Comparison of molecular markers for study of DNA polymorphism in Chickpea (*Cicer arietinum* L.)

A thesis
submitted for the partial fulfillment of the degree of
Master of Technology in
Biotechnology

By
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Accomplishments of this thesis are the result of benevolence of The Almighty.

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Dedicated To
my parents

CERTIFICATE

This is to certify that the dissertation entitled **"Comparison of molecular markers for study of DNA polymorphism in Chickpea (*Cicer arieinium* L.)"** submitted in partial fulfillment for the award of the degree of Master of Technology in Biotechnology, to the Jawaharlal Nehru Technological University, is a bonafide work carried out by **Ms. Mohita Aminha**, under our guidance and supervision.

The results embodied in this dissertation have not been submitted to any other university or institution for the award of any degree or diploma.

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SUMMARY

The DNA-based molecular markers have significant advantages over the morphological, protein or isozyme markers for genotype identification, genome analysis and mapping. DNA markers are neutral to various environmental factors, highly sensitive and highly reproducible. Therefore, my objective was to familiarize with various molecular marker techniques, and attempt to apply these to study chickpea genome. During this project work, I learnt techniques such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Random Amplified Microsatellite Polymorphism (RAMPO), Simple Sequence Repeats (SSR) and DNA Amplification Fingerprinting (DAF). Most of these techniques were used to decipher polymorphism at DNA level in chickpea. Some additional activities - computer applications like using MS Office, information search and retrieval using *Internet* - were also undertaken to increase my skills and knowledge.

To study chickpea genome, attempts were made to characterize a few cultivars and wild species of *C. arietinum* using RAPD, SSRs and DAF analysis. DNA was extracted from nine chickpea genotypes, and the two population sets (F_2 generations of GL 769 x ICCW 49 and Annigeri x ICCW 6). RAPD and SSR analyses were done with several primers. Clear differences are seen between nine genotypes with some of the primers. The study was expanded to the two sets of populations.

RAPD is simple, fast, free from hazardous materials, needs only small amounts of DNA, and well suited for use in large samples-throughput systems required for plant breeding. Although it has advantages over protein markers and RFLP, the uncertainty of reproducibility of RAPD markers, and their dominant nature limits its use.

RAPD analysis of 9 different genotypes in chickpea with 19 different primers revealed 103 PCR amplified products of which 76 were polymorphic. The polymorphism was observed with the following 13 primers A-04, A-07, A-09, A-10, A-12, A-13, A-14, A-15, A-16, A-17, A-18, A-19 and A-20. Among these primers, polymorphism was best revealed with primers A-04, A-07, A-12, A-13 and A-14. The primers A-04, A-07 and A-14 were selected for RAPD analysis of the progeny of GL 769 and ICCW 49 genotypes as these revealed good polymorphism in the parents. Among these primers, A-07, A-14 revealed better polymorphism than A-04 as more polymorphic bands were obtained with A-07 and A-14 compared to those in A-04. Cluster analysis based on RAPD data of nine chickpea genotypes was carried out using statistical software package GENSTAT and a dendrogram was constructed. The wild species ICCW6 and ICCW 49 formed a separate and distinct group from other cultivated genotypes.

DAF is a novel strategy to detect genetic differences among organisms, uses a thermostable DNA polymerase directed by usually one short (>5 bp) oligonucleotide primer of arbitrary sequence to amplify short segments of genomic DNA, and generate a range of DNA amplification products. DAF is rapid and sensitive and is independent of

cloning and prior genetic characterization. DAF uses polyacrylamide gel electrophoresis for better resolution and silver staining or radio-chemicals for detection. It can be used in laboratories with limited resources.

DAF analyses of 4 chickpea genotypes (GL 769, ICCW 49, Annigeri and ICCW 6) using increasing primer concentrations was done to choose the best primer concentrations, and also to compare the band patterns obtained with each primer concentration. The number of polymorphic bands obtained varied with primer concentration 0.8 µM primer 6 out of 10 bands (60%) were polymorphic; with 1.6 µM, 10 out of 15 bands (67%); with 2.4 µM, 13 out of 19 (69%); with 3.2 µM, 16 out of 23 (70%); and finally with 4.0 µM primer 17 out of 24 bands (71%) were polymorphic.

SSRs are PCR generated, and detection of polymorphism is quick using simple experimental procedures. They are co-dominant markers and segregate in a simple Mendelian manner. Primer sequences can be easily disseminated. Sequence-Tagged Microsatellites (STMS) are markers in which primers are constructed complementary to the short, unique sequences flanking microsatellite repeat sequence loci and which direct the amplification of the repeat. STMS analysis of 9 chickpea genotypes with 8 different microsatellite primer pairs procured from the University of Frankfurt, Germany. Of the 8 primer pairs, 5 primers (Tr29, Tr19, Tr26, Ta53 and Ta72) showed good polymorphism for the parents GL 769 and ICCW 49 and can be used to study polymorphism in the progeny GL 769 x ICCW 49. Primer pair Tr29 was used to screen the progeny of the cross GL 769 and ICCW 49. Three primer pairs Tr19, Tr26, Tr29 showed good

polymorphism for the parents Annigeri and ICCW 6 and can be used to screen for polymorphism in progeny Annigeri x ICCW6. Primer pair Tr26 was used to screen the progeny of the cross Annigeri and ICCW 6. Diversity among nine chickpea genotypes was also studied with STMS markers. Cluster analysis based on STMS data of nine chickpea genotypes was carried out using statistical software package GENSTAT and a dendrogram was constructed to characterize genetic relatedness and dissimilarity between genotypes. From these studies, it was observed that the wild species ICCW 6 and ICCW 49 formed a separate and distinct group from other cultivated genotypes, similar to RAPD cluster analysis.

With the limited experience gained as described above, now I can expect to use DAF and STMS to develop linkage map of chickpea and for screening germplasm.

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LEGENDS FOR THE FIGURES

LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrary Primed-Polymerase Chain Reaction
CAPS	Cleaved Amplified Polymorphic Sequence
cDNA	complementary DNA
DAF	DNA Amplification Fingerprinting
DNA	Deoxyribonucleic acid
ISSRs	Inter-Simple Sequence Repeats
MAAP	Multiple Arbitrary Amplicon Profiling
MAS	Marker-assisted Selection
PCR	Polymerase Chain Reaction
QTL	Quantitative trait
RAMPO	Random Amplified Microsatellite Polymorphism
RAPD	Rapid Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SCARs	Sequence Characterized Amplified Regions
SSR	Simple Sequence Repeats
STMS	Sequence Tagged microsatellite sites
STS	Sequence Tagged sites
VNTR	Variable Number Tandem Repeats

1. INTRODUCTION

1.1 Recombinant DNA-based biotechnology

The double helix architecture of DNA was elucidated by Watson and Crick in 1953. Twenty years later, discovery of restriction enzymes in 1973 helped to create recombinant DNA molecules *in vitro*. Several important techniques were developed during the last 2 decades. The implications of these powerful and novel methods of molecular biology, and their potential use in the genetic manipulation and improvement of microbes, plants and animals became increasingly evident during 1980s, and led to the birth of modern recombinant DNA based biotechnology. The first transgenic plant in which a bacterial gene had been stably integrated was produced in 1983, and by 1993 transgenic plants had been produced in most of the major crop species including cereals and legumes. These remarkable achievements have resulted in the production of crops that are resistant to potent but environmentally safe herbicides, or to viral pathogens and insect pests. In other instances, genes have been introduced that delay fruit ripening or increase in starch content or cause male sterility. Most of these manipulations are based on the introduction of a single gene - generally of the bacterial origin that regulates an important monogenic trait, into the chosen crop plant. Many of the engineered crops are now under field trials, and few have been already commercialized. Many more are expected to be commercially produced within the next few years.

The early successes in plant biotechnology led to the realization, that further improvement of crop plants using molecular tools would require a thorough understanding of the molecular basis of plant development, identification, quantitation,

and characterization of genes that regulate agronomically important multigenic traits. During the past ten years there has been a resurgence of molecular and related cellular studies in plants, including the molecular mapping of plant genomes. Simultaneously, a great deal of exciting and useful information has been generated about the molecular basis of important plant processes.

1.2 Genome analysis

The concept of DNA-based markers has revolutionized our ability to follow chromosome segments including minute regions, and has led to new opportunities such as map-based cloning and marker-assisted selection (MAS). Species with little genetic information available in past have now hundreds of genetic markers. In some cases, the map from one species can be transferred almost directly to another species, such as from tomato to potato. Genome analysis generates information about the coding and non-coding regions of organisms. The evolutionary history of any set of organisms, whether they are plants, or animals can be traced using molecular markers not confined by functional constraints common to coding sequences. Additional information on genomic structure, such as homologous chromosome identification, is forthcoming for several species. The suggestion of ancient polyploidy has been made even for maize, the most intensively studied plant species for genetics. The ancestral relationships of species and the pedigree relatedness of lines have been identified in hundreds of cases. Molecular markers now are profiled and used as identification tags useful in Plant Variety Rights legislation, and in forensics, or paternity analysis (Nybom *et al.* 1990a).

The increased focus on genome has also generated techniques, which permit the comparison of near-isogenic organisms. The application finds utility in the detection of

molecular markers closely linked or part of hitherto undefined genes. This blossomed into the field of proactive diagnosis for plant and animal characteristics and has altered plant breeding, animal husbandry, and human genetic-counselling. Pathogen identification now can occur prior to the onset of disease symptoms.

The detection of molecular markers are closely linked is a part of a general scheme for the isolation of genes, for which no more than the inheritance and the phenotype are known. Many medical and plant traits fall into this category. Without a gene product or a homologous probe, such genes may only be isolated and further studied by positional cloning.

Plant genome analysis is a subject of significance to the basic researcher, the student, the legal expert, legislators, plant breeders, and applied technologists. Recent advances in genomic analysis including RFLPs, DNA amplification markers, such as microsatellites are reviewed by their developers (Sharma *et al.* 1995). The need for genome analysis comes from the demands of modern plant biology in which genes for which no more than heritable phenotype is known.

1.3 Chickpea

Among legumes, chickpea (*Cicer arietinum* L.) ranks third in the world after dry beans (*Phaseolus vulgaris* L.) and dry peas (*Pisum sativum* L.), and first in the Mediterranean basin (FAO 1995). The crop is grown on more than 10 million ha in 45 countries. In the 23 most important chickpea growing countries with average annual chickpea area in each is more than 10,000 ha (FAO 1996). Chickpea has a deep root system, and is considered a hardy crop. Chickpea is the most important grain legume crop of Indian sub-continent, West Asia and North Africa. It is a diploid species with 2n = 2x = 16, and is self-

pollinated. It is a food legume of the tribe *Cicereae* and family *Leguminosae,* exhibits a wide range of variability for morphological markers. Chickpea is an important source of protein in human diet and plays a significant role in the management of soil fertility.

Like other legumes, chickpea produces nodules, and is efficient in fixing atmospheric nitrogen (N) in a plant-usable form through biological nitrogen fixation. The crop is highly efficient in uptake of phosphorus (P) from soils containing low amounts of available P. Thus, growing chickpeas instead of cereals (where only one crop is grown per year), or after the harvest of cereals (e.g., rice fallows) should be encouraged to maintain soil fertility. Despite its importance as a crop, the world average yield of chickpea is only 700 metric kg ha^{-1}. The principal reasons for low unstable yields are diseases (most importantly *Fusarium* wilt) and terminal drought.

During a consultancy visit to ICRISAT Center in 1988, Kenneth J. Frey, Iowa State University, Ames, Iowa, USA, called chickpea a 'recalcitrant' crop species, meaning that it was not very amenable to genetic yield improvement, in spite of the many efforts to breed for yield increase during the last three decades. Wide hybridization is one of the potential means of broadening the genetic base of a crop species. In the genus *Cicer,* annual species occur, some of which are cultivated (*C. arietinum* L.) and some are wild. While cultivated chickpea is of limited source of genetic variability, wild species of *Cicer* have many important traits like resistance to diseases and pests; high protein and amino acid content and such agronomic traits as early seedling vigor, and high pod and seed numbers. Of wild species, only *C. reticulatum* and *C. echinospermum* can be easily crossed with chickpea. Crosses of other species with chickpea have not yet been successful. However, some wild species can be crossed among themselves. The species

C. judaicum, C. pinnatifidum, C. bijugum and *C. cuneatum* hold special significance, because they possess useful traits such as resistance to diseases and fast vegetative growth. At ICRISAT Patancheru, attempt to introgress desirable traits from these species into chickpea through embryo rescue and tissue culture techniques is being carried out.

Nine chickpea genotypes of diverse origin and the whole progeny of the cross GL 769 and ICCW 49, Annigeri and ICCW 6 were screened for their frequency of DNA polymorphism using RFLP, RAPDs and SSRs. The information on DNA polymorphism provides an essential basis to plan future marker facilitated breeding programs. Based on experience to date, the traditional breeding alone cannot effectively solve these constraints, and the development of molecular marker technology becomes a necessity for further improvement of resistance and agronomic traits in chickpea.

2. OBJECTIVES

- To learn DNA fingerprinting techniques such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Microsatellites (SSRs), DNA amplification fingerprinting (DAF), use of radio-labeled biomolecules for genetic analysis.

- Review DNA-based molecular markers with particular reference to RFLP and PCR based methods, summarize the protocols used, providing an appreciation of technical difficulty and cost and highlighting the advantages and/or limitations of their use and finally outline the theoretical and practical considerations to be made when choosing a molecular marker or technique.

- Study degree of polymorphism among potential mapping parents, segregation for markers in mapping populations and evaluate the applications of marker-assisted screening of germplasm in breeding programmes and construction of linkage maps.

- Construct dendograms representing the gene distance between accessions to help identification of divergent parents for future crosses.

3. REVIEW OF LITERATURE

Plant breeding is a process of designing and pursuing a desirable end product (e.g., cultivar, hybrid, and synthetic variety) that represents an assemblage of desirable agronomic and economic traits. The traits may be simple (qualitative) or complex (quantitative) in their genetic control. Possession of adequate genetic information on major and minor traits and their interactions improves the efficiency and probability of success in developing an end product with the desired attributes.

Construction of a detailed genetic linkage map for the crop of interest will make available a precise but vast amount of information that plant breeders can use to identify, manipulate, and complement traits to their maximum advantage in a short time. In many respects, plant genome maps might be considered analogous to a road map. Specific chromosomes could be thought of as numbered highways, and genes located on specific chromosomes comparable to cities and streets within cities. Larger boundaries (country, state, etc) containing a network of roads may be compared to multiple loci over several chromosomes that govern quantitative traits (QTLs). An effective and efficient way to reach a desired destination is to use a well-developed road map.

It will require several years and a vast amount of resources to map the genomes of major crops. However, already the linkage maps based on molecular markers have been developed for several major crops; and MAS has also been initiated.

The three major areas having impact on plant breeding are gene action, foreign or exotic genes, and molecular markers.

3.1 Increased understanding and exploitation of gene action

3.1.1 Gene action models

The selection methods that were used by plant breeders for genetic improvement in plant species were developed around the theories and concepts of gene action models. These models have been developed in order to explain the phenotypic expression of traits and the genetic variation observed in populations. The discrete classes of gene action are additive, dominance (including recessiveness, partial, and overdominance), epistasis, and pleiotropy.

In the additive model, the phenotype of the hybrid is intermediate between the two parents. The epistatic gene action model is referred to as interallelic interaction such that the value of alleles present at one locus depends on which allele(s) is (are) present at another locus. In cases where a single gene controls more than one trait, the gene action is referred to as pleiotropy. High-resolution molecular genetic maps should lead to a better understanding and utilization of precise types of gene action and phenomena that result from them.

Transgressive segregation: Segregants in a F_2 population, whose phenotypic expression for the trait of interest goes beyond expression in one or both of the parents, are referred to as transgressive segregants (Briggs and Knowles, 1967). This is an extremely important phenomenon which breeders exploit for population improvement procedures in cross-pollinated plant species as well as procedures to develop pure-line varieties in self-pollinated species. It is based on additive gene action at individual loci (qualitative traits) and across multiple loci for quantitative traits. The strategy to take advantage of transgresssive segregation is to match parents which posses different "favorable" alleles

for the trait(s) of interest so that with crossovers and recombination it is possible to produce progeny that possess the strengths of both the parents with the least weaknesses of both. With a quantitative trait such as seed yield, several hundred or even thousand progeny have to be evaluated to ensure a reasonable probability of detecting those rare individuals that posses the maximum number of favorable with the minimum number of unfavorable alleles. This is one area where markers linked to QTLs that highly influence the trait would greatly enhance the accuracy while simultaneously reducing the number of evaluations required to detect progeny with superior gene combinations.

Hybrid Vigor or heterosis: In the early part of this century, maize (*Zea mays* L.) breeders discovered that inbreeding reduced vigor and production of the inbred stocks, but when some combinations of inbreds were crossed the F_1 hybrid had vigor and yielded substantially higher than the average of the two parents. Out of these studies the term "hybrid vigor" and subsequently "heterosis" were coined.

It became apparent that there was a strong association between heterozygosity and heterosis. That phenomenon has led to one of the best plant breeding success stories for genetic improvement of crop yields (Duvick, 1984). Hybrid varieties have revolutionized corn production significantly in USA and the approach has spread to other crops, including some that are naturally self-pollinated like chickpea (*Cicer arietinum* L.) and alfalfa. The genetic mechanisms for heterosis are still not clear, but the two most widely accepted theories are dominance and over-dominance (Crow, 1964). When inbred lines are crossed together, the F_1 hybrid is heterozygous at all loci for which the genotype of the inbred parents differs. The dominance theory basically states that the different dominant alleles contributed by the inbred parents mask the detrimental effects of the

recessive alleles, thus the hybrid has the best strengths of the parents expressed with their weaknesses masked.

The theory of over-dominance is that there is an inherent superiority of the heterozygote (interaction between the dominant and recessive alleles at each locus) compared to the dominant homozygote (interaction between the dominant alleles at each locus; Crow, 1964). With the development of genetic maps and genetic markers, it should become easier to study the effects of individual as well as sets of genes on the expression of traits (Paterson *et al.* 1991). In return, that information should contribute to a better understanding of the genetic basis of heterotic responses observed and how that can be used by plant breeders to "design" inbreds and inbred combinations to further improve performance of hybrids.

Epistasis: As described earlier, epistasis is the interaction between or among alleles at different loci (interallelic interaction). Because of the immediate complexity of the number of combinations of alleles and their effects that are possible with a small number of loci, it has been very difficult to assess epistatic combinations of alleles. For quantitative traits there are many different loci involved in the expression of the trait and there are many interactions taking place to give final expression. Even with genetic maps, it will continue to be very difficult to evaluate large numbers of combinations and the differences elicited with each change. On the other hand, the task will be easier to undertake when the location and functions of genes are better defined. This is where the application of computer and statistical techniques (e.g., Informatics) will greatly facilitate predicted outcomes through stimulating changes of interacting loci and alleles based on gene products and function (Casey 1992). It seems logical that epistatic gene action plays

a larger role than we now understand in the final expression of traits, but the degree of complexity will mean that increased understanding will still be slow at best.

Pleiotropy: It is very difficult to separate pleiotropy from linkage. Because of the large number of genes contained in crop species and the fact that some of them occur adjacent to each other on a chromosome results in some very tight linkages. These linkages give the appearance that two or more traits controlled by the same gene(s). Very tight linkages necessitate evaluations of a large number of progeny before a crossover type can be detected. With well developed genetic maps, it should be possible to separate some strong associations between traits that are due to linkages of a small number of genes or linkages of QTLs vs. genes that are pleotropic (Paterson *et al.*1991). With the information of gene location, function and activation it should be possible to inactivate some genes that are known to control one trait and determine if there is a corresponding lack of expression of the other trait(s). It would be particularly helpful in developing breeding strategies to know if strong associations between desirable and undesirable traits can be broken because they are linked, or cannot because they are due to pleiotropy. In cases of strong associations between two desirable traits pleiotropic control may be better than tight linkage; however, the best strategies to exploit the association would differ with the two scenarios.

3.2 Access to foreign or exotic genes

The rapid development of molecular techniques has opened up sources of genes/germplasm to plant breeding that have been unavailable previously through conventional techniques. This is a very exciting and potentially valuable mechanism for crop improvement in the future. Some examples of active research for transferring genes

from "foreign or exotic species" are: to visualize other possible important agronomic and economic traits such as: genes for tolerance to drought; extreme soil acidity or salinity; or transfer potential habit to important annual species, etc. Ideas of transferring genes among species, genera, kingdoms that seemed impossible or too difficult a few years ago are now within the realm of possibility. Once genes are transferred they become a part of the recipient's genome and can be subjected to further modifications and enhancements.

Wild relatives of crop plants are generally considered to have genes that would enhance the cultivated form. However, the utilization of that source of genetic variation is limited due to the difficulty in making the crosses and the sterility problems often encountered. Where full fertility is found between cultivated and exotic germplasm, transfer of desirable genes from the wild progenitor is often accompanied by closely linked genes with deleterious effects. Using RFLP, Tanksley *et al.* (1989) have shown that it is possible to select for desirable genes while retaining little unwanted DNA from the donor species in plants e.g., *Brassica.*

3.3 Molecular markers

In 1865 Mendel determined that genetic factors behave as discrete particles when passed from parent to offspring. His studies on pea plants marked the beginning of genetics, the discipline concerned with segregation of genes. In the early part of the twentieth century, scientists discovered that Mendelian 'factors' controlling inheritance, which we now call as genes, were organized in linear order on cytologically defined structures called chromosomes. Shortly thereafter Sturtevant produced the first chromosome map with segregation data derived from studies on Drosophila (Crow and Dove 1988). The markers

of this first genetic map were phenotypic traits scored by visual observation of morphological characteristics of the flies.

