and produce easily observable polymorphic markers. Polymorphisms between individuals result from changes in either the sequence of the primer binding site (e.g. point mutations), which prevent stable association with the primer, or from changes which alter the size or prevent the successful amplification of a target DNA (eg. insertions, deletions, inversions) and are visible as the presence or absence of a particular band. Size variants are only rarely detected and individual amplification products represent only one allele per locus. RAPD markers are dominant markers i.e. the homozygous genotype for the dominant-allele "band presence " (+/+) and heterozygous genotype (+/-) cannot be distinguished from each other. Dominant markers provide less information per locus than co-dominant markers but this limitation can be overcome by multilocus estimation using a large number of dominant markers (Gaiotto, Bramucci and Grattapaglia, 1997). RAPDs are dominant markers inherited in Mendelian fashion and can be generated without any prior knowledge of the target DNA sequence (Welsh and McClelland, 1990; Williams *et al.*, 1990; Welsh and McClelland, 1991).

A single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template, such as a plant genome (Williams *et al.*, 1990). Each amplification product is derived from a region of the genome that contains two short DNA segments with some homology to the primer. These segments must be present on opposite DNA strands and be sufficiently close to each other to allow DNA amplification to occur. Theoretically the number of amplified fragments generated by PCR depends on the length of the primer and the size of the target genome. This is based on the probability that a given DNA sequence (complementary to that of the primer) occurs on opposite strands in the genome, in opposite direction within a distance that is readily amplifiable by PCR. For most plants, primers that are nine to 10 nucleotides long are predicted to generate on average, two to 10 amplification products. The nucleotide sequence of each primer is chosen randomly with the constraints that it contains between 50 and 80 % guanine (G) and cytosine (C), because the G + C content of a primer should be similar to the G + C content to the analysed genome (this will maximise the frequency of binding sites and hence amplification products) (Hadrys, Balick and Schierwater, 1992). The primer should also not contain palindromic sequences of six or more nucleotides (Waugh and Powell, 1992). The short primers and low annealing temperatures ensure that several sites, randomly distributed throughout the genome give rise to amplification products. The presence of each amplified product therefore identifies complete or partial nucleotide sequence homology between the genomic DNA and the oligonucleotide primer at each end of the amplified product site. These products are easily separated by standard electrophoretic techniques and visualised by ultraviolet illumination of ethidium bromide stained agarose gels.

Available data indicates that RAPD technology is suitable for studies of genetic diversity and DNA fingerprinting (Welsh and McClelland, 1991; Vierling and Nguyen, 1992; Wilde, Waugh and Powell, 1992), for rapid identification of markers linked to important plant genes (Giovannoni, Wing, Ganal and Tanksley, 1991; Klein- Lankhorst, Vermunt, Weide, Liharska and Zabel, 1991; Martin *et al.*, 1991; Michelmore, Paran and Kesseli, 1991) and for the construction of high density genetic maps (Reiter *et al.*, 1992). Hu and Quiros (1991) studied RAPD markers of individual plants of broccoli and cauliflower (*Brassica oleracea* L.) cultivars and found all markers were consistent within each cultivar but varied from cultivar to cultivar.

Isabel, Tremblay, Michaud, Tremblay and Bousquet (1993) found no variation when RAPDs were used to evaluate the genetic integrity of a somatic embryogenesis derived population of black spruce (*Picea mariana*) whereas Yang, Tabei, Kamada, Kayano and Takaiwa (1999) detected somaclonal variation in cultured rice cells using digoxigenin-based RAPDs. More recently RAPDs have been used to distinguish between selected common bean (*Phaseolus vulgaris* L.) cultivars from three African countries: Tanzania, Uganda and Ethiopia (Mienie, Herselman and Terhlanche, 2000).

Other techniques that scan for DNA sequence divergence can be applied to individual amplification products derived from RAPD bands e.g. single-strand conformational polymorphism assays (SSCP) which rely on secondary and tertiary structure differences between denatured and rapidly cooled amplified DNA fragments that vary slightly in their DNA sequence. Different SSCP alleles are resolved on non-denaturing acrylamide gels, usually at low temperatures. The ability to resolve alleles according to size is dependant on the electrophoresis conditions i.e. gel concentration, etc. (Orita, Suzuki, Sekiva and Havashi, 1989). Denaturing gradient gel electrophoresis (DGGE) may also be used. This partially resolves denatured double-stranded DNA in precisely defined conditions of temperature and denaturation concentration. Different alleles denature to various extents under such conditions and migrate differently on DGGE acrylamide gels (He, Ohm and Mackensie, 1992; Myers, Silva, Procunier and Little, 1993).

Once a marker linked to a trait of interest is found, the RAPD assay may be turned into a more reproducible PCR-type assay based on secondary sequence. This may be done by allele-specific PCR (AS-PCR) where specific alleles or DNA sequence variants at the same locus are amplified. Specificity is achieved by designing one or both PCR primers so that they partially overlap the sites of sequence difference between the amplified alleles (Wu, Ugozzoli, Pol and Wallace, 1989). The allele specific ligation technique allows the discrimination of two alleles at a locus by providing two short synthetic oligonucleotides that ligate adjacent to each other on an amplified DNA fragment in the presence of DNA ligase. If one of the alleles contains a mutation overlapped by the 3' end of one oligonucleotide, its ligation to the oligonucleotide-bound 3' end is prevented. To deduce the identity of the unknown allele, differentially labelled oligonucleotide

pairs may be designed for each allele, and their ligation efficiency compared in the presence of the unknown allele.

Other PCR-based markers derived from RAPD markers are sequence characterised amplified regions (SCARs) where the amplified RAPD products are cloned and sequenced (Paran and Michelmore, 1993). This sequence is then used to design 24-mer specific oligonucleotide primers for each end of the RAPD fragment. A pair of SCAR primers then result in the amplification of a single major band, the same size as the cloned RAPD fragment, which represents a single, genetically defined locus. They may contain high-copy, dispersed genomic sequences within the amplified region. Polymorphism is retained as presence versus absence of the amplification product or appear as length polymorphisms that convert dominant RAPD loci into co-dominant SCAR markers. SCARs are advantageous over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions and they can potentially be converted into co-dominant markers. In addition SCARs provide information on the molecular basis of the polymorphism detected by RAPD markers. RAPD polymorphisms could be caused by differences in nucleotide sequence at the priming sites or by structural rearrangements within the amplified sequence. RAPD amplification is therefore likely to be initiated from genomic sites that do not perfectly match the primer sequence (Paran and Michelmore, 1993).

SCARs are similar to sequence tagged sites (STS) (Olsen, Hood, Cantor and Botstein, 1989). SCARs share the advantages of STS but are distinct in that they are primarily defined genetically and therefore can be used both as physical landmarks in the genome and as genetic markers. In addition, since SCARs contain repetitive DNA sequences within the amplified region as they are analysed by PCR their uniqueness is determined by the sequence and spacing of the primer sequences and not by hybridization (Paran and Michelmore, 1993).

1.2.3.2 Microsatellites

Another PCR-based methodology which requires the use of more specific primers namely microsatellite and telomere sequences is also available. Microsatellite repeats (also known as

simple sequence repeats (SSRs)) are hypervariable DNA sequences consisting of arrays of basic repeat units of two to eight base pairs (bp) probed with labelled oligonucleotides (Rus-Kortekaas, Smulders, Arens and Vosman, 1994). Microsatellites bind to mono, di-, tri- and tetra-nucleotide repeat sequences; in particular the dinucleotide repeats $(AC)_n$, $(AG)_n$ and $(AT)_n$, have been shown to be abundant, highly polymorphic and occur randomly dispersed in the DNA in all eukaryotic genomes tested (Jeffreys *et al.*, 1985, Weising, Driesel, Kahl, Zischler and Epplen, 1989). Akkaya, Bhagwat and Cregan (1992) found that simple sequence repeats are very polymorphic and that AT repeats are more numerous than AC repeats. Simple sequence repeats are analysed by PCR amplification of a short genomic region containing the repeated sequence, and size estimation of the repeat length by gel separation. Only a small quantity of DNA is required and agarose gels can be used for band separation but resolution of all alleles more often requires the use of acrylamide gels. A microsatellite fingerprint is made by hybridisation of a labelled probe to a Southern blot. The fingerprint obtained may contain numerous reproducible polymorphisms in one lane but the amount of work involved makes detection of microsatellite containing DNA more time consuming than amplification with RAPDs (Rafalski and Tingey, 1993; Rus-Kortekaas *et al.*, 1994).

The screening of enriched or non enriched genomic libraries is an expensive method (Rafalski, Vogel, Morgante, Powel, Andre and Tingey, 1996). Therefore, a less expensive enrichment technique known as inter-simple sequence repeat (ISSR) has been developed. This is a variation of the RAPD-PCR technique and it targets regions in the genome rich in microsatellite motifs (Zietkiewikz, Rafalski and Labuda, 1994). PCR fragments are cloned into commercially available plasmid T vectors (e.g. pGEM®) after which they are screened for SSR containing inserts and then sequenced to construct specific primers for SSR amplification. This has been used to develop SSR markers in *Eucalyptus sp.* (commonly referred to as gum trees) (van der Nest, Steenkamp, Wingfield and Wingfield, 2000). Despite the advantages of this technique, it is not being widely used yet as it is necessary to develop SSRs for each plant species separately. Universal cross species amplifying markers are thus being researched. Primer sequence conservation in the nuclear genome has been found in closely related species but more workable primers are obtained from the chloroplast genome due to the high conservation of chloroplast

genomes during evolution. These universal markers could then be developed for seed or pollen due to the uniparental transmission of chloroplasts (Breyne, Boerjan, Gerats, van Montague and van Gysel, 1997).

Telomere primers detect repeat areas of the telomeric regions of chromosomes (Kolchinsky and Gresshoff, 1994). Both of the target regions to which these primers bind are highly variable, i.e. PCR carried out in the presence of microsatellite and telomere sequences leads to the production of highly polymorphic fragment profiles (Tautz, Trick and Dover, 1989). Consequently these sequences have been successfully used to fingerprint cultivars of tomato (*Lycopersicon esculentum*) (Vosman, Arens, Rus-Kortekaas and Smulders, 1992) and soybean (*Glycine max* L.) (Kolchinsky and Gresshoff, 1994).

1.2.3.3 Cleaved amplified polymorphic sequence

Another assay recently developed is the cleaved amplified polymorphic sequence (CAPS) which has some of the advantages of the RFLP assay but not the disadvantages of the Southern blot analysis (Rafalski and Tingey, 1993). In this approach, partial DNA sequence information for the locus of interest is required to create a set of PCR primers which are used to amplify a segment of DNA at the locus using samples from several different individuals. The amplified bands are then digested with a number of restriction enzymes to identify RFLPs among the individuals. Because of the limited size of the amplification fragment, the polymorphisms may be more difficult to identify than RFLPs, but the technique has the advantage that many restriction enzymes can be tested on the amplified DNA.

Table 1.1 Properties of the different genetic marker systems (Rafalski and Tingey, 1993).

	RFLP	**RAPD**	**Microsatellites**	**CAPS**
Principle	endonuclease restriction Southern blotting hybridization	PCR amplification with random primers	PCR of simple sequence repeats	endonuclease restriction of PCR products
Type of polymorphism	single base changes insertions deletions	single base changes insertions deletions	changes in length of repeats	single base changes insertions deletions
Genomic abundance	high	very high	medium	high
Level of polymorphism	medium	medium	high	medium
Dominance	co-dominant	dominant	co-dominant	co-dominant
Amount of DNA required	2-10 µg	10-25 ng	50-100 ng	50-100 ng
Sequence information required	no	no	yes	yes
Radioactive detection required	yes/no	no	no	no
Costs	medium/high	low	high	medium/high

RFLP Restriction fragment length polymorphism
RAPD Random amplified polymorphic DNA
CAPS Cleaved amplified polymorphic sequence
DNA Deoxyribonucleic acid
PCR Polymerase chain reaction

1.2.3.4 Multiple arbitrary amplicon profiling

Multiple arbitrary amplicon profiling (MAAP) is an acronym which was suggested by Caetano-Anollés, Bassan and Gresshoff (1992) to encompass three techniques: random amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR) and DNA amplification fingerprinting (DAF). Each generate DNA profiles of varying complexity primarily defined by the sequence of the arbitrary primer used to direct amplification. RAPD describes the polymorphisms and AP-PCR and DAF describe the strategy used. The MAAP acronym therefore encompasses each variation of the overall strategy i.e. describes the underlying characteristics, the multiple, arbitrary nature of target sites and the amplification of a range of characteristic DNA products. Multiple arbitrary amplicon profiling therefore uses one or more oligonucleotide primer of

arbitrary sequence to initiate DNA amplification and generate characteristic fingerprints from anonymous genomes or DNA templates (Caetano-Anollés *et al.*, 1992). DNA amplification fingerprinting uses the simplest and least demanding amplification conditions and the shortest oligonucleotide primers of arbitrary sequence, which direct the enzymatic amplification of arbitrary stretches of DNA to generate complex but characteristic DNA fingerprints (Caetano-Anollés *et al.*, 1991; Bassam, Caetano-Anollés and Gresshoff, 1992). DNA amplification fingerprinting markers have been used for the fingerprinting of bacteria (Bassam *et al.*, 1992), to identify yeast artificial chromosomes (Caetano-Anollés, 1994; Kolchinsky and Gresshoff, 1994) and to study differential RNA populations that have been reversed transcribed (Rollinson and Stothard, 1994).

Table 1.2 Characteristics of the different MAAP techniques (Caetano-Anollés, 1994).

Characteristics	**DAF**	**AP-PCR**	**RAPD**
Primer length (nt)	5-15	18-32	9-10
Primer concentration (μM)	3-30	1-10	0.3
DNA concentration (ng μl^{-1})	0.01-1	0.1-5	1
Primer/template ratio	5-50 000	1-500	< 1
Annealing temperature (°C)	10-65	35-50	35-42
Amplification stringency	low to high	high to low	low
DNA separation	PAGE	PAGE	agarose
Visualisation	silver staining	radiolabeling	EtBr staining
Product resolution	high	intermediate	low
Number of products	10-100	3-50	1-10

DAF DNA amplification fingerprinting

AP-PCR Arbitrarily primed - polymerase chain reaction

RAPD Random amplified polymorphic DNA

nt number of nucleotides

DNA Deoxyribonucleic acid

PAGE Polyacrylamide gel electrophoresis

1.2.3.5 Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) (Vos, Hogers, Bleeker, Reaijans, van der Lee, Hornes, Friters, Pot, Peleman, Kuiper and Zabeau, 1995) also known as selective restriction fragment amplification (SRFA) (Zabeau and Vos, 1993), is another recently developed technique for fingerprinting genomic DNA. AFLPs are based on the selective amplification of a subset of genomic restriction fragments using PCR. DNA is digested with two restriction endonucleases and double stranded DNA adapters are ligated to the ends of the DNA fragments to generate template DNA for amplification. Thus the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. AFLPs have proven to be a highly reliable and reproducible technique with the DNA polymorphisms identified typically inherited in Mendelian fashion and therefore may be used for typing, identification of molecular markers and mapping of genetic loci (Vos *et al.*, 1995). AFLPs have been used for studying the genetic relationships of lettuce (*Lactuca spp.*) (Hill, Witsenboer, Zabeau, Vos, Kesseli and Michelmore, 1996); estimating the genetic variability in a *Eucalyptus urophylla* breeding population (Gaiotto and Grattapaglia, 1997); and for the genetic mapping of monterrey pine (*Pinus radiata*) (Cato, Corbett and Richardson, 1999).

The AFLP method has a high multiplex ratio i.e. a high number of markers generated in a single reaction (Rafalski *et al.*, 1996). Dominant and co-dominant markers can be analysed by scoring the different alleles of a particular locus. However, this is not obvious, implying that the information content is rather low (information content being the effective number of alleles that can be detected per marker in a set of individuals (Breyne *et al.*, 1997)). Another technique termed Selective Amplification of Polymorphic Loci (SAMPL) uses one AFLP primer and one self anchoring microsatellite to amplify random genomic sequences containing SSRs, combining the advantages of both AFLPs and SSRs to a single assay.

1.3 Choice of method

The choice of a particular method depends on the specific application it will be used for, and especially important is the kind of variation that each method will detect. The most common DNA fingerprint technologies differ substantially in (i) complexity of technological procedures; (ii) required amount of DNA; (iii) sequence information needed for a genome being scanned; (iv) analytical power of assigning genotypes relatedness; (v) expense in terms of labour and materials; and (vi) diversity of applications (Powell, Morgante, Andre, Hanafey, Vogel, Tingey and Rafalski, 1996). If the objective is to fingerprint or assess genetic diversity methods with a high multiplex ratio, AFLPs are suitable. They are also used for the building of genome maps, fine mapping of mutations and positional cloning which identifies markers to particular traits (Breyne *et al.*, 1997). Certain aspects of population biology (geneflow and paternity analysis) and plant breeding (hybridity determination), however, require methods with a high information content e.g. microsatellites. In this context, an intermediate method such as RAPD fingerprinting seems to have wide potential for applications in applied plant breeding as it requires the least in terms of technology, labour and expenses. When a network of European laboratories tested the reproducibility of RAPDs, AFLPs and SSR markers in plants (Jones, Edwards, Castaglione, Winfield, Sala, van de Wiel, Bredemeijer, Vosman, Matthes, Daly, Brettschneider, Bettini, Buiatti, Maestri, Malcevschi, Marmiroli, Aert, Volckaert, Rueda, Linacero, Vazquez and Karp, 1997), they found that RAPDs were the most difficult to reproduce between laboratories but that they were reproducible within laboratories. AFLPs revealed a single band difference and the SSR alleles were amplified in all the laboratories but small differences in sizing were observed. When Powell *et al.* (1996) compared RFLPs, RAPDs, AFLPs and SSRs for soybean germplasm analysis using cultivated and wild soybean, a high correlation was observed between RFLPs, AFLPs and SSRs. However, comparisons involving cultivated soybean only resulted in lower correlations between the marker systems, with RAPDs and AFLPs being more closely correlated.

1.3.1 Comparison of RAPDs and RFLPs

Restriction fragment length polymorphisms and RAPDs provide different types and amounts of genetic information. The most important advantage of RFLPs over RAPDs for evaluating germplasm is that the restriction fragment hybridises to a particular probe, is homologous and occurs in a specific genome. Maize RFLP probes are selected to represent single-copy genomic sequences (Helentjaris *et al.*, 1986) and therefore generally provide information on a single marker locus. RAPD markers, on the other hand, provide information at many loci (Welsh *et al.*, 1991) although large differences are observed across primers and species. Therefore, the benefit gained from using multiple-locus markers for fingerprinting or for determining relationships among genotypes is lost when monitoring genetic changes at specific loci as in the case of backcrossing or marker-assisted selection. RFLPs are co-dominant markers, inherited in Mendelian fashion (Botstein *et al.*, 1980), whereas RAPDs are usually dominant markers, i.e. the presence of a given RAPD band does not distinguish whether its respective locus is homozygous or heterozygous, thus preventing accurate detection of heterozygotes (Williams *et al.*, 1990). However, if it is necessary to identify heterozygous regions, two closely linked RAPD markers each amplified from a different parent may be used as a pair (Williams, Hendey, Rafalski and Tingey, 1993). The presence of a single RAPD band is diagnostic for a sequence totalling 18 bp in the target genome (nine bases at each end of the genomic sequence that is amplified). Hence an average of five amplified bands per primer means that each primer is diagnostic for five x 18 = 90 bp in the template. This is in contrast to a RFLP, which is diagnostic for only 12 bp per probe-enzyme combination.

RFLP methodology has been shown to be costly, time consuming and technically challenging, especially in species with large and complex genomes (Beckmann and Soller, 1983; Gale, Chao and Sharp, 1990). RAPD technology is more amenable to automation which increases the output with lower labour costs. Because RAPDs are based on PCR they require considerably less DNA than RFLPs (Welsh *et al.*, 1991). For maize, between 10 and 30 ng of DNA is needed per RAPD reaction, which is 30-200 times less than the amount needed for RFLP analysis (10 µg of DNA for five to 10 hybridisations). RAPDs therefore allow the use of much simpler procedures for

isolation of genomic DNA (Williams, Rande, Nair and Mohan, 1991). However, high purity of DNA is required and the samples must be free from contamination. RAPDs are detected more easily than RFLPs and can be comparable with RFLPs even in genomes with high levels of heterozygosity (Williams *et al.*, 1990; Hu and Quiros, 1991).

Ragot and Hosington (1993) conducted a study comparing RFLP and RAPD genotyping costs using three molecular marker protocols, viz: chemiluminescent RFLPs, radioactivity based RFLPs and RAPDs. RAPDs proved to be the most economical markers for small to medium sized projects with RFLPs more suitable for larger projects. The work of Martin *et al.* (1991) suggested that RFLPs also be used for large studies thus collaborating these findings; however, Rafalski and Tingey (1993) suggest that RFLPs are not suitable for large-scale agricultural applications. The relative merits of RAPDs appear to depend on the cost of *Taq* DNA polymerase which can account for up to 30% of the cost of each reaction. In maize several hundred public RFLP probes are available at no charge to researchers. Therefore the estimations of RFLP genotyping costs does not include the costs of probe development. However, probes for RFLPs must typically be developed for each species or group of related species when there is no RFLP history (Ragot and Hosington, 1993).

RAPDs are advantageous over RFLPs for characterisation of plant material because they are easier to detect than RFLPs. It is necessary though to proceed cautiously when interpreting RAPD data. RAPD markers give similar information to RFLP data when comparing genetic relationships within a species. However, interspecific genetic relationships based on RAPDs do not compare with those of RFLP markers, and this appears to be due to the non-homology of identically sized fragments assumed to be homologous. This occurs when scoring closely related species, where there is the possibility of errors occuring if fragments are scored as homologous. This may occur if genetically diverse accessions within species are compared. Thorman, Ferreira, Carmargo, Tivang and Osborne (1994) published that further investigation on the magnitude of this problem needs to be undertaken, before conducting a large scale study on germplasm relationships.

RAPD techniques may also be combined with RFLPs to increase the efficiency of genetic marker analysis. A RAPD marker known to be polymorphic may be labelled and used as a probe for RFLP analysis, thus eliminating the need for recombinant DNA cloning of probes in bacteria (Williams *et al.*, 1993). Polymorphic RAPD markers used as probes typically reveal RFLPs that are likewise polymorphic.

1.3.2 Comparison of RAPDs and microsatellites

Microsatellite-containing DNA is repetitive DNA that has been found to be highly polymorphic (Tautz, 1989). RAPDs on the other hand are thought to be generated randomly throughout the genome i.e. not exclusively in repetitive DNA. This implies a lower rate of polymorphism compared with microsatellites, but this has not been shown directly. Several characteristics of RAPDs may be taken to indicate that it is not known what sequences lead to amplified bands. Firstly, RAPD patterns always contain up to 10-15 bands, never more; secondly, addition of a second primer does not lead to more bands (Klein-Lankhorst *et al.*, 1991), and certainly not to a doubling of the number of bands amplified as would be expected with two unrelated primers; thirdly, the amplification process does not depend on the size of the genome, since both prokaryotic DNA and eukaryotic DNA resulted in comparable banding patterns despite a 100-fold or more difference in genome size (Rus-Kortekaas *et al.*, 1994).

When RAPD and microsatellites are compared in the same material, microsatellites are tandemly repeated DNA sequences that are mostly polymorphic. RAPD and microsatellite/telomer primers detect two different types of DNA that differ in their degree of variability and, therefore, their level of informativeness (Rafalski *et al.*, 1996). RAPDs detect a higher degree of band sharing between varieties suggesting that this methodology is more suitable for the determination of genetic relationships between cultivars. By contrast, microsatellite probes detect far more polymorphic loci and are therefore more appropriate for the identification of cultivars because more variation can be detected. The choice of whether to use RAPD or microsatellites to distinguish between cultivars or species consequently depends on the amount of genetic variation expected and the questions to be answered. The "slipping" of DNA polymerase has been

suggested by Tautz *et al.* (1989) to explain the high mutation rate of microsatellite DNA. This may be an exclusive phenomenon for short tandemly repeated sequences.

Both methods have drawbacks: RAPDs because of the need to test reproducibility rigorously and microsatellite fingerprinting because of the time and amount of work involved (Rus-Kortekaas *et al.*, 1994). A good technical solution would be to tag microsatellite bands with specific PCR primers known as sequence tagged sites (STS) (Olson *et al.*, 1989). A RAPD band may also be sequenced and tagged (i.e. sequence characterised amplified regions) providing a combination of speed and high levels of reproducible polymorphisms (Paran and Michelmore, 1993).

1.3.3 Comparison of RAPDs and AFLPs

Both RAPDs and AFLPs are PCR-based methods which generate dominant markers that do not require prior sequence data, with AFLPs able to selectively amplify sequences from a large number of restriction fragments. The AFLP assay under optimised conditions is extremely reproducible and generates a large number of polymorphic markers. Careful selection of the primer combination allows the generation of a large set of polymorphic markers in a single gel lane. Interpretation of data when markers across gels need to be scored is easier and more reliable with AFLPs compared to RAPD markers (Gaiotto *et al.*, 1997), although Eriksson (1997) states that the technique is limited in practice to the resolving power of the gel electrophoresis system. Reactions that generate more than 50-100 fragments are generally too complex to analyse. AFLPs function best when used in conjunction with an established breeding programme and documented multigeneration lines. Extra care and higher stringency in selecting markers must be taken when scoring RAPD markers across gels to avoid scoring errors with co-migrating or faint bands. The high robustness of the AFLP assay compared with RAPD is to be expected because specific PCR with longer primers is carried out at higher stringency. Both methods share the high cost inherent in PCR but AFLPs also share the lengthy time requirement of RFLP mapping (Eriksson, 1997). AFLPs and RAPDs have a different genetic base and sample different genomic regions. AFLPs are based on the use of restriction enzymes i.e. restriction sites and RAPDs are based on amplified arbitrary sequences (Gaiotto *et al.*, 1997).

There are indications that maize contains more active transposon systems than other species (Coe, Neuffer and Hoisington, 1988) contributing to the high degree of insertion/deletion type mutations whereas in other species single nucleotide mutations are the prominent mechanism for generating new alleles. RFLPs, AFLPs and RAPDs are all capable of detecting single nucleotide mutations as well as insertions or deletions. SSRs and AFLPs are becoming increasingly popular because of their overall uniformity but RAPDs will remain popular due to their simplicity and low developmental costs and RFLPs are attractive due to their co-dominance and easy transferability between genomes (Rafalski *et al.*, 1996).

1.3.4 The limitations of RAPD markers

An important constraint on the use of markers in plant breeding is the cost and time required to undertake such analyses and the lack of detectable polymorphism in certain crops. Improvement in the screening techniques will reduce costs and can be expected in the future but it is likely that large-scale application of marker selection to plant breeding will be restricted to large breeding organizations. Saturated genome maps for maize do exist but many have been put together by using wide crosses as the base material (Heun and Helentjaris, 1993). In a conventional breeding population the extent of polymorphism may be greatly reduced due to lack of genetic variability.

The RAPD reaction is based on DNA polymerase-mediated amplification and is therefore subject to variation in physical and chemical reaction parameters resulting in a sensitivity to amplification conditions. It is therefore important to carefully optimise the components of the reaction cocktail. Variation in the RAPD pattern represents allelic segregation at independent loci, and therefore the presence of DNA amplification artifacts and ambiguous products can obscure genetic analysis (Reidy, Hamilton and Aquardro, 1992). Artifactual non-genetic variation in RAPD analysis appears considerable if primer-template concentrations and annealing temperature are not carefully optimised (Muralidharan and Wakeland, 1993). A fraction of RAPD primers are non-functional i.e. unable to amplify DNA and produce profiles. When this amount is considerable, this adds to the expense and effort of primer screening (Caetano-Anollés, 1994). Band co-migration where different fragments of similar size co-migrate and obscure the detection

of polymorphic DNA is another problem. This problem may be ameliorated by using DNA separation techniques of high resolving power but ultimately co-migration of fragments can only be confirmed by fragment isolation and Southern hybridisation (Weaver, Caetano-Anollés, Gresshoff and Callahan, 1994).

Another major disadvantage of RAPD markers, particularly in population genetics studies, is their dominant nature and low allele number. More than 90% of RAPD markers are inherited in a dominant fashion with loci defined by two alleles (band either present or absent). Consequently, the RAPD reaction produces a fragment with template DNA from individuals that are either homozygous or heterozygous for an amplified allele. No fragment is produced in homozygous recessive allele because amplification is disrupted in both alleles. Therefore dominance prevents tests of random mating within populations because individual genotypes cannot be discerned (Apostol, Black, Reiter and Miller, 1996). Dominance also makes mapping in segregating F_2 populations inefficient, as linkage of markers in repulsion phase is difficult to demonstrate. Thus to use dominant RAPD markers in genome mapping, for any locus the zygosity of the parent(s) must be determined prior to conducting a full-scale segregation analysis with the progeny. When pedigrees are not available, which may often be the case, the zygosity of parents at a locus can only be deduced from the progeny (Carlson, Tulsieram, Glaubitz, Luk, Kauffeldt and Rudledge, 1991).

However, mapping data can be readily obtained from recombinant inbred lines, backcross progeny, double pseudotestcross, polyploids, double haploids, or haploid tissue. Low allele numbers decrease the likelihood that the marker will segregate in numerous crosses and be more informative, a drawback that is perhaps only compensated by a profuse generation of polymorphisms. RAPD markers often represent dispersed repetitive DNA, a characteristic that devalues their use as landmarks for genetic mapping. Some of these limitations may be overcome by converting RAPD markers into SCARs as the higher specificity of these PCR-based markers makes them more suitable for positional cloning (Paran and Michelmore, 1993).

1.3.5 Other uses of RAPD markers

A phenomenon often observed in plants regenerated from tissue culture is the occurrence of mutations (somaclonal variation). This has been used in certain crops to obtain novel traits but may also be undesirable when the objective is to produce genetically identical plants (Anastassopoulos and Keil, 1996). The phenotypic testing of tissue-culture derived plants for genetic stability is laborious and expensive, and RAPD markers have been shown to be able to detect tissue-culture induced mutants in a number of plant species (Brown, Lange, Kranz and Lorz, 1993; Isabel *et al.*, 1993). However, Anastassopoulos and Keil (1996) demonstrated that the RAPD technique is an inappropriate tool for rapid screening of *Alstroemeria spp.* for induced variation as somaclonal variation remains undetected.

