MOLECULAR CHARACTERIZATION OF PIGEONPEA (*Cajanus cajan* (L.) Millsp.) AND ITS WILD RELATIVES USING PCR GENERATED DNA MARKERS

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MILIND BALKRISHNA RATNAPARKHE

DIVISION OF BIOCHEMICAL SCIENCES

NATIONAL CHEMICAL LABORATORY

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DECLARATION

Certified that the work incorporated in the thesis entitled "**MOLECULAR CHARACTERIZATION OF PIGEONPEA (*Cajanus cajan* (L.) Millsp.) AND ITS WILD RELATIVES USING PCR GENERATED DNA MARKERS**" submitted by Mr. MILIND BALKRISHNA RATNAPARKHE was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

P K Ranjekar

(P.K. Ranjekar)
Research Guide

TO MY PARENTS

INTER-INSTITUTIONAL COLLABORATIVE RESEARCH EFFORT

RESEARCH WORK EMBODIED IN THIS THESIS WAS CARRIED OUT

AT

NATIONAL CHEMICAL LABORATORY
PUNE, (M.S.) INDIA

IN COLLABORATION WITH

WASHINGTON STATE UNIVERSITY
PULLMAN, USA

SCIENTISTS INVOLVED FROM EACH INSTITUTE

NCL	DR. Mrs. V.S. GUPTA	DR. P.K. RANJEKAR
WSU	DR. F.J. MUEHLBAUER	DR. T.W. OKITA
	DR. C.A. RAYAN	DR. V.R. FRANCESCHI

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MBRatnaparkhe

Milind B. Ratnaparkhe

LIST OF ABBREVIATIONS

AFLP	:	Amplified Fragment Length Polymorphism
ASAP	:	Allele Specific Associated Polymorphism
ASLP	:	Amplification Sequence Length Polymorphism
bp	:	Base pairs
BSA	:	Bulk Segregant Analysis
cM	:	centi Morgan
dATP	:	Deoxyadenosine 5' - triphosphate
dCTP	:	Deoxycytidine 5' - triphosphate
dGTP	:	Deoxyguanosine 5' - triphosphate
dTTP	:	Deoxythymidine 5' - triphosphate
EDTA	:	Ethylene Diamine Tetra Acetic acid
ICRISAT	:	International Crop Research Institute for Semi Arid Tropics
ISSR	:	Inter-simple sequence repeat
Kbp	:	Kilobase pairs
MAS	:	Marker Assisted Selection
NIL	:	Near-Isogenic Lines
PCR	:	Polymerase Chain Reaction
QTL	:	Quantitative Trait Loci
RAPD	:	Random Amplified Polymorphic DNA
RFLP	:	Restriction Fragment Length Polymorphism
SCAR	:	Sequence Characterised Amplified Region
SPAR	:	Single Primer Amplified Repeat
SDS	:	Sodium Dodecyl Sulphate
SSR	:	Simple Sequence Repeats
STMS	:	Sequence Tagged Microsatellite Site
STR	:	Short Tandem Repeats
TAE	:	Tris-Acetate-EDTA buffer
Tm	:	Melting temperature
Tris	:	Tris-hydroxymethyl amino methane
VNTR	:	Variable Number of Tandem Repeat

CHAPTER 1

DNA Marker Research in Legumes: A Brief Account

DNA MARKER RESEARCH IN LEGUMES: A BRIEF ACCOUNT

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INTRODUCTION

The ultimate purpose of genetic resource activities is to support present and future crop improvement work that aims to improve the productivity and quality of the crop. Crop cultivars with higher yield potential are the key to increased productivity. Besides the increased yield potential and yield stability, germplasm is improved for many characteristics which indirectly contribute to increased productivity. For such development or improvement of plant varieties, conventional plant breeding involves several methods including crossing, backcrossing, selfing and testing wherein, two parents having desirable characteristics are crossed in order to transfer a particular character from one parent to another. Identification of desired parental genotypes in germplasm collections is often limited by lack of knowledge of the organization of the germplasm resources.

Success in crop improvement work largely depends on access to well-classified and diverse genetic resources. The analysis of genetic diversity between or within different species, populations and individuals has played a key role in plant breeding programs. Simple and accurate determination of genetic relationships based on comparisons throughout entire genomes is needed in order to use the information most effectively. Determining genetic relationships in specific germplasm sources is also useful for identifying lines or populations that should be maintained to preserve maximum diversity in germplasm banks. Established methods for the characterization and measurement of genetic diversity have mostly relied on morphological, physiological, cytological markers as well as biometrical analysis of quantitative variation. A number of complications are faced when classical strategies are used to characterize genetic diversity. Hence during the past decade, the classical strategies are being complimented by molecular markers involving macromolecules like proteins and DNA. These markers, which are based on the polymorphism found in the proteins and DNA, have revolutionized the genetic analysis methods in crop plants. Today, various types of DNA markers and breeding strategies have been employed for the identification and utilization of agronomically important

traits in many crop systems.

DNA MARKERS: THEIR PROPERTIES AND ADVANTAGES

DNA markers are ubiquitous, innumerable, discrete, nondeleterious, inherited by Mendelian laws, unaffected by the environment, and are free of epistatic interactions (Beckman and Soller 1986; Tanksley *et al.*, 1989) and have following advantages over morphological and isozyme markers:

1. They are unlimited in number because the RFLP and PCR techniques are able to detect differences at the DNA level in both translatable and non translatable DNA regions (Helentjaris, 1991; Stuber, 1992).
2. They are phenotypically and environmentally neutral because sequence polymorphisms are detected directly at the DNA level and not by assaying gene products which may be influenced by alterations in gene coding sequence or by changes in the level of gene expression (Beckmann and Soller, 1986; Laurie *et al.*, 1992).
3. DNA markers mediated selection in breeding programs can be carried out in the off-season in a non destructive process to the original plant because only a small amount of tissue is required.
4. They enable selection for specific genotypes in a heterozygous form where the genotype cannot express because a dominant allele masks a recessive allele. This advantage can be achieved with markers which are co-dominant in nature.
5. They do not show either dominant recessive interaction or epistatis and are devoid of pleotrophic effect.
6. DNA markers in the genome can be considered as entry point to the genes. Thus, they facilitate the chromosome walking technique for gene cloning (Melchinger, 1990; Stuber, 1992; Laurie *et al.*,1992).
7. DNA markers are not tissue specific and can be used at the any stage of the plant growth.

DNA markers are extensively used for various applications in plant breeding procedures. They have proven to be powerful tools in the

assessment of genetic variation both within and between plant populations, and in finding the genetic relationships among accessions within a species. Characterization and quantification of genetic diversity, both within and between populations, provides a guidence in the choice of parents for breeding hybrids. The DNA markers tightly linked to various qualitative and quantitative traits can be used in Marker Assisted Selection (MAS) in breeding program. DNA markers are routinely used for genome analyses in a number of crop species (Aruna *et al.*, 1993; Chalmers *et al.*, 1994; Lanham *et al.*,1995; Abo-elwafa *et al.*, 1995, Schnell *et al.*, 1995).

TYPES OF DNA MARKERS

(1) Hybridization based markers

Restriction Fragment Length Polymorphisms (RFLPs)

RFLP markers detect variation in the position of the restriction endonuclease sites among genotypes. This causes alterations in the fragmentation pattern obtained by digestion with type II restriction enzymes. These variations are due to creation or abolishment of sites for restriction endonuclease cleavage or from other internal events such as deletion or insertion, translocation and inversion. RFLPs are co-dominant markers and are very reliable in linkage analysis and breeding. These markers have been used to investigate the degree of genomic variation within a crop species, and in phylogenetic and taxonomic studies. Single copy genomic DNAs or cDNAs have been used as molecular genetic markers to construct RFLP maps of crop plants such as rice (McCouch *et al.*, 1988; Saito *et al.*, 1991), maize (Helentjaris 1987), lettuce (Landry *et al.*, 1987), soybean (Keim *et al.*, 1990), oil seed (Diers *et al.*, 1994), tomato (Young and Tanksley 1989), potato (Gebhardt *et al.*, 1989) and wheat (Chao *et al.*, 1989).

Inspite of the usefulness of RFLPs for the construction of genetic map, there are some technical limitations for their usage. For example, several micrograms of DNA digested by restriction enzymes are required for southern blotting and hybridization. Moreover RFLP techniques are laborious, expensive, time consuming and require well trained personnel

(Gebhardt and Salamini, 1992; Rafalski *et al.*, 1991).

Microsatellites and minisatellites

Microsatellites, abundant in higher eukaryotic genomes, are tandem repeats with a basic motif of less than 6 bp and are also called as Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STR) (Jacob *et al.*, 1991). SSRs have been proven to be highly informative and are distributed throughout the genome (Wang *et al.*, 1994). The repeat number of microsatellites has been demonstrated to be highly variable in animals and to be inherited in a codominant manner (Litt and Luty 1989; Johansson *et al.*, 1992). The presence of microsatellites has been documented in many plants such as arabidopsis (Bell and Ecker 1994), barley (Saghai-Maroof *et al.*, 1994), brassica (Lagercrantz *et al.*, 1993), maize (Condit and Hubbell 1991; Senior and Heun 1993), rice (Wu and Tanksley 1993), soybean (Akkaya *et al.*, 1992; Morgante and Olivieri 1993) and wheat (Roder *et al.*, 1995). Microsatellite molecular marker linkage maps developed in crops such as barley, wheat, rice, soybean and arabidopsis are gaining more importance in recent years.

Minisatellite DNA regions contain a class of variable number of tandem repeat (VNTR) loci in which the repeated sequences are short (<65bp) and frequently GC rich (Jeffreys *et al.*, 1985a; 1985b; Nakamura *et al.*, 1987). The presence of numerous, dispersed, highly polymorphic minisatellites appears to be an ubiquitous feature of eukaryotic genomes (Ryskov *et al.*, 1988). Jeffrey's 33.6 and 33.5 minisatellite probes have been used for fingerprinting cultivars of Asian and African rice (Dallas 1988) while a human minisatellite probe, pV47 has been shown to be polymorphic in indica, japonica and wild rice (Ramakrishna *et al.*, 1995). A 15 bp repeat motif in the protein III gene of bacteriophage M13 appears to be extremely useful in detecting polymorphism (Vassart *et al.*, 1987) and serves as an universal marker for DNA fingerprinting (Ryskov *et al.*, 1988). It has been used for the first time in plants by Ryskov *et al.*, (1988) to identify polymorphic regions in barley, to detect minisatellite-like sequences in a few

gymnosperms and angiosperms (Rogstad *et al.*, 1988; Zimmerman *et al.*, 1989) and to assess genetic variability in Rosaceae (Nybom *et al.*, 1990) and ornamental plants (Tzuri *et al.*, 1991).

(2) PCR-based Markers

Randomly Amplified Polymorphic DNA (RAPDs)

RAPD markers as described by Williams *et al.*, (1990), and Welsh and McClelland (1990) are obtained by using single primer of arbitrary nucleotide sequence in Polymerase Chain Reaction (PCR) amplification. Since RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region (Williams *et al.*, 1990), these polymorphisms are usually noted as presence or absence of an amplification product from a single locus. Their advantage over RFLPs include the small amount of DNA required and a comparatively easier technical procedure.

RAPD markers have proven to be powerful tools in molecular assessment of genetic variation both within and between plant populations and in the elucidation of genetic relationships among accessions within a species. These markers are also useful in genetic mapping (Martin *et al.*, 1991; Reiter *et al.*, 1992; Barua *et al.*, 1993; Paran and Michelmore 1993; Williams *et al.*, 1993), genetic fingerprinting (Quiros *et al.*, 1991; Wilde *et al.*, 1992; Collins and Symons 1993; Graham *et al.*, 1994), phylogenetic analysis (Demeke *et al.*, 1992; Halward *et al.*, 1992; Kresovitch *et al.*, 1992; Kazan *et al.*, 1993; Yu and Pauls 1993) and parentage analysis (Welsh *et al.*, 1991; Roy *et al.*, 1992; Scott *et al.*, 1992). Inspite of numerous advantages, RAPDs have an inherent disadvantage of reproducibility amongst laboratories because of the reaction conditions i.e. low annealing temperature, ionic conc. and template quality.

DNA Amplification Fingerprinting (DAF)

A slightly different protocol for arbitrary primers is the DNA amplification fingerprinting (DAF) technique (Caetano-Anolles *et al.*, 1991)

which uses shorter primers, usually about 8 nucleotides in length, and produces large numbers of products. DAF involves enzymatic amplification of DNA directed by a single arbitrary oligonucleotide primer and can be used to generate complex and characteristic fingerprints. Each amplification product reflects single base pair changes in a particular primer-target site making DAF a powerful fingerprinting strategy, especially for organisms that are closely related.

Sequence Characterized Amplified Region (SCAR)

This technique, developed by Paran and Michelmore (1993), is derived from RAPD and circumvents the drawbacks inherent to RAPDs. The markers are generated by cloning and sequencing RAPD fragments of interest and designing 24-mer oligonucleotide primers that are complementary to the ends of the original RAPD fragment. Using these primers, single locus called SCAR can be specifically amplified. The SCARs have more advantages compared to RAPDs because of the reproducibility and co-dominant nature and can be used in map-based cloning, physical mapping and comparative mapping.

Inter-Simple Sequence Repeat (ISSR)

Recently, multilocus fingerprinting methods have been developed using oligonucleotides based on SSRs as primers in PCR amplification of genomic DNA (Zietkiewicz *et al.*, 1994; Wu *et al.*, 1994; Gupta *et al.*, 1994). Repeat anchored primers that amplify region between SSRs are useful in detecting polymorphism and have been used for fingerprinting closely related cultivars. Inter simple sequence repeat (ISSR) primers anneal directly to SSRs and thus require no knowledge of target sequence. Differences in experimental procedures include changing the sequence of the SSR motif, the length of primer, and the presence or absence of a short stretch of nucleotide residues at the 5' or 3' end. All the different primers can be used as a single primer or in combination with oligonucleotide primers of arbitrary sequence. Depending on the primer used, amplified products

consist of either only inter-SSR sequences or inter-SSR sequences plus SSR arrays. This technique enables amplification of DNA from different sources, providing information of many loci simultaneously. Experiments have shown the Mendelian nature of inheritance of fragments scored as dominant (Zietkiewicz *et al.*, 1994; Gupta *et al.*, 1994) or codominant markers (Wu *et al.*,.1994).

Sequence Tagged Microsatellite Site (STMS)

Microsatellite DNA sequences are an excellent source of polymorphisms in eukaryotic genomes and are well suited for genotyping and map construction. Polymorphisms appear because of variation in the number of tandem repeats in a given repeat motif at a particular locus. Detection of variation in the number of repeats at each particular locus requires information on the conserved sequences flanking the SSR. Most SSRs are dinucleotide repeat-based [$(AT)_n$, $(AG)_n$ and $(CA)_n$] microsatellite markers (Rafalski and Tingey 1993). Similar to $(CA)_n$ repeats in humans, $(AT)_n$ dinucleotide microsatellite repeats are relatively abundant and highly polymorphic in plants (Akkaya *et al.*, 1992; Morgante *et al.*, 1993). STMS markers are inherited in a Mendelian fashion and can be used for segregation and linkage analyses. In recent years, PCR amplification of microsatellites has been successfully applied to the analysis of plant genomes (Morgante and Olivieri 1993; Wu and Tanksley 1993; Panaud *et al.*, 1996, Chen *et al.*, 1997).

Direct Amplification of Minisatellite DNA (DAMD-PCR)

Minisatellite DNA region contains a VNTR where each repeat itself includes a short highly conserved core sequence. These repetitive DNA regions normally contain tandem repeats that vary in the number of repeat unit between the genotypes and thus are both referred to as variable number of tandem repeats (VNTRs) or hypervariable regions (HVRs). This form of HVR is found at numerous loci within the genome and generates high level of polymorphism between individuals (Jeffreys *et al.*, 1985 a, b). The core

sequences of several reported human minisatellites have been recently used as primers in PCR reaction, that efficiently amplify regions rich in minisatellites and VNTR loci in fish (Heath *et al.*, 1993). This PCR strategy referred to as the direct amplification of minisatellite DNA (DAMD-PCR) overcomes the difficulty of searching for VNTR loci with the heterologous probes.

Amplified Fragment Length Polymorphism (AFLP)

AFLP, developed by Zabeau and Vos (1993), combines the strengths of different marker systems and provides new opportunities for plant genome analysis. It is based on selective amplification of restriction enzyme digested DNA fragments with specific primers. Multiple bands are generated in each amplification reaction that contains DNA markers of random origin. In this technique, the DNA is digested with one or two restriction enzymes, and double stranded adapters are ligated to ends of the fragments to generate template DNA for amplification. Thus, the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. Analysis of DNA on denaturing polyacrylamide gels typically results in the production of 50 to 100 bands per individual sample. Polymorphism 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. Similar to RAPD analysis, AFLP assay requires no prior sequence knowledge, but detects a 10-fold greater number of loci than those detected by RAPD analysis. Thus, the AFLPs have the capacity to rapidly screen thousands of independent genetic loci and hence have added advantage when combined with Near-Isogenic Lines (NILs) or Bulk Segregant Analysis (BSA). 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 markers are gaining more importance in developing saturated genetic

linkage maps in various crop plants.

APPLICATIONS OF DNA MARKERS

(1) Genetic diversity analysis and germplasm collection

The modern agricultural practices to develop varieties are so far successful, but there is a reduction in the genetic diversity of the primary gene pool under cultivation. This will lead to sporadic breakdown of the crop systems due to their increasing susceptibility to pathogens. Hence awareness of genetic diversity and management of crop genetic resources are both important components in the plant improvement programs. The foundation of crop-based agriculture largely rests on the availability and knowledge of exotic plant genetic resources in germplasm collections. If germplasm collections could be systematically organized based on genetic relationships, then the efficiency of sampling and utilization of germplasm resources could be greatly improved. Establishment of a core collection based on genetic variation shown by accessions will be advantageous, but clear and detailed assessments of diversity within germplasm collections are not usually available. This information can be obtained using molecular markers, which have the advantage of sampling at the genome level directly.

The DNA markers make it possible to understand the processes and dynamics of biodiversity, its evolution and natural preservation in cultivars, landraces and wild species (Helentjaris *et al.*, 1985; Milligan *et al.*, 1994; Lande 1988). They can be effectively used in identification, classification and managing the germplasm collections (Virk *et al.*, 1995a, b; Howell *et al.*, 1994; Kazan *et al.*, 1993; Fukuoka *et al.*, 1992). The prospect of utilizing DNA marker technology for managing germplasm collections is reviewed by Kresovich and McFerson, 1992 and Bretting and Widrlechner,1995. To assess genetic variation, many reports are available in plants using various markers such as RFLPs (Helentjaris *et al.*, 1985; Tanksley *et al.*, 1989), RAPDs (Fukuoka *et al.*, 1992; Yu and Nguyen 1994), microsatellites (Akkaya *et al.*, 1992), DAF (Caetano-Anolles *et al.*, 1991), and SPARs (Gupta *et al.*, 1994). Information on the extent and distribution of genetic diversity will

assist in the development of efficient collection and sampling strategies and in the identification centers of diversity. However, this technology has not yet made a significant impact on the management and utilization of germplasm collections.

(2) Gene tagging and mapping

The most important application of DNA markers in plant breeding is through finding a tight linkage between genetic markers and genes for agronomic and quantitative traits such as disease resistance, male sterility, and self-incompatibility. Such linkage will allow molecular marker assisted selection (MAS) for the desired gene by assaying for the genetic marker rather than for the trait itself. This advantage becomes evident when the breeding goal is to incorporate disease resistance genes into susceptible varieties. The traditional method requires a cross with a parental stock that carries the resistance genes, followed by selection from the segregating population for an individuals that possesses the specific resistance gene. Such selection procedures require inoculation of the segregating population with the pathogen, which is usually hampered by the difficulty of ensuring uniform exposure to the inoculum in the field, such as with soil born pathogen and nematodes. Moreover, artificial preparation and application of inoculum is difficult and expensive. Also, the method needs the occurrence of environmental conditions that permit uniform infection for precise screening. In contrast, marker facilitated selection overcomes the previous mentioned problem because the genetic markers are phenotypically and environmentally neutral. In addition, marker facilitated selection offers the following advantages:

1. It allows screening for resistance to new pathogens that do not exist naturally in the test environment because of quarantine restrictions on their shipment and use for safety reasons.
2. It facilitates the transfer of recessive genes in a back crossing program because most markers are co-dominantly inherited. Therefore determination of heterozygotes does not require progeny testing as in the

classical procedure (Tanksley *et al.*, 1989; Helentijaris, 1991; Young, 1992; Stuber, 1992; Melchinger, 1990).

3. It is very effective in screening for several different disease resistance genes simultaneously with no need of inoculation to the breeding material finally leading to pyramiding of various genes in a single background.

Most mapping research in agriculture has concentrated on locating economically important genes such as disease resistance genes and polygenic characters. Many important genes have now been mapped in terms of tightly linked markers and some of the best examples are genes for resistance to nematode, fungal diseases, viral diseases and insects.

(3) QTL analysis

Another important application of mapping is the resolution of the quantitative traits into their genetic components. These traits include yield, maturity date, quality and stress tolerance such as drought, heat and cold. The traits show continuos phenotypic variation in their expression and are highly affected by environmental conditions because they are usually affected by numerous genes. Resolution of such complex traits into their genetic components allows treating these characters as single gene traits. Such achievements become feasible due to the development of the high density genetic map that permits identification, mapping and measurement of the effects of gene underlying quantitative traits.