Genetic studies undertaken so far focussed on morphological traits. Traits such as plant habit, leaf form and colour, flower colour, podding habit and seed coat colour were used for study of morphological traits. They are traditionally used in taxonomy, genetics, and breeding. Since most of these traits are recessive with detrimental effect (pleiotropic), these are not suitable as selectable genetic markers in breeding programme (Gaur and Slinkard 1990). Despite the wide variability for morphological traits, their less number and their expression is age and environment dependent.

Seed storage proteins were the first candidates as molecular markers to distinguish the germplasm lines, due to their ease of isolation and identification on gels. Next came the isozymes, which are still popular in some cases such as for study of diversity, or as a marker during introgression of alien germplasm. In many cases, such as for drought or disease resistance, planned indirect methods of selection (markers) for the traits of interest may be more desirable or effective than direct selection. Indirect methods may take the form of morphological markers, biochemical markers (isozymes), or DNA markers (RFLP, RAPD, DAF, SSR, and AFLP).

Some of the reasons for using indirect selection via associated markers may be:

- Lack of sufficient number of morphological markers
- To identify individuals in early stages of growth for discarding, to conserve resources or to identify individuals for crossing prior to flowering (e.g., backcrossing or population improvement program).

- Inaccurate direct measures of the trait expression (phenotype data) due to many loci involved (such as QTLs) or due to uneven inoculations, infections or infestations in screening nurseries.
- Difficulties in selecting for several traits simultaneously.

A major breakthrough occurred when it was realized that genetic maps could be constructed by using pieces of chromosomal DNA as direct markers for segregation pattern of chromosomal segments.

In eukaryotes, DNA is condensed with histone and non-histone proteins into thread-like structures called chromosomes. The number of chromosomes varies between species and occasionally within species. At the sub-chromosomal level, several types of organizations are observed. These can be summarized as follows:

- Gene-rich sectors: In large genomes, genes are found clustered in gene-rich sectors especially in regions close to the telomeres. In a number of cases, it is significant that the order of genes, in a sector is conserved between species ('gene synteny'). Genes in a gene-rich sector are interspersed with short repeat sequences, often including transposable elements.
- Tandem repeats: Multiple repeats of essentially the same sequence are found at many locations, especially around the centromeres, telomeres and interstitial locations. These arrays can consist of up to millions of repeat units. Tandem repeats vary according to size and sequence of the repeat unit, the number of repeats found and their distribution throughout the genome. They have therefore received considerable attention as molecular markers.

Thus, a molecular marker is a sequence of DNA or a protein which can be readily detected and whose inheritance can be monitored. It is the variation in, or *polymorphism* of, molecular markers, which can be used in genetic diversity and mapping studies.

3.3.1 Properties of molecular markers

The following properties would be generally desirable for a molecular marker:

- Highly polymorphic behavior.
- Codominant inheritance (which allows us to discriminate homo- and heterozygotic states in diploid organisms).
- Frequent occurrence in the genome.
- Even distribution throughout the genome.
- Selectively neutral behavior (i.e., no pleiotropic effect).
- Easy access (by purchasing or fast procedures) to get data.
- Easy and fast assay (e.g., by procedures amenable to automation).
- High reproducibility.
- Easy exchange of data between laboratories.

No molecular markers are available yet that fulfills all of these criteria. However, according to the kind of study to be undertaken, one can already choose between a variety of marker systems, as different markers explore different areas of genome, some more suited for specific purpose, some are species-specific and application dependent.

3.3.2 Protein based markers

The number of polymorphic morphological markers is limited, especially in intraspecific crosses, and their expression is influenced by environment. Therefore, more reliable

markers such as proteins or, more specifically, allelic variants of several enzymes, so-called isozymes (Tanksley & Orton 1983), other biochemical characteristics, such as lipids or sugars, had to be considered. The multiple forms of an enzyme are of two classes:

- Allozymes: The enzyme is coded for by different alleles *at one* gene locus.
- Isozymes: The enzyme is coded for by alleles *at more than one gene* locus.

For the generation of molecular markers based on protein polymorphisms the most frequently used technique is the electrophoretic separation of proteins on gels and staining.

Interpretation of banding patterns: The principle considerations here are:

- Whether the organism is homozygous or heterozygous at the gene loci.
- the quaternary structure of the enzymes (monomeric, dimeric etc).
- the number of gene loci.
- the number of alleles per locus.

Allozymes are controlled by codominant alleles, which means that it is possible to distinguish between homozygotes and heterozygotes. For monomeric enzymes (i.e., consisting of a single polypeptide), plants homozygous for that locus will produce one band whereas heterozygous individuals will produce two. For dimeric enzymes (i.e., consisting of two polypeptides), plants homozygous for that locus will produce one band whereas heterozygous individuals will produce three owing to random association of the polypeptides. With tetrameric enzymes, heterozygous individuals will produce five bands. For multimeric enzymes, where the polypetides are specified by different loci, the formation of isozymic heteromers can complicate the banding patterns considerably.

Advantages of isozymes

- The low cost of chemicals and labour.
- Ease of isolation.
- the user friendliness: many individuals can be scored for several allozyme loci within a short time span.
- Allozyme markers are codominant - both alleles in a diploid organism are usually clearly identifiable, and heterozygotes can be discriminated from homozygotes, which is a prerequisite for estimation of allele frequencies in population genetic studies.
- Confirms gene expression.

Disadvantages of isozymes

- A nucleotide substitution should result in amino acid substitution for detecting a new allele as a polymorphism.
- Restricts study of those parts of DNA that code for stainable enzymes.
- Analysis of Allozyme patterns of polyploids can be extremely difficult.
- Plant tissue has to be processed shortly after harvest since proteins are quite unstable.
- Allozymes differ in one or more physiological respects and therefore, may not be evolutionary neutral.
- Very few markers.
- Distantly related taxa difficult to study.
- Redundancy of genetic code not accounted.
- Cannot use old or stored tissues or fossils.

Applications of isozymes

1. Isozyme polymorphism has been used for characterizing/identifying genotypes, for studying population genetics, and for examining geographical patterns of variation.

2. Enzyme electrophoresis has also been very useful in genetic diversity studies, biochemistry, physiology, genetic breeding, etc. as it can directly reveal genetic polymorphism through demonstrating multiple forms of a specific enzyme. Over 30 enzyme systems have been used in plants, and for some crop plants the genes involved have been mapped.

3.3.3 DNA based markers

A major breakthrough occurred when it was realized that genetic maps could be constructed using pieces of chromosomal DNA as direct markers for segregation pattern of chromosomal segments (Botstein *et al.*1980). Because each individual's DNA sequence is unique, this information can be exploited for any study of genetic diversity and relatedness between organisms. A wide variety of techniques to visualize DNA sequence polymorphism have been derived from these techniques.

The term "*DNA fingerprinting*" is used to describe a method for the simultaneous detection of many highly polymorphic DNA loci by hybridization of specific multilocus "probes" to electrophoretically separated restriction fragments. In other words, DNA fingerprinting refers to any multilocus approach of visualizing DNA polymorphisms either by hybridization or Polymerase Chain Reaction (PCR). Jeffreys *et al.* 1985 originally introduced the term. In recent years, several modifications of the basic technique have appeared and related strategies have been developed. Most importantly DNA polymorphisms became detectable by the PCR. Some of the new marker methods are still called DNA fingerprinting, but "DNA profiling", "DNA typing" or more specific terms have also been introduced. According to this definition, DNA fingerprints are mainly obtained by either of two strategies.

- *"Classical" hybridization-based fingerprinting* involves cutting of genomic DNA with a restriction enzyme, electrophoretic separation of resulting DNA fragments according to size; and detection of polymorphic multilocus banding patterns by hybridization with a labeled complementary DNA sequence, a so called "probe."

  PCR-based fingerprinting involves the *in vitro* amplification of particular DNA sequences with the help of specifically or arbitrarily chosen oligonucleotides "primers" and a thermostable DNA polymerase; the electrophoretic separation of amplified fragments, and the detection of polymorphic banding patterns by such methods as staining.

3.3.4 Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis was one of the first techniques to be used widely to detect variation at the sequence level (Botstein *et al.* 1980). It examines the variation in size of specific DNA fragments following digestion with restriction enzymes. A large number of different restriction enzymes are commercially available. Digestion of a particular DNA molecule with such an enzyme results in a reproducible set of fragments of well-defined lengths. Point mutations within the recognition sequences as well as insertions and deletions will result in an altered pattern of restriction fragments and may thus bring about a screenable polymorphism between genotypes.

RFLPs have been used for the construction of linkage maps (Gill *et al.*1991; Kiss *et al.* 1993; McCouch *et al.* 1988; Helentjaris *et al.* 1986) and gene tagging (Young *et al.* 1988) in many crop species. RFLP analysis is used extensively in the construction of genetic maps and has been successfully applied to genetic diversity assessments, particularly in cultivated plants (Castagna *et al.* 1994; Jack *et al.* 1995). Use of RFLP

technique in chickpea has shown little molecular diversity (Udupa *et al.* 1993). In self pollinating legumes such as lentil, peanut and soybean, a very low level of polymorphism has been reported (Havey *et al.* 1989; Kochert *et al.* 1991; Keim *et al.* 1988).

This methodology is quite similar to hybridization-based fingerprinting which actually represents a special case of RFLP analysis. Genomic DNA is extracted, digested with restriction enzymes and separated by electrophoresis on a gel. The DNA from the gel is transferred to a nylon membrane (Southern Blotting) and species-specific fragments are made visible by hybridization with a labeled probe.

Analysis of results:

The result is ideally a series of bands on a gel, which can be scored for the presence, or absence of particular bands. Differences between genotypes are usually visualized as an altered pattern of DNA restriction fragments. This may result from the point mutations creating new restriction sites or loss of an existing site, reorganization of blocks of DNA, such as deletions or insertions, between restriction sites.

It is clear that the choice of the DNA probe-restriction enzyme combination is crucial in discriminating power of RFLP technology.

Traditional RFLP analysis makes use of probes obtained from the following sources:

1. Nuclear DNA probes: These probes are obtained from
   - genomic libraries. Total genomic DNA is digested with restriction enzymes (e.g., Pst I) and individual fragments are cloned into a bacterial or viral vector. Suitable probes are selected from this "anonymous" library for RFLP analysis.
   - cDNA (complementary DNA or copied DNA libraries) probes: The mRNA is isolated and transcribed into DNA using the enzyme reverse transcriptase. The

cDNA so obtained is cloned into vectors and used as a library for probes in RFLP analysis.

2. Cytoplasmic DNA: These are obtained from mitochondrial and chloroplast DNA libraries.

RFLP probes are

1.Locus specific- give rise to easily identified co-dominant markers.

2. Mainly species-specific.

Advantages of RFLP technique

- Results are highly reproducible between laboratories.
- RFLP markers usually show co-dominant inheritance.
- Discriminating power: can be at species/population level (single locus probes) or individual level (multi-locus probes).
- Simplicity of the method: given the availability of suitable probes, the technique can be readily be applied to any system.

Disadvantages of RFLP technique

- Time consuming and expensive to perform - technical expertise required.
- Where no suitable single-locus probes exist, it is time consuming and expensive to identify suitable marker-restriction enzyme combinations from genomic and cDNA libraries.
- Most RFLP work is carried using radioactive labeled probes, and therefore requires expertise in autoradiography. This can be a serious drawback in some situations where special facilities and permits are required to carry out the work.

3.3.5 DNA fingerprinting based on hybridization

The technique of classical DNA fingerprinting is methodologically derived from RFLP analysis and is mainly distinguished from the latter technique by the kind of probe applied to reveal polymorphisms. Two main differences exist between RFLP and hybridization based fingerprinting.

1. DNA fingerprinting makes use of multilocus probes, creating complex banding patterns, whereas RFLP probes are usually locus specific, resulting in an easy to screen co-dominant marker behavior.
2. DNA fingerprinting is mostly performed with non-species specific probes that recognize ubiquitously occurring sequences such as minisatellites, whereas RFLP probes are generally species-specific.

Two categories of such multilocus probes are mainly used. The first category comprises cloned DNA fragments or oligonucleotides which are complementary to so called "minisatellites" i.e., tandem repeats of a basic motif of about 10 to 60 bp. The second category is exemplified by oligonucleotide probes which are complementary to so called "simple sequences" or "microsatellites" i.e., tandem repeats of very short motifs, mostly 2-5 bp.

With both kinds of probes, a high degree of polymorphism between related genotypes is usually observed, which has been exploited for numerous studies in diverse areas of genome analysis.

3.3.6 Molecular markers based on DNA amplification

The analysis of nucleotide sequence variability has been revolutionized by the development of PCR. This technique allows us to amplify any DNA sequence of interest to high copy numbers, thereby circumventing the need of molecular cloning. Further advancements in this technique has evolved PCR-based markers such as Random Amplified Polymorphic DNA sequences (RAPD) to Amplified Fragment Length Polymorphisms (AFLP) and Simple Sequence Repeat markers (SSR) or microsatellites. Currently their potential for use in germplasm characterization, fingerprinting and also increasingly mapping, and ultimately in conservation are widely studied.

3.3.6.1 Random Amplified Polymorphic DNA (RAPD)

In a large variety of plants and animals it has been shown single arbitrary primers, 8 to 10 nucleotides in length, will produce one to few amplification products (Williams *et al.* 1990). The primers are generated with >50% G+C content to ensure efficient annealing, and with sequences that are not capable of internal pairing so as to avoid PCR artifacts. The PCR procedure allows specific amplification of DNA fragments ranging from 200 bp to 3000 bp in length, which can be visualized after electrophoresis by staining with ethidium bromide. Because a single primer allows amplification of multiple loci dispersed throughout the genome, RAPDs provide a rapid assay for nucleotide sequence polymorphism (Tingey *et al.*1992).

The key point about this technique is that nothing is known about the identity of the amplification products. The amplification products are however extremely useful as markers in genetic diversity studies. Other important features of the technique are:

- The number of fragments. Many different fragments are normally amplified using each single primer, and the technique has therefore proved a fast method for detecting polymorphism. The majority of commercially produced primers result in 6 to 12 fragments.
- Simplicity of the technique. RAPD analysis does not involve hybridization or autoradiography or high technical expertise. Only minute quantities of target DNA are required. Arbitrary primers can be purchased. Unit costs per assay are low. This has made RAPD analysis very popular.
- RAPD markers are dominant. Amplification either occurs at a locus or it does not, leading to scores presence or absence of bands. This means homozygotes and heterozygotes cannot be distinguished.
- Problems of reproducibility. RAPD does suffer from a sensitivity to changes in PCR conditions resulting in changes to some of the amplified fragments. Reproducible results can be obtained if care is taken to standardize the conditions used.

The various factors, which affect the reproducibility are

1. Primer: Primers can be purchased from several manufacturers [e.g.,*Operon Technologies* Inc-U.S.A; University of British Colombia (UBC)-Canada, or Pharmacia LKB]. Primer concentrations are generally optimal between 0.01 to 2.0 μM. In most species, the majority of RAPD primers result in fragment patterns with 6 to 12 fragments, while a few primers fail to amplify DNA. The G+C content has the highest prediction value; a high G+C content is positively co-related with primer strength.

2. Polymerase: A large number of brands and types of polymerases are available for PCR. Different polymerases often give to different RAPD products. Therefore, the initial choice of polymerase is important; switching to another type of enzyme is likely to render comparisons with previous experiments impossible.
3. Template concentration: The concentration of the genomic DNA should be determined accurately and the amount of DNA used in the assay should be uniform and well within the experimentally determined reproducibility ranges (usually 5 to 500 ng).
4. $MgCl_2$ concentration: Strong and reproducible bands are obtained over a wide range of $MgCl_2$ concentrations. A change in concentration often results in a qualitative change of fragment patterns.

Advantages of RAPD technique

- Fast method for detecting polymorphism.
- Simple, technically not demanding.
- Relatively cheap to perform (low unit costs).
- Avoids the need for hybridization with radioactive probes.

Disadvantages of RAPD technique

- Dominant markers.
- Problems with reproducibility - RAPD are sensitive to alterations in PCR conditions.
- Problems with interpreting band patterns e.g., problems of co-migration.

Applications of RAPD markers

1. Cultivar identification.
2. Genetic mapping.
3. Phylogenetic pedigree and linkage analysis.
4. Population differentiation.
5. Estimation of out crossing rates.
6. Identification of duplicates and the establishment of core collections within the germplasm.
7. To determine the extent and role of introgression in the evolution of the species.
8. To detect genetic variations at the intraspecific level between closely related cultivars.
9. Recently it was reported that RAPD primers detected polymorphism among plants generated from tissue culture.

Williams *et al.* (1990) used a shorter primer, 9 or 10 nucleotides in length, and low stringency cycles to reveal fewer amplification products (about 10 products) by agarose gel electrophoresis and ethidium bromide staining. This method (RAPD) demonstrated polymorphism between two lines each of the fungus *Neurospora crassa* and *Zea mays* and also between two lines of soybean, *Glycine max* and *G. soya.*

3.3.6.2 Other PCR techniques using single arbitrary primers

Other techniques like, Arbitrarily primed PCR (AP-PCR; Welsh and McClelland 1990), and DNA amplification fingerprinting (DAF; Caetano-Anolles *et al.*1991a) target multiple annealing sites without the requirement of prior knowledge of template sequence. Caetano-Anolles has encompassed all the analyses that use arbitrary primers

under the heading MAAP (Multiplicity and arbitrary nature of amplicons), that appropriately depicts the nature of this amplification strategy.

Welsh and McClelland (1990) used single arbitrary primers of a length comparable to that of PCR primers in an amplification reaction that used two cycles of low stringency (i.e., low annealing temperature) followed by a series of cycles of high stringency amplification. Amplification products were resolved by polyacrylamide gel electrophoresis and were detected by autoradiography. AP-PCR was used to distinguish various bacterial strains as well as three varieties of rice. Polyacrylamide gel electrophoresis and detection by autoradiography resolved between 3 and 20 products.

3.3.6.2.1 DNA Amplification Fingerprinting

Caetano-Anolles used one or more primers, as short as 5 nucleotides, but typically 7 or 8 nucleotides in length, and either high or low stringency cycles to produce relatively complex DNA profiles (Caetano-Anolles *et al.*1991a) when resolved by polyacrylamide gel electrophoresis and a highly sensitive DNA silver stain (Bassam *et al.* 1991). DAF uses low stringency amplification conditions so that primers can anneal arbitrarily at multiple sites on each template DNA strand. Although initiation of DNA synthesis occurs throughout the template, only those sequences in which priming sites are on opposite strands and in near proximity will be successfully amplified. Mismatch annealing also occurs to a variable extent and can produce less numerous "secondary" amplification products that are also characteristic of the template in study. Amplification in DAF is arbitrary but not random. DAF fingerprints have bands that fall into two categories, those that are phylogenetically conserved, and those that are individual-specific. This suggests that primer sites are randomly distributed along the target genome and flank both

conserved and highly variable regions. There is also wide variation in the degree of amplification between different fragments that is reproducible between experiments.

Applications of DAF: Within plants, DAF offers the possibility of identifying cultivars and near-isogenic lines. DAF method amplified DNA from a wide variety of organisms, including the fungus *Candida albicans* and several plant species as in different cultivars of soybean and of several turf grasses, e.g., *Zoysia*, varieties of rice and inbred lines of maize were also identified using polyacrylamide gel electrophoresis and autoradiography. DAF was used to separate cultivars of dogwood (*Cornus florida*) and differentiate from other *Cornus* species. Cultivars of peanut(*Arachis hypogea* L.) were not separated but wild *Arachis* species were readily identified when using agarose gel electrophoresis and ethidium bromide staining of amplified DNA. This point out both dearth of polymorphism between cultivated peanut accessions, and also possibly the limitation of using insufficiently sensitive techniques for the separation and staining of DNA.

Differences between DAF (Caetano *et al.* 1991) *and RAPD*

- Higher primer concentrations in DAF than in RAPD
- Shorter primers are used in DAF (5-8 nucleotides)
- Two-temperature cycle in DAF compared to three-temperature cycle in RAPD.
- More complex banding patterns with DAF than with RAPD.

3.3.6.2.2 Arbitrarily-Primed Polymerase Chain Reaction

Welsh and McClelland introduced AP-PCR. Arbitrarily Primed PCR is a method of creating genomic fingerprints from species, in which little is known about target sequence to be amplified. Strain-specific arrays of DNA fragments (fingerprints) are generated by PCR amplification using arbitrary oligonucleotides to prime DNA synthesis

from genomic sites, which they fortuitously match or almost match. Generally, two cycles of PCR are performed under conditions of low stringency with a single random primer, followed by PCR at high stringency with specific primers. DNA amplified is this manner can be used to determine the relatedness of species or for analysis of RFLP.