Plant breeding often involves the use of wide crosses with the introduction of genes from related species. This has lead to the introduction of many useful genes into new commercial varieties. Sometimes, however, the introduced chromosome segment containing the gene may have deleterious side effects (Law, 1995). RFLP, RAPD and even *in situ* hybridisation techniques help to monitor the process of transfer so that only small segments containing the desired gene are selected, therefore reducing the presense of adverse genes. There is a high probability that these introduced segments although reduced in size, will still carry polymorphic markers diagnostic for the desired gene (Law, 1995).

Comparative gene mapping using molecular probes may also be used in the effective mapping of related genes and sequences across groups of distantly related crop species. Cereals could then be considered as a group and the maps of maize may be related to those of wheat, barley (*Hordeum spontaneum* L.), rice, millet (*Panicum spp.*) and rye (*Secale cereale*). For some years it has been possible to identify homologous relationships between the chromosomes of wheat, rye and barley. Comparative mapping will allow this to be done in greater detail, and homologies within genes of these species as well as rice and maize are being recognised (Moore, 1995). Molecular techniques are also starting to provide tools to characterise the different stages of interaction between plants and potential pathogens (Michelmore, 1995).

1.4 RAPD methodology

1.4.1 DNA isolation

Plant DNA isolation methodology evolved rapidly from cumbersome, messy and often inefficient large scale phenol procedures which resulted in the isolation of only a small fraction of the available DNA and usually sheared the DNA to relatively low molecular weight (Bendict and McCarthy, 1970), to more efficient methods which relied on the isolation of organelles and the subsequent purification of DNA using ultracentrifugation in CsCl density gradients (Watson and Thompson, 1986). However, these procedures require a large amount of tissue and are generally both time-consuming and labour-intensive (Kamalay, Tejwan and Rufener, 1990) and are not well suited to assaying a large number of samples (Doyle and Doyle, 1987).

More recently a number of "miniprep" procedures have been developed (Dellaport, Wood and Hicks, 1985; Doyle and Doyle, 1987; Edwards, Johnson and Thompson, 1991; Deragon and Landry, 1992; Cheung, Hubert and Landry, 1993) which permit rapid isolation of DNA with a minimum of expense using a small amount of tissue. A plant DNA "miniprep" procedure for the rapid isolation of total nucleic acid must fulfil the criteria of simplicity, rapidity and low cost in order to facilitate the analysis of a large number of seedlings at a young age, and yielding DNA fragments of sufficient purity and length for RAPD analysis. DNA extraction must not be the time- limiting factor to fully exploit the potential of RAPD technology (Cheung *et al.*, 1993). Although most of these protocols are simple, they tend to be designed for a specific animal or plant, or more specifically a species or tissue type (Cheung *et al.*, 1993). The different procedures tend to work better for different plant groups, as might be expected considering the great diversity of plant secondary components which in many cases interfere with a particular method of DNA isolation (Doyle and Doyle, 1987).

When the end product of the DNA extraction procedure is to be used in a PCR application, the quality of the template DNA has a significant effect on the generation and resolution of amplified products (Reidy *et al.*, 1992). PCR has revolutionised the rapid analysis of mammalian genome

DNA but is less useful in the analysis of plant DNA due to difficulties in extracting nucleic acids from limited amounts of plant tissue (Edwards *et al.*, 1991). Micheli, Bova, Pascale and D'Ambrosio (1994) stated that the patterns of fragments amplified are, in a large part, a function of the sites on the template to which productive annealing of the oligonucleotide primer can occur, hence differences between DNA preparations that affect primer annealing could be one major source of inconsistency of RAPD patterns.

1.4.2 PCR optimisation

The PCR amplification process is dependent upon many components and their interaction (Devos and Gale, 1992). The inconsistent reproducibility of the RAPD technique has been identified as one of the major drawbacks in the application of this technique (Penner, Bush, Wise, Kim, Domier, Kasha, Lorche, Scoles, Molnar and Fedak, 1993). Sub-optimal PCR conditions may promote the appearance of PCR artefacts such as dimerisation, misincorporation and mismatch extension of primer template duplexes. It is therefore important to specify a set of reaction conditions in order to obtain reproducible results for a given species (Devos and Gale, 1992). Many laboratories have succeeded in optimising reaction conditions with respect to DNA, chemicals and equipment used. Resendez-Perez and Barrera-Saldana (1990) revealed that one of the factors which must be optimised to ensure highest sensitivity and specificity of PCR is the processor, which enables automatic performance of the PCR assay. The thermocycler must guarantee temperature homogeneity for all samples of an individual run and results between runs must be comparable. Yu and Pauls (1992) first optimised the PCR program for RAPD analysis resulting in a 2.5 h, 35 cycle program with a 5 sec denaturation step at 94 °C, a 30 sec annealing step at 36 °C and 60 sec extension step at 72 °C. This shortened reaction cycle could be indicative of *Taq* inactivation over time or that other components in the reaction mixture become limiting at high cycle numbers.

In order to be able to compare RAPD markers between different laboratories, Schierwater and Ender (1993) compared 13 commercially available thermostable polymerases. Little variation within amplification patterns of any given enzyme, even under slightly different reaction

conditions, was observed. However, between enzymes obvious differences were found in both number and relative amounts of amplified products. The enzymes also differed significantly with respect to sensitivity to slight changes in $MgCl_2$ concentration. Cipriani, Di Bella and Testolin (1996) found that the AmpliTaq Stoffel fragment and the *Taq* polymerase were both suitable for RAPD analysis. The AmpliTaq Stoffel fragment is a recombinant *Taq* which lacks exonuclease activity because of deletion of the N-terminal 289 amino acid portion. The Stoffel fragment produced smaller fragments in comparison to *Taq* polymerase. These results showed that the outcome of the RAPD fingerprint also depends on the type of polymerase used.

A variety of optimisation protocols are reported in the literature. Levi, Rowland and Hartung (1993) employed five $MgCl_2$ concentrations, three sources of *Taq* DNA polymerase at three concentrations, nine primer:template ratios and nine annealing temperatures, three durations of DNA denaturation temperatures and four types of thermocyclers in their optimisation programme. Javed Iqbal and Rayburn (1994) examined the concentrations of $MgCl_2$, template DNA and *Taq* DNA polymerase per 50 µl reaction to determine the optimum PCR reaction conditions of rye. This protocol had stringent conditions for annealing primer and template, whilst maintaining favourable conditions for DNA amplification. Wolff, Schoen and Peters-van Rijn (1993) used a fractional factorial experimental design for the rapid detection of factors that may influence the result of a RAPD reaction. Polymerase brand, thermal cycle brand, annealing temperature and primer were revealed to be the most important factors in obtaining good DNA yield and optimal fragment patterns. Optimal species-primer combinations must, however, be found by trial and error.

Optimising PCR-based technology is therefore a laborious task as many components can be altered in PCR reactions and not all processes and mechanisms are fully understood. Optimisation generally relies on the sequential investigation of each reaction variable. This approach often leads to prohibitively large and costly optimisation experiments in order to include all possible combinations. In practise the optimal conditions are rarely identified. Cobb and Clarkson (1994) describe an optimisation strategy based on the modified method of Taguchi (1986) which circumvents many of the problems associated with conventional optimisation strategies. The

Taguchi (1986) method uses a number of progressive trials. An initial experiment is often used to examine a number of factors in order to identify those that have a major effect. These controlling factors are then used to predict a combination that will lead to optimal performance. If these results are satisfactory then further experiments are unnecessary. These basic principles can also be applied to PCR, allowing determination of the many components that can influence the PCR reaction. Those factors which have principle effects on amplification are identified by using a single trial with a few reactions (Cobb and Clarkson, 1994).

PCR optimisation experiments normally require each variable to be tested independently. A trial investigating the interactions and effects of four reaction components each at three concentration levels would therefore require an experiment with 81 (i.e. 3^4) separate reactions. Using the Taguchi (1986) method an estimate of the effect of each component could be carried out using nine reactions. Factors which are thought likely to effect the process are arranged in an orthogonal array. In PCR each column represents individual reaction components and each row represents individual reactions (Cobb and Clarkson, 1994).

Loarce, Gallego and Ferrer (1996) reported on whether to use bulked or individual samples. Although the specific mechanisms involved in amplification using single arbitrary primers have not been characterised, there is probably competition among DNA sequences for the substrates in the reaction. In PCR, DNA with a greater homology to the primer may be amplified at the expense of other DNA (Gibbs, Nguyen and Caskey, 1989) and some DNA may be preferentially amplified under specific conditions (Kirkpatrick, Cowan and Dentine, 1991). Michelmore *et al.* (1991) determined that rare alleles were not detected when they comprised less than four % of the PCR mixture. These rare alleles could also occur when arbitrary primers were used. As a result, the amplification of a genetically mixed sample of individuals (bulk sample) may not produce the same fragments that would be generated by the individuals in a mixture (Sweeny and Danneberger, 1994).

1.4.3 Product visualisation

Electrophoresis is the tool used in DNA fingerprinting to separate the fragmented nucleic acids according to size. Nucleic acids are large negatively charged bio-molecules which possess ionisable groups (Strickberger, 1985). Molecules which have similar charge will have different charge:mass ratio when they have inherent differences in molecular weight. These differences are sufficient for a differential migration when ions in solution are subject to an electric field, a process termed electrophoresis. As DNA molecules have an essentially constant charge per unit mass, they therefore separate in supporting mediums almost entirely on the basis of size. A variety of supporting media offering different advantages are available e.g. sheets of absorbent paper, a thin layer of silica or alumina or a gel of starch agar, agarose or polyacrylamide. These do not absorb the nucleic acids. Agarose gels are most commonly used in RAPD analysis as they are inexpensive, simple to use and the DNA fragments may be stained with ethidium bromide and viewed under ultraviolet (UV) light (Strickberger, 1985).

Bassam, Caetano-Anollés and Gresshoff (1991) reported that fragments can be adequately resolved and visualised by polyacrylamide gel electrophoresis combined with silver staining of the nucleic acids with over 100 bands being detected depending on the primer and template combination. Highly sensitive detection of nucleic acids in the nanogram range has been achieved by the specific chemical reduction of silver ions. Methods for silver staining nucleic acids employ either a histologically derived procedure that uses ammoniacal solutions of silver or a photochemically derived reaction in which silver binds to nucleic acid bases and is then selectively reduced by chemical agents or light (Goldman and Merril, 1982). The silver-staining methods are as sensitive as radioisotopic methods. The optimised photochemically derived silver stain for protein staining and later applied to nucleic acids uses formaldehyde to selectively reduce silver ions to metallic silver under alkaline conditions (Bassam *et al.*, 1991). Yang *et al.* (1999) reported the use of digoxigenin (DIG)-based RAPDs (where the primer's 5' end is labelled with DIG and subject to electrophoresis of five % PAGE) to detect somoclonal variation in cultured rice cells.

1.4.4 Interpretation of RAPD results

In the interpretation of RAPD reaction results, the individual band intensity, size distribution of amplified products, and overall pattern should be the same for the same primer and the same DNA template but will vary between different genomic DNA samples and different primer pairs (Williams *et al.*, 1993). Fingerprints of related plants should display common bands as well as some differences in banding pattern due to DNA polymorphisms. The total number of bands as well as the number of polymorphisms will depend on the crop, variety, complexity of the genome and primer used. Some primers result in either too few or too many bands for a particular sample. It is therefore important to perform a control experiment. Some minor differences in band intensity and position may be observed from experiment to experiment but the overall pattern should match. With the scoring of bands, only distinct bands should be included in the data set. Each individual is scored for the presence (1) or absence (0) of every such band. This may be done by scoring against a molecular weight marker and assigning tentative identification numbers to each band based on approximate molecular weight (Lenny Williams, Goldson, Baird and Bullock, 1994).

1.4.5 Data analysis

DNA fingerprinting allows rapid assessment of the levels of genetic variation. Data analysis has received and is receiving a lot of attention, and there are many computer software programmes available to analyse gel data (Rollinson and Stothard, 1994). It is necessary to assume that the data to be analysed is unambiguous, that the DNA of individuals being compared is run in nearby lanes and/or with adequate controls, to minimise the errors in assigning fragment pairs, that any co-migration of non-allelic markers can be resolved either by differences in band intensity or from other information; that the marker loci are assumed to be unlinked and in Hardy-Weinberg equilibrium within and between loci; and that the same set of homologous loci are assayed completely for all individuals (Lynch, 1990). There is as yet no universally approved method for analysis of RAPDs. Some workers analyse all fragments, whilst others only use those of a certain size range. Bands may be scored on a presence vs. absence or sometimes account is given to

intensity. Most popular in the literature is the construction of similarity or distance tables between all possible pairs of profiles (Curnow, 1998), with subsequent construction of dendrograms by cluster analysis. The calculation of genetic distance of each individual component from all other groups is formed by the process of agglomeration/division. There are many algorithms for cluster analysis and different algorithms do not necessarily produce the same results for the same data set, therefore there is a large subjective component in the assessment (Manley, 1986). Statistics are mostly borrowed from those used in RFLP research (Rollinson and Stothard, 1994). DNA fingerprint similarity is generally defined as the fraction of shared bands, and similarity matrices can be calculated from the data on band presence or absence for each individual

Genetic distance or similarity can be determined using Jacquard similarity genetic distance (Gower, 1985), band sharing (Hendrick, 1992) and calculation of similarity coefficient which is commonly based on the proportion of fragments shared Nei and Li (1979). Similarities between cultivars may also be estimated by the Dice algorithm using the NTSYS.PC computer program (Rohlf, 1993). The Dice algorithm is identical to that of Nei and Li (1979) (Loarce *et al.*, 1996). The measure of band sharing can be determined in two ways i.e. using positive frequency only and both positive and negative frequencies. The presence/absence data matrix may also be analysed using the SAS procedure DISCRIM (SAS version 6, 1989). This procedure generates ordered canonical discriminant functions for the variables (bands). The means of the canonical discriminant functions may then be plotted.

Data may also be analysed by cluster analysis and principle co-ordinate analysis, thus verifying that results are not dependant on the type of analysis used. Dendrograms can be constructed from similarity matrices and hierarchical cluster analysis with an average link option using the GENSTAT programme (Genstat 5™, 1993). Similarity matrices can also be analysed in GENSTAT by principle co-ordinate analysis to generate a simpler display of relationships between individuals than a dendrogram (Lenny Williams *et al.*, 1994). The clustering procedure (unweighted pair group method with arithmetic averages, UPGMA) may also be employed to produce dendrograms (Loarce *et al.*, 1996) derived from distance matrices. There is a problem

with this type of analysis in that the genomic origins of the amplified sequences are unknown and there is no guarantee that apparently homologous fragments correspond to the same proportion of genomic DNA (Rollinson and Stothard, 1994). RAPD fragments originate from potentially all areas of the genome, both coding and non-coding and may be subject to different evolutionary constraints. This questions the UPGMA analysis as it assumes a universal molecular clock. There is still a need to develop a better framework for RAPD analysis.

Goodwin and Annis (1991) assigned double weights to strongly amplified fragments and single weights to weakly amplified fragments (Gabrial, Hunter, Kingsley, Miller and Lazo, 1988) in their analysis of bacteria. The analysis can combine the results of all primers or individual primers can be treated separately. The similarity coefficient can be converted to a distance value (d) using the formula d=1f, where f is the degree of similarity (Hillis and Moritz, 1990). In RAPD analysis it has been argued that bands of identical mobility could occasionally correspond. This seems to occur more frequently when comparisons between less closely related individuals is performed (Thormann *et al.*, 1994). Moreover, it has been reported that RAPD markers do not always arise from amplifications of single-sequence DNA (Devos and Gale, 1992). In this case, the probability of obtaining fragments of different mobilities from the same region of the genome is higher than when single-copy DNA is amplified. Thus amplification of repeated DNA would reinforce the association of two closely related varieties but the scoring of distinct fragments as identical would cluster together cultivars that should not, by pedigree, show relationships. Nevertheless, sampling errors due to the use of markers non uniformly-dispersed throughout the genome would also explain the observed differences (Dos Santos, Niehuis, Skroch, Tivang and Slocum, 1994). A high number of RAPDs must be analysed in order to obtain dendrograms that accurately reflect genetic relationships between the cultivars. The advantage of RAPDs over certain other marker types is that a larger number of fragments can be analysed with a single primer (Williams *et al.*, 1990).

1.5 Applications of polymorphic genetic markers in plant breeding.

Two major areas of application of polymorphic genetic markers exist, firstly: utilisation of genetic markers to determine genetic relationships, these applications include varietal identification, protection of breeders rights or pedigree assessment (Dweikat, MacKenzi, Levy and Ohm, 1993) and parentage determination (Welsh *et al.*, 1991; Pellisier Scott, Haymes and Williams, 1992). The second area is based on the use of genetic markers to identify and map loci involving monogenic or quantitative traits and to monitor these loci during introgression or selection programmes (Soller and Beckman, 1983).

1.5.1 Genetic relationships

1.5.1.1 Varietal identification

Varietal identification by DNA fingerprinting and the determination of relationships among individuals (for instance to assign individuals to heterotic groups) has long involved markers that can distinguish between a number (*n*) of strains or varieties (Soller and Beckman, 1983; Smith *et al.*, 1991b). Previous studies with protein markers (isozymes or seed storage proteins) showed the utility of using DNA markers for selecting parents for source populations. The choice of which fingerprinting technique to use is dependant on the application e.g. DNA typing, DNA marker mapping and the organism under investigation e.g. prokaryotes, plants, or animals. Ideally a fingerprinting technique should require no prior investments in terms of sequence analysis, primer synthesis or characterisation of DNA probes negating the use of RFLPs or microsatellites. As examples of the application of the technique, cultivar-specific DNA profiles in rye were developed using RAPDs (Javed Iqbal and Rayburn, 1994). Hu and Quiros (1991) studied RAPD markers of individual plants of broccoli and cauliflower cultivars and found that all the markers were consistent within each cultivar but varied from cultivar to cultivar. Devos and Gale (1992) and He *et al.* (1992) demonstrated the usefulness of RAPD analysis in the identification of wheat lines, and Wilde *et al.* (1992) used RAPD markers for the genomic fingerprinting of *Theobroma*

clones and Mienie *et al.* (2000) used RAPDs to distinguish between selected common bean cultivars.

1.5.1.2 Protection of breeders' rights

Due to the considerable effort and financial investment in developing new commercial plant varieties, there is an interest to protect these investments by obtaining plant variety protection. This requires the unambiguous identification of plant varieties (Sederhoff and Meagher, 1995). Rapid advances during the past decade of DNA fingerprinting techniques facilitating the genetic identification of plants has further increased the interest and debate in the private sector over ownership rights and plant propriety rights. Consequently DNA fingerprinting has become increasingly important in developing and enforcing plant propriety rights defining the boundary of ownership for existing and new genotypes from these lines. To prove misappropriation of a protected plant variety, it is necessary to show that the allegedly misappropriated variety is identical to or equivalent to the protected variety. Hence, a set of genetic markers is required that allows calculation of the likelihood that an unknown variety or population actually represents a given commercial variety (Soller and Beckman, 1983).

In the USA five general legal categories exist for plant protection: (i) trade secret law; (ii) contracts and licensing; (iii) plant patents for asexually reproduced plants; (iv) plant variety protection; and (v) utility patents (Brobovic, 2000; personal communication). The effectiveness of protection for each category is continually evolving and changing. Combinations of these major legal categories are used by some companies and different levels of protection result from them. The techniques and laws are constantly changing which results in a number of complex questions and decisions to be made by scientists and administrators. According to the Union for Protection of Breeders Rights (UPOV, 1991) breeders rights are currently being amended and modified in a number of countries.

A utility patent was issued to Du Pont that covers the use of RAPD markers. Du Pont then licensed exclusive rights to the use of this marker technique for certain species to an Australian

company, ForBio Ltd which would then license RAPD markers to individual laboratories at a specific charge for each RAPD reaction (ForBio Ltd has subsequently closed down). However, since RAPD reactions are PCR-based a license is required to use *Taq* polymerase. Du Pont decided not to charge fees for research carried out in universities or government laboratories that "has no commercial purposes". This distinction deserves consideration as it could set a precedent for protecting freedom of enquiry (Sederhoff and Meagher, 1995).

1.5.1.3 Parentage determination

Methods of parent selection may be divided into two broad categories, *a priori* (direct evaluation of the parents) and *a posteriori* (progeny testing) (Baenziger and Peterson, 1992). Plant breeding programs for annual crops have relied predominately on the latter, especially in the development of F_1 hybrid cultivars. *A priori* methods have been used more commonly for simply inherited traits and for the evaluation of exotic germplasm. However, the latter is being reconsidered from the results of theoretical examples and empirical studies in maize and tomato, which demonstrated the recovery of unexpected, favourable alleles from donor parents, alleles masked by *a priori* evaluation but detected by DNA markers in segregating populations (Lee, 1995). Parentage determination therefore requires a set of markers that allow positive parent identification. Soller and Beckman (1983) determined that 15 polymorphisms gives a combined probability of exclusion (CPE) of 0.95 if the female parent is scored and that the combined allelic frequencies are 0.5. If the female parent is not scored, then 22 polymorphisms are required for a CPE of 0.95. Reidy *et al.* (1992) raised concerns over the suitability of the RAPD method for determining parentage because of non-parental bands that appeared in the offspring of known pedigrees. However, Scott, Haymes and Williams (1992) used RAPD PCR for parentage analysis of burying beetles (*Nicrophorus tomentosus*) and found very low frequencies of non-parental bands.

1.5.2 Characterisation of monogenic disease resistance

Disease resistance genes are major components of many breeding programmes and genetic mapping studies. The advent of several fingerprinting technologies, as mentioned previously,

allows all regions of the genome to be assayed for molecular markers linked to disease resistance (Beckman and Soller, 1983). A broad genetic base for resistance to major and potentially serious diseases is a long term breeding objective. Marker analysis has located many disease resistances to gene clusters in the genome, supporting data from classical segregation analysis of resistance genes. Markers linked to many resistance genes are now known, but often the closest markers are not tightly linked (Table 1.3), therefore identification of additional markers is required before marker assisted selection or map based cloning is initiated (Michelmore, 1995). Progress in constructing genetic maps has been made using RFLP markers and many new ones are being assembled using both RAPD and microsatellite markers (Reiter *et al.*, 1992).

Table 1.3 Recent examples of mapping and identification of markers linked to disease resistance genes (Michelmore, 1995).

Host	**Pathogen/Pest**	**Gene**	**Method of identification**	**Type of Marker**	**Closest markers**
Maize	*Bipolaris maydis*	*rhm*	F_2 seg.	RFLP	0.5, 1
Barley	*Rhynchosporium secalis*	*Rh*	NILs, BSA	RAPD	7
Oats	*Puccinia graminis*	*Pg3*	NILs	RAPD	0
Wheat	*Puccinia recondita*	*LR9*	NILs	RAPD	0
Soyabean	Soyabean mosaic virus	*Rsv*	F_2 seg.	μsat., RFLP	0.5, 35.9
Lettuce	*Plasmopara lactucae-radicis*	*plr*	BSA	RAPD, RFLP	2, 3
Common bean	Common bean mosaic virus	*1*	NILs	RAPD	1 to 5

F_2 seg Segregating population

NILs Near-isogenic lines

BSA Bulk segregant analysis

RFLP Restriction fragment length polymorphism

RAPD Random amplified polymorphic DNA

Usat Microsatellite

In another application molecular markers that are distributed throughout the genome can be used to estimate the genetic contribution of each parent to each member of a segregating population. Individuals whose genome composition represents the recurrent parent may be selected for the

next cross, and this will accelerate the introgression of traits from genetically different germplasm sources (Rafalski and Tingey, 1993).

Selection for monogenic pest resistance traits is often practised in the first segregating generation (F_2) from a cross between susceptible and resistant parents (Michelmore, 1995). In such a population, selection or discard decisions based on linkage with a RAPD marker would be a function of: (i) the genotypic frequency for resistance; (ii) the recombination frequency between marker and resistant alleles; and (iii) the coupling repulsion phase status between marker and resistant allele. In the absence of meiotic abnormalities or gametic selection, the F_2 genotypic frequency for complete dominance or recessive resistance is 1:2:1 (resistance: segregating: susceptible). Therefore, a given resistance class is composed of individuals with one combination (from either parental gamete), or two recombinants (from each parental gamete or no recombinants between marker and resistance alleles (Haley, Afandor and Kelly, 1994a). Selection or discard decisions are a function of the coupling-repulsion status between marker and resistant allele and assume that: (i) individuals possess a RAPD marker linked in coupling with a resistance allele would be selected; or (ii) individuals that possess a RAPD marker linked in repulsion with a resistance allele (linked in coupling with the allele conferring susceptibility) would be discarded (Haley *et al.*, 1994a).

The underlying objective in selecting for a monogenic resistance trait, whether controlled by a dominant or recessive allele, is selection of genotypes homozygous (or heterozygous in some circumstances such as vegetatively propagated species or early segregating populations in self pollinating crops) for a particular resistant allele. Direct selection for a completely dominant resistant allele is dependant on homozygous and heterozygous resistant genotypes being clearly distinguished during progeny testing. Conversely, direct selection for completely recessive resistant alleles allows immediate fixation of the resistant locus. Indirect selection using a RAPD marker linked in coupling to either a dominant or recessive resistant allele negates the advantage of fixation for a recessive resistant allele and does not remove the requirement for progeny testing for dominant resistant allele because in both instances heterozygous individuals will be among those selected. A RAPD marker linked in repulsion with the dominant or recessive allele (linked in

coupling with the allele conferring susceptibility) results in improved efficiency in marker assisted selection (MAS) for homozygous resistant genotypes with a RAPD marker linked in coupling with the same resistant allele. Improved selection efficiency with repulsion-phase linkage has a direct result in the reduction of heterozygotes (segregating) and homozygous susceptible classes and an increase in the homozygous resistant class among a population selected from the F_2 generation (Haley *et al.*, 1994b).

The advantages of MAS have been well documented (Beckman and Soller, 1986; Lande and Thompson, 1990). Studies in tomato (Niehuis *et al.*, 1987) and maize (Stuber, 1989) have demonstrated that MAS is not limited to qualitative traits, i.e. traits controlled by one or a few genes, but is also applicable to quantitative and semi-quantitative traits (Carlson *et al.*, 1991). Marker assisted selection offers considerable advantages for backcross breeding: (i) indirect selection of desirable gene(s) from donor parents; (ii) selection for regions of recurrent parent genome unlinked to the introgressed region; and (iii) reduction of linkage drag of unwanted donor parent genome near the introgressed region(s) resulting in a decreasing number of generations to recover the parent phenotype (Lee, 1995).

1.5.2.1 Near-isogenic lines

The identification of markers tightly linked to resistance genes has been greatly facilitated by targeting molecular markers with contrasting near isogenic lines (NILs). Combining the use of RAPDs and NILs provides a route for quickly identifying markers linked to traits of interest (Waugh and Powell, 1992). NILs arise from backcross method to introgress a single (in)-complete dominant gene with desirable effects into a valuable cultivar (Young *et al.*, 1988; Michelmore *et al.*, 1991). It is assumed that after five to six backcrosses under selection for the phenotype of the recurrent parent (RP) with the desirable character, the genotype of the backcross product is nearly identical to that of the RP, except for the introgressed gene. Such lines are called isogenic lines but because there is linkage drag of a linkage block where donor genes are linked to the desired gene. These lines are called congenic, co-isogenic or near-isogenic

lines (NILs). The latter name is most common. True isogenic lines derive from a gene mutation in a pure line (Stam and Zeven, 1981).

The high probability that any polymorphism detected will be in the DNA surrounding the introduced gene, provides a powerful means of identifying markers that are linked to the trait of interest (Young *et al.*, 1988). Using the RAPD technique, Martin *et al.*, (1991) screened two tomato NILs that differed for the presence or absence of a gene (*pto*) conferring resistance to *Pseudomonas* with 144 random primers. A total of seven polymorphic amplification products were identified between the two lines. Four of these products were investigated by segregation analysis and three were confirmed to be linked to the *pto* gene. Near isogenic lines are often a product of co-ordinated plant breeding programmes and using this technique markers linked to many major genes particularly conferring resistance to plant pathogens have been assigned. Bulk segregant analysis (BSA) is also being used increasingly as it allows the rapid mapping of monogenic resistance genes using segregating populations.

1.5.2.2 Bulked segregant analysis

Based on similar principles to NILs, an alternative approach is to use bulked DNA from homozygous individuals of an F_2 population. This method termed Bulked Segregant Analysis (BSA) was developed by Michelmore *et al.* (1991) to rapidly identify markers linked to specific genes or regions of the genome when NILs are unavailable. Bulk segregant analysis may be applied to any defined genomic interval for which a population can segregate. This includes major gene loci, quantitative trait loci (QTL) and loci exhibiting only partial penetrance. Providing other markers flanking the region of interest exist, even chromosomal structures and functional loci e.g. centromeres which cannot be scored directly in a segregating population may be targeted by BSA (Giovanni *et al.*, 1991).

Two bulked DNA samples are generated from the segregating population of a single cross, with each pool containing individuals that are identical for a particular trait or genomic region but arbitrary at all unlinked regions. The two bulks are therefore dissimilar in the selected region but

seemingly heterozygous for all the other regions (Michelmore *et al.*, 1991). The two regions can be made from any genomic region and from any segregating population. The bulks may be screened for differences using either the RFLP or RAPD technique. Using BSA, Olaya, Abawi and Weeden (1996) identified two RAPD markers linked to resistance genes for *Macrophomina phaseolina* in common beans and Timmerman-Vaughan, McCallum, Frew, Weeden and Russel (1996) mapped 10 RAPD markers linked to QTLs controlling seed weight in pea (*Pisum sativum* L.). O'Donoughue, Chong, Weight, Fedak and Molnar (1996) used NILs and BSA to identify putative RFLP and RAPD markers for stem rust resistance in cultivated oat (*Avena sativa*).

Giovanni *et al.* (1991) used an extension of the RAPD-BSA method where the individual plants used to construct the bulk DNA were chosen on the basis that they contained alternative alleles of closely linked RFLP markers. The RFLP markers therefore defined the interval differing between bulks. The polymorphic PCR products were derived from within the defined interval or immediately adjacent to it. This approach is particularly attractive for focusing on particular chromosome regions where there are few existing markers.