TYPES OF MAPPING POPULATIONS

Genetic map construction and mapping studies in plants are commonly analyzed in either segregating or non-segregating populations. The F_2 or the first backcross generation derived from a cross between two homozygous parents differing at appropriate loci represents the segregating populations. Non-segregating populations include recombinant inbred lines (RILs), double haploid lines (DHLs) and near-isogenic lines (NILs) (Melchinger, 1990). A brief account of each type of mapping population is as follow:

(I) F_2 population

This segregating population is developed in plants by crossing two homozygous inbred lines to give the F_1, a heterozygous generation. The F_1 is selfed to produce the F_2 segregating population in which individual plants are the unit of segregation (Gebhardt and Salamini, 1992). Inspite of the simplicity in development of F_2 population, it has some limitations. This method assumes that there will be no further recombination between linkage groups in the subsequent selfing generations (Powell *et al.*, 1990). This assumption seems unlikely because the possibility of further recombination in subsequent generations cannot be eliminated. Therefore, early segregating generations of a cross may not represent the optimal evaluation of linkage between marker and trait. Another limitation of use of the F_2 generation in mapping studies is that quantitative traits are scored at only one time and only at one location. Therefore, it becomes difficult to obtain accurate estimates of the environmental and genetic components of variance (Sollar and Beckmann, 1990).

(II) Doubled haploid lines (DHLs)

Doubled haploids are developed from chromosome doubling of haploids resulting in the development of completely homozygous individuals from a heterozygous F_1 parent (Powell *et al.*, 1990). Commonly DHLs production involves culturing anthers or microspores from the F_1 plants followed by chromosome doubling either spontaneously or by colchicine treatment leading to the development of completely homozygous double haploid lines (Bentolila *et al.*, 1992). Doubled haploids are immortal reference populations for mapping and phenotypic evaluation (Kleinhofs *et al.*, 1993) and hence they can be propagated and evaluated under different environmental conditions. However, doubled haploids have some disadvantages including difficulty in development, genotype dependence and possible low vigor of the regenerated plants.

(III) Near isogenic lines (NILs)

Near isogenic lines are non segregating mapping populations which were described by Muehlbauer *et al.*, (1988) and Young *et al.*, (1988). Mapping using NILs is a rapid method to identify tight linkages between molecular markers and a large number of plant genes. NILs are developed by crossing a donor parent (DP) that carries a gene of interest to the recurrent parent (RP) which is a cultivated line selected because of its otherwise favorable traits. The resulting F1 is back crossed to the recurrent parent for 5-7 generations until the recurrent parent phenotype is recovered. After each backcrossed generation, only the F1 plant that has the phenotype of the desired gene from the donor parent is selected. The expected product of this repeated backcrossing process is a new line which is nearly isogenic to the recurrent parent except for the chromosomal segment containing the desired gene (Melchinger, 1990; Gebhardt and Salamini, 1992).

Utilization of NILs in mapping requires a lot of probe screening in order to identify polymorphic markers between the NIL and RP. Such markers should be located within the introgressed chromosomal segment that contains the target gene (Melchinger, 1990). Further a near isogenic line can map only one trait.

(IV) Recombinant inbred lines (RILs)

Recombinant inbred lines represent non segregating families that are produced by crossing two inbred lines followed by about 6-7 generations of selfing. After each generation of selfing, a single seed is randomly selected and planted for the next generation. In the final generation, near homozygosity is achieved and RILs become fixed for short linkage blocks of inbred alleles. Plants are harvested individually at the final generation and their seeds represent the RILs (Burr *et al.*, 1988; Snape, 1988; Burr and Burr, 1989). Since RILs represent a relatively stable homozygous population, individuals can be characterized for the parental alleles and the frequencies of recombination among them can be determined. Based on that, RILs can be used in constructing genetic maps. The advantages of

using RILs in mapping as reported by Bailey (1981) and Burr and Burr (1989) are: (a) RILs represent an immortal population in which segregation is complete or near complete. Thus, they can be propagated and tested in different environments. (b) RILs can be used to carefully map quantitative traits because several measurements of quantitative traits can be made on lines, as opposed to a single measurement in case of F_2 segregating population. The limitations of RILs are that they cannot be used to map traits that are monomorphic in the parental lines, development of RILs is time consuming and it is not possible in self-incompatible species (Burr and Burr, 1989; Gebhardt and Salamini, 1992).

STATUS OF DNA MARKERS IN IMPORTANT LEGUME CROPS

Legumes are the major source of proteins to a large proportion of the population and hence play a very important role in the human diet. The current global distribution of the main leguminous species grown for food and feed is 69 million hectares which includes major legumes such as pigeonpea, chickpea, pea, lentil, lupin, beans and other leguminous species. Within the last 5 years RFLP and PCR based markers have been described in legume crops such as soybean, pea, lentil, chickpea, sweet pea and faba bean. Recently numerous AFLP-based mapping experiments have been performed in the legume family. The status of DNA marker research in few legumes is described:

(I) Soybean

Keim *et al.*, (1990) developed a RFLP linkage map of soybean and reported association between DNA markers and quantitative trait analysis. Muehlbauer *et al.*, (1991) demonstrated RFLP mapping in soybean using near isogenic lines. The DAF methodology allowed the identification of a marker that co-segregated with the *nts* locus in F2 families derived from a cross between mutant soybean line and *G. soja* (Caetano-Anolles *et al.*, 1993). Akkaya *et al.*, (1992), reported the first study of microsatellite variation in soybean and analyzed SSR variation in 43 soybean accessions

where they observed from six to eight alleles at three SSR loci. Yu *et al.*, (1994) described the first genetic mapping of a gene conferring disease resistance in plants using a soybean microsatellite marker. Webb *et al.*, (1995) carried out the mapping of soybean cyst nematode race-3 resistant loci using RFLP markers. Rongwen *et al.*, (1995) demonstrated the utility of microsatellite DNA markers for soybean genotype identification. Akkaya *et al.*, (1995) further mapped 40 simple sequence repeats (SSR) in a soybean mapping population. Maughan *et al.*, (1995) studied microsatellite and amplified sequence length polymorphisms in cultivated and wild soybean and indicated that ASLPs may be useful as genetic markers in site directed mapping. Powell *et al.*, (1996) did comparision of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis of soybean. Yu *et al.*, (1996) demonstrated the divergence and allelomorphic relationship of a soybean mosaic virus (SMV) resistance gene (*Rsv*1) based on tightly linked DNA microsatellite and RFLP markers.

Pea

In peas, morphological markers have been used for some time as tags for certain virus resistance genes. Yu *et al.*, (1991) reported two RAPD markers linked to *En* , the gene conferring resistance to pea enation mosaic virus. Ellis *et al* (1991) reported a linkage map of pea comprising 151 markers, spanning approximately 1700 cM. Comparison of the lentil map with that available for pea has revealed that approximately 40% of the loci in pea are syntenic in lentil (Weeden *et al.*, 1992). Studies on isozyme loci in chickpea (Gaur and Slinkard, 1990) also indicated a significant similarity with the pea map. Timmerman *et al.*, (1993) reported the mapping of *sbm-1*, a gene conferring resistance to pea seed-born mosaic virus, using RFLP and isozyme markers. Further Timmerman *et al.*, (1994) did linkage analysis of *er-1*, a recessive gene for resistance to powdery mildew using RFLP and ASAP markers. Yu *et al.*, (1995) reported identification and mapping of two DNA markers linked to the gene conferring resistance to pea enation mosaic virus.

French bean

This bean is an important source of dietary protein for over half a billion people in Africa and Latin America. Vallejos *et al.*, (1992) reported a linkage map of french bean using RFLP markers which were distributed into 11 linkage groups covering 960 cM of the bean genome. Further a bulked segregant analysis approach was used by Miklas *et al.*, (1993) in an effort to identify RAPD markers for the *Up2* allele, conferring resistance to bean rust in the common bean. No recombination was observed between the marker and the resistant allele, suggesting that they were tightly linked. Haley *et al.*, (1994) reported the RAPD makers linked to the bean common mosaic virus (BCMV). The two RAPD markers were linked in coupling and in repulsion respectively and were useful for the selection of the resistant lines. Miklas *et al.*, (1996) demonstrated the recombination-facilitated RAPD marker-assisted selection for disease resistance in common bean. Johnson *et al.*, (1997) reported molecular tagging of the *bc-3* gene for introgression into Andean common bean.

Lentil

Lentil (*Lens culinaris* Medik.) is another important food legume in the Middle east., northern Africa, Asia and America. Even though lentil has been an important food legume for centuries, studies on DNA markers have been made only recently. Havey and Muehlbauer (1989) reported several linkages between isozyme, RFLP and morphological markers in lentil. A 560 cM linkage map consisting of 64 morphological, isozyme, and DNA markers has been developed from an inter specific cross (*Lens ervoides* X *L. culinaris*) (Weeden *et al.*, 1992). Extensive conservation of linkage relationships between pea and lentil genetic maps has been reported. Recently RAPD markers have been used to estimate intra- and inter-specific variations in the genus *Lens* (Abo-elwafa 1995) indicating that the intra-specific variation in lentil is lower than that in wild species.

Mungbean

Mungbean is widely cultivated in the Indian subcontinent has been introduced to Africa, West Indies and USA in the recent past. Young *et al.*, (1992) reported the use of RFLP for mapping of a major bruchid resistance gene. Hautea *et al.*, (1992) prepared a genome map of mungbean based on DNA markers. Further Hautea *et al.*, (1993) reported comparative genome analysis of mungbean and cowpea using RFLP mapping data. The study indicated that several large linkage blocks were maintained in both genomes. Young *et al.*, (1993) reported mapping of polygenic resistance to powdery mildew with RFLP markers.

GENESIS AND SCOPE OF PRESENT THESIS

Pigeonpea and chickpea are two most important legume crops in India and are the major source of protein in the human diet. The highest production of pigeonpea occurs in India, where it is the most widely grown legume after chickpea. At present very little information was available at the molecular level in these two legume crops when the work was initiated to study the applicability and use of DNA marker technology in pigeonpea and chickpea. The two major objectives of the thesis were:

(1) Assessment of genetic variability and DNA fingerprinting of pigeonpea accessions from India and from different geographic regions, and phylogenetic studies of the wild species of pigeonpea.

(2) Studies on inter-simple sequence repeat polymorphisms in chickpea and their association with the gene for resistance to fusarium wilt.

The work on pigeonpea was initiated in collaboration with Dr. M. R. Ven Murthy, Faculty of Medicine, University of Laval, Quebec, Canada and later continued at NCL, Pune, India. Six months extensive training on use of PCR generated DNA markers was undertaken in Dr. Murthy's laboratory. The pigeonpea accessions and wild relatives were kindly provided by International Crop Research Institute for Semi Arid Tropics (ICRISAT), Patancheru and Mahatma Phule Agriculture University, Rahuri. The pigeonpea accessions and their wild relatives were previously classified

using morphological characters and therefore no data were available on the classification of accessions using DNA markers. It was essential to find a suitable technique which can identify pigeonpea accessions and their wild relatives.

In India the productivity of chickpea is severely affected by the fusarium wilt disease. No DNA markers were available for screening chickpea genotypes for fusarium wilt resistance except for one RAPD marker. The work on chickpea was initiated for finding the DNA markers linked to the gene for resistance to fusarium wilt, under the McKnight Foundation funded project between National Chemical Laboratory, Pune, Washington State University, Pullman, USA and Mahatma Phule Agricultural University, Rahuri. I was the first graduate student selected under 'Career Development Program' supported by McKnight Foundation. Under this program two years extensive research and training was undertaken at WSU with Dr. F. J. Muehlbauer, USDA-ARS, Dr. T.W.Okita, Institute of Biological Chemistry (IBC) and Dr. V. Franceschi, Center for Electron Microscopy. The present thesis is the outcome of the work done at National Chemical Laboratory, Pune, University of Laval, Quebec, Canada and Washington State University, Pullman, USA.

CHAPTER II

DNA markers for genetic fingerprinting of pigeonpea accessions and its wild relatives

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DNA MARKERS FOR GENETIC FINGERPRINTING OF PIGEONPEA ACCESSIONS AND ITS WILD RELATIVES

INTRODUCTION

Pigeonpea

Pigeonpea (*Cajanus cajan* (L.) Millsp.) (2n=22) is the only cultivated species of the genus *Cajanus*, belonging to subtribe Cajaninae of family Leguminosae (van der Maesen, 1990). It is the sixth most important legume crop in the world and is an important source of dietary protein to the vegetarian population particularly in Indian subcontinent (Nene and Sheila 1992). Availability of genetic variability is a prerequisite to any genetic improvement program. There are over 12000 accessions of pigeonpea in the ICRISAT germplasm collection (Johnson *et al.*, 1993) which includes accessions of related wild species containing traits of known potential value for improving cultivated pigeonpea. So far pigeonpea classification based on morphology has been directed towards satisfying the rather isolated interests of taxonomists and agronomists. Recently, Remanandan *et al.*, (1988) measured the variability in more than 11,000 germplasm accessions assembled at ICRISAT from 52 countries. The difficulties encountered in accurate identification of cultivars are due to reliance on morphological characters. Pigeonpea, although a self pollinating crop, often shows cross pollination which creates problems in maintenance of pigeonpea germplasm (Gupta *et al.*, 1980). Moreover, outcrossing leads to ambiguities in the choice of an appropriate breeding method. Relative to its importance as a food crop, pigeonpea has received little attention in terms of molecular approaches to its genetic improvement.

Origin and distribution

Pigeonpea originated in India and spread quite early. A secondary center of diversity of pigeonpea is found in eastern Africa. Seventeen species of pigeonpea occur in the Indian subcontinent, and another 13 species are endemic to Australia. The Australian species are developed from ancestral complexes separated from the Asian group when Australia separated from main land Asia after the Pleistocene era. Africa harbors only one close relative of pigeonpea, *C. kerstingii* Harms, the other wild relative is

the widespread *C. scarabaeoides* (L.) Thouars which arrived in Africa recently (van der Maesen 1990). India and Myanmar account for 16 related species, one of which, *Cajanus cajanifolius* (Haines) van der Maesen, is considered as a progenitor of pigeonpea. The cultivated pigeonpea stands alone as a crop species in the subtribe, of which most species belong outside the pigeonpea gene pool, or at the most in the secondary gene pool, while several *Cajanus* species can be placed in the secondary gene pool. Apart from the pigeonpea, only one species *Cajanus scarabaeoides* (L.) du Petit-Thouars is common and widespread throughout South and Southeast Asia, the Pacific Islands, and northern Australia. Other regions where pigeonpea is grown are Southeast Asia, Africa, and the America. There is a substantial area of pigeonpea in Kenya, Uganda, and Malawi in eastern Africa, and in the Dominican Republic and Puerto Rico in Central America.

Genetic diversity

Pigeonpea shows a wide range of diversity for various morphological characters such as plant height, branching pattern, flowering habit, leaf, stem, flower, pod, and seed shapes, sizes and colours. In addition, several vegetative and reproductive variants that have arisen either spontaneously or induced by hybridization or mutagen treatment have been described by several workers. The morphological variation in pigeonpea is the greatest in Asia and especially in India. In order to effectively and economically maintain and utilize the world germplasm collection of pigeonpea that now numbers several thousands, but possibly includes many duplicates, we need to develop a more comprehensive pigeonpea classification. Reddy *et al.,* (1975) reported the variability in pigeonpea for some agronomic traits among a collection of 887 accessions. Laxman Singh and Shrivastava (1976) made a collection of 900 accessions of pigeonpea from central India, and reported variability in relation to adaptation. Reddy and Rao (1975) and Rao *et al.,* (1977) observed that natural variation in pigeonpea was also due to somatic variation, in addition to genetic variation and outcrossing. Recently, Remanandan *et al.,* (1988) measured the variability in more than 11,000

germplasm accessions assembled at ICRISAT from 52 countries representing a wide range of variability. The subtribe Cajaninae consists of 13 genera with several species. The biosystematic relationships of these genera are not yet completely understood. All the phylogenetic studies in subtribe Cajaninae have been based on the morphological and cytological data, breeding behavior, seed storage protein patterns and RFLP data (Deodikar and Thakar 1956; Kumar *et al.,* 1958; Ladizinsky and Hamel 1980; Reddy *et al.,* 1980; Pundir and Singh 1985a, b; Nadimpalli *et al.,* 1992). Ladizinsky and Hamel (1980) used seed protein electrophoresis to identify pigeonpea accessions; however, very little polymorphism was detected among them. Recently restriction fragment length polymorphisms (RFLP) have been used successfully for detecting genetic diversity among the wild species of pigeonpea (Nadimpalli *et al.,* 1992).

Wild species of pigeonpea

Within the genus *Cajanus* there are 32 species (van der Maesen 1986) with the majority from India and Australia and these species have been found to have the same chromosome number as the cultivated type (Dundas 1990). Several wild relatives of pigeonpea are cross compatible and produce fertile hybrids with pigeonpea (Deodikar and Thakar 1956). The transfer of new genes from other *Cajanus* species is well- advanced, with 11 wild species successfully crossed with *C. cajan*. High seed protein content has already been transferred to the cultivated type from *C. albicans*, *C. sericeus* and *C. scarabaeoides* var. Scarabaeoides. Difficulties in producing hybrids between pigeonpea and wild species have been encountered especially with *C. platycarpus*, and *C. crassus* var. crassus. *Cajanus platycarpus* is of great potential value for pigeonpea improvement possessing characters such as resistance to Fusarium wilt (*Fusarium udum* Butler) and Phytopthora blight (*Phythopthora drechsleri* Tucker f. sp. *cajani*), early maturity and annuality. A sectional division of the genus groups the species into six sections. Section *Cajanus* contains pigeonpea, and its closest relative *C. cajanifolius*. Sections *Atylia* Benth. and *Fruticosa* van der

Maesen include the remaining erect species, sections *Cantharospermum* (W. & A.) Benth. and *Volubilis* van der Maesen cover the climbing species, while section *Rhynchosoides* Benth. has three trailing species. The genus *Cajanus* is distributed in the old world, with 18 species in Asia, 15 in Australia, and one in Africa. Remarkable homology between pigeonpea and wild species confirms cogenericity. Several workers have attempted to rank the wild relatives of pigeonpea according to the closeness of their relationship to the cultivated type. Pundir and Singh (1985a, b) have assessed details of plant morphology, karyotype, crossability, meiotic behavior, and seed protein profile of nine related species. The study has shown that *C. cajanifolius* is the most closely related species of pigeonpea, followed by *C. lineatus*. Kollipara *et al.,* (1994) have reported the genetic variation of trypsin and chymotrypsin inhibitors in pigeonpea and its wild relatives. The migration pattern of the two major inhibitors indicate that *C. cajanifolius* is genomically closest to pigeonpea. A high degree of homology between chromosomes, among species which hybridize, has been reported (Deodikar and Thakar 1956; Roy and De 1965; Sikder and De 1967; Reddy 1981a; Dundas *et al.,* 1983). Nadimpalli *et al.,* (1992) have reported use of RFLP in the phylogenetic studies of pigeonpea and its wild relatives. Some of the *Rhynchosia* species are reported to be morphologically similar to pigeonpea. The species *Rhynchosia acutifolia* has now merged in genus *Cajanus* as *Cajanus acutifolius* (Yadavendra 1986). Similarly one species of *Dunbaria* has merged with genus *Cajanus* as *Dunbaria heyei*. The genera *Dunbaria, Flemingia* & *Paracalyx* have been found to be useful in qualitative characters.

Wild relatives of pigeonpea serve as an important source of disease and insect resistance that can be introgressed into the cultivated genotype. For example, the wild species *C. sericeus* has the elite character of resistance to sterility mosaic diseases while *C. scarabaeoides* has resistance to pod borer, *Helicoverpa armigera* (Remanandan 1990). Desirable traits available in readily crossable species include male sterility, stem blight resistance, high protein content, photoperiodic insensitivity and

resistance to pod borer, pod fly and bruchids (Remanandan, 1990). In addition to agriculturally valuable traits, wild species exhibit many properties which are important for survival in their natural environment.

Genome analysis of pigeonpea using DNA markers

Although pigeonpea is a very important legume crop, no DNA data were available till 1987. It was for the first time when Dabak *et al.*, (1988) carried out molecular characterization of pigeonpea genome with special emphasis to base composition, reassociation kinetics and restriction endonuclease cleavage analysis. During this analysis, pigeonpea genome revealed the presence of *Mob* I, *Hae* III and *Alu* I repeat families. Later Ranade *et al.*, (1989) identified the dispersed *Mbo* I repeat family in pigeonpea and a few other plant genomes.

The first report on the use of molecular markers was that of Nadimpalli *et al.*, (1992) where RFLP probes were used for studying the phylogenetic relationship of wild relatives of pigeonpea. It was further shown that the polymorphism was high among wild relatives as compared to that observed in few pigeonpea accessions. Recently Shivaramkrishnan *et al.*, (1997) reported the utility of RFLP for the study of cytoplasmic male sterility in pigeonpea. However there were no reports on the application of PCR based DNA markers in pigeonpea and hence the work initiated to study genetic variability in pigeonpea using PCR generated DNA markers in 34 pigeonpea accessions and 13 wild relatives.

The specific objectives of the study were:

(1) To identify pigeonpea accessions using RAPDs and arbitrary primed PCR.

(2) To assess genetic variability among accessions from India and from different geographic regions and to classify into different groups.

(3)To determine phylogenetic relationship among wild relatives of pigeonpea representing the genus *Cajanus*, *Rhynchosia*, *Flemingia* and *Dunbaria*

(4) To identify species and genus specific markers using RAPD.

(5) To isolate vicilin gene using PCR based approach and its application in the variability studies of pigeonpea and its wild relatives.

For convenience the results in this chapter are grouped in two sections. Section A includes the data on pigeonpea cultivars within India and from different geographical regions while section B pertains to the characterization of wild relatives of pigeonpea.

MATERIALS AND METHODS

Plant materials

Pigeonpea cultivars 'PT-14', 'T-21', 'ICPL75-125', 'TAT-14', 'BAHAR', 'MTH-12', 'ICPL-87', 'PT-31' and 'C-11' were from the Pulse Research Center, Mahatma Phule Agricultural University, Rahuri. The cultivars listed in Table 1 were obtained from the International Crops Research Institute for Semi Arid Tropics, ICRISAT, Patancheru, India. The wild relatives of pigeonpea listed in Table 2 and 3 were also provided by ICRISAT, Patancheru, India.