Oligonucleotides of 20 or more nucleotides, were used as primers (e.g., the M13 universal sequencing primer; the pBS reverse sequencing primer; and the Kpn-R, KA, KB, KM, KR, KX, and KZ primers). Two cycles with low stringency (allowing for mismatches) were followed by 30 to 40 cycles with high stringency. [^{32}P] dCTP was included in the last 20 to 30 cycles. Radiolabeled products were separated by polyacrylamide gel electrophoresis and made visible by autoradiography. The AP-PCR variant of the arbitrary PCR method is used the least, compared to RAPD and DAF. It is also the most complicated method and uses radioisotopes. However, it can be simplified by separating the fragments on agarose gels and using ethidium bromide staining for visualization.

Differences between AP-PCR (Welsh and Mc Clelland, 1990) *and RAPD*

- in AP-PCR the amplification is in three parts each with its own stringency and concentrations of constituents.
- high primer concentrations are used in the first PCR cycles.
- primers of variable length, and often designed for other purposes are arbitrarily chosen for use (e.g., M13 universal sequencing primer).

MAAP illustrates the importance of both amplification reaction conditions and the separation and detection procedure used to resolve the spectrum of amplified products into a characteristic and reproducible fingerprint pattern. MAAP usually relies on non-

stringent reaction conditions (other than annealing temperature) for the amplification of arbitrary target sites. When compared with other PCR protocols (Williams *et al.* 1990) over ten times more primer (3 μM) was required to reveal all amplification products. In the PCR, high primer concentration often results in increased primer mismatching and spurious annealing events. Therefore, higher concentrations favor the nonstringent reaction conditions typical of the amplification of arbitrary amplicons. Increasing primer concentration can also affect the interaction of symmetrical sequences in the formation of hairpin structures and concatemers. Primer concentrations used in DAF tend to give smears in RAPD analyses, or 7-mer and 8-mer primers that render complex profiles by DAF appear to produce no amplification products by RAPD. MAAP encompasses all these closely related techniques, but it is not commonly used.

Multiple Arbitrary Amplicon Profiling techniques.

Characteristics	DAF		
Resolution	High	Intermediate	Low
Products	10-100	3-50	1-10
Separation	Polyacrylamide	Polyacrylamide	Agarose
Visualization	Silver staining	Radiolabelling	EtBr staining
Primer length	5-15 nt	20-34 nt	9-10 nt
Primer conc.	3-30 mM	3 mM	0.3 mM
Stringency	Low or High	High or Low	Low

3.3.6.3 DNA markers based on sequence-tagged sites

As more sequence information is becoming available from different sources, which can be located in widely available databases, it can be used for developing new strategies for

the analysis of genetic variation. A sequence-tagged site (STS) is a general term given to a marker, which is defined by its primer sequences. STSs have been used extensively for mapping of the human genome.

Example of STSs:

- Sequence tagged microsatellites (STMS) also known as Simple Sequence Repeat Polymorphisms.
- Anchored microsatellite oligonucleotides including inter-simple sequence repeat (ISSR) primers.
- Sequence- characterized amplified regions (SCARs).
- Cleaved amplified polymorphic sequence (CAPS).

3.3.6.3.1 Sequence-Tagged Microsatellites

Primers can be constructed which are complementary to the short, unique sequences flanking microsatellite repeat sequence loci and which, in the PCR reaction, direct the amplification of the repeat. Since the repeat length is highly variable, this is an effective way of detecting polymorphisms. These markers generally have the following properties, which make them useful for population studies:

* usually define a single, multi-allelic locus
* co-dominant- homozygotes and heterozygotes can be distinguished
* highly reproducible results obtained

For high levels of discrimination, polyacrylamide gels are used which can detect single copy differences. It is also possible to combine the PCR reactions with different STMS primers in the same reaction tube (so-called multiplexing) which saves on time but this is only possible where the products of the different primers donot overlap in size.

In 1989 synthetic oligonucleotide probes that recognize simple repetitive DNA sequences were introduced to plant DNA fingerprinting. Weising (1992) demonstrated different DNA fragment patterns between three barley cultivars by hybridization to a $(GACA)_4$ probe as well as between three accessions of chickpea, *Cicer arietinum*, by hybridization to a synthetic digoxygenated oligonucleotide $(GATA)_4$ probe complementary to a microsatellite DNA sequence.(Serret *et al.* 1997). Presently laboratories at University of Frankfurt are in the process of isolating multiallelic, single-locus probes from chickpea. Such sequences will allow linkage analysis and genome mapping, both strategies aiming at the characterization and isolation of genes conferring tolerance (or also susceptibility) towards Ascochyta blight disease.

Advantages of Sequence-tagged microsatellites

- Since the repeat length is highly variable, this is an effective way of detecting polymorphisms.
- These markers generally have the following properties which make them useful for population studies.
- Co-dominant - homozygotes and heterozygotes can be distinguished.
- Highly reproducible results obtained.
- Usually define a single, multi-allelic locus.
- Highly abundant and polymorphic.

3.3.6.3.2 Sequence Characterized Amplified Regions

An example of STS, based on the RAPD technique is SCARs. These markers are generated by cloning and sequencing RAPD fragments, which are of particular interest. When the sequence is known, it is then possible to design primers which are longer than usual RAPD primers (24-mer oligonucleotides) and which are exactly complementary to the ends of the original RAPD fragment. When these primers are used in a PCR, single loci are identified which correspond to the original fragment. These loci are called SCARs. SCARs offer several advantages over RAPD and other arbitrarily primed methods, principally that the results are highly reproducible (longer primers used) and the markers are co-dominant.

3.3.6.3.3 Cleaved Amplified Polymorphic Sequence

In another technique called CAPS or PCR-RFLP, PCR primers are constructed for a particular locus. The PCR amplified product is digested with a restriction enzyme and visualized on an agarose gel using ethidium bromide staining. As with RFLP, polymorphisms are detected by differences in restriction fragment sizes.

3.3.6.3.4 Anchored microsatellite oligonucleotides

Variants of STMS technique have been developed using anchored microsatellite oligonuleotides as primers which direct the amplification of genomic DNA segments other than the repeat region itself. These approaches use oligonucleotides based on a SSR anchored to their 5' or 3' ends by 2 to 4 arbitrarily chosen nucleotides which trigger site-specific annealing. This initiates PCR amplification of genomic segments which are flanked by inversely oriented, closely spaced repeat sequences. Specifically, ISSR

primers are anchored to their 3' ends and amplify segments between ISSRs. Such anchored microsatellite markers are usually dominant.

3.3.6.4 Microsatellite DNA as a Genetic Marker

Litt and Luty introduced the term microsatellite in 1989 to characterize the simple sequence stretches amplified by PCR. These are also known as short tandem repeats (STRs) or simple sequence repeats (SSRs) of 1-5 bp and differ from minisatellites (often called VNTRs), which are repeated sequences having repeat units ranging from 11 to 60 bp in length. The minisatellites were first reported by Jeffreys *et al.* (1985) as tandemly organized repeats though their utility through PCR was suggested later. The microsatellites are randomly and more evenly dispersed in the genome than minisatellites, which are generally confined to telomeres. The tri- and tetra-nucleotide repeats are also common in human genome.

The DNA sequences flanking SSRs are known to be conserved in the same manner as those flanking minisatellites. These conserved sequences have been used for designing suitable primers for amplification of the SSR loci using PCR. Any such primer or a pair of primers, when used to amplify a particular SSR locus in a number of genotypes will reveal SSR polymorphism in the form of differences in the length of the amplified product, each length representing an allele at that locus. The length differences are attributed to the variation in the number of repeat units at a particular SSR locus, possibly caused by slippage during replication, and therefore provide a valuable source of polymorphisms for many purposes, including linkage analysis (Lathrop *et al.* 1985; Jeffreys *et al.* 1986; Nakamura *et al.* 1987; Wells *et al.*1989), identification of species

and cultivars (Weising *et al.* 1989, 1991a, 1992) and marker-assisted selection (Beckmann and Soller 1990).

Microsatellites have been found and used for genetic analysis in many a mammalian species and to a lesser extent in other eukaryotes, e.g., insects, birds, fish, mouse, cattle and plants. $(CA)_n$ repeat is one of the most frequently occurring microsatellites (several tens of thousands of copies) in human and many mammals, but is comparatively less frequent in plants. In contrast, $(AT)_n$ microsatellites are the most abundant dinucleotide repeats in plants. Further, greater abundance of $(GA)_n$ repeats than $(CA)_n$ repeats appears to be a consistent feature of plant genomes. Trinucleotides and tetranucleotides repeats are also found in plant genomes, the most frequent of them being $(AAG)_n$ and $(AAT)_n$.

A comparable number of minisatellites occurs in the tomato genome and some of the most polymorphic loci cloned in the tomato contain microsatellites (Brown & Tanksley 1993). Microsatellites with relatively low numbers are generally very abundant. In the rice genome $(GT)_n$ repeats every 480 kb (Wu & Tanksley 1993). An example is the SAT1 locus found in soybean in which 25 alleles were found at this single locus. In humans as many as 80 alleles have been documented at one locus. Since microsatellites can find more alleles at a locus than RFLP's, former is more informative.

SSRs offer a potentially attractive combination of features that are useful as molecular markers:

- SSRs have been reported to be highly polymorphic in plants, and thus highly informative, providing many different alleles for each marker screened, even among closely related individuals.

- SSRs can be analyzed by a rapid, technically simple, and inexpensive PCR-based assay that requires only small quantities of DNA.
- SSRs are co-dominant, and simple Mendelian segregation has been observed.
- SSRs are both abundant and uniformly dispersed in both human and plant genomes.

Basically, three different methods are applied to plant genome analysis using repeat sequences:

(i) Minisatellites (repeat units of 9-20 nucleotides) can be hybridized to restricted and electrophoretically separated DNA blotted onto nylon membrane (Jeffreys *et al.* 1985).

(ii) Microsatellites (repeat units of 1-5 nucleotides) can be hybridized to DNA in dried gels (Ali *et al.* 1986; Epplen 1988). Alternatively, microsatellites can be cloned, sequenced, and amplification fragment length polymorphisms detected by PCR, using oligonucleotides from the surrounding monomorphic DNA sites as primers (Litt and Luty 1989) later called as sequence-tagged microsatellite sites (STMS; Beckmann and Soller 1990). Like RFLP, they are co-dominant markers, but are more informative. For analysis of STMS, tri- and tetra-nucleotide motifs gained more and more attention compared with mono- and di- nucleotide repeats, because the former group present a clearer banding pattern after PCR and gel electrophoresis (Hearne *et al.* 1992).

Microsatellite DNA markers are useful in many types of studies. They can be used in pedigree analysis to determine kinship among individuals, fingerprinting, forensics, genetic mapping and phylogenetic analysis. Genetic mapping was done particularly in crop species with low polymorphism such as wheat and soybean. Since microsatellite DNA changes rapidly during the course of evolution, and is not influenced by selection, phylogenetic analysis can be conducted and also can be used as an

evolutionary timeclock by measuring the gain or loss of repeats in a genera over evolutionary time and can possibly detect when speciation occurs. One single disadvantage of microsetellites is homeoplaisy.

Advantages of Microsatellites

- Highly variable and abundant.
- Rich source of allelic diversity.
- Analysis using RFLP or PCR techniques.
- Anchored SSR for fingerprinting.

Disadvantages of Microsatellites

- Origin of variation unknown.
- Map location hard to define.

3.3.6.5 Random Amplified Microsatellite Polymorphism

A combination of RAPD amplification of DNA and subsequent hybridisation using microsatellites, known as random amplified microsatellite polymorphism (RAMPO) has recently been reported to be efficient for detecting of more variability in plant and fungal DNA samples (Richardson *et al.* 1995). A similar technique, where a microsatellite – anchored primer was combined with the random primer during PCR amplification has helped to locate new loci in barely RFLP map (Becker and Heun, 1995). The repetitive sequences in the genome are not involved in gene expression and represent in most cases the introns. Further, they are generally not accessible to RFLP probes generated from low copy DNA sequences. The use of repeat sequences (di-, tri- or tetra- nucleotides) as a probe that hybridizes to repetitive sequences in the genome has uncovered a great deal of variation. The detection of these microsatellites however, involves the intricacies of

RFLP detection. Further, probe hybridization requires technical expertise, or detailed sequence information to design primers for PCR. The recent technique of RAMPO is based on RAPD amplification of genomic DNA and subsequent detection of microsatellites in blotted DNA using the labeled simple repeat sequences as probes.

The simplicity of operation of this technique allows quick detection of microsatellite loci in hybridization blots. No previous knowledge of sequence is required, and random genomic regions can be amplified with each RAPD primer. Additionally, a single blot of the amplified products can be repeatedly hybridized to repeat several sequence probes. Thus the application of DNA markers is entering into an exciting era of new applications in plant genetics, crop environment, and conservation of biodiversity.

Advantages of RAMPO

- High sensitivity and faster analysis.
- Highly variable.
- Prior sequence information not required to confirm use of marker.
- Uses RAPD amplified or PCR amplified DNA.
- Blot can be probed with multiple repeats.

Disadvantages of RAMPO

- Uses radiolabeled primers.
- Detailed inheritance study required.
- Microsatellite hybridization may be more reliable than minisatellite.

Comparison of RFLP and RAPD markers with microsatellites

Characteristic			Microsatellites
Principle involved	DNA blot hybridization	PCR amplification with random primers	PCR amplification of simple sequence repeat loci
Type of polymorphism	Single base changes; insertions; deletions	Single base substitutions insertions; deletions	Variation in number of repeat motifs
Genomic abundance	High	Very high	Medium
Level of polymorphism	Medium	Medium	High
Inheritance	Codominant	Dominant	Codominant
Amount of DNA required	5-10 µg	10-25ng	50-100ng
Sequence information required	No	No	Yes
Radioactive detection required	Yes/No	No	No
Development costs	Medium	Medium	High
Start up costs	Medium/High	Low	High
Detection	Autoradiography; biotin labeling	Ethidium bromide; silver staining	Ethidium bromide; silver staining

3.3.6.6 Amplified Fragment Length Polymorphism

The AFLPs were initially named to rhyme with RFLP as "Amplified Fragment Length Polymorphism" but subsequently it was realized that AFLP involves the detection of "presence or absence" of restriction fragments rather than differences in their lengths. The AFLP approach was developed by a private company *Keygene* in Netherlands led by Dr. Marc Zabeau, which holds the patent for this technology (Vos *et al.* 1995).The primary reason for the rapid acceptance of AFLP technology is due to its ability to detect a large number of polymorphic DNA markers rapidly and in a reproducible manner. These fingerprints may be used as a tool for determining the identity of a specific DNA sample or to assess the relatedness between samples. Fingerprints are also used as source for genetic markers to generate linkage maps or to identify molecular markers linked to

phenotypic traits and/or genetic loci. Polymorphisms detected in DNA fingerprints obtained by restriction cleavage 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. The DNA polymorphisms identified using AFLP are typically inherited in Mendelian fashion, and may therefore be used for typing, identification of molecular markers, and mapping of genetic loci.

The AFLP approach is conceptually simple and combines both RFLP and PCR techniques. The various steps involved are:

Restriction Endonuclease Digestion: To prepare an AFLP template, genomic DNA is isolated digested with two restriction endonucleases simultaneously. This step generates the required substrate for ligation and subsequent amplification.

The restriction fragments for the amplification are generated by two restriction endonucleases: *Eco*RI and *Mse*I. *Eco*RI has a 6-bp recognition site, *Mse*I has a 4 bp recognition site. When used together, these enzymes generate small DNA fragments that will amplify well and are in the optimal size range(< 1 kb) for separation on denaturing polyacryamide gels. Due to primer design and amplification strategy, these *Eco*RI-*Mse*I fragments are preferentially amplified (rather than *Eco*RI - *Eco*RI and *Mse*I-*Mse*I).

The success of the AFLP technique is dependent upon the complete restriction digestion. Therefore, much care should be taken to isolate high quality genomic DNA, intact without contaminating nucleases or inhibitors.

Ligation of adapters: Following heat inactivation of the restriction endonucleases, the genomic DNA fragments are ligated to *Eco*RI and *Mse*I adapters to generate template DNA for amplification. This common adapter sequences flanking variable genomic DNA sequences serve as primer binding sites on these restriction fragments. Using this strategy, it is possible to amplify many DNA fragments without having prior sequence knowledge.

Amplification results: PCR is performed in two consecutive reactions. In the first reaction called preamplification, genomic DNA are amplified with AFLP primers each having one selective nucleotide. The PCR products of the preamplification reaction are diluted and used as a template for the selective amplification using 2 AFLP primers, each containing 3 selective nucleotides. The 5' end of the *Eco*RI selective primer is ^{32}P- or ^{33}P-labeled using T_4 Polynucleotide kinase before amplification. This two step amplification strategy results in consistently cleaner and more reproducible fingerprints with the added benefit of generating enough template DNA for thousands of AFLP reactions.

The most important factor determining the number of restriction fragments amplified in a single AFLP reaction is the number of selective nucleotides in the selective primers. Plants having genomes ranging in size from $5x10^8$ to $6x10^9$ bp, including tomato, corn, soybean, cucumber, lettuce, barley, cotton, oilseed rape, potato, sunflower, pepper and brassica. The number of fragments amplified per sample/ primer pair averages 50, but may range from as low as 10 to ~100 depending on the sequence context of the selective nucleotides, and the complexity of the genome.

A second factor in determining the number of restriction fragments is the C and G composition of the selective nucleotides. In general, the more Cs and Gs used as selective nucleotides in the amplification primers, the fewer the DNA fragments amplified. Also, the smaller the genome being analyzed, the fewer fragments and the simpler the fingerprint.

Separation of amplified fragments on denaturing polyacrylamide gels: Products from the selective amplification are separated on a 6% denaturing polyacrylamide (sequencing) gel. The resultant banding pattern obtained after autoradiography can be analyzed for polymorphisms either manually or using analytical software.

Interpretation of results: Individual band intensity, size distribution of amplified products, and overall pattern should be the same for AFLP analysis with the same primer pairs and the same DNA template, and will vary between different genomic DNA samples and different primer pairs. Fingerprints of related plants should display common bands, as well ad 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 the primer pair used. Some primer pair combinations may result in either too few or too many bands for a particular sample.

The primary reason for the superiority of AFLP technique is that it detects very large number of DNA bands enabling simultaneous identification of many polymorphic markers. Routinely about 50-100 bands are observed in each lane of a gel and this enables rapid creation of very high-density genetic maps rapidly. For instance, in genomes such as barley with large genome with low polymorphism rate, the use of AFLP

approach enabled scientists to develop a more informative and enriched genetic map (Becker *et al.* 1995). The AFLP does not necessarily offer higher rates of polymorphism but is more efficient than RFLP, RAPD or microsatellite approaches of detecting polymorphic DNA. AFLPs detect more point mutations than RFLPs, enable detection of very large number of polymorphic DNA markers than RFLP or RAPDs, and are simpler than microsatellites as no prior sequence information is needed.

The AFLP markers are dominant markers similar to RAPDs but *Keygene* scientists are developing densitometric software that may discriminate between heterozygotes and homozygotes based on allelic density. Imaging software is also being developed by *Keygene* to analyze the AFLP bands which can be difficult to be done manually. Although AFLP approach is highly informative, a few criticisms of this technique includes: the use of multiple procedures, expensive, cumbersome and laborious protocol. Although the use of radioactivity to detect DNA in AFLPs is one major drawback that may limit its use, Guohao He at the Center for Plant Biotechnology Research at Tuskegee University and Dr. Susan McCouch at Cornell University have developed non-radioactive silver staining protocols to detect AFLP markers with no major loss in sensitivity.

Advantages of AFLP

- AFLP combines the advantages of RFLP and RAPDs.
- It requires less amount of DNA and is faster than RFLP.
- It reveals several polymorphic fragments in a single reaction.
- Highly sensitive.
- Highly reproducible.
- Widely applicable.

- Discriminates heterozygote (when gel scanner is used).

Disadvantages of AFLP

- Expensive.
- Technically demanding.
- Normally uses radio-isotopes.
- Problems in interpreting banding pattern e.g. co-migration of fragments, uncertainty in assigning equivalence of bands when comparing individuals.

3.4 Applications of DNA marker technologies

It is evident that the development of DNA markers has revolutionized the construction of genetic maps in plants and the utilization of genetic maps in studies of plant evolution, systematics, and plant breeding. DNA fingerprinting can be applied in plants and fungi, especially in the fields of identification (e.g., genotypes, strains, and cultivars), paternity analysis, estimation of genetic relatedness, and genome mapping. DNA markers allow direct access to any part of a plant genome, and they liberate researchers from having to deal with plant genes through the fog of phenotype, many steps away from the gene itself. Technology for the utilization of DNA markers is evolving rapidly at the present time, and further advances are sure to occur soon. Some of these will involve making the process of developing and utilizing DNA markers technically simple, less expensive, and more capable of automation. To be practical on a large scale for plant breeding applications, and particularly in developing countries, the detection procedures for DNA markers need to be developed which do not require the use of radio-isotopes, southern blots, DNA sequencing gels and the like. PCR based methods such as RAPD analysis seem to provide part of the answer, but these procedures are still very expensive because

of high reagent costs. Simplified DNA analysis seem to be possible with PCR, and even tissue squashes may suffice for DNA isolation (Langridge *et al.* 1991).