1.5.3 Characterisation of quantitative polygenic disease resistance

The study of the relationship between markers and traits of economic value is important to determine the usefulness of markers as breeding tools. Many studies have considered the relationship between genetic markers and quantitative traits, in the case of segregating populations such as F_2, backcross, or recombinant inbreds (Lande and Thompson, 1990; Beavis, Grant, Albertsen and Fincher, 1991; Bar-Hen, Charcosset, Bourgoin and Guiard, 1995). Use of such populations allows the construction of marker genetic maps, because the statistical associations between markers can be expressed as a function of their chromosome location. These maps can be used to assign chromosomal locations to the loci involved in the variation of a quantitative trait (i.e. QTLs (quantitative trait loci)). The study of the relationship between genetic markers and quantitative traits for other types of germplasm (elite line collections, populations undergoing recurrent selection) is also important for breeding purposes, even if the study cannot

yield conclusions about the location of loci (Bar-Hen *et al.*, 1995). Identification of disease resistance QTLs is no different to the genetic dissection of other QTLs (Table 1.4).

Genomic regions contributing to resistance have been identified using regression analysis, interval mapping or both. Some aspects of plant-pathogen interactions can, however, complicate QTL dissection of disease resistance where different pathogen virulences may introduce additional variation. Some QTLs are additive e.g. resistance to grey leaf spot of maize, whereas others are dominant or over-dominant. In addition, recessive QTLs are occasionally found. The relationship between the determinants of quantitative resistance and single genes conferring complete resistance (major genes) is unclear (Michelmore, 1995).

Table 1.4 Dissection of quantitative trait loci determining quantitative disease resistance (Michelmore, 1995).

Host	**Pathogen/Pest Disease**	**No. of markers Genome coverage**	**Population size Method of analysis**	**No. of QTL Effects**
Maize	*Cercospora zeae-maydis* Grey leaf spot	87, 87, 78 RFLP 77, 74, 79%	139, 193, 144 ANOVA	F_2/F_3 9 * 4 to 26, =24 to 58 (3 populations) 7
	Exserohilum turcicum Northern leaf blight	103 RFLP Complete	150 F_2/F_3 MMOTL	7-18, Sum = 29 to 24 (Three traits) 4 to 5
	Gibberella zeae Stalk and ear rot	95 RFLP, 19 RAPD complete	112 F_2/F_3 MMQTL	Sum = 20
Soyabean	*Heterodera glycines* Soyabean cyst-nematode	36 RFLP, 7 RAPD	56 F_2/F_3 ANOVA	3 21-40, Sum = 52
Barley	*Erysiphe graminis* Powdery mildew	155 RFLP, 3 others Complete	113 DH MMQTL	2 Sum = 20
	Puccinia striiformis f.sp hordei Strip rust	61 RFLP 78 RFLP 70 %	28 F_1 Diallele ANOVA 110DH MMQTL	5 or 6 2 10, 57

RFLP Restriction fragment length polymorphism
RAPD Random amplified polymorphic DNA
DH Double haploid
ANOVA Analysis of variance
MMQTL Molecular marker quantitative trait loci

The defence mechanisms controlled by major genes have been exploited highly successfully by plant breeders in affording resistance to a number of maize diseases. The ease and speed with

which major genes can be incorporated into elite breeding material has ensured that major gene resistance especially in the short term is regarded as very important, although genetic breakdown can occur (Michelmore, 1995).

1.5.3.1 Grey leaf spot

Grey leaf spot (GLS) of maize is a foliar disease in South Africa caused by the fungus *Cercospora zeae maydis* (Alexopoulos and Mims, 1979). It is an aggressive fungal pathogen, that is widely adaptable and can severely damage maize foliage, reduce grain yield and increase the incidence of lodging. It is therefore considered one of the most destructive of the maize diseases. This disease has become of increasing agricultural importance in the warmer, humid areas of the USA (Donahue, Stromberg and Myers, 1991) (and presumably other countries which have favourable conditions for its development). Conservation tillage practices allowing plant debris and stubble to remain on the field from season to season increase the incidence of the disease which has serious yield-reducing effects. The continuation of these tillage practises therefore depends on, among other solutions, the development of cultivars more resistant to this disease (Gevers and Lake, 1994). While GLS can be effectively controlled with fungicide and various chemicals, this is expensive and the economical implications must be considered (Smit and Flett, 2000).

Host plant genetic resistance has the potential to be the most effective and economical method of preventing losses due to GLS. Resistance to GLS is a highly heritable, quantitative trait with genes that act primarily in an additive manner (Thompson *et al.*, 1987; Elwinger, Johnson, Hill and Ayers, 1990; Donahue *et al.*, 1991; Gevers and Lake, 1994;). However, expression of resistance is complicated by genotype-environment interactions (Saghai-Maroof *et al.*, 1993). The occurrence of resistant or tolerant hybrids have been reported but few if any can be classified as highly resistant (Thompson *et al.*, 1987). Identification of additional GLS resistant inbreds and information on the inheritance will help facilitate the development of resistance elite hybrids (Ulrich, Hawk and Carroll, 1990).

DNA RAPD analysis would provide a potentially powerful tool for identifying the quantitative trait loci responsible for controlling resistance to GLS. An easily scored marker linked to the

gene(s) conferring a particular resistant phenotype would therefore represent an important tool for plant breeders. Furthermore, information on the chromosomal and genetic map location of a gene provides an alternative route to gene isolation and cloning. A future development could involve the construction of new resistant genotypes using gene transfer and plant regeneration techniques in conjunction with conventional plant breeding techniques.

1.5.3.2 Leaf blight

There are five diseases of maize caused by the fungi formerly placed in the genus *Helminthosporium* (Alexopoulos and Mims, 1979). One of these is northern leaf blight which is considered a major foliar disease of maize in many parts of the world and has been responsible for serious epidemics and yield losses in the major maize producing regions of the world (Carson, 1995). Northern maize leaf blight is caused by the ascomycete *Setospharia turcica* Syn.= *Helminthosporium turcicum*. The symptoms of northern leaf blight are grey-green, elliptical or 'cigar' shaped lesions on the leaf, from three to 150 mm long. As the lesions mature they may become tan and have distinct zones of sporulation. The disease usually begins as a few scattered distinct lesions on the lower leaves, progressing to the upper leaves as the epidemic progresses. The disease can develop quite rapidly after anthesis, resulting in the complete blighting of leaves. Late in the season, severely infected fields may appear to have been killed by early frost (Carson, 1995).

Northern leaf blight is most common and potentially destructive in maize growing regions throughout the world wherever cool to moderate temperatures and moist conditions prevail (Carson, 1995). Losses to northern leaf blight are distinctly related to the amount and duration of lost leaf tissue during the grain filling period. If substantial leaf tissue has been lost one to two wks after anthesis, then grain yield reduction in excess of 40% can occur. Harvest losses are due to direct decrease in kernel weight and indirect harvest losses are due to an increase in stalk rots and stalk lodging in severely infected plants. Control of northern leaf blight is primarily through the use of resistance hybrids. Crop rotation and tillage practises that bury maize residues may also reduce the amount of primary inoculum (Carson, 1995).

Resistance to *Helminthosporium* in maize is one of two types: polygenic (partial resistance that is not race specific), and monogenic resistance controlled by one of four described single dominant genes at different loci and designated *Ht1, Ht2. Ht3, Ht4* and *HtN* (Gevers, 1975; Hooker, 1981). The *HtN* resistance is an exception in that it is not characterised by chlorotic lesions but rather by a delay in disease development until after flowering (Raymundo, Hooker and Perkins, 1981). Resistance conferred by the *HtN* gene is expressed as a very long latent period and fewer lesions. *HtN* which is thought to originate from the tropical variety Pepitilla has been mapped to the long arm of chromosome 8, distal to *Ht2* (Simcox and Bennetzen, 1993).

1.6 Conclusion

From the literature it is apparent that DNA markers are established as another tool for use during the many phases of crop improvement. The utility of this technology varies considerably with the application and context of the crop and culture (Lee, 1995). Advances in DNA technology, basic knowledge of plant biology and experience will increase the absolute efficiency of DNA markers. The identification of comparative advantages and appropriate integration of molecular technology will require the careful analysis of information. Molecular markers facilitate the preservation and exploitation of germplasm, allow marker assisted selection, and facilitate the generating of particular combinations of resistance genes (Michelmore, 1995). Markers that flank a gene determining a trait of agronomic interest can be used to track that trait in genetic crosses. In another application molecular markers that are distributed throughout the genome can be used to estimate the genetic contribution of each parent to each member of a segregating population. Individuals whose genome composition represents the recurrent parent may be selected for the next cross, this will accelerate the introgression of traits from genetically different germplasm sources (Rafalski and Tingey, 1993). Increasing success in cloning resistance genes from numerous species will allow characterisation of the diversity of mechanistic classes that exist and the genetic changes that generate variation in specificity. In the intermediate term, molecular markers will allow characterisation and manipulation of genes determining quantitative resistance and facilitate cloned genes to be used for novel transgenic approaches.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant material

The different maize inbreds, single crosses, a first and sixth generation backcross and an F_2 population used in this study (Table 2.1) were locally developed and provided by Dr Hans Gevers (retired plant breeder, formerly of the Agricultural Research Council Summer Grain Sub-centre, Pietermaritzburg). The inbreds, single crosses, backcrosses and F_2 populations are hereafter collectively refered to as genotypes which is a broader application of the term. For determination of markers linked to leaf blight resistance, the near-isolines K0315Y (resistant to leaf blight) and D0940Y-1 (susceptible to leaf blight) were crossed to produce a F_1 population which was selfed to produce a F_2 population of 14 individuals. Details of the crossing procedure and culture of rescued F_1 and F_2 embryos is provided in Appendix D.

Table 2.1. Pedigree and source of the six maize inbreds, two single crosses, two backcrosses and F_2 population used in this study.

Genotype	**Pedigree**	**Source**
Inbred A	NPPES1	C190 BA
Single cross AB	PAN 473 (suspected NPPES1*M162W)	PANNAR 91
Inbred B	M162W	WNIP 2569
1st gen. Backcross ABxB	(NPPES1*M162W)*M162W	01/480b*478
F2 population AB(F_2)	(NPPES1*M162W) F_2	01/479a(P#'s
Single cross BC	Suspected M162*R108W	01/139*133,134
Inbred C	R108W	WNO P256
Inbred K	K054W	MI 91/529-536
Inbred S	S0173W	MI 91/689-692
Inbred K-1	K0315Y (resistant*D0940Y)BC^6	052 P 646
Inbred D	D0940Y	052 P 660

As RAPDs involve changes at the DNA level, greenhouse material should not differ from field grown material when identifying polymorphisms (King *et al.*, 1990). The 11 maize genotypes were cultivated under controlled environmental conditions in a greenhouse. Prior to planting, the greenhouse was sprayed with Redspider-Cide® (2 mℓ ℓ^{-1}; a.i. tetradifon) and Malathion® (2.5 mℓ ℓ^{-1}; a.i. mercaptothion). Soil and compost (Gromor®) in a ratio of 1:1 were used as

potting medium. Each 3 ℓ pot was fertilized with 10 g of 2N:3P:2K(22). Four seeds of each genotype were planted per pot, with four pots per genotype and the pots were arranged randomly on benches in the greenhouse. One week post-emergence, the seedlings were fertilized with 2 g of 3N:2P:1K and 2 g of superphosphate per pot. The seedlings were watered daily and supplied with macro and micro nutrients over a seven day cycle when they were watered with 0.4 ℓ of a 0.1 % (w/v) solution of Chemicult® (hydroponic nutrient powder) and 0.3 ℓ of a 0.2 % (v/v) solution of Trelmix ® (trace element solution): day 1 Chemicult ®; and day 4 Trelmix ®

The growing maize plants were sprayed with Redspider-Cide ® and Malathion ® as required to control redspider and aphids. Benlate ® (a.i. benomyl) was applied to prevent the plants from becoming infected with damping off (caused by various pathogenic organisms). Shortly before maturity, the plants were given a booster of 5 g of 2N:3P:2K(22).

2.2. DNA isolation

The method of Honeycutt, Sobral, Kiem and Irvine (1982) for sugarcane was adopted and modified for the isolation of DNA from young, unexpanded maize leaf tissue (leaf roll). DNA was extracted from freshly harvested leaf roll material of either seedlings or young shoots of all the genotypes being cultivated in the greenhouse. Using the lid of a sterile Eppendorf tube, leaf material from the youngest leaf was punched out. Leaf roll tissue from three individual plants of each genotype was composited to provide a 3 mg sample. This tissue sample was then immediately suspended in 400 μℓ of ice-cold homogenisation buffer (50 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 0.5 mM spermidine, 1 % (w/v) polyethylene glycol (PEG 8000), 0.1 % (v/v) 2-mercaptoethanol and 0.35 M sucrose) in an Eppendorf tube. The tissue was homogenised by grinding it in an Eppendorf tube with a disposable pestle supplied by Eppendorf until no intact pieces of tissue were visible. The tube was then vortexed briefly for 5 sec. The homogenate was filtered through a layer of sterile damp mutton cloth into another sterile Eppendorf tube and centrifuged, at 4 °C, at 5000 g for 20 min. The supernatant was discarded and the cell pellet resuspended in 100 μℓ of cold wash buffer (50 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5 mM spermidine, 0.1 % (v/v)

2-mercaptoethanol and 0.35 M sucrose) and placed on ice. To this was added 20 µℓ of 5 M NaCl, 10 µℓ of 10 % (w/v) sodium dodecyl sulphate (SDS) and 13 µℓ of 10 % (w/v) cetyltrimethylamonium bromide (CTAB). The filtrate was stirred gently after each addition using a disposable pipette. The filtrate was incubated at 60 °C for 30 min and then allowed to cool to room temperature (approximately 15 min). An equal volume (150 µℓ) of chloroform:isoamyl alcohol (24:1) was added and the tube gently inverted until the phases were completely emulsified. The tube was then centrifuged at 3500 g for 10 min. The aqueous (top) layer was transferred to a new tube and the step repeated. After the final collection of the aqueous phase, an equal volume of cold isopropanol (-20 °C) was added and mixed gently. The tubes were left at room temperature until the DNA strands formed and clustered together (approximately 10 min). The DNA was then spooled or lifted out using a sterile modified "Pasteur hook", drained briefly and the DNA released into 75 µℓ TE Buffer (10 mM Tris-HCl (pH 7.6) and 1 mM EDTA (pH 8.0)) and left to dissolve. The DNA stock solutions were stored at -20 °C.

2.2.1 Template DNA concentration calculation

The working range of template DNA concentration was investigated by two methods: (i) ultraviolet spectrophotometry; and (ii) gel electrophoresis

2.2.1.1 Ultraviolet spectrophotometry

Nucleic acid yield was calculated based on the observation that 1 mg of DNA in low salts absorbs 25 optical density (OD) units at 260 nm. The OD_{260}/OD_{280} absorbance ratios of 1.8 or greater indicate levels of purity. DNA concentration was calculated using the following formula:

DNA concentration (µg mℓ$^{-1}$) = (OD x dil x E^) / tv.....Equation 1

where, OD = optical density (260 nm)

dil = dilution of the stock solution

E^ = A constant (50 µg mℓ$^{-1}$ for double stranded DNA)

tv = total volume (mℓ)

2.2.1.2 Gel electrophoresis

The quantity of the DNA was estimated by agarose gel electrophoresis. A standard gel of 0.8 % (w/v) agarose (Boehringer Mannheim®) with 0.5 times TBE running buffer (8 mM Tris-borate, 89 mM boric acid and 2 mM EDTA) was run containing 250 ng samples of DNA and the DNA standard, uncut Lambda (Boehringer Mannheim®). The integrity of the DNA was assessed at the same time. The gels were run at 5.6 V cm^{-1} and stained with ethidium bromide (0.5 μg $m\ell^{-1}$), followed by destaining in distilled water. The concentrated stocks were diluted in sterile distilled water to give working stocks of 3 ng $\mu\ell^{-1}$ which were stored at 4 °C and used for up to 4 wk.

2.3. Primers

Random decamer (ten-base pair) oligonucleotide primer kits OPA1-20, OPB1-20, OPC1-20, OPV1-20, OPW1-20 and OPX1-20 (Operon Technologies Inc., Alameda, California, USA) were used. The primers were characterised by an arbitrary sequence, while satisfying the imposed condition of 50-70 % G+C content and contained no inverted repeats (for DNA sequences see Appendix B). The primers were diluted in sterile double distilled water to a final concentration of 5 pmol $\mu\ell^{-1}$ and stored at -20 °C.

2.4 DNA amplification

Following optimisation of the PCR technique (Williams *et al.*, 1990) (Chapter 3), PCR was carried out in 25 μℓ reaction volume containing 1x reaction buffer (10 mM Tris-HCl (pH 9), 50 mM KCl, 0.1 % (v/v) Triton X-100) (Promega ®), 0.001 % (w/v) gelatin, 2.5 mM $MgCl_2$, 0.1 mM each of dATP, dCTP, dGTP and dTTP (Promega ®), 0.4U *Taq* DNA polymerase (Promega®), 5 picomoles of primer (Operon Technologies) and 30, 12 or 3 ng of template DNA. Thin-welled tubes (NT Labs) were used to perform the PCR reactions, and all reactions were performed strictly according to the following scheme: (i) the DNA (diluted in ddH_2O) was put in the tubes, which were then kept on ice; (ii) a master mix (buffer, $MgCl_2$, dNTP, gelatin) was prepared using ultra-pure water and vortexed thoroughly; (iii) the master

mix was divided up and the primers mixed in thoroughly; and (iv) *Taq* was added, the mixture inverted carefully and finally 20 μℓ was added to each tube.

The reactions were overlaid with two drops of mineral oil (Sigma®) to avoid evaporation. Amplification was carried out in a Hybaid® (1991) thermocycler for 40 cycles with the first step at 92 °C for 2 min, followed by 40 cycles of 92 °C for 1 min, 35 °C for 1 min and 71 °C for 2 min, with the fastest available transitions between each temperature. After 40 cycles there was a final elongation step of 5 min at 71 °C. Before removing the reaction products, they were held at 25 °C for at least 5 min. Occasionally the amplification products were stored at 4 °C for up to 24 h before electrophoresis.

2.5 Electrophoresis of amplification products

2.5.1 Agarose gel electrophoresis

The amplification products were analyzed by agarose gel electrophoresis in 2 % (w/v) agarose gels containing 2 μℓ 50 $m\ell^{-1}$ of ethidium bromide (10 mg $m\ell^{-1}$). A volume of 20 μℓ of the sample was loaded with 5 μℓ loading buffer (0.25 % (m/v) bromophenol blue, 15 % (v/v) Ficoll) Buffer Type II (Sambrook, Fritsch and Maniatis, 1989) and run in 1 x TAE buffer (2 M Tris (pH 8.0), 57.1 % (v/v) glacial acetic acid, 0.5 M EDTA (pH 7.0)) at 5.6 V cm^{-1}. Molecular weight markers (MWM) III, lambda DNA digested with *Hind*III and *Eco*R1 (21226bp, 5148bp, 4973bp, 4268bp, 3530bp, 2027bp, 1904bp, 1584bp, 1375bp, 947bp, 831bp and 564bp) and V, pBR322 digested with *Hae*III (587bp, 540bp, 504bp, 458bp, 434bp, 267bp, 234bp, 213bp, 192bp, 184bp and 124bp) (Boehringer Mannheim ®) were used as molecular standards. The gel was visualised under UV light (312 nm) and photographed with slide film (Agfa ®Iso 400) using a Konica Autoflex T4 camera. At a later stage the slides were photographed with photo film (Kodak gold Iso 200) using a Pentax camera and slide duplicator adapter and developed as normal print photographs. For the purposes of this thesis the photographs were then scanned using a PC scanner and saved as JPEG files which were then imported into Lotus Word Pro documents.

2.5.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was conducted using 0.45 mm thick slab gels of 5 % (w/v) acrylamide, 10 M urea, 10 % (w/v) ammonium persulphate and TEMED in a Mighty Small gel electrophoresis unit (Hoeffer®). The ratio of acrylamide to the cross linker piperazine diacrylamide was 20:1. All solutions were prepared in deionized water (Milli-Q synthesis®). The gels and the running buffer were prepared in TBE buffer (100 mM Tris-HCl, 83 mM boric acid and 1 mM Na_2.EDTA (pH 8.3)). The samples were loaded in 5 μℓ 5 M urea and 0.02 % (w/v) bromophenol blue in double distilled water. A volume of 4 μℓ of each amplification reaction was loaded and electrophoresised at 100 V until the dye front had reached the end of the gel.

The silver staining procedure of Bassam *et al.* (1991) was used. The gels were fixed with 10 % (v/v) acetic acid for 20 min, rinsed three times with distilled water and impregnated with silver solution (0.1 % (w/v) Ag NO_3 and 0.056 % (w/v) HCOH) for 30 min. The gels were then rinsed with distilled water and developed at 8-10°C in alkaline solution (3 % (w/v) Na_2CO_3, 0.056 % (w/v) HCOH and 0.0002 % (w/v) $Na_2S_2O_3.5H_2O$) for five to 10 min. The reaction was stopped with 10 % (v/v) acetic acid for 5 min. The gels were then soaked for 10 min in distilled water before being dried at room temperature (approximately 25 °C). All the steps were performed in plastic containers and the liquids removed by suction with a pipette. The developer and $AgNO_3$ solutions were prepared immediately before use. Impregnation with silver nitrate was under normal overhead fluorescent room lighting. The developer solution was replaced if a dark precipitate formed during image development.

2.6 Statistical analysis

Each amplified product was considered as a unit character. The RAPD banding profiles were scored manually for the presence (1) or absence (0) of a given amplification product of each genotype. Only intensely stained bands were scored. Degree of polymorphism between cultivars was calculated using an index of genetic distance (1-F). F values were obtained by using the method of Nei and Li (1979) as follows:

Degree of similarity $F = (2.N_{ab})/(N_a+N_b)$.....Equation 2

where, N_{ab} = Number of bands shared between genotypes 'A' and 'B'

N_a = Number of bands in genotypes 'A'

N_b = Number of bands in genotypes 'B'

This method of computation was chosen over other general similarity indices because of the increased weighting of bands versus non-matches. Two software programmes were used: (i) Genstat 5™ release 4.1 (1993); and (ii) PAUP (Phylogenetic Analysis Using Parsimony) 4.0 beta version developed by Swafford (1998) to produce similarity matrices. Cluster analysis of the markers was conducted using the hierachial cluster procedure and UPGMA (unweighted pair group method with arithmetric averages) methods respectively to produce dendrograms (Appendix C).

CHAPTER 3
RESULTS AND DISCUSSION OF THE OPTIMISATION OF DNA ISOLATION AND RAPD AMPLIFICATION PROTOCOLS

3.1 Optimisation of DNA isolation protocol

3.1.1 Introduction

Plant DNA can be successfully isolated from fresh tissue, lyophilized material, dehydrated or desiccated tissue stored in silica gel. Fresh, young leaf tissue is preferable since it may contain less polyphenolic and terpenoid compounds than older tissue. These brown-coloured compounds are released upon cell lysis and irreversibly adhere to DNA, often inhibiting PCR amplification (Jobes, Hurley and Thein, 1995). One of the main advantages reported for the RAPD technique is that it requires only small amounts of template DNA which need not be ultra pure in terms of protein contamination (Caetano-Anollés *et al.*, 1991). Several plant DNA extraction procedures have been reported in the literature (Saghai-Maroof, Soliman, Jorgensen and Allard, 1984; Dellaport *et al.*, 1985; Doyle and Doyle, 1987; Kamalay *et al.*, 1990; Edwards *et al.*, 1991; Deragon and Landry, 1992; Cheung *et al.*, 1993). The basis of these extraction protocols is the detergent lysate technique, first described for the isolation of microbial DNA (Marmur, 1961). Variations include the addition of a pseudocleared lysate procedure and precipitation of nucleic acids by the cationic detergent CTAB (Ralph and Bellany, 1964); an organic extraction step of phenol (Kirby, 1968) and chloroform-isoamyl alcohol (Sevag, Lackman and Smolens, 1938); ribonuclease and proteinase digestion steps (Marmur, 1961). The use of the time-consuming and expensive caesium chloride (CsCl) density gradients to purify the DNA as developed by Mandel, Shildkraut and Marmur (1968) are omitted.

3.1.2 Preliminary evaluation of five DNA isolation protocols

Five published procedures (Saghai-Maroof *et al.*, 1984; Dellaport *et al.*, 1985; Doyle and Doyle, 1987; Edwards *et al.*, 1991; Cheung *et al.*, 1993) were tested initially (Table 3.1,

Appendix A1-A5). These protocols all contained Tris, EDTA, NaCl, SDS (except protocols three and five), BME (except protocols four and five), ammonium acetate (except protocols two, four and six), chloroform:isoamylalcohol (except protocols two, four and five and protocol one also includes phenol), isopropanol and ethanol (except protocols four and six). In addition, protocol one included a proteinase K digestion and ether extraction (ether is thought to remove phenol which may interfere with PCR amplification (Saghai-Maroof *et al.*, 1984)), protocol two included potassium and sodium acetate and RNAse, protocol three included RNAse and protocol five sodium metabisulphite and sarcosyl. In protocol four, DNA is harvested directly after physical disruption of the plant tissue. The DNA was not sufficient and consistent to support RAPD amplification, and cross contamination between samples could not be avoided or inhibitors of enzymatic action were co-purified with it. The sodium metabisulfite used in protocol five is thought to increase the salt concentration in the extraction buffer which presumably "salts out" several PCR inhibitors and sarcosyl replaces SDS for cell lysis. The tissue disruption techniques rendered the DNA extraction process simple, fast and inexpensive with RNAse also included in TE buffer. The five protocols had varying amounts of starting material and all yielded sufficient DNA for PCR but the purity of the samples as determined by the absorbance readings were not acceptable except for protocol five which proved to be a rapid method where a number of samples could be performed simultaneously. However, variations in DNA yield were observed between samples, and slight variations in band intensity and faint DNA bands were seen upon RAPD amplification.

Table 3.1 Summary of the five types of DNA isolation protocols initially tested for DNA purity and yield.

Protocol	**Type of extraction**	**Tissue**	**Amount of starting material (mg)**	**Extraction time (h)**	**Purity A_{260}/A_{280}**	**DNA yield $\mu g\ \mu\ell^{-1}$**
1	maxi	seed	400	6	0.86-1.12	0.217-0.467
2	mini	leaf	1,000	4	1.29-1.65	0.508-1.407
3	mini	leaf	500-1500	3-3.5	1.45-1.85	0.157-0.376
4	micro	leaf disc	1	0.5-1	1.59-1.80	0.044-0.231
5	micro	leaf disc	1	2.5	1.60-2.00	0.080-0.950

3.1.3 Development of a modified DNA isolation procedure

None of the above mentioned protocols appeared to meet all of the requirements of an efficient DNA diagnostic system for a maize breeding programme i.e. quick and simple DNA extraction from a small amount of plant tissue, the capacity to analyse a large number of samples, uniform DNA yield and elimination of cross contamination (Deragon and Landry, 1992). In addition, the different extraction methods produced DNA of widely different purity. It is reported in the literature that DNA isolated with significant quantities of polysaccharides or phenolics does not make suitable PCR template and DNA containing moderate levels of impurities often produces blurred or faint RAPD phenotypes. In addition, differences between DNA preparations that affect primer annealing could also result in irreproducibility of RAPD patterns (Jobes *et al.*, 1995). Because the amplification process requires small amounts of template DNA, extraction procedures that emphasise purity rather than quantity are usually more appropriate to RAPD research. The techniques used for disruption of the plant tissue, the elimination of plant cell contaminants and the time taken to perform each extraction were identified as major problems with all five protocols. The protocol developed by Honeycutt *et al.* (1982) (hereafter referred to as the Honeycutt protocol), for the extraction of DNA from sugarcane (Harvey, 1994 personal communication), was then tested. Preliminary results of the DNA concentration and purity were good (Table 3.2) but a large amount of tissue was required (+/- 6 g) and it was decided to modify this protocol for use in this study.

The Honeycutt protocol was modified to a micro-extraction protocol. Leaf tissue disks of diameter 10 mm were punched out using an Eppendorf tube lid and placed into the tube. This ensured uniform size and also reduced the possibilities of contamination arising from handling the tissue for PCR analysis (Edwards *et al.*, 1991). The small amount of tissue required allowed molecular analysis of plants at an early growth stage. The leaf disks were macerated using an Eppendorf pestle to break open the cells, with the release of chlorophyll into the homogenisation buffer being a good indication of effective breakage of the plant cell wall. The addition of chilled homogenisation buffer and immediate placing on ice caused quick freezing which immediately inactivates DNAses.

Table 3.2 Ultraviolet spectrophotometry of DNA extracted from 11 maize genotypes using the unmodified Honeycutt *et al.* (1982) protocol.

Genotype	**Absorbance (260nm)**	**DNA concentration ($\mu g\ \mu\ell^{-1}$)**	**Purity A_{260}/A_{280}**
Inbred A	0.2705	0.541	1.86
Single cross AB	0.2655	0.531	1.92
Inbred B	0.4033	0.8066	1.92
Backcross ABxB	0.7164	1.4328	1.81
AB(F2) population	0.4786	0.9572	1.81
Single cross CB	0.2329	0.4658	1.83
Inbred C	0.1663	0.3326	1.83
Inbred K	0.4245	0.849	1.89
Inbred S	0.2513	0.5026	1.9
Inbred K-1	0.9891	1.9782	1.89
Inbred D	0.7859	1.5718	1.91

In the elimination of plant cell contaminants the components of the homogenisation and wash buffer in the Honeycutt protocol differed markedly from the other protocols tested. In addition to containing Tris, the homogenisation buffer contains EDTA which removes the Mg^{2+} ions that are essential for the preservation of the overall structure of the cell and inhibit cell enzymes which could destroy the DNA (Brown, 1990), and mercaptoethanol which causes unwinding of the protein helixes. This buffer also contains spermidine, a low weight polyamine, that at physiological pH 7 carries positive charges which provide a basis for binding interaction with DNA and possibly RNA. This contributes to the stability of the double helix and helps prevent possible DNA shearing (Strickberger, 1985). Polyethylene glycol (PEG) is included in the buffer which takes advantage of the inverse relationship between macromolecular size and the concentration of PEG required for precipitation. The wash buffer was identical to the homogenisation buffer but contains 25 mM EDTA and no PEG. Additional chemicals which are separately added to the wash buffer are: NaCl which causes alkaline denaturation and deproteinization (this would also denature any bacterial DNA present) and a high molar concentration of NaCl is reported to inhibit co-precipitation of polysaccharides and DNA (Jobes *et al.*, 1995); SDS a detergent that saponifies lipids thereby disrupting cell membrane integrity which increases the release of DNA from the cell; and CTAB which is a cationic detergent that aids in precipitation of the nucleic acids. Isopropanol aids in precipitation which separates high molecular weight DNA from polysaccharides (Marmur, 1961).