DNA extraction:

Plant DNA was extracted from young leaves by CTAB method (Rogers and Bendich, 1988) with a few modifications. Young leaf tissue was ground in liquid nitrogen and mixed with 15 ml of CTAB extraction buffer (2% hexadecyltrimethyl ammonium bromide, 100mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 1% Polyvinyl pyrrolidone). The homogenate was then incubated at 60°C for 15 minutes, emulsified with an equal volume of chloroform: isoamyl alcohol mixture (24:1) and centrifuged at 10,000 rpm for 10 minutes. To the supernatant an equal volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0) was added, mixed gently and centrifuged at 10,000 rpm for 10 minutes. The pellet was dissolved in high salt TE buffer (1M NaCl, 10mM Tris-HCl, pH 8.0, 1mM EDTA) and was precipitated with twice the volume of chilled ethanol. The precipitate was washed with 70 % ethanol, dried and redissolved in TE buffer. DNA was quantified in a fluorometer (Hoefer instrument model TKO-

100) according to the supplier's instructions.

PCR primers

Table 4 lists the primers used for arbitrary primer PCR. Apart from these primers, RAPD primers (Kit A) from Operon Technologies was also used for the analysis.

PCR amplifications

Randomly amplified polymorphic DNA (RAPD)

PCR amplification was performed as described by Williams *et al.*, (1990) with a set of twenty oligonucleotides from kit A (OPA1-OPA20) synthesized by Operon Technologies Inc. (Almeda, California, USA). The reaction mixture (25 µl) contained 10 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 100 µM each of dATP, dTTP, dCTP and dGTP (Pharmacia), 5 pmoles of primer, 15 ng of genomic DNA and 0.3 units of *Taq* polymerase. Each reaction was overlaid with 100 µl of mineral oil to prevent evaporation. Amplification was carried out in a Perkin Elmer 9600 thermocycler for 45 cycles, each consisting of a denaturation step of 1 minute at 94°C, followed by an annealing step of 1 minute at 36°C and an extension of 2 minutes at 72°C using the fastest available temperature transitions. The last cycle was followed by 5 minutes at 72°C to ensure that the primer extension reaction was completed. To reduce the possibility of cross contamination and variation in the amplification reaction, mastermixes were always used.

Arbitrary Primed PCR (AP-PCR)

For 20 mer primers the PCR amplification conditions were: denaturation for 1 minute at 94°C, annealing at 42°C for 1 min., extension at 72°C for 2 min. for 2 cycles followed by 1 min. at 94°C, 1 min. at 55°C, and 2 min. at 72°C for 40 cycles. The last cycle was followed by 5 min. at 72°C. The reaction mixture (25 µl) contained 10 mM Tris HCl, pH 8.3, 50 mM KCl,

1.5 mM $MgCl_2$, 0.001% gelatin, 100 μM each of dATP, dTTP, dCTP and dGTP (Pharmacia), 10 pmoles of primers, 25 ng of genomic DNA and 0.5 units of *Taq* polymerase. Each reaction was overlaid with 100 μl of mineral oil to prevent evaporation. Primers listed in Table 7 (A1 to A4) were used for the AP-PCR amplifications.

Agarose gel electrophoresis

The RAPD products (25) μl were loaded on 1.4 % agarose gel for electrophoresis in 1 X TBE (89 mM Tris-HCl, pH 8.3, 89 mM boric acid, 5 mM EDTA) at 50 V for 6 h, stained with ethidium bromide and visualized by illumination under UV light.

Data analysis

Pairwise comparisons of accessions based on the presence or absence of unique and shared fragments produced by PCR amplifications were used to generate a similarity matrix using the formula of Nei and Li (1979) and was used to construct a dendrogram by an unweighted pair group method with arithmetical averages (UPGMA; Sneath and Sokal, 1973). A Multidimensional Scaling (MDS) procedure of SAS (SAS Institute, 1988) was employed to gain a view of the relative distances between the accessions in two dimensional space.

Table 1

Pigeonpea accessions from different geographic regions

No.	Cultivars	Origin
A	ICP 125-1	India
B	PT 37	Maharashtra, India
C	PT 25	Maharashtra, India
D	BDN 1	Maharashtra, India
E	UQ-60	India
F	JA 274	Madhya Pradesh, India
G	Anm 449	Bihar, India
H	Khandwa Local-153	Madhya Pradesh, India
I	ICP 8094	Nigeria
J	EC 109884	Sri Lanka
K	N-291	Uttar Pradesh, India
L	P 3203	Uttar Pradesh, India
M	R-60-187-2	Madhya Pradesh, India
N	Granada-1	Peru
O	DSLR 17	India
P	Norman	USA
Q	Brazil-465	Brazil
R	Kanpur local	Uttar Pradesh, India
S	UC 1381-1	Nigeria
T	Prabhat	New Delhi, India
U	Code No 2	Trinidad & Tobago
V	EC-109890	Sri Lanka
W	EC-109893	Sri Lanka
X	ST-1515-186-1	Bihar, India

Table-2

Wild relatives of pigeonpea

Serial no.	Species	Accession no.	Origin
1	*Cajanus acutifolius*	IBS 2419	Australia
2	*Cajanus albicans*	JM 3023	Karnataka, India
3	*Cajanus goensis*	JM3501	Kerala, India
4	*Cajanus grandifolius*	PR 4256	Australia
5	*Cajanus lineatus*	PR 5227	Kerala, India
6	*Cajanus reticulatus*	IBS2156	Australia
7	*Cajanus sericeus*	JM 1961	Maharashtra, India
8	*Cajanus volubilis*	JM4208	Burma
9	*Dunbaria ferruginea*	PR 5709	Tamilnadu, India
10	*Rhynchosia rothii*	JM 3410	Tamilnadu, India
11	*Rhynchosia bracteata*	JM 3952	Andhra Pradesh, India
12	*Rhynchosia melacophylla*	ICPW 295	Unknown*
13	*Flemingia stricta*	NKR 186	Andhra Pradesh, India
14	*Cajanus cajan*-1	ICPL 8094	India
15	*Cajanus cajan*-2	ICPL 317	India

* Source ICRISAT

Table 3

Wild relatives used for genus and species specific markers

Serial no.	Species	Accession no.	Origin
1	*Cajanus cajan*-1	ICP6443	India
2	*Cajanus cajan*-2	ICP7220	India
3	*Cajanus albicans*-1	ICPW22	India
4	*Cajanus albicans*-2	ICPW25	India
5	*Cajanus goensis*-1	ICPW32	India
6	*Dunbaria ferruginea*-1	ICPW176	India
7	*Dunbaria ferruginea*-2	ICPW178	India
8	*Rhynchosia rothii*-1	ICPW256	India
9	*Rhynchosia rothii*-2	ICPW258	India
10	*Rhynchosia bracteata*-1	ICPW214	India
11	*Rhynchosia bracteata*-2	ICPW215	India
12	*Flemingia stricta*-1	ICPW202	India
13	*Flemingia stricta*-2	NKR186	India

Table 4

Vicillin gene amplification primers

Primer No.	Sequence
1	CCATCCATCCAGAGTACTAC
2	CACTACGGACAACTAGGAGT
3	CTCTCCATGAGCTGTTACAC
4	CGGACAATGTCATAAGCAGC
5	ATACCTGCAAGGAGGTTCCT
6	TTCTAACCAAGACGACAGTG
7	ACATTGAAGGAGGCCTCTAG
8	GGCAGTACATGAATCTGAGG
9	CTATCTAGCACAGAAGCCCA
10	TTCGAGGAGATCAACAGGGT
11	GGAGCTCTCTTTTTGTGCCAC
12	CGCCATACTCGTCTTGGTGG

RESULTS

SECTION A

Genetic fingerprinting of pigeonpea accessions and their relationships

Optimization of amplification conditions

Reproducibility of RAPD products appears to be highly dependent on several parameters such as primer to template concentration ratio, annealing temperature, and magnesium concentration. Factors affecting the RAPD amplifications were studied in pigeonpea and these are summarized as follows:

(1) The annealing temperature was especially critical. Below 35°C, many bands were amplified, however, they were difficult to reproduce in replicated experiments. The number of bands decreased when temperature was raised above 40°C. Annealing temperature above 45°C did not give any amplification. For all the experiments, annealing temperature of 36°C was used.

(2) One of the most important variables is the concentration of genomic DNA. Since different DNA extraction methods produce DNA of widely different purity, it is necessary to optimize the amount of DNA used in the reaction. Assays to optimize the template concentration were conducted over a range of 0.5 to 200 ng. A constant band pattern was obtained at a template concentration between 5 and 30 ng. Ultimately a template DNA concentration of 15ng/25µl was selected for PCR amplification.

(3) Use of *Taq* polymerase above 2 units/reaction increased the background smear. Finally a ratio of 0.3 units/reaction was used for PCR amplifications.

(4) Another important variable was the concentration of magnesium which affected both, the polymerase activity and the efficiency of annealing. Different concentrations of $MgCl_2$ have been successfully employed on DNA from the same species. In this study, a low magnesium concentration (1.5 mM) in the PCR reaction was found to be optimal for the purpose of producing clear and reproducible DNA fingerprints. As the magnesium concentration was increased, some bands were amplified more efficiently while others were amplified less efficiently.

(5) Primer concentration of 5 pmoles was selected for the amplification. At lower concentration, it became difficult to detect the amplification

products in the stained agarose gel and at higher concentration smearing of the bands was observed.

(6) No effect of dNTP concentration on the relative intensity of the amplified band was observed and therefore 100 μM each of dATP, dTTP, dCTP and dGTP was used for PCR amplifications.

To determine the degree of heterogeneity within the population, DNA was extracted from different individuals of the same accession and used for PCR amplifications. The RAPD pattern was found to be identical in all cases. Successive amplifications were performed routinely on DNA extracted from leaves pooled from at least ten individuals of the same accession. Fragments with identical mobility were considered to be the same fragment. Figure 1 shows a representative picture of PCR amplification using primer OPA-1. The co-migrating bands provided an internal control to monitor the reproducibility of the amplification patterns.

Identification of pigeonpea accessions

Ten elite cultivars of pigeonpea, as mentioned in 'Plant materials' of 'Material and Methods' section, that are widely cultivated in India were selected for identification at DNA level. In an attempt to examine the potential of RAPD markers for their ability to identify pigeonpea cultivars, a set of primers (OPA kit from Operon Technologies) was used to amplify DNA from different accessions of pigeonpea. In total, 16 primers were found to be polymorphic, while 3 primers were monomorphic and the other primers did not show amplification. The number of bands for each primer which produced a polymorphic band pattern varied from 3 (OPA-6) to 11 (OPA-18) in the size range of 0.25 to 3 kb. Few primers amplified only one or two monomorphic bands among different cultivars. These primers appeared to amplify conserved sequence which existed in all the pigeonpea cultivars examined.

The choice of primers selected in the present study was based on the ability of a primer to produce polymorphic bands as well as many non-variant bands among different genotypes suggesting that amplification was

consistent over a wide range of genotypes. Easily detectable and well resolved bands were those which were reproducible over repeated runs with sufficient intensity to determine presence or absence of bands. Quantitative differences in band intensity were also observed. Since the reproducibility of quantitative differences might not be consistent, only the presence of band versus absence were analyzed in the study. Different fingerprints were obtained when different primers were used on the same individuals and no bands were produced in the control experiment with no template DNA. Some amplified bands were common to all individuals, while others were present in some individuals and were thus informative. Bands of high (>3 kb) and low (< 0.2 kb) molecular weight were not scored since they could not be reproducibly amplified in replicated experiments.

Since RAPD markers are dominant, a locus was considered to be polymorphic if the presence and absence of the band were observed in various individuals and monomorphic if the bands were present among all individuals. A representative electrophoretic pattern of PCR-amplified DNA fragment of pigeonpea cultivars using primer OPA-13 is shown in Figure 2. In general, no single primer was able to distinguish between all the cultivars. However, amplifications by different primers were informative and produced a cultivar-specific pattern. The marker is designated as the Operon primer number, followed by molecular weight in base pairs of the amplified band. The markers OPA1-610, OPA2-1090, OPA6-872, OPA7-1904, OPA11-603, OPA12-2027, OPA12-584, OPA15-603 and OPA17-400 were unique to different individuals. One fragment, OPA11-320 was unique to two cultivars, namely 'ICPL-87'and 'C-11'. Fragments OPA15-900 and OPA12-872 were specific to 'PT-31' and 'DPPA85-12'. Other fragments were present in more than two cultivars and were still polymorphic. The reproducibility of these results was evaluated by replicating the RAPD analysis on all the accessions with primer OPA-11, OPA-13 and OPA-18. Table 5 gives the details of the polymorphic bands specific to pigeonpea cultivars.

Figure 1: Agarose gel electrophoretic pattern of PCR amplification products obtained with different pigeonpea cultivars using primer OPA-1. M represents the phi X 174 *Hae* III marker. Lanes 1-10 represent pigeonpea cultivars PT-14, T-21, PT-31, DPPA 85-12, ICPL 75-125, TAT-14, BAHAR, MTH-12, ICPL-87 and C-11, respectively.

Figure 2: PCR amplification products obtained with different pigeonpea cultivars using primer OPA-13. The sizes of the marker bands are given for comparison. Lanes 1-10 represents pigeonpea cultivars PT-14, T-21, PT-31, DPPA 85-12, ICPL 75-125, TAT-14, BAHAR, MTH-12, ICPL-87 and C-11, respectively.

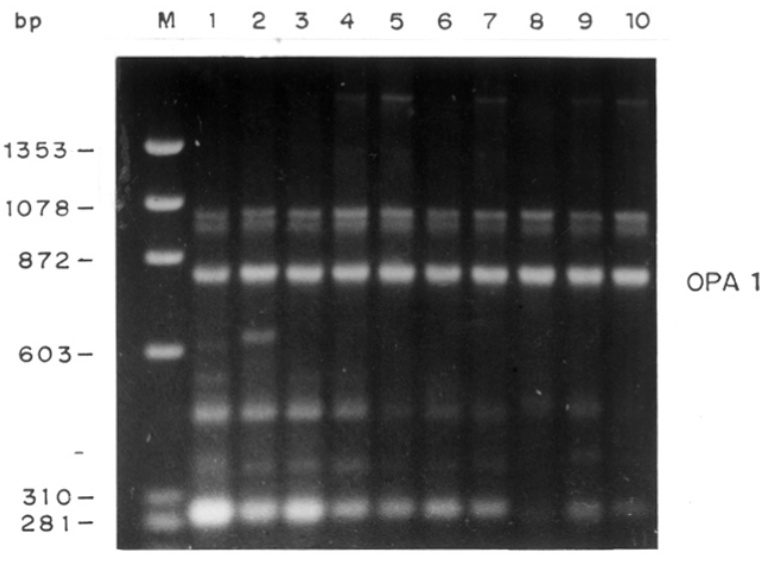

bp
M 1 2 3 4 5 6 7 8 9 10
1353 –
1078 –
872 –
603 –
310 –
281 –
OPA 1

Fig. 1

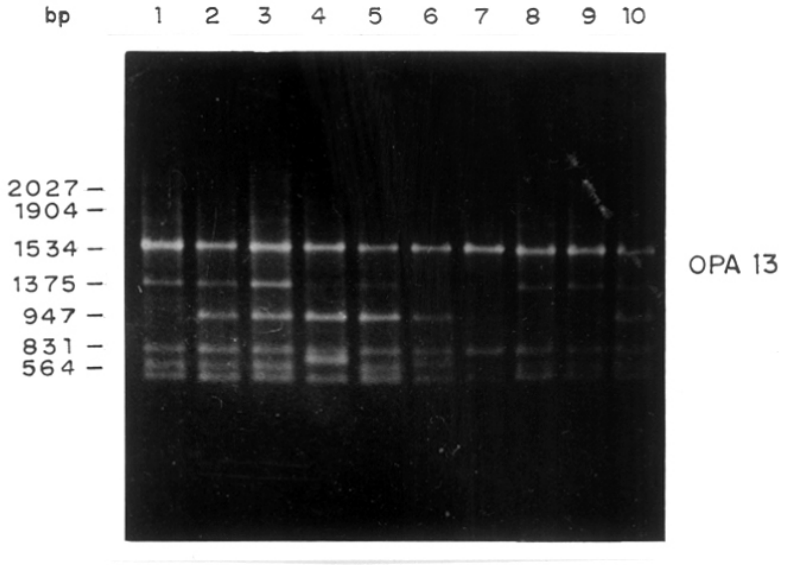

bp
1 2 3 4 5 6 7 8 9 10
2027 –
1904 –
1534 –
1375 –
947 –
831 –
564 –
OPA 13

Fig. 2

TABLE 5

Pigeonpea cultivar specific DNA markers

Cultivar	Marker
T-21	OPA1-610
PT-14	OPA2-1090
BAHAR	OPA6-872
ICPL75-125	OPA7-1904
PT-31	OPA11-603
ICPL-87	OPA12-2027
MTH-12	OPA12-584
C-11	OPA15-603
TAT-14	OPA17-400
EC-109893	OPA1-1078
P-3203	OPA5-1353
EC-109884	OPA11-1078
ICP-8094	OPA14-300
EC-109890	OPA16-1078
Code No. 2	OPA16-603
N-291	OPA17-1375
ST-1515-186-1	OPA10/20-224
R-60-187-2	OPA11/12-271

Genetic similarity within pigeonpea cultivars from India

For this analysis 10 pigeonpea cultivars were subjected to PCR amplifications with 16 RAPD primers. Pairwise comparisons were made between all the genotypes and the average genetic similarity obtained with each primer was estimated. The similarity index was calculated as $F=2N_{ab}/(N_a+N_b)$ where N_{ab} is the number of bands shared in common between individuals A and B while N_a and N_b are the total number of bands observed in lane A and in lane B, respectively (Nei and Li, 1979). All the accessions fell in the range of 0.7-0.9 (Table 6) indicating little polymorphism at the DNA level among various accessions. This may be due to the predominantly self-pollinating nature of pigeonpea, although some degree of outcrossing has been reported. A dendrogram constructed from the similarity index values for 10 accessions from Indian origin is shown in Figure 3. It indicates three different clusters which are further divided into subgroups. The first cluster consisted of Bahar and DPPA 85-12, while the second cluster included C-11, ICPL 75-125 and T-21. ICPL-75-125 and T-21 form a subgroup and are more closely related to each other than to C-11. The third cluster shows two subgroups; the first consists of TAT-14 and PT-31 while the other subgroup consists of ICPL-87, MTH-12 and PT-14. In the latter subgroup MTH-12 and PT-14 are the most closely related on the basis of similarity, 0.91.

Identification of pigeonpea cultivars from different geographic regions

To study the genetic similarity between diverse pigeonpea accessions, 24 cultivars (Table 1) were collected from different geographical regions representing different countries. These genotypes show large amount of morphological variation and have important agronomic characters. All the primers gave many amplification products in the range of 3-11. Details of the 23 primers that were selected and also the number of bands amplified for all cultivars with each primer are shown in Table 7. A total of 168 different RAPD products were amplified. The extent of similarity between individual accessions was primer dependent. For example, two cultivars Prabhat and

Table 6.

Similarity matrix among pigeonpea cultivars from Indian origin.

	C-11	ICPL-87	MTH-12	BAHAR	TAT-14	ICPL-75-125	DPPA-85-12	PT-31	T-21	PT-14
PT-14	0.79	0.87	0.91	0.72	0.79	0.76	0.72	0.78	0.76	1
T-21	0.80	0.76	0.76	0.71	0.76	0.88	0.71	0.77	1	
PT-31	0.75	0.77	0.78	0.72	0.84	0.76	0.70	1		
DPPA-85-12	0.71	0.73	0.74	0.85	0.71	0.72	1			
ICPL-75-125	0.79	0.75	0.76	0.72	0.75	1				
TAT-14	0.74	0.76	0.79	0.70	1					
BAHAR	0.72	0.72	0.71	1						
MTH-12	0.76	0.87	1							
ICPL-87	0.76	1								
C-11	1									

Figure 3

Dendrogram constructed from the similarity index values for different pigeonpea cultivars from India.