3.4.1 Molecular markers for estimating genetic diversity

Over the years, the methods for detecting and analyzing genetic diversity have expanded from Mendelian analyses of discrete morphological and cytological variants, to statistical analyses of quantitative variation, to biochemical assays, and finally, to molecular assays. The molecular study of genetic variation has revealed a number of previously unsuspected genetic phenomena and it has raised a host of questions and applications for population genetics (reviewed by Clegg and Epperson, 1985). The primary focus will be on molecular diversity within populations or at the intraspecific level.

Molecular genetic techniques, both on their own and in combination with other biotechnological approaches, are beginning to have a significant impact on plant genetic resources conservation and use. Initially, the molecular techniques were used largely for the analysis of specific genes, for understanding gene action, gene mapping and the development of gene transfer technologies. More recently, the techniques have been applied to problems of direct relevance for understanding the distribution and extent of genetic variation within and between species.

Genetic diversity - caused by selection and various mutational and sexual events - rests on genome changes ranging from a single base-pair exchange to rearrangments of entire chromosomes. In closely related genomes, differences may occur once in every 100 bp (Soller & Beckmann 1983). These DNA polymorphisms are exploited by an ever-increasing number of molecular marker techniques for the differentiation between individuals, accessions and species of plants, pathogens and pests. Their higher resolution

compared with all other markers makes them a valuable tool for varietal and parental identification for the protection of breeders rights.

DNA markers further add to the repertoire of tools for the determination of the evolutionary relationship between plant species and families. For example, using repetitive DNA (Jung *et al.* 1993) was able to elucidate the evolutionary relationship between several species in *Beta*.

DNA fingerprinting with minisatellites (Jeffreys *et al.* 1985) or simple synthetic oligonucleotides (Tautz & Renz 1984) has also found widespread application in the differentiation of species. Even a minisatellite-like sequence present in the genome of the M13 phage has been found useful (Rogstadt *et al.* 1988; Weising & Kahl 1990). This probe was used to examine the gene flow and genetic diversity in coastal seagrass populations in California, revealing more sexual than clonal propagation in ecologically important and genetically heterogeneous species (Alberte *et al.* 1994) Using human minisatellite probes, molecular taxonomy has possible with crop species such as rice (Dallas 1988), tomato (Brown & Tanksley 1993) and grape (Thomas *et al.* 1993). Microsatellites have also been used in various genera (Weising *et al.* 1989, 1991a), including *Brassica* (Poulsen *et al.* 1994), *Beta* (Schmidt *et al.* 1993), *Cicer* (Weising *et al.* 1992; Sharma *et al.* 1995), *Musa* (Kaemmer *et al.* 1992) and *tomato* (Kaemmer *et al.* 1995).

DAF studies revealed a much higher level of diversity of Douglas fir in coastal and interior regions of Canada than observed in earlier allozyme studies (Carlson *et al.* 1994).

Molecular markers allow the relationships between chromosomes of related species to be determined. By examining the segregation of heterologous DNA markers, chromosomes of different species can be ordered into synthenic groups so that the probes derived from one organism can be used in related organisms. For example, comparative genetic mapping with RFLP markers has shown that tomato and potato are nearly identical in the order of marker loci (Bonierbale *et al.* 1988; Tanksley *et al.* 1992). Conservation of loci has also been found between maize and sorghum (Whitkus *et al.* 1992) and between rice, wheat and barley chromosomes (Ahn *et al.* 1993).

3.4.2 Marker-assisted Breeding (MAS)

The use of molecular markers enables the breeder to connect the gene action underlying a specific phenotype with the distinct regions of the genome in which the gene resides. Once markers for an interesting trait are established, these should allow the prediction of the yield or resistance of individual offspring derived from a cross, solely by the markers distribution pattern in the offspring's genome. Molecular markers then would have considerable impact on breeding economically important crops, because they provide, together with genetic engineering techniques, access to hitherto unavailable genetic resources for crop improvement programmes. Besides the exploitation of genomic polymorphisms for germplasm utilization and protection of varieties, the breeder's interest in molecular markers currently focuses on three major issues:

1. The acceleration of the introgression of single resistance genes for plant pathogens such as viruses, bacteria, fungi, nematodes or insects, from wild species or cultivated donor lines into otherwise superior cultivars.

2. The accumulation (pyramiding) of major and/or minor resistance genes into cultivars to generate multiple and more durable (horizontal) resistances against several pathotypes of the same pathogen (Serret *et al.* 1997).
3. The improvement if the agronomic value of crops by breeding for quantitatively inherited traits, such as yield, fruit solids and protein content, or drought and cold tolerance.

Molecular mapping and tagging of agronomically important genes using RFLP and RAPD markers were carried out in three different crops: rice, mustard and chickpea. In rice, tagging of genes for resistance to gall midge and blast was accomplished. Molecular mapping of cooking quality traits in rice is in progress. For fingerprinting rice cultivars, suitable probe-enzyme combinations were identified. In mustard, a partial RFLP linkage map was constructed and one of the yellow seed-coat colour loci mapped. Potential use of RAPD markers to identify heterotic groups among mustard accessions was demonstrated.

3.4.2.1 Resistance breeding

The main advantage of using molecular markers for the introgression of resistance genes to cultivars is a gain in time (Tanksley *et al.* 1989; Melchinger 1990). Gene introgression is normally conducted by crossing a resistant donor line with an agronomically superior cultivar, only retaining the desired resistance gene. The use of DNA markers could speed up this process by three plant generations, allowing selection of the resistant offspring that contain the lowest amounts of the donor genome in every generation (Tanksley *et al.* 1989).

Quantitative Trait Loci (QTLs) - a challenge for genomic analysis: Many agronomically interesting traits, such as yield or tolerance to abiotic stresses, are controlled by polygenes, with every gene contributing only a few percent to the expression of the trait. Tagging of polygenes with molecular markers requires a saturated linkage map with a marker spacing of no more than 20 cM intervals throughout the genome in order to tag any gene of interest with a selection fidelity of 99% and at least 250 F_2 individuals from a cross between parental lines that differ markedly with respect to the trait in question (Paterson *et al.* 1988; Tanksley 1993). It is unlikely that this spacing of isozyme markers throughout the genome will be achieved in many crop plants. However, this level of saturation and distribution of RFLP markers is nearly attained in crops such as maize and tomato (Helentjaris *et al.*1985, 1988; Tanksley and Hewitt 1988).

Attributes of populations amenable to molecular marker applications for quantitative traits are:

- the number of segregating marker loci available in the population or material of interest
- the distribution or uniformity of spacing of the marker loci
- the level of linkage disequilibrium in the population

If only a few marker loci are available, a population such as F_2 derived from the cross of two homozygous lines may be preferred because linkage disequilibrium is maximized in this generation. Although an F_2 is advantageous for detecting QTLs with a minimum number of markers, large genomic regions would probably be represented by specific marker loci in this generation. Thus, there is a high probability that genotypic classes at an individual marker locus may be reflecting the effects of multiple QTLs.

High-density gene maps can be used effectively to locate genes that affect quantitative variation (Michelmore and Shaw 1988; Lander and Botstein 1989). This method involves the comparison of segregating progenies with constrasting alleles at numerous loci. Where significant differences are detected between individuals differing at marker loci, conclusions can be made about the linkage between the markers and loci that influence the expression of the quantitative trait. It has some drawbacks when a single locus is used in the analyses. First the offspring are tested for the trait and their genotype determined for every marker locus. Then the likelihood that the observed data rely of the presence of a QTL is calculated, against that no QTL is present, using specially designed computer software such as *MAPMAKER* (Lander *et al.* 1987; Paterson *et al.* 1988).

3.4.3 Map-based cloning of agronomically interesting genes

Reverse genetics: The detection and cloning of distinct genes of unknown sequence and function, when only their involvement in specific traits and their chromosomal location is known, has been termed "reverse genetics". In, contrast to conventional approaches, where a gene is cloned on the basis of its known product or sequence and then localized to a chromosomal region, this strategy starts with the localization of a gene on a specific chromosomal region by determining the linkage of the phenotype it specifies to a set of flanking molecular markers. These linked markers are then used as starting points for physically mapping the gene-flanking region with pulsed field gel electrophoresis and rare cutting restriction enzymes. Large scale restriction site mapping is necessary because physical and genetic distances between markers may vary over several orders of magnitude (Sehgal *et al.* 1992). This could cause severe problems if the cloning of the

region is intended. Physical maps are especially useful in polyploid crops such as soybean, where duplicated sequences could prevent the assignment of markers to a single distinct location (Funke *et al.* 1993).

The utility of maps and molecular markers will continue to increase. The ability to rapidly construct genetic maps has made possible applications that were unimaginable using conventional mapping techniques. Comparative mapping of different crop plants (Bonierbale *et al.* 1988) will provide useful information about the location of important genes, because it is likely that there will be enough conservation of synthetic blocks so that genes located in one species will have the same flanking markers in another species. Comparative mapping of crop plants and their wild relatives will be a valuable tool for phylogenetic analysis (Jung *et al.*1993) as well as being useful in introgression studies.

3.4.4 Constructing a linkage map with DNA markers

The mapping population: The most critical decision in constructing a linkage map with DNA markers is the mapping population. F_2 populations or backcross populations derived from crosses between inbred parent lines have been used in the construction of molecular maps in plants. The use of inbred lines simplifies genetic analysis because the phase (coupling or repulsion) of the markers is completely known. F_2 populations provide more mapping information for a given number of plants when codominant markers are analyzed, since two recombinant chromosomes can be scored in each plant (Allard 1956; Tanksley *et al.* 1988b; Reiter *et al.* 1992). F_2 populations provide a sex- averaged map because chromosomes from both the male and female parent are scored. Backcross populations can provide a male or female map depending on which sex was the recurrent parent. One of the greatest advantages of molecular markers is that a virtually unlimited

number of markers can be mapped using a single segregating population. As long as the same set of F_2 or backcross plants is used, the database of mapped markers accumulates. If the mapping population is lost, the mapping information must be transferred to a new population by scoring some of the same markers that have been previously mapped. Thus the database of mapped markers for a given segregating population becomes a valuable resource for mapping of new markers and the need for construction of permanent mapping populations.

Backcross and F_2 populations constructed from inbred lines are segregating populations but are not a permanent resource in most plants. However, perennial plants or plants which can be reproduced asexually, such as alfaalfa (Brummer *et al.* 1991) or a rice population derived from an interspecific cross, where one of the parents is perennial, constitute a permanent mapping population.

For markers, which are not inherited in a dominant-recessive fashion, such as RAPD markers, recombinant inbred lines provide as much efficiency in mapping as do segregating populations. With F_2 mapping populations, the results of meiosis in one generation are being scored, but recombinant inbred lines are the results of a series of meiosis, which give more opportunities for recombination. RIL can be developed quickly in self-pollinating crops like chickpea by following the single-seed descent method from an F_2 of a hybrid population to the F_6 or F_7. At the later generation, the RILs become homozygous and fixed for linkage blocks within the genome. Each RIL is then fully characterized for molecular markers and traits of interest. Thus a map derived from recombinant inbreds will have higher resolution than one derived from an F2 or backcross population (Burr *et al.* 1988).

Design of Test Populations: An appropriate design of a test population is a crucial step in the development of markers for agronomical traits. Crosses of wild species with cultivated lines have generally been found useful for the generation of genetic maps, because of the relatively high degree of morphological, isozyme and DNA polymorphisms in the wild species. Such crosses are essential in crops, such as tomato, where relatively few polymorphisms are detected within cultivated lines. For example, crosses between cultivated tomato (*Lycopersicon esculentum*) and its wild relatives *L. penneli* (Wing *et al.* 1994) or *L. pimpinellifolium* (Sarfatti *et al.* 1989) have helped in generation of linkage maps and the identification of an RFLP marker linked to *Fusarium oxysporum* resistance gene.

Theoretically, a large F_2 generation is most informative for genome mapping (Melchinger 1990), especially if the map is at an early stage and only a few markers are mapped. However, an F_2 has three major drawbacks as far as the development of markers for agronomically interesting traits is concerned :

1. Same individuals tested for the trait also have to be used for linkage analysis. This can cause serious problems, since after extensive testing, for instance with a pathogenic fungus, some of the plants will be too affected to provide enough DNA for linkage analysis.
2. After completion of their life cycle, plants die and (especially the pheno- and geno-typically characterized individuals) will no longer be available for backcrosses or further genetic analysis.
3. Most multilocus markers, including RAPD and mini and microsatellites are dominant markers, whose homo- or hetero- zygous state cannot be determined. An F2 does not

allow these two possibilities to be distinguished and much information is therefore lost.

3.4.5 Establishment of a high-density linkage map

Plant breeders now have access to computerized data bases (genetic linkage maps, gene product and function data, performance data etc.) for conducting simulated matings of various potential parents and only make those that give the maximum expression of transgressive segregation, heterosis, epistasis, and pleiotropy.

Steps involved in establishing a high-density gene map for any crop would be as follows:

1. Crosses between lines that differ for qualitative traits, isozyme markers and RFLP.
2. Genetic analyses of patterns of inheritance and gene interaction of the progenies. This is usually done in the F_2 but recently the use of recombinant inbreds has received greater attention.
3. Detection of abnormal joint segregation ratios among genes and calculation of linkage estimates.
4. Placement of linked segments into a linear arrangement corresponding to chromosomes or at least to linkage groups that might later be assigned to specific chromosomes.

When a genome map is completed for a given crop it should be possible to determine:

1. The number of loci involved in the control of important agronomic traits and design more efficient breeding strategies in terms of procedures, population sizes, selection intensities, etc., to obtain maximum genetic gain.
2. Major loci that interact together in a synergistic fashion and use those combinations to assemble, or avoid, gene combinations in developing improved varieties.

3.4.6 Advantages and Limitations of Linkage maps

Linkage maps based on hybridization exhibit advantages as well as limitations as compared to maps derived from other types of markers. Advantages include the high level of polymorphism detected, the rapid screening of the genome with few probes, the availability of universally applicable probes without cloning, and the higher reproducibility as compared to RAPDs. However, this approach may be limited by extensive clustering of simple repeats or by high mutation rates leading to unexpected fragments in the progeny (Jeffreys *et al.* 1988). Inclusion of parental and F_1 DNA in the segregation analysis, and the preferred use of accession rather than (presumably less stable) individual-specific markers will help to minimize this problem. A disadvantage shared with RAPDs is that the allelic state of a fingerprint band is usually unknown, and its occurrence has to be treated as a dominant rather than a codominant marker.

3.4.7 Other Uses of high density linkage maps

Conservation of linkage groups between closely related genera appears to be quite common and has been observed in the *Gramineae* (maize and sorghum), *Solanaceae* (potato, tomato, and pepper), and *Brassica* (cabbage, turnip and rape) (Tanksley *et al.* 1989). Similar conservation of linkage groups have been observed in *Lens* and *Pisum* of the *Viceae* (Weeden *et al.* 1988) and in *Cicer* (Muehlbauer and Weeden 1989).

Conservation of linkage groups between closely related genera facilitates mapping efforts and may indicate potential sites for important genes. Also, substitutions of entire chromosomes from one genus to another have been suggested as a possibility.

Gene cloning for the eventual development of transgenic plants is at present a remote possibility for *Cicer* and many other crops. Prospects for transgenic plants depend

on determinations of the gene products to enable cloning of the gene, and upon the presence of systems to introduce the foreign gene. Identification of closely linked RFLP markers may permit cloning; however, even small map distances translate into great distances at the DNA level.

3.5 Status of molecular marker technology in chickpea research

The ease with which a genetic map can be developed and applied to a target crop species depends on the genetic complexity of the species and the extent of DNA polymorphism present in the species. Some plant species, such as maize (Smith 1988), potato (Gebhardt *et al.*1989) and *Brassicas* (Figdore *et al.* 1988) exhibit a high degree of DNA polymorphism even within commercially exploitable germplasm pools of maize (Lee *et al*, 1989; Dudley *et al.* 1991; Messmer *et al.* 1991; Melchinger 1991). However, other plants such as soybean (Apuya *et al.* 1988); Keim *et al.* 1989) and tomato (Miller and Tanksley 1990) and hexaploid wheat exhibit relatively low frequencies of RFLPs. Thus, the first step in developing genetic maps with DNA markers has been to examine the frequency of DNA polymorphism within species to identify suitable parents showing sufficient DNA polymorphisms for efficient mapping. Genetic mapping in generally monomorphic species like sorghum, groundnut etc has usually been achieved by using wide crosses between highly divergent parental genotypes, sometimes even using different species (Paterson *et al.* 1991). The low frequency of DNA polymorphism within a species can also limit the utilization of mapped DNA markers in crosses that are of agronomic importance, but involves more genetically monomorphic parents. Recently, SSR marker technology has been developed and used for genome mapping and DNA fingerprinting in different plant species, such as rice (Wu and Tanksley 1993; Yang *et al.*

1994 Chen *et al.* 1997), wheat (Roder *et al.* 1995), barley (Saghai Maroof *et al.* 1994), maize (Senior *et al.* 1993) Soybean (Cregan *et al.* 1994; Morgante *et al.* 1994 Akkaya *et al.* 1995) and tomato (Broun and Tanksley 1996). For these reasons, it is important to establish the frequency of DNA polymorphism within a species before engaging in a plant improvement program using molecular markers.

The application of molecular markers helps in breeding, particularly for traits in which screening is difficult. At present, there is no published genetic map of chickpea incorporating DNA markers, but mapping projects are underway. The future success of using mapped RFLP and RAPD markers in breeding programs will greatly depend on the degree of genetic variation in the germplasm under investigation.

3.5.1 Mapping in chickpea

The map of a crop species can greatly increase the efficiency of genetic and breeding studies. A gene map is needed to accelerate the crop improvement processes in chickpea (Muehlbauer,1989). Genetic studies undertaken so far in chickpea focussed on morphological traits such as plant habit, leaf form and colour, flower colour, podding habit and seed coat colour which resulted in the establishment of a few linkages between these traits as reported by Muehlbauer and Singh 1987. They are traditionally used in taxonomy, genetics, and breeding. Since most of these traits are recessive with detrimental effect (pleiotropic), these are not suitable as selectable genetic markers in breeding programme (Gaur and Slinkard 1990). Despite the wide variability for morphological traits in C. *arietinum*, their less number and their expression is age and environment dependent.

Seed storage proteins were the first candidates as molecular markers to distinguish the germplasm lines, due to their ease of isolation and identification on gels. Next came the isozymes which are still popular in some cases such as for study of diversity, or as a marker during introgression of alien germplasm. Studies on inheritance and linkage of isozyme genes in C. *arietinum* and two closely related wild species, C. *reticulatum* Lad. the proposed progenitor (Ladizinsky and Adler 1976), and C. *echinospermum* Davis were carried out.(Gaur and Slinkard 1990b). Studies on inheritance and linkage of isozyme genes in C. *arietinum* and two closely related wild species, C. *reticulatum* Lad. the proposed progenitor (Ladizinsky and Adler 1976), and C. *echinospermum* Davis were carried out.(Gaur and Slinkard 1990b). However, this map is still in preliminary stage, mainly due to the low level of polymorphism displayed by isozymes and RFLPs at molecular level (Van Rheenen, 1992). DNA amplification based markers, RAPDs (Williams *et al.* 1990) or AP-PCR (Welsh *et al.*1990) or DAF (Caetano-Anolles *et al.* 1991) have also allowed construction of high-density genetic maps (Reiter *et al.* 1992) and saturation of already existing genetic maps in a few plant species (Sharma *et al.* 1994). In chickpea, the occurrence of considerable interspecific DNA polymorphism as revealed by RAPD analysis has facilitated construction of a partial linkage map. The genetic variability in agronomically important chickpea accessions (*Cicer arietinum L.)* as detected by single-locus RFLP probes, RAPD and isozyme markers, was found to be rather low. Lack of adequate polymorphism in cultivated chickpea was observed even with powerful techniques like AFLP. RAMPO analysis has also been carried out in chickpea genotypes. (Banerjee *et al.*1997).

Now onwards one can expect microsatellite markers to take over as the major type of DNA marker for mapping, and fingerprinting since they are PCR-based, highly polymorphic, and co-dominant. In chickpea genome, microsatellites are reported to be highly polymorphic (Weissing *et al.* 1992) and could be used effectively for linkage map construction.Those detected by in-gel hybridization with simple repetitive oligonucleotide probes such as $(GATA)_4$ probably have repeat unit numbers much greater than 20. They are present in at least 200 loci in the chickpea genome (Sharma *et al.* 1995). A comparable number of minisatellites occurs in the tomato genome and some of the most polymorphic loci cloned in the tomato contain microsatellites (Brown & Tanksley 1993).

After a long history of search for suitable markers for genome mapping in chickpea and its pathogen *Ascochyta rabiei,* STMS were identified as the only type of markers that would not only allow tagging of specific genes in test crosses but could also to be applied in routine breeding (Udupa *et al.* 1997). These markers are robust, highly informative, PCR-based, can distinguish heterozygotes from homozygotes, and can be used for automation. These types of markers are becoming increasingly popular in plant genome analysis and map development, and gradually regarded as the standards for applications in marker- assisted breeding. The markers will also be very useful for genetic diversity studies (Ayad *et al.* 1997). They form the backbone of the most advanced available genetic map of chickpea. Further, STMS may be used to exploit the until now inaccessible gene pool of chickpeas wild relatives. The generation of these markers by Winter *et al.* (under publication), and their application resulted in the first

integrated molecular marker map of the chickpea genome, which will soon be ready for practical applications.