The final step in the protocol involving ethanol induced precipitation of the DNA was substituted with a step in which DNA was spooled out of the solution using a glass hook. Micheli *et al.* (1994) showed that ethanol precipitable contaminants in DNA are a major cause of irreproducibility. These workers stated that the spooling of the DNA may free it of the material that causes the RAPD variability as exhibited by centrifuged sample DNA. In extracting DNA for RAPD analysis using the five protocols there appeared to a be correlation between the method used to collect ethanol precipitated DNA and the reproducibility of the RAPD pattern. Of the protocols tested, only the Honeycutt protocol and the modified protocol yielded sufficient DNA that could be spooled out. The other methods only yielded DNA after centrifugation even though certain of these protocols had a substantial amount of starting material. More reproducible RAPD profiles were obtained, using the standard Williams *et al.* (1990) protocol, when DNA was spooled out compared to variable profiles when DNA was collected by centrifugation. This variability could also have been caused by the fact the RAPD reactions conditions had not yet been optimised. Contaminating RNA may be partly responsible for the variability observed with the centrifuged samples, but either the presence of very short DNA fragments or shortened templates or both may also lead to RAPD variability. This supports the hypothesis that ethanol precipitable contaminants and possibly also isopropanol precipitated contaminants include very low weight DNA and or RNA which in some way alters the formation of productive template primer complexes (Micheli *et al.*, 1994). The 70% ethanol wash prevents the DNA from going into solution and removes any salts still present.

The advantages of the modified DNA extraction protocol are that expensive enzymes, liquid nitrogen and phenol were not required. The technique isolates total genomic DNA (nuclear, chloroplast and mitochondrial) and is relatively simple and rapid. This allows for multiple extractions to be carried out in a relatively short period of time and therefore is cost efficient, yielding uniform high molecular weight DNA from small amounts of plant tissue, in adequate amounts and of sufficient purity and good quality for RAPD analysis. In addition, an extraction control to test for the absence of DNA contaminants in the solutions used for DNA extraction was performed. A control extraction of distilled water was carried through the entire procedure. This control was the last one handled in each step of the procedure, to offer

the best chance of detecting DNA carry-over among samples. No DNA contaminants were observed.

3.1.4 Yields of total nucleic acid and DNA

One of the most important variables in the RAPD amplification reaction is the concentration of genomic DNA. Hence it is important to determine DNA concentration of each extraction prior to amplification. The representative yield and purity of total nucleic acid was estimated by UV spectrophotometry. This enabled the specific DNA concentration of the template DNA and the purity of the sample to be determined. Although CTAB has been reported to cause obscurance of absorbance readings at 260 nm, due to the interference of residual CTAB in the final DNA solution (Jobes *et al.*, 1995), this was not observed as all of the 260 nm readings were within acceptable norms. DNA yields of between 0.3326 and 1.9782 μg $\mu\ell^{-1}$ per 6 g leaf roll (Table 3.2) were obtained for the Honeycutt protocol and 0.2985 and 0.4605 μg $\mu\ell^{-1}$ per 3 mg leaf disc (Table 3.3) when the modified protocol was used. These results compared favourably with those obtained by Harvey and Botha (1996) and Honeycutt *et al.* (1982) for the macro-protocols. As each RAPD reaction requires approximately 25 ng (Williams *et al.*, 1991) the modified protocol yielded sufficient DNA for 50 reactions per extraction.

Table 3.3 Ultraviolet spectrophotometry of DNA extracted from 11 maize genotypes using the modified protocol.

Genotype	**Absorbance (260nm)**	**DNA concentration (μg $\mu\ell^{-1}$)**	**Purity A_{260}/A_{280}**
Inbred A	0.557	417.75	1.82
Single cross AB	0.525	384	2.01
Inbred B	0.512	393.75	1.9
Backcross ABxB	0.492	369	1.75
AB(F2) population	0.398	298.5	1.81
Single cross CB	0.419	314.25	1.91
Inbred C	0.614	460.5	1.8
Inbred K	0.466	349.5	1.92
Inbred S	0.434	325.5	1.79
Inbred K-1	0.577	432.75	1.86
Inbred D	0.54	405	2

The relative purity of the isolated preparations generally did not vary widely, but the occasional occurance of an aberrant absorbance profile preceded problems with PCR amplification and was consequently repeated. The extracted DNA was reasonably free of contaminants as shown by the A_{260}/A_{280} ratios between 1.79 and 2.01 (Table 3.3), which are an improvement on the ratios of 1.60 to 1.78 obtained by Harvey and Botha (1996) and 1.64 to 1.86 obtained by Honeycutt *et al.* (1982).

3.1.5 Assessment of the integrity of the extracted DNA sequences

Kamalay *et al.* (1990) stated that differences in the quality of extracted total nucleic acid i.e. A_{260}/A_{280} calculation and the exact concentration of DNA in each preparation as measured spectrophotometrically showed that spectrophotometric readings may not necessarily be completely accurate. They also maintained that as it is necessary to know the exact amount of DNA used in each RAPD assay to achieve reproducibility, this amount should be quantified by agarose gel electrophoresis using uncut Lambda as a standard. This allows for standardization of the DNA in the sample and acts as a second check. The size heterogeneity of the extracted DNA was therefore assessed by agarose gel electrophoresis.

Under neutral conditions, the modal double strand size of the isolated DNA was approximately 21226 bp (Figure 3.1; lanes 3, 5, 6, 7 and 8) when compared to the co-migration of molecular weight standards (lanes 2 and 9). Given an estimated haploid genome size of $5x10^9$ bp and a diploid chromosome number of 2n=20 for maize, the average chromosomal DNA length is therefore $2.5x10^8$ bp. The isolation of maize genome in the $2x10^4$ bp range therefore suggests that approximately 12 500 random breaks were introduced into the chromosomal DNA.

Lanes 1 2 3 4 5 6 7 8 9

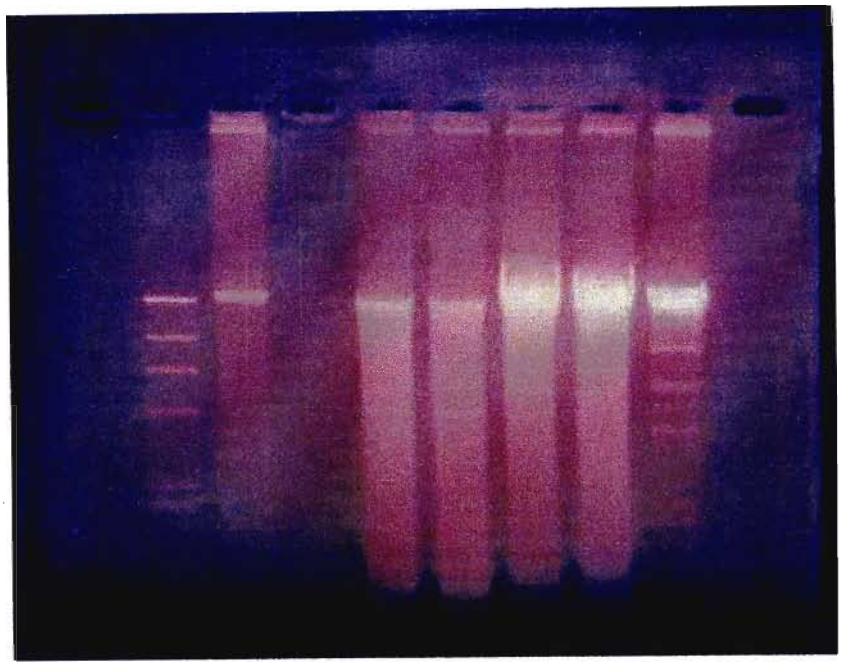


Figure 3.1 Assessment of DNA integrity of maize inbred A by electrophoresis (250 ng DNA at 5 V cm^{-1} for 3 h). Lane 1 template control, lanes 2 and 9 molecular weight marker II (Lambda DNA digested with Hind III), lane 3 genomic Lambda DNA (48502 bp), lane 4 extraction control and lanes 5 and 6 nucleic acid extractions of maize leaf tissue from the modified protocol and lanes 7 and 8 nucleic acid extractions of maize leaf tissue from the Honeycutt.

3.2 Optimisation of the RAPD amplification conditions

3.2.1 Introduction

Despite the simplicity of PCR, successful amplification is a result of a balance among the many experimental variables. The standard RAPD amplification conditions reported by Williams *et al.* (1990) are: 25 µℓ volumes containing 10 mM Tris.Cl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$. 0.001 % (w/v) gelatin, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM primer, 25 ng of DNA and 0.5 U of *Taq* polymerase, overlaid with mineral oil. It is, however, reported in the literature that the reproducibility of RAPD fingerprints can be quite problematic (Penner *et al.*, 1993; Wolff *et al.*, 1993), for example the sensitivity of fragment amplification can be due to minor changes in the composition of the reaction cocktail or cycle condition. If the RAPD

phenotype produced is very sensitive to even one of the PCR components then reproducibility is seriously impaired. Since many of the conditions of the RAPD reaction procedure may influence the result, the reaction conditions must be well defined in order to obtain reproducible patterns. The number of fragments amplified is a function of the sites on the template to which productive annealing of the oligonucleotide primer can occur (Williams *et al.*, 1993).

The optimisation of PCR-based technology is a laborious task as many components can be altered in PCR reactions and not all of the processes and mechanisms are fully understood. Wolff *et al.* (1993) used a factorial design to optimise the generation of RAPDs in chrysanthemum (*Dendranthema grandiflora*). Levi *et al.* (1993) optimised RAPD reaction conditions by employing five $MgCl_2$ concentrations; three sources of *Taq* DNA polymerase at three concentrations; nine primer:template ratios and nine annealing temperatures; three durations of DNA denaturation temperatures; and four numbers and patterns of cycles. Javed Iqbal and Rayburn (1994) optimised the concentrations of $MgCl_2$ from 1.0 to 5.0 mM; template DNA from 12.5 to 150 ng; and *Taq* DNA polymerase from 0.5 to 3.0 units per 50 μℓ reaction.

In this study, each PCR amplification was conducted as an experiment, with control reactions to test the purity and viability of the reagents. The types of controls used were: (i) a 'no template' control to test for the presence of contamination in the reagents i.e. a reaction with all reagents except template DNA, was included in each experiment to test the reagents for contaminating DNA; and (ii) a positive control of template AB and primer OPA-04 which was used to test the performance of the buffers, enzyme, temperature cycle and other parameters. A RAPD reaction was only assumed to be reproducible when the reaction was repeated in separate experiments three times and always found to be identical.

3.2.2 Thermocycler performance

Initial experiments using the protocol of Williams *et al.* (1990) revealed inconsistencies in RAPD profiles, even between aliquots of the same reaction cocktail that were incubated in adjacent wells of the same thermocycler. In addition, the full temperature cycle took up to 8 h

to complete. These inconsistencies were attributed to a defective Hybaid thermocycler. Linz (1990) also attributed RAPD inconsistencies to the thermocycler used. *Taq* polymerase activity is optimal at 72 °C. In a typical PCR the enzyme has a half-life of more than 2 h at 92.5 °C, but only 40 min at 95 °C, and less than 6 min at 97.5 °C (Kocher and Wilson, 1991). A faulty thermocycler could therefore result in all the enzyme being used up before the cycle was completed.

Another thermocycler of the same manufacturer and model, in which the thermo-sensor had been equilibrated, was then tested. The replacment thermocycler took a shorter time to complete the cycle (approximately 4 h) which appeared to remove any differences between well performance and produced more consistent results. Although the replacement thermocycler was shown to be reading temperatures 2 °C higher than was programmed, this temperature difference was taken into account in the programming. Later testing of the temperature sensor of the first thermocycler showed that the sensor was damaged and incorrect temperature readings were being taken.

Low amplification of the DNA fragments was still observed using the replacment thermocycler, and as DNA used in the experiments had normal spectrophotometric absorbance (A_{260}/A_{280} =1.8-2.0) and was intact, the inconsistencies in this experiment could not be attributed to poor DNA quality. It was therefore necessary to optimise the RAPD experimental conditions for obtaining reproducible results which was a prerequisite before being able to obtain results in the fingerprinting and molecular marker components of this study.

3.2.3 Choice of *Taq* DNA polymerase and buffer

Schierwater and Ender (1993) reported that different thermostable DNA polymerases may produce different RAPD products. Therefore, three *Taq* DNA polymerases sourced from Boehringer Mannheim®, Promega® and Perkin Elmer were compared. The AmpliTaq® Stoffel fragment was obtained as a trial aliquot but all the PCR reactions failed, this is possibly due to poor transport or storage conditions affecting enzyme activity. The other polymerases are both derived from *Thermus aquaticus* and initially produced similar (but not identical)

RAPD profiles for the same DNA. Standard reaction parameters (Williams *et al.*, 1991) with reaction buffers provided by the *Taq* enzyme manufactures, primer OPA-04 and an amount of 10 ng of DNA of genotype AB were used. An amount of 0.5 U of each polymerase were used per reaction, of the Promega ® *Taq* polymerase (5 U $\mu\ell^{-1}$), 0.1 $\mu\ell$ was used per reaction and of the Boehringer Mannheim® *Taq* (1U $\mu\ell^{-1}$), 0.5 $\mu\ell$ was used per reaction. All the reactions were repeated in triplicate and run simultaneously in the thermocycler. Control reactions without template DNA did not produce amplification products for either enzyme. For each primer/template polymerase combination, clearly reproducible fingerprint patterns were achieved, but for the different combinations quantitative differences in the amplification patterns were found, both in the relative amounts i.e. band intensity and number of amplified products. It was therefore decided to repeat the reactions but using the same reaction buffer (Promega ®) for each *Taq* polymerase. No variation within the amplification patterns of the two enzymes was then observed (Figure 3.2, lanes 1-6).

Lanes 1 2 3 4 5 6

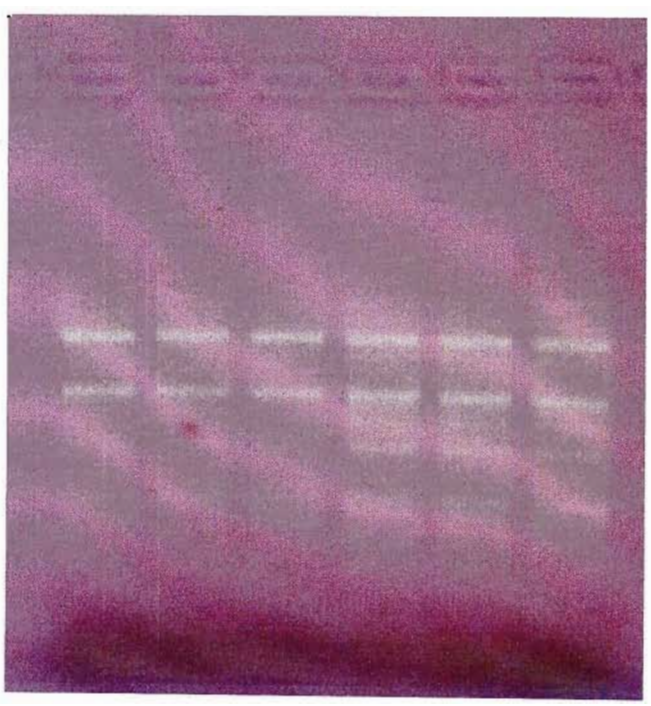


Figure 3.2 RAPD fingerprint patterns of two thermostable DNA polymerases with the same amplification buffer. Lanes 1-3 Promega® *Taq* and lanes 4-6 Boehringer Mannheim® *Taq*.

Schierwater and Ender (1993) stated that the exact reasons for the differences in the amplification patterns of the polymerases are not known, but that the activity and specificity of the different polymerases may depend on slight differences in temperature and reaction preferences, which affect the outcome of possible competition reactions between the DNA template amplified in the first and most critical cycle. However, from these results it was shown that the reaction buffer is more critical to the reproducibility of the reaction. These results are more in agreement with those obtained by Levi *et al.* (1993) who published that the combination of buffer type, annealing temperature and duration of incubation at 94 °C were most critical for obtaining a high amplification rate and reproducible phenotypes. *Taq* is a highly hydrophobic protein and tends to precipitate from aqueous solutions. The addition of non-ionic detergent emulsifiers (e.g. Triton X-100) helps to maintain full activity, both in storage solutions and in the amplification reaction (Kocher and Wilson, 1991). Promega® reaction buffer contains 500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25 °C) and 1 % Triton-X, whereas Boerhinger Mannheim® buffer contains only 500 mM KCl and 100 mM Tris-HCl. In addition 0.001 % gelatin was included in the reaction buffers used in these experiments. Gelatin is a heterogenous mixture of water-soluble carrier proteins of high molecular weight. It is thought that Triton-X and gelatin possibly decrease the relative volume of the reaction cocktail and thus maximise template-primer interaction and may also stabilise DNA/DNA hybrids (Levi *et al.*, 1993). The pH of the reaction buffer is also important; *Taq* appears to have a pH optimum of 7.0 - 7.5 at 72 °C. Normally a Tris buffer with pH 8.0 is used. The Promega ® *Taq* and reaction buffer kit was chosen for the remainder of the experiment as this enzyme was cheaper and the cost of the analyses was an important consideration in this study.

3.2.4 Template DNA concentration

One of the most important variables is the concentration of genomic DNA. It is necessary to optimise the amount of DNA used in the RAPD assay to achieve reproducibility and good band intensity. Too much DNA may result in gel smears or in a lack of clearly defined bands in the gel. Conversely, too little DNA often gives unreproducible results. Keeping all the other reaction parameters equal, the standard RAPD conditions (Williams *et al.*, 1990), with 0.5 U *Taq* (Promega ®) and 0.1 % gelatin were used. The following maize DNA amounts were amplified: 3 ng, 6 ng, 9 ng, 12 ng, 15 ng, 18 ng, 21 ng, 24 ng, 27 ng and 30 ng using

primers OPA-04, OPB-10 and OPC-18 on three of the 11 maize genotypes, namely AB, S and C. Each reaction was amplified in triplicate to assess the reproducibility of the reaction. Near identical RAPD phenotype template DNA profiles were obtained (Figure 3.3). A DNA concentration of 15 ng (lanes 15-17) per reaction was chosen for the remaining optimisation experiments and for the molecular marker screening experiments as this banding profile was the most consistent.

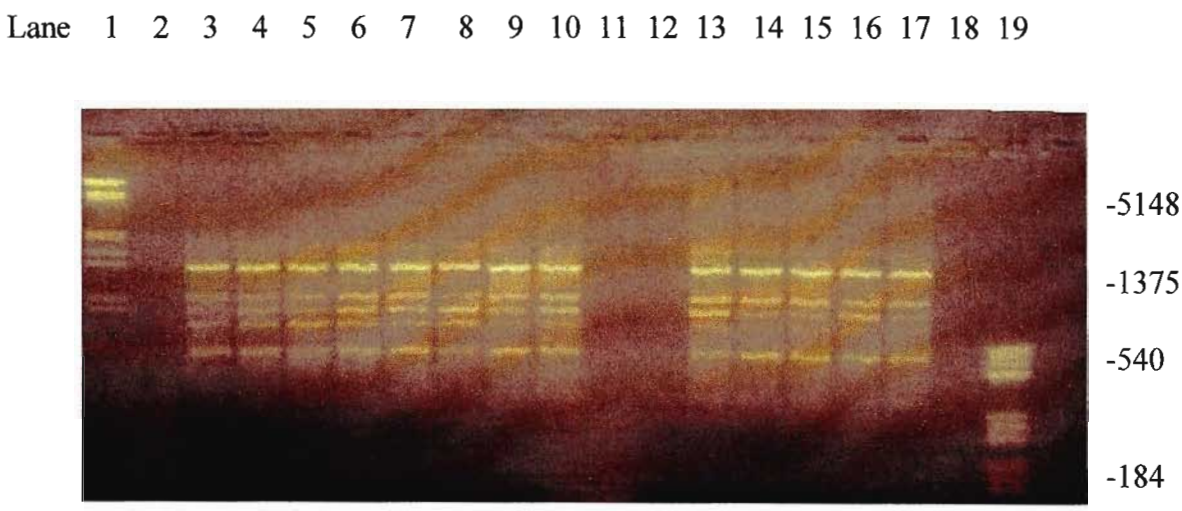


Figure 3.3. Effect of template DNA concentration on the RAPD reaction. DNA from maize single cross AB was amplified with primer OPA-04. Lane 1 contained MWM III, lane 2 control reaction, lanes 3-5 3 ng DNA, lanes 6-8 6 ng DNA, lanes 9-11 9 ng DNA, lanes 12-14 12 ng DNA, lanes 15-17 15 ng DNA and lane 19 MWM V.

3.2.5 Reaction components optimised separately

3.2.5.1 Effect of *Taq* polymerase concentration

In the determination of the optimal enzyme concentration, both the cost of *Taq* DNA polymerase and the generation of clear and stable amplification products must be considered (Devos and Gale, 1992). The recommended concentration of *Taq* is 20 U $m\ell^{-1}$ which is reported to work well for most plant and animal species (Williams *et al.*, 1993). It was, however, decided to optimise this parameter for this application. Using standard RAPD

reaction conditions (Williams *et al.*, 1990), five *Taq* DNA polymerase amounts (0.2 U, 0.4 U, 0.8 U, 1.0 U and 1.2 U) were tested using primers OPA- 04, OPB-08 and OPC-12 on three maize genotypes AB, S and K-1. Each reaction was amplified in triplicate to assess the reproducibility of the reaction. Although similar banding patterns were obtained for all concentrations (Figure 3.4), 0.4 U (lanes 12-14) gave the most consistent and reliable banding patterns. As it is also considerably cheaper to use a small amount of enzyme, it was decided to use this concentration for the remainder of the experiments.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Figure 3.4 Effect of *Taq* DNA polymerase concentration on the RAPD reaction. DNA from maize single cross AB was amplified with primer OPA-04. Lane 1 MWM V, lane 2 control reaction, Lanes 3-5 1.2 U *Taq*, lanes 6-8 1.0 U *Taq*, lanes 9-11 0.8 U *Taq*, lanes 12-14 0.4 U *Taq*, lanes 15-17 0.2 U *Taq* and lane 19 MWM III.

3.2.5.2 Effect of primer concentration

Williams *et al.* (1993) reported that primer concentrations between 0.1 and 2.0 μM are optimal. At lower concentrations it becomes difficult to detect amplification products in ethidium bromide stained agarose and at higher concentrations smearing of the bands may be evident. Using the reaction conditions optimised thus far, five primer concentrations (1, 2.5,

5, 7.5 and 10 pmol) were tested using three primers OPA-04, OPB-10 and OPC-18 on three maize genotypes AB, S and K-1. Primer OPC-18 (Figure 3.5) resulted in a higher background and a higher number of amplification products at the 10 pmol concentration (lanes 3-5). At lower primer concentrations (lanes 12-14) the reactions produced fewer PCR products but the bands were more intense and not always reproducible (lanes 15-17). There appeared to be a narrow working range of primer concentration, with the limits of the range partially dependant on the template DNA and sequence of the primer. The 5 pmol primer concentration (lanes 9-11) gave the most reproducible results and was therefore chosen for the remaining experiments.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

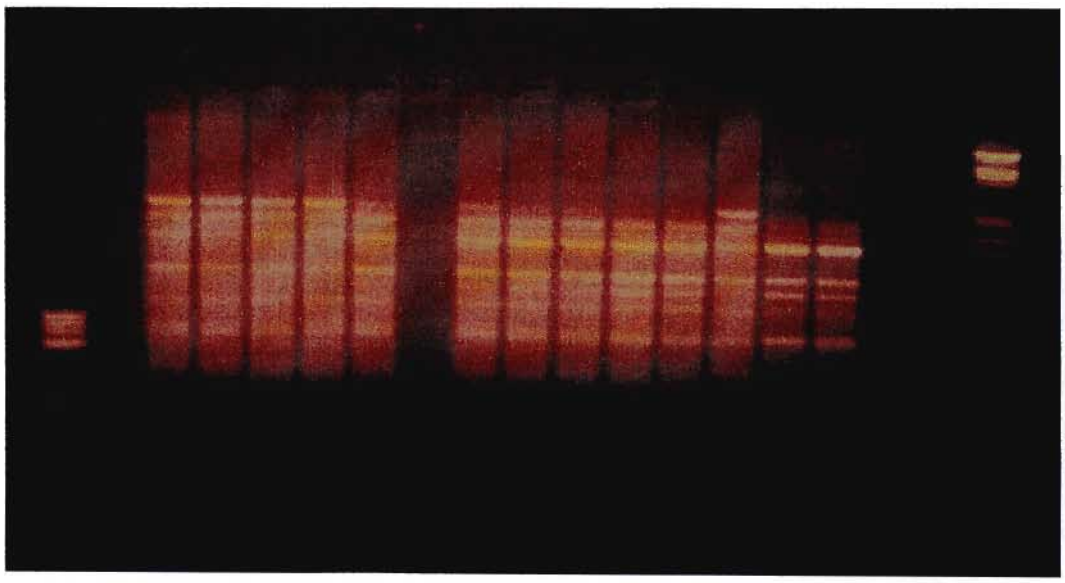


Figure 3.5 Effect of primer concentration on the RAPD reaction. DNA from maize inbred K-1 amplified with primer OPC-18. Lane 1 MWM V, lane 2 control reaction with no DNA, lanes 3-5 10 pmol, lanes 6-8 7.5 pmol, lanes 9-11 5.0 pmol, lanes 12-14 2.5 pmol, lanes 15-17 1 pmol and lane 19 MWM III.

Hadrys *et al.* (1992) reported that primer size determines the degree of specificity in genome scanning, where primers of short length amplify an unreasonably large number of sequences and larger primers amplify too few sequences to be routinely informative. As all the primers used in this study were decamers, the effect of primer length was not tested but most studies using standard RAPD conditions show 10 bp to be an efficient size. Williams *et al.* (1993) reported that a length of less than nine nucleotides resulted in failed reactions, whilst the DNA

amplification fragment (DAF) technique developed by Caetano-Anollés *et al.* (1992) employs primers as small as five nucleotides in length with good success.

All the primers tested had a G + C content of 50 - 70 %, therefore the effect of G + C content was not tested. However, Williams *et al.* (1993) showed that primers with a G + C content less than 40 % resulted in failed reactions; 40-60 % produced reactions with a few bands, but the best results are obtained with 70-90 %, 80 % being the optimum. A 100 % G + C content produced reactions with diffuse bands. Hadrys *et al.* (1992) stated that a primer with a G + C content similar to the G + C content of the analysed genome will maximise the frequency of binding sites and hence the amplified products. Yu and Pauls (1992) also reported an interaction between the time taken for annealing and the GC content of the primer; however, as the G + C content of the primers used in this study was not investigated, this could not be validated

The interaction of primer with template DNA also affect RAPD reactions. A fraction of the primers are non-functional i.e. unable to amplify DNA or produce adequate profiles. These non-functional primers therefore add to the expense and effort of primer screening. This number may be considerable in RAPD analysis (Caetano-Anollés, 1994). The fingerprinting and marker aided selection sections (Chapters 4 and 5) provide evidence of the effect of primer x DNA interaction.

3.2.5.3 Effect of magnesium concentration

Taq requires free Mg^{2+} ions for activity. Nucleotides and EDTA chelate divalent cations significantly decreasing the concentration of free magnesium. Mg^{2+} also influences the hybridisation of primers to template DNA and may prevent primer extension by the polymerase. The Mg^{2+} concentration in the PCR reaction must therefore be a few millimolar units higher than the nucleotide concentration (Kocher and Wilson, 1991). However, high concentrations of Mg^{2+} can lower the specificity of primer-template interaction and increase the possibility of mis-priming and the production of 'primer-dimers' (Hill and Steward, 1992). The Mg^{2+} concentration has also been reported to affect the relative intensity of the bands in the PCR reaction (Williams *et al.*, 1993).

Using the reaction conditions optimised thus far, five $MgCl_2$ concentrations (0.5 mM, 1.5 mM, 2.5 mM, 3.5 mM and 4.5 mM) were chosen to investigate the effects of increasing magnesium concentration on the RAPD reaction (Figure 3.6). These concentrations were tested using primers OPA-04, OPB-10 and OPC-18 on the three maize genotypes AB, S and K-1. Each reaction was run in triplicate to test the reproducibility. The $MgCl_2$ concentration appeared to alter the size and distribution of the RAPD products. The reactions for 0.5 mM $MgCl_2$ (lanes 15-17) did not work with no bands visible. Minor changes were observed for the range 1.5 mM to 3.5 mM (lanes 8-12), whereas when the Mg^{2+} concentration was increased there was a shift from amplification of a number of large fragments towards a number of smaller fragments, the amplification of band 'a' increased which is similar to that reported by Williams *et al.* (1993). The bands for 4.5 mM $MgCl_2$ appeared slightly diffuse. The optimum magnesium concentration was therefore chosen to be 2.5 mM $MgCl_2$ (lanes 10-12).

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

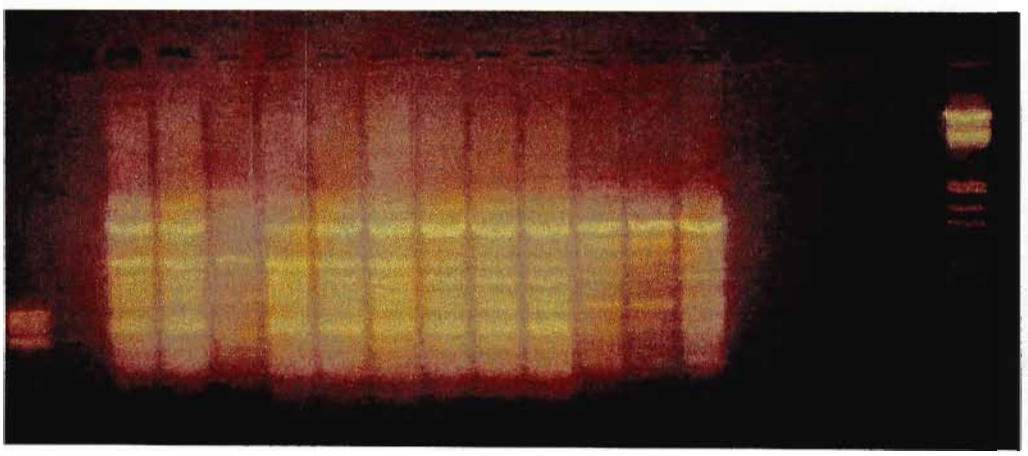


Figure 3.6 Effect of magnesium concentration on the RAPD reaction. DNA from maize inbred S was amplified with primer OPB-10. Lane 1 contains MWM V, lane 2 blank, lanes 3-5 4.5 mM $MgCl_2$, lanes 6-8 3.5 mM $MgCl_2$, lanes 9-11 2.5 mM $MgCl_2$,, lanes 12-14 1.5 mM $MgCl_2$,, lanes 15-17 0.5 mM $MgCl_2$,, lane 18 is a control reaction without DNA (2.5 mM $MgCl_2$) and lane 19 MWM III.