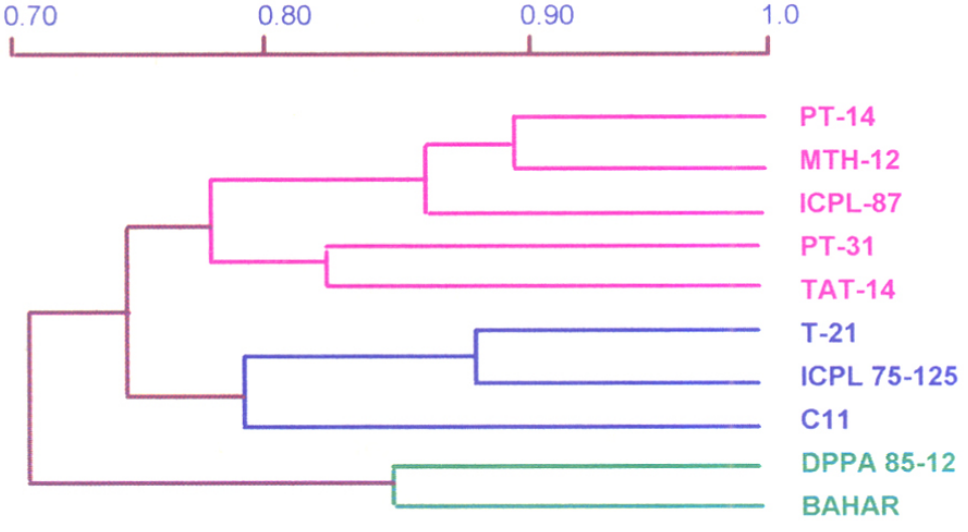


Table 7 Number of polymorphic bands among pigeonpea cultivars from different geographic regions

Primer	Nucleotide sequence	No. of Bands	No. of Poly. bands
OPA-1	CAGGCCCTTC	9	4
OPA-3	AGTCAGCCAC	8	3
OPA-4	AATCGGGCTG	3	2
OPA-5	AGGGGTCTTG	8	4
OPA-7	GAAACGGGTG	5	3
OPA-8	GTGACGTAGG	6	3
OPA-10	GTGATCGCAG	8	4
OPA-11	CAATCGCCGT	7	3
OPA-14	TCTGTGCTGG	9	5
OPA-16	AGCCACCGAA	10	6
OPA-17	GACCGCTTGT	6	3
OPA-18	AGGTGACCGT	11	4
OPA-20	GTTGCGATCC	5	3
OPA 5+17	AGGGGTCTTG + GACCGCTTGT	4	3
OPA 10+20	GTGATCGCAG + GTTGCGATCC	9	4
OPA 11+12	CAATCGCCGT + TCGGCGATAG	8	3
OPA 14+19	TCTGTGCTGG + CAAACGTCGG	11	6
OPA 15+20	TTCCGAACCC + GTTGCGATCC	8	4
OPA 16+20	AGCCAGCGAA + GTTGCGATCC	11	6
20 mer A1	CTCTCCATGAGCTGTTACAC	8	4
20 mer A2	CGGACAATGTCATAAGCAGC	5	3
20 mer A3	ATACCTGCAAGGAGGTTCCT	4	1
20 mer A4	TTCTAACCAAGACGAACATC	5	2

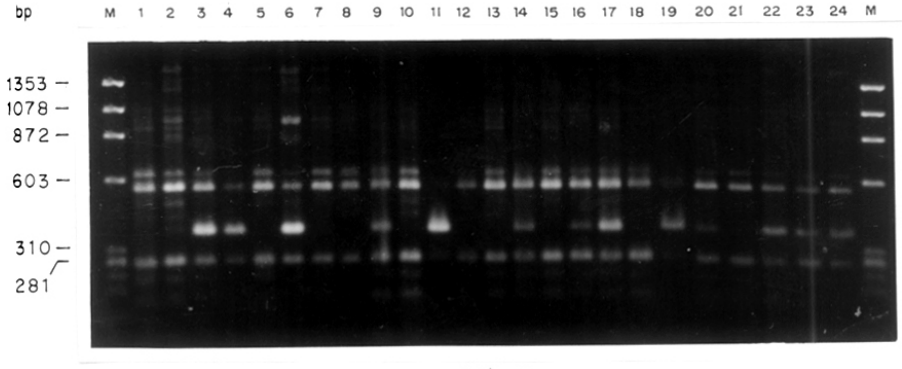
EC-109890 had only one band in common when primer OPA-14 was used, whereas the extent of similarity was higher when primer OPA-17 was used. Comparisons of band patterns among 24 pigeonpea accessions indicated discrimination of all the cultivars where many cultivar specific bands were also amplified. The bands OPA1-1078, OPA5-1353, OPA11-1078, OPA14-300, OPA16-1078, OPA16-603, OPA17-1375, OPA11\12-271, and OPA10\20-224 were unique to different individuals. Table 5 shows various markers specific for pigeonpea accessions.

Two primer PCR for genetic fingerprinting

All the 24 genotypes from different geographic regions were subjected to two primer PCR analysis. Some oligonucleotides primed the amplification of only a few bands from pigeonpea when single primers were used; however, when these primers were used in pairs, several new bands were obtained. When data for each primer were analyzed, the primer combination OPA5\OPA17, OPA10\OPA20, OPA11\OPA12, OPA14\OPA19, OPA15\OPA20 and OPA16\OPA20 produced unique patterns for all the 24 individuals. Figures 4-7 show typical electrophoretic patterns using primers OPA5\OPA17, OPA14\OPA19, OPA15\OPA20 and OPA16\OPA20 respectively. For detecting polymorphism between closely related cultivars, use of polyacrylamide gels was also tested. However, not much difference was visible on the gel except for a few minor bands. Since minor bands are more vulnerable to changes in reaction conditions, agarose gel electrophoresis was used for all the primers. In the two primer PCR, several new bands occurred in the low molecular weight region which allowed the differentiation of many closely related cultivars. This indicates that arbitrary primers can be used in pairs to amplify segments of genomic DNA in pigeonpea and are useful for detecting polymorphism.

Figure 4 : Agarose gel electrophoretic pattern of RAPD amplification products obtained with different pigeonpea cultivars using primer OPA5\OPA17. M represents the phi X 174 *Hae* III marker. Lanes 1-24 represent pigeonpea cultivars ST-1515-186-1, EC-109893, EC-109890, Code No. 2, Prabhat, UC 1381-1, Kanpur local, Brazil-465, P-3203, Norman, R-60-187-2, DSLR-17, Granada-1, N-291, EC-109884, ICPL-8094, Khandwa-local-153, ANM-449, JA-274, UQ-60, BDN-1, PT-25, PT-37, ICPL-125-1, respectively.

Figure 5 : PCR amplification products obtained with different pigeonpea cultivars using primer OPA14\OPA19. Lanes 1-24 represent pigeonpea cultivars ST-1515-186-1, EC-109893, EC-109890, Code No. 2, Prabhat, UC-1381-1, Kanpur local, Brazil-465, P-3203, Norman, R-60-187-2, DSLR-17, Granada-1, N-291, EC-109884, ICPL-8094, Khandwa local-153, ANM-449, JA-274, UQ-60, BDN-1, PT-25, PT-37, ICPL-125-1, respectively.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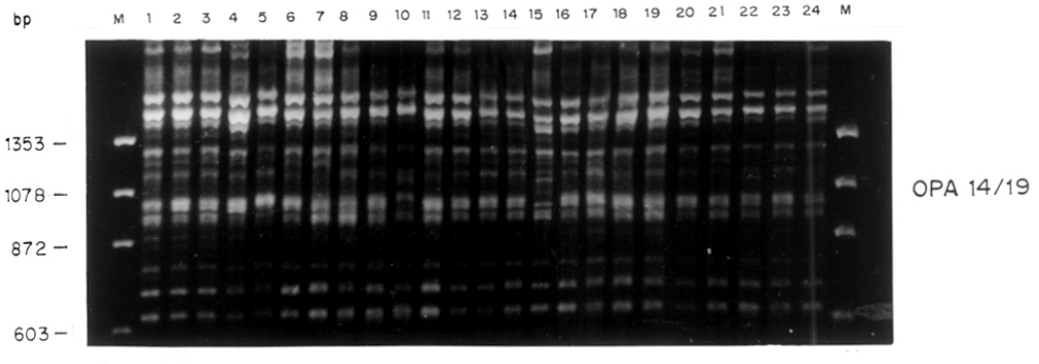
1353
1078
872
603
310
281
OPA 5/17

Fig.4


bp
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1353
1078
872
603
OPA 14/19

Fig.5

Fig. 6 : Agarose gel electrophoretic pattern of RAPD amplification products obtained with different pigeonpea cultivars using primer OPA 15\OPA20. M represents the phi X 174 *Hae* III marker. Lanes 1-24 represent pigeonpea cultivars ST-1515-186-1, EC-109893, EC-109890, Code No. 2, Prabhat, UC 1381-1, Kanpur local, Brazil-465, P-3203, Norman, R-60-187-2, DSLR-17, Granada-1, N-291, EC-109884, ICPL-8094, Khandwa-local-153, ANM-449, JA-274, UQ-60, BDN-1, PT-25, PT-37, ICPL-125-1, respectively.

Fig. 7 : PCR amplification products obtained with different pigeonpea cultivars using primer OPA 16\OPA20. Lanes 1-24 represent pigeonpea cultivars ST-1515-186-1, EC-109893, EC-109890, Code No. 2, Prabhat, UC 1381-1, Kanpur local, Brazil-465, P-3203, Norman, R-60-187-2, DSLR-17, Granada-1, N-291, EC-109884, ICPL-8094, Khandwa local-153, ANM-449, JA-274, UQ-60, BDN-1, PT-25, PT-37, ICPL-125-1, respectively.

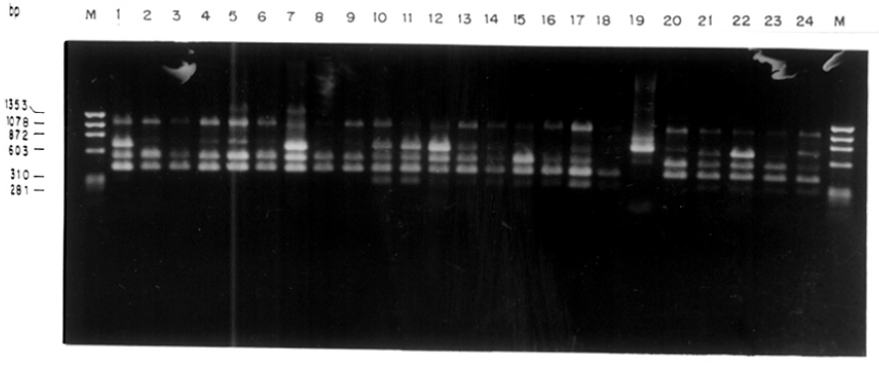

bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 M
1353
1078
872
603
310
281
OPA 15/20

Fig.6

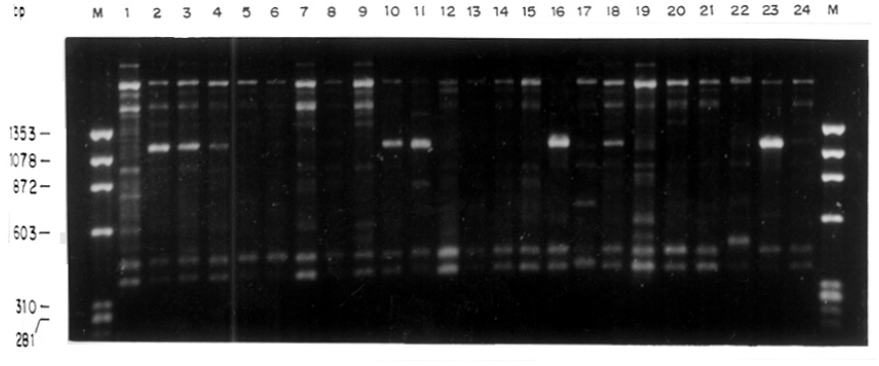

bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 M
1353
1078
872
603
310
281
OPA 16/20

Fig.7

Genetic similarity among accessions from different geographical regions

The similarity index was calculated for the cultivars from different geographic origins and their genetic relationship was estimated (Table 8). The largest genetic distance value was observed between the genotype Khandwa local-153 and EC-109890; whereas, the smallest genetic distance value was observed between PT-25 and PT-37. The genetic distance obtained for each pairwise combination among 24 genotypes was used to construct a dendrogram (Figure 8). Cluster analysis indicated that there was low level of diversity among the pigeonpea cultivars used in this study, which was expected based on the self pollinating nature of pigeonpea. Although many small groups are formed, three major groups can be considered. 'Khandwa local-153' was the most distinct cultivar. Many Indian cultivars grouped together, however, genotype 'R-60-187-2' and 'P-3203' showed the greatest similarity to genotype 'Granada-1' which was the only other genotype from Peru. The Indian genotypes 'PT-25' and 'PT-37' were similar whereas the genotype 'Kanpur local' showed more similarity with 'Brazil-465' than any other Indian genotypes. Cultivars 'EC-109884' 'EC-109890' and 'EC-109893' from Sri-Lanka showed more similarity to accessions from Indian origin.

Multidimensional scaling

Multidimensional scaling (MDS) was employed to gain a view of the relative distances between the accessions in two dimensional space (Fig. 9). From fig. 9 , it is clear that the accessions form one large group with no major divisions. Cultivar Khandwa local was observed to be the most distant in the multi dimensional scaling.

Table 8.

Similarity matrix for pigeonpea cultivars from different geographic regions. Lanes 1-24 indicate pigeonpea accessions ST-1515-186-1, EC-109893, EC-109890, Code No. 2, Prabhat, UC 1381-1, Kanpur local, Brazil-465, Norman, DSLR-17, Granada-1, R-60-187-2, P-3203, N-291, EC-109884, ICPL-8094, Khandwa local-153, ANM-449, JA-274, UQ-60, BDN-1, PT-25, PT-37, ICPL-125-1, respectively.

	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
1	0.80	0.76	0.72	0.77	0.79	0.80	0.77	0.71	0.77	0.76	0.74	0.77	0.80	0.78	0.72	0.84	0.81	0.88	0.87	0.87	0.80	0.80	0.84	1
2	0.74	0.71	0.73	0.79	0.83	0.76	0.79	0.75	0.79	0.81	0.72	0.75	0.78	0.72	0.84	0.85	0.79	0.81	0.88	0.84	0.84	0.84	1	
3	0.82	0.86	0.84	0.79	0.83	0.78	0.81	0.70	0.72	0.74	0.80	0.77	0.82	0.78	0.81	0.86	0.83	0.83	0.80	0.83	0.83	1		
4	0.80	0.75	0.73	0.77	0.84	0.72	0.77	0.72	0.74	0.77	0.77	0.81	0.85	0.80	0.85	0.82	0.83	0.82	0.81	0.88	1			
5	0.83	0.81	0.79	0.81	0.84	0.80	0.81	0.74	0.79	0.83	0.81	0.79	0.72	0.84	0.85	0.85	0.86	0.88	0.85	1				
6	0.78	0.75	0.73	0.80	0.84	0.72	0.78	0.75	0.79	0.81	0.75	0.77	0.80	0.76	0.83	0.82	0.83	0.81	1					
7	0.81	0.79	0.76	0.82	0.82	0.77	0.78	0.72	0.78	0.78	0.86	0.84	0.88	0.83	0.84	0.87	0.89	1						
8	0.85	0.83	0.80	0.87	0.86	0.79	0.82	0.73	0.80	0.84	0.82	0.82	0.89	0.85	0.86	0.85	1							
9	0.88	0.86	0.86	0.85	0.85	0.82	0.85	0.78	0.83	0.83	0.82	0.83	0.92	0.85	0.91	1								
10	0.87	0.87	0.87	0.84	0.82	0.83	0.84	0.80	0.86	0.84	0.86	0.88	0.89	0.87	1									
11	0.80	0.82	0.79	0.77	0.75	0.73	0.83	0.72	0.85	0.85	0.85	0.92	0.93	1										
12	0.82	0.80	0.79	0.81	0.79	0.77	0.81	0.74	0.85	0.86	0.90	0.93	1											
13	0.77	0.77	0.78	0.78	0.76	0.74	0.83	0.73	0.85	0.85	0.89	1												
14	0.78	0.79	0.82	0.80	0.78	0.73	0.80	0.74	0.82	0.86	1													
15	0.79	0.79	0.78	0.78	0.80	0.76	0.83	0.77	0.87	1														
16	0.81	0.81	0.80	0.76	0.76	0.72	0.83	0.78	1															
17	0.77	0.73	0.73	0.73	0.78	0.74	0.78	1																
18	0.84	0.84	0.85	0.81	0.85	0.84	1																	
19	0.80	0.78	0.79	0.75	0.79	1																		
20	0.89	0.82	0.81	0.89	1																			
21	0.84	0.88	0.86	1																				
22	0.86	0.95	1																					
23	0.91	1																						
24	1																							

Figure 8

Dendrogram constructed from the similarity index values for pigeonpea accessions from different geographical regions.

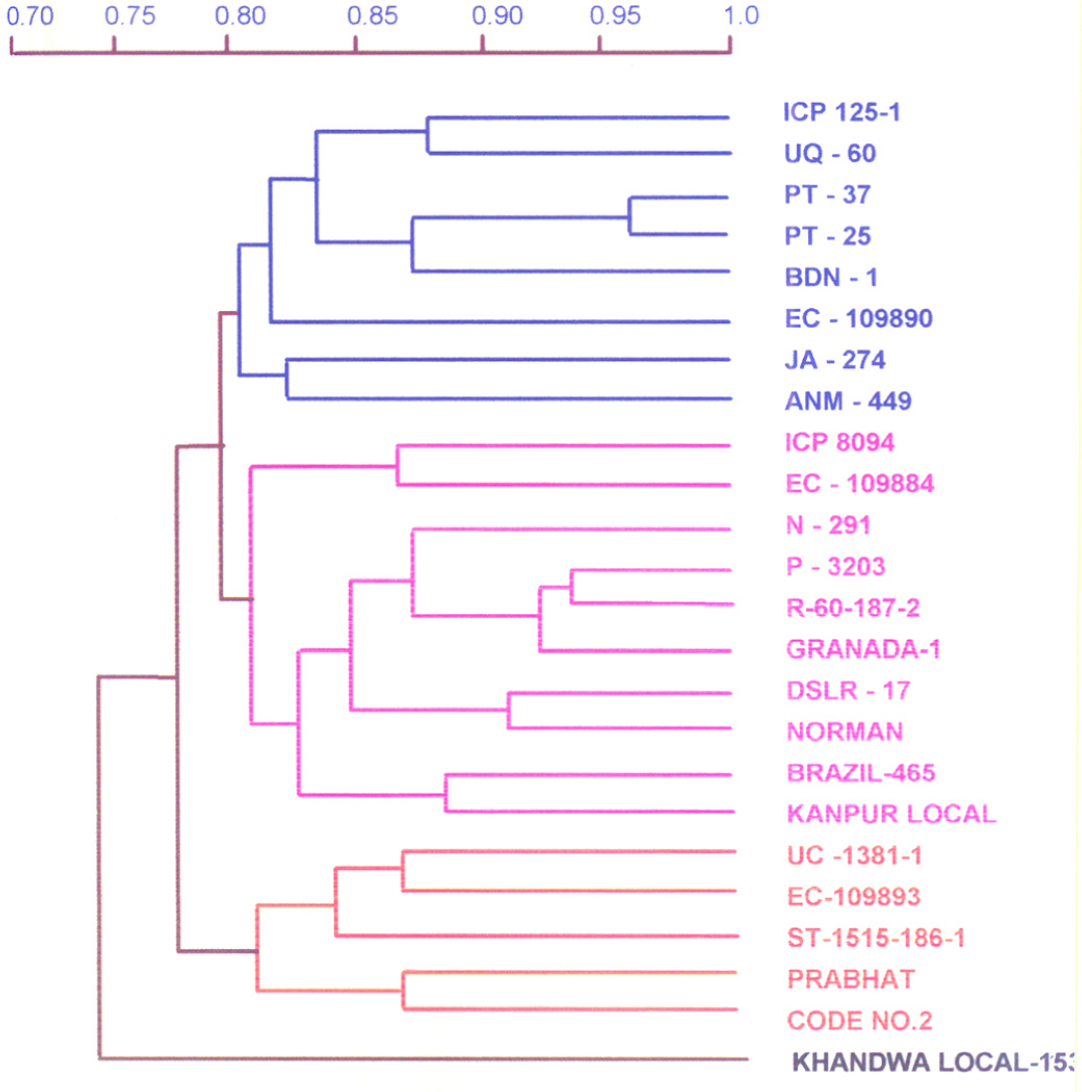


Fig 9: Multidimensional Scaling (MDS) of pigeonpea accessions from different geographic regions. A-X represent accessions as indicated in Table 1.

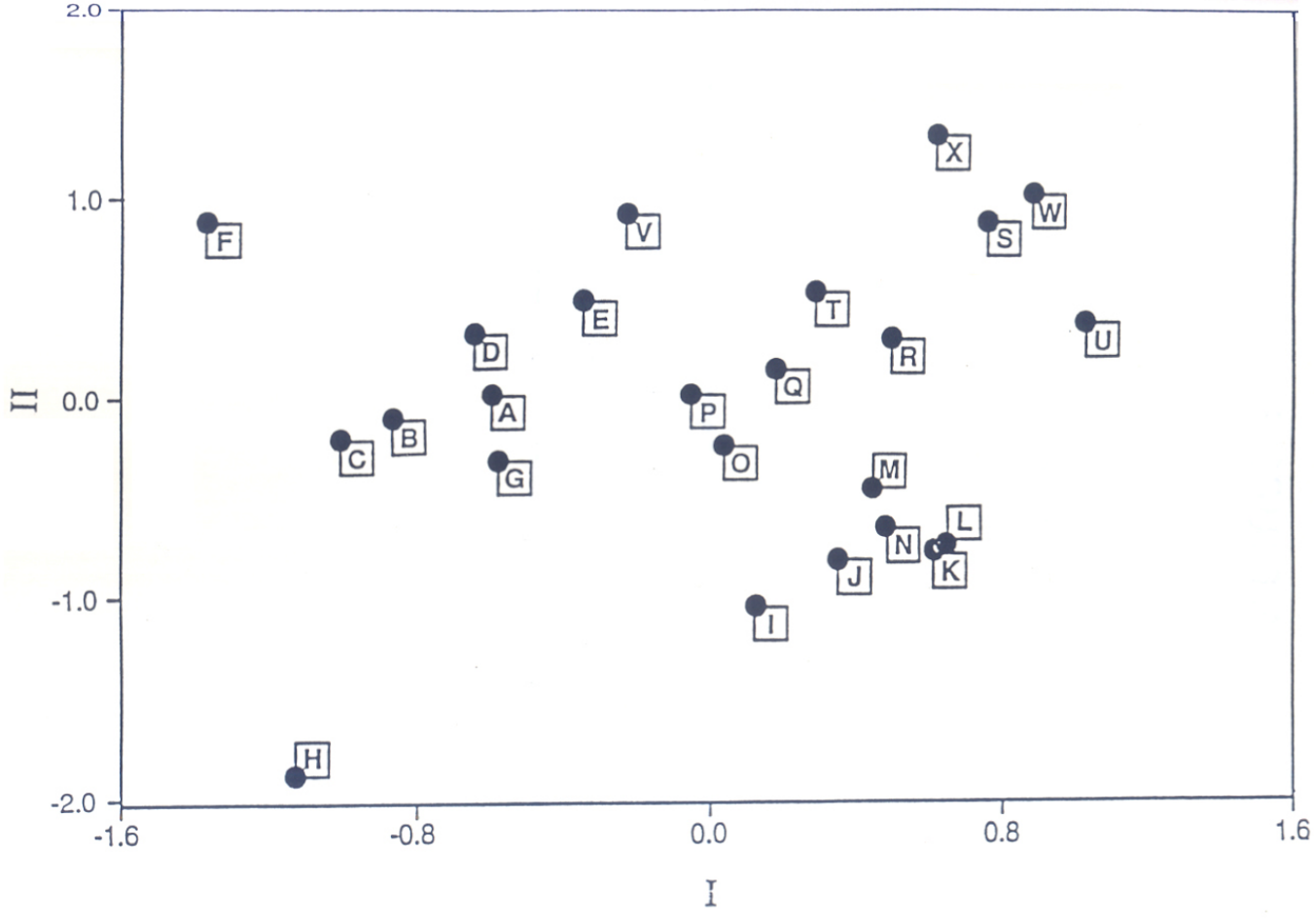


Fig 9

RESULTS

SECTION B

Molecular Characterization of wild relatives of pigeonpea using DNA Markers

Genetic variability among wild species of pigeonpea

Table 2 enumerates the wild relatives of pigeonpea representing the genus *Cajanus*, *Rhynchosia*, *Flemingia* and *Dunbaria*.. A total of 15 such genotypes were selected to examine the potential of RAPDs for detecting inter-specific and inter-generic variation.. Figures 10-11 show the electrophoretic pattern of PCR products amplified with primer OPA-11 and OPA-18 respectively. Unlike in pigeonpea cultivars, extensive polymorphism is detected among wild genotypes. From Fig. 10-11 it can be seen that each wild species has a distinct banding pattern distinguishing each genotype from the other. There are relatively few bands in each lane which are easily scorable. In Fig. 12, the bands in each lane are few and distinct and are better separated form each other as compared to Fig 10-11. A characteristic feature of Fig..13 is the presence of a band OPA3-500 which is specific to all the species in genus *Cajanus* and is absent in all the other genera.