Recently, highly polymorphic microsatellites became the markers of choice for linkage mapping and population studies. They are currently following two main strategies to exploit the variability of microsatellites and adjacent sequences for genetic studies in chickpea.

1.) In an approach referred to as oligonucleotide fingerprinting, microsatellite-complementary oligonucleotides were employed as multilocus probes for in-gel hybridization. A total of 38 different probes representing di-, tri- and tetranucleotide repeats were used to analyze variability between and within four accessions of C. *arietinum*. Hybridization signals were obtained with 35 probes. While the abundance and level of polymorphism of different target sequences varied considerably, distinct, intraspecifically informative banding patterns were obtained with the majority of probes and all restriction enzymes tested. No obvious correlation existed between abundance, fingerprint quality, and sequence characteristics of a particular motif.

2.) In a recently developed strategy called microsatellite-primed polymerase chain reaction (MP-PCR), microsatellite-complementary oligonucleotides serve as single PCR primers for genomic DNA templates. They tested the general applicability of MP-PCR by amplifying DNA samples from tomato, chickpea and two related annual *Cicer* species with a variety of di-, tri- and tetranucleotide repeat primers. Most but not all primers generated distinct fingerprint-like banding patterns after agarose gel electrophoresis and ethidium bromide staining of the amplification products. Since the method proved to be sensitive to reaction conditions in a way similar to RAPD analysis,

they increased the PCR specificity by the introduction of a modified "touch-down" protocol. In chickpea, touchdown MP-PCR generated highly reproducible banding patterns which predominantly revealed interspecific polymorphisms.

At present, there is very little information available on genetic diversity within *Cicer arietinum* and no comprehensive survey has been reported. However, in recent years, following the introduction of molecular markers in plant genetic research, considerable effort has been made to gain a better understanding of chickpea genetics and evolution, and important data have been gathered. Annual growth habit, diploid chromosome complement, and the relatively small chromosome number of 2n=16 make chickpea a relatively simple genetic system that can be studied using molecular markers and classical genetic principles. An additional advantage that should and will be exploited for chickpea is the apparent conservation of certain linkage groups between the *Cicereae* and *Vicieae* tribes.

Conserved linkage groups between *Pisum,* an extensively mapped genus, and *Lens*, are currently being used effectively to extend the lentil gene map. Similarly conserved segments of the genome have been discovered between *Lens* and *Cicer.*

3.5.2 Future prospects for chickpea improvement

Many laboratories have now begun investigations into the genomic organization of *Cicer arietinum* and related species. Much of the work has been encouraged, supported, and coordinated by ICRISAT, ICARDA in Syria and the USA. A concerted effort is currently underway to map the *Cicer* genome at Saskatoon, Canada and at Pullman, Washington USA using both conventional markers, isozyme loci and RFLP. Other programs involved in chickpea RFLP mapping and other diversity analysis are also participating in this

informal network. Interactions between developed and developing country programs have been encouraged and initiated. This group plans to select a common set of well spaced DNA probes to be mapped in all programs so that integration of separate maps will be facilitated.

The beginning of linkage groups have emerged and additional loci are currently been added. Analysis of the inheritance of *Ascochyta* blight resistance, tagging of important genes, identification of quantitative loci, and marker-assisted introgression between desi and kabuli types and wild species to the cultigen are considered to be the primary benefits to be derived from this mapping effort. These areas could eventually represent breakthroughs for chickpea crop productivity. Greatly improved genetic maps, particularly those derived from RFLP and RAPD programs, can contribute immensely to future chickpea improvement by plant breeders. These investigations, based on microsatellite obtained through both database searches and random screening of genomic libraries, have demonstrated that the high level of polymorphism intrinsic to this marker system may improve the genetic analysis of plant species with medium or low genetic variability. Furthermore, the ease and speed of genetic analysis based on SSRs enhance the ability to make a greater number of SSRs available to the scientific community, at least for most of the species of social and economical value, such as sorghum, for which SSRs are not yet available. Due to the initial high cost and time required, the production of a suitable number of SSRs in chickpea, as well as in other species, can only be obtained through the effort of several laboratories.

From the review of literature, it is evident that mapping of genomes is very advantageous and provides us information about the various genes which are associated

with traits of agronomic and economic importance. In view, of the advantages conferred by plant genome maps based on molecular markers, the objective of this present study was framed to familiarize myself with the various molecular marker technologies, especially with reference to chickpea.

4. MATERIAL AND METHODS

4.1 Plant material

The genotypes used for the study are listed in **Table 1.** Their description is given in **Table 2.**

S.No.	Genotype	Variety	Type	Scientific name
1.	ICCV 2	Kabuli	Cultivar	*Cicer arietinum* L.
2.	JG 62	Desi	Cultivar	*Cicer arietinum* L.
3.	ICCV 88202	Desi	Cultivar	*Cicer arietinum* L.
4.	ICCV 92504	Desi	Cultivar	*Cicer arietinum* L.
5.	Pant G 114	Desi	Cultivar	*Cicer arietinum* L.
6.	GL 769	Desi	Cultivar	*Cicer arietinum* L.
7.	ICCW 49	Desi	Wild	*Cicer reticulatum* L.
8.	Annigeri	Desi	Cultivar	*Cicer arietinum* L.
9.	ICCW 6	Desi	Wild	*Cicer reticulatum* L.

Table 2. Description of the nine chickpea genotypes.

S.No	Genotype	Comments
1.	ICCV 2	High yielding, short duration, susceptible to collar rot, early maturity, resistant to *Fusarium* wilt, single-poded, small-seeded, white-coloured seed.
2.	JG 62	High yielding, medium duration, resistant to collar rot, medium maturity, susceptible to *Fusarium* wilt, double-poded, brown-coloured seed.
3.	ICCV 88202	High yielding, short duration, tolerant to *Fusarium* wilt, cold susceptible & early line used in breeding & in development of RILs.
4.	ICCV 92504	High yielding, short duration, tolerant to *Fusarium* wilt, cold tolerant line used in breeding & in development of RILs.
5.	Pant G 114	High yielding, long duration, tolerant to *Fusarium* wilt, cold tolerant line used in breeding & in development of RILs.
6.	GL 769	High yielding, long duration, resistant to *Fusarium* wilt, cold-susceptible
7.	ICCW 49	Low yielding, Short duration, resistant to *Fusarium* wilt, cyst nematode, leaf miner, Ascochyta blight, and Botrytis gray mold, cold tolerant.
8.	Annigeri	High yielding, short duration, resistant to *Fusarium* wilt, cold- susceptible, twin-poded.
9.	ICCW 6	Low yielding, short duration, resistant to *Fusarium* wilt, cyst nematode, leaf miner, *Ascochyta* blight, and *Botrytis* gray mold, cold tolerant.

Two sets of populations were also used for studying DNA polymorphism

1.GL 769 x ICCW 49 F_2 generation No of progeny 1-18.

2.Annigeri x ICCW 6 F_2 generation No of progeny 1-68.

4.2 DNA isolation and purification

Step 1: Extraction of genomic DNA

Genomic DNA was extracted from the young leaves of greenhouse and field-grown chickpea plants listed in the Table 1. The CTAB method of DNA extraction was followed (Saghai-Maroof *et al.* 1984). Fresh young leaves (5.5 - 6.0 g) were harvested, lyophilized in liquid nitrogen and stored at -70^{0}C. The leaves were pulverized to fine powder in a mortar and pestle, and 10 ml of freshly prepared CTAB buffer (1.0 M Tris, pH 8.0, 5.0 M EDTA, 2% β-mercaptoethanol, 2% CTAB) at 65^{0}C was added to freeze-dried, ground tissue in a 30 ml Falcon tube, mixed well on a rotating shaker and incubated for 2 hours at 65^0 C with occasional mixing The tubes were taken out from water bath, cooled to room temperature, and 10 ml chloroform-isoamyl alcohol (24:1) was added and mixed gently by inverting for 5-6 times and centrifuged using swing bucket rotors at 6000 rpm for 20 min at room temperature. The aqueous phase was transferred to a new 30 ml tube to which 10 ml chloroform-isoamyl alcohol was added and mixed gently 5-6 times. Next, the extract was centrifuged at 6000 rpm for 20 min at 2^{0}C and the aqueous phase was transferred to 30 ml corex tube. After chilling, 10 ml of isopropanol was added to the aqueous extract, and mixed gently by inversion for several times, and kept at -20^{0}C for 15-20 minutes. DNA was spooled with glass hook. The spooled DNA was washed in a 5 ml corex tube containing 2 ml of 76% ethanol, 0.2 M NaOAc (Washing Buffer I) followed by a 100% ethanol wash. The tubes were inverted on a paper-towel and allowed for all ethanol to evaporate. The DNA was treated with 2 ml of $T_{50}E_{10}$ containing RNase (0.2 μg/ml) and incubated at 37^{0}C for one hour.

Step 2: Purification of genomic DNA

After the RNase treatment, 200 µl of 5M NaCl was added, shaked gently and incubated at 4^0C for 15-20 minutes. The tubes were next centrifuged at 6000 rpm at 2^0C for 20 minutes. The aqueous phase was transferred to a 5 ml corex tubes. Next, 2 ml of phenol:chloroform (1:1) was added and centrifuged at 2500 rpm for 10 minutes at 2^0C. Equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous phase and centrifuged at 2500 rpm for 10 minutes at 2^0C. For precipitation of DNA, 200 µl of 2.5 M sodium acetate pH 5.2 was added to the aqueous phase, mixed well and 2 ml of absolute alcohol was added and again mixed well, incubated at -20^0C for 15-20 minutes. The precipitated DNA was spooled with a glass hook into a 1.5 ml eppendorf tube and washed with 76% and then 100% alcohol. $T_{10}E_1$buffer (10mMTris.Cl, 1mM EDTA pH 8.0; 300 µl) was added to dissolve the pellet and stored at 4^0C until further use.

Step 3: Quantification of DNA

The quantity and purity of the DNA samples were determined spectrophotometrically by measuring the absorbance at 260 and 280 nm with a *SHIMADZU* UV 160A spectrophotometer. The ratio of OD_{260} to OD_{280} provides some information about the purity of the DNA samples. DNA was quantified considering that 1.0 OD unit at 260 nm is equivalent to 50 µg of DNA per ml (Sambrook *et al.* 1989). A major disadvantage of this method of DNA estimation is that is that RNA, oligonucleotides, proteins and other contaminants interfere with the measurement.

Step 4: Ethidium Bromide Staining for DNA Quantification

An aliquot of genomic DNA was run on 0.8% ethidium bromide stained agarose gel. The dye intercalates into the DNA double helix, and the intensity of florescence induced by

UV light is proportional to the amount of DNA in the lane. Comparison with a standard λ Hind III digest of λ marker DNA, gives an estimate of the amount of DNA in the samples.

This technique also allows (1) DNA quantitation

(2) Estimation of the extent of contamination by RNA (which usually runs ahead)

(3) Evaluation of DNA quality (the extent of degradation).

4.3 RFLP analysis

Step 1: Restriction digestion of genomic DNA

Genomic DNA was digested with two restriction enzymes, *Eco R 1* and *Hind III.*

ECOR1 Restriction Sites Sequence: 5' G AATTC 3'
3' CTTAA G 5'

HIND III Restriction Sites Sequence: 5' A AGCTT 3'
3' TTCGA A 5'

Protocol for Restriction digestion:

Concentration of genomic DNA: 10-15 μg

Reagents for 10 reactions:	
Restriction buffer	50 μl
Restriction enzyme (20 units/μl)	30 μl
SDW	20 μl
Total reaction volume	100 μl

Master-mix was made and 10 μl was dispensed into 9 reaction tubes 10μl each, and template DNA added such that the final concentration of DNA was 10-15 μg. Reaction tubes were briefly centrifuged and incubated at 37^0C for overnight for complete

digestion of chickpea genomic DNA. The samples were run on the 0.8% agarose gel at 40 V. After the run, the gel was stained with ethidium bromide and then de-stained in distilled water for about 30 min. The gel was then transferred on to the vacuum blot apparatus to transfer the DNA fragments to the nylon membrane. This was performed in two stages:

Step 2: Southern Blotting

The nylon membrane was cut according to the gel size (Amersham Hybond N) and it was marked. The gel was carefully transferred on to the membrane and the depurination using 0.25 M HCl, denaturation using 0.4 N NaOH and neutralization reactions were carried out for 20 min each. The transfer was done in 20x SSC solution for one hour.

After the transfer, the DNA was cross-linked to the nylon membrane, followed by baking at 80^0C for one hour. The blot was wrapped in *Saran wrap*, preserved at 4^0C. The preserved blots were ready for the hybridization.

4.3.1 Labeling of probe

Random-primer labeling of inserts was performed as described by Feinberg and Vogelstein (1983). A total of 20 ng purified insert DNA was used as the probe. It was denatured for 5 min in a boiling water bath, flash-cooled, and final volume was made upto 50 µl as shown below and incubated at 37^0C for 3 hr. The reaction was stopped using 0.5 M EDTA and the sample was diluted to 200 µl with distilled water. The unincorporated radioactive material was removed by using spin columns packed with Sephadex G-50. The radioactivity of 2 µl aliquot was monitored before and after purification to calculate the percent of incorporation of the label.

Chickpea clone 33-1 was labeled with (α-^{32} P) dCTP in the following way:

DNA	5 µl
Buffer	5 µl
dNTP without dCTP	6 µl
dCTP (α-^{32}P)	5 µl
Klenow	2 µl
SDW	27µl
Total volume	50 µl

The samples were incubated at 37^0 C for 1 hour before stopping the reaction by adding 200mM EDTA (amount of EDTA to be added was adjusted according to the reaction volume). The samples were denatured at 95^0 C for 5 min and immediately transferred to ice and kept for 5 min; after 5 min, λ DNA marker cut with Hind III was added to the probe.

4.3.2 Visualization of DNA band of interest using the radioactive probe

Step 3: Prehybridization and hybridization

Prehybridization was carried out in boxes containing prehybridization solution (200 ml 20x SSPE, 5 gm SDS, 50 ml 100x Denhart's reagent, 20 ml salmon sperm DNA (10 mg/ml), distilled water to make volume 1 litre at 65^0C for 4 hr. The labeled probe was denatured for 5 min in boiling water bath, flash-cooled and added to the same prehybridization solution. The hybridization was carried out in hybridization oven for 16 hr at 65^0C with constant agitation (rotation).

20x SSPE (1litre)		
NaCl	3.6 M	210 g
$Na_2HPO_4 7H2O$	0.2 M	53.6 g
EDTA	0.02M	7.44 g

100x Denhart's reagent (1litre)	
Ficoll 400	20 g
Polyvinylpyrolidone(PVP)	20 g
BSA fraction V	20 g

Step 4: Posthybridization processing of Southern blots

After 16 hr hybridization the excess probe was removed by washing the blots in solution 1(2x SSC, 0.5% SDS) for 15 min and in solution II (0.1X SSC, 0.1% SDS) for 15 min at 65^0C with constant agitation. The blots were wrapped in *Saran wrap* and exposed to X-ray film (*Kodak*) at -80^0C for 4-5 days, before developing the film

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4.4 RAPD analysis

Table 3. Sequence of *Operòn* primers used for RAPD analysis.

Primer ID	Sequence (5'-3')
A-01	CAG GCC CTT C
A-02	TGC CGA GCT G
A-03	TGC CGA GCT G
A-04	AAT CGG GCT G
A-05	AGG GGT CTT G
A-06	GGT CCC TGA C
A-07	GAA ACG GGTG
A-09	GGG TAA CGC C
A-10	GTG ATC GCA G
A-11	CAA TCG CCG T
A-12	TCG GCG ATA G
A-13	CAG CAC CCA C
A-14	TCT GTG CTG G
A-15	TTC CGA ACC C
A-16	AGC CAG CGA A
A-17	GAC CGC TTG T
A-18	AGG TGA CCG T
A-19	CAA ACG TCG G
A-20	GTT GCG ATC C

RAPD-PCR was performed according to the protocols of Williams *et al* (1990). Random 10-mer primers employed in this study were purchased from *Operon Technologies*, USA.

Step 1: PCR amplification

PCR reaction was performed under a laminar hood with 25 µl of a total reaction mixture containing 25 ng of genomic DNA, 2.5 µl 10x PCR Buffer, 1.5 µl 25 mM $MgCl_2$, 1µl 2.5 mM dNTP, 1 µl 10 µM RAPD primer and 0.4 µl *Taq* polymerase (*Gibco BRL* 5 U/µl) volume was made upto 25 µl with sterile distilled water. A control without template DNA was included in each set of reactions with a single primer. The amplification reaction was performed in a *Perkin Elmer GeneAmp 9600* thermal cycler programmed for 40 cycles with the following tempreature profile:

First cycle:	Denaturation at 94^0C for 2 min.
	Primer annealing at 40^0C for 1 min.
	Primer extension at 72^0C for 2 min.
Next 38 cycles:	Denaturation at 94^0C for 1 min.
	Primer annealing at 40^0C for 1min.
	Primer extension at 72^0C for 2 min
Last cycle:	Denaturation at 94^0C for 1 min.
	Primer annealing at 40^0C for 1 min.
	Primer extension at 72^0C for 5 min.

Step 2: Electrophoresis

The amplified DNA fragments were mixed with 2 µl of 6x loading dye (25 mg Xylene cyanol and 1.5 g Ficoll type 400 for 10 ml). PCR products were electrophoresed on 1.5% Agarose (*Sigma*) gels at a voltage of 25 V overnight. The gels were stained with ethidium bromide (5 mg/ml) and photographed under UV illumination.

Step 3: Scoring of gels

The presence of a DNA band was scored as 1 and absence as 0. The polymorphism in an accession was detected as presence of a band which is not shared with a different accession.

Step 4: Cluster analysis

Similarity index matrices were generated based on the proportion of common restriction digestion fragments between two genotypes (Nei 1987) using

$$F = \frac{2\,M_{xy}}{M_x + M_y}$$

Where 'F' is the similarity index, M_x is the number of bands in genotype x, M_y is the number of bands in accession y, and $2M_{xy}$ is the number of bands common to both x and y. Cluster analysis of data for nine chickpea genotypes was carried out using the statistical software package GENSTAT.

4.5 RAMPO ANALYSIS

The RAPD amplified DNA fragments using primers A-11, A-12, A-13, A-14 were blotted onto Hybond N^+ nylon membrane and preserved at 4^0C. The gel was washed with distilled water and the electrophoretically separated DNA samples was depurinated by rinsing the gel in 0.25 N HCl for 15 min followed by denaturation in 0.4N NaCl for 45 min. The DNA was transferred on to nylon membrane (*Zeta-Probe GT Blotting Membrane, BioRad*) at 5 inches Hg Vacuum for 2 h using 20x SSC (3M sodium chloride, 0.3 M trisodium citrate) as transfer buffer. After completion of transfer, the blots were rinsed in 3x SSC and UV crosslinked using UV crosslinker (*Stratagene*).

4.6 DNA Amplification Fingerprinting (DAF)

The protocol of Caetano-Anolles *et al* (1990) was followed. RAPD primer A-06 5'-GGT CCC TGA C-3'that showed single band in RAPD analysis was selected for DAF analysis.

Step 1: Primer labeling

This was performed by phosphorylating the 5' end of the RAPD primers with [γ^{32}P]ATP and T_4 polynucleotide kinase. *T_4 PNK dilution:* T_4 PNK was diluted in 1:10 ratio in dilution buffer supplied in the USB kit, i.e. 0.5 µl enzyme mixed with 4.5 µl dilution buffer.

Labeling of primer OPA-6 (concentration of each primer -15 ng/µl) 3 µl of 2.5mM RAPD primer, 1 µl 10x T4 PNK buffer, 1 µl diluted T_4 PNK (1.5units), 4 µl distilled water, 1 µl γ -P^{32}ATP (10 µCi) were mixed to make final volume to 10µl. The above reaction mixture was incubated at 37^0C for 1 hour and the enzyme T_4 PNK was denatured by heating at 70^0C for 10 minutes, and the reaction mixture was chilled quickly on ice and frozen till further use.

Step 2: PCR amplification

A set of 5 master mixtures (6.7 µl) were prepared for each primer, Sufficient for all samples plus one negative control to which water was added instead of DNA. Master mix containing 2.5µl 10 x PCR buffer, 2µl 25 mM $MgCl_2$, 1ul dNTP, 1 unit of *Taq* polymerase (*Gibco BRL*) and 1µl each of γ-^{32}P ATP labeled A-06 was prepared. Different volumes of unlabelled primers 2, 4, 6, 8 and 10 µl were added to the above 5 master mixtures in sequence and the final volume was made upto 23 µl with double distilled water and 2 µl of DNA (5 ng) was added. PCR was performed in *Perkin Elmer 9600* thermocycler programmed for 40 cycles with the following same RAPD temperature profile.After amplification, vials were stored at –20°C.