3.2.5.4 Effect of deoxynucleotide tri-phosphate concentration

The Km for deoxyribonucleotides (dNTPs) is about 1.5 μM, but higher concentrations are reported to increase processivity (Kocher and Wilson, 1991). The mis-incorporation rate of the *Taq* polymerase may be altered by nucleotide concentration. Very low concentrations or unbalanced ratios of dNTPs are reported to lead to mis-incorporation. However, at very high concentrations of dNTPs, error rates are also much higher, therefore it is necessary to optimise the dNTP concentration (Bell and DeMarini, 1991). Nucleotides are remarkably resistant to heat and have a half life of more than 40 thermal cycles in a PCR amplification (Kocher and Wilson, 1991).

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

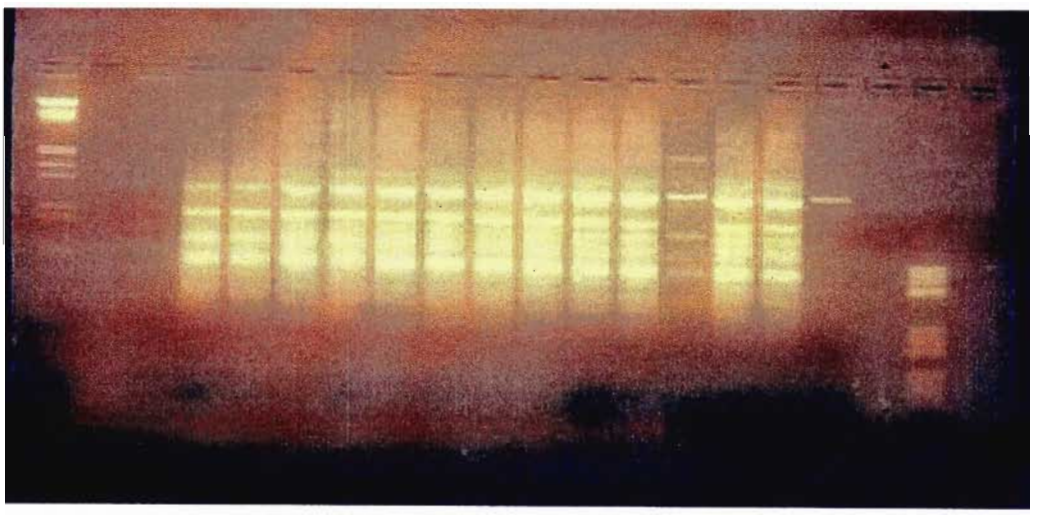


Figure 3.7 Effect of dNTP concentration on the RAPD reaction. DNA from maize inbred S was amplified with primer OPB-10. Lane 1 MWM III, lane 2 control reaction without DNA, lanes 3-5 0.5 mM , lanes 6-8 1.0 mM, lanes 9-11 2.0 mM, lanes 12-14 2.5 mM, lanes 15-17 3.0 mM and lane 19 MWM V.

Using the reaction conditions optimised thus far, five dNTP concentrations (0.5 mM, 1.0 mM, 2.0 mM, 2.5 mM and 3.0 mM) were tested using primers OPA-04, OPB-10 and OPC-18 on three maize genotypes AB, S and K-1. Each reaction was replicated in triplicate. No big differences was noted between the samples but at a lower concentration 0.5 mM the intensity of the bands in the gel appeared to be weaker (Figure 3.7, lanes 3.5). The 1.0 mM

concentration of dNTPs (lanes 6-8) was chosen as the optimum for the remaining experiments.

3.2.5.5 Effect of annealing temperature

It is reported that each primer-template combination has an optimum annealing temperature (Wolff *et al.*, 1993). However, for the purposes of this study it was necessary to have standard conditions for each primer. Therefore, using the reaction conditions optimised thus far, four different annealing temperatures (T_0) 20 °C, 35 °C, 37 °C and 40 °C were tested using the primers OPA-04, OPB-10 and OPC-18 on three maize genotypes AB, S and K-1. Each reaction was amplified in triplicate to test the reproducibility. The samples were placed in the thermocycler and amplified for one cycle of 92 °C for 2 min, followed by 40 cycles of 92 °C for 1 min, T_0 for 1 min and 72 °C for 2 min (where the annealing temperature T_0 was as indicated as above). The 20 °C annealing temperature reactions did not work (Figure 3.8, lanes 3-5). The 37 °C and 40 °C (lanes 9-14) temperatures gave similar banding profiles whilst 35 °C (lanes 6-8) gave the highest number of bands as well as the clearest bands.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

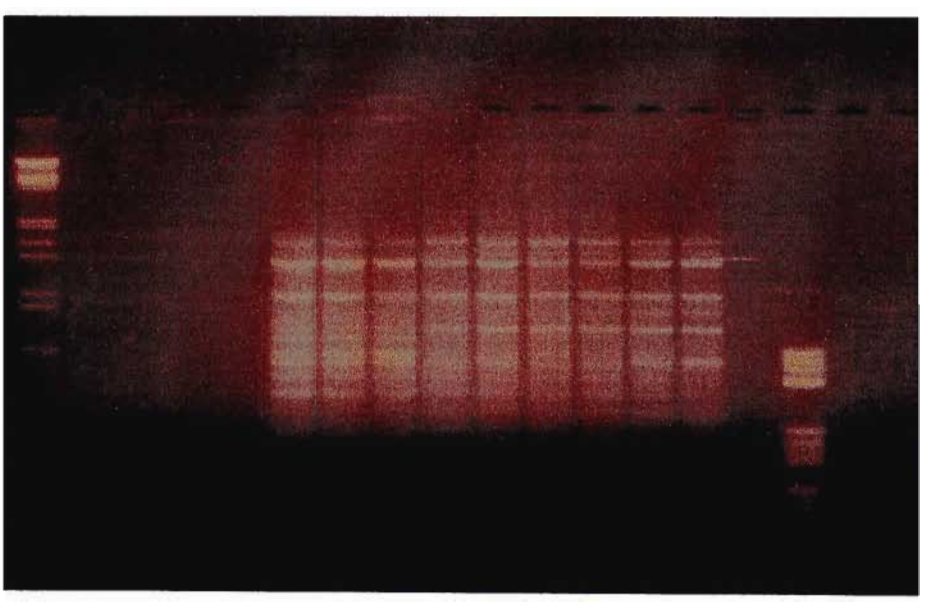


Figure 3.8 Effect of annealing temperature on the RAPD reaction. DNA from maize inbred K was amplified with primer OPC-18. Lane 1 MWM III, lanes 3-5 20 °C, lanes 6-8 35 °C, lanes 9-11 37 °C, lanes 12-14 40 °C and lane 16 MWM V.

This experiment illustrates quantitatively the dependance of amplification on annealing temperature. The 35 °C temperature (lanes 6-8) was chosen as the optimal annealing temperature and used for the fingerprinting and marker studies. A postulated reason for using lower annealing temperature is that the incidence of scoring errors appears less; however, there is insufficient data to document this theory (Wolff *et al.*, 1993).

3.2.5.6 Effect of incubation time and number of cycles

Yu and Pauls (1992) examined the length of each step and the number of cycles and reported that 35 cycles with a 5 sec denaturation step, a 30 sec annealing step and a 60 sec extension step gave improved results. Using the reaction conditions optimised thus far, incubation times of 5, 30 and 60 sec at 92 °C; 50, 60 and 70 sec at 35 °C; and 60, 90 and 120 sec at 71 °C were tested systematically following an initial denaturation step of 91 °C for 2 min. The temperature cycles program of Yu and Pauls (1992) gave inconsistent results for the maize genome, possibly due to maize having a larger genome than alfalfa and therefore requiring longer denaturation, annealing and extension steps. The 60, 90 and 120 sec combination gave the most consistent results, which is in agreement with the standard incubation times of Williams *et al.* (1990) so it was decided to use these incubation times. Four different numbers of cycles were also tested, namely 35, 40, 45 and 50 cycles. As there were no observable differences between the number of cycles except the minor increase in the intensity of some bands, it was decided to use 40 cycles as this number is reported most frequently in the literature. A final elongation step of 5 min at 71 °C was also included. A summary of the optimisation experiments conducted in this section is given in Table 3.4.

Table 3.4 Summary of the RAPD reaction conditions evaluated and the optimum chosen.

Variable	**Concentrations or conditions evaluated**	**Optimum**
Buffer	1 or 2	2
Gelatin (%)	0, 0.01, 0.1	0.01
dNTPs (mM)	100, 200	200
Primer (pmol)	1, 5, 10	5
Template (ng)	3, 6, 9, 12, 15, 18, 21, 24, 27, 30	12
Taq polymerase (U)	0.5, 0.75, 1.0, 1.5, 2	0.5
$MgCl_2$ (mM)	0.5, 1.5, 2.5, 3.5, 4.5	2.5
Annealing temperature (°C)	20, 35, 36, 37, 42	35
Incubation time (sec) 92 °C (denaturation) 35 °C(annealing) 71 °C(elongation)	 5, 30, 60 50, 60, 70 60, 90, 120	 60 60 120
No. cycles	35, 40, 45, 50	40

3.2.6 RAPD reaction components optimised in combination

3.2.6.1 Introduction

PCR optimisation relies on the sequential investigation of each reaction variable. In 1994, Cobb and Clarkson published an optimisation approach for the PCR reaction (based on the model of Taguchi (1986) for optimising industrial reactions) which optimises the reaction components in combination. Provided that three concentrations are used for each reaction component tested, the number of experiments required (E) is calculated from the equation $E = 2k+1$, where k is the number of factors to be tested. Each component occurs at one of three predetermined levels (A, B and C). These must be chosen so that they are sufficiently separated to determine their effects on the reaction. The product yield for each reaction is then used to estimate the effects of the individual components on amplification. This is done using the signal-to-noise ratio (SNL):

$$SNL = -10 \log(1/n \text{ SUM } 1/y^2) \quad \text{.....Equation 3}$$

where n is the number of levels and y is the yield. For each component the optimal conditions are those that give the largest SNL. The reaction can be further refined by using the

polynomial regression from the SNL values for each component to obtain curves whose maximum represents the reaction optima (Cobb and Clarkson, 1994). RAPD products are scored according to a desired characteristic. The largest number of scorable bands covering the greatest size range is optimal as larger products are easier to size without resorting to polyacrylamide gel electrophoresis. RAPD profiles are scored according to the equation:

$P = (r \times s)+1$ .....Equation 4

where P is the product yield, r is the number of products and s is the size range which is either up to 1kb (s=1) or up to 2kb (s=2). Reactions giving a smear of amplification products or no amplification products at all are given a score of one. These scores are used as target yields to calculate the SNL which are then used to estimate the optimal conditions.

3.2.6.2 Combination of PCR components experiment

Optimisation of a standard reaction using the Cobb and Clarkson (1994) principles was tested. Tables 3.5 and 3.6 give the concentration levels of the components and the orthogonal arrays for the four variables, each at three levels, used respectively. The nine optimisation reactions were replicated in triplicate. Amplification profiles were obtained for every one of the nine component combinations (Figure 3.9).

Table 3.5 Concentration levels for components used in a Taguchi (1986) array for optimisation.

Components	**Levels**		
	A	**B**	**C**
Primer concentration (pmol)	1	5	10
$MgCl_2$ (mM)	1.5	2.5	3.5
DNTP (mM)	0.1	0.2	0.4
Taq polymerase (U)	0.5	1	1.5

Table 3.6 Orthogonal arrays (Taguchi, 1986) for the four reaction components used, each at three levels.

Reactions	Primer (pmol)	$MgCl_2$ (mM)	dNTPs (mM)	*Taq* pol (U)
1	1	1.5	0.1	0.5
2	1	2.5	0.2	1
3	1	3.5	0.4	1.5
4	5	1.5	0.2	1.5
5	5	2.5	0.4	0.5
6	5	3.5	0.1	1
7	10	1.5	0.4	1
8	10	2.5	0.1	1.5
9	10	3.5	0.2	0.5

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

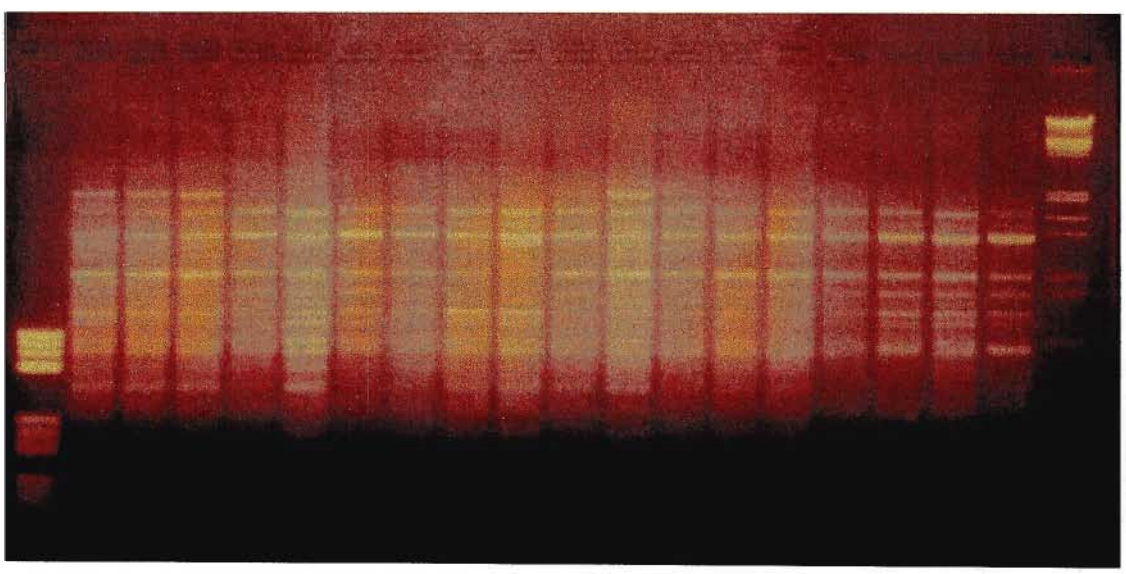


Figure 3.9 Amplification profiles obtained for each of the nine component combinations. DNA from maize inbred K was amplified with primer OPC-18. Lane 1 MWM V, lanes 2-4 reaction 6, lanes 5-7 reaction 5, lanes 8-10 reaction 4, lanes 11-13 reaction 3, lanes14-16 reaction 2, lanes 17-19 reaction 1 and lane 20 MWM III

RAPDs were scored according to the number and distribution of products for each reaction. The largest number of scorable bands covering the greatest size range was optimal as larger products are easier to size without resorting to polyacrylamide gel electrophoresis. Table 3.7 gives the results of the RAPD profiles when scored according to Equation 3, the band size range which was up to 2 kb (s=2). This introduces a yield bias for products of high molecular

weights. This data was then used to calculate the SNL values Equation 2 (Table 3.8) to establish the optimal reaction conditions.

Table 3.7 The yield obtained for each reaction component combination tested.

Reaction	**Number of Bands (r)**	**Yield (p)**
1	8	17
2	11	23
3	14	29
4	9	19
5	9	19
6	13	27
7	14	29
8	15	31
9	14	29

Table 3.8 The signal to noise ratio (SNL) values obtained for every component level.

	A	**B**	**C**
Primer conc	26.616	26.375	29.433
$MgCl_2$	26.068	27.212	29.031
dNTPs	26.068	27.212	29.031
Taq pol	26.068	28.286	27.782

Two general assumptions are made when using the Taguchi (1986) method: (i) that the optimal level for the PCR component lies within the range tested; and (ii) that the optimal level does not form a discreet, tightly defined peak within the range tested but not represented by any one of the levels used. The choice of absolute values to be tested for each component is chosen based on the prior knowledge of PCR amplification from target DNA. Table 3.9 gives a summary of the optimum reaction components obtained for the conditions optimised separately and in combination.

Table 3.9 Optimised reaction conditions obtained using the reaction components analysed separately (a) and the reaction components analysed in combination using the Taguchi-model (b).

	$MgCl_2$ (mM)	**DNA (ng)**	**Primer (pmol)**	**dNTPs(mM)**	***Taq* pol (U)**
a	2.5	12	5	200	0.4
b	3.5	-	10	400	1

As cost was an issue and there was no significant improvement in profiles obtained for the components optimsed in combination the lower concentrations of the components optimised separately were chosen for the remaining experiments.

3.2.7 Appropriate visualisation of the RAPD profile

In RAPD studies relatively few amplification products are resolved and the fingerprints produced are fairly simple to interpret. It is, however, important to quantify the degree of amplification achieved before characterising the fragment further (Kocher and Wilson, 1991). The homogeneity of the amplified DNA is most conveniently assessed by electrophoresis through an agarose gel. However, Caetano-Anollés *et al.* (1991) using DNA amplification fingerprinting (DAF), with arbitrary primers as short as five nucleotides, produced relatively complex DNA profiles with polyacrylamide gel electrophoresis and silver staining, whereas agarose gel electrophoresis and ethidium bromide staining of amplification products detected only a few major fragments. Therefore a considerable loss of information could occur if suitable fragment separation and detection procedures were not employed.

The optimised silver staining procedure of Bassan *et al.* (1991) was then compared to agarose gel electrophoresis with ethidium bromide staining to determine which method gives appropriate visualisation of the RAPD profile. RAPD reactions were run using the optimised protocol with primers OPA-01 to OPA-10 using template DNA from maize genotypes A, AB, B, K, S, K-1 and D. Aliquots from the same reaction sample (25 µℓ) were loaded onto the two different gel types. A volume of 20 µℓ was loaded onto 2 % (w/v) agarose gel with 5 µℓ loading buffer (20 % (w/v) sucrose and 0.2 % bromophenol blue in 1x TBE) and 4 µℓ was loaded onto polyacrylamide gel with 5 M urea and 0.02 % bromophenol blue. The reactions were run on the same day in the same area of the laboratory. No reactions were observed for primer OPA-06 on either gel. This is consistent with other reactions run, possible due to the unavailability of priming sites on the maize DNA for primer OPA-06. Polyacrylamide gel electrophoresis with silver staining resulted in complex banding patterns with numerous bands (Figure 3.10). These PAGE fingerprints included a range of bands which varied widely in intensity and were often so close together (lane 5) that the bands were difficult to distinguish from one another making them difficult to analyse. The stronger staining of smaller fragments

relative to larger ones was also observed. This has been has been reported to be related to physical factors during staining (Goldman and Merril, 1982).

Lanes 1 2 3 4 5 6 7 8

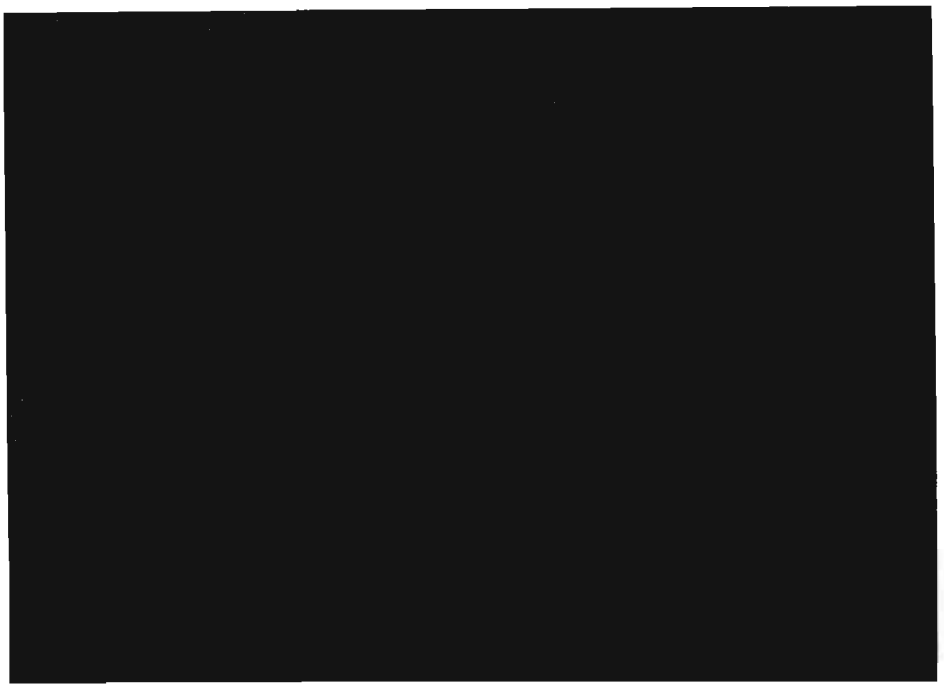


Figure 3.10 Polyacrylamide gel electrophoresis of DNA from seven of the 11 maize genotypes amplified with primer OPA-04. Lane 1 A, lane 2 AB, lane 3 B, lane 4 ABxB, lane 5 AB(F2), lane 6 BC and lanes 7 C.

The agarose gels with ethidium bromide staining produced clear resolution of both major and minor bands with consistent reproducibility of banding patterns (Figure 3.11) and yielded a number of bands ranging from two to nine per reaction. Therefore agarose gels still yielded sufficient bands for an informative fingerprint. Thus it was decided to use agarose gel electrophoresis with ethidium bromide for all further detection of RAPD products in the following experiments.

Lanes 1 2 3 4 5 6 7

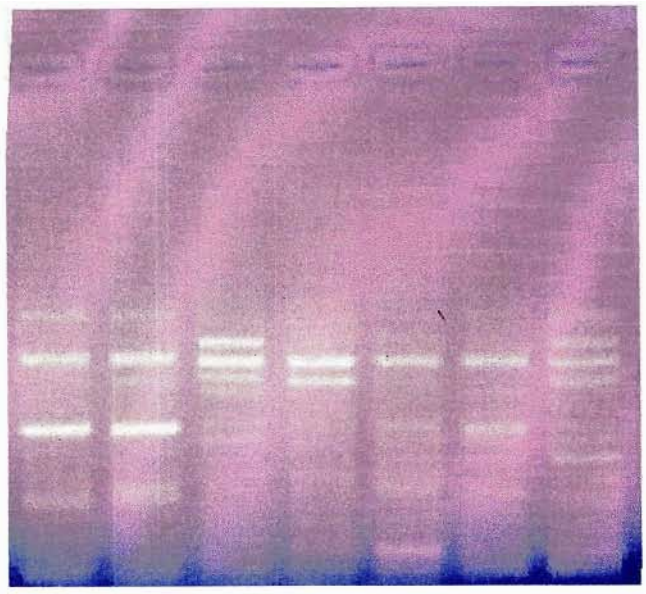


Figure 3.11 Agarose gel electrophoresis of DNA from seven of the 11 maize genotypes amplified with primer OPA-04. Lane 1 A, lane 2 AB, lane 3 B, lane 4 ABxB, lane 5 AB(F2), lane 6 BC and lanes 7 C.

3.3 Evaluation of RAPD markers amplified from bulked samples versus individual samples, and determination of the optimum number of individuals to include in a bulk for genotype analysis.

Having established reproducibility of the amplification profiles, the occurrence of polymorphism between plants of the same genotype were examined. In single crosses AB and BC no individual plant polymorphisms were identified among the resulting amplification profiles. These results indicate the DNA stability of these genotypes. This is to be expected as they are single cross F_1 hybrids and all individual plants of the same single cross are theoretically genetically identical to each other. However, the analysis of maize inbreds A, B and more so in C, revealed a few polymorphisms among the individual plants tested. The inbreds surprisingly appear to be genetically less stable than the single cross hybrid. Maize inbreds are highly homozygous, therefore there should be no polymorphism between plants of the same inbred unless there is a mutation event. As maize are reported to have a higher

degree of transposon events (Gourmet and Rayburn, 1996), there is a possibility that RAPDs are detecting these insertion and deletion events. It is also possible that inbred C is not as homozygous as inbreds A or B. The backcross ABxB and F_2 generation AB(F_2) revealed more polymorphisms between individual plants which is expected as segregation would be taking place.

The capacity of DNA-based molecular markers to detect more genetic variation, compared with isozyme or morphological markers does have the drawback that more intra-cultivar variation may now be detected. In the context of cultivar identification, this sensitivity to DNA sequence variation needs to be reduced by diluting the number of rare alleles. The allelic variation detected between pools of samples is then the result of several individuals sharing the same alleles. Melchinger *et al.* (1990) reported using a bulk of five individuals maize inbred plants for their RFLP study of genetic diversity and Harvey and Botha (1996) stated that DNA from at least three separate sugarcane plants from the same variety must be pooled to ensure that sugarcane variety-specific as opposed to individual plant-specific banding profiles are obtained. One would not expect polymorphisms between individual plants of a sugarcane variety as sugarcane is vegetatively propogated and therefore each plant is a clone of an original genotype. However, there is a known tendency for genetic variation to develop in polyploids such as sugarcane due to mutations.

It was decided to determine the optimal number of individuals to use in a bulked sample of maize. Bulked samples were prepared by: (i) pooling equal amounts of already extracted genomic DNA purified from two, three, five and 10 different individuals to determine the optimum number of individuals to be combined in each bulk; and (ii) extracting and purifying DNA from leaf samples bulked prior to DNA extraction comprising of two, three, five and 10 individuals per genotype using one leaf disk from each individual. Each pool was constructed twice using a different set of individuals for each of the genotypes. Aliquots of 25 ng from the combined samples were used in the RAPD reaction.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

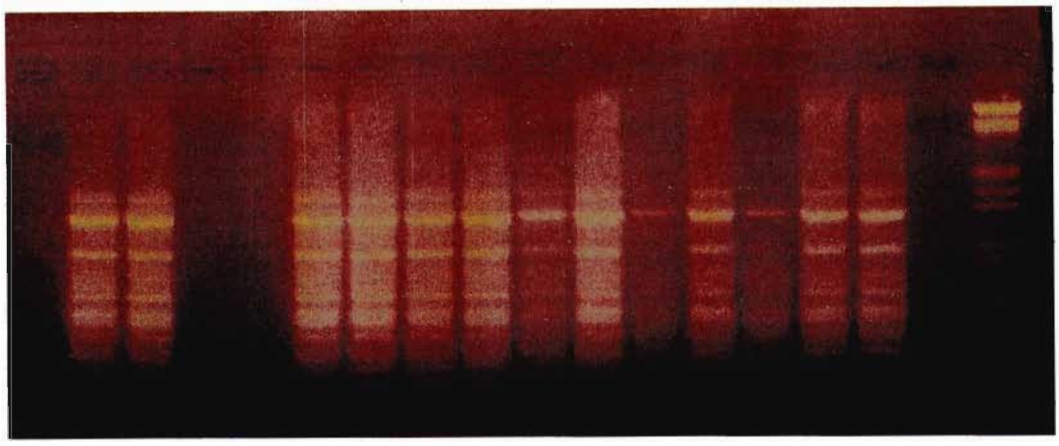


Figure 3.12 Amplification profiles obtained for bulk samples of DNA isolations and bulked leaf samples. Maize inbred K amplified with primer OPC-18. Lane 1 and 2 10, lanes 3 and 4 5, lanes 5 and 6 3, lanes 7 and 8 2, lanes 9 and 10 10, lanes 11 and 12 5, lanes 13 and 14 3, lanes 15 and 16 2 and lane 17 MWM III.

Minor differences were observed between the genomic DNA pools (Figure 3.12; lanes 9-16) and the pools of bulk leaf samples (lanes 1-8). The profiles obtained for the leaf bulk of three individuals was the most clear (lanes 5 and 6). It was therefore decided to bulk leaf samples of three individual plants of a genotype prior to DNA extraction. The RAPD markers generated would then amplify only DNA sequences shared among most of the individuals of a given genotype considering that inbreds, single crosses and segregating populations were being compared. Since bulked samples of DNA were used to produce RAPDs, a mixture of sequences with different degrees of homology with the primer could be amplified. The final quantity of amplifications therefore depends upon the sequence frequency in the sample. Competition between these could mean that only a reduced number of sequences of all those possible are effectively resolved as defined bands in the gel. This competition could also occur in the amplification of single plant DNA but to a lesser extent due to the lower number of sequences homologous to the primer.

To determine the level of resolution provided by the amplification of bulked DNA samples, individual plants of maize inbred C was also analysed. DNA from six individual plants was separately amplified with primer OPA-04 (Figure 3.13). More background and a larger

number of resolved fragments were observed in the individual fragments (lanes 1-6) than in the result obtained with bulk DNA sample (lane 7). In the analysis of bulk DNA sample, only frequently seen fragments in the individual plants were observed. Fragments seen at frequencies below 8 % were not amplified in the bulk sample. This result is slightly lower than the 10 % described by Michelmore *et al.* (1991) and the 14 % of Loarce *et al.* (1996).

Lanes 1 2 3 4 5 6 7

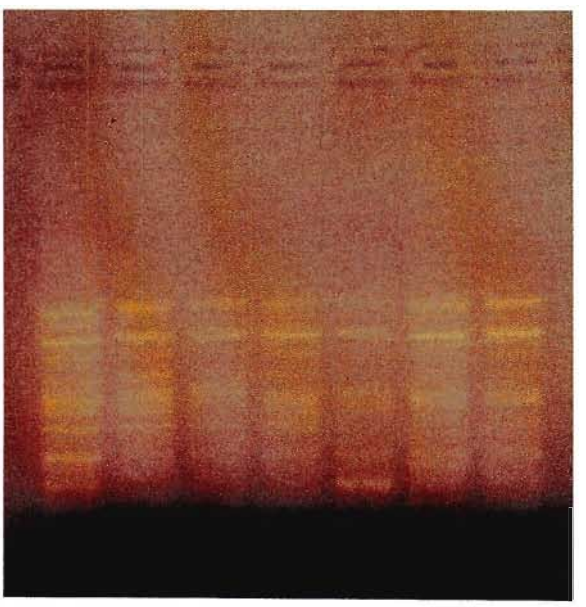


Figure 3.13 Amplification profiles obtained for individual plants and a bulk sample of maize inbred A amplified with primer OPA-04. Lane 1 individual 1, lane 2 individual 2, lane 3 individual 3, lane 4 individual 4, lane 5 individual 5, lane 6 individual 6 and lane 7 bulk sample.