The overall results of the RAPD data with wild pigeonpea genotypes can be summarized as follows:

(1) A total of 274 polymorphic RAPD products were produced by the PCR amplifications.

(2) Most of the primers generated polymorphic bands in the range of 10-25 except OPA 4 which resulted into 8 polymorphic bands.

(3) Table 9 gives the number of polymorphic bands amplified by various primers.

(4) Many bands were common between the species, while numerous unique bands were also obtained.

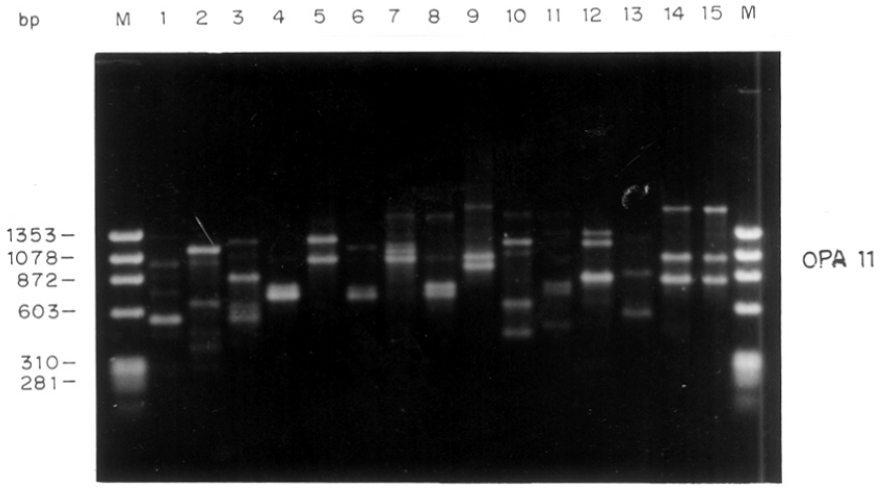
(5) Species could clearly be discriminated with as few as 1 well selected primer or with 5-10 randomly chosen fragments.

Phylogenetic relationships among wild relatives of pigeonpea

The similarity matrix among the wild relatives was generated on the basis of shared RAPD amplification products, which included information of all 274 bands. The similarity matrix between the wild species ranged from 0.22 to 0.88, indicating a large amount of genetic variation between the

Fig 10: Agarose gel electrophoresis of DNA fragments amplified from different wild relatives of pigeonpea using primer OPA-11. M represents the phi X 174 *Hae* III marker. Lanes 1-15 represent species *C. acutifolius, C. albicans, C. goensis, C. grandifolius, C. lineatus, C. reticulatus, C. sericeus, C. volubilis, D. ferruginea, R. rothii, R. bracteata, R. melacophylla, F. stricta*, *C. cajan*-1, *C. cajan*-2.

Fig 11: PCR amplified DNA fragments from different wild relatives of pigeonpea using primer OPA-18. Sizes of the marker bands are given for comparison. Lanes 1-15 represent species *C. acutifolius, C. albicans, C. goensis, C. grandifolius, C. lineatus, C. reticulatus, C. sericeus, C. volubilis, D. ferruginea, R. rothii, R. bracteata, R. melacophylla, F. stricta*, *C. cajan*-1, *C. cajan*-2.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1353
1078
872
603
310
281
OPA 11

Fig. 10

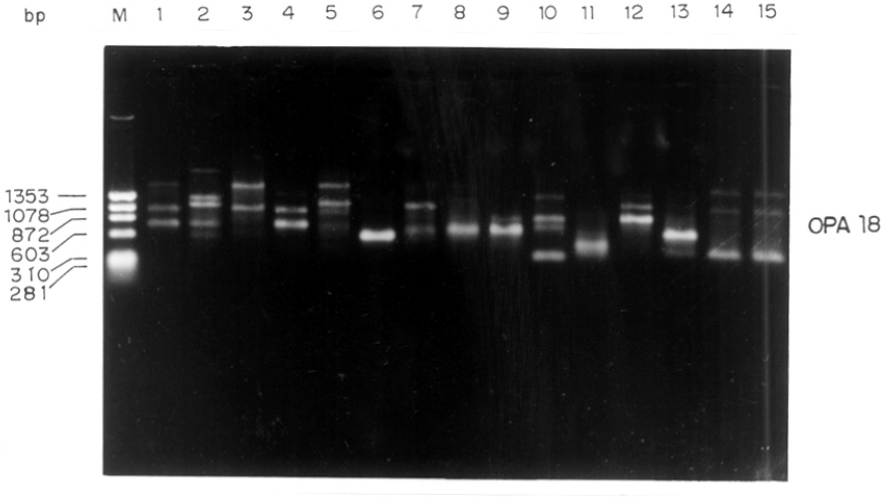

bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1353
1078
872
603
310
281
OPA 18

Fig. 11

Fig 12: Agarose gel electrophoresis of DNA fragments amplified from different wild relatives of pigeonpea using primer OPA-15. M represents the phi X 174 *Hae* III marker Lanes 1-15 represent species *C. acutifolius, C. albicans, C. goensis, C. grandifolius, C. lineatus, C. reticulatus, C. sericeus, C. volubilis, D. ferruginea, R. rothii, R. bracteata, R. melacophylla, F. stricta*, *C. cajan*-1, *C. cajan*-2.

Fig 13: PCR amplified DNA fragments from different wild relatives of pigeonpea using primer OPA-3. Sizes of the marker bands are given for comparison. Lanes 1-15 represent species *C. acutifolius, C. albicans, C. goensis, C. grandifolius, C. lineatus, C. reticulatus, C. sericeus, C. volubilis, D. ferruginea, R. rothii, R. bracteata, R. melacophylla, F. stricta*, *C. cajan*-1, *C. cajan*-2.

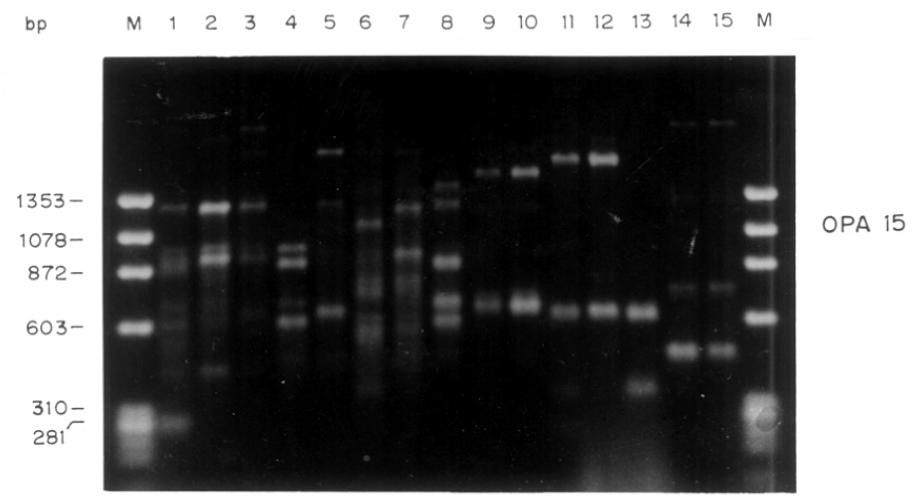

bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
1353
1078
872
603
310
281
OPA 15

Fig.12

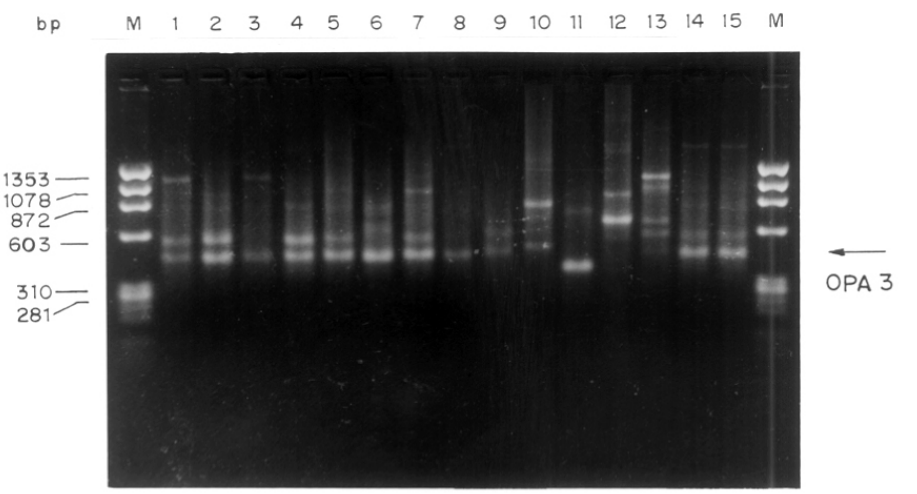

bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M
1353
1078
872
603
310
281
OPA 3

Fig.13

Table 9

Number of polymorphic bands among wild relatives of pigeonpea.

Primer	Number of polymorphic bands
OPA-1	12
OPA-3	11
OPA-4	8
OPA-5	13
OPA-6	14
OPA-7	22
OPA-8	25
OPA-10	18
OPA-11	15
OPA-12	16
OPA-13	12
OPA-15	21
OPA-16	25
OPA-17	10
OPA-18	11
OPA-20	14

species (Table 10), and was used for constructing the dendrogram (Fig. 14). The dendrogram suggests that *C. albicans* clusters with *C. lineatus* and *C. sericeus*; with *C. lineatus* being more closely related to *C. sericeus* than to *C. albicans*. Previous reports show that all 3 species are crossable with *C. cajan* and a few characters have already been transferred from *C. sericeus* and *C. albicans* to *C. cajan* (Remanandan 1990). These results also indicate that the Australian species *C. acutifolius*, *C. reticulatus* and *C. grandifolius* are less closely related to *C. cajan* and other Indian species. All the *Rhynchosia* species group together while *F. stricta* is observed to be the most distinct. Species *C. goensis*, grouped with *C. volubilis* and showed greater similarity with *D. ferruginea*.

Species and genus specific markers among wild relatives of pigeonpea

To explore the presence of species and genus specific markers, two accessions for each species as listed in Table 3 were used for the analysis. The bands present in both the accessions and absent in all the other species were considered as species specific markers. The primers which showed polymorphism in the previously described wild species were used for amplifications. The analysis conducted with 16 primers generated 120 scorable fragments, of which 67 were monomorphic in at least two species. The number of bands for each primer varied from 10 (OPA-16) to 23 (OPA-5). The size of the amplified fragments ranged from 250 bp (OPA-3) to 3100 bp (OPA-13). The RAPD reaction resulted in numerous fragments being amplified, many of them were common among different species. However extensive polymorphism was observed among the wild species with several of the primers studied. Only easily resolved bright DNA bands were considered as markers. Conditions were kept constant for the experiments to ensure that comparisons between amplification profiles could be made.

Different RAPD markers were identified that gave genus and species specific amplifications. The markers were specific to *C. albicans*, *C. cajan*, *R. rothii, R. bracteata* and *D. ferruginea*. This polymorphism could be caused by differences in nucleotide sequence at the priming binding sites or

by structural rearrangement within the amplified sequence. Primer OPA-3, OPA-13, OPA-15 amplified a number of bands, often against a background of less strongly amplified minor fragments. While some of these bands were common among the species, differences between species were apparent not only in the major bands but also in many of the minor fragments.

Two representative pictures of PCR amplified DNA fragment from different wild species of pigeonpea using OPA-5 and OPA-20 are shown in Figs 15 and 16, respectively. As seen in these figures, the banding pattern of the accessions within species share many common bands while a few accession specific bands are amplified. For example a 0.9 kb fragment is amplified from *R. rothii* using primer OPA-5 which fails to show any amplification from *R. bracteata*. The primer OPA-5 amplified a fragment of 1350 bp specific for *C. cajan* which is absent in the individuals of other species (Fig 15). The primer OPA-5 also amplified a marker for *C. albicans* and was not present in *C. cajan* (Fig 15). In contrast, primer OPA-20 amplified a 0.8 kb fragment from *R. bracteata* but fails to show amplification with *R. rothii* DNA. The co-amplification of two bands of 600 and 625 bp is observed in *D. ferruginea* with primer OPA-20 The amplification revealed that fragment OPA5-900 was specific to *R. rothii* while OPA 20-800 to *R. bracteata* species (Figure 16).

In this study, a single primer was not able to produce markers for all the species., however, combination of two or more primers could produce many species specific bands. The divergent RAPD pattern among the different wild species indicated the extent of genetic variation that existed in these genotypes.

Variability studies in pigeonpea using vicilin gene primers

Seed storage proteins have been widely used for studying the variability among plant accessions. Recently genes coding for seed storage protein have shown polymorphism in number of crop species. For example, in wheat the gene coding for glutenin has shown the polymorphism between different cultivars (Smith *et al.*, 1994). In this study, the gene coding for a 50

kDa subunit of the 7S (vicilin) seed storage proteins was amplified using PCR. The PCR primer sequences were derived from the genomic clones of phaseolin which is a member of small vicilin gene family (Slightom *et al.*,1983). For this purpose, primer pairs spanning different exons of the vicilin gene were used. From the sequence of the phaseolin gene, 12 different primers were synthesized. The phaseolin gene consists of six exons and five introns. Since exons were more conserved than introns during evolution, all the primers were selected from the exonic region of the phaseolin gene. They were selected with an average G+C content of around 50 % and a random base distribution. Sequences containing stretches of polypurines and polypyrimidines were avoided. The sequences of 12 primers are as shown in Table 4. PCR amplifications were done at the annealing temperature of 45°C and the amplification products were electrophoresed on 1% agarose gel. Different amplification products were amplified using different primer combinations. The PCR amplification of vicilin gene in overlapping fragments is shown in Figure 17.

In the present work, amplification of the vicilin gene from different pigeonpea cultivars and wild species did not show variability. This indicates that seed storage proteins are highly conserved among the pigeonpea accessions and between the wild species. However, further studies will be needed to determine the variability in other vicilin gene families since many genes are present in the plant species.

Table 10

Genetics similarity among wild relatives of pigeonpea. Lanes 1-15 represent species species *C. acutifolius, C. ąlbicans, C. goensis, C. grandifolius, C. lineatus, C. reticulatus, C. sericeus, C. volubilis, D. ferruginea, R. rothii, R. bracteata, R. melacophylla, F. stricta*, *C. cajan*-1, *C. cajan*-2.

	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
1	0.28	0.30	0.27	0.33	0.23	0.22	0.30	0.31	0.24	0.31	0.31	0.37	0.38	0.37	1
2	0.28	0.28	0.33	0.32	0.29	0.28	0.29	0.34	0.32	0.29	0.28	0.59	0.61	1	
3	0.33	0.35	0.29	0.26	0.29	0.29	0.33	0.29	0.27	0.29	0.29	0.69	1		
4	0.29	0.27	0.30	0.24	0.32	0.34	0.27	0.25	0.31	0.27	0.29	1			
5	0.38	0.39	0.43	0.40	0.41	0.43	0.39	0.43	0.59	0.62	1				
6	0.41	0.41	0.38	0.40	0.42	0.36	0.40	0.40	0.65	1					
7	0.40	0.41	0.40	0.43	0.37	0.41	0.42	0.39	1						
8	0.51	0.52	0.49	0.49	0.51	0.65	0.81	1							
9	0.50	0.51	0.46	0.53	0.48	0.62	1								
10	0.47	0.48	0.53	0.51	0.50	1									
11	0.54	0.55	0.59	0.76	1										
12	0.50	0.49	0.58	1											
13	0.52	0.51	1												
14	0.85	1													
15	1														

Figure 14

Dendrogram constructed from the similarity index values for different wild species.

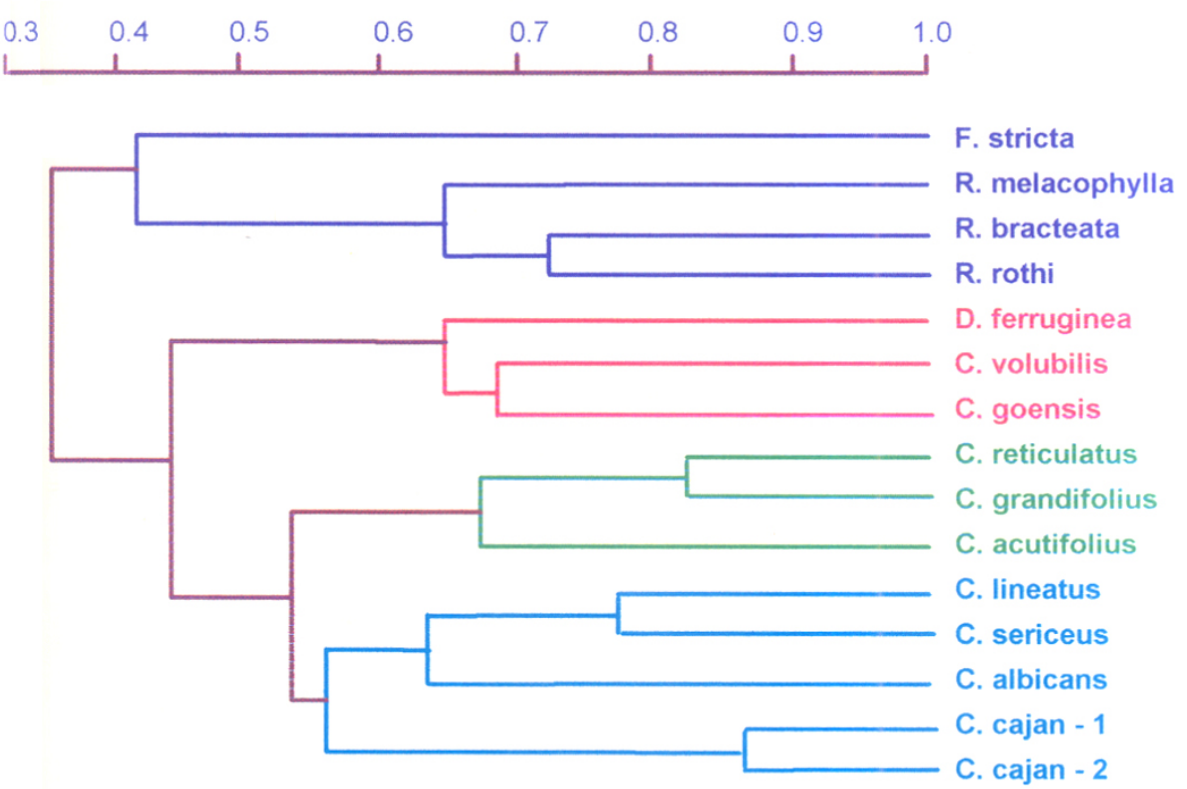


Fig. 15: Species specific markers amplified with primer OPA-5. Lanes 1-13 show *C. cajan*-1, *C. cajan*-2, *C. albicans*-1, *C. albicans*-2, *C. goensis*-1, *D. ferruginea*-1, *D. ferruginea*-2, *R. rothii*-1, *R. rothii*-2, *R. bracteata*-1, *R. bracteata*-2, *F. stricta*-1, *F. stricta*-2 respectively.

Fig. 16: Species specific markers amplified with primer OPA-20. Lanes 1-11 show *C. cajan*-1, *C. cajan*-2, *C. albicans*-1, *C. albicans*-2, *D. ferruginea*-1, *D. ferruginea*-2, *R. rothii*-1, *R. rothii*-2, *R. bracteata*-1, *R. bracteata*-2, *F. stricta*-1, respectively.