Step 3: Electrophoresis

The amplified samples (25 µl) were mixed with an equal volume of formamide dye (98% deionised formamide, 10 mM EDTA pH 8.0; 0.025% bromophenol blue and 0.025 % xylene cyanol as tracking dyes). The resulting mixtures were heated for 5 minutes at 90°C and then quickly cooled on ice. Each sample (6 µl) was loaded on a 6% denaturing sequencing polyacrylamide gel (19:1 acrylamide: bis; 7.5M urea, 10x TBE buffer).

6% Polyacrylamide gel composition: 15 ml of 40% acrylamide; 10 ml10x TBE and45 g urea and the final volume was made upto 100 ml with distilled water.Electrophoresis was performed at constant power of 1500 volts for three hours. and the gels were dried and exposed to X-ray film.

Step 4: Scoring of gels

The presence of a DNA band was scored as 1 and absence as 0. The polymorphism in an accession was detected as, presence of a band which is shared with a different accession analyzed.

4.7 MICROSATELLITES

Table 4. List of primers used for SSRs:

Ta5	Not shared by University of Frankfurt
Tr19	Not shared by University of Frankfurt
Tr23	Not shared by University of Frankfurt
Tr26	F: AACAACTTCCTCTTATTTTCCA R: CAGTAAAAATCAGCCCAAAC
Tr29	F: GCCCACTGAAAAATAAAAAG R: ATTTGAACCTCAAGTTCTCG
Ta53	Not shared by University of Frankfurt
Tr56	F: TTGATTCTCTCACGTGTAATTC R: ATTTTGATTACCGTTGTGGT
Ta72	Not shared by University of Frankfurt

Step 1: PCR amplification

The total reaction mixture of 20 µl consisting of 25 ng of genomic DNA, primer 6 µl (primer concentration is 15 pmol/µl obtained from Germany, dNTP mix (2.5 mM each) 2µl, 10 x PCR buffer 2µl, 25mM $MgCl_2$ 1.2µl, Taq polymerase (5U/µl) 0.2 µl and the final volume was made upto 20 µl with double distilled water.

All PCR reactions were performed in a *Perkin Elmer 9600* thermocycler.

Program: Each pair of primers were initially screened for amplification of a specific product from chickpea genomic DNA using the following programs:

First cycle: 96^0C 2 min

35 cycles : 96^0C 20 sec
55^0C 50 sec
60^0C 50 sec
followed by cooling at 4^0C.

Step2: Electrophoresis procedure

After amplification, 5 µl of 6x Loading buffer (0.25% xylene cyanol and 15% Ficoll 400) was added to each sample. The samples were loaded on ethidium bromide stained 2% Nusieve agarose gels and run at constant voltage of 40 V for 4 hours and photographed under UV illumination.

Step 3: Scoring of gels

Polymorphism was recorded by scoring the presence or absence of a particular mobility. Segregation in the mapping population was recorded by scoring the presence or absence of the band that correspond to either of the parents.

Step 4: Cluster analysis

Similarity index matrices were generated based on the proportion of common restriction digestion fragments between two genotypes (Nei 1987) using

$$F = \frac{2 M_{xy}}{M_x + M_y}$$

Where 'F' is similarity index, M_x is the number of bands in genotype x, M_y is the number of bands in accession y, and M_{xy} is the number of bands common to both x and y. Cluster analysis of SSR data for 9 chickpea genotypes was carried out using the statistical software package GENSTAT.

5. RESULTS AND DISCUSSION

5.1 Detection of genetic variability in chickpea using RAPDs

RAPD analysis of seven cultivars of *Cicer arietinum* L. and two wild species of *Cicer reticulatum* L. (ICCW 49 and ICCW 6) has revealed a total of 103 DNA bands amplified with 19 primers tested, out of these 76 were polymorphic.

Of the 19 primers,13 which showed polymorphism are A-04, A-07, A-09, A-10, A-12, A-13, A-14, A-15, A-16, A-17,A-18, A-19 and A-20. Among these primers, polymorphism was best revealed with primers A-04, A-07, A-12, A-13 and A-14. The fragments obtained were in the size range of 300 bp to 3000 bp. On an average about 5-6 fragments were obtained for each primer. Two primers A-02 and A-06 produced single high intensity bands and no polymorphism was observed. A-02 produced thick and A-06 produced thin single band. Of the 19 primers tested, primers (A-01, A-02, A-06, A-09, A-11 and A-19) did not produce any polymorphism but showed amplification (Fig.1-5). Primer A-05 produced no amplification.

RAPD analysis of chickpea progeny from the cross GL 769 and ICCW 49 was done using primers A-04, A-07 and A-14. Of the three, A-07 and A-14 were better than A-04 as these revealed more polymorphic bands compared to those in A-04. (Fig. 6).

Primer A-04 was used to screen whole progeny from the cross Annigeri and ICCW 6 that showed most bands in progeny were inherited from Annigeri.

A dendrogram based on degree of similarity, from the RAPDs data placed the genotypes into 2 distinct groups. The genotypes ICCV2, GL 769, Pant G 114, Annigeri,

ICCV 88202, ICCV 92504, and JG 62 formed a separate group which can be further divided into four sub-groups. The sub-group I ICCV 88202, ICCV 92504 and JG 62; sub-group II Annigeri, sub-group III GL 769 and Pant G114 and ICCV2 in sub-group 4. The wild species ICCW6 and ICCW 49 formed a separate distinct group indicating the diversity when compared with other groups (Fig.12).

Similarity matrix based on RAPD analysis of 9 chickpea genotypes using single linkage cluster analysis.

**** SIMILARITY MATRIX *****

1	----								
2	69.9	----							
3	69.9	80.8	----						
4	66.7	85.7	85.7	----					
5	58.4	71.1	73.3	78.5	----				
6	55.0	73.7	71.4	78.8	71.1	----			
7	50.6	62.2	58.3	63.6	59.8	62.2	----		
8	66.2	74.4	70.0	79.3	71.8	70.0	63.1	----	
9	47.7	48.4	50.0	50.5	44.6	48.4	63.9	56.2	----
	1	2	3	4	5	6	7	8	9

***** SINGLE LINKAGE CLUSTER ANALYSIS *****

Table 5. RAPD data of nine chickpea accessions G1 ICCV 2; G2 JG 62; G3 ICCV 88202; G4 ICCV 92504; G5 PANT G 114; G6 GL 769; G7 ICCW 49; G8 Annigeri; G 9 ICCW 6.

N- Non polymorphic P*- polymorphic

A01-B1	1	1	1	1	1	1	1	1	1	NP
A01-B2	1	1	1	1	1	1	1	1	1	NP
A01-B3	1	1	1	1	1	1	1	1	1	NP
A01-B4	1	1	1	1	1	1	1	1	1	NP
A01-B5	1	1	1	1	1	1	1	1	1	NP
A02-B1	1	1	1	1	1	0	1	1	1	P*
A02-B2	1	1	1	1	1	0	1	1	1	P*
A02-B3	1	1	1	1	1	0	1	1	1	P*
A03-B1	1	1	1	1	1	1	1	1	1	NP
A03-B2	1	1	1	1	1	0	1	1	1	P*
A03-B3	1	1	1	1	1	1	1	1	1	NP
A03-B4	1	1	1	1	1	1	1	1	1	NP
A04-B1	1	1	1	1	1	0	0	1	0	P*
A04-B2	1	1	1	1	1	1	0	1	1	P*
A04-B3	1	1	1	1	1	1	1	1	1	NP
A05-B1	0	0	0	0	0	0	0	0	0	NP
A06-B1	1	1	1	1	1	1	1	1	1	NP
A07-B1	1	1	1	1	0	1	1	1	1	P*
A07-B2	1	1	1	1	0	1	0	1	0	P*
A07-B3	1	1	0	1	1	1	0	1	0	P*
A07-B4	1	1	1	1	1	1	1	1	1	NP
A07-B5	1	1	1	1	1	1	1	1	1	NP
A07-B6	0	0	0	0	0	0	0	0	1	P*
A07-B7	1	1	1	1	1	1	1	1	1	NP
A07-B8	1	1	1	1	1	1	0	1	0	P*
A09-B1	1	1	1	1	0	1	1	1	1	P*
A09-B2	1	1	1	1	0	1	1	1	1	P*
A09-B3	1	1	1	1	0	1	1	1	1	P*
A09-B4	1	1	1	1	0	1	1	1	1	P*
A09-B5	1	1	1	1	0	1	1	1	1	P*
A09-B6	1	1	1	1	1	1	1	1	1	NP

Primer-band	G1	G2	G3	G4	G5	G6	G7	G8	G9	Remarks
A10-B1	0	0	0	1	1	1	1	1	0	P*
A10-B2	0	0	0	0	0	0	0	0	1	P*
A10-B3	1	1	1	1	1	1	1	1	1	NP
A10-B4	1	1	1	1	1	1	1	1	0	P*
A10-B5	0	0	0	0	0	0	0	0	1	P*
A10-B6	1	1	1	1	1	1	1	1	0	P*
A10-B7	1	1	1	1	1	1	0	1	0	P*
A10-B8	1	1	1	1	1	1	0	1	0	P*
A10-B9	0	0	0	0	0	0	1	0	0	P*
A11-B1	1	1	1	1	1	1	1	1	1	NP
A11-B2	1	1	1	1	1	1	1	1	1	NP
A11-B3	0	0	0	0	0	0	0	0	1	P*
A11-B4	0	0	0	0	0	0	1	0	1	P*
A11-B5	1	1	1	1	1	1	1	1	1	NP
A11-B6	0	0	0	0	0	0	0	0	1	P*
A12-B1	1	1	1	1	1	1	1	1	1	NP
A12-B2	1	1	1	1	1	1	0	1	0	P*
A12-B3	0	1	1	1	1	1	0	1	0	P*
A12-B4	1	0	0	0	0	0	0	0	0	P*
A12-B5	0	0	0	0	0	0	1	0	1	P*
A13-B1	0	1	1	1	1	1	1	0	0	P*
A13-B2	0	1	1	1	1	1	1	0	0	P*
A13-B3	0	1	1	1	1	1	1	0	0	P*
A13-B4	0	1	1	1	1	1	1	0	1	P*
A13-B5	0	1	1	1	1	1	1	0	0	p*
A13-B6	0	1	0	1	0	0	0	0	0	P*
A13-B7	0	1	1	1	1	1	1	1	1	P*
A13-B8	0	1	1	1	1	1	1	1	1	P*
A13-B9	0	1	1	1	1	1	0	1	1	P*
A13-B10	0	0	0	0	0	0	0	1	1	P*
A13-B11	0	0	0	0	0	0	0	1	0	P*
A13-B12	0	1	1	1	1	1	1	1	1	p*
A14-B1	1	1	1	1	1	0	1	1	0	P*
A14-B2	1	1	1	1	1	0	1	1	0	P*
A14-B3	1	1	1	1	1	0	1	1	1	P*
A14-B4	1	1	1	1	1	1	0	1	0	P*
A14-B5	1	1	1	1	1	1	1	1	1	NP
A14-B6	0	0	0	0	0	0	1	0	1	P*

Primer –band	G1	G2	G3	G4	G5	G6	G7	G8	G9	Remarks
A15-B1	0	1	0	1	1	1	1	1	0	P*
A15-B2	0	1	0	1	1	1	1	1	1	P*
A15-B3	0	0	0	0	0	1	1	0	1	P*
A15-B4	0	1	0	1	0	1	0	0	0	P*
A15-B5	0	1	1	1	0	1	1	1	1	P*
A15-B6	0	0	1	1	0	1	0	0	0	P*
A16-B1	1	1	1	1	1	1	0	1	0	P*
A16-B2	1	1	1	1	0	0	0	1	1	P*
A16-B3	0	0	1	1	0	0	0	0	0	P*
A16-B4	1	1	1	1	1	1	1	1	1	NP
A16-B5	1	0	0	0	0	0	0	0	1	P*
A16-B6	1	0	0	0	0	0	1	0	1	P*
A17-B1	0	0	0	0	0	0	1	1	0	P*
A17-B2	0	0	0	1	0	0	1	1	1	P*
A17-B3	0	0	1	1	1	1	1	1	1	P*
A17-B4	0	0	0	0	0	0	0	0	1	P*
A17-B5	0	0	0	0	0	0	1	0	1	P*
A17-B6	1	1	1	1	1	1	1	1	1	NP
A18-B1	1	0	1	1	1	0	0	0	0	P*
A18-B2	1	0	1	1	1	1	0	1	1	P*
A18-B3	1	0	1	1	1	1	0	0	0	P*
A18-B4	1	0	0	0	0	0	1	0	1	P*
A18-B5	1	1	1	1	1	1	1	1	1	NP
A19-B1	0	0	0	0	0	0	0	0	1	P*
A19-B2	1	1	0	1	0	1	1	1	0	P*
A19-B3	0	0	0	0	0	0	0	0	1	P*
A19-B4	1	1	0	1	1	1	1	1	1	P*
A19-B5	0	0	0	0	0	0	0	0	1	P*
A20-B1	0	0	0	0	1	1	1	1	1	P*
A20-B2	0	0	0	0	1	1	1	1	0	P*
A20-B3	0	0	1	1	1	1	1	1	1	P*
A20-B4	0	0	0	0	0	0	0	0	1	P*
A20-B5	0	0	0	1	1	0	0	1	0	P*
A20-B6	0	0	0	1	1	1	1	1	1	P*

Table 6. RAPD analysis of chickpea progeny from the cross GL 769 x ICCW 49 with primers A-04, A-07, A-14.

Primer	Distance from the well cm	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	P1	P2
A- 04	0.5	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
	0.7	1	1	0	0	0	0	1	1	1	1	0	1	1	1	0	1	1
	0.9	0	1	0	0	0	0	1	1	1	1	1	0	1	1	1	0	1
	1.1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A-07	0.6	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	0.8	1	0	1	0	0	0	1	1	0	1	0	0	0	0	1	0	1	0	1	0
	0.9	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1.1	1	1	0	1	1	1	1	!	1	1	1	1	1	1	1	1	1
	1.3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	1.6	0	1	1	1	0	1	1	1	1	1	0	1	0	0	1	1	1	1	0	1
A-14	1.9	1	0	1	0	0	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0
	2.0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	2.2	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1	1	1	1	0	1

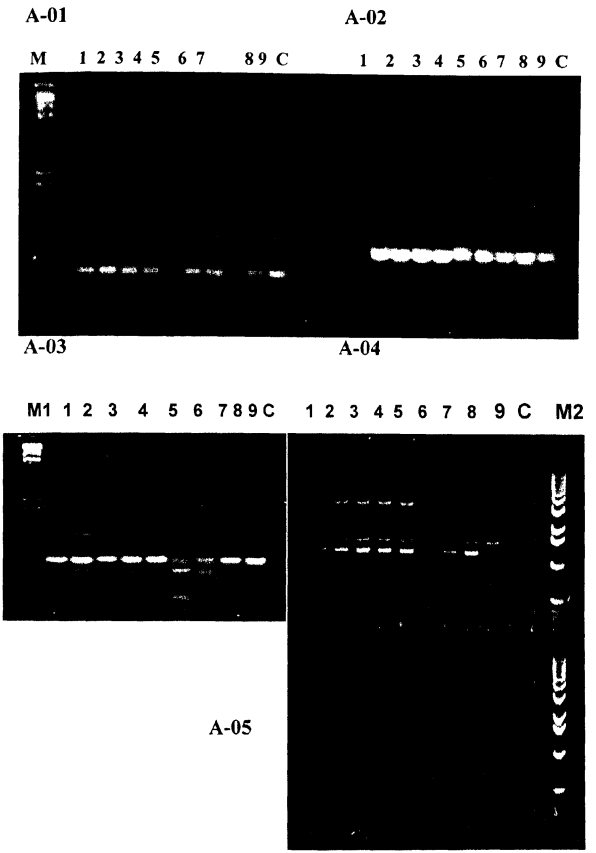


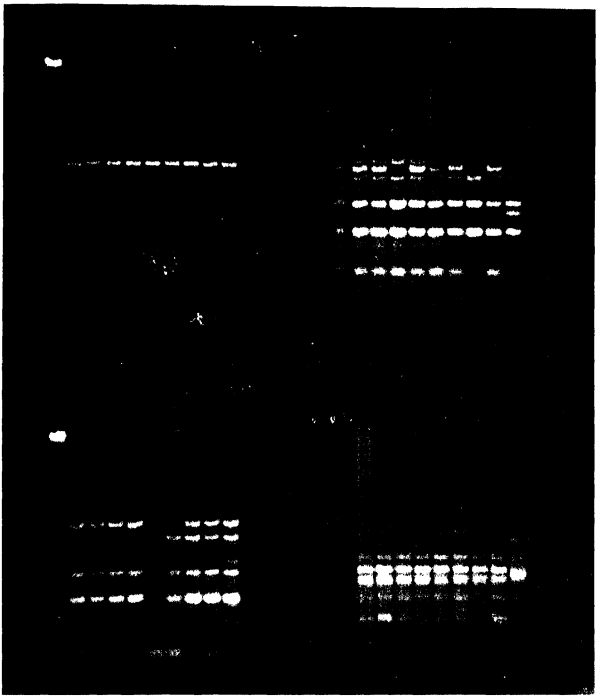
Figure1: RAPD profile of nine chickpea genotypes using primers A-01, A-02, A-03, A-04 and A-05.

A-06

M 1 2 3 4 5 6 7 8 9 C

A-07

1 2 3 4 5 6 7 8 9 C



A-09

A-10

Figure 2: RAP3D profile of nine chickpea genotypes using primers A-06, A-07, A-09 and A-10. M: Lambda Hind III marker, C: Control.

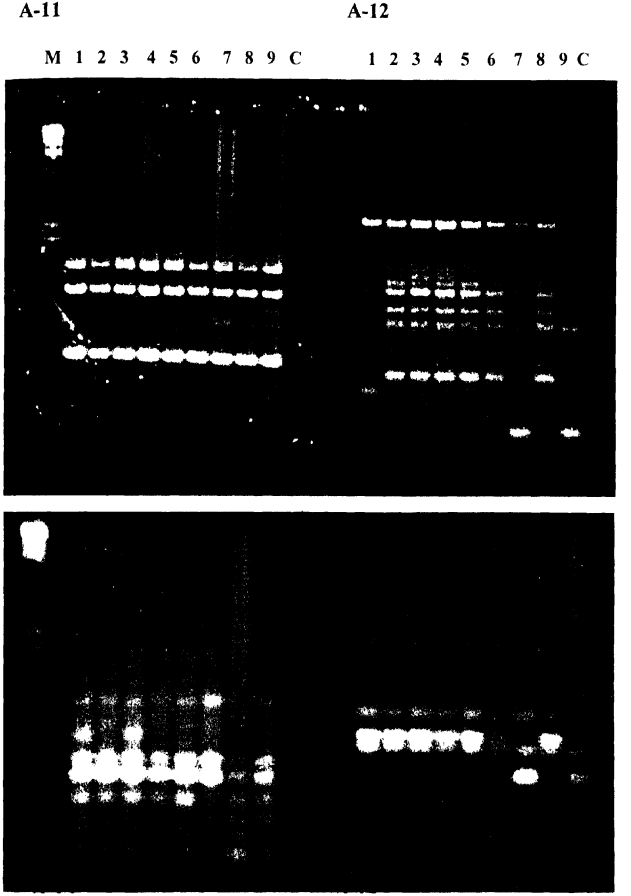


Figure 3: RAPD profile of nine chickpea genotypes using primers A-11, A-12, A-13 and A-14.M: Lambda Hind III marker, C: Control.

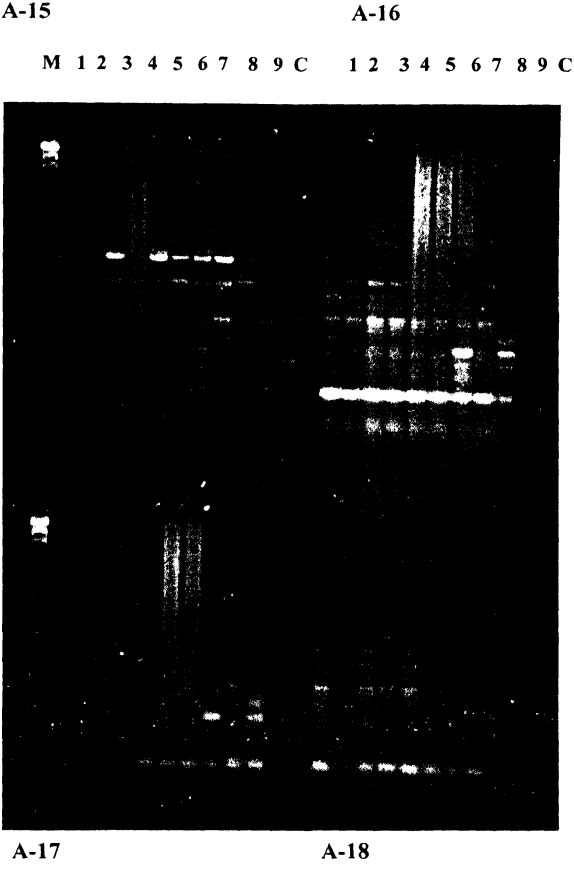
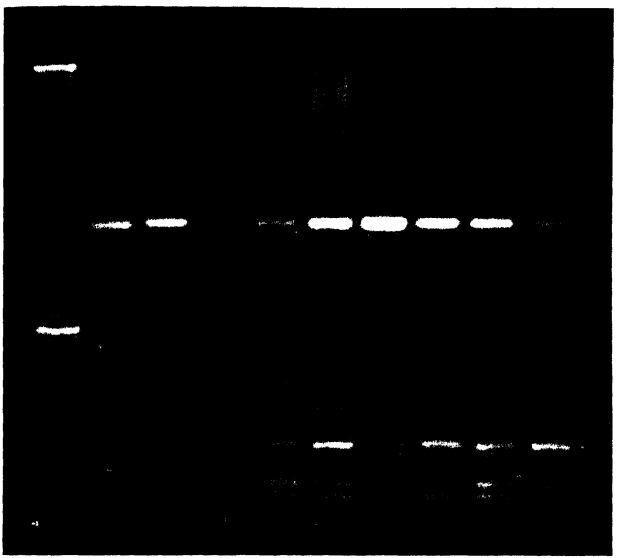


Figure 4: RAPD profile of nine chickpea genotypes using primers A-15, A-16, A-17 and A-18.