CHAPTER 4
RESULTS AND DISCUSSION OF THE DETERMINATION OF THE GENETIC RELATIONSHIP BETWEEN SEVEN MAIZE GENOTYPES USING RAPD MARKERS

4.1 Introduction

The production of RAPD markers with PCR amplification requires consistency in reaction conditions in order to obtain reproducible results. Following optimisation of the DNA isolation and RAPD protocols (Chapter 3), the RAPD results reported in this chapter were found to be extremely consistent. The PCR conditions specifically $MgCl_2$, primer, dNTP, *Taq* polymerase and DNA concentrations were kept constant throughout all experiments to ensure that comparisons between amplification profiles could be made. For each genotype the PCR reactions were run in duplicate to check the consistency of the amplification. The pedigree and sources of the seven maize genotypes A, AB, B, ABxB, AB(F_2), BC and C characterised by RAPD analysis are provided in Table 2.1. Using these seven maize genotypes the level of polymorphism, inheritance of markers bands and determination of genetic diversity between these genotypes was investigated.

4.2 Polymorphism detected by RAPD markers

A total of 60 oligonucleotide primers from Operon® kits A, B and C were screened for their ability to generate RAPD markers (details of kits provided in Appendix B). Eighteen of the primers resulted in no amplification indicating that they had no homology with maize DNA. Forty-two primers produced a total of 233 fragments, an average of 5.5 loci per primer. Twenty-four of the primers did not give reproducible or easily distinguishable amplification products. Only 18 primers (Table 4.1) gave fragments that were present in both replicates of the same genotype. These primers were scored as they produced the most clearly resolved banding patterns with at least a few polymorphisms detectable between the genotypes. Many other authors report a similar selection of only a subset of primers (Connolly, Godwin, Cooper

and Dehaey, 1994; Rus-Kortekaas *et al.*, 1994; Harvey and Botha, 1996), although this may exaggerate the magnitude of the calculated DNA diversity. Also listed in Table 4.1 are the number of bands amplified for each primer.

Table 4.1 Primers used in this study, their sequence, number of bands obtained, and number of variable bands among them.

Primer	**Sequence (5'-3')**	**Amplification products**	
		Total bands	Variable bands
OPA-01	CAGGCCCTTC	8	8
OPA-02	TGCCGAGCTG	8	5
OPA-05	AGGGGTCTTG	5	0
OPA-07	GAAACGGGTG	4	2
OPA-18	AGGTGACCGT	9	7
OPB-01	GTTTCGCTCC	7	5
OPB-07	GGTGAGGCAG	7	7
OPB-10	CTGCTGGGAC	5	4
OPB-18	CCACAGCAGT	6	5
OPB-19	ACCCCCGAAG	4	4
OPC-01	TTCGAGCCAG	6	6
OPC-02	GTGAGGCGTC	6	6
OPC-04	CCGCATCTAC	6	2
OPC-05	GATGACCGCC	5	3
OPC-08	TGGACCGGTG	5	3
OPC-10	TGTCTGGGTC	4	3
OPC-18	TGAGTGGGTG	7	6
OPC-19	GTTGCCAGCC	8	6

The 18 primers produced a total of 110 genetic loci, 81 % of which were polymorphic. The size of the scored fragments varied from 300 to 2500 bp relative to the molecular weight standards. The number of fragments amplified ranged from four to nine per reaction depending on the primer used, with an average of 6.1 per reaction. Not all 18 primers revealed polymorphisms. One of the primers, OPA-05, resulted in amplification profiles that were identical across all the populations (Figure 4.1, lanes 13-19). This monomorphic primer appeared to amplify conserved sequences which exist in all the maize genotypes examined. Such conserved sequences may be useful in determining the sequence divergence of cereal genomes (Devos and Gale, 1992).

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

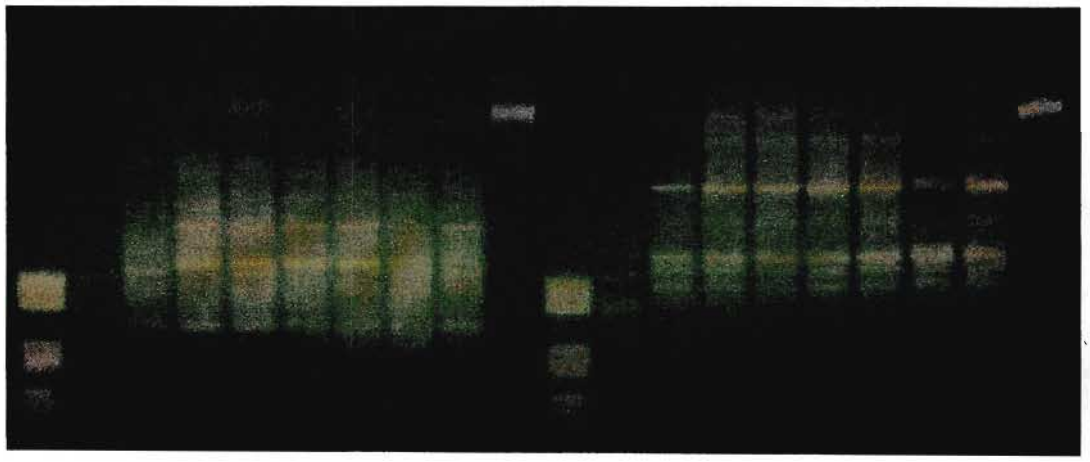


Figure 4.1 RAPD profiles of the seven maize genotypes amplified with primers OPA-07 and OPA-05 respectively. Lanes 1 and 11 MWM V, lanes 2 and 12 negative control with no DNA, lanes 3 and 13 inbred C, lanes 4 and 14 single cross BC, lanes 5 and 15 population AB(F2), lanes 6 and 16 backcross ABxB, lanes 7 and 17 inbred B, lanes 8 and 18 single cross AB, lanes 9 and 19 inbred A and lanes 10 and 20 MWM III.

The primers of primary interest in this study were ones that could be used for genotype identification. Such primers, for example OPA-02 (Figure 4.2, lanes 2-8), resulted in polymorphic amplification profiles between the genotypes.

Lanes 1 2 3 4 5 6 7 8

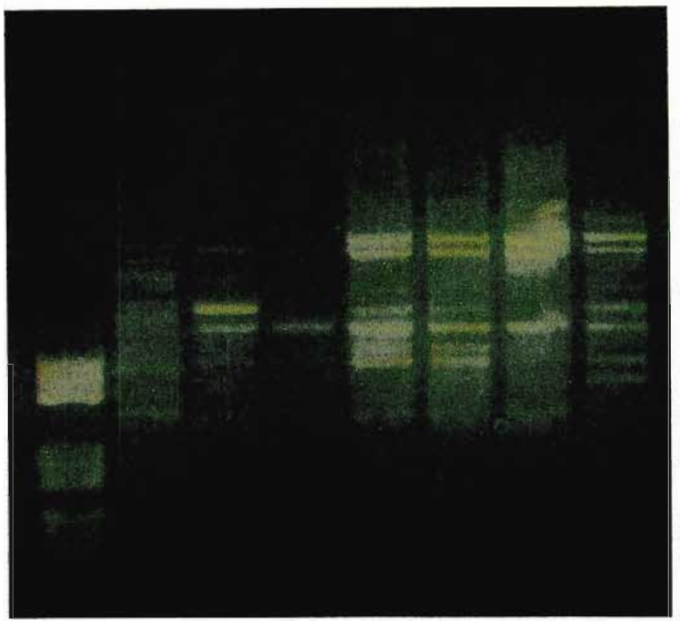


Figure 4.2. RAPD profiles of the seven maize genotypes amplified with primer OPA-02. Lane 1 MWM V, lane 2 inbred C, lane 3 single cross BC, lane 4 population AB(F2), lane 5 backcross ABxB, lane 6 inbred B, lane 7 single cross AB, lane 8 inbred A.

The fragment profiles obtained with the RAPD primers were scored manually as present (1) or absent (0) (Table 4.2). Five of the 18 primers screened produced population specific markers i.e. bands monomorphic within the population where they are present but polymorphic between populations. Primer OPA-01 revealed a fragment unique to maize inbred B (indicated by arrow < in lane 5 Figure 4.3); primer OPB-07 revealed a fragment unique to maize inbred A; primer OPC-01 revealed a fragment unique to the F2 population AB(F2); and primers OPC-05 and OPC-08 revealed two fragments unique to the single cross BC. Therefore four out of the seven cultivars were distinguishable from each other on the basis of their amplification profiles alone. The rest of the genotypes, while not greatly dissimilar, had easily distinguishable profiles. Thus, to reliably distinguish between the genotypes a number of primers are needed (Figures 4.4).

Table 4.2. Manual scoring of the presence (1) or absence (0) of RAPD generated bands using 18 primers on seven maize genotypes.

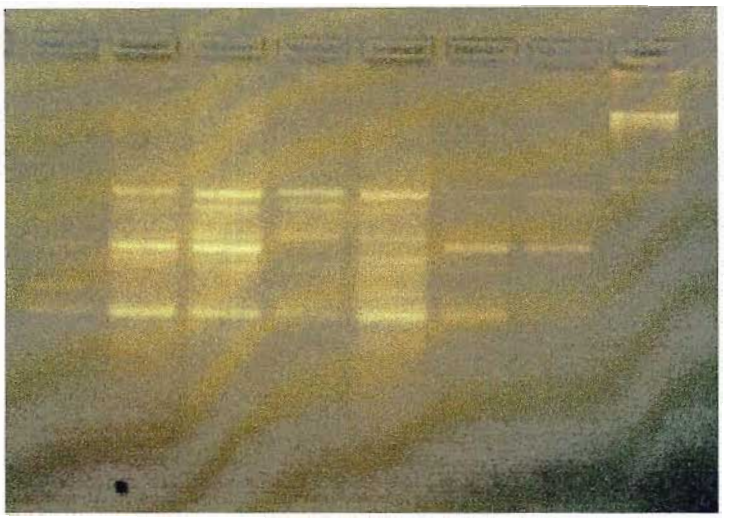
Primer	A	AB	B	ABxB	AB(F2)	BC	C
OPA-01	1	1	1	1	1	1	0
	1	1	1	1	1	1	0
	0	0	1	1	1	0	0
	1	1	0	0	1	1	1
	0	0	1	0	1	1	0
	0	0	1	0	1	1	0
	0	0	1 *	0	0	0	0
	0	1	1	1	1	1	1
OPA-02	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	0	1	1	0	1	0
	1	1	1	1	1	1	1
	0	0	1	1	0	0	1
	1	1	1	1	0	0	0
	1	1	1	1	0	0	1
	0	1	1	1	0	1	1
OPA-05	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
OPA-07	1	1	1	1	1	1	0
	1	1	1	1	1	1	1
	1	0	0	0	1	0	1
	1	1	1	1	1	1	1
OPA-18	1	0	1	1	1	1	0
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	0	1	1	1	0	1	1
	1	1	1	1	1	1	0
	0	0	1	1	0	1	1
	0	1#	0	0	0	1	1
	1	1	1	1	0	1	0
	1	1	0	1	1	0	1
OPB-01	0	0	1	1	0	1	0
	1	1	1	1	1	1	1
	0	1	1	1	0	1	0
	1	1	0	1	1	0	0
	0	1	1	1	0	0	0
	1	1	1	1	1	1	1
	0	1	1	1	1	1	0
OPB-07	0	1	0	1	1	0	0
	1 *	0	0	0	0	0	0
	1	1	1	1	1	1	0
	1	1	0	0	0	0	1
	0	1	1	1	1	0	1
	1	1	1	1	1	0	1
	1	1	1	1	1	0	0
OPB-10	1	1	1	1	1	0	1
	0	0	1	1	0	0	0
	1	1	1	1	1	0	0
	1	1	1	1	1	0	1
	1	1	1	1	1	1	1

OPB-18	1	0	1	1	1	1	0
	1	0	1	1	1	1	1
	1	1	1	1	1	1	0
	1	1	1	1	1	0	1
	1	1	1	1	1	0	1
	1	1	1	1	1	1	1
OPB-19	1	1	1	1	1	0	0
	1	1	0	0	0	0	0
	1	1	1	1	1	1	0
	1	1	1	1	1	0	1
OPC-01	1	0	1	1	1	0	1
	1	1	1	1	1	0	1
	1	1	1	1	1	0	0
	1	1	1	1	1	0	1
	0	0	0	0	1 *	0	0
	1	1	0	0	0	1	1
OPC-02	1	0	0	0	0	1	1
	1	1	1	1	1	0	1
	1	1	1	1	1	1	0
	1	0	1	1	1	0	1
	0	0	0	0	0	1	1
	1	1	1	1	1	0	1
OPC-04	1	0	0	0	0	1#	0
	1	1	1	1	1	0	1
	1	0	1	1	1	0	0
	1	1	1	1	1	1	0
	1	1	1	1	1	1	0
	1	1	1	1	1	0	1
OPC-05	0	0	0	0	0	1* #	0
	1	1	1	1	1	0	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
OPC-08	1	1	0	1	1	1#	0
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	0	0	0	0	0	1* #	0
	1	0	1	1	1	1	1
OPC-10	1	1	1	1	1	0	1
	0	1	1	1	1	0	0
	0	1	1	1	1	1	1
	1	1	1	1	1	1	1
OPC-18	1	1	1	1	1	1	0
	1	1	0	1	1	0	0
	1	1	1	1	1	1	0
	1	0	0	0	0	1#	0
	1	1	1	1	1	1	1
	1	1	0	1	1	0	1
	1	1	1	1	1	0	1
OPC-19	1	1	1	1	1	0	0
	1	1	1	1	1	1	0
	1	1	1	1	1	1	1
	1	1	1	1	1	1	1
	1	1	1	1	1	0	1
	0	1	1	1	1	0	1
	0	0	0	0	0	1	1
	1	1	1	1	1	0	1

* genotype specific marker

non-parental band

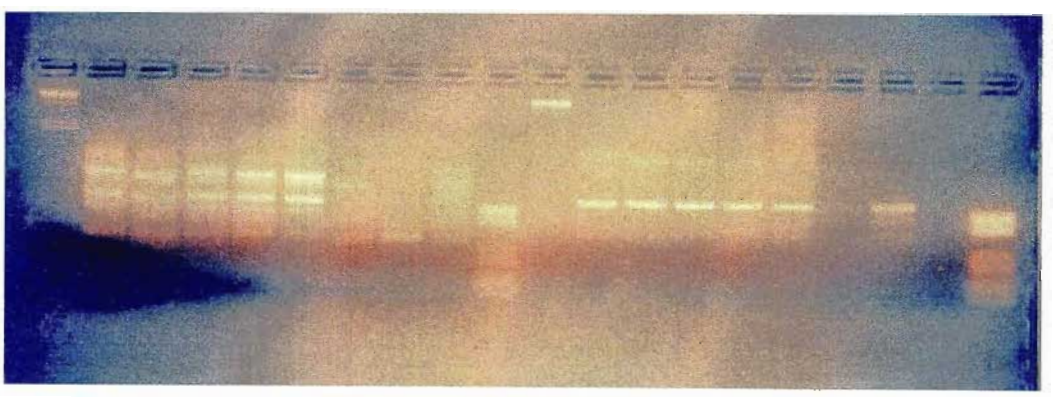
Lanes 1 2 3 4 5 6 7 8



<

Figure 4.3 RAPD profiles of seven maize genotypes amplified with primer OPA-01. < shows maize inbred B specific fragment in lane 5. Lane 1 inbred C, lane 2 single cross BC, lane population AB(F_2), lane 4 backcross ABxB, lane 5 inbred B, lane 6 single cross AB, lane 7 inbred A and lane 8 MWM III.

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



#

Figure 4.4 RAPD profiles of seven maize genotypes amplified with primers OPB-18 and OPB-19 respectively. Lanes 1 and 11 MWM III, lanes 2 and 12 inbred A, lanes 3 and 13 single cross AB, lanes 4 and 14 inbred B, lanes 5 and 15 backcross ABxB, lanes 6 and 16 population AB(F_2), lanes 7 and 17 single cross BC, lanes 8 and 18 inbred C, lanes 9 and 19 negative control with no DNA and lanes 10 and 20 MWM V.

4.3. Cluster analysis of the seven maize genotypes A, AB, B, ABxB, AB(F_2), BC and C.

Cluster analysis based on the estimates of genetic similarity was used to determine the relationships of the maize genotypes. Two algorithms for cluster analysis detailed in Chapter 2 (software and programme details provided in Appendix 3) were used in this study. Firstly, hierachial cluster analysis calculated from the similarity coefficient (Nei and Li, 1979) using Genstat 5™ release 4.1 (Table 4.3 and Figure 4.5) and secondly, the UPGMA (unweighted pair group method arithmetic average) method using PAUP (Phylogenetic Analysis Using Parsimony) 4.0 beta version (Swafford, 1998) (Table 4.4 and Figure 4.6). The relationships between the genotypes are graphically represented as dendrograms (Figures 4.5 and 4.6).

Table 4.3 Similarity matrix for seven maize genotypes using Genstat 5™ release 4.1 software (1993).

	A	AB	B	AB* B	AB(F2)	BC	C
A	1						
AB	0.852	1					
B	0.816	0.844	1				
AB*B	0.859	0.898	0.95	1			
AB(F2)	0.871	0.864	0.874	0.904	1		
BC	0.667	0.658	0.701	0.675	0.653	1	
C	0.72	0.738	0.714	0.726	0.72	0.585	1

Table 4.4 Polymorphism among seven maize genotypes evaluated by pairwise marker difference using PAUP 4.0 beta version software (Swafford, 1998).

	A	AB	B	AB* B	AB(F2)	BC	C
A	-	0.23	0.29	0.23	0.2	0.45	0.38
AB	25	-	0.25	0.16	0.21	0.46	0.35
B	32	27	-	0.08	0.2	0.42	0.4
AB*B	25	18	9	-	0.15	0.46	0.39
AB(F2)	22	23	22	17	-	0.47	0.38
BC	50	51	46	51	52	-	0.49
C	42	39	44	43	42	54	-

- above the diagonal: total character differences and below the diagonal: mean character differences (adjusted for missing data)

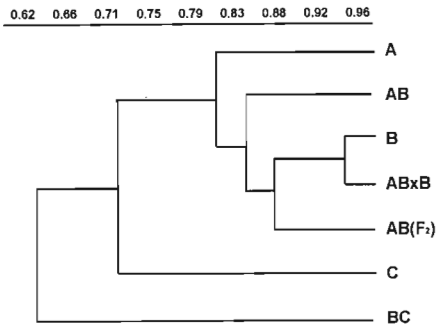


Figure 4.5 Dendrogram of seven maize genotypes A, AB, B, ABxB, AB(F_2), BC and C generated using Genstat 5^{TM} release 4.1 software (1993).

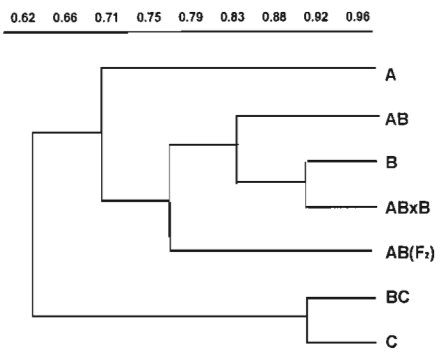


Figure 4.6 Dendrogram of seven maize genotypes A, AB, B, ABxB, AB(F_2), BC and C produced using PAUP 4.0 beta version software (Swafford, 1998).

All the genotypes were distinguishable from each other based on their amplification profiles, irrespective of the method of statistical analysis. Using Genstat 5^{TM} release 4.1 software (1993) a genetic similarity of 0.62 between genotypes BC and A was obtained. BC was observed to be the most distinct genotype. A genetic similarity of 0.96 between genotypes B and ABxB was obtained. This result is in agreement with the hypothesised close relationship of these genotypes. A should be closely related to AB, and so should B as these are the hypothesised inbred parents of the single cross. A should be closely related to ABxB, but B more so as ABxB is a backcross to B. A and B should also be related to AB (F_2) because it is the segregating F_2 of the single cross AB. B should be related to BC and so should C as these

are the hypothesised inbred parents of the single cross. C should be less related to AB, ABxB and AB(F_2), With respect to genotype identification using the PAUP 4.0 beta version software (Swafford, 1998) analysis both BC and C were found to be consistently different from the other genotypes.

4.4 Inheritance of polymorphic fragments and interpretations concerning parentage

Parentage was examined in terms of RAPD fragment transmission and parental contribution to offspring. The breeder suspected that AB (PAN 473) was the result of a cross between A (NPPES1) and B (M162W), and that BC was the result of crossing B (M162W) and C (R108W). It is expected that all the RAPD bands present in the single cross progeny should be present in either or both of the inbred parents. The converse situation where the presence of one or more RAPD bands in a particular single cross is not matched in either parent was observed. One band marked #, generated with primer OPA-18 (Figure 4.7, lane 2) for single cross AB, was not present in either inbred parent A or B: this could be the result of an incongruous band (also observed by Reidy *et al.* (1992) arising from the inconsistency inherent in the RAPD technique). Five bands were generated in the single cross with suspected parentage B x C that were not present in either inbred parent B or C. Two of these fragments were also the genotype specific fragments for single cross BC (Figure 4.8, lane 7). It is possible such incongruous fragments could arise as a result of recombination of chromosomal material, but this is unlikely where more than one primer binding site is implicated. It is more likely that either inbred B and/or inbred C are not the parents of the single cross BC. All bands present in backcross ABxB were present in either parent AB or B.

Very rarely the control lane (Figure 4.8, lane 9) showed the presence of DNA contamination. As the fragments were not the same size as DNA amplified from any of the genotype these fragments were possibly due to primer dimers or indicated the presence of contaminants in the PCR reagents, new reagents were then used for further experiments.

Lanes 1 2 3 4 5 6 7 8 9 10

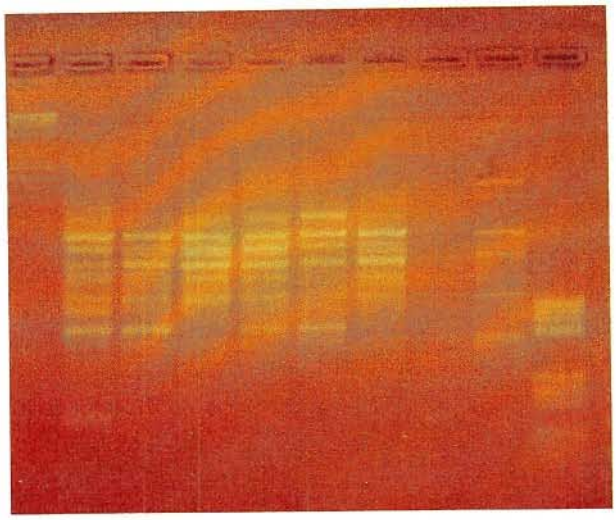
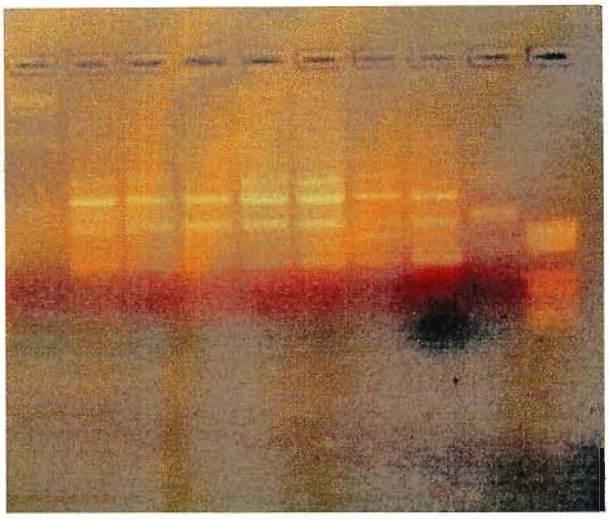


Figure 4.7. RAPD profiles of seven maize genotypes amplified with primer OPA-18. Lane 1 MWM III, lane 2 single cross AB, lane 3 inbred A, lane 4 inbred B, lane 5 backcross ABxB, lane 6 population AB(F_2), lane 7 single cross BC, lane 8 negative control without DNA, lane 9 C and lanes 10 MWM V.

Lanes 1 2 3 4 5 6 7 8 9 10



\#

* #

Figure 4.8. RAPD profiles of seven maize genotypes amplified with primer OPC-08. The # represents two non parental bands and * represents the genotype specific band. Lane 1 MWM III, lane 2 inbred A, lane 3 single cross AB, lane 4 inbred B, lane 5 backcross ABxB, lane 6 population AB(F_2), lane 7 single cross BC, lane 8 inbred C, lane 9 control lane with no DNA and lane 10 MWM V.

The estimates of genetic similarity based on the formula of Nei and Li (1979) showed good agreement with the breeders hypothesis of parentage for single cross AB that inbred A (unknown) is the parent in hybrid AB. This does not provide absolute confirmation of documented parentage since the analysis is based on transmission patterns only. RAPD markers have been shown to be useful in several inheritance studies to date in spite of earlier doubts based on inconsistencies in behaviour as dominant characters with expected Mendelian segregation (Echt *et al.*, 1992; Reiter *et al.*, 1992). The work of Heun and Helentjaris (1993) was specifically designed to address the problem concerning certain RAPD fragments being classed as 'unambiguous polymorphisms' (simple presence /absence of specific fragments) or 'quantitative polymorphisms' (exhibited a variation in the intensity of a fragment). Both these types of RAPD markers were shown to behave as dominant markers in maize F_1 hybrids.

Since the DNA samples used in these experiments consisted of a bulk sample of DNA extracted from three individual plants, a low intensity for any particular fragment may be explained by the lesser representation of that specific sequence in the bulk sample of DNA. Intensity was therefore not taken into account in this study and fragments from different genotypes showing identical mobility were considered to represent the same genetic locus. This could introduce a bias in the estimation of genetic distances between genotypes. The fragment pattern of a genotype will be composed of fragments amplified from sequences with a high frequency of occurance in the sample. It is more probable that the rare sequences presented in a bulk DNA sample would be amplified when DNA from individual plants was analysed. Therefore, two genotypes whose differences were due to poorly represented sequences in both genotypes would show a stonger similarity when bulk DNA samples were used compared. The opposite would occur for two distantly related genotypes i.e. two genotypes whose similarities were reduced to poorly represented sequences of low common occurance would show a lower genetic distance using bulked DNA samples than using DNA from individual plants (Loarce *et al.*, 1996).

CHAPTER 5
RESULTS AND DISCUSSION
OF RAPD SCREENING FOR PUTATIVE MARKERS FOR DISEASE RESISTANCE IN LISTED MAIZE POPULATIONS

5.1 RAPD screening for markers for grey leaf spot resistance

The intention of this study was to identify a putative marker linked to GLS. To that end two non-isogenic maize inbreds K054W (K) and S0173W (S), resistant and susceptible to GLS, respectively were screened with 20 primers (Operon® primer Kit A). Since these two inbreds are not isogenic lines the polymorphisms revealed could not necessarily be linked to GLS resistance. As no normally distributed population for resistance and susceptibility to GLS was readily available, it was not possible to conduct a bulk segregant analysis (Michelmore *et al.*, 1991). The results of the GLS screening are provided in Appendix B.1 and briefly summarised here. Three primers failed to amplify products in both genotypes, six primers only amplified in one of the genotypes, seven primers produced identical profiles in genotypes and four primers produced polymorphic profiles. Primers OPA-01 and OPA-07 resulted in two and one polymorphic fragments in inbred K respectively and OPA-15 and OPA-16 produced one polymorphic fragment each in inbred S.

5.2 RAPD screening for markers for leaf blight resistance

As two near isogenic lines (NILs) for leaf blight (*Helminthosporium spp.*) resistance were available, K0315Y (K-1) resistant and D0940Y (D) susceptible, RAPD screening for leaf blight resistance markers was conducted. D0940Y is the recurrent, susceptible parent of K0315Y. K0315Y is a sixth generation backcross recovery of D0940Y and is resistant to *Helminthosporium* spp. Therefore K0315Y is virtually a complete isoline of D0940Y differing only in its resistance to *Helminthosporium* spp which appears to be controlled by the major gene HtN i.e. a single locus (Gevers, 1994 personal communication). Crossing K0315Y (HH) and D0940Y (hh) results in a F1 of Hh and an F2 of 1HH:2Hh:1hh i.e. a segregation of 3 resistant:1 susceptible.

The linkage of RAPD polymorphisms to genetic regions of interest have been determined most successfully using pairs of backcross derived NILs (Paran *et al.*, 1991), the principle being the identification of markers located in a linkage block surrounding the introgressed gene (Melchinger *et al.*, 1990). The linkage relationship between the polymorphic fragment (putative marker) needs to be verified by segregation analysis, since polymorphic regions unlinked to the trait, also could be present in the introgressed NIL.

5.2.1 Incidence of polymorphism between K0315Y and D0940Y

One hundred and twenty decamer primers (Operon kits A,B,C,V,W,X;) were used for a comparative RAPD-PCR analysis of genomic DNA extracted from the NILS. The results are given in Appendix D and summarised in Table 5.1.

Table 5.1. Summary of RAPD analysis of maize inbreds K0315Y and D0940Y.

Number of primers used	120
Number of loci characterized	341
Number of primers scored	10
Number of polymorphisms identified	14
Polymorphic fragments characteristic of K0315Y	5
Polymorphic fragments characteristic of D0940Y	9
Frequency of polymorphism	0.04

Fourteen primers failed to give amplification products, 16 primers did not give reproducible or easily distinguishable amplification products therefore a total of 30 primers were not scored. From the remaining 90 primers a total of 341 discrete products ranging in size from 0.4 kbp to 3.5 kbp were amplified, approximately 3.8 bands per primers (Appendix B). These were considered to represent distinct genetic loci. A majority of the products were monomorphic in both K0315Y and D0940Y; however, 10 primers (Table 5.2) produced polymorphic fragments that were present in the one inbred and absent in the other.

Table 5.2 List of primers generating polymorphic fragments, their sequences, number of polymorphic bands and in which maize inbred.