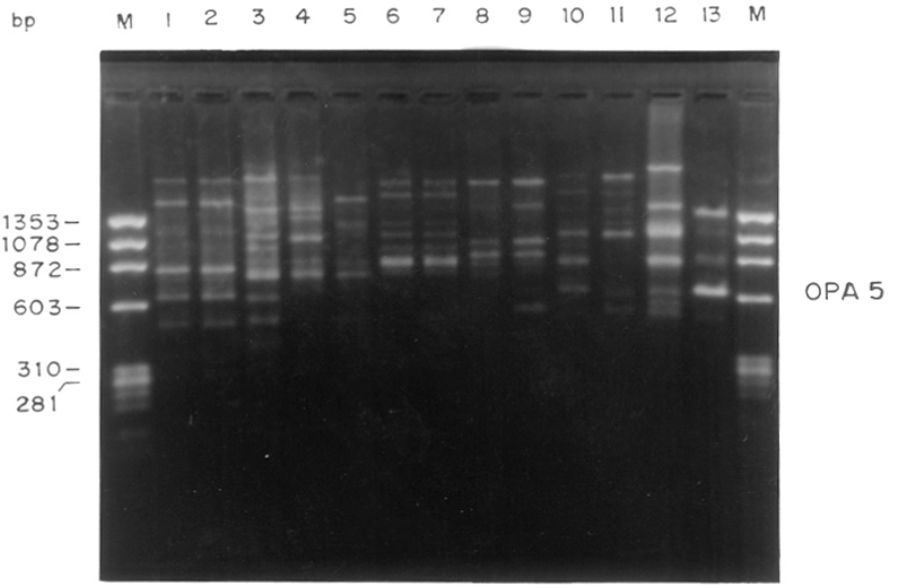

bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
1353
1078
872
603
310
281
OPA 5

Fig. 15

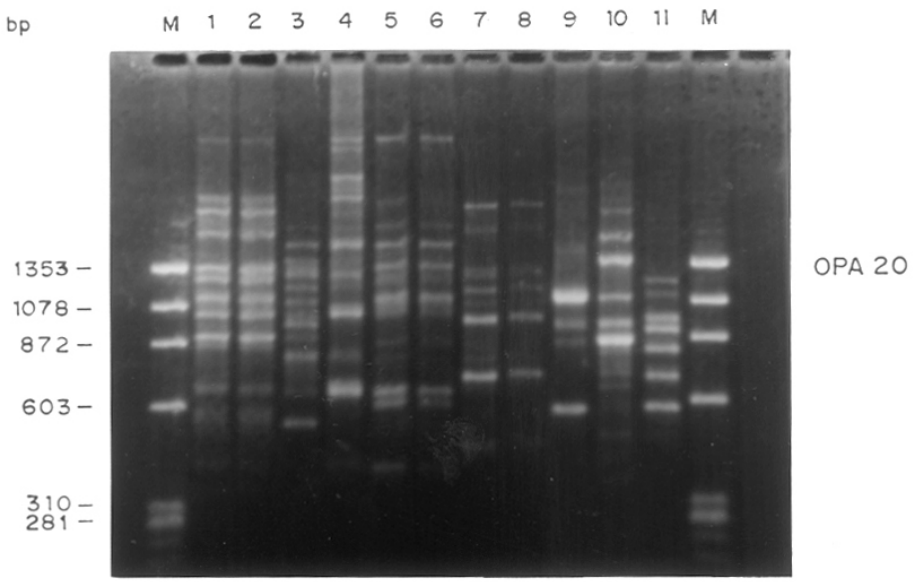

bp
M 1 2 3 4 5 6 7 8 9 10 11 M
1353
1078
872
603
310
281
OPA 20

Fig. 16

Fig 17: Vicilin gene amplification from pigeonpea. M represents the phi X 174 *Hae* III marker. Lanes 1-4 show the vicilin gene amplified in overlapping fragments.

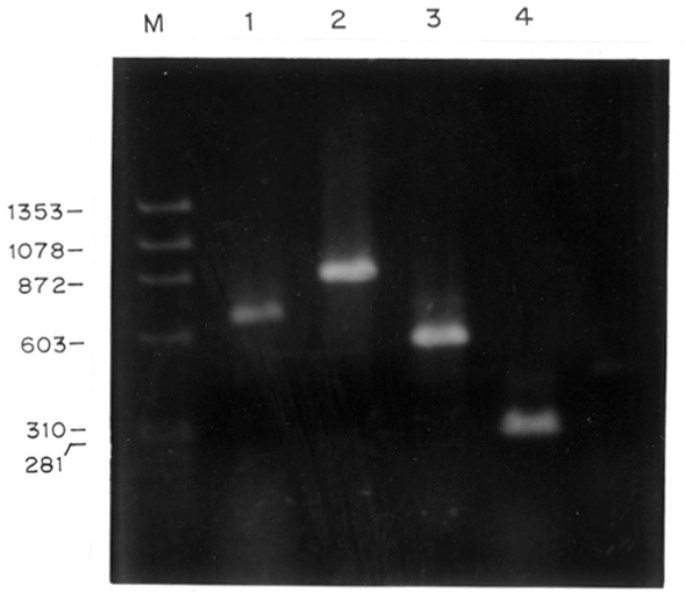


Fig.17

DISCUSSION

Utility of PCR based markers in identification and classification of pigeonpea accessions from India and from different geographic regions

For identification and classification of pigeonpea accessions, 10 elite cultivars from Indian origin and 24 accessions from different geographic origins were used. All the pigeonpea accessions showed genetic similarity between 0.7-0.9 indicating little polymorphism at the DNA level among various accessions. This may be due to the predominantly self-pollinating nature of pigeonpea. However, the degree of polymorphism detected in the present study using PCR based approach is comparatively higher than that previously reported using RFLP markers in pigeonpea (Nadimpalli *et al.*, 1992). Many other crop species have also shown higher polymorphisms using PCR based markers than RFLP markers (Link *et al.*, 1995). This may be due to high resolving power of PCR based markers in detecting polymorphism. In this study, the number of primers tested were sufficient to generate discrete amplification profiles for each genotype analyzed. However, a large number of amplification products are required when there is very little genetic diversity among the selected genotypes as is observed with some of the closely related pigeonpea accessions such as PT-25 and PT-37. In order to evaluate large number of accessions, the procedure employed must be both, simple and fast. RAPD markers could be very useful for evaluating germplasm because they are easier to detect than RFLPs. These markers do not require DNA sequence information or synthesis of specific primers. The ease of detecting RAPD markers makes them an attractive choice for determining genetic relationships.

Biosystematic relationships in subtribe Cajaninae

Characterization of wild relatives of pigeonpea using PCR based DNA markers suggests that these markers are extremely useful in establishing genetic relationships among wild relatives of pigeonpea and in the

identification of species specific markers. For example, one important finding is that *C. lineatus* is more closely related to *C. sericeus* than to *C. albicans*. Similarly the Australian species *C. acutifolius*, *C. reticulatus* and *C. grandifolius* are less closely related to *C. cajan* and other Indian species. This is supported from the view that the hybrids between pigeonpea and the Australian species tend to have a higher level of meiotic abnormalities (Dundas 1984). The results indicate the utility of RAPD approach in resolving biosystematic relationships in subtribe Cajaninae.

The phylogenetic relationships within the genus *Cajanus* have been earlier reported by Nadimpalli *et al.*, (1992). In this study sufficient amount of polymorphism is detected among the species to group them into different clusters. It is quite interesting that number of genus and species specific markers have also been identified. By including more species and accessions of different wild relatives of pigeonpea many new markers can be identified. Since RAPD markers are easier and faster to use, these markers may be preferred in genome analysis. In PCR based approach, DNA extracted from single leaf of a seedling can be used for such analysis and large number of genotypes can be surveyed in a short period of time.

The present collection of wild relatives of pigeonpea at ICRISAT consists of 270 accessions of 47 species belonging to six genera. These accessions serve as an important source of disease and insect resistance for introgression into the cultivated species. In addition to agriculturally valuable traits, wild species exhibit many properties which are important for survival in their natural environment. To transfer such traits from these species to cultivated pigeonpea, breeders need to understand the inheritance pattern of the traits in inter-specific crosses. Desirable traits available in readily crossable species include male sterility, stem blight resistance, high protein content, photoperiodic insensitivity, resistance to pod borer, pod fly and bruchides (Remanandan, 1990).

Application of genetic diversity and DNA fingerprinting in pigeonpea breeding

Information of genetic diversity of pigeonpea germplasm has several applications for plant breeding. Some of the specific applications are as follow:

(1) Selection of inbred parents or testers for maximizing heterotic response in pigeonpea.

(2) Identification of lines or populations that should be maintained to preserve maximum genetic diversity in germplasm banks.

(3) Assessment of the purity and stability of the genotypes entering into the breeding programs.

(4) Organization of genetic resources into related groups to make decisions regarding choice of parents. Knowledge of genetic relationship when complemented by phenotypic data can reveal sources of desirable characteristics in more closely related genotypes.

(5) Grouping genotypes to different heterotic groups, which can be of great relevance in assessing combining ability and developing maximum heterosis in pigeonpea. A high level of heterosis for yield has been observed in crosses between morphologically unrelated pigeonpea cultivars (Shrivastava *et al.*, 1976). The estimate of genetic diversity can, therefore, be used in allocating the cultivars to different heterotic groups.

(6) Utility in parent selection, germplasm management and germplasm protection.

CHAPTER III

DNA markers for tagging of genes conferring resistance to fusarium wilt in chickpea

Part of this chapter has been accepted as a paper for publication in Theoretical and Applied Genetics 1998

DNA MARKERS FOR TAGGING OF GENES CONFERRING RESISTANCE TO FUSARIUM WILT IN CHICKPEA

INTRODUCTION

Chickpea genome and mapping efforts
Fusarium wilt of chickpea
Present thesis work on gene tagging in chickpea

MATERIAL AND METHODS

Plant material
Chickpea DNA extraction
Inter-simple sequence repeat (ISSR) amplifications
Agarose gel electrophoresis
Amplification fragment length polymorphism
Polyacrylamide gel electrophoresis
Culture preparation and inoculation procedures for *Fusarium oxysporum* in chickpea
Data analysis

RESULTS

Optimization of amplification conditions
Selection of ISSR markers and their inheritance
Marker UBC-855_{500} linked to the gene for resistance to fusarium wilt race 4
ISSR marker UBC-855_{500} is linked to marker CS-27_{700}
Marker UBC-825_{1200} linked to the gene for resistance to fusarium wilt
Gene for resistance to fusarium wilt race 4 and 5 are present in same linkage group
AFLP markers in chickpea
Bulked segregant analysis

DISCUSSION

Mendelian segregation of ISSR markers
Advantages of ISSR Markers UBC-855_{500} and UBC-825_{700} for fusarium wilt screening
Genes conferring resistance to fusarium wilt
Cluster of fusarium wilt disease resistance genes in chickpea

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop in the world, and ranks first in the Indian subcontinent and the Mediterranean basin. Unlike cereals, the productivity of this crop has not been markedly improved through conventional breeding. Average yields of chickpea vary from 600-700 kg\ha in developing countries to about 1500 kg\ha in developed countries (FAO, 1993). Yields under irrigated conditions average about 2000 kg\ha (FAO, 1993). The annual growth rate of chickpea production has been very slow at 1.9% and yields have risen only 0.6% annually. The main bottleneck for increasing chickpea yields is susceptibility to many diseases and pests particularly the fungal diseases caused by *Fusarium oxysporum* and *Ascochyta rabiei.* The productivity of chickpea has not been improved through conventional breeding mainly because of narrow genetic variability and limited genetic information. Programs to breed resistant and high yielding cultivars are underway, but these are time consuming and it is hard to pyramid resistance genes by conventional means.

The genus *Cicer* contains 9 annual and 34 perennial species. According to the review by Ladizinsky *et al.,* (1988) crossability and fertility of hybrids in inter-specific crosses have been used as a basis to classify the nine annual species into four crossability groups. The species *C. arietinum* L., *C. reticulatum* Lad. and *C. echinospermum* Davis belongs to the first crossability group. Hybrids could be obtained only between the species belonging to the same group. No hybrid was obtained in crosses between *C. arietinum* and species in other crossability groups (Ladizinsky and Adler, 1976 and Pundir and van der Maesen, 1983). The main focus of international and national research programs is improvement of yield and stability of chickpea. Muehlbauer *et al.*, (1990) and Singh *et al.*, (1985) suggested resistance breeding to biotic (Ascochyta blight and Fusarium wilt) and abiotic stresses by transferring genes from wild types to the cultivars.

Chickpea genome and mapping efforts

Chickpea is a self-pollinated diploid crop, and has a genome size of (2C) 3.29 × 10^6 kb (Ohri and Pal, 1991). Genetic studies in chickpea have been based mainly on inheritance of morphological traits such as plant habit, leaf form and colour, flower colour, podding habit, seed coat colour, disease resistance and several quantitative inherited traits (Muehlbauer and Singh, 1987). Although some of the morphological traits have been identified to be linked, a linkage analysis and assignment of these traits to the various linkage groups have not been worked out. RFLP analysis has revealed that chickpea genome is rich in low copy sequences. Weising *et al.*, (1992) have used oligonucleotide fingerprinting to differentiate different accessions of *Cicer* species and have shown that simple repetitive motifs are present in chickpea genome. Further Sharma *et al.*, (1995) demonstrated the abundance and polymorphism of various di-, tri- and tetra nucleotide tandem repeats in chickpea.

Cultivated chickpea shows only low level of polymorphism (Kazan and Muehlbauer, 1991). Therefore existing chickpeas maps, comprising isozyme and morphological markers are mainly based on the inter-specific crosses between *C. arietinum* and its wild relatives *C. reticulatum* or *C. echinospermum,* (Gaur and Slinkard, 1990; Kazan *et al.,* 1993). Currently linkage groups are being established for *Cicer* which consist of morphological traits, isozymes, RFLPs, RAPD and disease resistance loci. To date there are ten linkage groups containing morphological and molecular markers. Expansion of the genetic map of *Cicer* will help breeders make selections of economically important traits early in their programs using markers linked to the trait of interest. Compared to some other crops, the gene map for *Cicer* could be established rather quickly. The advantages, according to Muehlbauer *et al.,* (1990) are its diploid chromosome compliment, a relatively small chromosome number (2n=16), and the synteny chickpea shares with other legume crops like pea and lentil. The establishment of molecular marker technology and the generation of genetic map would be desirable for marker assisted selection and the

positional cloning of the resistance genes in chickpeas.

The first linkage map of the *Cicer* had four linkage groups consisting of 13 isozyme loci (Gaur and Slinkard, 1988). They were *Aat-p:ENP:Pgm-p, Amy:Aat-m:Est-3: Pgd-p:Pgm-3, Lap:Acp-1:Adh-2, and Aco-m:prx-3.* Subsequently, *Est-10* to linkage group I, 4 isozymes (*Ald-pl*, *Glu*-3, *Gal-2* and *Est*-2) to linkage group II, and *Gal -3* and *Prx-3* to linkage group IV, were added (Gaur and Slinkard, 1990b). Besides these additions, three more linkage groups were established using both morphological and isozymic loci, namely; *gr:Aco-c:Pep-3:bplv:slv* to linkage group V, *Gpi-c:Fdh* to linkage group VI and *Est-4:Me-1* to linkage group VII (Gaur and Slinkard, 1990). Unfortunately, *Cicer arietinum* exhibits few number of polymorphisms at isozymic loci (Oram *et al.,* 1987 and Gaur and Slinkard, 1990b). Thus, application of isozymic markers in chickpea mapping appears to be limited. The reason could be: the highly self pollinating nature of the species and genetic bottle necks (Kazan and Muehlbauer, 1991). Inter-specific crosses between cultivated and wild germplasm are being used to expand the existing chickpea map and it has already proved to be useful since new linkage groups have been added to the existing map (Gaur and Slinkard, 1990 and Simon and Muehlbauer, 1991). In all cases F_2 population were used to determine the linkage groups. To date recombinant inbred lines (RILs) have not been utilized to map genes of interest in *Cicer*. However, Burr *et al.,* (1988) and Tahir (1990) used RILs to map several genes and added new linkage groups in maize and lentil respectively. The use of RAPDs and RFLPs was of recent introduction to expand the *Cicer* map (Simon and Muehlbauer, 1991 and 1997). Currently, the chickpea map consists of 9 morphological, 28 isozymic and 44 RAPDs and 8 RFLP loci (Simon and Muehlbauer, 1997). The authors considered the reported homologies of linkage group between *Cicer* and *Viceae* (Pea) while arranging linkage group in *Cicer* (Weeden *et al.,* 1992).

Fusarium wilt of chickpea

Fusarium wilt caused by *Fusarium oxysporum* Schlecht emd.: Fr. f.

sp. *ciceris* (Padwick) Matuo & K. Sato, is a major disease next to Ascochyta blight with yield losses ranging from 10-90 % (Nene and Sheila, 1992, Gupta *et al.,* 1986, Shrivastava *et al.,* 1984). It is even more important disease than Ascochyta blight in Eastern Africa, South Asia, India, and Mexico (Faris and Gowda, 1990) specifically in latitudes ranging from 0-30 degree (Van Rheenen, 1991 and Haware *et al.,* 1990). Even though chemicals have a significant role in controlling fusarium wilt, the cost involved prevents its widespread use. The most economical method to control the disease is through breeding for disease resistant cultivars. The existence of pathogenic races in *Fusarium oxysporum* f. sp. *ciceris* is well established. Races 1, 2, 3 and 4 were reported from India (Haware and Nene, 1982) while races 0, 5 and 6 were identified from Spain (Jimenez-Diaz *et al.,* 1989). Fusarium wilt of race 1 reportedly concluded to be controlled by single recessive gene (Kumar and Haware, 1982) and by at least two genes (Upadhyaya *et al.,* 1983a, 1983b). More recently a third locus was also identified which independently delayed wilting (Singh *et al.,* 1987). Several genetic studies in various plant species have indicated that disease resistance genes are often clustered as multiallelic series at a locus or as a multiple linked loci (Kesseli *et al.,* 1993). For the genes to be closely linked, they should show that they could be recombined and also should show that they control different primary functions. If they control the same function the genes are considered functionally allelic (Shepared and Mayo, 1972). Durable and effective breeding program can be established when the genetics of inheritance is well defined and the chromosomal locations of the genes are mapped.

Present work in gene tagging

The objective of the present study was to identify the PCR generated DNA markers linked to the Fusarium wilt resistance gene that could be used in selection of wilt resistance lines in early stage of breeding program.

The specific objectives of this thesis were:

(1) To study the inheritance of inter-simple sequence repeat (ISSR)

polymorphisms in the recombinant inbred lines (RILs) developed from a cross of *C. arietinum* (ICC4958) and *C. reticulatum* (PI489777)

(2) To identify the ISSR markers linked to the gene for resistance to fusarium wilt

(3) To develop amplification fragment length polymorphism (AFLP) in chickpea for their applicability in gene tagging and in linkage map construction.

MATERIAL AND METHODS

Plant material

The chickpea RIL's were developed at the USDA-ARS, Grain Legume Genetics and Physiology Unit, Washington State University, Pullman, WA.

Chickpea DNA extraction

DNA was isolated from the parents and RILs from vegetative buds and leaf tissues using the microprep method of Doyle and Doyle (1987). One gram of each leaf sample was submerged in liquid nitrogen and then ground to fine powder. The powder was quickly transferred to a tube containing 7.5 ml of ice cold extraction buffer (0.35 M sorbitol, 0.1 M Tris, 5mM EDTA, pH 7.5). The tube was briefly shaken and 7.5 ml of nuclei lysis buffer (2M NaCl, 0.2 M Tris, 50 mM EDTA, 2% CTAB, pH 7.5) was then quickly added, followed by 3 ml of 5 % sarkosyl solution. Sample sets were incubated in a 65°C water bath for 20 minutes. After incubation, the tubes were allowed to cool for a few minutes and 18 ml of chloroform/isoamyl alcohol (24:1) was added to each tube. The tubes were then centrifuged at 500x g for 15 minutes. The aqueous layer was removed and extracted again with 15 ml chloroform mixture. Finally, DNA was precipitated with two volumes of chilled ethanol and was suspended in one ml of TE buffer.

Inter-simple sequence repeat (ISSR) amplifications

PCR amplifications were performed for 35 cycles with denaturation at 94°C for 1 min, annealing at 50°C for one minute and extension at 72°C for 2

minutes. The reaction mixture (25 µl) contained 10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 200 µM each of dATP, dTTP, dCTP and dGTP (Pharmacia), 0.24 µM of primers, 30 ng of genomic DNA and 1 unit of *Taq* polymerase. Each reaction was overlaid with 100 µl of mineral oil to prevent evaporation. For AT rich primers annealing at 36°C was always used.

PCR primers

Table 11 lists the microsatellite primers used for the amplification of the chickpea DNA.

Agarose gel electrophoresis

The PCR products 25µl were loaded on 2% agarose gel for electrophoresis in 1 X TBE buffer (89 mM Tris-HCl, pH 8.3, 89 mM boric acid, 5 mM EDTA) at 50 V for 6 h, stained with ethidium bromide and visualized by illumination under UV light.

Amplified fragment length polymorphism (AFLP)

The AFLP analysis was followed by the procedure described by Zabeau and Vos (1993). This involved cutting genomic DNA with two restriction enzymes, a 6-base cutter *Eco*RI and 4-base cutter *Mse*-I, ligating it with adapters, amplifying a subset of *Mse*I-*Eco*RI fragments with primers that match the adapters and contained the additional selective nucleotide at the 3' end and finally separating the fragments on denaturing polyacrylamide gel.

Restriction endonuclease digestion

The restriction enzyme reaction was carried out with 500 ng genomic DNA of each line to which was added 5 U *Eco*RI, 5 U *Mse*I, 5µl of 5 X reaction buffer and water to give total volume of 25 µl. The reaction was incubated for two hours at 37°C. Further reaction was heated to 70°C to inactivate the enzymes.

Ligation of adapters

For ligation, 24 µl of adapter ligation solution was used with 1 µl of T4 DNA ligase and was incubated at 20°C for 2 hours. Further 1:10 dilutions were performed with TE buffer and ware used for pre-amplification reactions.

Pre-amplification reactions

Pre-amplification reactions were carried out with AFLP primers each having one selective nucleotide. Amplifications were carried out with 40 µl of pre-amplification buffer, 5 µl of diluted DNA , 1 units of *Taq* polymerase, and 5 µl of 10X PCR buffer for AFLP. The pre-amplification reaction was performed for 20 cycles with denaturation at 94 °C for 30 sec., annealing at 56 °C for 60 sec. and extension at 72 °C for 60 sec. After the completion of reaction, 1:50 dilutions were performed with TE buffer and ware used for the second selective amplification.

Primer labelling

Primer labelling was performed with 18 µl of *Eco*RI primer, 10 µl of 5X kinase buffer, 20 µl of γ-^{32}P ATP (3000 Ci/mmol) and 1 unit of T4 polynucleotide kinase. The reaction was incubated for one hour at 37 °C. Finally the enzyme was heat inactivated at 70 °C for 10 minutes.

Selective AFLP amplifications

Selective AFLP reactions were performed with 5 µl of labelled primer, 45 µl of *Mse*I primer, 20 µl 10 X buffer for AFLP, 1 unit *Taq* polymerase, 5 µl of primer /dNTP mix and 10 µl of *Taq* DNA polymerase buffer, 79 µl of AFLP grade water and was used for 10 AFLP amplifications. PCR amplifications were carried out as described in Zabeau and Vos (1993).