A 19

M 1 2 3 7 8 9 C

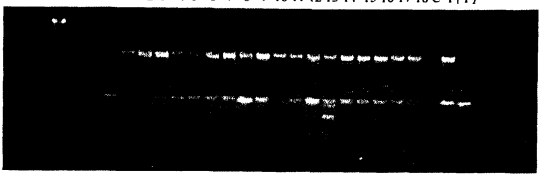


A-20

Figure 5: RAPD profile of nine chickpea genotypes using primers OPA 19 and 20. **M:** Lambda Hind III marker, **C:** Control.

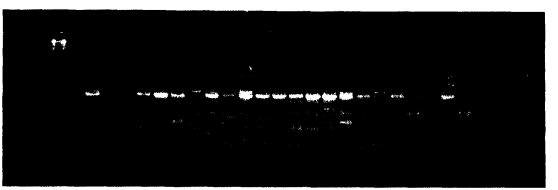
A-04

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 C P_1 P_2



A-07

M P_1 P_2 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 C P_1 P_2



A-14

M P_1 P_2 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 C P_1 P_2

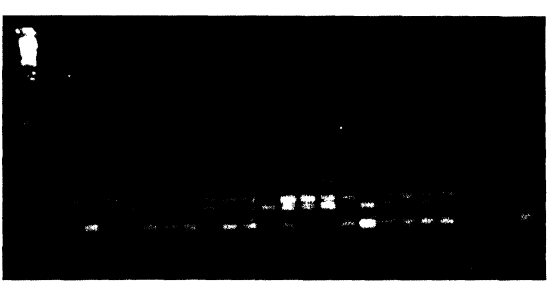
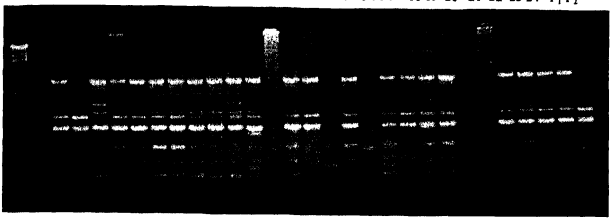


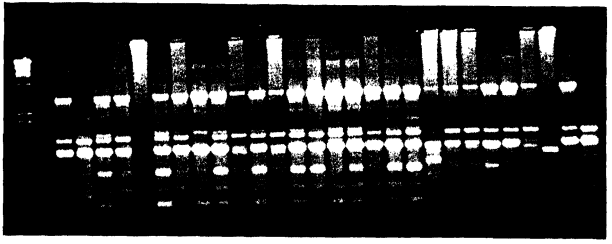
Figure 6: RAPD profile of the progeny GL 769 X ICCW 49 using the primers A-04, A-07 and A-14. **M** Lambda Hind III marker, $\mathbf{P_1}$ GL 769, $\mathbf{P_2}$ ICCW 49.

A-04

M P_1 P_2 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 P_1 P_2



M P_1 P_2 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 P_1 P_2



P_1 P_2 49 50 51 52 53 54 55 56 57 58 59 P_1 P_2 60 61 62 63 64 65 66 67 68 C

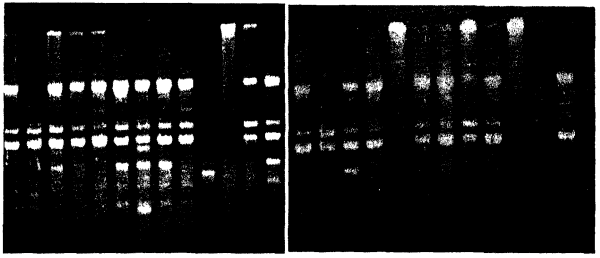
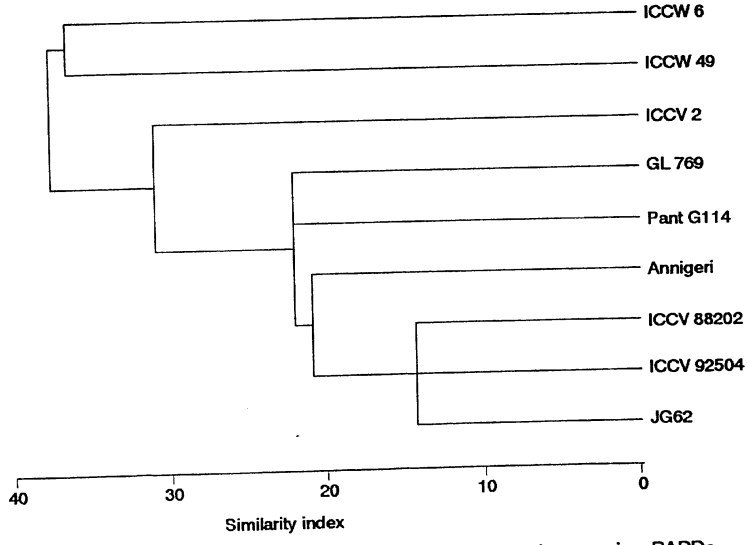


Figure 7: RAPD profile of the progeny Annigeri x ICCW 6 with primer A-04:M Marker;P_1 Annigeri; P_2 ICCW 6, 1-68 progeny of Annigeri x ICCW 6, **C**-Control



Dendrogram showing diversity among 9 chickpea genotypes using RAPDs

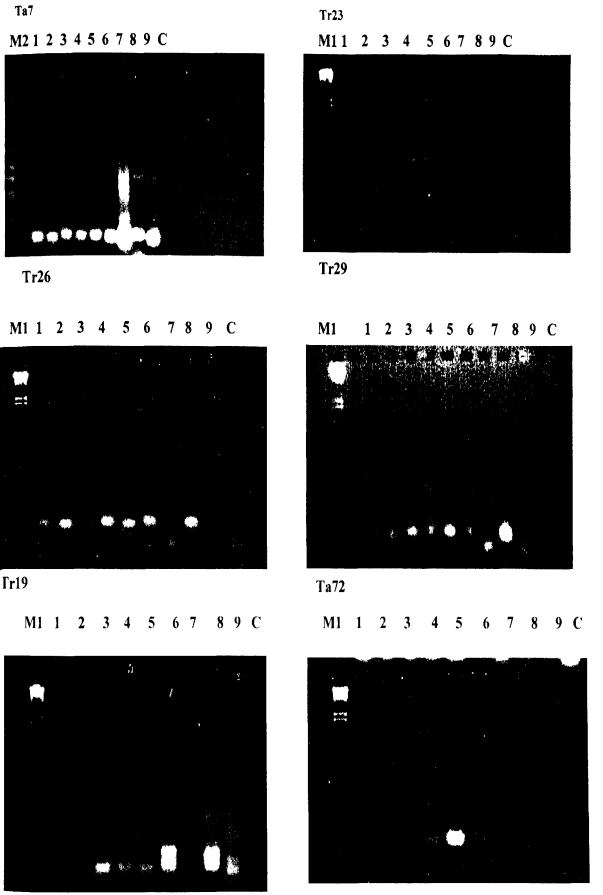


Figure 8 : SSRs (Microsatellites) profile of nine chickpea genotypes with primers Ta7, Tr23, Tr26, Tr29, Tr19 and Ta72; M1 Lambda Hind III marker , M2 1kb ladder.

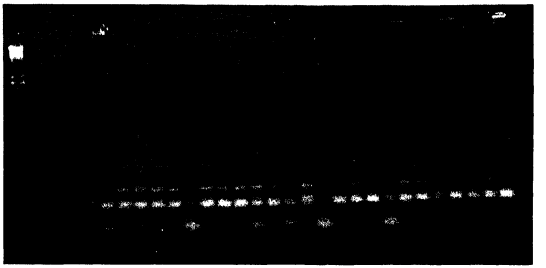
Tr29

M P_1 $P_2$1 2 3 4 5 6 7 8 9101112131415161718 C $P_1$$P_2$

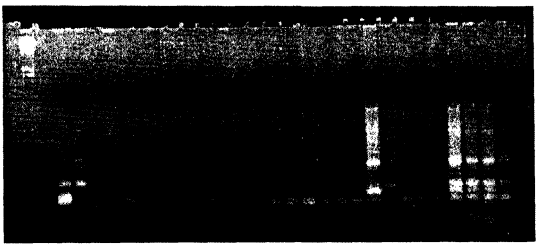
Figure 9: SSRs (Microsatellite) polymorphism in progeny GL769 x ICCW 49 using primer pair Tr29; **M** Lambda Hind III marker; **P1** GL 769; **P2** ICCW 49.

1-18 Progeny of the cross GL 769 x ICCW 49.

M P_1P_2 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



M P_1P_2 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50



M P_1P_2 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 6768

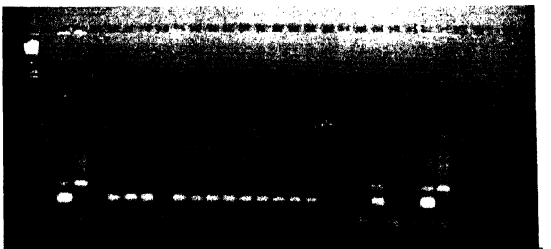
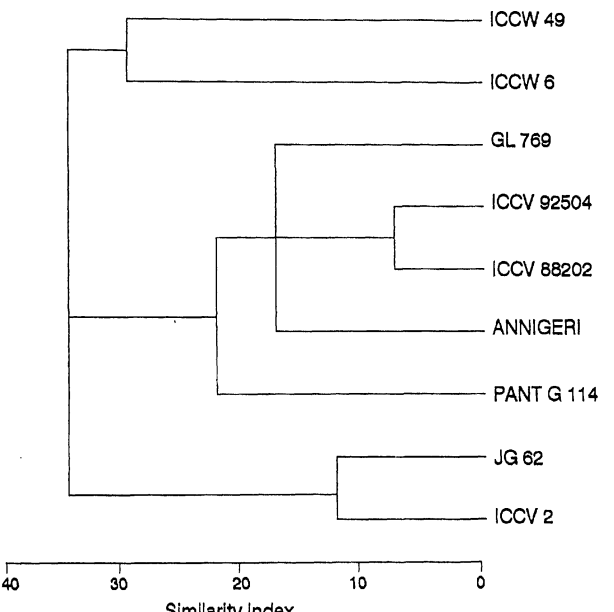


Figure10: SSR polymorphism in progeny from the cross Annigeri x ICCW 6; M Lambda Hind III marker; 1-68 progeny.



Dendrogram based on microsatellite data of 9 chickpea genotypes

5.2 Detection of variability among chickpea genotypes using microsatellites

For microsatellite analysis, 9 genotypes of chickpea were screened with 8 different microsatellite primer pairs procured from University of Frankfurt, Germany. Of the 8 primer pairs, 5 primers (Ta29, Ta19, Tr26, Ta53 and Ta72 showed good polymorphism for the parents GL 769 and ICCW 49 and can be used to study polymorphism in the progeny of GL 769 x ICCW 49. T23 produced faint bands. Among the 5 primer pairs, Tr26 and Ta29 were better than the other three in distinguishing many of the chickpea genotypes. Hence Ta29 was used to screen the whole progeny of the cross GL 769 and ICCW 49. Of the 18 homozygotes, 16 were of GL 769 type and 2 were of ICCW 49 type shown in Table 8.

Three primers Ta19, Tr26, Ta29 showed good polymorphism for the parents Annigeri and ICCW 6, and can be used to screen for polymorphism in progeny of Annigeri x ICCW6. Tr26 was selected to screen for polymorphism in the cross Annigeri and ICCW 6, 33 heterozygotes and 28 homozygotes and 7 produced no bands.

Similarity matrix based on microsatellite analysis of 9 chickpea genotypes using single linkage cluster analysis.

***** SIMILARITY MATRIX *****

1	----								
2	88.9	----							
3	66.7	66.7	----						
4	72.2	72.2	94.4	----					
5	61.1	72.2	72.2	77.8	----				
6	50.0	50.0	83.3	77.8	55.6	----			
7	50.0	38.9	50.0	44.4	33.3	44.4	----		
8	50.0	61.1	83.3	77.8	77.8	77.8	44.4	----	
9	44.4	44.4	66.7	61.1	50.0	50.0	72.2	61.1	----
	1	2	3	4	5	6	7	8	9

Cluster analysis of microsatellite markers placed the genotypes into 3 major groups. The genotypes JG 62 and ICCV 2 (group I), and the wild genotypes ICCW 49 and ICCW 6 (group III) formed distinct groups, while the genotypes GL 769, ICCV 92504, ICCV 88202, Annigeri and Pant G 114 formed a separate group (Group III) which can be divided into three subgroups at the level 22. Subgroup I include ICCV 92504 and ICCV 88202; Subgroup II Annigeri and GL 769; and Pant G114 was clustered into Subgroup III. The wild species ICCW6 and ICCW 49 formed a separate distinct group indicating the diversity when compared with other groups. The cluster analysis of RAPD data also indicated the diversity of wild species when compared with other genotypes.

Table 7. SSR analysis of 9 chickpea genotypes.

Primer	Distance (Cm)	G1	G2	G3	G4	G5	G6	G7	G8	G9
T5-B1	3.0	0	1	0	0	1	0	0	1	0
T5-B2	3.6	0	0	0	0	1	0	1	1	1
T7-B1	3.0	1	1	1	1	1	1	1	1	1
T19-B1	3.0	0	0	1	1	1	1	0	1	0
T19-B2	3.1	1	1	0	0	0	0	0	0	0
T19-B3	3.8	0	0	0	0	0	1	1	0	0
T23-B1	1.4	1	1	0	1	1	0	0	0	0
T26-B1	2.5	1	1	1	1	1	1	0	1	0
T26-B2	2.8	0	0	1	1	0	1	1	1	1
T29-B1	3.2	1	1	1	1	1	1	0	1	0
T29-B2	3.5	0	0	0	0	0	0	1	0	1
T53-B1	1.9	0	0	0	0	0	1	0	1	0
T53-B2	2.1	0	0	1	1	1	1	0	1	1
T53-B3	2.2	0	0	0	0	0	0	0	0	1
T53-B4	2.7	1	1	1	1	1	0	1	1	1
T72-B1	1.6	0	0	0	0	1	0	0	0	0
T72-B2	2.1	0	1	1	1	1	1	0	1	1
T72-B3	2.2	0	0	0	0	0	0	1	0	0

1 - Band present; 0 - Band absent.

Table 8. SSR data of chickpea progeny from the cross GL 769 x ICCW 49 with primer pair Tr29.

Primer	Distance from the well Cm	P1	P2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	P1	P2
Tr29	1.5	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0
	1.7	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1

1:1 heterozygotes

1:0 homozygotes of the type GL 769

0:1 homozygotes of the type ICCW 49

TABLE 9. SSR data of chickpea progeny from the cross Annigeri and ICCW 6 with primer pair Tr26.

1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1
P_1	P_2	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1
P_1	P_2	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
1	1	0	0	0	0	0	0	1	1	1	0	0	0	1	1	1	1	0
1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
P_1	P_2	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68
1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
1	0	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	0

1:1 heterozygotes

1:0 homozygotes of the type ICCW 6

5.3 Detection of variability among chickpea genotypes using DNA amplification fingerprinting.

Primer giving single band in RAPDs i.e., OPA6 was selected for DAF analysis. Increasing primer concentrations were used to choose the best primer concentrations and also to compare the band patterns obtained with each primer concentration. The number of polymorphic bands obtained varied with increasing primer concentrations with 0.8 μM, 6 out of 10 bands (60%); with 1.6 μM, 10 out of 15 bands (67%); with 2.4 μM, 13 out of 19 (69%); with 3.2 μM, 16 out of 23 (70%) and finally with 4.0 μM, 17 out of 24 (71%) bands were polymorphic.

Table 10. DAF analysis of four chickpea genotypes
NP Non polymorphic P* Polymorphic

Primer	Distance from the well (cm)	G6	G7	G8	G9	Remarks
A-06 0.8 µM	0.4	0	0	0	1	P*
	2.1	1	1	1	1	NP
	2.5	1	1	1	1	NP
	2.9	0	0	1	0	P*
	3.1	1	1	1	0	P*
	3.3	0	0	0	1	P*
	3.7	1	1	1	1	NP*
	4.3	1	1	1	1	NP*
	5.4	0	0	0	1	P*
	5.7	0	0	1	0	P*
1.6 µM	2.1	1	1	1	1	NP
	3.6	1	1	1	1	NP
	4.0	0	0	1	0	P*
	4.1	0	1	0	1	P*
	4.3	1	1	1	1	NP
	4.5	1	0	1	0	P*
	5.3	0	0	1	0	P*
	5.7	1	1	1	1	NP
	6.9	1	0	0	0	P*
	8.2	0	0	1	0	P*
	9.2	0	0	1	0	P*
	9.5	0	0	0	1	P*
	10.4	0	1	0	0	P*
	13.3	0	0	0	1	P*
	16.0	1	1	1	1	NP
2.4µM	2.1	1	1	1	1	NP
	2.6	1	0	1	0	P*
	3.0	1	1	1	1	NP
	3.4	1	1	1	1	NP
	3.7	1	1	1	1	NP
	4.4	1	1	1	1	NP
	4.7	0	1	0	1	P*
	5.5	1	0	0	0	P*
	5.7	0	1	0	0	P*
	5.8	0	0	1	1	P*
	6.2	0	0	1	0	P*
	6.6	0	1	0	0	P*
	6.7	0	0	1	0	P*
	6.8	0	1	0	0	P*
	8.1	0	1	0	0	P*
	8.7	0	0	1	0	P*
	9.3	0	0	1	0	P*
	13.3	0	0	0	1	P*

	16.0	1	1	1	1	NP
3.2 μM	2.0	1	1	1	1	NP
	2.7	1	1	1	1	NP
	3.0	1	1	1	1	NP
	3.4	1	1	1	1	NP
	3.6	1	1	1	1	NP
	4.0	0	1	0	0	P*
	4.3	1	1	1	1	NP
	5.4	0	1	1	0	P*
	5.7	1	1	0	1	NP*
	6.1	1	0	1	1	P*
	6.2	0	0	1	0	P*
	6.4	0	0	0	1	P*
	6.9	0	1	0	1	P*
	7.5	1	0	0	0	P*
	8.0	1	1	0	0	P*
	8.2	0	0	1	0	P*
	9.5	1	0	0	0	P*
	10.1	1	1	0	0	P*
	11.5	0	1	0	0	P*
	11.8	0	0	0	1	P*
	12.2	1	1	1	0	P*
	13.3	0	0	0	1	P*
	16.0	1	1	1	1	NP
4 μM	2.0	1	1	1	1	NP
	2.1	1	0	1	1	P*
	2.7	1	1	1	1	NP
	3.0	1	1	1	1	NP
	3.4	1	1	1	1	NP
	3.7	1	1	1	1	NP
	4.3	1	1	1	1	NP
	5.8	1	1	1	0	P*
	5.9	1	0	0	1	P*
	6.3	1	1	1	0	P*
	6.5	0	0	0	1	P*
	6.7	0	0	1	0	P*
	7.0	0	1	0	0	P*
	7.2	0	0	1	0	P*
	7.4	1	0	0	0	P*
	8.0	1	0	0	0	P*
	8.4	1	0	0	0	P*
	8.7	1	0	0	0	P*
	10.4	0	0	1	0	P*
	11.5	0	0	0	1	P*
	11.7	1	0	0	0	P*
	11.9	0	0	0	1	P*

	13.3	0	0	0	1	P*
	16.0	1	1	1	1	NP

5.4 Conclusions

Consistent results have been obtained with different type of marker techniques used to investigate a set of chickpea genotypes. RAPD marker can be the marker of choice when screening large number of samples, but their dominant nature, problems with reproducibility and interpretation of band patterns limits its use.

DAF requires little amounts of DNA, higher primer concentration, stringent conditions in PCR and use of sequencing gels and radioactive detection for higher resolution, sensitivity and discrimination. Comparable levels of variation was observed with either RAPDs and DAF.