Primer designation	Sequence (5'-3')	Polymorphic fragment generated	Maize inbred
OPA-02	TGCCGAGCTG	1	D
OPA-04	AATCGGGCTG	1	K
OPB-01	GTTTCGCTCC	2	K,D
OPB-16	TTTGCCCGGA	2	2K
OPV-01	TGACGCATGG	1	D
OPV-08	GGACGGCGTT	2	2D
OPV-16	ACACCCCACA	1	D
OPW-04	CAGAAGCGGA	1	D
OPW-13	CACAGCGACA	1	D
OPX-01	CTGGGCACGA	2	K,D

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

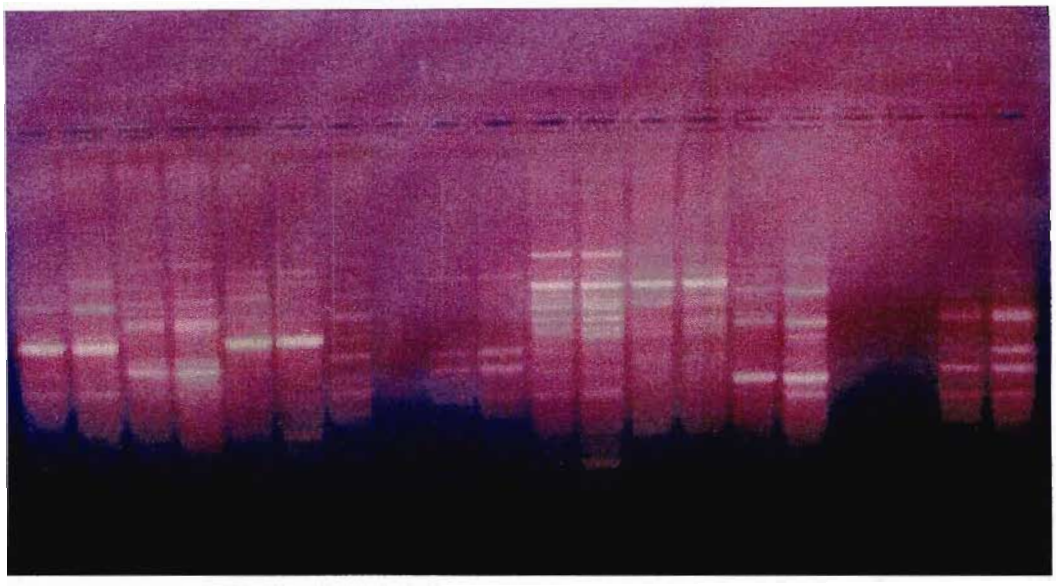


Figure 5.1 DNA of near isogenic maize lines K0315Y (K-1) and D0940Y (D), amplified with primers OPV-01 to OPV-10. Lane1 K-1 OPV-01, lane 2 D OPV-01, lane 3 K-1 OPV-02, lane 4 D OPV-02, lane 5 K-1 OPV-03, lane 6 D OPV-03, lane 7 K-1 OPV-04, lane 8 D OPV-04, lane 9 K-1 OPV-05 lane 10 D OPV-05, lane 11 K-1 OPV-06, lane 12 D OPV-06, lane 13 K-1 OPV-07, lane 14 D OPV-07, lane 15 K-1 OPV-08, lane 16 D OPV-08, lane 17 K-1 OPV-09, lane 18 D OPV-09, lane 19 K-1 OPV-10 and lane 20 D OPV-10.

Figure 5.1 shows two polymorphisms produced by primers OPV-01 in inbred D (lane 2) and OPV-08 in inbred D (lane 16). Figure 5.2 shows the monomorphic bands produced by primer OPC-10 (lanes 19 and 20).

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

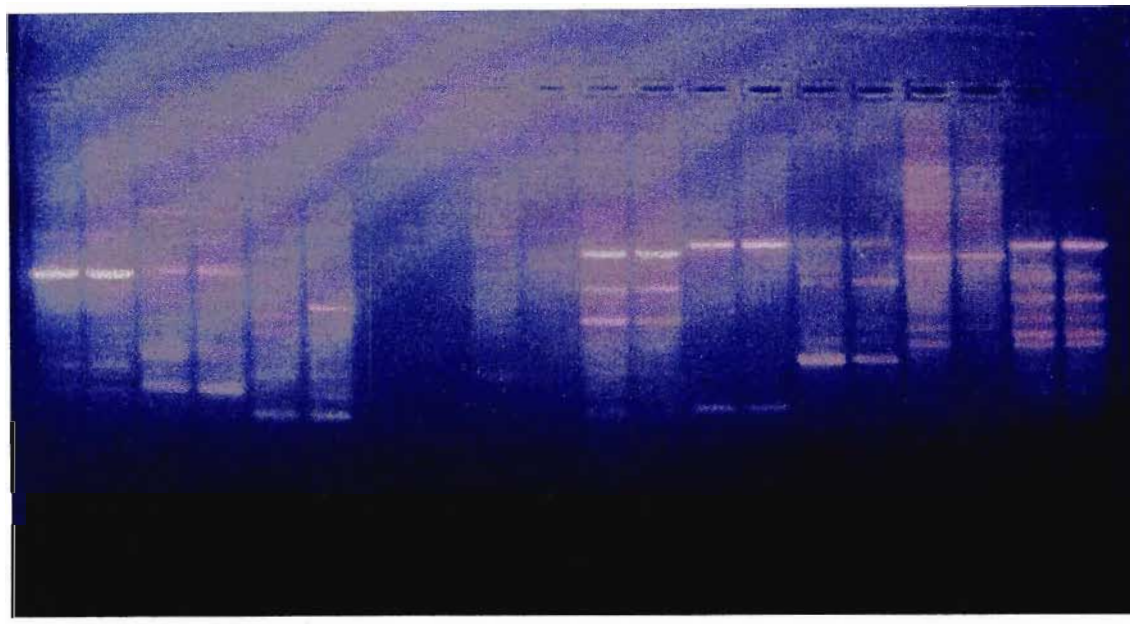


Figure 5.2 DNA of near isogenic maize lines K0315Y (K-1) and D0940Y (D) amplified with primers OPC-01 to OPC-10. Lanel K-1 OPC-01, lane 2 D OPC-01, lane 3 K-1 OPC-02, lane 4 D OPC-02, lane 5 K-1 OPC-03, lane 6 D OPC-03, lane 7 K-1 OPC-04, lane 8 D OPC-04, lane 9 K-1 OPC-05, lane 10 D OPC-05, lane 11 K-1 OPC-06, lane 12 D OPC-06, lane 13 K-1 OPC-07, lane 14 D OPC-07, lane 15 K-1 OPC-08, lane 16 D OPC-08, lane 17 K-1 OPC-09, lane 18 D OPC-09, lane 19 K-1 OPC-10 and lane 20 D OPC-10.

The amount of polymorphism revealed by the 120 primers represents a frequency of variation between the inbreds of 4 %. Five of the 14 polymorphic fragments were shown to be consistently present in sixth generation backcross K-1 and absent in inbred D (i.e. in coupling phase with the resistant gene), whilst nine showed the reverse occurrence (i.e. in repulsion phase with the resistant gene). The low incidence of polymorphism between K-1 and D indicated a 95.8 % sequence similarity which was in keeping with the close relationship of these near-isogenic lines. This low level of variation is a positive feature as it increases the likelihood of any one of polymorphisms identified being linked to major phenotypic difference

of interest between the two lines. Figure 5.3 shows the presence of a polymorphic band in inbred D produced by primer OPA-02 (lane 4) and a polymorphic band in inbred K-1 produced by primer OPA-04 (lane 8).

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

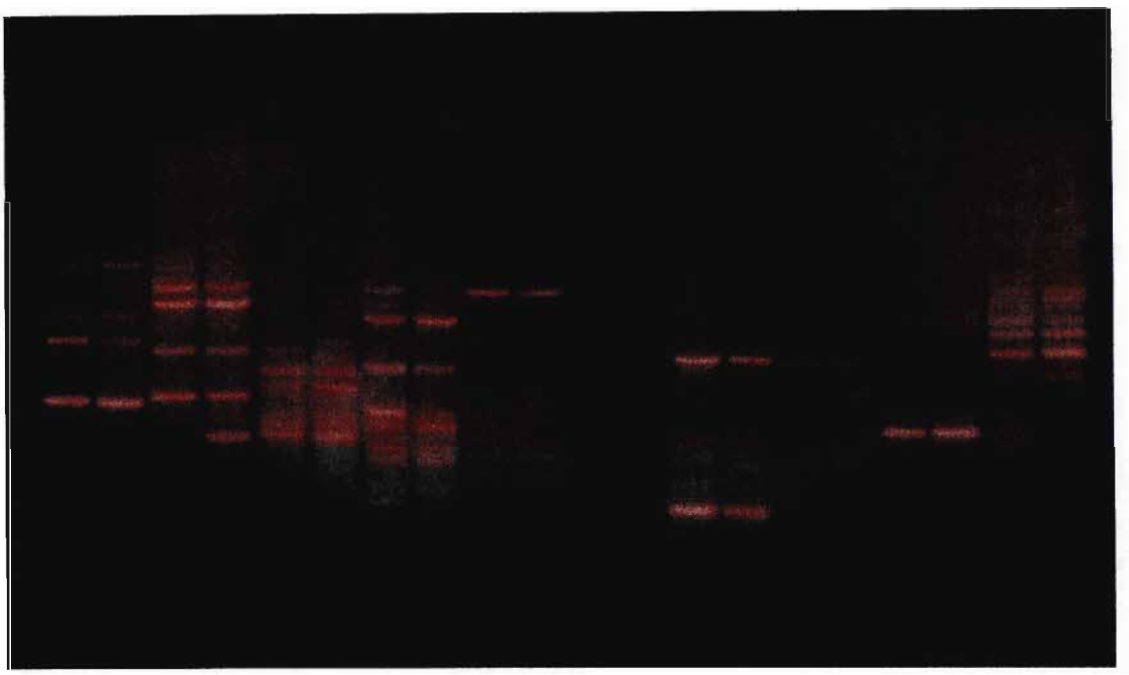


Figure 5.3 DNA from near isogenic maize lines K0315Y (K-1) and D0940Y (D) amplified with primers OPA-01 to OPA-10. Lane1 K-1 OPA-01, lane 2 D OPA-01, lane 3 K-1 OPA-02, lane 4 D OPA-02, lane 5 K-1 OPA-03, lane 6 D OPA-03, lane 7 K-1 OPA-04, lane 8 D OPA-04, lane 9 K-1 OPA-05, lane 10 D OPA-05, lane 11 K-1 OPA-06, lane 12 D OPA-06, lane 13 K-1 OPA-07, lane 14 D OPA-07, lane 15 K-1 OPA-08, lane 16 D OPA-08, lane 17 K-1 OPA-09, lane 18 D OPA-09, lane 19 K-1 OPA-10 and lane 20 D OPA-10.

Figure 5.4 shows the presence of a polymorphic band in inbred D produced by primer OPW-04 (lane 8) as well as the occurance of failed PCR reactions e.g. inbred D amplified with primer OPW-07 (lane 14).

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

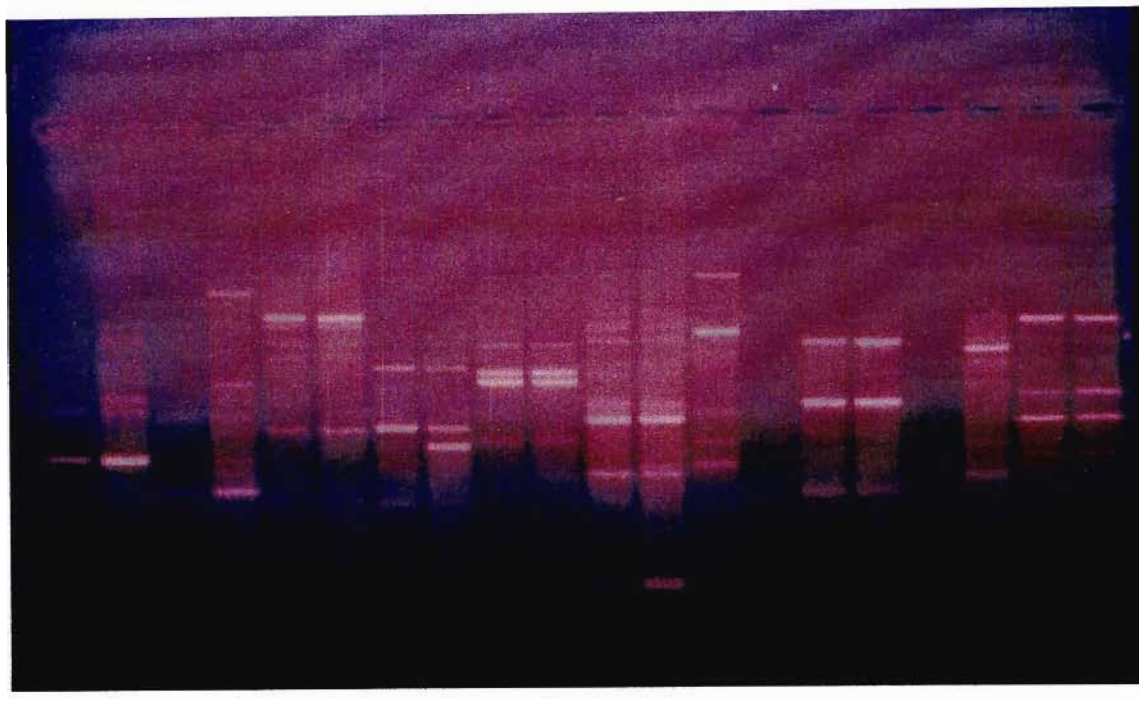


Figure 5.4 DNA of near isogenic maize lines K0315Y (K-1) and D0940Y (D) amplified with primers OPW-01 to OPW-10. Lanel K-1 OPW-01, lane 2 D OPW-01, lane 3 K-1 OPW-02, lane 4 D OPW-02, lane 5 K-1 OPW-03, lane 6 D OPW-03, lane 7 K-1 OPW-04, lane 8 D OPW-04, lane 9 K-1 OPW-05, lane 10 D OPW-05, lane 11 K-1 OPW-06, lane 12 D OPW-06, lane 13 K-1 OPW-07, lane 14 D OPW-07, lane 15 K-1 OPW-08, lane 16 D OPW-08, lane 17 K-1 OPW-09, lane 18 D OPW-09, lane 19 K-1 OPW-10 and lane 20 D OPW-10.

5.2.2 Evaluation of polymorphic fragment transmission inheritance in an F_2 population

The five primers which generated polymorphic fragments in genotype K were used to screen the F_2 mini-population developed by embryo rescue (Appendix D). The 14 plantlets comprising the F_2 population were then scored for the presence or absence of each polymorphic fragments (Figure 5.7). The segregation results showed individual polymorphism expression differed both in clarity and stability across the F_2 population (Table 5.3). A varying degree of infidelity could be ascribed to the nature of either the primer or template. However, all primers are decamers (ten nucleotides) with a similar G + C content of 60-70% so the primers are unlikely to be responsible for the effect (Williams *et al.*, 1993). Inherent variability

of the genomic target sequence or physical accessibility factors could account from spurious or masked expression in the manner of 'epistatic effects' of genetic background as proposed by Heun and Heletjaris (1993). Huckett and Botha (1996) proposed that stable polymorphisms probably represent parts of the genome which are conserved across several generations of breeding and are relatively uncomplexed with proteins or nucleotides in DNA.

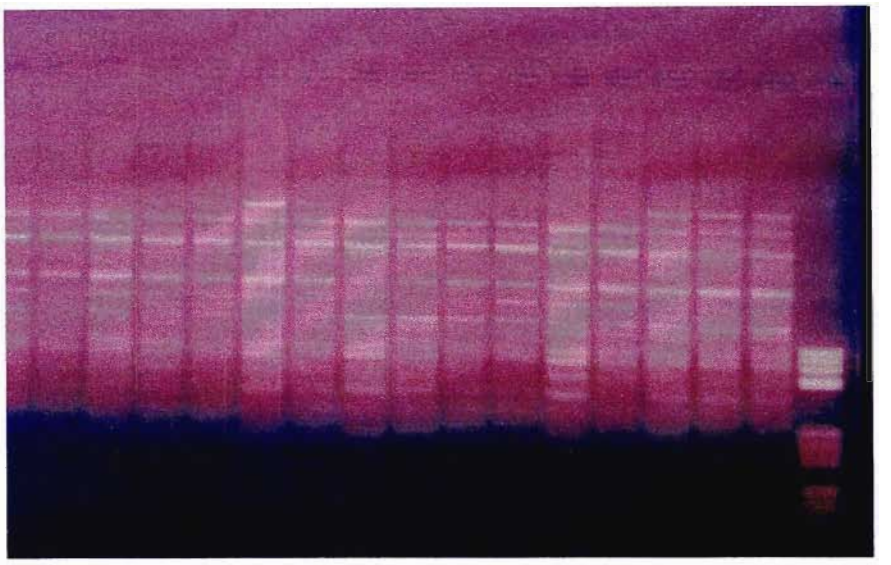
Polymorphic RAPD loci were given the designations using the nomenclature of Michelmore *et al.* (1991) and Miklas, Stavely and Kelly (1993) in which the subscript indicates the size (bp) of the fragment generated. The OPB-01_{627} fragment, a product of primer OPB-01 was reproducibly generated in parent K0315Y and in 10 of the 14 F_2 population showing a normal 3:1 segregation (Table 5.3) for resistance and was therefore considered to be putatively linked to the *HtN* resistance gene (represented by # in Figure 5.5; lanes 1, 3, 5, 6, 7, 8, 9, 10, 11, 12 and 13). The OPX-01_{831} product of primer OPX-01 showed similar reliability in that it was reproducibly generated in parent K0315Y but only in a proportion of the population i.e. in seven of the 14 F_2 population showing a 1:1 segregation (represented by * in Figure 5.6; lanes 3, 8, 9, 10, 12, 13, 14 and 15). The two polymorphic fragments OPB-16_{434} and OPB-16_{1325}, products of primer OPB-16 were absent in all 14 F_2 individuals and OPA-04_{700} was scored in all 14 of the 14 F_2 population showing no segregation.

Table 5.3 Polymorphic fragment segregation in F_2 mini-population

Primer	**Segregation ratios**
OPA-04_{700}	no segregation
OPB-01_{627}	3:1
OPB-16_{434}	absent
OPB-16_{1325}	absent
OPX-01_{831}	1:1

Although the F_2 population is small it is acceptable on the basis that a 3:1 segregation ratio is expected in the F_2 generation as HtN resistance is monogenically inherited.

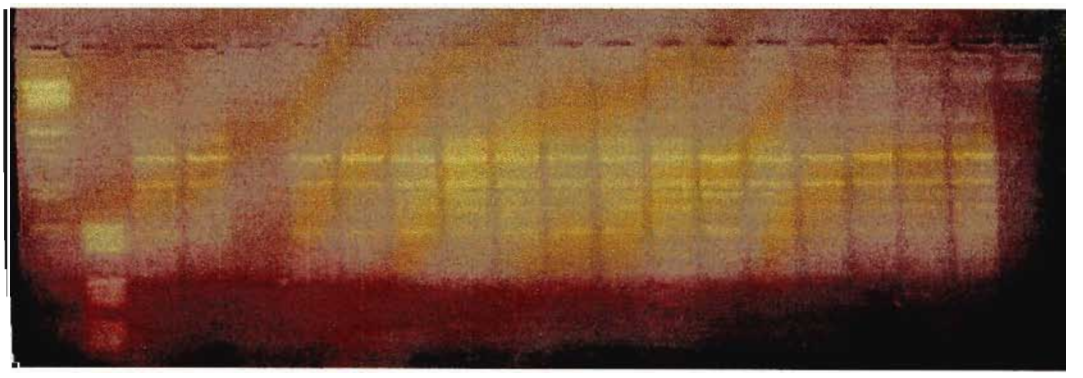
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17



#

Figure 5.5 RAPD profiles of a F_2 population of 14 maize plants amplified with primer OPB-01. The # represents the segregating 627 bp fragment. Lane 1 inbred K0315Y, lane 2 inbred D0940Y, lanes 3-16 14 individuals making up the F_2 population and lane 16 MWM V.

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



*

Figure 5.6. RAPD profiles of a F_2 population of 14 maize plants amplified with primer OPX-01. The * represents the segregating 831 bp fragment. Lane 1 MWM III, lane 2 MWM V, lane 3 inbred K0315Y, lane 4 inbred D0940Y, lane 6-19 14 individuals making up the F_2 population and lane 20 control lane with no DNA.

To confirm linkage of the 627 base pair marker identified by primer OPB-01 for leaf blight resistance, a larger F_2 population would need to be screened and the population would also need to be scored phenotypically for resistance or susceptibility to the disease to determine if the genotypic and phenotypic segregation corresponds. If linkage was confirmed then the development of a sequence characterised amplified region (SCAR) as a stable replacement for the RAPD marker would increase the efficiency of this marker.

CHAPTER 6

REVIEW OF RESULTS AND FINAL CONCLUSIONS

DNA markers are becoming established as another tool for plant breeding programmes. The utility of the technology varies considerably with the application and crop. Advances in DNA technology, basic knowledge of plant biology and experience will increase the absolute efficiency of DNA markers. DNA technology is rapidly changing whereas plant breeding methodology is relatively stable and whilst DNA markers have great utility in basic research, their utility in commercial plant breeding programmes remains to be established and verified. There is still considerable hesitation among plant breeders in applying the technology but it is generally regarded that developments in DNA technology can assist plant breeders in their breeding programmes. It is important to clearly establish the advantages of the appropriate adoption of fingerprinting techniques combined with careful analysis of the data generated.

A number of different approaches for using primers of arbitrary sequence in the polymerase chain reaction have been developed with a view to analyzing genetic variation. In this study the random amplified polymorphic DNA (RAPD) technique (Williams *et al.*, 1990) was evaluated, which employs primers ten nucleotides in length for DNA fingerprinting. Relative to other techniques currently available for DNA analysis, RAPDs are relatively simple and inexpensive, and a large number of samples can be analyzed in a relatively short period of time. A great disadvantage of the technique is its sensitivity to different reaction conditions and the time it takes to optimise these parameters. In addition once optimised the protocol is not necessarily transferable between laboratories.

High reproducibility is an essential requirement for the suitability of a marker system for genetic fingerprinting. Following optimisation, RAPD markers can fulfil this requirement. Different DNA extraction methods produced DNA of widely different purity therefore it was necessary to modify a DNA isolation protocol which was also converted into a micro-extraction protocol to produce good quality DNA for this application. Spooling of the DNA also appeared to free it of contaminants. Additional fragments were apparent when DNA from individual plants of the same genotype e.g. an inbred was amplified, therefore leaf

samples from three plants of each genotype were bulked before extraction to give template DNA which gave consistent RAPD results. Repeated experiments using the same DNA samples then showed little or no variation. The RAPD protocol components were also optimised both separately and in combination to obtain the most suitable concentrations of the PCR reaction variables. The components optimised in combination gave higher concentrations which increased the overall costs of the reactions with no significant improvement in quality of RAPD profiles generated. Therefore the optima determined for the reaction components individually optimised were chosen for the remaining studies. Once the RAPD technique is optimised a researcher should be able to generate reproducible DNA profiles for samples in less than 24 h.

The ability of the RAPD technique to distinguish between genotypes was shown in tests conducted on seven selected maize genotypes. With the average of 5.5 bands per primer, a less complex DNA profile is generated using RAPDs compared to other techniques where profiles consisting of over 100 bands have been observed. Less complex DNA profiles significantly facilitate the scoring of individual bands in the profiles. Despite the presence of fewer bands per profile, these results show that the variation of RAPD profiles between individual maize genotypes is sufficiently high to allow them to be distinguished. When highly related maize genotypes were compared e.g. ABxB and B, genetic distance values of 0.95 were obtained indicating that closely related genotypes could be distinguished from each other. However, more extensive studies analyzing a wider range of crosses would be needed to establish whether highly related maize genotypes can always be distinguished with confidence using RAPD markers. For the other genotypes e.g. BC and C, genetic distance values of 0.585 were obtained indicating that the respective genotypes compared were relatively unrelated showing C was not a parent of the single cross BC. The genetic distance values appear to correctly reflect the genetic distance background of the samples analyzed, as demonstrated by cluster analysis and the results graphically represented as dendrograms. The seven maize genotypes analyzed could be distinguished with RAPDs but the number of genotypes tested was small and further analysis would be important in order to complete the standardisation of the procedure for routine identification of maize genotypes. Screening of a large number of primers is also important to ensure analysis is based on a statistically adequate number of informative primers (i.e. primers revealing a variety of unique polymorphic sites

between genotypes). Therefore, the primers found to be useful for this study would not necessarily be appropriate for identifying other germplasm, additional primers would have to be screened. If RAPDs were not able to distinguish between genotypes then an additional technique would need to be evaluated e.g. AFLPs which, although technically more complex and expensive, generate a greater number of polymorphisms.

The high percentage of band sharing produced by the RAPD technique does make it suitable for specific investigations e.g. parental and pedigree relationships in maize, which makes selected use of stably expressed RAPD markers that are transmitted across a number of generations. Furthermore, by implication such markers if linked to traits of interest could be used directly in a plant breeding selection programme. Confirmed linkage between DNA markers and the gene of interest must be determined. In this study, RAPDs were evaluated for their ability to distinguish between two near isogenic lines (NILs) for leaf blight (*Helminthosporium* spp.) resistance. Polymorphisms between the NILs were identified but only one polymorphism showed a 3:1 segregation when tested in a small F_2 population of 14 individuals resulting from a cross between the two NILs. To confirm linkage of the 627 base pair marker identified by primer OPB-01 for leaf blight resistance, a larger F_2 population would need to be screened and the population would also need to be scored phenotypically for resistance or susceptibility to the disease to determine if the genotypic and phenotypic segregation corresponds. If linkage was confirmed then the development of a sequence characterised amplified region (SCAR) as a stable replacement for the RAPD marker would increase the efficiency of this marker.

The results reported in this thesis indicated that the RAPD technique has numerous potential applications in a maize breeding programme. The production of genetic markers by the RAPD technique has several apparent advantages over other fingerprinting techniques. The analysis of DNA profiles is simpler than the analysis of complex bands generated by restriction endonuclease based fingerprinting techniques such as RFLPs. The RAPD assay is faster and less labour intensive than Southern blot hybridizations of RFLPs and does not require the specific nucleotide sequence information of microsatellites or the complex polyacrylamide gel electrophoresis of AFLPs.

Despite the development of newer technologies, RAPDs are still being widely used as is evident from the following recent publications: Gaiotto *et al.* (1997) used RAPDs to estimate the outcrossing rates in a breeding population of *E. urophylla*; Wu *et al.* (1999) used RAPDs to study the nuclear DNA diverse population to differentiate the phylogenic relationships in California closed cone pines; Young *et al.* (1999) used RAPDs to detect somoclonal variation in cultured rice cells; Dvorak *et al.* (2000) used RAPDs to assess the evolutionary relationships in the *Oocarpae* and *Australes* subsections; and Mienie *et al.* (2000) used RAPDs analysis to characterise six African dry bean cultivars.

The experience gained during this study has enabled the author to optimise the RAPD technique for use in a wheat breeding programme screening for molecular markers for aluminium tolerance (Warburton, Malan and Wentzel, 1997). Current work by the author includes using RAPD fingerprinting to verify the integrity of Mondi Forests' *Eucalyptus* clonal hedges (Edwards, Payn, Blakeway and Janse, 2000), characterising selected South African *Eucalyptus* clones using RAPDs, AFLPs and microsatellites (Edwards, Blakeway, Chiswell, van der Nest, Wingfield and Janse, 1999; Edwards, Blakeway, Chiswell, van der Nest, Wingfield and Janse, submitted in 2000). The decision has been taken to use RAPDs to fingerprint all commercially released *Eucalyptus* clones in the Mondi Forests tree improvement programme as well as to determine the relationship between *Pinus patula, P. greggi* var. *greggi* and *P. greggi* var. *australis* and *P. taeda* (Edwards, Chiswell, Blakeway, Vermaak, Kietzka and Janse, 2000).

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APPENDICES

APPENDIX A DNA ISOLATION PROTOCOLS

A.1 Isolation of genomic DNA from single seeds (Saghai-Maroof *et al.*, 1984)

Maize kernels were soaked in a small amount of CTAB extraction buffer (1 M Tris (pH 7.5), 5 M NaCl, 0.5 M EDTA and 14 M BME) overnight to soften the kernels. The embryos were then excised using sterilised tweezers and a scalpel, taking care to remove all the white (triploid) endosperm. Samples of approximately 400 mg were weighed out and ground to a fine pulp using a pestle and mortar. This finely ground material was then transferred to a 15 mℓ polypropylene tube and 9 mℓ of CTAB extraction buffer, 450 µℓ of proteinase K and 1 mℓ SDS (10x concentration) was added. The tube was incubated for 60 min at 65 °C in a water bath and mixed gently by inversion every 10 min. After incubation the tubes were allowed to cool to room temperature before the addition of 4.5 mℓ phenol:chloroform:isoamyl alcohol (25:24:1) to each tube. The tubes were then rocked gently on a orbital shaker for 10 min. Following this the tube was spun in a table top centrifuge for 10 min at 2800 rpm. The top aqueous layer was then transferred to a new 15 mℓ polypropylene tube taking care not to remove the bands between the top CTAB layer and the phenol layer. This step was then repeated. The top aqueous layer was then pipetted into a 50 mℓ polypropylene centrifuge tube containing two thirds volume isopropanol and 10 % $CH_3COO.Na$. This was mixed gently by inversion and left overnight at -20 °C for the DNA to precipitate out. The tube was then centrifuged in a Beckman® JA20 rotor at 4 °C for 30 min at 1500 rpm. This resulted in the formation of white pellets at the base of the tube. The clear supernatant was discarded and the white pellet was dried in a vacuum desiccator for 30 min. The pellets were washed by the addition of 2 mℓ 70 % EtOH and allowed to stand for 20 min with occasional gentle mixing to loosen the pellet, the ethanol was then discarded and the pellet again dried by vacuum for 30 min. Using a small pipet tip the DNA was gently transferred to a minifuge tube containing 300 µℓ TE Buffer (1 M Tris (pH 8.0) and 0.5 M EDTA (pH 8.0)). Gentle inversion of the tube ensured that the DNA dissolved in the TE buffer. A volume of 300 µℓ of saturated ether was added, the mixture gently mixed by inversion and spun briefly in a centrifuge to clarify the

two layers. The top ether layer was discarded and the step repeated. The minifuge tube was then placed with the cap open in a water bath at 65 °C for 15 min to evaporate off residual ether. Samples were stored at -20 °C.