Polyacrylamide gel electrophoresis

The reaction products were analyzed on 4.5 % denaturing polyacrylamide gels. The gel solution consisted of 42 g urea, 10 ml 10 x

TBE buffer, 4.275 g acrylamide, 0.225 g bisacrylamide, and water to make 100 ml; 1 X TBE was used as electrophoresis buffer. Gels were run at a constant 120W for 1.5-2 h, dried on a gel drier, and exposed to x-ray film.

Culture preparation and inoculation procedures for *Fusarium oxysporum* in chickpea

Inoculum was prepared from a single-spored fungal isolate grown on sterile filter paper placed on potato-dextrose-agar (PDA) as described in Tullu (1996). When the filter paper was completely colonized by a fungus, only colonies representative of wild type were aseptically removed and placed in a fresh petri dish to dry for 5 days in a laminar flow hood. The dried filter paper was cut into pieces aseptically using a pair of sterile scissors. Pieces of filter paper were then used to prepare the primary inoculum. The conidia concentration was adjusted to $1x\ 10^6$ spores ml^{-1} with a hemacytometer. Twelve to twenty seeds of each RIL were grown in the glasshouse (21-26°C) in single rows in plastic trays filled with sterile coarse perlite. When the seedlings reached the 3-4 nodal stage, they were carefully removed from the perlite, pruned while submerged in the spore suspension, and after about 5 minutes in the spore suspension, were replanted into the perlite. Plants were then scored as susceptible or resistant over the next 2 months.

Data analysis

The data was scored for all the ISSR markers and for the fusarium wilt resistance for race 4 and 5 and the analysis was done using MAPMAKER program (Lander *et al.*, 1987).

Table-11

List of microsatellite primers

801 ATA TAT ATA TAT ATA TT
802 ATA TAT ATA TAT ATA TG
803 ATA TAT ATA TAT ATA TC
804 TAT ATA TAT ATA TAT AA
805 TAT ATA TAT ATA TAT AC
806 TAT ATA TAT ATA TAT AG
807 AGA GAG AGA GAG AGA GT
808 AGA GAG AGA GAG AGA GC
809 AGA GAG AGA GAG AGA GG
810 GAG AGA GAG AGA GAG AT
811 GAG AGA GAG AGA GAG AC
812 GAG AGA GAG AGA GAG AA
813 CTC TCT CTC TCT CTC TT
814 CTC TCT CTC TCT CTC TA
815 CTC TCT CTC TCT CTC TG
816 CAC ACA CAC ACA CAC AT
817 CAC ACA CAC ACA CAC AA
818 CAC ACA CAC ACA CAC AG
819 GTG TGT GTG TGT GTG TA
820 GTG TGT GTG TGT GTG TC
821 GTG TGT GTG TGT GTG TT
822 TCT CTC TCT CTC TCT CA
823 TCT CTC TCT CTC TCT CC
824 TCT CTC TCT CTC TCT CG
825 ACA CAC ACA CAC ACA CT
826 ACA CAC ACA CAC ACA CC
827 ACA CAC ACA CAC ACA CG
828 TGT GTG TGT GTG TGT GA
829 TGT GTG TGT GTG TGT GC
830 TGT GTG TGT GTG TGT GG
831 ATA TAT ATA TAT ATA TYA
832 ATA TAT ATA TAT ATA TYC
833 ATA TAT ATA TAT ATA TYG
834 AGA GAG AGA GAG AGA GYT
835 AGA GAG AGA GAG AGA GYC
836 AGA GAG AGA GAG AGA GYA
837 TAT ATA TAT ATA TAT ART
838 TAT ATA TAT ATA TAT ARC
839 TAT ATA TAT ATA TAT ARG
840 GAG AGA GAG AGA GAG AYT
841 GAG AGA GAG AGA GAG AYC
842 GAG AGA GAG AGA GAG AYG
843 CTC TCT CTC TCT CTC TRA
844 CTC TCT CTC TCT CTC TRC
845 CTC TCT CTC TCT CTC TRG
846 CAC ACA CAC ACA CAC ART
847 CAC ACA CAC ACA CAC ARC
848 CAC ACA CAC ACA CAC ARG
849 GTG TGT GTG TGT GTG TYA
850 GTG TGT GTG TGT GTG TYC
851 GTG TGT GTG TGT GTG TYG
852 TCT CTC TCT CTC TCT CRA
853 TCT CTC TCT CTC TCT CRT
854 TCT CTC TCT CTC TCT CRG
855 ACA CAC ACA CAC ACA CYT
856 ACA CAC ACA CAC ACA CYA
857 ACA CAC ACA CAC ACA CYG
858 TGT GTG TGT GTG TGT GRT
859 TGT GTG TGT GTG TGT GRC
860 TGT GTG TGT GTG TGT GRA
861 ACC ACC ACC ACC ACC ACC
862 AGC AGC AGC AGC AGC AGC
863 AGT AGT AGT AGT AGT AGT
864 ATG ATG ATG ATG ATG ATG
865 CCG CCG CCG CCG CCG CCG
866 CTC CTC CTC CTC CTC CTC
867 GGC GGC GGC GGC GGC GGC
868 GAA GAA GAA GAA GAA GAA
869 GTT GTT GTT GTT GTT GTT
870 TGC TGC TGC TGC TGC TGC
871 TAT TAT TAT TAT TAT TAT
872 GAT AGA TAG ATA GAT A
873 GAC AGA CAG ACA GAC A
874 CCC TCC CTC CCT CCC T
875 CTA GCT AGC TAG CTA G
876 GAT AGA TAG ACA GAC A
877 TGC ATG CAT GCA TGC A
878 GGA TGG ATG GAT GGA T
879 CTT CAC TTC ACT TCA
880 GGA GAG GAG AGG AGA
881 GGG TGG GGT GGG GTG
882 VBV ATA TAT ATA TAT AT
883 BVB TAT ATA TAT ATA TA
884 HBH AGA GAG AGA GAG AG
885 BHB GAG AGA GAG AGA GA
886 VDV CTC TCT CTC TCT CT
887 DVD TCT CTC TCT CTC TC
888 BDB CAC ACA CAC ACA CA
889 DBD ACA CAC ACA CAC AC
890 VHV GTG TGT GTG TGT GT
891 HVH TGT GTG TGT GTG TG
892 TAG ATC TGA TAT CTG AAT TCC C
893 NNN NNN NNN NNN NNN
894 TGG TAG CTC TTG ATC ANN NNN
895 AGA GTT GGT AGC TCT TGA TC
896 AGG TCG CGG CCG CNN NNN NAT G
897 CCG ACT CGA GNN NNN NAT GTG G
898 GAT CAA GCT TNN NNN NAT GTG G
899 CAT GGT GTT GGT CAT TGT TCC A
900 ACT TCC CCA CAG GTT AAC ACA

RESULTS

Optimization of amplification conditions

In order to obtain stable and reproducible amplification of PCR products as judged from the agarose gel electrophoresis, it was essential to optimise the PCR amplification conditions. Strong and compact bands on agarose gel facilitate easy and accurate scoring of the gel electrophoretic data. Three main parameters, which played important role to get such results, are summarized in the following paragraphs

The template concentration in the range of 0.5 to 200 ng. was used to determine the optimum concentration for the PCR assay. A constant band pattern was obtained at different template concentrations. A template DNA concentration of 30ng /25 μl was selected for PCR amplification.

The annealing temperature for the PCR amplification was found to be critical. Some (AT)n primers had much lower melting temperature (20-30°C) and primers with high GC content had a melting temperature above 60°C. Three temperatures 45°C, 50°C, 55°C were examined for all the primers and 36°C for (AT) rich primers. An annealing temperature of 50°C gave good amplification with most of the primers while 45°C gave more background and smear. Many additional bands were amplified at low annealing temperatures in addition to bands amplified at higher stringency. This may be due to the amplification obtained by the primer template mismatch at lower stringency. (AT) rich primers worked well at 36 °C. For routine PCR work, annealing temperature of 50°C was always used. Fig 18 shows the representative picture of PCR-amplified DNA fragment using primer UBC-843.

Another important variable was the concentration of magnesium, which affected the efficiency of annealing. As the magnesium concentration was increased, some bands were amplified more efficiently while others were amplified less efficiently. A magnesium concentration of 2.5 mM in the PCR reaction was found to be optimal for the purpose of producing clear and reproducible DNA bands.

Selection of ISSR markers and their inheritance

During parental screening, 38 primers out of 100 were found to be polymorphic, and 26 were monomorphic, the other primers did not show amplification. The SSR sequences are selected because these sequences are abundantly present in the chickpea genome and provide amplification at higher annealing temperature. Moreover, the PCR products obtained are stable and highly reproducible.

From the primers that gave amplification products in our initial screen, 22 primers were selected that gave the clearest bands (Table 12). A total of 190 bands were amplified out of which 31 were scored; unstable and weak bands were not scored. The number of bands for each primer, which produced a polymorphic band pattern, varied from 2 (UBC-843) to 16 (UBC-888) in the size range of 200 bp (UBC-885) to 3 kb (UBC-840). Thirty one bands were generated by 22 primers, of which 29 segregated in a 1:1 ratio.

The polymorphism with inter-simple sequence repeat was primer dependent. Of the 22 primers studied in the population, the $(GA)_n$ primers were most abundant while primers with $(TG)_n$ repeat gave rise to the largest number of polymorphic loci followed by $(AC)_n$ repeat. Table 12 gives details of sequences of the 22 primers that were selected.

Marker UBC-855_{500} linked to the fusarium wilt resistance gene

The 131 RILs were inoculated with race 4 of fusarium wilt. The scoring for disease reaction of each inbred line was either resistance (0-10% wilted plants) or susceptible (90-100% wilted plants). However, a few lines had an intermediate type of reaction (11-89%) and were not used for the analysis.

We surveyed 131 RILs with 100 ISSR primers and identified that UBC-855_{500} amplified a DNA region closely associated with the gene for resistance to fusarium wilt race 4. The electrophoretic pattern of the PCR-amplified DNA fragment using primer UBC-855 is shown in Fig 19. The marker is designated as the primer number, followed by molecular weight of

the band in base pairs. The amplification pattern revealed that UBC-855_{500} was amplified in all the susceptible lines and was absent in the resistant lines. However, the marker was also amplified in five resistant lines. The amplification of the UBC-855_{500} in the resistant lines was most likely due to recombination between the gene and the marker. The genotypic data were then used to determine the degree of linkage of the marker and its relative position to the locus conferring resistance to race 4 of fusarium wilt. Linkage analysis was performed using the Mapmaker program (Lander *et al.*, 1987) with Lod score >7.0. Goodness of fit to the expected 1:1 Mendelian ratio for each segregating locus was determined by Chi-square.

ISSR marker UBC-855_{500} is linked to RAPD marker CS-27_{700}

The earlier work of Mayer *et al* .,(1997) and Tullu (1996) identified two markers CS-27_{700} and UBC-170_{550} that amplified fragments linked to fusarium wilt race 1 resistance with 7 % recombination in RILs developed from a cross between C-104 and WR-315. Both these RAPD markers were located 7 cM from the resistance genes and were on the same side. The marker CS-27_{700} was scored on the 131 RILs and was found to co-segregate in coupling with the UBC-855_{500}. The CS-27_{700} locus was mapped to linkage group 6 of the *Cicer* genome (Simon and Muehlbauer 1997). The UBC-855_{500} was shown to be 5.5 cM from the CS-27_{700} RAPD marker. This indicates that the genes for resistance to race 1 and 4 are also closely linked. Such linked markers and a complete genetic map can be used to investigate if there are one or more additional genes segregating in the population which can account for the different classes of resistance (Upadhyaya *et al.* 1983; Kumar and Haware 1982).

Table 12

Segregation of ISSR fragments in chickpea RILs developed from a cross of ICC-4958 and PI 489777. "a" indicates band presence, "b" indicates band absence, *R* purine, *Y* pyrimidine; *N* any nucleotide; *B* indicates C, G, or T; *D* as A, G, or T; *H* as A, C, or T; and *V* as A, C, or G

Primer	Sequence	Fragment Size(bp)	Observed ratio a:b (1:1)	χ^2	p
UBC807	AGAGAGAGAGAGAGAGT	829	71:60	0.4618	0.25-0.50
UBC809	AGAGAGAGAGAGAGAGG	1400	65:66	0.0038	0.90-0.95
UBC810	GAGAGAGAGAGAGAGAT	1100	62:69	0.1870	0.50-0.75
UBC811	GAGAGAGAGAGAGAGAC	1600	65:66	0.0038	0.90-0.95
UBC823	TCTCTCTCTCTCTCTCC	940	70:61	0.3091	0.50-0.75
UBC823	TCTCTCTCTCTCTCTCC	1800	71:60	0.4618	0.25-0.50
UBC825	ACACACACACACACACT	900	67:64	0.0343	0.75-0.90
UBC825	ACACACACACACACACT	1100	63:68	0.0594	0.75-0.90
UBC830	TGTGTGTGTGTGTGTGG	1300	73:48	0.8587	0.25-0.50
UBC840	GAGAGAGAGAGAGAGAYT	1000	53:78	2.3580	0.10-0.25
UBC840	GAGAGAGAGAGAGAGAYT	1300	67:64	0.0343	0.75-0.90
UBC842	GAGAGAGAGAGAGAGAYG	1200	65:66	0.0038	0.95-0.97
UBC843	CTCTCTCTCTCTCTCTRA	1050	95:36	13.2862	< 0.005
UBC855	ACACACACACACACACYT	375	64:67	0.0343	0.75-0.90
UBC856	ACACACACACACACACYA	1800	63:68	0.0954	0.75-0.90
UBC858	TGTGTGTGTGTGTGTGRT	400	65:66	0.0038	0.95-0.97
UBC859	TGTGTGTGTGTGTGTGRC	1800	60:71	0.4618	0.25-0.50
UBC860	TGTGTGTGTGTGTGTGRA	725	60:71	0.4618	0.25-0.50
UBC864	ATGATGATGATGATGATG	425	83:48	4.6755	0.02-0.05
UBC866	CTCCTCCTCCTCCTCCTC	900	61:70	0.3091	0.50-0.75
UBC868	GAAGAAGAAGAAGAAGAA	700	77:54	2.0190	0.10-0.25
UBC880	GGAGAGGAGAGGAGA	1050	74:57	1.1030	0.25-0.50
UBC880	GGAGAGGAGAGGAGA	1400	72:59	0.6450	0.25-0.50
UBC880	GGAGAGGAGAGGAGA	1600	74:57	1.1030	0.25-0.50
UBC884	HBHAGAGAGAGAGAGAG	450	68:63	0.0954	0.75-0.90
UBC884	HBHAGAGAGAGAGAGAG	1150	60:71	0.4618	0.25-0.50
UBC884	HBHAGAGAGAGAGAGAG	1600	80:51	3.2099	0.05-0.10
UBC885	BHBGAGAGAGAGAGA	1100	78:53	2.3850	0.10-0.25
UBC885	BHBGAGAGAGAGAGA	1500	79:52	2.7824	0.05-0.10
UBC888	BDBCACACACACACA	600	63:68	0.0954	0.75-0.90

Fig. 18 : PCR-amplified inter-simple sequence repeat patterns on 2 % agarose gels. M represents marker pBR 322 *Bst*N1 digest. Lanes 1& 2 represents *C. arietinum* (ICC4958) and *C. reticulatum* (489777) while 3-12 represents chickpea RILs amplified with primer UBC-843.

Fig. 19 : PCR-amplified inter-simple sequence repeat patterns on 2 % agarose gels. M represents marker pBR 322 *Bst*N1 digest. Lanes 1& 2 represents *C. arietinum* (ICC4958) and *C. reticulatum* (489777) while 3-12 represents chickpea RILs amplified with primer UBC-855. Marker linked with fusarium wilt is indicated by an arrow.

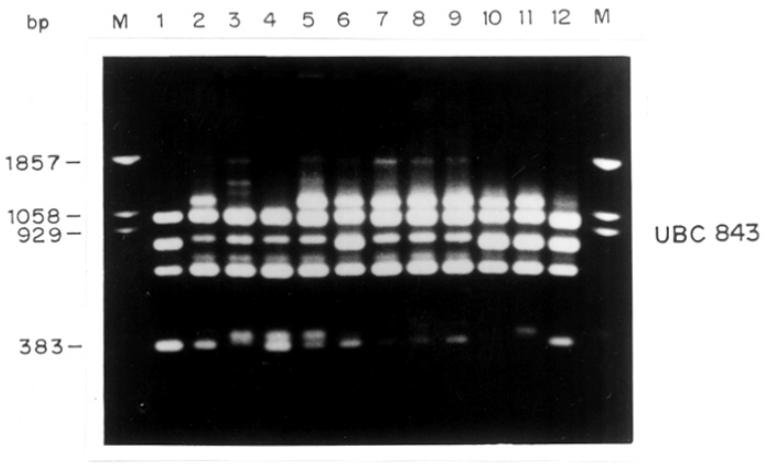

bp
M 1 2 3 4 5 6 7 8 9 10 11 12 M
1857–
1058–
929–
383–
UBC 843

Fig. 18

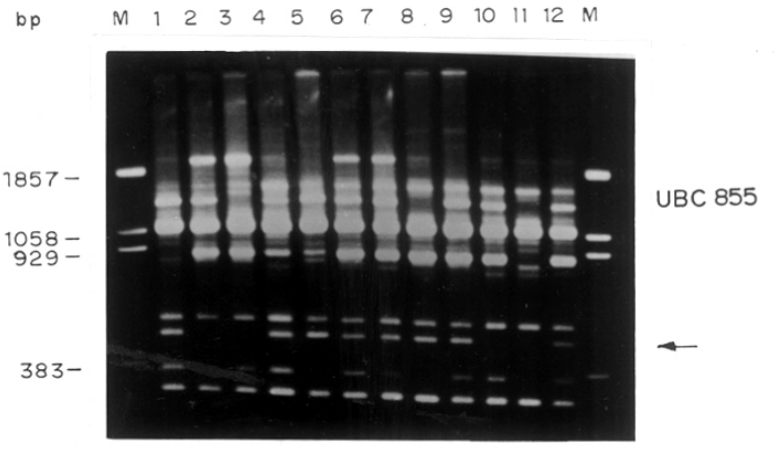

bp
M 1 2 3 4 5 6 7 8 9 10 11 12 M
1857–
1058–
929–
383–
UBC 855

Fig. 19

Marker UBC-825$_{1200}$ linked to fusarium wilt resistance gene

We had previously demonstrated that a simple sequence repeat $(AC)_8YT$ when used directly in PCR, amplified a marker, UBC-855$_{500}$, which was linked to the gene for resistance to fusarium wilt race 4. The aim of the present study was to find the suitability of the $(AC)_n$ sequences for providing guidelines for selecting new primers for its use in marker enrichment in the desired region. For this we studied the inheritance of inter-simple-sequence-repeat (ISSR) polymorphism in the same cross. Based on a $(AC)_8YT$ sequence, which amplified marker linked to the disease resistance gene we selected other SSR sequences with variation at the 3' and 5' anchors containing AC repeats. The study indicated that other SSR with AC repeats also amplified fragments that were linked to the fusarium wilt resistance genes. The SSR $(AC)_8T$ amplified a marker UBC-825$_{1200}$ which was located 5.0 cM from the gene for resistance to fusarium wilt race 4 and was closer than the UBC-855$_{500}$ and CS-27$_{700}$ markers (Mayer 1997). Repeat $(AC)_8YG$ amplified a fragment UBC857$_{800}$ which was located 5.5 cM from UBC-855$_{500}$ and was associated with the fusarium wilt resistance gene. A one or two nucleotide change at the 3' end in the sequence resulted in the shifting of marker. $(GA)_n$ and $(AC)_n$ repeats were also found to be in the same linkage group. To study the inheritance of the complimentary sequences, we also selected $(TG)_n$ repeats with different anchors. The repeat $(TG)_8RG$ amplified the fragment UBC-860$_{600}$ from the resistant lines as compared to the $(AC)_n$ repeats and was associated with the gene for resistance for race 4.

Genes for resistance to fusarium wilt race 4 and 5 are present in same linkage group

We also studied the inheritance and segregation of gene for resistance to fusarium wilt race 5. The 131 RILs were inoculated with race 5 and the segregation data was scored. The data indicates that the gene for resistance to race 4 and 5 are present in same linkage group. The genes for resistance to fusarium wilt race 1, 2, 4, and 5 were also found to be in cluster in the cross of WR-315 and C-104 (Tullu 1997). We observed the

abundance of dinucleotide and trinucleotide repeats at the fusarium wilt disease resistance gene cluster. In addition to $(AC)_n$ and $(TG)_n$ repeats, the trinucleotide $(ATG)_6$, $(CTC)_6$ and $(GAA)_6$ were also present at the fusarium wilt resistance gene cluster. The markers amplified with trinucleotide repeat, UBC 864_{425}, UBC-866_{900} and UBC-868_{700} were associated to the gene for resistance to fusarium wilt and were in the same linkage group. Table 13 shows the sequences of repeats and the size of the amplified markers linked to the resistance gene. The position of ISSR markers with respect to the gene for resistance to race 4 (Foc 4) and 5 (Foc 5) are shown in Fig 20. The size of the amplified band and the segregation data indicated that the markers were amplified from the independent loci and were not the same fragment. Our study showed that markers linked to various genes could be rapidly identified using ISSR directed approach and the presence of dinucleotide repeats could be exploited for fine mapping of fusarium wilt resistance gene cluster.

Our study indicated that markers amplified with ISSR co-segregated and were often present in the same linkage group. For example two fragments amplified by a marker UBC-880_{1500} and UBC-880_{1700} cosegregated at a distance of 5.8 cM and were in the same linkage group. The markers UBC-856_{1800} and UBC-859_{1900} amplified by the complementary repeats $(AC)_8YA$ and $(TG)_8RC$ respectively co-segregated and were clustered (data not shown).

AFLP markers in chickpea

To evaluate the usefulness of AFLP markers in genetic mapping experiments, polymorphic markers were analyzed using the 131 recombinant inbred lines. With the available *Eco* RI +3 and *Mse* I +3 primers, 16 different primer combinations were analyzed with the two parents of the mapping population. This survey revealed that for each primer combination the number of bands visible on the gel ranged between 15 and 68 with a mean of 35 bands.