STMS usually define a single, multi-allelic locus, co-dominant and highly reproducible. Therefore, it can be concluded that STMS can be markers of interest and best to reveal polymorphism provided many STMS primers are available and information about primer sequences is easily exchanged between laboratories. Costs, time of project and sufficient expertise must be taken into account before taking up new projects. Undoubtedly, there is still a long and winding road to be followed before the ultimate markers for detecting genetic diversity and relatedness in plants are developed.

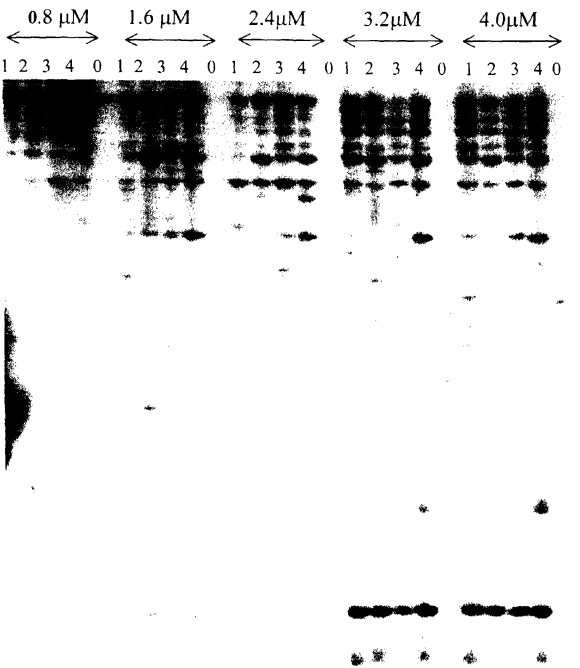


Figure 11: DAF profile of 4 chickpea genotypes: LANE 1 GL769 , LANE 2 ICCW 49, LANE 3 Annigeri, LANE 4 ICCW 6, LAGNE 0 Control

6. REFERENCES

Ahn S, Anderson JA, Sorrells ME & Tanksley SD 1993. Homologous relationships of rice, wheat and maize chromosomes. Molecular and General Genetics 241: 483-490.

Allard RW 1956. Formulas and tables to facilitate the calculation of recombinant values in heredity. Hilgardia 24: 235-278.

Alberte RS, Zimmermann GK & Fain SR 1994. Assessment of genetic diversity of seagrass populations using DNA fingerprinting: implications for population stability and management. Proceedings of the National Academy of Sciences of the United States of America 91: 1049-1053.

Ali S, Muller CR & Epplen JT 1986. DNA fingerprinting by oligonucleotide probes specific for simple repeats. Human Genetics 74: 239-243.

Apuya NR, Fraiser BL, Kiem P, Roth EJ & Lark KG 1988. Restriction fragment length polymorphism as genetic marker in soyabean, *Glycine max* (L.) Merill. Theoretical and Applied Genetics 75: 889-901.

Ayad WG, Hodgkin T, Jaradat A & Rao, VR. 1997. Molecular genetic techniques for plant genetic resources. Report of an IPGRI Workshop, 9-11 October, 1995. International Plant genetic Resources Institute, Rome, Italy.

Bassam BJ, Caetano-Aholles G & Gresshoff PM 1991. A highly sensitive silver stain method. Analytical Biochemistry 196: 80-83.

Becker JP, Vos M, Kuiper F, Salamini & Heun M 1995. Combined mapping of AFLP and RFLP markers in barley. Molecular and General Genetics 249: 65-73.

Bierwerth S, Kahl G, Weigand F & Weising K 1992. Oligonucleotide fingerprinting of plant and fungal genomes: a comparison of radioactive, colorigenic and chemiluminescent detection methods. Electrophoresis 13: 115-122.

Binelli G, Gainfranceshchi L Pe ME, Taramino G, Busso C, Stenhouse J & Ottavino E 1992. Similarity of maize and Sorghum genomes as revealed by maize RFLP probes. Theoretical and Applied Genetics 84: 10-16.

Bonierable M, Plaisted RL & Tanksley SD 1988. RFLP maps of potato and tomato based on a common set of clones reveal modes of chromosomal evolution. Genetics 120: 1095-1103.

Botstein D, White RL, Skolnik M & Davis RW 1980. Construction genetic linkage map in man using restriction fragment length polymorphisms. American Journal of Human Genetics 32: 314-331.

Briggs FN & Knowles PF 1967. Introduction to plant breeding. Reinhold, New York.

Brummer EC, Kochert G & Bouton JH 1991. RFLP variation in diploid and tetraploid alfaalfa. Theoretical and Applied Genetics 83: 89-93.

Broun P & Tanksley SD 1996. Characterization and genetic mapping of simple repeat sequences in tomato genome. Molecular and General Genetics 250: 39-49.

Brown P & Tanksley SD 1993. Characterization of tomato DNA clones with sequence similarity to human minisatellites. Plant Molecular Biology 23: 231-242.

Caetano-Anolles G, Bassam BJ & Gresshoff PM 1991b. DNA amplification fingerprinting: a strategy for genome analysis. Plant Molecular Biology Reporter 9: 295-305.

Caetano-Anolles G, Bassam BJ & Gresshoff PM 1994. MAAP using short oligonucleotide primers. Plant Genome analysis Ed Gresshoff PM CRC Press, Boca Raton 29-45.

Carlson JE, Hong YP, Brown GR & Glaubitz JC 1994. FISH DNA amplification markers and conifers. Plant Genome Analysis Ed Gresshoff PM CRC Press, Boca Raton 69-82.

Casey D 1992. Primer on molecular genetics U.S. Department of Energy, Washington, D.C.

Clegg MT & Epperson BK 1985. Recent developments in population genetics. Advanced genetics 23: 235-269.[6,9,14].

Cregan PB, Bhavwat AA, Akkayya MS & Rongwen J 1994. Microsatellite fingerprinting and mapping of soybean. Methods in Molecular and Cell Biology 5: 49-61.

Crow JF & Dove WF 1988. A diamond anniversary: the first chromosome map. Genetics 118: 389-391.

Crow JW (Ed) 1964. *In*: heterosis. Hafner, New York.

Dallas JF 1988. Detection of 'DNA fingerprints' of cultivated rice by hybridization with human minisatellite DNA probe. Proceedings of the National Academy of sciences of the United States of America 85: 6831-6835.

Dudley JW, Saghai Maroof MA, & Rufener GK 1991. Molecular markers and grouping of parents in maize breeding programs. Crop Science 31: 718-723.

Duvick DN 1984. Genetic contributions to yield grains of five major crop plants. Ed Fehr WR special Pub. No. 7, Crop science Society of America, Madison, WI 15-47.

Figdore SS, Kennard WC, Song KM, Osborn TC 1988. Assessment of the degree of restriction fragment length polymorphism in *Brassica*. Theoretical and Applied Genetics 75: 833-840.

Funke RP, Kolchinsky A & Gresshoff PM 1993. Physical mapping of a region in the soybean (*Glycine max*) genome containing duplicated sequences. Plant Molecular Biology 22: 437-446.

Gaur PM, Slinkard AE (1990a). Inheritance and linkage of isozyme coding genes in chickpea. Journal of Heredity 81: 455-461.

Gaur PM, Slinkard AE (1990b). Genetic control and linkage relations of additional isozymes markers in chickpea. Theoretical and Applied Genetics 80: 648-656.

Gebhardt C, Blomendhal U, Debener T, Salamini F & Ritter E 1989. Identification of 2n breeding lines and 4n varieties of potato (Solanum *tuberosum* sp.) with RFLP fingerprints. Theoretical and Applied Genetics 75: 16-22.

Gupta PK, Balyan HS, Sharma PC & Ramesh B 1996. Microsatellites in plants: A new class of molecular markers. Current Science 70: 45-54.

Havey MJ & Muelbauer FJ 1989. Linkages between restriction fragment length, isozyme and morphological markers in lentil. Theoretical and Applied Genetics 77: 395-401.

Hajj Moussa E, Millan T, Gil J & Cubero JI 1996. Variability and genome length estimation in chickpea (*Cicer arietinum* L.) revealed by RAPD Analysis. Journal of Genetics & Breeding. 51: 83-85.

Hearne CM, Grosh S & Todd JA 1992. Microsatellites for linkage analysis of genetic traits. Trends in Genetics 8: 288-294.

Helentjaris T, Slocum M, Wright S, Schaefer A & Nienhuis J 1986. Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphism. Theoretical and Applied Genetics 72: 761-769.

Jeffreys AJ, Wilson V & Thein SL 1985. Hypervariable 'minisatellite' regions in human DNA. Nature 316: 67-73.

Jung C, Pillen K, Frese LS & Melchinger AE 1993. Phylogenetic relationships between cultivated and wild species of genus *Beta* revealed by 'DNA fingerprinting'. Theoretical and Applied Genetics 86: 449-457.

Kaemmer D, Afza R, Weising K, Kahl G & Novak FJ 1992. Oligonucleotide and amplification fingerprinting of wild species and cultivars of Banana (*Musa* sp.). Bio/Technology 10:1030-1035.

Kaemmer D, Weising K, Beiermann B, Borner T, Epplen JT & Kahl G 1995. Oligonucleotide fingerprinting of tomato DNA. Plant Breeding 114: 12-14.

Kahl G, Ramser J, Weising K, Winter P, Huttel B, Geistlinger J, Sharma P, Morjane H, Harrabi M, Weigand F, & Saxena MC 1995. Junk DNA: Not so junky after all. Pages 201-203 In: Induced Mutations and Molecular Techniques for Crop Improvement. International Atomic Energy Agency, Vienna.

Kahl G, Kaemmer D, Weising K, Kost S, Weigand F, & Saxena MC 1994. The potential of gene technology and genome analysis for cool season food legume crops: theory and practice. Pages 705-725 In: Expanding the production and use of cool season food legumes. Eds: Muehlbauer, FJ, Kaiser, WJ. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Kazan K, Muehlbauer FJ, Weeden NF, Ladizinsky G 1993. Inheritance and linkage relationships of morphological and isozyme loci in chickpea (Cicer *arietinum* L.). Theoretical and Applied Genetics 86: 417-426.

Keim P, Shoemaker RC & Palmer RG 1989. Restriction fragment length polymorphism diversity in soybean. Theoretical and Applied Genetics 77: 786-792.

Kiss GB, Csanadi G, Kalo P & Okresz L 1993. Construction of a basic genetic map for alfaalfa using RFLP, RAPD, isozyme and morphological markers. Molecular and General Genetics 238: 129-137.

Ladizinsky G & Alder A 1976. The origin of chickpea Cicer arietinum L. Euphytica 25: 211-217.

Lander E, Green P, Abrahamson J, Barlow A, Daly M, Lincoln S & Newburg L 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1: 174-181.

Lander ES & Botstein B 1989. Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps. Genetics 121: 185-199.

Langridge U, Schwall M & Langridge P 1991. Squashes of plant tissue as substrate for PCR. Nucleic Acids Research 19: 6954.

Lee M, Godshalk EB, Lamkey KB & Woodman WW 1989. Association of restriction fragment length polymorphisms among maize inbred with agronomic performance of their crosses. Crop Science 29: 1067-1071.

McCouch SR, Kochert G, Yu ZH, Khush GS, Coffman WR & Tanksley SD 1988. Molecular mapping of rice chromosomes. Theoretical and Applied Genetics 76: 815-829.

Melchinger AE 1990. Use of Molecular markers in breeding for oligogenic disease resistance. Plant Breeding 104: 1-19.

Messmer MM, Melchinger AE, Lee M, Woodman WL, Lee EA & Lamkey KR 1991. Genetic diversity among progenitors and elite lines from the Iowa Stiff Stalk Synthetic (BSSS) maize population: Comparison of allozyme and RFLP data. Theoretical and Applied Genetics 83: 97-107.

Michelmore RW & Shaw DV 1988. Character dissection. Nature 335: 672-673.

Miller JD & Tanksley SD 1990. RFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycoperiscon*. Theoretical and Applied Genetics: 437-448.

Morgante M, Rafalski a, Biddle P, Tingey S &Oliveri AM 1994. Genetic mapping and varaibility of seven soybean simple sequence repeat loci. Genome 37: 763-769.

Muehlbauer FJ, Simon CJS, Speath C & Haddad NI 1989. Genetic improvement of chickpea: Key factors to be considered for a breakthrough in productivity. In: Proceedings of the second International workshop on chickpea improvement, Dec 4-8, ICRISAT, India. pp. 209-216.

Nakamura Y, Leppert M, O'Connell & Fujimoto 1987. Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 235: 1616-1622.

Nei M 1987. Molecular evolutionary genetics. Columbia University Press New York.

Nybom H & Schaal BA (1990a). DNA fingerprints applied to paternity analysis in apples (*Malus* x *domestica*). Theoretical and Applied Genetics 79: 763-768.

Paran, Goldman I, Tanksley SD & Zamer D 1995. Recombinant inbred lines for genetic mapping in tomato. Theoretical and Applied Genetics 90: 542-548.

Paterson A, Lander E, Hewitt JD, Peterson S, Lincoln SE & Tanksley SD 1988. Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature 335: 721-726.

Paterson AH, Tanksley SD & Sorells ME 1991. DNA markers in plant improvement. Advances in Agronomy 46: 39-90.

Poulsen GB, Kahl G & Weising K 1994. Differential abundance of simple repetitive sequences in species of *Brassica* and related brassicacae. Plant Systematics and Evolution 190: 21-30.

Rafalski JA, Hanafey MK, Tingey SV & Williams JGK 1994. Technology for molecular breeding RAPD markers, Microsatellites and Machines. Plant Genome Analysis. Ed Gresshoff PM CRC press. Boca Raton pp 19- 27.

Reiter RS, Williams J, Feldman KA, Rafalski JA, Tingey SV & Scolnik PA 1992. Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and RAPDs. Proceeding of the National Academy of Sciences of the United States of America 89: 1477-1481.

Richardson T, Cato S, Ramser J, Kahl G & Weising, K. 1995. Hybridization of microsatellites to RAPD: a new source of polymorphic markers. Nucleic Acids Research 23: 3798-3799.

Roder MS, Plaske J, Konig SU, Bolner A, Sorrels ME, Tanksley SD & Ganal MK 1995. Abundance, variability and chromosomal location of microsatellites in wheat. Molecular and General Genetics 246:327-333.

Rogstadt SH, Patton II JC & Schaal BA 1988. M13 repeat probe detects DNA minisatellite-like sequences in gymnosperms and angiosperms. Proceedings of the National Academy of Sciences of the United States of America 85: 9176-9178.

Saghai-Maroof MA, Soliman KM, Jorgenson RA and Allard RS 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendalian inheritance, chromosomal location, and population dynamics. Proceedings of the National Academy of Sciences of the United States of America 81: 8014-8018.

Schmidt T, Boblenz, K & Weising K 1993. Use of highly repeated DNA polymorphisms for genome diagnosis and evolutionary studies in genus *Beta*. DNA fingerprinting: State of the science, Eds Pena SDJ, Chakabrorty R, Epplen JT & Jeffreys AJ Basel: Birkhauser Velag 454-459.

Singh KB 1987. Chickpea breeding. pp 127-162 in The chickpea. Eds Saxena MC and Singh KB Wallington, Oxon, UK: CAB International.

Singh KB 1997. Experience with pyramiding of ascochyta blight resistant genes in kabuli chickpea. DNA Markers and Breeding for Resistance to Ascochyta Blight in Chickpea. Proceedings of the Symposium on "Application of DNA Fingerprinting for Crop Improvement: Marker-assisted selection of Chickpea for Sustainable Agriculture in the Dry Areas". Eds Udupa SM and Weigand F 11-12 April 1994, Aleppo, ICARDA, Syria.

Senior ML & Heun M 1993. Mapping microsatellites and polymerase chain confirmation of the targeted repeats using a CT primer. Genome 36: 884-889.

Sharma PC, Winter P, Bunger T, Huttel B, Weigand F, Weising K & Kahl G 1995. Abundance and polymorphism of di-, tri-, and tetranucleotide tandem repeats in chickpea (*Cicer arietinum* L.). Theoretical and Applied Genetics 90: 90-96.

Sharma PC, Winter P, Bunger T, Huttel B, Weigand F, Weising K & Kahl G Detection of DNA polymorphism in chickpea (*Cicer arietinum* L.) using oligonucleotide probes. Agricultural Biotechnology- 2nd Asia Pacific Conference pp 107-115.

Sharma PC, Hüttel B, Kahl G, Winter P, Gardner RC & Weising K 1995. The potential of microsatellites for hybridization and PCR-based fingerprinting of chickpea (*Cicer arietinum* L.) and other species. Electrophoresis 16: 1755-1761.

Sharma PC, Winter P, Bünger T, Hüttel B, Weigand F, Weising K & Kahl G 1995. Abundance and polymorphism of di-, tri- and tetranucleotide tandem repeats in chickpea (*Cicer arietinum* L.). Theoretical and Applied Genetics 90: 90-96.

Smith JSC 1988. Diversity of United States hybrid maize germplasm; isozymic and chromatographic evidence. Crop Science 28: 63-69.

Soller M & Beckmann JS 1983. Genetic polymorphism in varietal identification of genetic improvement. Theoretical and Applied Genetics 67: 25-33.

Soller M & Beckmann JS 1990. Towards a unified approach to genetic mapping of eukaryotes based on sequence tagged microsatellite sites. Bio/Technology 8: 930-932.

Serret MD, Udupa SM & Weigand F 1997. Assessment of genetic diversity of cultivated chickpea using microsatellite-derived RFLP markers: Implications for origin. Plant Breeding 116: 573-578.

Tanksley SD & Orton TJ 1983. Isozymes in plant genetics and breeding. Amesterdam: Elsevier Science.

Tanksley SD 1993. Mapping polygenes. Annual Review of Genetics 27: 205-233.

Tanksley SD, Ganal MW, Price JP, De Vincente MC, Bonierbale MW, Broun P, Fulton TM, Giovannoni H, Grandillo S, Martin GB, Messeguer R. Miller JC, Miller L, Paterson AH, Pineda O, Roder M, Wing RA, Wu W & Young ND 1992. High-density molecular linkage maps of tomato and potato genomes; biological inferences and practical applications. Genetics 132: 1141-1160.

Tanksley SD, Young ND, Paterson AH & Bonierbale MW 1989. RFLP mapping in plant breeding: new tools for an old science. Bio/Technology 7: 257-264.

Tanksley SD & Hewitt J 1988. Use of molecular markers in breeding for soluble solids content in tomato. Theoretical Applied Genetics 75:811-823.

Tao Y, Manners JM, Ludlow MM & Henzell RG 1993. DNA polymorphisms in grain sorghum (*Sorghum bicolor* (L.) Moench). Theoretical and Applied Genetics 86: 679-688.

Thomas MR, Matsumoto S, Cain P & Scott NS 1993. Repetitive DNA of grapevine: classes present and sequences suitable for cultivar identification.Theoretical and Applied Genetics 86: 173-180.

Tautz and Renz M 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. Nucleic Acids Research 12: 4127-4138.

Udupa SM, Anuradha Sharma, Sharma RP and Pai RA 1993. Narrow Genetic Variability in *Cicer arietinum* L. as revealed by RFLP Analysis. Journal of Plant Biochemistry and Biotechnology Vol. 2, 83-86, July 1993.

Udupa SM & Weigand 1997.Genotyping with microsatellite and RAPD markers resolves pathotype diversity in the ascochyta blight pathogen of chickpea. Pages 67-77 in DNA Markers and Breeding for Resistance for Ascochyta Blight in Chickpea. Proceedings of the Symposium on "Application of DNA Fingerprinting for Crop Improvement: Marker-assisted selection of Chickpea for Sustainable Agriculture in the Dry Areas". Eds Udupa SM and Weigand F 11-12 April 1994, Aleppo, ICARDA, Syria.

Van Rheenen HA 1992. Biotechnology and chickpea breeding. International Chickpea Newsletter 26: 14-17.

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 21: 4407-4414.

Waugh R & Powell W 1992. Using RAPD markers for crop improvement. Trends in Biotechnology 10: 186-191.

Weising K, Beyermann B, Ramser J & Kahl G 1991a. Plant DNA fingerprinting with radioactive and dioxigenated oligonucleotide probes complementary to simple repetitive sequences. Electrophoresis 12: 159-169.

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

Weising K, Nybom H, Wolff K, Meyer W 1995. DNA fingerprinting in plants and fungi. CRC Press, Boca Taton.

Weising K, Weigand F, Driesel A, Kahl G Zischlrt H & Epplen JT 1989. Polymorphic simple GATA/GACA repeats in plant genomes. Nucleic Acids Research 17: 10128.

Welsh J & Mc Clelland M 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18: 7213-7218.

Whitkus R, Doubly J & Lee M 1992. Comparative genome maps of sorghum and maize. Genetics 132: 119-1130.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA & Tingey SV 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18: 6531-6535.

Winter P & Khal G 1995. Molecular marker technologies for plant improvement. World Journal of Microbiology and Biotechnology 11: 438-448.

Wu KS & Tanksley SD 1993. Abundance, polymorphism and genetic mapping of microsatellites in rice. Molecular and General Genetics 241: 225-235.

Yang GP, Saghai-Maroof MA, Xu Quifa Zhang CG & Biyashev RM 1994. Comparative analysis of microsatellite DNA polymorphism in landraces cultivars of rice. Molecular and General Genetics 245: 187-194.