A.2 Maize DNA miniprep (Dellaport *et al.*, 1985)

One to 2 g of leaf tissue was frozen in liquid nitrogen and, using a mortar and pestle ground well to produce a fine powder. This was then transferred to a 50 mℓ tube and 15 mℓ of extraction buffer (100 mM Tris pH 8.0, 50 mM EDTA (pH 8.0), 500 mM NaCl, 10 mM BME) was added. Then 1 mℓ of 20 % (w/v) SDS was added and the mixture incubated at 65 °C for 10 min. Five mℓ 5 M potassium acetate was then added, the sample mixed and placed on ice for 10 min. Following this it was centrifuged at 4 °C for 20 min at 10 000 rpm. The solution was poured through a miracloth filter (Calbiochem) into a new sterilised tube containing 10 mℓ isopropanol, mixed gently and incubated at -20 °C for 30 min. The DNA was then pelleted at 4 °C for 20 min at 10 000 rpm. The supernatant was poured off and the tube was drained for a few minutes before resuspending the DNA pellet in 0.7 mℓ 50x TE (50 mM Tris, 10 mM EDTA (pH 8.0)). This DNA solution was then transferred to a sterile eppendorf tube containing 7 µℓ RNAse and incubated at 37 °C for 1 h. The sample was centrifuged in a high speed microfuge for 15 min and the supernatant transferred to a new sterile Eppendorf tube containing 75 µℓ 3 M sodium acetate (pH 5.2) and mixed; 0.5 mℓ isopropanol was added and kept at room temperature for 5 min. The DNA was then pelleted in a high speed microfuge for 5 min, the supernatant removed and the pellet washed with 75 % ethanol and then dried briefly before resuspending in 200 µℓ TE buffer overnight at 4 °C.

A.3 CTAB total DNA isolation (Doyle and Doyle, 1987)

Leaf tissue (0.5-1.5 g) was finely ground using a mortar and pestle with 7.5 mℓ CTAB isolation buffer (100 mM Tris-Cl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2 % (w/v) CTAB and 0.2 % (v/v) BME). This was then poured into a 30 ml cortex tube and the mortar was rinsed with 0.5 mℓ CTAB isolation buffer, which was also added to the tube. The sample was incubated at 60 °C for 30 (15-60) min with occasional mixing by gentle swirling, then 5 mℓ of

chloroform:isoamyl alcohol (24:1) was added and mixed gently and thoroughly. This was then spun in a centrifuge at 6000 g for 10 min at room temperature. The aqueous layer was removed using a wide-bore pipette and transferred to a clean tube; 2/3 volume of cold isopropanol was added and mixed gently to precipitate nucleic acids. The nucleic acids were spooled into 20-25 mℓ wash buffer (76 % ethanol, 10 mM ammonium acetate) for 20 min. The nucleic acids were then spun down or spooled out and allowed to air dry before being adding 1 mℓ resuspension buffer (10 mM ammonium acetate, 0.25 mM EDTA) or TE buffer (10 mM Tris-Cl (pH 7.4), 1 mM EDTA (pH 8.0)). RNAse was then added to a final concentration of 10 μg $mℓ^{-1}$ and incubated at 37 °C for 30 min. The sample was then diluted with two volumes sterile distilled water or TE buffer and 7.5 M ammonium acetate was added to a final concentration of 2.5 M and the sample mixed well before 2.5 volumes of cold ethanol was added to precipitate the DNA. The DNA was then spun down at 9000 rpm for 10 min in a refrigerated centrifuge or benchtop centrifuge. The supernatant was discarded and the pellet air dried before being resuspend in TE buffer.

A.4 Simple rapid method for preparation of plant genomic DNA for PCR analysis (Edwards *et al.*, 1991)

Leaf tissue was collected using the lid of a sterile Eppendorf tube and macerated using an Eppendorf pestle in the original tube at room temperature without buffer for 15 sec. A 400 μℓ volume of extraction buffer (200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA and 05 % (w/v) SDS) was added and the sample vortexed for 5 sec. The extract was centrifuged at 13 000 rpm for 1 min and 300 μℓ of the supernatant transferred to a new Eppendorf. A 300 μℓ volume of isopropanol was added and the sample mixed and left at room temperature for 2 min. The supernatant was then centrifuged at 13 000 rpm for 5 min, the supernatant removed and the pellet vacuum dried for 30 min. The pellet was then dissolved in 100 μℓ TE and stored at 4 °C.

A.5 Simple and rapid DNA microextraction method for plant, animal and insect. Suitable for RAPD and other PCR analyses (Cheung *et al.*, 1993)

A 5 mm diameter leaf disc of plant leaf tissue was immersed in 160 µℓ extraction buffer (200 mM Tris-HCl (pH 8.0), 70 mM EDTA, 2 M NaCl and 20 mM sodium metabisulfite) in a sterilised microcentrifige tube. Tissue breakage was achieved by physical grinding with a pestle until no intact pieces of tissue remained. Cells were further lysed by the addition of 40 µℓ 5 % (w/v) sarcosyl solution and incubated at 60 °C for 1 h. The lysate was then centrifuged for 15 min at 16 000 g to remove cell debris. The clear supernatant was transferred to a new tube and 90 µℓ of 10 M ammonium acetate and 200 µℓ of isopropanol added at room temperature for 15 min to precipitate DNA. Total DNA was then pelleted by centrifugation at 16 000 g for 15 min in the microfuge and then washed with 70 % ethanol dried briefly and resuspended in 50 µℓ TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) with RNAse added at 10 µg $mℓ^{-1}$.

A.6 Maxi DNA extraction (Honeycutt *et al.*, 1982)

Six g of leaf roll from four wk old maize seedlings was cut into slices and immediately suspended in 40 mℓ of homogenisation buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 0.5 mM spermidine, 1 % (w/v) PEG 8000, 0.1 % (v/v) BME and 0.35 M sucrose) kept on ice. This was then homogenised for two min using a dounce homogenizer and the homogenate filtered through two layers of sterile, damp mutton cloth into a 50 mℓ sterile plastic centrifuge tube. The filtrate was then centrifuged at 8000 rpm for 20 min in a Beckman JA rotor precooled to 4 °C. The supernatant was discarded and the pellet resuspended in 10 mℓ wash buffer (50 mM tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 0.5 mM spermidine, 0.1 % (v/v) 2-mercaptoethanol and 0.35 M sucrose) and placed on ice. Two mℓ 5 M NaCl was added and mixed in gently with a disposable pipette, then 1 mℓ 10 % (w/v) SDS added and gently mixed by stirring with the pipette tip and 1.3 mℓ 10 % (w/v) CTAB added and gently mixed by stirring with the pipette tip. The mixture was then incubated at 60 °C for 30 min and then allowed to cool at room temperature. An equal volume of 24:1 chloroform:isoamyl alcohol was added (14.3 mℓ) and the tube gently inverted until the phases were completely

emulsified. The solution was then centrifuged a 6500 rpm for 10 min at 4 °C and the aqueous phase transferred to a new tube and the step repeated. An equal volume of cold isopropanol was then added and the phases mixed until DNA strands formed and clustered together at room temperature. The DNA was spooled or lifted out using a Pasteur hook, drained briefly and the DNA released into 0.75 mℓ of TE (10 mM Tris-HCl (pH 7.6) and 1 mM EDTA (pH 8.0)) buffer and dissolved overnight at 4°C.

APPENDIX B PRIMER SEQUENCES

B.1 Sequences of Operon® primers (OPA) used in RAPD fingerprinting of the 11 maize genotypes.

Primer	Sequence (5'-3')	Result*	No. of loci*	K#	S#	K-1#	D#
OPA-01	CAGGCCCTTC	scored	8	5	3	4	4
OPA-02	TGCCGAGCTG	scored	8	-	-	4	5
OPA-03	AGTCAGCCAC	Inbred A failed	4	1	1	5	5
OPA-04	AATCGGGCTG	AB(F_2) and C failed	4	4	-	8	7
OPA-05	AGGGGTCTTG	scored	5	6	6	2	2
OPA-06	GGTCCCTGAC	all genotypes failed	0	3	3	-	-
OPA-07	GAAACGGGTG	scored	4	2	1	2	2
OPA-08	GTGACGTAGG	ABxB and C failed	3	7	7	3	3
OPA-09	GGGTAACGCC	A, ABxB and C failed	4	4	-	6	6
OPA-10	GTGATCGCAG	all genotypes failed	0	5	5	7	7
OPA-11	CAATCGCCGT	C failed	4	-	3	3	3
OPA-12	TCGGCGATAG	C failed	5	-	-	-	-
OPA-13	CAGCACCCAC	C failed	5	4	-	5	5
OPA-14	TCTGTGCTGG	C failed	6	3	-	1	1
OPA-15	TTCCGAACCC	all genotypes failed	0	4	5	1	1
OPA-16	AGCCAGCGAA	all genotypes failed	0	2	3	3	1
OPA-17	GACCGCTTGT	all genotypes failed	0	4	4	3	3
OPA-18	AGGTGACCGT	scored	9	-	-	4	4
OPA-19	CAAACGTCGG	A and C failed	4	3	3	1	1
OPA-20	GTTGCGATCC	ABxB, BC and C failed	6	-	4	3	3

* Results of fingerprinting study for seven maize genotypes A, AB, B, ABxB, AB(F_2), BC and C, indicating whether a PCR reaction failed for a particular genotype or if the primer was scored and the number of bands or loci scored.

Results of marker assisted selection screening for markers for grey leaf spot resistance K054W (K) and S0173W (S) and for leaf blight resistance K0315Y (K-1) and D0940Y (D) showing number of loci scored.

B.2 Sequences of Operon® primers (OPB) used in RAPD fingerprinting of the nine maize genotypes.

Primer	Sequence (5'-3')	Result*	No. of loci*	K-1#	D#
OPB-01	GTTTCGCTCC	scored	7	4	4
OPB-02	TGATCCCTGG	all genotypes failed	0	3	2
OPB-03	CATCCCCCTG	all genotypes failed	0	6	6
OPB-04	GGACTGGAGT	all genotypes failed	0	-	-
OPB-05	TGCGCCCTTC	all genotypes failed	0	-	-
OPB-06	TGCTCTGCCC	C failed	7	-	-
OPB-07	GGTGACGCAG	scored	7	2	2
OPB-08	GTCCACACGG	all genotypes failed	0	-	1
OPB-09	TGGGGGACTC	all genotypes failed	0	1	-
OPB-10	CTGCTGGGAC	scored	5	4	4
OPB-11	GTAGACCCGT	all genotypes failed	0	-	-
OPB-12	CCTTGACGCA	BC failed	5	3	1
OPB-13	TTCCCCCGCT	AB(F_2), BC and C failed	3	4	4
OPB-14	TCCGCTCTGG	BC and C failed	6	6	6
OPB-15	GGAGGGTGTT	BC and C failed	5	3	3
OPB-16	TTTGCCCGGA	BC and C failed	2	5	3
OPB-17	AGGGAACGAG	BC and C failed	7	3	3
OPB-18	CCACAGCAGT	scored	6	4	-
OPB-19	ACCCCCGAAG	scored	4	4	4
OPB-20	GGACCCTTAC	BC and C failed	9	-	5

* Results of fingerprinting study for seven maize genotypes A, AB, B, ABxB, AB(F_2), BC and C, indicating whether a PCR reaction failed for a particular genotype or if the primer was scored and the number of bands or loci scored

Results of marker assisted selection screening for markers for leaf blight resistance K0315Y (K-1) and D0940Y (D) showing number of loci scored.

B.3 Sequences of Operon® primers (OPC) used in RAPD fingerprinting of the nine maize genotypes

Primer	Sequence (5'-3')	Result*	No. of loci*	K-1#	D#
OPC-01	TTCGAGCCAG	scored	6	3	3
OPC-02	GTGAGGCGTC	scored	6	3	3
OPC-03	GGGGGTCTTT	all genotypes failed	0	6	6
OPC-04	CCGCATCTAC	scored	6	-	-
OPC-05	GATGACCGCC	scored	5	2	2
OPC-06	GAACGGACTC	BC and C failed	4	3	3
OPC-07	GTCCCGACGA	all genotypes failed	0	7	5
OPC-08	TGGACCGGTG	scored	5	3	-
OPC-09	CTCACCGTCC	BC and C failed	7	5	5
OPC-10	TGTCTGGGTG	scored	4	3	4
OPC-11	AAAGCTGCGG	all genotypes failed	0	2	1
OPC-12	TGTCATCCCC	all genotypes failed	0	6	6
OPC-13	AAGCCTCGTC	all genotypes failed	0	4	4
OPC-14	TGCGTGCTTG	BC and C failed	6	3	-
OPC-15	GACGGATCAG	BC failed	6	-	-
OPC-16	CACACTCCAG	all genotypes failed	0	5	5
OPC-17	TTCCCCCCAG	C failed	5	7	7
OPC-18	TGAGTGGGTG	scored	7	3	-
OPC-19	GTTGCCAGCC	scored	8	2	2
OPC-20	ACTTCGCCAC	BC failed	6	-	-

* Results of fingerprinting study for seven maize genotypes A, AB, B, ABxB, AB(F_2), BC and C, indicating whether a PCR reaction failed for a particular genotype or if the primer was scored and the number of bands or loci scored

Results of marker assisted selection screening for markers for leaf blight resistance K0315Y (K-1) and D0940Y (D) showing number of loci scored.

B.4 Sequences of Operon® primers (OPV) used in RAPD screening of the two maize near isogenic lines

Primer	**Sequence (5'-3')**	**No. of loci K-1[#]**	**No. of loci D[#]**
OPV-01	TGACGCATGG	5	5
OPV-02	AGTCACTCCC	6	6
OPV-03	CTCCCTGCAA	5	4
OPV-04	CCCCTCACGA	5	1
OPV-05	TCCGAGAGGG	3	3
OPV-06	ACGCCCAGGT	9	9
OPV-07	GAAGCCAGCC	6	6
OPV-08	GGACGGCGTT	5	6
OPV-09	TGTACCCGTC	-	-
OPV-10	GGACCTGCTG	4	4
OPV-11	CTCGACAGAG	-	-
OPV-12	ACCCCCCACT	7	-
OPV-13	ACCCCCTGAA	-	-
OPV-14	AGATCCCGCC	5	5
OPV-15	CAGTGCCGGT	51	1
OPV-16	ACACCCCACA	6	7
OPV-17	ACCGGCTTGT	-	5
OPV-18	TGGTGGCGTT	2	2
OPV-19	GGGTGTGCAG	5	5
OPV-20	CAGCATGGTC	5	5

[#] Results of marker assisted selection screening for markers for leaf blight resistance K0315Y (K-1) and D0940Y (D) showing number of loci scored.

B.5 Sequences of Operon® primers (OPW) used in RAPD screening of the two maize near isogenic lines

Primer	Sequence (5'-3')	**No. of loci K-1#**	**No. of loci D#**
OPW-01	CTCAGTGTCC	2	4
OPW-02	ACCCCGCCAA	-	8
OPW-03	GTCCGGAGTG	5	5
OPW-04	CAGAAGCGGA	3	4
OPW-05	GGCGGATAAG	4	4
OPW-06	AGGCCCGATG	6	6
OPW-07	CTGGACGTCA	5	-
OPW-08	GACTGCCTCT	3	3
OPW-09	GTGACCGAGT	-	1
OPW-10	TCGCATCCCT	3	3
OPW-11	CTGATGCGTG	1	1
OPW-12	TGGGCAGAAG	6	6
OPW-13	CACAGCGACA	6	6
OPW-14	CTGCTGAGCA	-	-
OPW-15	ACACCGGAAC	1	1
OPW-16	CAGCCTACCA	3	3
OPW-17	GTCCTGGGTT	2	2
OPW-18	TTCAGGGCAC	4	4
OPW-19	CAAAGCGCTC	7	3
OPW-20	TGTGGCAGCA	6	6

Results of marker assisted selection screening for markers for leaf blight resistance K0315Y (K-1) and D0940Y (D) showing number of loci scored.

B.6 Sequences of Operon® primers (OPX) used in RAPD screening of the two maize near isogenic lines

Primer	**Sequence (5'-3')**	**No. of loci K-1[#]**	**No. of loci D[#]**
OPX-01	CTGGGCACGA	3	4
OPX-02	TTCCGCCACC	-	-
OPX-03	TGGCGCAGTG	3	-
OPX-04	CCGCTACCGA	2	2
OPX-05	CCTTTCCCTC	-	2
OPX-06	ACGCCAGAGG	3	1
OPX-07	GAGCGAGGCT	-	-
OPX-08	CAGGGGTGGA	6	6
OPX-09	GGTCTGGTTG	3	-
OPX-10	CCCTAGACTG	4	4
OPC-11	GGAGCCTCAG	4	4
OPC-12	TCGCCAGCCA	2	2
OPX-13	ACGGGAGCAA	4	4
OPX-14	ACAGGTGCTG	3	3
OPX-15	CAGACAAGCC	3	3
OPX-16	CTCTGTTCGG	3	3
OPX-17	GACACGGACC	3	4
OPX-18	GACTAGGTGG	1	1
OPX-19	TGGCAAGGCA	1	1
OPX-20	CCCAGCTAGA	3	3

[#] Results of marker assisted selection screening for markers for leaf blight resistance K0315Y (K-1) and D0940Y (D) showing number of loci scored per genotype.

APPENDIX C SOFTWARE PROGRAMMES USED FOR STATISTICAL ANALYSIS TO CHARACTERISE SEVEN MAIZE GENOTYPES

C.1 Programme for analysis of data using Genstat 5™ release 4.1 (1993)

"Programme for hierachial cluster analysis of DNA fingerprint data"

The programme uses the following formula to calculate DNA fingerprint similarity for two genotypes i and j:

Fingerprint similarity: s(ij)=2n(ij)/(n(i)+n(j))

Where i and j are 2 genotypes, n(i) is the number of bands present in i, n(j) is the number of bands present in j, and n(ij) is the number of bands common to i and j.

```
Combine data across gels
"
job'DNA fingerprint - Primer x'
Factor[levels=7]genotype; values=!((1...7)110)
Factor[levels=18;labels=!t(a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r)]gel;\
Values=!(56(1),56(2),35(3),28(4),63(5),49(6),49(7),35(8),42(9),\
        28(10),42(11),42(12),42(13),35(14),35(15),28(16),\
        49(17),56(18))
pointer band
open 'u:/tir/nicci/combine.txt';channel=2; filetype=input
read{ch=2]band[1...7]
close ch=2
"
Create 7x110 matrix where there is one row per genotype and all bands are concentrated
across gels to form columns"
Matrix[rows=7;col=110]M
Equateband;newstructures=M"reads data in row by row"
"
Calculate 7x7 similarity matrix using formula s(ij)"
symmetric[rows=7]S
```

```
& num "matrix to store numerator of similarity calc"
& den "matrix to store denominator of similarity calc"
Calc num=2 *M*+T(M)
Matrix[ros=110;col=7;values=770(1)]ones
        "110x7 vector containing 1s"
Calc den=(M*+ones)+T(M8+ones)
CalcS=num/den"element by element division"
PrintT(M);fieldwidth=7;dec=0
PrintS;fieldwidth=7;dec=3
"
Perform hierachial cluster analysis of genotypes"
Hcluster[print=dendogram;method=averagelink;cthreshold=1]S;\
Threshold=95;groups=genegrp;perutation=geneperm;\
Amalgamations=geneamal
endjob
Stop
```

C.2 Programme for reading data into PAUP 4.0 beta version (Swafford, 1998)

```
#NEXUS [dark bands]
Begin data;
Dimensions NTAX=7 NCHAR=110
Format Symbols="01";
Matrix
A
1111110 etc...
;
Endblock;
Begin PAUP;
End;
```

APPENDIX D PRODUCTION OF A F_2 POPULATION FROM A CROSS BETWEEN K0315Y AND D0940Y USING THE *IN VITRO* TECHNIQUE OF EMBRYO RESCUE

D.1 Introduction

Embryo rescue is an *in vitro* technique which can help remove the effects of seed dormancy and seed maturity (Raghavan, 1976). Tissue culture uses nutrient media which must contain inorganic salts required by a growing plant, a carbon or energy source, growth regulators and vitamins. Other components which may be added for specific purposes include organic nitrogen compounds, tricarboxylic acid compounds and plant extracts (Gamborg, 1991). The Murashige-Skoog (MS) salt combination (Murashige and Skoog, 1962) are most widely used, especially in plant regeneration procedures. In maize, fertilisation occurs between 16 and 24 h after pollination, depending on temperature and silk length. The zygote does not divide until c.a. 10-12 h after fertilisation. Fourteen days after fertilisation, when the embryo is approximately 1 mm or more in length, it possesses a prominent shoot apical meristam surrounded by a coleoptile ring on its anterior surface and is backed by a scutellum. The first leaf primordia arises c.a. 16 d after pollination on the lower side of the apical meristem as a crescent shaped bulge. The embryo is now referred to as a stage one embryo (Randolf, 1936). The development stage of the embryo is important and should be determined by embryo length (base to tip of scutellum) rather than days post pollination (Armstrong, 1994). The physiological state of the embryos at the time of excision and culture is also important (Lu, Vasil and Vasil, 1983).

D.2 Materials and Methods

D.2.1 Hand pollination of maize plants

To produce the segregating F_2 population, the F_1 population was first established from a cross made between the near-isogenic lines K0315Y (K-1) female parent and D0940Y (D) pollen parent grown in a greenhouse. Pollen was transferred from inbred D (susceptible to leaf blight) to six generation backcross recovery K-1 (resistant to leaf blight) at silk emergence. Upon appearance of the first tassels and before the silks were extruded, the ears were covered

with plastic bags (20.5 x 10 cm) to prevent uncontrolled pollination. When the first day's silks were visible, the tip of the husks and silks were cut off squarely (cutting back), but avoiding cutting off the tip of the cob inside. This was done to ensure that a full set of seeds per ear would be obtained from hand pollination. When the silks had emerged to form a thick brush, they were ready for pollination. Viable pollen was collected in brown paper bag placed over the tassels, by carefully bending the plant so that the top open end of the brown bag, covering the tassel, was higher than the bottom. The bag and tassel were sharply shaken and the tassel then carefully withdrawn. For each plant, the plastic bag covering the silks was removed and a small amount of pollen was shaken out of the paper bag onto the silks. The silks were then re-covered with a plastic bag. Controlled self pollination of the F_1 plants was conducted to produce immature F_2 embryos which were grown until the plantlets had sufficient leaf material for DNA extraction.

D.2.2 Embryo rescue and plantlet generation

Embryo rescue was performed for both the F_1 and the F_2 population in order to decrease the amount of time conventionally taken to generate progeny populations. The method of Vasil and Vasil (1991) was used for the embryo rescue of the F_1 and F_2 populations. Developing ears were removed 18 d after pollination. The outermost husks of the ear were stripped off and the remaining portion (ear surrounded by three to four husks) was sterilised with 70 % (v/v) ethanol. The rest of the procedure was performed on a laminar flow bench. The remaining husks and silks were aseptically removed from the sterilised ear. The top third of the developing caryopsis was sliced off with a scalpel. The embryos (stage one i.e. 0.5 to 3.0 mm in length) were removed using a sterile modified spatula and placed with their embryo-axis (i.e. with the rounded scutellar surface exposed) in contact with modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962; Appendix D.4) (1/2 strength MS medium + 3 % (m/v) sucrose + 15 g ℓ^{-1} agar (Dodds and Roberts, 1985) in sterile test tubes. This orientation induces germination of the embryo. The test tubes were sealed with Parafilm "M". The embryos were incubated for 72 h in the dark at 21 °C and then placed in a growth cabinet with a 14 h light photoperiod at 21 °C.

After two to three wk of incubation, once a good root system had developed on the rescued embryos, they were carefully removed from the medium and the plantlets were transferred to pots containing sterilised sand and were given nitrogen in the form of liquid NH_4NO_3, at a rate of 200 kg ha^{-1}. To harden off the plants, the pots were placed on laboratory benches and covered with plastic bags for 7 d, then the plastic bags were removed for a further 7 d after which the plants were transferred to the glasshouse. Initially, the F_1 plantlet root systems were washed and then the plants were placed in pots containing soil. The F_2 plantlets were planted in the soil with the medium still surrounding the roots and these plants appeared to survive better.

D.3 Results and Discussion

D.3.1 Greenhouse cultivation of maize plants

The conditions prevailing during the different periods of growth of maize plants (fertilizer, irrigation water, rainfall, temperature and photoperiod) affect the physiological state of developing plantlets and immature embryos of mature plants (Lu *et al.*, 1983). The experimental plants were grown under controlled greenhouse conditions, this ensured that the stages of plant development could be well controlled. All the plants used for embryo rescue were potted in a mixture of soil and compost which had a high water retention capacity and although nutrient levels could not be completely controlled the seedlings produced were vigorous. Some genotypes germinated better than others possibly due to the age of the seed.

D.3.2 Plantlet regeneration of rescued embryos

Fifty immature embryos were isolated from four maize ears to produce the F_1 plants. Of these only 50 % regenerated in culture, 42 % rooted and 12 % survived (Table D1). To produce the F_2 population controlled self pollinations of ears from the six F_1 plants were performed. Fifty eight immature embryos isolated from 10 maize ears with 55 % regeneration, 46 % rooting and 24 % survival (Table D2). The rate of embryo development depends on temperature (Sheridan and Clark, 1994). The embryos took longer to develop in the colder months than in the warmer months even though the plants were grown in a glasshouse set at 24 °C. Embryo

development may thus also have been influenced by photoperiod or day length. The larger isolated embryos germinated better than the smaller embryos. Callus initiation was initially observed on some of these embryos, but this callus initiation was limited and eventually ceased. The F_1 plantlets had a high mortality rate of 86 % which may be due to damage of the roots incurred during washing. To improve the survival rate, the F_2 plantlets were placed in soil with the modified MS medium still surrounding the roots which resulted in a lower mortality rate of 76 % than those with washed roots. This is not normally recommended in tissue culture as the medium might become contaminated and cause rotting.

Table D.1 Number of F_1 plants obtained from four control pollinations.

Maize ear	No. isolated	No. regenerated	No. rooted	No. hardened off
1	12	9	7	2
2	15	7	6	0
3	9	5	4	1
4	14	4	4	3
Total	50	25	21	6

Table D.2 Number of F_2 plantlets obtained from controlled self pollination of six F_1 individuals

Maize ear	No isolated	No. regenerated	No. rooted	No. Hardened off
1	8	6	5	2
2	12	5	4	1
3	9	7	6	5
4	13	6	5	0
5	11	5	4	3
6	5	3	3	3
Total	58	32	27	14

D.4 Conclusion

The technique of embryo rescue can be used to generate F_1 and F_2 populations of small numbers quicker than using the conventional method but the high percentage of mortality indicates that hardening off procedures need to be optimised.

D.5 Stock solutions for half strength Murashige-Skoog (MS) medium (Murashige and Skoog, 1962)

Stock 1A.	g $2\ell^{-1}$
KNO_3	190

The potassium nitrate was added to 1500 mℓ of distilled H_2O and stirred until dissolved. The pH was adjusted to 5.8 with 1 M NaOH and the volume brought up to 2 ℓ. The solution was sterilised by autoclaving at 121 °C (103.5 kPa) for 15 min.

Stock 1B.	g ℓ^{-1}
NH_4NO_3	165

The ammonium nitrate was added to 500 mℓ of distilled H_2O and stirred until dissolved. The pH was adjusted to 5.8 with 1 M NaOH and the volume brought up to 1 ℓ. The solution was sterilised by autoclaving at 121 °C (103.5 kPa) for 15 min.

Stock 2.	g ℓ^{-1}
$MgSO_4.7H_2O$	37
$ZnSO_4.7H_20$	0.86
$MnSO_4.4H_20$	1.69
$CuSO_4.5H_2O$	0.0025

The magnesium sulphate.7 hydrate, zinc sulphate.7 hydrate, manganese sulphate.1.hydrate and cupric sulphate.5 hydrate were added to 500 mℓ of distilled H_2O and stirred until dissolved. The pH was adjusted to 5.8 with 1 M NaOH and the volume brought up to 1 ℓ. The solution was sterilised by autoclaving at 121 °C (103.5 kPa) for 15 min.

Stock 3A.	g ℓ^{-1}
$CaCl_2.2H_20$	44
$CoCl_2.6H_2O$	0.0025

The calcium chloride.2 hydrate and coboltous chloride.6 hydrate was added to 500 mℓ of distilled H_2O and stirred until dissolved. The pH was adjusted to 5.8 with 1 M NaOH and the volume brought up to 1 ℓ. The solution was sterilised by autoclaving at 121 °C (103.5 kPa) for 15 min.

Stock3B.	g ℓ^{-1}
KI	0.08

The potassium iodide was added to 500 mℓ of distilled H_2O and stirred until dissolved. The pH was adjusted to 5.8 with 1 M NaOH and the volume brought up to 1 ℓ. The solution was sterilised by autoclaving at 121 °C (103.5 kPa) for 15 min.

Stock 4.	g ℓ^{-1}
KH_2PO_4	17
H_3BO_3	0.62
$NaMoO_4.2H_2O$	0.025

The potassium phosphate (dibasic), boric acid and sodium molibdonate.2 hydrate were added to 500 mℓ of distilled H_2O and stirred until dissolved. The pH was adjusted to 5.8 with 1 M NaOH and the volume brought up to 1 ℓ. The solution was sterilised by autoclaving at 121 °C (103.5 kPa) for 15 min.

Stock 5.	g ℓ^{-1}
Na_2EDTA	3.724
$FeSO_4.7H_2O$	2.784

The ethylene dianine tetra-acid disodium salt was boiled vigorously for 1-2 min in 200 mℓ distilled H_20 and added to the Ferrous sulphate already dissolved in 200 mℓ H_20. The solution was sterilised by autoclaving at 121 °C (103.5 kPa) for 15 min.

Stock 6.	g ℓ^{-1}
Myo-inositol	10
Glycine	0.2
Thiamine.HCl	0.01
Nicotinic acid	0.05
Pyridoxine.HCl	0.05

The vitamins are dissolved in distilled H_2O and the pH adjusted to 5.8 with 1 M NaOH. The solution is filter sterilised through a 0.22 μm Millipore filter and stored at 4 °C

Final composition ½ strength MS media (1 ℓ)

Stock 1A	50 mℓ
Stock 1B	5 mℓ
Stock 2	5 mℓ
Stock 3A	5 mℓ
Stock 3B	5 mℓ
Stock 4	5 mℓ
Stock 5	50 mℓ
Stock 6	5 mℓ
Sucrose	30 g
Agar	15 g
Final pH	5.7
Distilled H_2O	870 mℓ