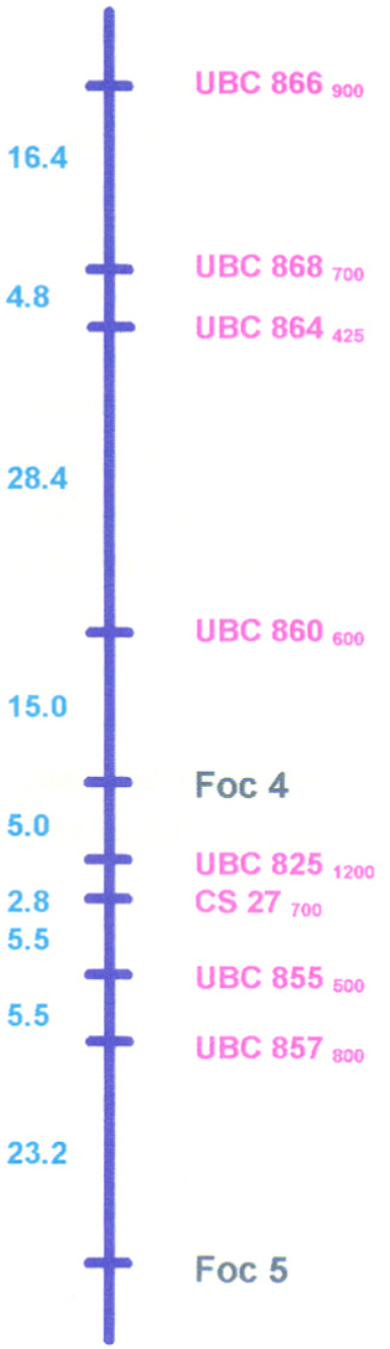
Table 13

Segregation of ISSR markers associated with the gene for resistance to fusarium wilt. *R* indicates purine while *Y* as pyrimidine.

Primer No.	Sequence	Fragment Size	χ 2	*P*
UBC-825	ACACACACACACACACT	1200	1.36	0.25-0.50
UBC-855	ACACACACACACACACYT	500	4.0	0.02-0.05
UBC-857	ACACACACACACACACYG	800	3.3	0.05-0.10
UBC-860	TGTGTGTGTGTGTGTGRA	600	4.6	0.02-0.05
UBC-864	ATGATGATGATGATGATG	425	2.0	0.10-0.25
UBC-866	CTCCTCCTCCTCCTCCTC	900	0.52	0.25-0.50
UBC-868	GAAGAAGAAGAAGAAGAA	700	1.3	0.25-0.50

Figure 20

Position of ISSR markers with respect to the gene for resistance to fusarium wilt race 4 (Foc 4) and 5 (Foc 5). The distances are indicated in cM.



The degree of polymorphism using AFLP markers was higher than the RFLP and RAPD markers. The capacity to reveal many polymorphic bands in one lane is the major advantage of the AFLP markers as compared to the RAPDs, RFLPs and microsatellite markers. Segregation analysis with the AFLP markers indicated Mendelian inheritance and agreement with expected 1:1 ratio in the RILs. The fact that alleles revealed by the AFLP technique segregated as expected indicates they are heritable and that the loci defined can be used for linkage map construction. AFLP were found to be highly reproducible as compared to RAPD markers. Since the amplification conditions involve specific primers and highly stringent amplification conditions, the reproducibility was high. At present there is no report on the use of AFLP markers in chickpea. This study indicated that AFLP can be used very effectively in chickpea genome mapping. Since chickpea is a self-pollinating crop and has a very low genetic diversity, the use of AFLP will be essential for linkage map development using a narrow crosses. The AFLP can also be used for detecting the genetic diversity among chickpea accessions and in establishing the relationship among wild species. AFLP linkage maps have been developed in the other legume crops such as soybean and pea. The high degree of homology among the pea and chickpea genome can be useful in comparative genome mapping.

Bulk Segregant analysis

Two bulks were prepared from the 6 resistant and 6 susceptible lines for fusarium wilt race 4. The DNA of all the 6 lines was mixed in equal concentration. In addition to the bulked DNA, 4 resistant and 4 susceptible lines were also used for the AFLP analysis. The amplification of the DNA with primer AGG-CTA indicated band present in all the resistant lines and the resistant bulk, and was absent in the susceptible lines and in the susceptible bulk. The markers showed association with the fusarium wilt race 4 resistant genes. Fig. 21 show a AFLP picture amplified by primer EcoRI AGG- *Mse*I CTA. Scoring of the marker in 131 RILs will provide the further details about the marker.

Fig 21: Bulked Segregant analysis for fusarium wilt in chickpea using AFLP. Lanes 1 and 2 indicates parents *C. arietinum* (ICC4958) and *C. reticulatum* (PI 489777), lanes 3-4 indicate susceptible bulk and resistant bulk for fusarium wilt, lanes 5-8 indicates susceptible RILs while lanes 9-12 indicate resistant RILs. The marker is indicated by an arrow.

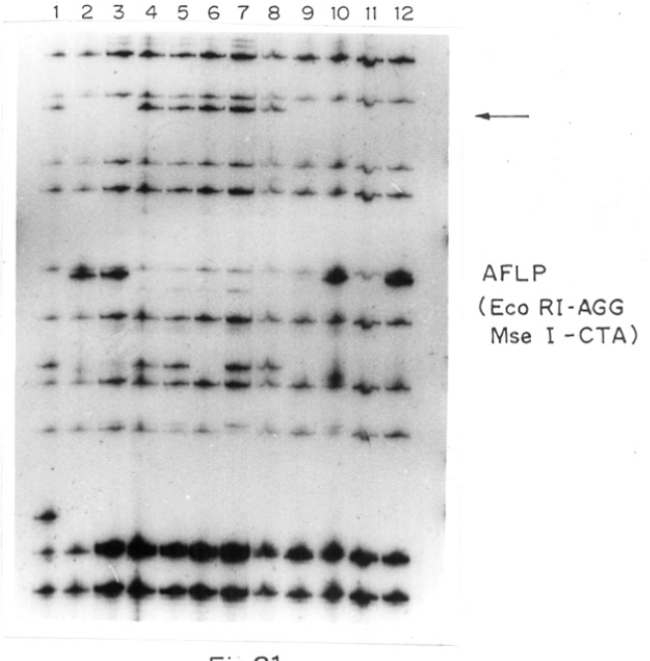


Fig.21

DISCUSSION

Mendelian segregation of ISSR markers

The ISSR loci we studied have shown virtually complete agreement with the expectations of Mendelian segregation, which is necessary for their use in genome mapping and population studies. The Mendelian inheritance of the ISSR markers indicated that most lines were relatively unbiased and showed low heterozygosity. The lines would thus appear to constitute a suitable population for the mapping of major disease resistance genes. The choice of the parents for the construction of the interspecific mapping population was based on the fact that there is very little polymorphism within chickpea accessions. The availability of RILs as permanent mapping populations will greatly facilitate the mapping of other genes.

The degree of polymorphism detected in the present study is comparatively higher than previously reported using RFLP markers (Simon and Muehlbauer 1997). This may be due to the high resolving power of PCR based markers for detecting polymorphism. The probability of finding a marker within a specified distance of a target gene depends on the genome size, the number of primers screened, and sequence divergence between the region spanning the target gene. Since chickpea has little genetic diversity, it was necessary to screen many ISSR primers to identify a marker linked to the gene for resistance to fusarium wilt race 4. In our study, 90% of PCR products were common between *C. arietinum* and *C. reticulatum* indicating that microsatellites were highly conserved in both the species. This is not surprising considering the large amount of structural and functional homology that exists between the two species. In this study, the markers showing skewed ratios have been amplified from *C. reticulatum*. The possible reason for the skewed ratios might be the preferential chromosome elimination (Goshen *et al.*, 1982) or selective elimination of particular zygotes (Zamir & Tadmor 1986).

Advantages of ISSR markers, UBC-855_{500} and UBC-825_{1200} for fusarium wilt screening

The markers $UBC855_{500}$ and UBC 825_{1200} have number of advantages over RFLP and RAPD markers for use in high resolution mapping and marker assisted indirect selection of traits in plant breeding applications. The ISSR marker procedure is simple, quick and reliable and it is possible to process many samples per day as compared to RFLP. The amplification conditions with ISSR markers are very stringent which allow high reproducibility as compared to RAPD markers. The ISSR markers can provide valuable information for the selection of fusarium wilt resistant lines. Further ISSR markers are closer to the resistant gene than previously identified RAPD markers, $CS\text{-}27_{700}$ and $UBC\text{-}170_{550}$, which are at a distance of 7 cM from fusarium wilt race 4. The ISSR marker can also be used to develop sequence tagged microsatellite sites (STMS) at the fusarium wilt locus.

There are many reports of the microsatellite markers linked to disease resistance genes. Yu *et al.*,(1996) have reported the association of microsatellite marker linked to the soyabean mosaic virus (SMV) resistance gene (*RSV1*). Finding of SSR markers linked to the disease resistance gene is very expensive and involves screening the library, sequencing the clone and synthesizing the primers. This has prevented the broad use of microsatellites in plants. Our study has demonstrated that microsatellite sequence can be used directly in the PCR reaction and can be used to find the linked marker to the disease resistance gene. This approach is faster and cheaper than southern hybridization and oligonucleotide fingerprinting. The polymorphism is detected within the region spanned by the microsatellite primer and also within the amplified region between the primer binding sites.

Genes conferring resistance to fusarium wilt

Identifying fusarium wilt race specific resistance genes and transferring them to adapted backgrounds are major challenges. To-date,

only genes conferring resistance to race 1 have been reported. For race 1, three genes are reported to control resistance; complete resistance is conferred when any two of the loci are present, while partially recessive alleles in a homozygous conditions delay wilting (Singh *et al.*, 1987) Screening technique for evaluating fusarium wilt resistance have already been developed and standerdized. However, they are costly in terms of time and effort. Several sources of fusarium wilt resistance have been reported, and it is possible that the resistance in these lines involves distinct genes. At present, no information is available on the chromosomal location of race specific resistance genes in the *Cicer* genome. Our study indicated that genes for fusarium wilt race 4 and race 5 are present in same linkage group. Further the markers UBC-855_{500} and UBC-825_{1200} can be used for selecting the fusarium wilt resistant lines.

Cluster of fusarium wilt disease resistance genes

The clustering of host resistance genes conferring resistance to pathogenic fungi has been documented in many plant species. In soybeans, Lohnes *et al.*, (1993) have reported that two resistance genes (*Rmd* and *Rps2*) are closely linked to each other, and to a non-nodulation gene (*Rj2*). In lettuce (*Lactuca sativa* L.) 13 resistance genes for downy mildew (*Bremia lactucae* Regel) have been mapped to four clusters (Hulbert and Michelmore, 1985). The other examples are rust resistance genes (Rp) on chromosome 10 of maize (Hooker 1985) and rust resistance gene clustered in the L group of flex (Shepared and Mayo 1972; Islam *et al.*, 1993). Our study has indicated that the genes for resistance to fusariumn wilt race 4 and 5 are linked. The clustering of resistance genes at a specific chromosomal region is advantageous in a breeding programs. This block of genes can be transferred to an adapted background via backrossing and the desired trait can be selected using the ISSR markers. These results also indicate the presence of SSR at the disease resistance gene cluster and that fragments amplified by SSR often co-segregated indicating cluster of the microsatellite repeat. The clustering of microsatellites was also reported in tomato where

mapping of GATA and GACA containing mcirosatellite loci showed that they were not distributed randomly throughout the genome but were often clustered in the same chromosomal region (Arens *et al.*, 1995; Broun and Tanksley 1996). The remarkably high level of polymorphism around the disease resistance gene cluster may suggest an association between the molecular mechanism of disease resistance and rapid sequence divergence in plants (Yu *et al.*, 1996, Sudupak *et al.*, 1993). The multiple genes for disease resistance are thought to be due to the duplication of the ancestral gene. It might be possible that the region surrounding to the gene containing SSR repeats is duplicated and thus provided amplifications using homologous sequences. The shift in the position in the marker varied according to the anchors present at the 3' end and 5' end of the SSR sequence. The anchors at the 5' end had a small change in the position of the marker. By changing the sequence of the short nucleotide residues at the ends, many primers with different anchors can be synthesized which may amplify markers more-closely linked to the disease resistance gene. Additional experiments with *in situ* hybridization would provide detailed information on association of various SSR with disease resistance gene clusters.

In summary our results indicate that inter-simple sequence repeats can be used as highly informative markers for genome mapping and gene tagging. The ISSR directed approach in combination with bulked segregant analysis (BSA) has wide application in plant and animal genome mapping . It can be extremely useful in (1) identifying the markers at clusters of disease resistance genes (2) filling large gaps in linkage maps (3) developing the sequence tagged microsatellite sites and (4) marker enrichment at desired regions.

Thesis Summary

Genetic fingerprinting of pigeonpea (*Cajanus cajan* (L.) Millsp.) accessions and their classification using arbitrary primed PCR

The genetic relationships among pigeonpea accessions were analyzed by amplifying DNA using arbitrary oligonucleotide primers in (PCR) polymerase chain reaction. Twenty-three primers gave reproducible amplification patterns in the range of 0.2 to 3 kb. Sufficient level of polymorphism was detected which not only allowed the identification of pigeonpea accessions of diverse origins but also their classification into different groups. The similarity matrix within pigeonpea accessions ranged between 0.7 to 0.9 indicating little polymorphism within accessions.

The highest genetic distance value was observed between 'Khandwa local-153' and 'EC-109890' whereas the lowest genetic distance value was observed between 'PT-25' and 'PT-37'. Multi-dimensional scaling (MDS) was employed to gain a view of the relative distances between the accessions in two-dimensional space, which indicated that all the accessions formed one large group with no major divisions.

Result from the dendrogram suggested that many Indian accessions grouped together, however 'Khandwa local-153' was the most distinct cultivar. Accession 'Norman' from USA grouped with 'DSLR-17' whereas 'Granada' from Peru grouped with 'P-3203' and 'R-60-187-2'. Accessions from Sri-Lanka 'EC-109893', 'EC-109884', 'EC-109890' showed more similarity to the accessions from India. 'Code no. 2' from Trinidad and Tobago grouped with 'Prabhat'.

Molecular characterization of wild relatives of Pigeonpea

(a) Genetic relationship among wild relatives

Genetic relationship among wild relatives of pigeonpea was established using randomly amplified polymorphic DNA (RAPD) markers. Extensive polymorphism was detected among all the species, which were distinguishable from each other, based on their amplification profiles.

Similarity matrix was calculated using the RAPD data and a dendrogram was constructed. The similarity matrix among wild relatives

ranges from 0.22-0.85 indicating extensive polymorphism among wild species. The results based on the dendrogram suggested that *C. albicans* clustered with *C. lineatus* and *C. sericeus*, with *C. lineatus* being more closely related to *C. sericeus* than to *C. Albicans*.

Australian species, *C. acutifolious*, *C. reticulatus* and *C. grandifolius* were less closely related to *C. cajan* and other Indian species.

(b) Identification of genus and species specific markers

Different RAPD markers were identified that had genus and species specific amplifications. DNA markers were specific to *C. albicans, C. cajan, R. rothii, R. bracteata* and *D. ferrugineae* .

Marker OPA 3-500, was found to be specific to all the species in the genus *Cajanus* and was absent in all the other genera.

(c) Isolation of vicilin gene from pigeonpea

The gene coding for a 50 kDa subunit of the 7S (Vicilin) seed storage protein was isolated using PCR. The PCR primer sequences were derived from the genomic clones of phaseolin (a member of small vicilin gene family) and was used for the amplification of vicilin gene in pigeonpea. The amplification of vicilin gene in pigeonpea accessions and wild relatives showed similar pattern indicating that the gene was highly conserved.

Inheritance of inter-simple sequence repeat polymorphisms and their association with Fusarium wilt resistance gene in chickpea

(a) Inheritance of simple sequence repeat polymorphisms

The inheritance of inter-simple sequence repeat (ISSR) polymorphism was studied in a cross of cultivated chickpea (*Cicer arietinum* L.) and a closely related wild species (*C. reticulatum* Lad.). The ISSR loci showed virtually complete agreement with expected Mendelian ratios. Twenty-two primers were used for analysis and yielded a total of 31 segregating loci.

(b) Linkage of ISSR marker to the gene for resistance to fusarium wilt

Two ISSR marker linked to the gene for resistance to fusarium wilt race 4 was identified. The marker UBC-855_{500} was found to be linked to the gene for resistance to fusarium wilt race 4. The marker co-segregated with CS-27_{700}, a RAPD marker previously shown to be linked to the gene for resistance to fusarium wilt race 1. Further marker UBC-825_{1200} was found to be linked to fusarium wilt at 5.0 cM. This is the first report of the ISSR marker linked to any disease resistance gene in a crop plant.

The study indicted that genes for resistance to fusarium wilt race 4 and 5 were present in the same linkage group.

(c) Amplification fragment length polymorphisms (AFLP) in chickpea

AFLP revealed high level of polymorphisms among chickpea accessions as compared to the RAPDs, RFLP and microsatellite markers. Segregation analysis with the AFLP markers indicated Mendelian inheritance and agreement with expected 1:1 ratio in the RILs. Bulked segregant analysis identified a marker using primer combination AGG-CTA for potential use in fusarium wilt screening

Conclusions

The overall study indicates that PCR based DNA markers are highly informative in the genome analysis of pigeonpea and its wild species. These markers can be used for the identification and classification of accessions, in developing genetic relationships among wild species and for finding genus and species specific markers. The results demonstrate the utility of RAPD in resolving biosystematic relationships in subtribe Cajaninae. This could contribute to a better characterization of genetic relationship among wild relatives and also for determining genetic variation among the species. The study on chickpea genome indicates that inter-simple-sequence-repeat polymorphisms are useful in genome mapping and can be used in gene tagging and in the construction of linkage map in chickpea. An ISSR marker linked to the gene for resistance to fusarium wilt race 4 is identified. The

marker UBC-855_{500} and UBC-825_{1200} can be used for the selection of fusarium wilt resistance. AFLP markers can be more useful than other marker systems particularly for studies with chickpea.

Future Prospectives

1. The PCR generated DNA markers can be used for genetic analysis of pigeonpea accessions and for establishment of germplasm core collection of pigeonpea and its wild species. Studies on identification of new markers specific to wild relatives can be investigated.
2. Vicilin gene can be used for isolation of other vicilin gene families, which will be useful in the development of allele specific primers for variability studies of pigeonpea germplasm.
3. In chickpea, markers linked to other races of fusarium wilt can be identified. ISSR markers can be used for the linkage map construction of chickpea. Marker UBC-855_{500} and UBC-825_{1200} can provide valuable information for the development of sequence tagged microsatellite sites (STMS) at the desired locus. The AFLPs can be used for finding marker linked to fusarium wilt and ascochyta blight resistant gene.

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CURRICULUM VITAE

M.B. RATNAPARKHE

Academic qualifications

Ph.D (Thesis submitted)	(1998)	University of Pune	Pune
M.Sc (Biotechnology)	(1991)	Devi Ahilya University	Indore
B.Sc (Agriculture)	(1989)	Jawaharlal Nehru Agri. Univ.	Jabalpur

*Ph.D. thesis title
Molecular characterization of pigeonpea (*Cajanus canjan* (L.) Millsp.) and its wild relatives using PCR generated DNA markers.

Address

Permanent

A51\55 Devi Deep Appt.
NDA Road, Bawdhan
Pune

Temporary

Plant Molecular Biology Unit
Division of Biochemical Sciences
National Chemical Laboratory
Pune

References

Dr. P.K.Ranjekar
Plant Molecular Biology Unit
Division of Biochemical Sciences
National Chemical laboratory
Pune-411008, India

Dr.(Mrs.) V.S. Gupta
Plant Molecular Biology Unit
Division of Biochemical Sciences
National Chemical Laboratory
Pune-411008, India

Dr. F.J. Muehlbauer
303 Johnson Hall
Washington state University
Pullman, USA

Dr. T.W. Okita
Institute of Biological Chemistry
Washington State University
Pullman, USA

Awards/Scholarships

1. Junior and Senior Research Fellowships of CSIR (1991- 1995)
2. GATE Fellowships (1991)
3. Visiting Fellowship, University of Laval, Quebec, Canada (March 1993 to August 1993)
4. Visiting Fellowship, McKnight Foundation, Washington State University, Pullman, USA. (January 1996 to January 1998)

Publications

(1) Genetic fingerprinting of pigeonpea (*Cajanus cajan* (L.) Millsp.) and its wild relatives using RAPD markers.(1995). **M.B. Ratnaparkhe**, V.S. Gupta, M. R. Ven Murthy, P.K. Ranjekar. Theoretical and Applied Genetics 91:893-898

(2) Inheritance of inter simple sequence repeat polymorphisms and their linkage with fusarium wilt resistance gene in chickpea.(1998) **M.B.Ratnaparkhe**, D.K.Santra, A. Tullu, F.J.Muehlbauer. Theoretical and Applied Genetics (In Press)

(3) Identification and classification of pigeonpea accessions using arbritrary primed PCR (Communicated to Plant Breeding). **M.B. Ratnaparkhe**, V.S. Gupta, V.J. Sant, P.K. Ranjekar

(4) Inter-simple sequence repeat (ISSR) polymorphisms are useful in the identification of DNA markers linked to the cluster of the disease resistance genes (Communicated to TAG) **M.B.Ratnaparkhe**, M. Tekaeglu, F.J.Muehlbauer.

(5) Isolation of Vicilin gene from pigeonpea using PCR (Under Preparation) **M.B. Ratnaparkhe**, V.S. Gupta, M. R. Ven Murthy, P.K. Ranjekar.

(6) Species specific markers in pigeonpea and its wild relatives. (Under Preparation) **M.B. Ratnaparkhe**, V.S. Gupta, P.K. Ranjekar

(7) Inheritance and segregation of AFLP markers in chickpea (Under Preparation)